

Readers of PCNA modifications

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Abstract The eukaryotic sliding clamp, proliferating cell nuclear antigen (PCNA), acts as a central coordinator of DNA transactions by providing a multivalent interaction surface for factors involved in DNA replication, repair, chromatin dynamics and cell cycle regulation. Posttranslational modifications (PTMs), such as mono- and polyubiquitylation, sumoylation, phosphorylation and acetylation, further expand the repertoire of PCNA's binding partners. These modifications affect PCNA's activity in the bypass of lesions during DNA replication, the regulation of alternative damage processing pathways such as homologous recombination and DNA interstrand cross-link repair, or impact on the stability of PCNA itself. In this review, we summarise our current knowledge about how the PTMs are “read” by downstream effector proteins that mediate the appropriate action. Given the variety of interaction partners responding to PCNA's modified forms, the ensemble of PCNA modifications serves as an instructive model for the study of biological signalling through PTMs in general.

Keywords PCNA · Ubiquitin · SUMO · Phosphorylation · Acetylation · Posttranslational modifications · DNA replication · Genome stability

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Introduction

Posttranslational protein modifications (PTMs) generally modulate the functions of their target proteins by creating, blocking or modifying interaction surfaces. If recognised in an intramolecular manner, the modification leads to a change in the target's conformation. More often, however, PTMs affect the interactions between their targets and other cellular factors that function as effectors by translating the modification into a biological action (Fig. 1). Although some modifiers, such as the phosphate group, may act in part by affecting the surface charge properties of the modified substrate, PTMs are commonly recognised by means of dedicated domains or motifs that bind specifically to the modifier. Such recognition motifs often exhibit low-affinity binding, allowing an effective interaction only in combination with a basal affinity of the effector for the unmodified target protein. In this manner, a high degree of selectivity for the modified substrate is achieved and interactions with other target proteins carrying the same modification are avoided.

The effects of histone modifications on chromatin structure are paradigmatic for how PTMs diversify the properties and interactions of their targets (Kouzarides 2007). In fact, when considering the staggering number of possible nucleosome variants resulting from a combinatorial placement of several different PTMs (phosphorylation, acetylation, methylation, ubiquitylation and sumoylation) onto the histone octamer, the term “histone code” seems an appropriate description of the resulting complexity (Jenuwein and Allis 2001). Research in this area has coined the terms “writers” and “erasers” for the enzymes that attach and remove the modifiers, respectively, and “readers” for the effector proteins that recognise the modified histones and transmit the biological signal.

In this review, we will discuss demonstrated and potential readers of the PTMs of one of the central regulators of DNA

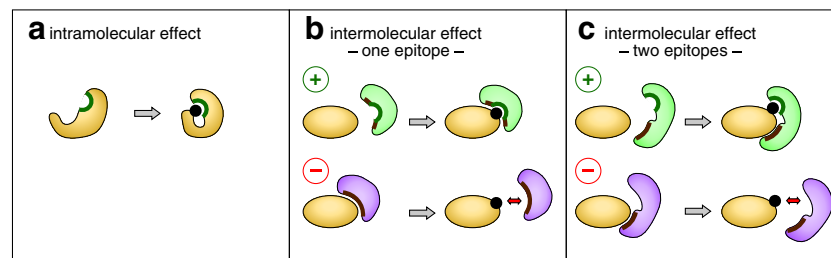


Fig. 1 Posttranslational modifications and their recognition by effector proteins. **a** Intramolecular recognition of the modifier, leading to a conformational change in the target protein. **b** Intermolecular recognition involving a single interaction site. In cases where the PTM enhances binding of the effector (*plus symbol*), the modifier is recognised

in the context of the target protein as a composite epitope. Dissociation (*minus symbol*) is induced by direct influence at the interaction site. **c** Intermolecular recognition involving two interaction sites. Binding is enhanced (*plus symbol*) by bipartite recognition and disrupted (*minus symbol*) by interference at a second site

metabolism, the eukaryotic sliding clamp, proliferating cell nuclear antigen (PCNA). The protein is a homotrimeric, ring-shaped molecule that encircles DNA and acts as a binding platform for the replicative DNA polymerases as well as components of other pathways involved in DNA repair, chromatin dynamics and cell cycle regulation (Kelman 1997). Even in its unmodified form, PCNA interacts with dozens of proteins (Moldovan et al. 2007). Interactions usually involve the so-called interdomain connector loop (IDCL) of PCNA and a motif termed PCNA-interacting peptide (PIP box) on the interaction partner (Warbrick 1998). In higher eukaryotes, a second PCNA-interacting motif, called APIM (AikB homologue 2 PCNA-interacting motif), has also been described, although the region on PCNA to which this motif binds remains to be characterised (Gilljam et al. 2009).

Phosphorylation of PCNA has been reported as early as 1994 (Prosperi et al. 1994), and we now know that PCNA is also subject to ubiquitylation, sumoylation and acetylation (Fig. 2). Over the past few years, tremendous progress has been made in describing the biological consequences of some

of these PTMs and identifying their readers (Table 1, Fig. 3). Here we will briefly describe each modification, review our current understanding of how it is recognised by downstream effector proteins and discuss unresolved questions.

Readers of PCNA ubiquitylation

Since its discovery a decade ago (Hoegge et al. 2002), PCNA ubiquitylation has emerged as a prominent marker for replication problems associated with DNA damage or replication fork stalling (Bergink and Jentsch 2009; Ulrich 2009; Ulrich and Walden 2010). By means of its downstream effectors, ubiquitylated PCNA controls several aspects of damage tolerance, defined as a mechanism that allows the replication machinery to bypass or avoid lesions in the template DNA. The modification affects a highly conserved lysine (K) residue, K164, and is detectable in all eukaryotic species that have been analysed, including budding and fission yeast, mammalian and avian cells, *Xenopus laevis* egg extracts and probably plants (Hoegge et al. 2002; Leach and Michael 2005; Arakawa et al. 2006; Frampton et al. 2006; Anderson et al. 2008). It is

Fig. 2 PCNA modifications and their consequences. Modification sites on PCNA and ubiquitin are indicated according to their approximate location. Ubiquitin: *black balls*, SUMO: *grey balls*, phosphate: *red ball*, acetyl group: *blue ball*. Note that the site(s) of acetylation have not been determined

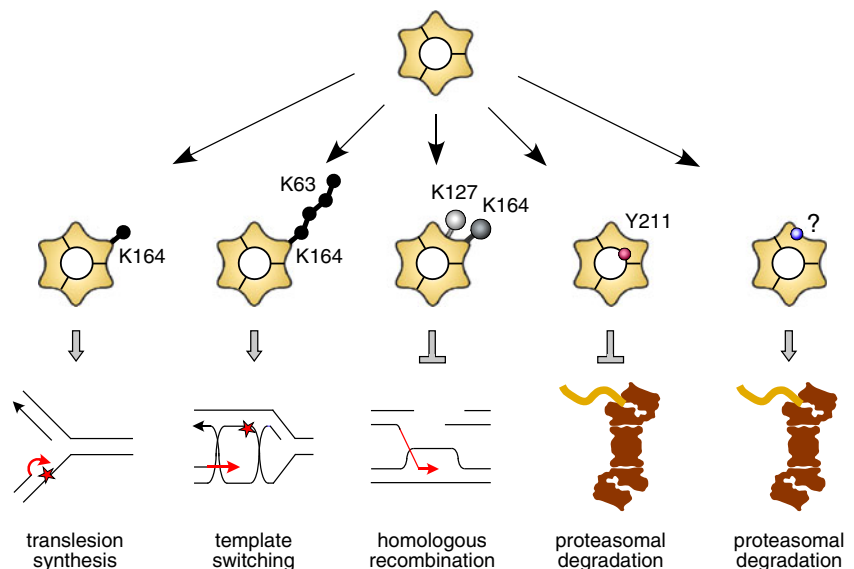


Table 1 PCNA modifications and their effectors

Modification	Effector	Effect	Domains	Pathway	References
Ubiquitylation	Y family polymerases	Binding	PIP UBZ, UBM	DNA damage bypass	Stelter and Ulrich 2003 Bienko et al. 2005 Plosky et al. 2006
	FANCL	Binding (?)	DRWD	ICL repair	Song et al. 2010; Geng et al. 2010; Palle and Vaziri 2011
	SNM1A	Binding	PIP UBZ	ICL repair	Yang et al. 2010
	C1ORF124/Spartan/DVC1	Binding (?)	PIP UBZ	DNA damage bypass	Centore et al. 2012 Davis et al. 2012 Ghosal et al. 2012 Juhasz et al. 2012 Machida et al. 2012 Mosbech et al. 2012
	Mgs1	Binding	UBZ	DNA damage bypass	Saugar et al. 2012
	ZRANB3	Binding	PIP, APIM NZF	DNA damage bypass	Ciccia et al. 2012 Weston et al. 2012
	hELG1	Binding	?	PCNA deubiquitylation	Lee et al. 2010
Sumoylation	Srs2	Binding	PIP-like SIM	Inhibition of recombination	Papouli et al. 2005 Pfander et al. 2005
	PARI	Binding	PIP SIM	Inhibition of recombination	Moldovan et al. 2012
	scElg1	Binding	PIP-like SIM	Genome maintenance	Parnas et al. 2010
	scRad18	Binding	SIM	PCNA ubiquitylation	Parker and Ulrich 2012
	Eco1	Dissociation (?)	PIP-like	Cohesion establishment	Moldovan et al. 2006
Phosphorylation	CRL4 ^{C^{DT2}(?)}	Dissociation	?	PCNA stability	Wang et al. 2006 Lo et al. 2012
Acetylation	MTH2	Dissociation	?	PCNA stability	Yu et al. 2009

mediated by a group of ubiquitin conjugation factors collectively called the *RAD6* pathway (Lawrence 1994). These include its founding member, the ubiquitin-conjugating enzyme (E2) Rad6, which—in complex with the ubiquitin protein ligase (E3) Rad18—monoubiquitylates PCNA and the heterodimeric E2 complex Ubc13-Mms2 (or Ubc13-UEV1 in mammals), which cooperates with the E3 Rad5 (or one of the mammalian homologues, SHPRH and HLTF) in the extension of the monoubiquitin unit to a K63-linked polyubiquitin chain (Hoege et al. 2002). In vertebrates, the deubiquitylating enzyme USP1 removes ubiquitin from PCNA (Huang et al. 2006); the isopeptidase Ubp10 was recently shown to fulfil an analogous function in budding yeast (Gallego-Sanchez et al. 2012). Ubiquitylation is induced by conditions that lead to replication fork stalling (but not collapse) and involve the exposure of single-stranded (ss)DNA. In yeast, Rad18 is rate limiting for both mono- and polyubiquitylation of PCNA (Davies et al. 2008), but the conditions that control the activation of Ubc13-Mms2 and Rad5 or determine the balance between the two modifications have not been determined.

Damage-tolerant DNA polymerases

A straightforward mechanism to process lesions during DNA replication is the use of specialised, damage-tolerant DNA polymerases that can accommodate non-native templates in their active sites. This reaction, named translesion synthesis (TLS), allows replication to be completed in the presence of damage, but is at the same time a predominant source of damage-induced mutations, generated as a consequence of the low fidelity of these polymerases on damaged as well as undamaged templates (Pages and Fuchs 2002; Lehmann et al. 2007). Ubiquitylation was first implicated in the activation of TLS polymerases by yeast genetics, when a gene encoding one of these enzymes was cloned and identified as a member of the *RAD6* pathway (McDonald et al. 1997). When PCNA was found to be ubiquitylated in response to DNA damage (Hoege et al. 2002), genetic experiments in budding yeast again provided the first evidence that mono-, but not polyubiquitylation of PCNA was required for TLS and damage-induced mutagenesis (Stelter and Ulrich 2003).

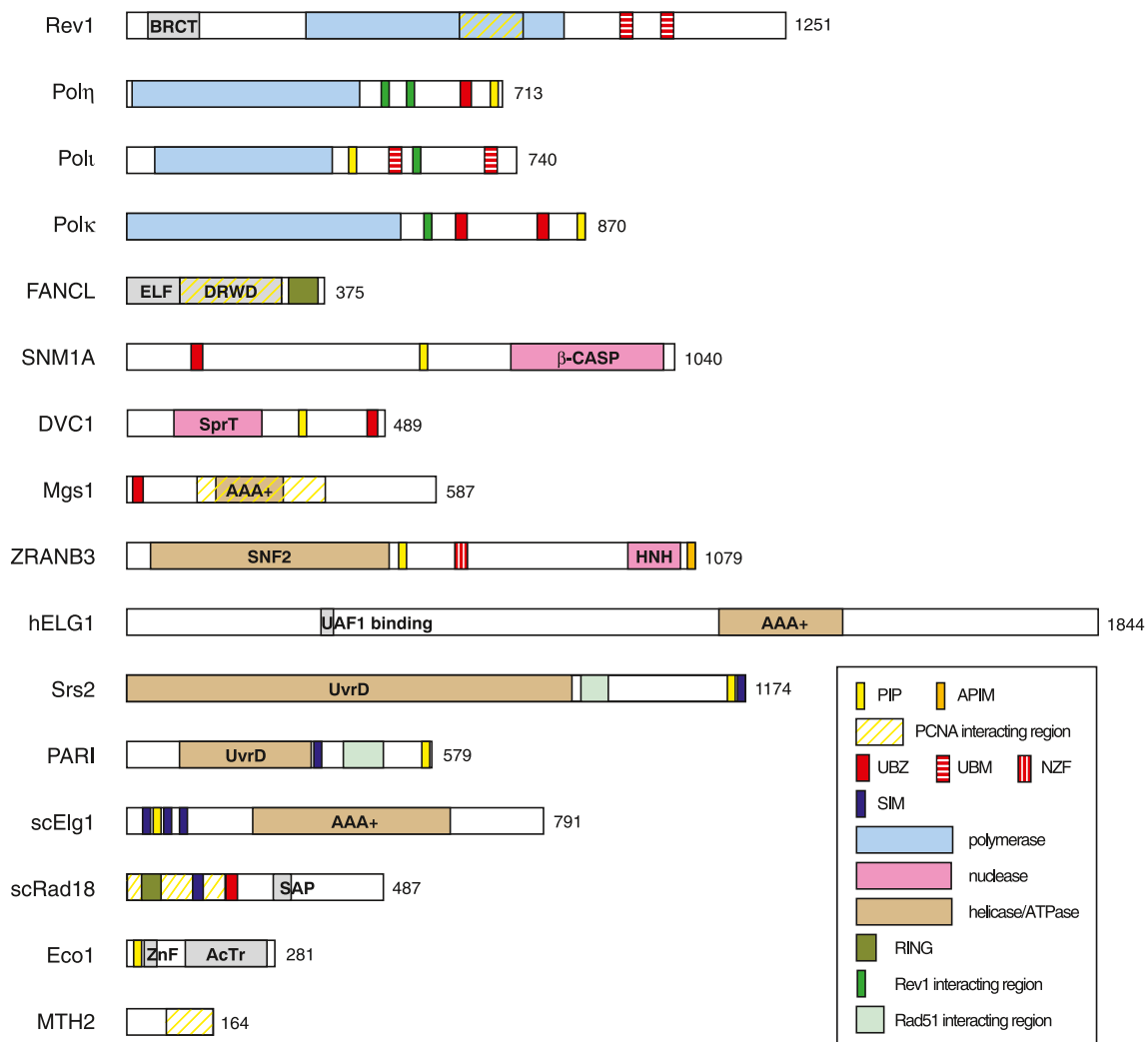


Fig. 3 Readers of PCNA modifications. Domain structures of downstream effectors of modified PCNA are indicated schematically, according to Table 1. Note that CRL4 is omitted from this figure, as the details of its interaction with PCNA have not been elucidated

The molecular basis for this requirement was subsequently elucidated with the discovery of ubiquitin-binding domains (UBDs) in a subset of damage-tolerant DNA polymerases (Kannouche et al. 2004; Watanabe et al. 2004; Bienko et al. 2005; Plosky et al. 2006).

Based on phylogenetic relationships, DNA polymerases have been classified into several families. Among them, the members of the Y family are characterised by their low fidelity and their ability to bypass DNA lesions (Ohmori et al. 2001). In budding yeast, there are two members, polymerase η (Pol η) and Rev1. Mammalian cells additionally encode polymerases ι (Pol ι) and κ (Pol κ). All Y family polymerases interact with PCNA: whereas Pol η , Pol ι and Pol κ contain classical PIP boxes, Rev1 interacts with PCNA through an alternative motif (Sharma et al. 2011). In addition, one or two UBDs were identified in all eukaryotic members of this family (Bienko et al. 2005; Plosky et al. 2006). They serve as prototypes for two distinct classes: the

ubiquitin-binding zinc finger (UBZ) and the helical ubiquitin-binding motif (UBM). Mutation of conserved residues within these domains abolishes TLS and damage-induced mutagenesis in yeast and prevents damage-induced association of the mutant polymerases with PCNA in mammalian cells (Bienko et al. 2005; Guo et al. 2006; Parker et al. 2007). A similar defect in TLS is observed when PCNA ubiquitylation is prevented by mutation of K164 or depletion of Rad18. In vitro, the modified form of PCNA was shown to activate Pol η - and Rev1-dependent lesion bypass (Garg and Burgers 2005), and in mammalian cell extracts, monoubiquitylation likewise promoted the exchange of the replicative polymerase δ (Pol δ) for a TLS polymerase during the replication of UV-damaged DNA (Zhuang et al. 2008; Masuda et al. 2010). Hence, PCNA ubiquitylation activates TLS by selectively enhancing the affinity of the damage-tolerant polymerases and thus recruiting them to their sites of action.

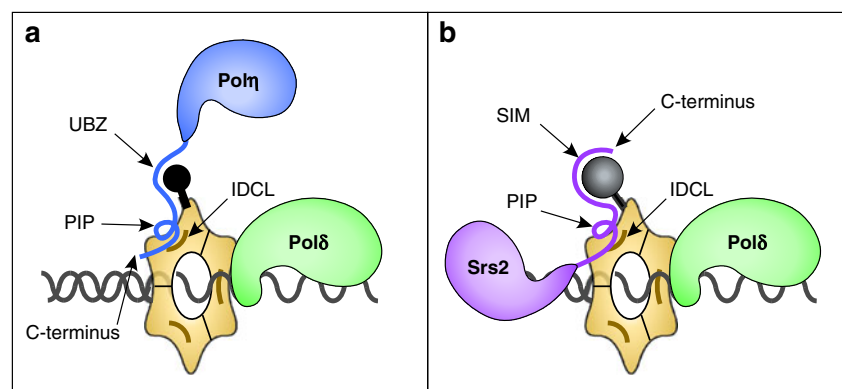
Structural information is available for the catalytic core of Pol η , its PIP box bound to PCNA, its UBZ domain in complex with ubiquitin and a recombinant construct mimicking the ubiquitylated form of PCNA (Trincao et al. 2001; Bomar et al. 2007; Hishiki et al. 2009; Freudenthal et al. 2010). This has allowed the modelling of a complex between Pol η and the ubiquitylated clamp (Freudenthal et al. 2010), in which the concept of how the polymerase acts as a reader of modified PCNA is nicely illustrated (Fig. 4a): the long, flexible C terminus of Pol η , harbouring the UBZ and PIP motifs in close proximity, is able to reach over the surface of PCNA such that the two domains simultaneously contact the ubiquitin moiety and the IDCL of PCNA. This leaves the catalytic core free to either reside at the side or back face of the clamp or to swing around and occupy the primer terminus, thus potentially replacing a stalled replicative polymerase at this position.

Although the crystal structure of the PCNA-ubiquitin mimic revealed two preferred positions of ubiquitin on the surface of PCNA that occlude the interaction site of ubiquitin with the TLS polymerases, biophysical data obtained with a native, enzymatically generated conjugate in solution are more consistent with an open and flexible conformation where the ubiquitin moiety is highly mobile and therefore freely available for interactions (Hibbert and Sixma 2012). Furthermore, the notion that ubiquitylation of PCNA does not interfere with replicative functions such as interactions with Pol δ , ligase I and flap endonuclease (Garg and Burgers 2005) lends support to an expanded “toolbelt” model (Pages and Fuchs 2002) where ubiquitin primarily acts as an interaction module to augment the scope of PCNA’s binding partners without affecting its overall conformation. This model is also consistent with the observation that the attachment site of ubiquitin on PCNA is apparently not crucial for activity, such that an in-frame fusion of ubiquitin to the amino terminus of PCNA supports TLS in the absence of endogenous PCNA ubiquitylation (Parker et al. 2007; Ramasubramanian et al. 2010; Zhao and Ulrich 2010).

While the basic mechanism of ubiquitin-dependent TLS is now well accepted, several controversial issues remain. Why

do some TLS polymerases, such as Pol ι and Rev1, harbour two UBDs, whereas one is sufficient in Pol η and Pol κ ? In mammalian Pol ι and Rev1, both UBM domains contribute to ubiquitin binding (Bienko et al. 2005; Plosky et al. 2006). In contrast, one of the UBMs of budding yeast Rev1 was found to be dispensable for function (Guo et al. 2006), and the isolated domain apparently does not even interact with ubiquitin (Bomar et al. 2010). This indicates that a single UBM can be sufficient for TLS. The relative importance of ubiquitin versus PCNA binding has also been debated. On the one hand, mutation of the PIP box in human Pol η results in a rather mild damage sensitivity (Bienko et al. 2005; Gueranger et al. 2008), suggesting either the existence of a secondary PCNA binding site (Acharya et al. 2008) or a predominance of the UBZ–ubiquitin interaction in the recruitment. On the other hand, another study found that PIP- and UBZ motifs need to cooperate for efficient TLS by Pol η (Bienko et al. 2010). Finally, a number of reports have postulated that ubiquitin binding by Pol η in both yeast and human cells is dispensable for TLS (Acharya et al. 2007; Acharya et al. 2008; Acharya et al. 2010). These seemingly contradictory results may have arisen from the specific experimental conditions that were used in the latter studies: in order to inactivate the UBZ domain of yeast Pol η , mutations were introduced into the zinc-coordinating residues, assuming that they would disrupt ubiquitin binding. As it was later shown, this was a misconception, as the somewhat unusual UBZ domain of budding yeast Pol η does not actually require zinc binding for structural integrity (Woodruff et al. 2010). In the human system, even a full deletion of the UBZ domain did not prevent recruitment of the mutant protein into UV-induced foci; however, in this case PCNA levels were artificially elevated by overexpression, which resulted in an enforced recruitment of the TLS polymerase even independent of the UBZ domain (Sabbioneda et al. 2009). Despite these criticisms, there is good evidence that the Y family polymerases are not entirely ubiquitin-dependent in vertebrates: in chicken DT40 cells, Rev1 activity requires PCNA ubiquitylation for processing of postreplicative gaps, but not when operating in a fork-associated mode (Edmunds et al. 2008). In mouse

Fig. 4 The expanded toolbelt model of PCNA interactors, illustrated by the recruitment of effector proteins to modified PCNA via tandem recognition motifs. **a** Pol η interacts with ubiquitylated PCNA via juxtaposed UBZ and PIP motifs. **b** Srs2 interacts with sumoylated PCNA via juxtaposed PIP and SIM motifs



embryonic fibroblasts, both Pol η and Rev1 were found to exhibit measurable, albeit weak, activity even in a mutant devoid of PCNA ubiquitylation (Hendel et al. 2011). Hence, the contributions of the UBDs to the recruitment of the TLS polymerases probably do not follow an all-or-nothing scenario, but rather provide a significant enhancement of an otherwise inefficient process. Considering the intricate mutual interactions among the Y family polymerases and their affinities for native PCNA and DNA, this is not all that surprising.

Fanconi anemia proteins

Damage-tolerant polymerases of the Y family perform the bulk of TLS across a variety of DNA lesions, including adducts affecting both strands of the DNA, called DNA interstrand cross-links (ICLs). These can serve as templates for TLS after their release from one of the two strands via nucleolytic incisions. Although there is evidence that replication-associated ICL repair can be mediated by TLS independently of PCNA monoubiquitylation (Hicks et al. 2010), the modification has been shown to contribute to a replication-independent pathway of ICL repair in yeast and chicken cells via recruitment of Rev1. In this reaction, which in yeast is observable in haploid G1 cells, Rev1 in cooperation with Pol ζ is thought to mediate the filling of gaps resulting from excision of the lesion from one of the strands (Sarkar et al. 2006; Shen et al. 2006). In *X. laevis* egg extracts, this activity appears to be taken over by Polk, recruited by means of its UBZ domain (Williams et al. 2012). An additional pathway of ICL repair has evolved in metazoans, and intriguingly, this system, the Fanconi anemia (FA) pathway, also appears to be influenced by PCNA monoubiquitylation.

The FA pathway is a DNA repair system that responds to replication fork problems in general, but its action becomes most obvious in the resolution of ICLs, where it coordinates several DNA transactions, such as TLS, homologous recombination and nucleotide excision repair (Ulrich and Walden 2010; Kim and D'Andrea 2012). Central to its regulation is the monoubiquitylation of the chromatin-associated proteins FANCD2 and FANCI by a multisubunit E3, the FA core complex. FANCL, its catalytic subunit, is capable of monoubiquitylating FANCD2/I in the absence of the other subunits *in vitro* when supplemented with the cognate E2, UBE2T (Alpi et al. 2008). Ubiquitylated FANCD2/I activate their own set of downstream effectors, which include UBD-containing proteins (Garner and Smogorzewska 2011). Intriguingly, several studies have recently implicated the PCNA-specific E3, Rad18, in the FA pathway (Zhang et al. 2008; Geng et al. 2010; Park et al. 2010; Song et al. 2010; Palle and Vaziri 2011; Williams et al. 2011). There is good agreement that inactivation of Rad18 prevents full FANCD2 ubiquitylation and recruitment to the chromatin (Song et al. 2010; Palle and Vaziri 2011). According to one report,

replacement of endogenous PCNA with a non-ubiquitylatable mutant caused the same defect (Geng et al. 2010; Song et al. 2010) observed a spontaneous recruitment of FANCA and FANCD2 to chromatin upon expression of a linear fusion of ubiquitin to PCNA, strongly implying that Rad18 mediates its effect on the FA pathway via ubiquitylation of PCNA. An even more direct impact was demonstrated by (Geng et al. 2010) in an *in vitro* ubiquitylation assay, where the presence of ubiquitylated PCNA stimulated the catalytic activity of FANCL towards FANCD2/I. As FANCL directly interacts with PCNA via its central DRWD domain (Geng et al. 2010), these data suggest that FANCL is a genuine reader of ubiquitylated PCNA that gains its activity by recognition of the modification. Whether FANCL harbours a UBD that would serve this purpose is currently unknown. Interestingly, FANCD2 itself contains a PIP box (Howlett et al. 2009), suggesting that PCNA may serve as an interaction platform that coordinates activation of the FA pathway.

Although attractive, this model is not undisputed, as conflicting results were obtained by other laboratories: Williams et al. (2011) reported that depletion of Rad18, but not abolition of PCNA ubiquitylation prevented efficient FANCD2/I ubiquitylation, suggesting a PCNA-independent function of Rad18 in the FA pathway. Likewise, Palle and Vaziri (2011) observed that treatment with camptothecin, a topoisomerase poison that causes replication fork collapse, triggered FANCD2 ubiquitylation without causing PCNA ubiquitylation, again implying that the two pathways act independently and in parallel. Whether factors such as insufficient depletion of native PCNA or the use of different genotoxic agents are responsible for these discrepancies remains to be determined.

SNM1A/Pso2

Another potential reader of ubiquitylated PCNA that participates in ICL repair is the nuclease SNM1A, or its yeast homolog Pso2/Snm1, which was first identified in screens for mutants sensitive to cross-linking agents (Brendel et al. 2003). Pso2 acts downstream of the incision step and may participate in the initial processing of the excised region surrounding the cross-link in order to facilitate subsequent TLS or homologous recombination (Cattell et al. 2010). Human SNM1A contains a canonical PIP box and interacts with PCNA (Yang et al. 2010), and both human and yeast proteins harbour a UBZ domain (Hofmann 2009; Yang et al. 2010) have demonstrated a UBZ-dependent enhanced affinity of SNM1A for a ubiquitin–PCNA fusion compared to native PCNA, and the protein localises to damage-induced foci in a PIP-, UBZ- and Rad18-dependent manner, thus strongly suggesting that its recruitment is directly mediated by ubiquitylated PCNA. Although strictly speaking this model has yet to be proved, it is consistent with the observation that

in yeast ICL-induced PCNA ubiquitylation in haploid G1 cells is observed at the step when Pso2 is acting, following the initial, nucleotide excision repair-mediated incision step (Sarkar et al. 2006).

C1orf124/DVC1/Spartan

Based on the candidates described above, the presence of a PIP box in combination with a UBZ motif appears to be a strong indicator of a function downstream of ubiquitylated PCNA. As a consequence, the previously uncharacterised human protein C1orf124 has recently attracted the attention of several laboratories (Centore et al. 2012; Davis et al. 2012; Ghosal et al. 2012; Juhasz et al. 2012; Machida et al. 2012; Mosbech et al. 2012). The protein, subsequently named Spartan (for SprT-like domain at the N terminus) or DVC1 (for DNA damage-targeting VCP adaptor C1orf124), contains an N-terminal SprT-like domain, related to a family of proteases, followed by an SHP box that mediates interaction with the ubiquitin-dependent chaperone p97 (or VCP), a canonical PIP box and a C-terminal UBZ domain. While there is general agreement between the studies that C1orf124 is recruited to sites of replication problems in a UBZ- and PIP-dependent manner and protects cells from DNA damage-induced mutagenesis, the mechanistic basis for its function in the damage response remains controversial.

A number of researchers reported a preferential interaction of C1orf124 with ubiquitylated PCNA (Centore et al. 2012; Ghosal et al. 2012; Juhasz et al. 2012) and observed that its recruitment to damage-induced foci was dependent on Rad18 (Centore et al. 2012; Juhasz et al. 2012; Machida et al. 2012) or even K164 of PCNA (Machida et al. 2012). Depletion of the protein reduced the amount of ubiquitylated PCNA and chromatin-associated Rad18 and Pol η (Centore et al. 2012; Ghosal et al. 2012), which was interpreted either as a function in a positive feed-forward regulation of the modification itself (Centore et al. 2012) or as a protection from USP1-mediated PCNA deubiquitylation (Juhasz et al. 2012; Ghosal et al. 2012) speculated that C1orf124 might exert a protective effect on PCNA ubiquitylation by sequestering Rad18 and/or ubiquitylated PCNA away from the segregase activity of p97. Common to all these models is a genuine function of C1orf124 as an interactor and reader of ubiquitylated PCNA and a positive contribution to TLS.

However, recent reports by Mosbech et al. (2012) and Davis et al. (2012) have challenged this viewpoint. Although they also observed a requirement of both PIP and UBZ domains for efficient localisation of C1orf124 to foci, this recruitment was found to be independent of Rad18, thus arguing against a selective recognition of ubiquitylated PCNA and instead suggesting a UBZ-mediated binding to other, yet unidentified ubiquitin conjugates at stalled replication forks. Furthermore, and in contrast to the model described above,

depletion of C1orf124 enhanced the association of the Y family Pol η with chromatin, implicating the protein in the downregulation of TLS rather than its activation (Davis et al. 2012; Mosbech et al. 2012). In this scenario, the segregase activity of p97 could possibly mediate the extraction or even degradation of PCNA-associated Pol η . In conclusion, while a contribution of C1orf124 to the regulation of TLS appears likely, neither its mechanism nor its net effect has been rigorously demonstrated. The lack of a yeast homologue complicates a genetic analysis that might otherwise have clarified the protein's mechanism of action. Moreover, the relatively unspecific affinity of the UBZ domain for K48-, K63- and linear polyubiquitin chains (Centore et al. 2012; Davis et al. 2012; Mosbech et al. 2012) does not allow definitive conclusions about its relevant modified targets at this time. Hence, future studies will have to elaborate whether or not C1orf124 is a reader of ubiquitylated PCNA.

Mgs1/WRNIP1

Compared to our relatively detailed understanding of TLS activation by monoubiquitylated PCNA, hardly anything is known about the consequences of PCNA polyubiquitylation. Genetic evidence in yeast has implicated this modification in an error-free pathway of damage avoidance that likely involves template switching, but the mechanistic aspects of the reaction are unexplored (Hoege et al. 2002; Zhang and Lawrence 2005). Considering that the K63-linked polyubiquitin chains do not trigger proteasomal degradation of PCNA (Zhao and Ulrich 2010), identification of proteins that recognise the polyubiquitylated form of PCNA might prove crucial to unravel the mechanism of error-free damage bypass. One such candidate is the AAA+ATPase Mgs1 from budding yeast. The protein contributes to genome stability in multiple ways, and both deletion and overexpression of the gene affects recombination and mutation rates (Hishida et al. 2001; Branzei et al. 2002; Hishida et al. 2002). Although no obvious PIP box has been identified, Mgs1 interacts with PCNA (Hishida et al. 2006), and it harbours an N-terminal UBZ domain that selectively enhances its affinity for ubiquitin-PCNA fusions in vivo and in vitro, with a marked preference for longer chains (Saugar et al. 2012). Genetic analysis has confirmed that the UBZ domain is functionally linked to ubiquitylated PCNA and contributes to the recruitment of Mgs1 to chromatin when PCNA is ubiquitylated in response to replication fork stalling (Saugar et al. 2012). Consistent with these data, the mammalian homologue, WRNIP1 (Werner helicase-interacting protein 1), is recruited to RPA-associated nuclear foci in a UBZ-dependent manner (Crosetto et al. 2008). These observations clearly identify Mgs1 as a reader of ubiquitylated PCNA.

Yet, a number of important issues remain unresolved. First, we still do not understand Mgs1's function in DNA damage

bypass. The protein exhibits DNA-dependent ATPase and strand annealing activities (Hishida et al. 2001) and it appears to exert a negative impact on the PCNA–Pol δ interaction (Branzei et al. 2002; Vjeh Motlagh et al. 2006; Saugar et al. 2012), but it is unknown which of these activities is relevant for damage bypass. Second, the genetic relationship between the UBZ domain and PCNA is a composite of mono- and polyubiquitin-dependent functions, and its affinity for ubiquitylated PCNA is not limited to the K63-linked polyubiquitylated form, but extends to monoubiquitin and linear, i.e. head-to-tail-linked polyubiquitin chains, which are inactive in the error-free pathway of damage avoidance (Zhao and Ulrich 2010; Saugar et al. 2012). Mgs1 is therefore likely to affect both TLS and template switching. Finally, a number of phenotypes of *mgs1* mutants are independent of the UBZ domain, such as accelerated ageing, elevated spontaneous mutation rates and a synthetic lethality with *rad6* and *rad18*. In summary, Mgs1 clearly acts as a downstream effector of mono- and polyubiquitylated PCNA, but neither is its function exclusively mediated by ubiquitylated PCNA, nor is it likely the only factor with an influence on error-free template switching.

ZRANB3/AH2

Preferential interaction with polyubiquitylated PCNA was recently reported for another ATPase, ZRANB3 (zinc finger, Ran-binding domain containing 3), also called AH2 (annealing helicase 2) (Ciccina et al. 2012). The protein harbours a helicase domain of the SNF2 family in combination with an HNH endonuclease motif (Flaus et al. 2006). Biochemical analysis has identified ATP-driven DNA rewinding, fork regression and D-loop dismantling activities as well as an ATP-dependent, structure-specific endonuclease activity (Yusufzai and Kadonaga 2010; Ciccina et al. 2012; Weston et al. 2012). Interaction with PCNA was found to be mediated by a canonical PIP box and an APIM motif in a partially redundant manner (Ciccina et al. 2012). Consistent with a function in resolving replication problems, interaction with PCNA mediates ZRANB3's localisation to laser-induced DNA damage foci in vivo, and depletion of the protein results in a mild sensitivity to replication stress, an accumulation of sister chromatid exchanges and a defect in replication fork restart after DNA damage (Ciccina et al. 2012; Weston et al. 2012; Yuan et al. 2012). ZRANB3 also contains a UBD of the NZF (Npl4 zinc finger) type, which binds to polyubiquitin chains, but not monoubiquitin, displays a preference for K63- over K48-linked chains, mediates the preferential binding to polyubiquitylated PCNA and contributes to its damage-induced localisation (Ciccina et al. 2012; Weston et al. 2012). Notably, ZRANB3 foci colocalise with WRNIP1, and WRNIP1 can be found in ZRANB3 immunoprecipitates (Ciccina et al. 2012).

Taken together, these data strongly suggest a function of ZRANB3 downstream of polyubiquitylated PCNA. In support of this model, retention of the protein in foci was found to be reduced upon depletion of RAD18 or UBC13 (Ciccina et al. 2012). A rigorous genetic analysis that takes into account the functions of UBC13 and the Rad5 homologs HLTf and SHPRH will be necessary in order to verify whether ZRANB3 is indeed a mediator of error-free damage avoidance, and if so, by what mechanism it influences template switching. Meanwhile, a better characterisation of the NZF domain may provide further insight. In contrast to the UBZ domain of Mgs1/WRNIP1, which is not per se polyubiquitin or linkage specific and may attain a preference for polymeric chains mainly by means of oligomerisation, NZF domains often exhibit genuine specificity for polyubiquitin. This is due to a two-sided binding mode, where two hydrophobic patches within the domain engage in simultaneous interactions with two molecules of ubiquitin (Kulathu et al. 2009; Sato et al. 2009). In this manner, the closely related NZF domain from TAB2 gains exquisite selectivity for the K63-linkage.

Surprisingly, no convincing homolog of ZRANB3 has been detected in lower eukaryotes. Considering that the ubiquitin conjugation factors operating on PCNA are highly conserved from yeast to humans, one would not expect the downstream events to have completely diverged. Hence, the question of how polyubiquitylated PCNA induces error-free damage bypass still remains unresolved.

hELG1

An interesting mechanistic facet of DNA damage bypass concerns the regulation of PCNA deubiquitylation, which in human cells is mediated by the isopeptidase USP1 in complex with its co-factor, UAF1 (Cohn et al. 2007). In a screen for USP1 interaction partners, the RFC1-like protein ELG1 was recently identified (Lee et al. 2010). Whereas RFC1 itself—in complex with the small subunits RFC2, RFC3, RFC4 and RFC5 called replication factor C—is responsible for loading PCNA onto the DNA during replication, ELG1 functions as the large subunit of an alternative clamp loader complex and has been broadly implicated in the maintenance of genome stability (Majka and Burgers 2004; Aroya and Kupiec 2005). ELG1 forms a complex with the small RFC subunits and physically interacts with PCNA (Kanellis et al. 2003), and Lee et al. (2010) observed that human ELG1 co-localised with PCNA at sites of replication fork stalling. Depletion and overexpression experiments demonstrated a negative effect of ELG1 on PCNA ubiquitylation, which depended on the presence of USP1. This effect was found to be mediated by a SUMO interaction motif (SIM) in the N terminus of ELG1 that was responsible for interaction with a SUMO-like domain in UAF1 but did not require the small RFC subunits,

suggesting that in this context ELG1 promotes PCNA deubiquitylation by serving as a structural link between USP1-UAF1 and PCNA rather than acting catalytically as a clamp loader (Yang et al. 2011). Alternatively, ELG1 could promote genome stability by recruiting USP1-UAF1 for deubiquitylation of a different substrate, and failure to do so could indirectly cause enhanced PCNA ubiquitylation due to the accumulation of replication problems. While this scenario has not formally been excluded, a direct recruitment of USP1-UAF1 to PCNA by ELG1 appears more likely. However, whether ELG1 actually selectively recognises the ubiquitylated form of PCNA, or how else it might be directed towards the relevant pool of PCNA is unknown. Hence, at this point it remains to be determined whether human ELG1 is a genuine reader of ubiquitylated PCNA or merely an adaptor for an eraser. Meanwhile, two recent reports have provided good evidence that in cooperation with the small RFC subunits ELG1 acts as a PCNA unloader (Kubota et al. 2013; Lee et al. 2013). However, this activity is unrelated to ELG1's effects on PCNA ubiquitylation, and while the unloading function has turned out to be highly conserved, the impact of ELG1 on PCNA modifications appears to have significantly diverged from yeast to humans (see below).

Readers of PCNA sumoylation

SUMO modification of PCNA was first observed in budding yeast as a damage-independent modification affecting predominantly K164 and to a minor extent a second site, K127 (Hoege et al. 2002). Conjugation is mediated by the sole SUMO-specific E2, Ubc9. At K164, this requires the SUMO E3 Siz1, whereas K127 sumoylation proceeds without an E3 in vitro and is mediated by Siz2 in vivo (Hoege et al. 2002; Parker et al. 2008). Similar to ubiquitylation, the modification is strongly enhanced by loading of PCNA onto DNA (Parker et al. 2008). In vivo, the residence on DNA appears to be both necessary and sufficient for PCNA to be sumoylated, which results in a replication-associated modification pattern (Hoege et al. 2002; Parker et al. 2008). The isopeptidase Ulp1 is responsible for deconjugation (Stelter and Ulrich 2003). Although sumoylation at K164 has been observed in several other species or model systems, such as chicken DT40 cells, *X. laevis* egg extracts and recently mammalian cells, the modification appears to be much less abundant than in budding yeast, and not much is known about its regulation (Leach and Michael 2005; Arakawa et al. 2006; Gohler et al. 2008; Gali et al. 2012; Moldovan et al. 2012).

Srs2

Preventing PCNA sumoylation suppresses the damage sensitivity of budding yeast mutants defective in damage bypass. This was originally interpreted as an antagonistic relationship

between SUMO and ubiquitin (Hoege et al. 2002). However, the effect was only observable in mutants unable to ubiquitylate PCNA, and it required the presence of homologous recombination factors. Taken together, these properties were reminiscent of mutants isolated previously as *srs2* (suppressor of *rad6*) (Papouli et al. 2005; Pfander et al. 2005). *SRS2* encodes a helicase that contributes to DNA double-strand break repair, replication and damage signalling (Marini and Krejci 2010). It acts mostly by eliminating toxic recombination intermediates, via disrupting or preventing the formation of presynaptic Rad51 filaments. Genetic epistasis between *srs2* mutants and loss of PCNA sumoylation indicated a function in the same pathway, and *Srs2* was indeed found to preferentially interact with the sumoylated forms of PCNA (Papouli et al. 2005; Pfander et al. 2005). Consistent with an effector function, association of *Srs2* with replication intermediates positively correlated with the extent of PCNA sumoylation, while a negative correlation was found with Rad51 (Papouli et al. 2005). At the same time, loss of PCNA sumoylation resulted in an enhancement of spontaneous mitotic cross-overs and intrachromosomal recombination, suggesting that *Srs2*, recruited via interaction with sumoylated PCNA, exerts its antirecombinogenic action even during undisturbed replication (Pfander et al. 2005; Robert et al. 2006). Its effect on damage bypass can be viewed as a precautionary measure that prevents damage processing by homologous recombination, thereby allowing the ubiquitin-dependent pathway to proceed.

Preferential interaction of *Srs2* with sumoylated PCNA is achieved by means of a SIM in the very C terminus of the protein such that deletion of this sequence selectively abolishes those functions of *Srs2* relating to its action at replication forks (Le Breton et al. 2008). The molecular basis for this interaction is illustrated by an X-ray structure of PCNA sumoylated at K164 in complex with a C-terminal fragment of *Srs2* (Armstrong et al. 2012). In this structure, recognition of sumoylated PCNA is achieved by two adjacent, but separate interaction motifs for PCNA and for SUMO, respectively (Fig. 4b). Interaction with PCNA is mediated by a PIP-like motif that differs somewhat from the canonical version in terms of sequence and conformation, whereas the SIM–SUMO interaction closely resembles that of other known examples. Intriguingly, the distance between the SIM and PIP-like motifs in the structure is incompatible with their originating from the same molecule of *Srs2*, which suggests that two *Srs2* fragments are cross-bridging two molecules of sumoylated PCNA in the crystal. However, molecular modelling indicates that a single *Srs2* can interact simultaneously with PCNA and SUMO (either on K164 or on K127) if the SUMO moiety on PCNA is allowed to adopt a different conformation.

The genetic and biochemical evidence identifies *Srs2* as an important reader of sumoylated PCNA. However, this function

appears to be specific to budding yeast: although Srs2 homologues can be identified in other fungi, such as *Schizosaccharomyces pombe*, a C-terminal SIM is conserved only in a small group of budding yeasts related to *Saccharomyces cerevisiae* (Fig. 5). Consistently, deletion of *S. pombe* *SRS2* does not suppress the damage sensitivity of mutants defective in PCNA ubiquitylation (Frampton et al. 2006).

PARI

Higher eukaryotes appear to lack a genuine Srs2 homologue altogether, although several unrelated helicases has been identified in vertebrates that may cover various aspects of Srs2's antirecombinogenic function (Krejci et al. 2012). PARI is a helicase of the UvrD family, thus distantly related to Srs2. It can remove Rad51 from single-stranded DNA when present in stoichiometric amounts, and depletion of the protein causes an increase in mitomycin C-induced chromosome aberrations, hypersensitivity to the drug and elevated levels of spontaneous and damage-induced homologous recombination (Moldovan et al. 2012). Based on the identification of a SIM and a PIP box, which mediate a preferential interaction with PCNA–SUMO fusions in vitro, the protein has been proposed to act as a vertebrate version of Srs2. Function of PARI in vivo requires both motifs (Moldovan et al. 2012), and a permanent fusion of SUMO1 to the C terminus of PCNA inhibits homologous recombination (Gali et al. 2012), suggesting that PARI may indeed act downstream of PCNA in an Srs2-like manner. However, PCNA sumoylation occurs at very low levels in mammalian cells, as detection requires overexpression of an epitope-tagged SUMO allele (Gali et al. 2012; Moldovan et al.

2012). This is not entirely consistent with a function in protection of replication forks from hyperrecombination and stands in contrast to the yeast system, where a significant portion of PCNA is sumoylated during S phase (Parker et al. 2008). Not much is known about the regulation of PCNA sumoylation in humans, and further work will clearly be necessary to confirm a genetic link to the function of PARI.

scElg1

Similar to the effect of *srs2* on damage bypass mutants, deletion of budding yeast *ELG1* has been reported to suppress the sensitivity of mutants deficient in PCNA polyubiquitylation (Parnas et al. 2010). As with *SRS2*, the toxic effect of Elg1 activity in the absence of ubiquitylated PCNA was dependent on Siz1 and the capacity of PCNA to be sumoylated. Consistent with these observations, Parnas et al. (Parnas et al. 2010) found a preferential interaction of Elg1 with sumoylated PCNA, mediated by three SIMs and a motif distantly related to the PIP box in the N-terminal domain of Elg1. Mutation of the SIMs or PIP-like motifs individually had little effect on Elg1 function; however, deletion of both SIMs and PIP in combination resulted in a substantial damage sensitivity.

How Elg1 acts downstream of sumoylated PCNA remains to be determined. Although unloading activity was recently demonstrated, this was independent of PCNA sumoylation (Kubota et al. 2013). A synergistic effect of *elg1* with *srs2* indicates that the two proteins do not act in the same pathway, even though both respond to SUMO. Notably, deletion of *ELG1* causes an accumulation of PCNA in its sumoylated form on chromatin, suggesting that the protein might act as

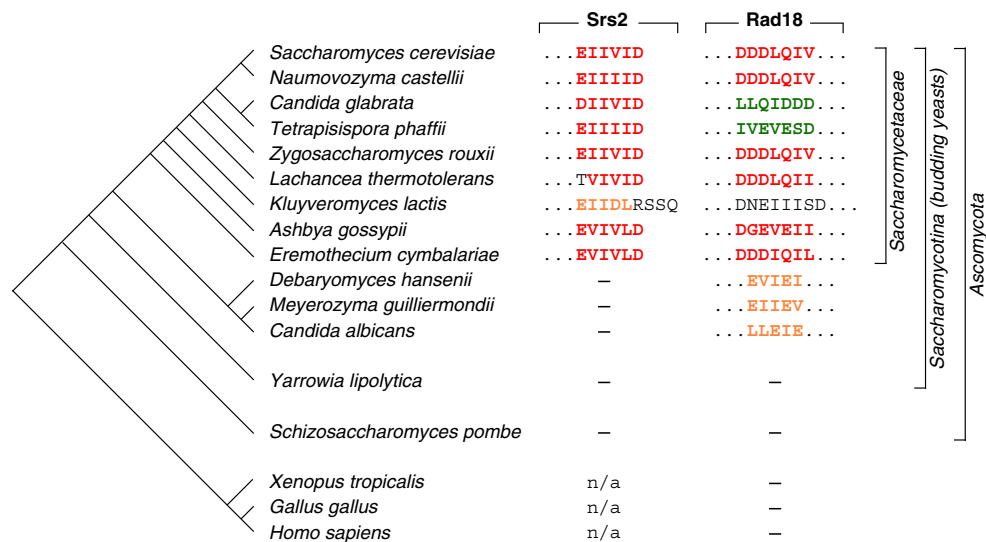


Fig. 5 Conservation of SIMs in Srs2 and Rad18. A phylogenetic tree of yeasts and selected vertebrates indicates the presence or absence of SIMs. Red high-probability SIM, based on position and homology to the *S. cerevisiae* genes. Green high-probability SIM in reverse orientation. Orange putative SIM, possibly at a different location. Note that the C-

terminal carboxylate in Srs2 from *S. cerevisiae* is engaged in the interaction with SUMO (Armstrong et al. 2012), making it unlikely that the motif in *K. lactis* Srs2 acts as a SIM. The phylogenetic tree (not to scale) was adapted from Dujon (2010) and Kurtzman and Robnett (2003). A detailed sequence alignment is provided in supplementary Figure S1

an unloader for sumoylated PCNA (Parnas et al. 2010). However, biochemical evidence for this activity is lacking and alternative models have not been excluded. The observation that mutation of PIP and SIM motifs results in a phenotype weaker than that of an *elg1* deletion implies that the protein has additional functions unrelated to sumoylated PCNA. Moreover, Elg1's SIMs might not be exclusively dedicated to PCNA-bound SUMO, as a two-hybrid screen revealed interactions with other sumoylated and SIM-containing proteins, mediated by poly-SUMO chains (Parnas et al. 2011).

Rad18

The notion that PCNA sumoylation is targeted predominantly toward the same lysine as ubiquitylation raises the question of how the transition between the replication-associated sumoylated form of PCNA to the damage-induced ubiquitylated form is accomplished. Biochemical analysis of the E3 Rad18 from budding yeast has now revealed an interesting cross-talk between the two modifiers that suggests a relevant mechanism (Parker and Ulrich 2012). Through a canonical SIM, Rad18's activity towards PCNA is strongly boosted by the presence of SUMO on the clamp. Interestingly, SUMO can either reside on K164 of one of the subunits or be appended as a fusion to PCNA's N terminus, indicating that its position on PCNA is irrelevant for its activating effect on Rad18 and suggesting that the transition from sumoylated to ubiquitylated PCNA may well involve a doubly modified trimer. In vivo, mutation of the SIM in Rad18 or selective inhibition of PCNA sumoylation (by deleting *SIZ1* in combination with mutating K127 to R) causes a substantial reduction in damage-induced PCNA ubiquitylation and a corresponding damage sensitivity (Parker and Ulrich 2012). Hence, sumoylated PCNA appears to be the physiological substrate of Rad18, and the modification not only recruits Srs2 and potentially Elg1, but it also facilitates damage-induced ubiquitylation. Like many other SIM-containing proteins, Rad18 itself is sumoylated in a SIM-dependent manner, but the modification sites or their significance for Rad18 function have not been elucidated. Notably, the enhancement of Rad18 activity by PCNA sumoylation is not conserved in mammals. In fact, based on sequence alignments it appears to be restricted to a small number of species closely related to *S. cerevisiae*. Here, the presence of the SIM in Rad18 exhibits a good correlation with the conservation of the SIM-containing C terminus of Srs2 (Fig. 5), suggesting that the recognition of sumoylated PCNA by Srs2 and Rad18 may have co-evolved.

Eco1

Although posttranslational modifiers very often promote the binding of downstream effector proteins, there are examples where the modification interferes with the association of a

binding partner. A similar scenario was suggested for the interaction of the budding yeast cohesion establishment factor Eco1 with PCNA. Eco1 acts by acetylation of a cohesin subunit, Smc3, during S phase (Skibbens 2009), and its interaction with PCNA, mediated by a PIP-like motif in its extreme N terminus, was reported to be essential for establishment of sister chromatid cohesion (Moldovan et al. 2006). In this context, PCNA sumoylation appears to counteract Eco1's activity, as abolition of the modification was found to suppress the temperature sensitivity of *eco1* mutants. However, although in vitro interaction assays revealed a competition between Eco1 and the SUMO-E2 Ubc9 for PCNA binding (Moldovan et al. 2006), sumoylation of K127 or K164 of PCNA does not interfere with the association of Eco1 (Armstrong et al. 2012), suggesting that the impact of SUMO-PCNA on Eco1 activity is likely mediated indirectly through an unknown mechanism.

Readers of other PCNA modifications

While the number of readers of ubiquitylated and sumoylated PCNA is steadily growing, we know much less about the consequences of other posttranslational modifications of PCNA, such as phosphorylation and acetylation. Both modifications have been detected in mammalian cell extracts (Prosperi et al. 1994; Naryzhny and Lee 2004), but their significance and regulation are not yet fully understood.

PCNA phosphorylation

Mass spectrometric analysis of PCNA isolated from human cells identified tyrosine (Y)211 as a phosphorylation site (Wang et al. 2006). The kinase responsible for this modification was found to be EGF receptor (EGFR), which plays an essential role in cell proliferation. Consistent with this function, Y211 phosphorylation, which was detected mainly in the chromatin-associated fraction, correlated with proliferation. Mutation of Y211 to phenylalanine caused polyubiquitylation and proteasomal degradation of PCNA, while phosphorylation stabilised the protein. Intriguingly, although ubiquitylation was directed at K164 of PCNA, the modification did not involve the set of conjugation factors required for damage-induced ubiquitylation, but instead the Cullin 4-based ubiquitin ligase CRL4, which is known for assembly of K48-linked ubiquitin chains (Lo et al. 2012). Phosphorylation at Y211 was found to interfere with the E3-PCNA interaction, thus explaining its stabilising effect. It is noteworthy that PCNA itself acts as a co-factor for CRL4-dependent ubiquitylation of several cell cycle regulators, such as CDT1 and p21 (Abbas and Dutta 2011). In these contexts the CUL4 subunit cooperates with the substrate adaptor CDT2; whether the same adaptor is used in the degradation of PCNA has not been

determined. It is also unclear how CRL4- and PCNA-dependent degradation of other targets is compatible with Y211 phosphorylation, or how this modification is regulated. However, an EGFR-dependent control over PCNA stability is consistent with the essential contribution of the clamp to cell proliferation via DNA synthesis. It should be mentioned that Y211 is unlikely to be the only phosphorylation site on PCNA; in fact, modification of Y114 has recently been linked to the development of adipose tissue by an unknown mechanism (Lo et al. 2013).

PCNA acetylation

Not only phosphorylation, but also acetylation seems to impinge on PCNA stability, although the two signals apparently respond to different stimuli. Critical to the acetylation-dependent mechanism is the mammalian protein MTH2 (MutT homologue 2). In *Escherichia coli*, MutT prevents incorporation of the nucleotide triphosphate 8oxo-dGTP into DNA by hydrolysis to 8oxo-dGMP, thus protecting the cell from the potentially mutagenic effect of the oxidised guanine (Maki and Sekiguchi 1992). Human MTH2 was identified based on its homology to MutT and exhibits similar enzymatic activity (Cai et al. 2003). MTH2 was found to bind to PCNA, but intriguingly, this interaction was abolished selectively by treatment with ultraviolet (UV) radiation, whilst ionising or oxidative damage had no effect (Yu et al. 2009). UV irradiation also caused the acetylation of PCNA, and treatment with trichostatin A (TSA), an inhibitor of deacetylation enzymes, likewise triggered the release of MTH2 from PCNA, indicating that PCNA acetylation interferes with the MTH2–PCNA interaction and thereby induces the UV-dependent dissociation. TSA treatment or MTH2 depletion reduced the half-life of PCNA in a proteasome-dependent manner, suggesting that interaction with MTH2 stabilises PCNA. Consistent with this model, PCNA degradation coincided with reduced DNA synthesis and cell cycle progression after UV irradiation, which was rescued by PCNA overexpression. Taken together, these data suggest that PCNA acetylation, via inhibiting MTH2 association, controls PCNA stability and thereby cell proliferation in response to DNA damage. However, many issues remain to be resolved, such as the residues affected by acetylation, the enzymes involved and the mechanism that induces the modification, the question of why the response seems to be limited to UV, and whether this function of MTH2 is at all related to its role in preventing mutagenesis by hydrolysis of 8oxo-dGTP.

Outlook

Despite the diversity of phenomena controlled by PCNA modifications, some common concepts about the mechanisms

of PCNA's readers emerge. First of all, and perhaps expectedly, most PCNA modifications exert their effects in the context of DNA, consistent with PCNA's clamp-like action. With the possible exception of acetylation, which has not been sufficiently characterised, PCNA modifications are all associated with DNA-bound PCNA. In the case of sumoylation, loading appears to be both necessary and sufficient for the modification (Parker et al. 2008), whereas ubiquitylation is induced by a recruitment of Rad18 to loaded PCNA via interaction with RPA-coated ssDNA (Davies et al. 2008; Niimi et al. 2008), and phosphorylation by EGFR also occurs preferentially on chromatin-associated PCNA (Wang et al. 2006).

Second, the characterisation of PCNA effectors has revealed important information about how ubiquitin and SUMO are recognised in conjunction with their target proteins. In contrast to phosphorylation-specific interactions, where the residues interacting with the phosphate moiety are usually embedded in the substrate binding surface, recognition of ubiquitylated or sumoylated proteins appears to largely follow a modular mode, involving separate and independent elements for binding to the target and the PTM. This is best illustrated by the molecular models of ubiquitylated and sumoylated PCNA interacting with the C-terminal tails of Pol η and Srs2, respectively (Freudenthal et al. 2011; Armstrong et al. 2012) (Fig. 4). The modularity is consistent with the notion that the ubiquitin- or SUMO-interaction motifs are often juxtaposed to the PIP motif (Fig. 3). In fact, the toolbelt model according to which PCNA interactors are loosely tethered to the clamp rather than engaging in extensive interactions predicts that an unstructured peptide harbouring the relevant interaction motifs would probably be most suited as flexible tether. It remains to be seen whether the combinatorial use of standard recognition modules is common to other effectors of ubiquitylated or sumoylated proteins. The recent identification of a histone binding motif in the ubiquitin-binding RING finger proteins RNF168 and RNF169, which confers specificity for ubiquitylated histones, suggests that bipartite recognition modules is likely a conserved feature in the ubiquitin system (Panier et al. 2012). As an upshot of this notion, however, simply assuming a preferential association with the modified form of a given protein based on the presence of both a substrate- and a PTM-specific recognition motif in an interaction partner would be unacceptable. Even in vitro studies suggesting a preferential interaction might be misleading in the absence of firm genetic evidence linking the conjugation of the modifier to the action of the reader.

Finally, the use of ubiquitylated and sumoylated PCNA as an interaction platform for multiple different PTM-specific effectors indicates a diversification of functions in metazoans. For example, the link of PCNA ubiquitylation to

the Fanconi anemia pathway, C1ORF124 or ZRANB3 seems to be without precedent in fungi, suggesting that these effectors were added later in evolution. It is likely that even now the list of readers of the modified forms of PCNA is not yet complete.

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