7. Regulation of translesion DNA synthesis by PCNA monoubiquitylation and beyond

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Abstract. Translesion DNA synthesis (TLS) is a DNA damage tolerance process that cells have elaborated to cope with a wide variety of chemicals and physical cues that constantly challenge the integrity of the DNA by generating damage. In particular during S-phase, DNA lesions that escape repair inhibit the progression of the replicative DNA polymerases resulting in a prolonged arrest of the replication fork. This “replication stress” represents a threat to genomic stability because it can lead to fork collapse by generating double-strand breaks (DSBs) and chromosomal rearrangements by homologous recombination (HR). Recruitment of translesion DNA polymerases mediates lesion-bypass at arrested forks by catalyzing DNA synthesis across blocking lesions. The most widely appreciated model for regulation of TLS DNA polymerases recruitment is based on reversible posttranslational modification of the replication factor

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PCNA through monoubiquitylation (PCNA\textsuperscript{monoUb}). In this model, Y-family DNA polymerases are recruited to chromatin-bound PCNA via a low affinity PCNA interaction motif (PIP box) and through interaction with ubiquitin, which increases their affinity for PCNA specifically upon DNA damage. Since PCNA\textsuperscript{monoUb} is already engaged in DNA replication and because its homotrimeric structure allows in theory simultaneous interaction with only three proteins at a time, this mechanism raises several conceptual questions on how PCNA interaction with translesion DNA polymerases is regulated. In this chapter, we will first describe the regulation of TLS by monoubiquitylation of PCNA, followed by a more detailed discussion on a second level of regulation by the ubiquitin-proteasome system that has emerged from recent advances in the regulation of PCNA-protein interactions.

The Y-family of DNA polymerases: Enzymes specialized for TLS

Enzymatic activity of Y-family DNA polymerases

Alterations of the chemical composition of DNA can result from exposure of living organisms to sunlight and cosmic rays, two major sources of ultraviolet (UV) light and ionizing radiation respectively, as well as from various man-made chemicals, which are responsible for most exogenous DNA damage. In addition, spontaneous non-enzymatic reactions of DNA with molecules of water (hydrolysis) and reactive oxygen species, by-products of cellular metabolism (oxidation), take place at significant rates \textit{in vivo} and account for an important part of the endogenous DNA damage [1].

At the turn of the twenty-first century, a large number of evolutionary-related DNA polymerases specialized in TLS were discovered in all three kingdoms of life, defining a new superfamily of DNA polymerases, called the Y-family [2] (and Figure 1). These enzymes differ from the replicative DNA polymerases by their low processivity and the lack of 3'→5' proofreading exonuclease activity, resulting in low-fidelity DNA synthesis on undamaged DNA [3, 4]. The ability of these DNA polymerases to bypass bulky lesions is believed to facilitate the restart of arrested replication forks. In higher eukaryotes, the principal TLS polymerases are members of the Y-family, together with the B-family member Pol ζ (zeta) [5].

Although TLS is generally beneficial to cell survival by allowing the release of stalled forks, it involves low-fidelity DNA synthesis that can compromise the integrity of the genome and therefore needs to be tightly regulated [6]. The human genome encodes four Y-family DNA polymerases, including Pol η (eta), Pol κ (kappa), Pol ι (iota), and Rev1. Each enzyme has evolved a catalytic mechanism adapted for TLS across a specific set of DNA lesions. Pol η is the only known eukaryotic DNA
polymerase capable of both efficient and accurate synthesis of DNA \textit{in vitro}, across the major UV-photoprodut, the thymine-thymine cyclobutane-pyrimidine dimer (CPD) \cite{7, 8}. This unique property is explained by its unusually large active site capable of accommodating and stabilizing the thymine dimer in the correct orientation for pairing with incoming adenines \cite{9, 10}. In humans, inactivation of the Pol \( \eta \)-coding gene (POLH) gives rise to the inherited autosomal recessive disorder xeroderma pigmentosum variant (XPV) \cite{8}, characterized by altered skin pigmentation and increased incidence of UV-induced skin cancer \cite{11}. Pol \( \eta \) is therefore an important natural barrier against tumorigenesis arising from chronic UV-exposure (see also the chapter from Masutani and Hanaoka). Pol \( \kappa \) is specialised in error-free bypass of bulky \( N^2 \)-guanine adducts generated by chemicals such as the polycyclic hydrocarbon benzo[a]pyrene, present in automobile fumes and cigarette smoke, and reactive aldehydes, by-products of lipid peroxidation such as acrolein \cite{12-16}. Pol \( \iota \) has evolved in higher eukaryotes as a backup TLS polymerase whose function partially overlaps with that of Pol \( \eta \) \cite{17, 18}. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{timeline.png}
\caption{Timeline of the principal TLS milestones since the discovery of the first Y-family DNA polymerase. CPDs: cyclobutane pyrimidine dimers; NER: nucleotide excision repair; MMR: mismatch repair. Numbers indicated years.}
\end{figure}
However, Pol ı is highly error-prone on template T and misincorporates G opposite T, 3 to 10 times more often than A [19]. This may represent a selective advantage for TLS across thymine-cytosine CPDs, where the cytosine base frequently undergoes deamination to uracil [20], because Pol ı preferentially inserts the “correct” G opposite U [21]. The polymerases η and ı also play an important role in tolerance of oxidative DNA damage by catalyzing error-free TLS across the major oxidative lesion, the 8-oxoG [22-25]. Rev1 is not a true DNA polymerase, but rather a deoxycytidined transferase that is only capable of incorporating C opposite template G or abasic sites [26]. To bypass abasic sites, Rev1 has evolved a unique template-independent catalytic mechanism whereby the base is flipped out of the DNA double-helix, and an arginine residue in the enzyme active site forms hydrogen bonds with the incoming C instead [27].

**TLS is coupled to DNA replication and repair**

In prokaryotes, reconstitution of TLS activity *in vitro* suggests that the restart of stalled forks requires replacement of high-fidelity replicative DNA polymerases by polymerases specialized for TLS, in a process known as “DNA polymerase switch” [33]. The sliding β-clamp, structurally related to PCNA, has a central role in this process by acting as an auxiliary protein for the TLS polymerase [34, 35]. Once the lesion has been bypassed, DNA synthesis switches back to high-fidelity replicative DNA polymerases. DNA fiber assays in human and avian cells also indicate that Pol η is required for the progression of replication forks past UV-lesions [36, 37]. Alternatively, blocking lesions could by bypassed by a repriming event, leaving gaps in newly synthesised DNA, which would be repaired later during a process of postreplication repair [38-41]. Genetic evidence from budding yeast shows that TLS can be uncoupled from DNA replication, however at the expense of a reduced fidelity of DNA synthesis [42, 43]. Whether TLS occurs at stalled forks or behind the forks is still a matter of debate and it may be possible that both mechanisms operate in vertebrate cells [44, 45]. In addition to bypassing a variety of chemically-altered DNA bases, TLS polymerases also synthesize DNA across non-canonical DNA structures that are difficult to replicate, such as non-B DNA sequences formed within common fragile sites and G-quadruplexes [46-49]. These structures represent natural pause sites for replication forks and require TLS to restart arrested forks. Several Y-family DNA polymerases have been also implicated in DNA repair outside S phase. For example, Pol η is required for error-free repair of 8-oxoG:A mismatches by the mismatch repair pathway [25, 46].
**Regulation of TLS by PCNA**

All eukaryotic Y-family DNA polymerases contain an N-terminal catalytic domain and a C-terminal regulatory region comprising several PCNA-protein and protein-protein interaction motifs, creating the possibility for a wide diversity of regulatory mechanisms. The importance of this region was first ignored when Pol η was identified by complementation of XP-V cells, because its C-terminus was shown to be dispensable for TLS across thymine-thymine CPDs in *cell-free* extracts [50]. The subsequent sequencing of the *POLH* gene revealed that some XP-V individuals carry a deletion of the last ~200 amino acids, which results in a non-functional allele, highlighting the essential role of the Pol η C-terminus for its function *in vivo* [51, 52]. The C-terminus of all eukaryotic Y-family DNA polymerases was later shown to contain several highly conserved motifs mediating interactions with PCNA.

**PCNA**

PCNA is a member of the sliding clamps, a group of sequence-unrelated ring-shaped proteins found in all domains of life, which bind DNA topologically by embracing the double-helix and function as auxiliary proteins for DNA polymerases [53]. By forming a mobile ring around DNA to which replicative DNA polymerases can attach, PCNA is believed to increase their processivity. At arrested forks, PCNA also coordinates the recruitment of translesion DNA polymerases, and can stimulate the enzymatic activity of Y-family DNA polymerases [54]. However, and in contrast to the replicative polymerase δ whose processivity is strongly increased upon binding to PCNA [55], the processivity of Pol η remains very low (less than 10 nt). The exact mechanism of polymerases replacement during TLS is still not fully understood, but likely involves multiple levels of regulation of PCNA-protein interactions.

Most DNA polymerases interact with PCNA through a highly conserved PCNA-Interaction Protein (PIP box) motif found in all members of the Y-family of TLS polymerases (polymerases η, ι, κ in human; Rad30/Pol η in budding yeast), with the exception of Rev1, which interacts with PCNA through a BRCT domain [56, 57]. The PIP box tethers DNA polymerases to PCNA by docking into a hydrophobic pocket located under the interdomain-connecting loop of PCNA [58, 59]. In addition, Y-family DNA polymerases also bind to an ubiquitin group attached to PCNA for efficient targeting to the replication machinery [51, 60, 61]. In higher eukaryotes, a second PCNA-interaction motif termed AlkB homologue 2 PCNA-interacting motif (APIM) was also identified [62], characterised by the consensus...
B-Ψ/W-Ψ/A-B (where B is a basic residue like Lys, Arg; Ψ is an aromatic residue and A is a hydrophobic residue). Functional APIM motifs were found in several human DNA repair factors, such as the oxidative demethylase AlkB, the endonuclease and translocase ZRANB3, and the DNA helicase FBH1 [62-64], indicating that this new motif may specify PCNA-protein interactions during the DNA damage response. Remarkably, putative APIM motifs were found in more than 200 human proteins [62].

The RING finger E3 ubiquitin ligase Rad18 and the E2 ubiquitin-conjugating enzyme Rad6, members of the RAD6 epistasis group of genes required for tolerance to DNA damage, catalyse PCNA\textsuperscript{monoUb} in response to various DNA-damaging agents, including UV light, cisplatin, benzo[a]pyrene, the alkylating agent methyl methanesulfonate (MMS), and the oxidizing agent hydrogen peroxide [25, 60, 65-68]. The ubiquitin moiety is attached to PCNA on the Lys164 residue highly conserved from yeast to humans [65]. RPA-coated single-stranded DNA produced by the functional uncoupling of replicative DNA polymerases from the helicase, has been proposed as required for Rad18 recruitment in yeast [69], although in vertebrate cells evidence for this regulation is lacking. A recent report suggests that chromatin structure may also affect PCNA\textsuperscript{monoUb}. In this study, the transcription repressor ZBTB1 is shown to be implicated in Rad18 recruitment and PCNA\textsuperscript{monoUb} probably by facilitating chromatin remodelling at site of DNA lesions, although an indirect effect on transcriptional regulation cannot be excluded [70]. In mammalian cells, two additional E3 ubiquitin ligases, CRL4\textsuperscript{Cdt2} and RNF8, have been reported to contribute to PCNA\textsuperscript{monoUb} at Lys164, but to what extent they play a role in TLS regulation is still unclear [71, 72]. Following DNA damage, eukaryotic Y-family DNA polymerases bind to PCNA\textsuperscript{monoUb} through evolutionary conserved C-terminal ubiquitin-binding domains (UBD), and this interaction was proposed to regulate their recruitment at stalled forks [61, 73]. Notably, binding to PCNA does not immobilize TLS polymerases that remain highly mobile inside replication factories and could probe DNA for a suitable substrate-lesion [74]. Additional contacts with PCNA mediated by the PIP box (or the BRCT domain of Rev1) are also required for retention of TLS polymerases at sites of DNA synthesis [51, 56, 75-77]. These interactions occur independently of PCNA\textsuperscript{monoUb} because ubiquitin is located on the back face of the clamp and does not mask the PIP box-interacting molecular surface [78]. This is consistent with a “tool-belt” model, where the ubiquitin creates an additional docking site for TLS polymerases on PCNA, thus increasing their affinity for the clamp, and could trigger a polymerase switch.

PCNA\textsuperscript{monoUb} is eventually reversed by the deubiquitylating isopeptidase USP1, however several hours following DNA damage [79, 80]. This implies
that additional mechanisms regulating PCNA interactions must operate at the fork to limit error-prone DNA synthesis by TSL polymerases.

**Beyond PCNA\textsuperscript{monoUb}: The role of the CRL4\textsuperscript{Cdt2} ubiquitin-proteasome pathway**

Not only PCNA is at the crossroad of damage tolerance and DNA repair pathways, but it also functions as a mobile molecular platform on which the E3 ubiquitin ligase CRL4\textsuperscript{Cdt2} meets and polyubiquitylates its substrates [81]. Recent evidence highlights a role for this enzyme in mediating recruitment of TLS DNA polymerases. CRL4\textsuperscript{Cdt2} is a Cullin RING E3 ligase multiprotein complex that has emerged as an important regulator of genome stability [82]. In various species, CRL4\textsuperscript{Cdt2} contributes to the preservation of chromosome integrity by targeting for proteasomal degradation important cell cycle-regulated proteins, including the licensing factor Cdt1, the Cyclin-Dependent Kinase (CDK) inhibitor p21, the methyltransferase Set8, the ribonucleotide reductase inhibitor Spd1 (in fission yeast), Pol \eta in worms, and more recently the FBH1 DNA helicase and the small subunit (p12) of replicative DNA polymerase \delta in humans [64, 83-86]. The licensing factor Cdt1 is essential for loading of the replicative helicase MCM2-7 at replication origins [87, 88]. By coupling Cdt1 destruction to the progression of DNA replication through chromatin-bound PCNA, CRL4\textsuperscript{Cdt2} prevents harmful DNA rereplication [89-92].

CRL4\textsuperscript{Cdt2} substrates have an unusual PIP box that functions as a degradation signal upon binding to PCNA [93, 94]. This specialized PIP box differs from the canonical PIP box by the presence of an additional TD motif that confers high-affinity PCNA-binding, and a basic residue four amino acids downstream (B+4) required for recruitment of CRL4\textsuperscript{Cdt2} (Figure 2), which together define what was termed a « PIP degron » of signature (Q/N-x-x-\Psi-T-D-x-x-9-9-x-x-R/K, where \Psi is a hydrophobic residue, and 9 is an aromatic amino acid) [93]. The B+4 residue is generally considered as the hallmark of PIP degrons, but a cluster of basic residues upstream of the PIP box can also contribute to proteasomal degradation [95, 96]. The protein-protein interaction surface at the PCNA-PIP degron peptide interface is recognised by the substrate receptor Cdt2 that recruits CRL4 to PCNA through interaction with the adaptor protein Ddb1 [93, 97]. PCNA also participates in the molecular recognition of the degradation signal via a pair of highly conserved acidic residues (Glu124 and Asp122 in humans) located in its interdomain-connecting loop [85, 96], probably required for
Figure 2. Primary structure of PIP boxes and PIP degrons. Sequence comparison of PIP boxes and PIP degrons from various organisms. The consensus residues of the canonical PIP box are shown in red, and the additional residues required for formation of a PIP degron are shown in blue. The boxes highlight clusters of basic residues required for CRL4\textsuperscript{Cdt2}-dependent degradation. Stars denote N- and C-termini. The abbreviations used are as follow: Hs: \textit{H. sapiens}; Xi: \textit{X. laevis}; Ce: \textit{C. elegans}; Sp: \textit{S. pombe}.

positioning the B+4 residue in the correct orientation with respect to Cdt2 (Figure 3). Once CRL4\textsuperscript{Cdt2} is bound to the PCNA-PIP degron complex, the central $\alpha$-helical scaffold protein (Cullin 4A or 4B) brings in proximity the substrate and the RING finger protein Rbx1/2 that catalyses ubiquitin transfer from the E2 ubiquitin-conjugating enzymes (UBC) to the substrate. Different E2 UBCs can participate in this reaction depending on the substrate: the UBCH8 cooperates with CRL4\textsuperscript{Cdt2} for targeting p21 and Set8, whereas UBE2G1 and UBE2G2 are involved in the polyubiquitylation of Cdt1 [98]. In addition to its role during DNA replication, CRL4\textsuperscript{Cdt2} is also activated outside S phase in response to various types of DNA damage [83],
and at least during nucleotide excision repair (NER) the polyubiquitylation reaction depends upon PCNA engaged in DNA repair [99, 100]. It was originally proposed that CRL4Cdt2-mediated destruction of Cdt1 may delay the initiation of DNA synthesis when cells encounter DNA damage in G1 phase [101]. However, considering that Cdt1 is dispensable for DNA replication after loading of MCM2-7 on chromatin [87, 102], which occurs in telophase as soon as mitotic CDK activity drops [103, 104], it is unlikely that this mechanism may be relevant to the control of origin licensing in G1.

**Figure 3. Structure of human PCNA in a complex with the p21 PIP degron.**
(a) Molecular structure of the PCNA homotrimer and that of the p21 PIP degron. The surface bound by the PIP degron of p21 is shown in the box. The PIP degron adopts a $3_{10}$ helical conformation, positioning the methionine residue M4 and tyrosine residue Y8 in the hydrophobic pocket [58]. The hydrophobic amino acids that form the pocket are indicated (numbers correspond to their respective positions in human PCNA). Acidic residues that form ionic contacts with the R+4 and R+5 residues are also indicated. (b) Molecular interactions of p21 PIP degron with the interdomain-connecting loop of PCNA. A single PCNA monomer bound by the PIP degron and details of the interactions are shown. For clarity, only the R+4 residue in the PIP degron is shown. The guanido group of R+4 forms ionic contacts with the $\gamma$-carboxyl group of Glu124 (orange dotted line) and hydrogen bonding with the $\alpha$-carbonyl group of Asp122 (black dotted line). The images were generated using PDB accession number 1AXC [58] and Swiss PDB viewer.
Furthermore, this mechanism cannot explain why all other CRL4\textsubscript{Cdt2} substrates need to be degraded in G1 following DNA damage [83]. Alternatively, it is possible that this degradation pathway may regulate PCNA-protein interactions.

**Destruction of PIP degron-bearing proteins regulates TLS**

More than fifty DNA replication and repair proteins, chromatin-modifying factors, and cell-cycle regulators are known to interact with chromatin-bound PCNA through conserved PIP boxes [57, 105]. As new PCNA-interacting proteins are constantly identified it becomes more and more puzzling to understand how all these interactions are regulated. In theory, the homotrimeric ring-shaped structure of PCNA could provide an interaction surface for up to three proteins at a time, implying that many proteins are likely to compete for interacting with the clamp [57]. Posttranslational modifications of PCNA though ubiquitin and SUMO represent an important mechanism for regulation of PCNA-protein interactions [106], and modifications of PCNA partners by phosphorylation, methylation, and ubiquitylation may represent an additional level of regulation [107, 108]. The recently identified PIP degrons have opened even more possibilities.

Interestingly, PIP degrons have evolved high PCNA-binding affinity, mediated by an evolutionary conserved TD motif (see below and Figure 2), giving them a priority access to the sliding clamp to ensure very efficiently degradation by CRL4\textsubscript{Cdt2} [93]. This feature may also limit error-prone TLS through competitive interactions with PCNA. Several recent studies suggest that in mammals TLS is also regulated by a pre-established natural competition between PIP degron-containing proteins and TLS polymerases, further fine-tuned by CRL4\textsubscript{Cdt2}-mediated destruction [64, 109, 110]. In support of this model, expression of nondegradable substrates of CRL4\textsubscript{Cdt2}, which sustains interaction with PCNA, including p21, Cdt1, or FBH1, impairs the localisation of Y-family DNA polymerases to sites of DNA synthesis, substantially increases the cellular sensitivity to UV light, and to different extents results in genomic instability [64, 109, 110]. Importantly, these effects are independent from the function of p21 or Cdt1 in cell cycle regulation, and depend upon interaction with PCNA [109-111]. Consistent with the role of Pol \( \eta \) in mediating bypass of UV-lesions at the replication fork [37], nondegradable p21 also impairs the progression of replication forks in UV-irradiated cells [109]. Hence, CRL4\textsubscript{Cdt2}-mediated destruction of p21, and possibly of other substrates, is required for TLS in S phase. Conversely, it was previously shown that p21 increases the accuracy of TLS
Regulation of translesion DNA synthesis by PCNA monoubiquitylation and beyond

events in a PIP box-dependent manner, however at the expense of lower lesion-bypass efficiency [112], suggesting that competitive binding of p21 and TLS polymerases to PCNA may reduce error-prone TLS. Even though at first it may seems paradoxical that p21 inhibits error-prone TLS and must be destroyed to allow TLS, these studies suggest that the equilibrium of PCNA-protein interactions is fine-tuned regulated by CRL4Cdt2-mediated destruction to control TLS accuracy. Since Cdt1 and p21 are degraded throughout S phase [92, 113, 114], this regulation may be important in the early stages of the initiation of DNA replication when PCNA is loaded at RNA/DNA primers synthesised by Pol α/primase. Loading of PCNA at the primer/template junction by the clamp loader RF-C leads to dissociation of Pol α and its replacement by the more processive polymerases δ. At this step, CRL4Cdt2 substrates are probably bound to PCNA, waiting to be polyubiquitylated, and can compete out TLS polymerases. This may be an efficient mechanism to prevent the incorporation of low-fidelity Y-family DNA polymerases into the replisome, which would result in slow and mutagenic DNA synthesis (Figure 4). In this model, the regulated activity of CRL4Cdt2 defines a time-window during the early steps of DNA synthesis when TLS polymerases are actively excluded from the replisome.

**Figure 4. Model for regulation of TLS polymerases recruitment during the initiation of DNA synthesis.** DNA replication begins with the synthesis of an RNA/DNA primer by Pol α/primase, followed by its dissociation and loading of PCNA at the primer/template junction by RF-C. Pol δ is then recruited to PCNA to initiate processive DNA synthesis. During this step, all substrates of CRL4Cdt2 are tethered to PCNA through their high-affinity PIP degrons, forming a « PIP degron shield » that prevents low-fidelity TLS polymerases to access the clamp. The time necessary for their polyubiquitylation and subsequent extraction from PCNA defines a time-window when TLS polymerases are excluded from the fork.
Although TLS is traditionally considered as a mechanism employed to deal with obstructive lesions encountered by replication forks, emerging evidence indicates that Y-family DNA polymerases can also be implicated in DNA repair outside S phase [6]. In some specific cases when both DNA strands are damaged, classical excision-repair mechanisms that rely on high-fidelity DNA polymerases are inadequate, and TLS polymerases are required for efficient and error-free lesion bypass. Several Y-family DNA polymerases were shown to function in different PCNA-dependent repair pathways. For example, Pol η functions in mismatch repair (MMR) of oxidative DNA lesions, and a less well characterized non-canonical MMR pathway activated by various DNA lesions rather than mismatches [25, 115]. Pol κ has been implicated in NER and single-strand break repair [116-118]. The recruitment of Pol η and Pol κ to sites of DNA repair depends on PCNA monoUb, which occurs to some extent in the G1 phase [25, 117]. The MSH2 and MSH6 proteins components of the mismatch-recognition complex MutSα are required for PCNA monoUb following H2O2-induced DNA damage, indicating that PCNA modification is associated with DNA repair [25]. Because the CRL4Cdt2 ubiquitin-proteasome pathway can also function in a manner coupled to DNA repair [99, 100]. it is possible that degradation of high-affinity PIP degron-containing proteins may regulate the switch of high-fidelity DNA polymerases to error-prone TLS polymerases during repair synthesis. Consistent with this possibility, non-degradable Cdt1, that sustains interaction with PCNA, impairs the localisation of Pol κ to DNA repair factories in G1 phase [110]. Hence, removal of PIP degrons from PCNA may be a general mechanism that cells employ to control the interplay of repair factors on chromatin-bound PCNA.

Currently, evidence for a role of CRL4Cdt2 in TLS regulation only exists in mammals. However, the widespread distribution of PIP degrons in fission yeast and Metazoans suggests that this mechanism may also be conserved in other species, though not in S. cerevisiae where PIP degrons have not been found.

Hierarchical interactions on PCNA: PIP degrons versus non-canonical PIP boxes

The observation that the CRL4Cdt2 ubiquitin-proteasome pathway can regulate the localisation of Y-family DNA polymerases at sites of DNA damage raises one question: why PIP degron-containing proteins do not also affect the binding of other replication factors to PCNA through PIP box motifs, such as the replicative DNA polymerase δ, or the flap structure-specific exonuclease Fen1? To answer this question, it is necessary to take
into account that interactions with PCNA can be hierarchical, because PIP boxes are highly adaptable and minor variations in their amino acid composition can dramatically change the strength of binding [59]. Since an extensive comparison of the binding affinities of all PCNA partners does not exist, we modelled the PIP box/PIP degrons of several PCNA partners into the hydrophobic pocket of PCNA, and calculated the corresponding interaction free energies ($\Delta G_{\text{int}}$) of each complex (Table I). The obtained thermodynamic dissociation constants ($K_D$) reveal roughly three hierarchical categories of clamp-binging peptides: I) PIP degrons, characterized by strong PCNA-interaction, II) canonical PIP boxes that have moderate affinity for PCNA, and III) non-canonical PIP box motifs that have suboptimal PCNA-binding affinity. The differences in interaction-affinity can be explained in terms of structure. The evolutionary conserved threonine residue within the TD motif (T5) confers strong PCNA-binding affinity [93], and for instance, mutation of this residue completely abolishes the ability of Cdt1 to impair the localisation of Pol $\eta$ at sites of DNA damage. However, how the T5 residue increases the PCNA-binding affinity of PIP degron motifs is currently unknown. It is noteworthy to mention that the TD motif is not conserved in fission yeast CRL4Cdt2 substrate, where other residues may contribute for strong interaction. In the PCNA-p21 complex, the interaction is further strengthened by an extensive antiparallel $\beta$-sheet formed between the residues downstream of the PIP degron and the interdomain-connecting loop of PCNA [58, 59]. In contrast, Pol $\eta$, $\iota$, and $\kappa$ have evolved a non-canonical PIP box that lacks the consensus glutamine residue (Q1), which weakens the interactions with the backbone of PCNA [119] (and Figure 2). Furthermore, the position of their PIP box at the extreme C-terminus of the protein makes impossible additional contacts with the interdomain-connecting loop, which would otherwise stabilize the complex. For these evolutionary reasons, the PIP box motifs of Y-family DNA polymerases have a suboptimal PCNA-binding affinity compared to those of other PCNA partners [119]. In a model for hierarchical interactions on PCNA, Y-family DNA polymerases have the lowest binding-affinity (Table I) and are therefore prone to expulsion from the trimeric clamp by other partners, and especially by strong-interacting PIP degron-containing proteins, whereas the binding of replication factors having canonical PIP boxes would be less affected. These competitive interactions could explain why Pol $\eta$ cannot spontaneously displace Pol $\delta$ in the absence of PCNA$_{\text{monoUb}}$.

The functional significance of the weak interaction-affinity of Y-family DNA polymerases for PCNA was elegantly addressed by replacing the Pol $\kappa$ PIP box with the PIP degron of p21. Of note, the B+4 residue was mutated to avoid proteasomal degradation. Remarkably, the resultant Pol $\kappa$-PIP$^{p21}$ chimera
Table I. Hierarchical classification of PCNA-interacting motifs based upon the dissociation constants and free Gibbs PIP/PCNA binding energies. PIP boxes of different PCNA substrates were locally aligned against the sequence of the wild-type peptide (p21 C-terminal region) bound to Human PCNA (pdbId: 1AXC, X-ray structure, 2.6Å resolution), then side chains of the substrates were modelled over the wild-type peptide (backbone and C-β) coordinates using Build Model FoldX command. For the resulting PCNA/PIP complexes, Gibbs interaction relative free energies were calculated by Analyze Complex FoldX command. Ranking of the interaction energies resulted in three major groups: I) Strong II) Moderate III) Low for non-canonical PIIP boxes (as those found in Y-family DNA polymerases).

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<th>ΔG (kcal/mol)</th>
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<td>I</td>
<td>p21</td>
<td>-20.84</td>
<td>5.2 × 10⁻¹⁸</td>
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<td></td>
<td>Cdt1</td>
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<td>1.3 × 10⁻¹⁰</td>
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<td>4.4 × 10⁻⁴</td>
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escapes all regulation mechanisms and localises in replication factories in an ubiquitin-independent manner, and induces genomic instability [120]. This study highlights the importance of hierarchical interactions on PCNA to prevent inappropriate targeting of TLS polymerases at replication forks. Prokaryotic Y-family DNA polymerases also rely on short clamp-binding sequences for interaction with the β-clamp, but additional posttranslational modifications of the clamp through ubiquitin are not required for efficient interaction [34, 35]. Hence, the independent evolution of clamp-binding peptides in eukaryotes might have seen an advantage in low-affinity PIP box motifs as a way to reduce error-prone TLS and to allow additional levels of regulation to build up.

Integration of CRL4Cdt2 with other mechanisms

Recently, the human DNA helicase FBH1 and the small subunit (p12) of the replicative DNA polymerase δ were also shown to contain a PIP degron, further enlarging the regulatory duties of CRL4Cdt2. However, and in contrast
to other CRL4\textsuperscript{Cdt2} substrates, FBH1 and p12 are degraded mainly in response to DNA damage though not very efficiently in S phase [64, 86, 121]. This might be due to the non-canonical sequence of their PIP degrons (FBH1 lacks the TD motif, whereas p12 lacks the conserved Q/N residue at position 1). In addition to the PIP degron, FBH1 also interacts with PCNA through an APIM peptide and can impair the recruitment of Pol η at sites of DNA synthesis in a manner dependent upon both interaction motifs [64]. The APIM peptide probably compensates for the lack of a TD motif in the PIP degron of FBH1 by increasing its interaction-affinity for PCNA and allowing competition with Pol η. The function of FBH1 is still not completely understood, but is believed to regulate HR by resolving recombination intermediates [122, 123]. Because Pol η also functions in HR by extending the invading strand in recombination intermediates in a reaction that also involves PCNA [124-127], it is not clear if competitive binding of FBH1 to PCNA interferes with TLS or HR. Besides DSBs repair, the HR pathway is also required for the recovery of collapsed forks [128]. It is then tempting to speculate that CRL4\textsuperscript{Cdt2}-mediated removal of FBH1 might regulate both TLS and the repair of broken forks, by limiting the anti-recombinase activity of FBH1 and at the same time promoting the interaction of Pol η with PCNA.

The small p12 subunit of the human heterotetrameric Pol δ is also degraded in response to DNA damage in a PIP degron- and CRL4\textsuperscript{Cdt2}-dependent manner in mammalian cells [86, 121], while in the yeast \textit{S. cerevisiae} the catalytic subunit of Pol δ is degraded by a CRL4\textsuperscript{Cdt2}-independent mechanisms that involves the DEF1 protein (see chapter by Makarova and Burgers). Interestingly, p12 was shown to regulate the fidelity of DNA synthesis by modulating the 3' → 5' proofreading exonuclease activity of Pol δ and its capacity to extend mismatched primers [129, 130]. In the absence of p12, the capacity of Pol δ to synthesize DNA across 8-oxoG and other non-blocking lesions is reduced and its proofreading activity is largely increased. This raises the interesting possibility that the destruction of p12 can reduce the TLS activity of Pol δ, preventing replication error when cells encounter DNA damage.

One aspect of the CRL4\textsuperscript{Cdt2} ubiquitin-degradation pathway that still remains underexplored is the extraction of polyubiquitylated substrates from chromatin-bound PCNA. In the case of Cdt1, this process involves the AAA+ chaperon p97 (also called VCP) that functions as a segregase to extract ubiquitylated Cdt1, and is required for its degradation by the proteasome [100]. Several groups have independently shown that p97 is recruited to sites of DNA synthesis following DNA damage through interaction with the ubiquitin-binding adaptor protein Spartan (also called...
DVC1) [131-134]. Spartan binds PCNA\textsuperscript{monoUb} via an evolutionary-conserved PIP box and an ubiquitin-binding zinc finger, but its role remains somehow contradictory. Because downregulation of Spartan by RNA interference results in retention of Pol \( \eta \) at sites of DNA synthesis following UV-irradiation, it was proposed that Spartan may recruit p97 at stalled forks to facilitate extraction of Pol \( \eta \), preventing excessive TLS [131, 132]. In contrast, two other studies reported both reduced PCNA\textsuperscript{monoUb} and localization of Pol \( \eta \) to sites of DNA damage in cells lacking Spartan [133, 134], suggesting a protective role against USP1-mediated PCNA deubiquitylation [134]. The possibility that p97 may function in extracting both Cdt1 and Pol \( \eta \) from PCNA is interesting in light of the recent finding that removal of Cdt1 from PCNA is required for recruitment of Pol \( \eta \) [110]. Further studies will be necessary to establish in what way the p97 chaperon and its adaptor Spartan regulate the interplay of DNA replication and repair factors on PCNA.

**Conclusion**

DNA repair and damage-tolerance pathways were originally considered as linear or branched cascades, but it now becomes increasingly clear that the crosstalk between these parallel pathways is an important feature of the cellular response to genomic insults. Hence, these pathways appear highly coordinated within the eukaryotic cell cycle in order to guarantee the precise and faithful duplication of the DNA. The restart of paused forks is a complex process that needs the coordination of multiple events including, repriming, translesional bypass of DNA lesions, and fork remodeling. As in all networks, the presence of a master hub where many factors are present, allows integration of multiple signals from each player. The sliding clamp PCNA is ideal for this function because it is a venue for many enzymes that function to restart and repair arrested forks. Protein-protein interactions are another important component of biological networks. The PIP box and its related PIP degron are an interesting example of protein-protein interaction motifs where duplication and divergent evolution gave rise to distinct cellular functions, allowing one partner to be targeted for degradation short after interaction with PCNA, while the other partner remains stable on the clamp. The widespread distribution of the PIP box among repair factors creates a natural state of competition for interaction with PCNA, which allows signals from different pathways to be integrated. This dynamic network is fine-tuned regulated by proteasomal degradation and modification of PCNA through ubiquitin to allow rapid and adaptable responses to replication stress.
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