Elg1, an alternative subunit of the RFC clamp loader, preferentially interacts with SUMOylated PCNA

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Replication-factor C (RFC) is a protein complex that loads the processivity clamp PCNA onto DNA. Elg1 is a conserved protein with homology to the largest subunit of RFC, but its function remained enigmatic. Here, we show that yeast Elg1 interacts physically and genetically with PCNA, in a manner that depends on PCNA modification, and exhibits preferential affinity for SUMOylated PCNA. This interaction is mediated by three small ubiquitin-like modifier (SUMO)-interacting motifs and a PCNA-interacting protein box close to the N-terminus of Elg1. These motifs are important for the ability of Elg1 to maintain genomic stability. SUMOylated PCNA is known to recruit the helicase Srs2, and in the absence of Elg1, Srs2 and SUMOylated PCNA accumulate on chromatin. Strains carrying mutations in both Elg1 and Srs2 exhibit a synthetic fitness defect that depends on PCNA modification. Our results underscore the importance of Elg1, Srs2 and SUMOylated PCNA in the maintenance of genomic stability.

Introduction

PCNA is a homotrimeric ring that encircles the double-stranded DNA (Krishna et al, 1994) and has an important function in DNA replication, enhancing the processivity of replicative DNA polymerases during DNA synthesis (Krishna et al, 1994; Eissenberg et al, 1997; Chilkova et al, 2007). In addition, PCNA constitutes a general platform for docking of DNA-processing enzymes involved in Okazaki fragment maturation, DNA repair, chromatin remodelling and sister chromatid cohesion (Warbrick, 2000; Majka and Burgers, 2004; Aroya and Kupiec, 2005; Lengronne et al, 2006; Moldovan et al, 2006).

In response to DNA damage, PCNA can be modified with ubiquitin at lysine 164, a process mediated by the E2/E3 pair Rad6 and Rad18 (Hoega et al, 2002). Mono-ubiquitination of PCNA was shown to enhance the binding of the translesion synthesis polymerases to PCNA in yeast and in human beings, resulting in an error-prone repair mechanism (Bienko et al, 2005; Parker et al, 2007; Acharya et al, 2008). Alternatively, PCNA can be further poly-ubiquitinated on the same lysine residue by a mechanism that additionally requires Ubc13-Mms2 (E2 heterodimer) and Rad5 (E3). Ubiquitin subunits in this chain are linked through the lysine 63 residue; the biochemical function of this poly-ubiquitin modification is still unknown; it coordinates a type of repair that is essentially error free (Blastyak et al, 2007; Branzei et al, 2008). Mutations in lysine 164 of PCNA, in RAD18 or in RAD5 cause high sensitivity to DNA damaging agents such as MMS and UV, emphasizing the importance of these modifications for DNA repair in living cells. Interestingly, the same residue (lysine 164) can be modified by another small protein named small ubiquitin-like modifier (SUMO). This modification takes place during S-phase (Hoega et al, 2002) or after high doses of DNA damage. An additional residue, lysine 127, can also be SUMOylated, but not ubiquitinated (Hoega et al, 2002). It was shown that the Srs2 helicase, which regulates homologous recombination, preferentially interacts with SUMOylated PCNA and that this interaction is harmful in the presence of DNA damage when PCNA cannot be ubiquitinated (Papouli et al, 2005; Pfander et al, 2005). However, it is not known whether other proteins also interact with SUMOylated PCNA and what is the function of this modification during ongoing replication. Recently, it was found that PCNA can also undergo polySUMOylation; the importance of this modification is still under investigation (Parker et al, 2008; Windecker and Ulrich, 2008).

A second way of regulating PCNA activity is by loading or unloading it from the DNA. This activity can be carried out by the replication-factor C (RFC) complex that binds PCNA, opens the homotrimeric ring and loads it on the template/primer junction in an ATP-dependent manner (Tsurimoto and Stillman, 1989, 1991). The clamp (PCNA) and clamp loader (RFC) are conserved throughout the evolutionary scale. Homologous proteins that share the same function and structure with RFC and PCNA were found in bacteria (Stukenberg et al, 1991), archaea (De Felice et al, 1999), yeast (Fien and Stillman, 1992) and other eukaryotes (Kelman and O’Donnell, 1995). In the last decade, three homologues of Rfc1, the large subunit of the clamp loader, were found in the yeast Saccharomyces cerevisiae. Each of these three proteins, Rad24, Ctf18 and Elg1, was found to bind to the small RFC subunits (Rfc2-5) forming RFC-like complexes. In contrast to Rfc1, these
alternative large subunits are not essential for vegetative growth; however, they have an important function in maintaining genome stability (reviewed in Aroya and Kupiec, 2005).

_**ELG1**_ was found in several genome-wide screens in yeast (Scholes et al., 2001; Bellaoui et al., 2003; Ben-Aroya et al., 2003; Huang et al., 2003; Banerjee and Myung, 2004; Smolikov et al., 2004). The _elg1_ mutants exhibit increased recombination rates (Ben-Aroya et al., 2003; Ogiwara et al., 2007), chromosome loss (Ben-Aroya et al., 2003) and gross chromosomal rearrangements (Smith et al., 2004). They have elongated telomeres (Smolikov et al., 2004) and elevated levels of Ty transposition (Scholes et al., 2001). Thus, mutations in _ELG1_ cause increased levels of genomic instability. Orthologues of Elg1 were identified in many organisms, including human beings. _hELG1_ has been recently shown to have an important function in maintaining genome stability in S-phase (Sikdar et al., 2009). Targeted gene knockdown of _hELG1_ resulted in spontaneous foci formation of _g-H2AX, 53BP1_ and phosphorylated ATM that usually mark chromosomal breaks, and to increased levels of recombination and chromosomal aberrations such as chromosomal fusions and inversions (Sikdar et al., 2009). _hELG1_ was also found to link DNA replication stalling with apoptosis (Ishii et al., 2005).

In this study, we have examined how Elg1 interacts with PCNA and we have identified the activity by which Elg1 contributes to genomic stability. We found that Elg1 preferentially interacts with SUMOylated PCNA through SUMO-interacting motifs (SIMs) and that this interaction affects the repair process chosen by the cells. The sensitivity of _elg1_ strains to DNA damage and the predicted function of Elg1 in DNA replication suggest that the alternative clamp loaders are responsible for the switch between DNA replication and DNA repair and for selecting the repair mechanism after PCNA modification.

### Results

**Deletion of ELG1 suppresses defects in the error-free branch of the post-replicative repair pathway**

By genetic analysis, the Elg1 clamp loader complex has earlier been linked to the maintenance of genome stability. To gain insights into the precise function of Elg1 in the DNA damage response, we tested the DNA damage sensitivity of the _Aelg1_ mutant on its own and in combination with different DNA repair mutants.

When chronically exposed to the DNA alkylating drug MMS, _Aelg1_ mutants displayed a mild sensitivity (Figure 1A). Surprisingly, the deletion of _ELG1_ clearly suppressed the (higher) sensitivity to MMS of _rad5_, a well-characterized mutant of the post-replicative repair pathway (Figure 1A). _Rad5_ is a large protein that contains a helicase domain and an E3 ubiquitin ligase domain (Chen et al., 2005; Gangavarapu et al., 2006). Supplementary Figure S1A shows that deletion of _ELG1_ suppresses a _rad5_ E3 ubiquitin ligase mutant ( _rad5-E3_), but has an additive relation with the helicase mutant ( _rad5-h_). We thus conclude that the suppressive effect of _elg1_ with respect to _rad5_ results from the polyubiquitination activity of this enzyme. A similar suppressive effect was obtained for strains deleted for _MMS2_ and _UBC13_, which encode the E2 poly-ubiquitin conjugation enzymes responsible for PCNA poly-ubiquitination and for the _ubi-K63R_ mutant, which is unable to support PCNA poly-ubiquitination (Supplementary Figure S1A). This suppression was specific for the error-free ( _Rad5_ -dependent) pathway and not for the error-prone pathway, as shown by the additive interaction with the error-prone translesion polymerase _ζ_ (Supplementary Figure S2).

We thus conclude that in the presence of DNA damage, Elg1 activity becomes toxic if PCNA cannot be poly-ubiqui-

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**Figure 1** Genetic interactions between _ELG1_ and PCNA depend on PCNA modification. Serial dilutions of yeast cultures on minimal SD-complete plates with or without methylmethane sulphonate (MMS). Ten different MMS concentrations were used for each experiment; the informative ones are shown. (A) Deletion of _ELG1_ suppresses the sensitivity of _Δrad5_ to DNA damage. (B) _elg1_ suppression effect cannot be seen on a _pol30-RR_ background. (C) _elg1_ suppression effect cannot be seen in the absence of the _Siz1_ SUMO ligase.
tinated; deletion of the ELG1 gene under these circumstances suppresses the sensitivity to DNA damage. One possibility is that the toxic activity of the alternative clamp loader containing Elg1 depends on the modification that PCNA undergoes when poly-ubiquitination is inhibited. If this were the case, the suppression by elg1 should be abolished when PCNA cannot be modified. Indeed, in pol30-RR strains (PCNA mutants that cannot be modified neither by ubiquitin nor SUMO on lysine 127 and 164), deletion of ELG1 no longer suppresses the sensitivity to MMS of rad5 mutants: the triple rad5 pol30-RR elg1 mutant is not more resistant than the rad5 pol30-RR double mutant strain (Figure 1B; note that pol30-RR by itself partially suppresses the rad5 phenotype; Papouli et al., 2005). More specifically, the suppressive effect of elg1 was dependent on SIZ1, the E3 SUMO ligase that is responsible for the SUMOylation of lysine 164 of PCNA (Supplementary Figure S3). It can also be seen (Figure 1B; Supplementary Figure S1C) that in the absence of SUMOylation (pol30-RR or pol30-K164R), Elg1 activity is needed for DNA damage resistance. We thus conclude that the toxic effect caused by Elg1 in the absence of PCNA poly-ubiquitination depends on PCNA SUMOylation.

Physical interactions between Elg1 and PCNA
To prove that the genetic relations between elg1 mutants and modified PCNA result from direct physical contact between the proteins, we examined the interaction between full-length Elg1 and various PCNA mutants using a yeast-two-hybrid assay (James et al., 1996). Figure 2A shows that Elg1 binds PCNA. A similar level of binding was observed with versions of PCNA carrying mutations in lysine 127, lysine 164 or both (Figure 2A). The lack of effect of PCNA modifications could in principle be due to the fact that only a small fraction of PCNA is being modified and the two-hybrid assay is not sensitive enough to measure this change. In contrast, when we expressed prey constructs that contain either SUMO or ubiquitin fused to pol30-RR (a gift from H Ulrich; Parker et al., 2007) in the presence of Elg1 baits, the SUMO-PCNA fusion allows growth even under very stringent conditions (plates lacking adenine, Figure 2) indicating that indeed Elg1 binds with high affinity to the fusion SUMO-PCNA. Moreover, Figure 2A shows that Elg1 also binds to SUMO in a yeast-two-hybrid assay.

Additional proof of the interaction between Elg1 and PCNA comes from immunoprecipitation experiments: Antibodies against PCNA were able to co-precipitate Myc-tagged versions of Elg1 under normal conditions (Figure 2B), confirming earlier observations obtained with overexpressed PCNA (Kanellis et al., 2003). Next, we precipitated Elg1 to find out if it interacts with modified PCNA (Figure 2C). These experiments were carried out in the presence of 0.3% MMS in ulp1-1 strains to maximize the SUMOylation signal (Hoege et al., 2002; Stelter and Ulrich, 2003). Elg1 co-precipitates with modified and unmodified PCNA. The same sample was probed with an anti-SUMO antibody proving that Elg1 interacts with SUMOylated PCNA (Figure 2C). It is important to notice that the SUMOylated PCNA is strongly enriched in the

Figure 2
Elg1 interacts physically with PCNA and SUMO. (A) Two-hybrid interaction between Elg1 fused to GAL4-binding domain (BD) and a number of constructs containing PCNA or SUMO fused to the GAL4-activating domain (AD). (B) Co-IP of an Elg1-13Myc protein with PCNA. After IP with IgG as a negative control or with anti-PCNA antibody, western blots were carried out with anti-PCNA or anti-myc antibodies. (C) Co-IP of an Elg1-13Myc protein with Histidine-tagged PCNA in ulp1-1 strains. The yeast culture was treated with 0.3% MMS for 1 h before lysis. The asterisk denotes a faint additional SUMOylation of PCNA at an unknown location that is sometimes detected.
pellet compared with the whole cell extract (WCE), emphasizing that the Elg1-containing alternative clamp loader preferentially interacts with the modified form of PCNA. To find the SUMOylated form with which Elg1 interacts, we introduced ELG1-13MYC into strains with Pol30 treated at lysine 127, lysine 164 or both. We found that Elg1 preferentially precipitated with all forms of SUMOylated PCNA (Figure 2C).

**N-terminus of Elg1 mediates the physical interaction with SUMOylated PCNA**

To get better insights into the mechanism that enables Elg1 to interact with PCNA and in particular with SUMOylated PCNA, we carried out a deletion analysis of Elg1. We divided the Elg1 protein into a central AAA domain, bearing most of the homology to Rfc1 (Ben-Aroya et al, 2003), an N-terminus and a C-terminal domain. Figure 3 shows that both PCNA and SUMO bind exclusively to the N-terminus of Elg1 (Figure 3A and B). The fact that Elg1 binds PCNA through its N-terminus is surprising, as crystal structure studies have shown that the interactions of RFC with PCNA are mediated by the AAA domain of Rfc1 (Jeruzalmi et al, 2001). As expected, we found that the N-terminal domain of Elg1 preferentially binds to the SUMO-PCNA fusion, as seen with the full-length Elg1 protein (Supplementary Figure S4). When we examined by yeast two hybrid the interaction between the N-terminus of Elg1 and SUMO in a pol30RR strain, this interaction was significantly reduced (data not shown). This means that the major interaction of Elg1 with SUMO is mediated by a different modification of lysine 127 and 164 of PCNA (although other lysine residues on PCNA or other SUMOylated protein may also interact with Elg1).

Next, we carried out pull-down experiments with recombinant GST-Elg1(Nter) and lysates of yeast cells treated with different concentrations of MMS. GST-Elg1(Nter) but not GST alone pulled down PCNA (Figure 3C). Importantly, the N-terminal domain of Elg1 preferentially interacts with SUMOylated PCNA (Figure 3C and D) and the amount of SUMOylated PCNA pulled down increased with increasing MMS concentration. Quantitation of the results (Figure 3E) shows a clear enrichment of SUMOylated PCNA (all forms) in the pellet compared with the WCE fraction. The higher level of SUMOylated PCNA in the presence of DNA damage may enhance the interaction between Elg1 and PCNA. To examine whether the interaction between the N-terminus of Elg1 and SUMOylated PCNA is direct or mediated by other cellular components, we examined the interactions in vitro. We performed an in vitro SUMOylation reaction of PCNA (see Materials and methods) followed by a pull-down experiment with GST or GST-Elg1(Nter) (Figure 3F). Again, a clear preferential interaction with SUMOylated PCNA could be detected. This experiment clearly shows that the interaction between Elg1 and SUMOylated PCNA is direct and does not depend on additional cellular factors.

**SUMOylated PCNA accumulates in the chromatin fraction of Δelg1 strains**

To better understand the effect of Elg1 on SUMOylated PCNA, we compared the amount of PCNA in the chromatin fraction of wild type versus Δelg1 strains. Figure 4A shows that in elg1 mutants, unmodified PCNA accumulates in the chromatin fraction (a ~three-fold increase). It was earlier found that such an accumulation occurs during S-phase and that PCNA SUMOylation also occurs mainly in S-phase (Hoeger et al, 2002; Parker et al, 2008). Thus, a possible explanation for the increased PCNA accumulation in elg1 mutants might be a prolonged S-phase. However, the difference in cell cycle distribution in wt and elg1 mutants is very small and cannot account for the effect seen (Supplementary Figure S5). Interestingly, the chromatin accumulation of SUMOylated PCNA was much more prominent than the unmodified form (Figure 4B and C). In contrast, ubiquitinated PCNA accumulated only to levels similar to those of the unmodified form (Supplementary Figure S6). In the absence of DNA damage, wt strains exhibit low levels of SUMOylated PCNA in the chromatin fraction; in contrast, elg1 mutants show a strong accumulation (Figure 4B and C). This result fits well with the preferential binding of Elg1 to SUMOylated PCNA in the yeast-two-hybrid assay (Figure 2A; without damage) and the pull-down assay (with damage; Figures 2 and 3) suggesting that the alternative clamp loader containing Elg1 might negatively regulate SUMOylated PCNA on chromatin.

**Genetic interactions between elg1 and srs2**

In earlier publications, it was suggested that PCNA-SUMO and Srs2 work in the same pathway and that Srs2 preferentially binds to SUMOylated PCNA (Papouli et al, 2005; Pfander et al, 2005). In Figure 4, we show that in elg1 mutants SUMOylated PCNA accumulates in the chromatin fraction; as Srs2 preferentially interacts with SUMOylated PCNA, we expect to find an accumulation of Srs2 in the chromatin fraction in elg1 mutants, in which the alternative clamp loader is not active. Indeed, this prediction is confirmed (Figure 5A). This finding raises the question of whether Elg1 and Srs2 have similar modes of action. To address this question, we have examined the amount of SUMOylated PCNA in the chromatin fraction in srs2 mutants. Supplementary Figure S7 shows that deletion of the SRS2 gene does not lead to an accumulation of SUMOylated PCNA in the chromatin fraction. We also found no difference in the amount of Elg1 at the chromatin in srs2 mutants (data not shown). These results contradict the possibility that Elg1 and Srs2 compete on regulating the amount of SUMOylated PCNA.
on the chromatin. To get a better understanding of the mechanism by which these proteins control genomic stability, we examined the genetic interactions between elg1 and srs2. Notably, the double mutant srs2 elg1 is sick and grows poorly compared with the single mutants (Figure 5B). We, therefore, examined whether PCNA modification may affect
this synthetic genetic interaction. Figure 5C shows that the pol30-RR allele improves the viability of elg1 srs2 strains. The same result was obtained in the presence of DNA damage: the high sensitivity of elg1 srs2 strains to MMS is suppressed when PCNA cannot be modified (Figure 5D). This effect did not result from the inability to ubiquitinate PCNA because a rad18 mutation (which prevents ubiquitination) could not suppress the growth defect or the sensitivity to MMS of an elg1 srs2 double mutant strain (Figure 5E). Thus, SUMOylation of PCNA in the absence of either protein is toxic, suggesting a model in which SUMOylated PCNA needs to be removed from the fork to allow survival. Recruitment of Srs2 promotes its clearance by allowing damage bypass or repair; alternatively, Elg1 may unload the modified PCNA molecules from the chromatin thus enabling other DNA repair mechanism to act. When both Elg1 and Srs2 are missing, SUMOylated PCNA stays at the fork and becomes toxic.

**Three SIM motifs and a PCNA-interacting protein box enable the interaction of Elg1 with SUMOylated PCNA**

To better understand the mechanism that enables Elg1 to interact with SUMOylated PCNA, we further defined, by deletion analysis, the regions of Elg1 that mediate the interaction with SUMO and PCNA. Two regions, between amino acids 67 and 94 and between residues 116 and 143, were important for the interactions with SUMO (Figure 6A), as deletion of each of them reduced the interaction. Careful examination of these regions uncovered three sequences similar to the earlier defined SIM, which enables proteins to interact with SUMO non-covalently (Minty et al., 2000; Song et al., 2004; Kerscher, 2007). This motif consists of a series of hydrophobic amino acids usually containing valine and isoleucine, followed by negatively charged amino acids. As shown in Figure 6C, each of the suspected regions contains a SIM motif. Next, we took the same approach to find the region of Elg1 that interacts with PCNA (using a version that cannot be modified by SUMO). Yeast-two-hybrid analysis defined a region located between amino acids 43 and 67 (Figure 6B). PCNA-binding motifs, termed PCNA-interacting protein (PIP) motifs, have been described for many proteins (Warbrick, 1998; Moldovan et al., 2007). Analysis of the Elg1 sequence in this region revealed a motif with general resemblance to the canonical PIP box (Figure 6D). Interestingly, the sequence found in the N-terminus of Elg1 is most similar to the PIP sequence of Rfc1 (Figure 6D; Bowman et al., 2004).

To examine whether the SIM and PIP motifs indeed contribute to the interactions with SUMO and PCNA, we mutated
them (mutations are detailed in Supplementary Table S2) and tested the mutants in the yeast-two-hybrid assay. Mutation of each SIM motif reduced the ability to bind SUMO, and the motifs acted in an additive manner: the double SIM mutants showed a stronger effect and a triple SIM mutant showed no interaction at all (Figure 6E). According to Figure 6E, mutations in the PIP box cause a significant reduction in the interaction between the N-terminus of Elg1 and PCNA. In addition, mutations in either the PIP or the SIM motifs reduce the interaction with the fusion construct of SUMO-PCNA (Supplementary Figure S8).

Using pull-down experiments, we also found that mutations in the SIM motifs of a version of the amino terminus of Elg1 strongly reduced the interactions with PCNA and with SUMOylated PCNA (Figure 6F). In conclusion, these results uncover the motifs that enable Elg1 to interact with SUMOylated PCNA showing a novel mechanism that involves three SIM motifs and a PIP box.

Next, we wanted to examine whether the SIMs and PIP motifs at the N-terminus of Elg1 have biological significance. First, we measured the sensitivity to MMS of yeast strains mutated in these motifs. In this assay, we did not find an effect for the SIM motifs or the PIP motif separately; however, when we used a strain in which both SIM and PIP motifs were mutated, a clear sensitivity to MMS was observed (Figure 7C), suggesting alternative functions in PCNA binding. This sensitivity was somewhat lower than that of an elg1 null mutant, suggesting that other domains also contribute to the activity of Elg1. Figure 7D shows a clear suppression of rad5 by mutation of the SIM motif, and an even stronger suppression by the double SIM, PIP mutant. As in the case of the null mutant (Figure 1C), the suppression effect by the SIM, PIP mutant could not be observed in the pol30-RR background, in which PCNA cannot be SUMOylated (Figure 7E). From all the results presented, we conclude that the PIP and SIMs motifs mediate, in an additive manner, the physical interactions between Elg1 and SUMOylated PCNA. These interactions are responsible for most of the DNA damage sensitivity of rad5 mutants and are also responsible for the contribution of Elg1 to yeast resistance to DNA damage.
Discussion

In this work, we found that elg1 suppresses the sensitivity to DNA damage of rad5 mutants and other post-replication repair mutants such as ube31 and mms2. We show that the suppression is due to the inability of these strains to poly-ubiquitinate PCNA (Figure 1; Supplementary Figure S1); under these circumstances, the presence of an active Elg1 protein is toxic. This toxicity required SUMOylation of PCNA, as no suppression effect could be seen on the background of siz1 or pol30-RR (which is mutated at the SUMOylation sites of PCNA; Figure 1B and C). We provide evidence for binding of PCNA by Elg1 and show that this interaction is mediated by the covalent modification of PCNA by SUMO. We base this conclusion on the two-hybrid assay results, the pull-down assay with the N-terminus of Elg1, the Co-IP experiments (Figures 2 and 3) and the in vitro assay with purified SUMOylated PCNA (Figure 3F). We have also identified three SIM motifs and a PIP box in the N-terminus of Elg1 that mediate the interaction (Figure 6). Moreover, our results show that in the absence of Elg1, SUMOylated PCNA accumulates in the chromatin fraction (Figure 4).

Figure 6 SIM and PIP motifs mediate the interaction between Elg1, PCNA and SUMO. Interaction between different fragments of the N-terminus of ELG1 fused to GAL4 BD and (A) SUMO or (B) PCNA-RR fused to GAL4 AD. (C) The location of the PIP box (bright grey) and SIM motifs (dark grey) in the N-terminus of Elg1. (D) Sequence alignment between proteins that contain PIP boxes. (E) Elg1 interactions with SUMO and PCNA are reduced in SIM and PIP mutants, accordingly. Two-hybrid interactions between SUMO or PCNA-RR and the N-ter Elg1 carrying various mutations. The SIM alleles were I28A (SIM1), I93 (SIM2), I121, 122AA (SIM3) and combinations thereof; the PIP mutants were either SV57, 58AA (PIP57) or VV58, 59 (PIP58). (F) Pull down with the N-terminus of elg1 with or without mutation in the three SIM motifs. The yeast lysates were treated with 0.3% MMS for 1 h.
Taking into account the homology of \( \text{ELG1} \) to the large subunit of the clamp loader/unloader complex RFC, the interaction of Elg1 with SUMOylated PCNA is mediated by the SIM and PIP motifs. A Co-IP experiment with \( \text{ELG1} \) mutants or wt cells tagged with 13-MYC or in strains in which \( \text{ELG1} \) is not tagged (as a control). The SIM allele used is the triple mutant I28A, 193K, 11121, 122AA; the PIP allele is SV57, 58AA, and the SIM-PIP (SP) allele is a combination of all the mutations. The yeast lysates were treated with 0.3% MMS for 1 h. (B) Western blot analysis to detect the protein amount of Elg1 mutants \textit{in vivo} using anti-Myc antibody or anti-PGK antibody as loading control. (C, D) Sensitivity to MMS of elg1 mutants in \( \text{RAD5} \) and \( \text{rad5} \) mutant background, accordingly. (E) No effect on the sensitivity to MMS by elg1 mutants could be observed in a \( \text{pol30-RR} \) background.

Earlier publications suggested that PCNA modification can occur only after the homotrimer PCNA ring encircles the DNA (Parker \textit{et al}, 2008). The switching-back mechanism, however, has not been explored yet. We propose a function for Elg1 in the clearance of SUMOylated PCNA, perhaps in parallel to mechanisms that cleave out the modification. The exact function of each type of PCNA modification is being currently intensively investigated (Moldovan \textit{et al}, 2007). For example, it was found that PCNA SUMOylation occurs mainly during S-phase, suggesting a function for SUMOylation of PCNA in the regulation of DNA replication (Hoege \textit{et al}, 2002; Parker \textit{et al}, 2008). Recently, Branzei \textit{et al}...
earlier finding that the pathways for repair and viability are different. This model is consistent with the idea that Srs2 accumulates in the chromatin fraction, leaving HR as the only alternative replication. When Elg1 is not active, SUMOylated PCNA is present, allowing recruitment of Srs2, which affects the repair mechanism at the damage site. After repair is completed (or as a step in the repair process), Elg1 may take off SUMOylated PCNA and Srs2 to allow the continuity of replication. When Elg1 is not active, SUMOylated PCNA remains on the chromatin, leaving HR as the only alternative pathway for repair and viability. This model is consistent with earlier findings that the affinity of Elg1 for PCNA changes during the DNA replication fork. If the replication fork stalls, the replicative DNA polymerase must be first activated, and, after the completion of repair, a switch-back event should take place to enable productive continuity of replication (Ulrich, 2009). Recently, Zhuang et al. (2008) suggested that the switching back from the TLS Pol η to the replicative Pol δ is not spontaneous and either de-modification of PCNA or unloading of the modified PCNA are needed for the continuity of the repair.

Other than Elg1, Srs2 is the only known protein that preferentially binds SUMOylated PCNA (Papoulis et al., 2005; Pfander et al., 2005). Similarly to Elg1, Srs2 also contains SIM motifs that are essential for the interaction with SUMOylated PCNA (Le Breton et al., 2008). We found that in the absence of Elg1 not only SUMOylated PCNA, but also Srs2 accumulates in the chromatin fraction (Figures 4 and 5A). These results suggest a model in which SUMOylation of PCNA (by Siz1) allows recruitment of Srs2, which affects the repair mechanism chosen at the damage site. After repair is completed (or as a step in the repair process), Elg1 may take off SUMOylated PCNA and Srs2 to allow the continuity of replication. When Elg1 is not active, SUMOylated PCNA remains on the chromatin, leaving HR as the only alternative pathway for repair and viability. This model is consistent with earlier findings that Elg1 is synthetic lethal with genes involved in HR (Aroya and Kupiec, 2005).

The discovery of PCNA modification (Hoege et al., 2002) suggested a way in which competing PCNA-dependent DNA-acting activities could be regulated. An important question that remains open is how the affinity of the various interactors may be affected by PCNA modification. We have identified a sequence in the Elg1 protein that binds PCNA and carries homology to the PIP motif (Xu et al., 2001; Moldovan et al., 2007). In addition, the affinity of Elg1 to SUMOylated PCNA is enhanced by three SIM motifs. The in vivo and in vitro interactions shown and the finding of the motifs in Elg1 that enable contact with SUMOylated PCNA strengthen the possibility that the interaction between Elg1 and SUMOylated PCNA is direct. Interestingly, the three SIM motifs are located in the N-terminus of Elg1 in a relatively small fragment (from AA 26 to AA 123) and the PIP box that enables the interaction with PCNA is embedded between them (Figure 6). Thus, our results present a possible mechanism of recruitment of Elg1 according to PCNA modification.

Increased affinity of a TLS polymerase to modified PCNA has been recently shown to be mediated both by PIP motifs and UBM/UBZ domains that enable proteins to interact with ubiquitin non-covalently (Bienko et al., 2005). Interestingly, in this case, pol η is ubiquitinated, raising the possibility that covalent modification of the polymerase may help regulate the non-covalent interaction with ubiquitinated PCNA. We have noticed that Elg1::MYC migrates as a double band (Figure 7B and see also Davidson and Brown, 2008). We are currently analysing the nature of this modification and its potential function in regulating interactions with other proteins.

Evidence for SUMOylation of PCNA was found in yeast, chicken DT40 cells (Arakawa et al., 2006) and Xenopus laevis egg extracts (Leach and Michael, 2005). Recently, it has been found that hELG1 has an important function in maintaining genomic stability during S-phase in human cells (Sikdar et al., 2009) and that the phenotypes of HeLa cells carrying sRNA directed against hELG1 are very similar to those of elg1 yeast cells. Sequences resembling SIM motifs can be found at the N-terminus of hELG1; their interactions with SUMOylated proteins and with SUMOylated PCNA will be the subject of future studies.

Materials and methods

Yeast strain and plasmids

A list of all strains used appears in Supplementary Table S1.

Plasmids

pGBU9 (pGBT9 in which TRP1 was replaced by URA3) was used in yeast two-hybrid experiments. JY28 (Bellouai et al., 2003) was the source of our ELG1::13MYC::KANMX strains. To construct the plasmids used in the pull-down assays, ELG1’s N-terminus (AA 1–230) was cloned into pGEX3. Mutations in the PIP and SIM motifs in the pGEX3-NTD, a pGEM that contains ELG1::13MYC::KANMX and two-hybrid plasmids were created using PCR reaction with long primers that contain the relevant mutation as indicated in Supplementary Table S2, dissection with Dpn1 and transformation to bacteria.

Plasmids carrying PCNA mutants and SUMO and ubiquitin fusions in pGAD424 are a generous gift from Helle Ulrich (Parker et al., 2007). The SUMO-PCNA 2 µ plasmid is kind gift from Hideo Shinagawa (Hishida et al., 2006).

DNA damage sensitivity

Serial 10-fold dilutions of logarithmic yeast cells were spotted on fresh SD-complete plates with or without MMS (Sigma) and incubated at 30°C for 3 days.

Two-hybrid assay

To detect two-hybrid interaction, yeast strain PJ69 (James et al., 1996) was co-transformed with one LEU2-marked plasmid containing genes fused to the GAL4-activating domain (pACT or pGAD424) and one plasmid containing genes fused to the GAL4-DNA-binding domain (pGBU9). Yeast cultures were grown in SD-URA-LEU medium and spotted on SD-URA-LEU plates, SD-ADE plates and SD-HIS plates. Cells were incubated for 3 days at 30°C.

Cell lysis

Cells were lysed as described earlier (Hanna et al., 2001) with small modifications. 200 OD of logarithmic cells were washed and cells were resuspended in B60. The tubes were vortexed 12 times for 45 s, with 45 s intervals on ice.

Co-IP

Cell lysates were pre-treated with Invitrogen (Oregon, USA) Dynal beads and incubated with 20 µl of anti-Myc antibodies (Santa Cruz) or 7 µl anti-PCNA antibodies (generous gifts by Peter Burgers and Alain Verreault). After 2 h incubation at 4°C, 100 µl beads were added, and further incubated for an hour. Beads were collected, washed eight times and boiled with 50 µl sample buffer. Western blot analysis was performed using anti-PCNA, anti-SUMO (a gift from Phil Hieter) and anti-Myc antibodies. Ten per cent of yeast lysate (WCE) was taken for an Ni-NTA pull down (Ulrich, 2009) when HIS-PCNA modification was examined, to avoid non-specific bands in the western blot with polyclonal anti-PCNA.

GST pull down

Recombinant GST-Elg1 N-terminus (AA 1–230) with or without mutation in the PIP and SIMs motifs was overexpressed in bacteria by incubating overnight in the presence of IPTG at 16°C. A binding assay was carried out as described (Uzunova et al., 2007). Bacterial cells were lysed and incubated with glutation beads (Sigma) for 2 h at 4°C. After five washes with PBS + 0.1% Triton, the beads were incubated for 2 h at 4°C with yeast cell lysate. The beads were collected, washed and boiled with 50 µl sample buffer. Ten percent of the yeast lysate (WCE) was used for nickel-NNTA pull down (Ulrich, 2009) when HIS-PCNA modification was examined.
**In vitro SUMOylation assay**

E1 (Aos1/Uba2), E2 (Ubc9), E3 (Siz1) and SUMO (SMT3) were purified as described earlier by the Johnson laboratory (Johnson and Gupta, 2001); His-PCNA was purified as described for other substrates (Johnson and Gupta, 2001). The SUMOylation reaction was carried out in 40 μl of buffer containing 50 mM NaCl, 20 mM HEPES pH 7.5, 5 mM MgCl2, 1 mM DTT, 1500 ng E1, 1500 ng E2, 4 μg E3, 1500 ng SUMO, 3 μg of PCNA and ATP to a final concentration of 2.5 mM. After 2 h at 30°C, the reaction mix was resuspended in 1 ml of binding buffer (Yunus and Lima, 2009) and subjected to GST pull down as described above.

**Chromatin assay**

Fractionation of soluble and chromatin-associated proteins was performed as described earlier (O’Shaughnessy et al., 2006), with a few modifications. Shortly, 7.5 × 106 cells/300 ml YPD medium were grown for 16 h to log phase, then treated with 0.2%, 0.3% of MMS (Sigma) or without MMS for 1 h. Cells were washed and incubated for 2 h at 37°C with 20 μg/ml of Zymolase T-100, next lysed with 0.25% Triton X-100. A total of 100 μl lysates were separated to supernatant and chromatin fractions by sucrose gradient. WCE, supernatant (Sup) and chromatin pellets (CHR) were subjected to chromatography and western blot analysis as described earlier, using PCNA- and SUMO-specific antibodies (Ulrich, 2009).

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


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