A structure–function analysis of the yeast Elg1 protein reveals the importance of PCNA unloading in genome stability maintenance

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Received April 08, 2016; Revised December 01, 2016; Editorial Decision December 21, 2016; Accepted January 12, 2017

ABSTRACT

The sliding clamp, PCNA, plays a central role in DNA replication and repair. In the moving replication fork, PCNA is present at the leading strand and at each of the Okazaki fragments that are formed on the lagging strand. PCNA enhances the processivity of the replicative polymerases and provides a landing platform for other proteins and enzymes. The loading of the clamp onto DNA is performed by the Replication Factor C (RFC) complex, whereas its unloading can be carried out by an RFC-like complex containing Elg1. Mutations in ELG1 lead to DNA damage sensitivity and genome instability. To characterize the role of Elg1 in maintaining genomic integrity, we used homology modeling to generate a number of site-specific mutations in ELG1 that exhibit different PCNA unloading capabilities. We show that the sensitivity to DNA damaging agents and hyper-recombination of these alleles correlate with their ability to unload PCNA from the chromatin. Our results indicate that retention of modified and unmodified PCNA on the chromatin causes genomic instability. We also show, using purified proteins, that the Elg1 complex inhibits DNA synthesis by unloading SUMOylated PCNA from the DNA. Additionally, we find that mutations in ELG1 suppress the sensitivity of rad5Δ mutants to DNA damage by allowing trans-lesion synthesis to take place. Taken together, the data indicate that the Elg1–RLC complex plays an important role in the maintenance of genomic stability by unloading PCNA from the chromatin.

INTRODUCTION

Accurate replication of the genome is essential for preserving cellular integrity. During the process of DNA replication, secondary structures and lesions may lead to the stalling of the replication fork (1,2) and failure in dealing with such events results in genomic instability. A key component of the DNA replication and repair machineries is the replication clamp PCNA. PCNA is a homotrimeric ring that is encoded by the POL30 gene. It slides along the DNA during replication and serves as a docking platform for polymerases and other proteins that participate in DNA replication and repair processes (3). Additionally, PCNA undergoes post-translational modifications, such as ubiquitination and SUMOylation, during normal DNA replication and upon DNA damage. These modifications have a role in directing the cell toward one of the DNA damage bypass or repair pathways [reviewed in (3)]. Mono-ubiquitination of PCNA on lysine 164 by Rad6 and Rad18 promotes an error-prone Trans-Lesion Synthesis (TLS) repair pathway carried out by specific polymerases (4). Further poly-ubiquitination of the same residue by the E2 ubiquitin ligase Mms2/Ubc13 and the E3 ubiquitin ligase Rad5 summons an error-free damage bypass pathway that involves a transfer of information from the replicated chromatid (5,6). SUMOylation of PCNA K164 and/or K127 by the SUMO ligase Siz1 enhances the binding of the Srs2

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helicase, which disturbs Rad51 filament formation and thus inhibits homologous recombination (HR) (7–9).

The RFC complex, composed of a large subunit (Rfc1) and four small subunits (Rfc2–5), has the ability to load and unload PCNA onto DNA in an ATP-dependent manner (10). This activity is required at the firing of each origin of replication and for the formation of each Okazaki fragment on the lagging strand, as well as for efficient DNA repair. PCNA and its loader are essential and structurally conserved in all living organisms (11). In eukaryotic cells, three alternative clamp loaders (RFC-like complexes or RLCs) were identified. The RLCs share the four small subunits (Rfc2–5) of the canonical Rfc1 complex, but differ in the large subunit, which share homology with Rfc1. In yeast, the three identified RLCs are Elg1, Ctf18 and Rad24. These three alternative subunits are, in contrast to Rfc1, not essential for cell viability. Nevertheless, the RLCs have important roles in maintaining genomic stability (12,13). Ctf18 plays a role in sister chromatid cohesion, Rad24 is involved in the activation of the DNA damage checkpoint, and Elg1 has been implicated in many aspects of maintaining genomic stability (14–16). ELG1 deletion results in increased sensitivity to genotoxic agents, elevated rates of spontaneous recombination, gross chromosomal rearrangements, loss of sister chromatid cohesion and elongated telomeres (13). Elg1 has been shown to interact genetically and physically with a variety of genes and proteins that are involved in DNA replication and repair, as well as with components of the SUMO pathway (17). The Elg1–RLC interacts with PCNA, particularly with its SUMOylated form, and has the ability to unload it from the chromatin (18–21). It has been recently shown that the unloading activity of the Elg1–RLC clamp is dependent on Okazaki fragment ligation (21), and that it occurs in S and G2/M phases of the cell cycle (22). The human orthologue of ELG1, ATAD5, has been shown to play a role in regulating the lifespan of DNA replication protein complexes by modulating PCNA levels on the chromatin (23). Homozygous ATAD5 mutations in mice are embryonically lethal, whereas over 95% of heterozygotes develop tumors. Moreover, mutations in ATAD5 were also found in several types of human cancers, implicating a tumor-suppressing function for this gene (24). ATAD5 has been shown to be involved in the Fanconi Anemia DNA repair pathway by facilitating the de-ubiquitination of FANC1/FANC D2 heterodimer (25,26). The yeast Elg1 interacts both genetically and physically with the yeast orthologs of the Fanconi Anemia repair pathway (27).

Although it has already been established that Elg1 is an unloader of PCNA, it is still not clear whether the phenotypes of elg1Δ cells are a consequence of PCNA retention on the chromatin. To address this issue, we have used homology modeling to predict the structure of Elg1, and generated various mutants that vary in their PCNA unloading ability. By examining different phenotypes of the elg1 mutants we found that the sensitivity of elg1 mutants to DNA damage can be correlated with their ability to unload PCNA from the chromatin, and that the various roles of Elg1 in genome stability maintenance involve the unloading of both modified and unmodified PCNA. Our results also suggest a role for the retention of mono ubiquitinated PCNA on the chromatin in the suppression of MMS sensitivity of rad5Δ mutants by deletion of ELG1.

MATERIALS AND METHODS

Elg1 homology modeling

The Elg1 AAA+ region was modeled using similarity to the three-dimensional structure of Rfc1 from the yeast RFC–PCNA complex (PDB id: 1SXJ) (10). Elg1 and Rfc1 sequences were first aligned using pairwise comparison of corresponding profile hidden Markov models with HHsearch (28). The Elg1 structural model was then generated from the resulting sequence-structure alignment using Modeller (29). A model for Elg1 interaction with PCNA was obtained by overlaying the modeled Elg1 AAA+ region onto the Rfc1 subunit within the RFC–PCNA complex and subsequently removing the Rfc1 structure.

Yeast strains and plasmids

All yeast strains used are derivatives of MK166 (30), and are shown in Table 1. Mutants were created using standard yeast manipulation techniques. Yeast cells used in this study were grown at 30°C in either YPD medium or in Synthetic Dextrose (SD) medium supplemented with essential nutrients as required.

Site specific mutagenesis

A DNA fragment carrying full length ELG1::MYC marked with a KANMX cassette was cloned into pGEM-T Easy Vector (Promega) and subjected to PCR using different forward and reverse primers containing the desired mutations (Table 2). Amplified PCR products were transformed into DH5α cells after digestion with DpnI (NEB). Plasmids were isolated and sequenced.

To transfer the mutation into the yeast genome, the mutagenized plasmids were digested with NotI (NEB) and transformed into elg1::HphMX cells. Transformed cells were selected on G418 plates, G418 resistant, hygromycin sensitive colonies were subjected to DNA sequencing to confirm the presence of the particular mutation, and the lack of spurious ones. In the elg1-linker allele the sequence of Elg1 between positions 289 and 320 was replaced by a five amino acid linker (Gly-Cys-Ala-Cys-Gly). The elg1-SIM mutant harbors the following mutations: I28A, I93K, I121,122AA.

Recombination rate assay

Recombination rates were measured as described previously (31), using the strain MK166 which allows to measure Ty recombination and DRR events.

Protein purification

Elg1–RLC and its mutant forms. Elg1–RLC was purified from Saccharomyces cerevisiae BJ4646 strain transformed with plasmids pBL448 (encoding GST-ELG1 under control of GAL1 promoter) and pBL422 (encoding RFC2, RFC3, RFC4, RFC5 under control of GAL1 promoter), respectively. The purified proteins were then used for the preparation of GST-Elg1 and Rfc1 complexes.
which were generously provided by Peter Burgers, according to the protocol of (32) with minor modifications. Briefly, a fresh overnight culture was diluted 8-fold in fresh synthetic medium lacking tryptophan and uracil, and containing galactose (2%, w/v), glycerol (3%) and lactate acid (3%) and was incubated for 22–24 h at 30 °C.

Elg1–RLC and its mutants were purified as follows: 70–100 g of yeast paste was lysed by cryo-milling. The resulting powder was dissolved in 200 ml of lysis buffer C, consisting of 50 mM Tris-HCl (pH 7.5), 10% sucrose (w/v), EDTA (10 mM), dithiothreitol (3 mM), nonidet-P40 (0.01%, v/v), NaCl (150 mM) and protease inhibitors (aprotinin, chymostatin, leupeptin, pepstatin A, benzamidine, each at 5 µg/ml). Solid ammonium sulphate was then added to the lysate to a final concentration of 300 mM and the mixture was stirred for 25 min at 4 °C. Afterward, 45 µl of 10% Polymixin P per 1 ml of the lysate was added, and the mixture was gently stirred at 4 °C for 10 min. The crude lysate was clarified by centrifugation (100 000 × g for 90 min). Next, Elg1–RLC complex was precipitated from the cleared lysate using ammonium sulphate (0.35 g/ml), and the precipitate was kept overnight at −80 °C. The next day, the precipitate was dissolved in buffer T (25 mM Tris–Cl, 10% (v/v) glycerol, 5 mM EDTA, pH 7.5). The conductivity of the sample was adjusted to be equal of buffer T containing 150 mM NaCl, supplemented with 20 mM glutathione (reduced form) and 0.05% ampholytes. Fractions containing the complex were pooled and incubated for 3 h at 4 °C with 5–15 µg of PreScission protease. Elg1–RLC complex was then loaded onto 1 ml MonoS column equilibrated with buffer T containing 100 mM NaCl and 0.05% ampholytes.

### Table 1. Strains used in this study

| Reference | Genotype | Strain name |
|-----------|----------|-------------|
| (29)      | lys2::Tet Sup ade2-1 (0c) can1-100 (0c) ura3-52 leu2-3,112 trp1-901 HIS5::lys2::ura3::his4::TRP1::his4 | MK166 |
| This study | elg1:HphMX | MK10935 |
| This study | ELG1-13Myc:: KanMX | MK11534 |
| This study | elg1-386 | MK11850 |
| This study | elg1-386 | MK11851 |
| This study | elg1-I27A, I93K, II121,122AA | MK12140 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK14904 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK14946 |
| This study | elg1-I289-319 deletion and an insertion of GCACG-13MYC::KanMX | MK15340 |
| This study | elg1-Sim1 | MK11293 |
| This study | elg1-Sim1 | MK13238 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13389 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13396 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13391 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13395 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13397 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13398 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13402 |
| This study | elg1-I27A, I93K, II121,122AA, TT386/7/13MYC::KanMX | MK13740 |
| This study | elg1-I289-319 deletion and an insertion of GCACG-13MYC::KanMX | MK15524 |
| This study | elg1-HphMX | MK11938 |
| This study | elg1-HphMX | MK13396 |
| This study | elg1-HphMX | MK13394 |
| This study | elg1-HphMX | MK13398 |
| This study | elg1-HphMX | MK13661 |
| This study | elg1-HphMX | MK13662 |
| This study | elg1-HphMX | MK13676 |
| This study | elg1-HphMX | MK15402 |
| This study | elg1-HphMX | MK15740 |
| This study | elg1-HphMX | MK15438B |
| This study | elg1-LEU2 | MK13078 |
| This study | elg1-LEU2 | MK13081 |
| This study | elg1-LEU2 | MK13083 |
| This study | elg1-LEU2 | MK13087 |
| This study | elg1-LEU2 | MK14902 |
| This study | elg1-LEU2 | MK15520 |
| This study | elg1-LEU2 | MK15807 |
| This study | elg1-LEU2 | MK16076 |
| This study | elg1-LEU2 | MK17212 |
| This study | elg1-LEU2 | MK7218 |
| This study | elg1-LEU2 | MK7383 |
| This study | elg1-LEU2 | MK7392 |

### Table 2. List of primers used to generate the Elg1 mutations through site directed mutagenesis

| Oligonucleotide | Sequence (5′→3′) |
|-----------------|----------------|
| Elg1193K        | Forward ATGACGAGCAGATGTAGTCTTAAAGTAATCTAGTGATAAGAGTCC |
| elg1-sim1       | Reverse GGAATCTTATCATGCTTATTGTTAAGTGATATCATGTCGAT |
| Elg1I27A,122AA  | Forward AGCATGAAGATGATATTTCTGCCGCTTCCACATCGAGAATAAC |
| elg1-sim2,3     | Reverse GATTCTGATCAGTGGAAGGGCGGAAAGATCTCTGATGCT |
| elg1TT386,387DD | Forward ATATGCGATGCAATATGGTAGTTGCCGTCGGATGGGAAAGT |
| elg1-386/7/DD   | Reverse GAGAATCTTATCATGCTTATTGTTAAGTGATATCATGTCGAT |
| elg1TT386,387AA | Forward ATATGCGATGCAATATGGTAGTTGCCGTCGGATGGGAAAGT |
| elg1-386/7/AA   | Reverse GAGAATCTTATCATGCTTATTGTTAAGTGATATCATGTCGAT |
| elg1-linker     | Forward CTACCTCTGGAGGAACTGCTAATGGCAAATATGGTGAGGTCG |
| elg1-linker     | Reverse GGAACAAAATCAGCGTCATATTGTTGCCGTCGGATGGGAAAGT |

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After 10 ml wash with buffer T containing 100 mM NaCl, 0.05% ampholytes, 5 mM MgCl₂ and 100 μM ATP, the complex was eluted with 20 ml linear gradient of 100–500 mM NaCl. Fractions containing Elg1–RLC were pooled and concentrated in a Vivaspin concentrator and stored in 2 μl aliquots at −80°C.

**PCNA and Polymerase δ purification.** PCNA and the polymerase δ complex were expressed and purified using the procedure described previously (33).

**RFC complex purification.** The RFC complex was purified from *Escherichia coli* using the procedure described by (34).

**D-loop extension assay**

The D-loop assay was performed essentially as described previously (35). Briefly, the fluorescent D1 oligonucleotide (90-mer; 3 μM nucleotides) was incubated for 5 min at 37°C with Rad51 (1 μM) in 10 μl buffer R (35 mM Tris–HCl pH 7.4, 2 mM ATP, 2.5 mM MgCl₂, 50 mM KCl, 1 mM DTT and ATP regenerating system consisting of 20 mM creatine phosphate and 20 μg/ml creatine kinase). Rad54 (150 nM) was added to the reaction in 1 μl, followed by a 3 min incubation at 23°C. The reaction was initiated by addition of the pBluescript replicative form I (50 μM base pairs) in 1.5 μl and incubated for 5 min at 32°C.

The primer extension reaction was assembled as described mixture containing 12 μl from the D-loop reaction was supplemented with 660 nM RPA, 10 nM PCNA or SUMO-PCNA, 0.3 nM RFC and 33 nM Polδ in buffer O (20 mM Tris–HCl pH 7.5, 5 mM DTT, 150 mM KCl, 40 μg/ml BSA, 8 mM MgCl₂, 5% glycerol and 75 μM each of dGTP and dCTP). PCNA loading reaction was incubated at 30°C for 5 min. The reaction was stopped by cooling on ice followed by addition of increasing concentrations (10, 50, 250 nM) of Elg1–RLC. Alternatively and where indicated, Srs2 (20 nM) and other indicated proteins (Ulp1, 15 nM) were incorporated. The reaction was continued at 30°C for an additional 5 min. DNA synthesis was initiated by addition of buffer O containing 75 μM dTTP and 0.375 μCi [α-32P]dATP. After 10 min extension at 30°C, the reactions were stopped, deproteinized and loaded onto a 8% (w/v) agarose gel. The gel was either directly analyzed for fluorescent DNA species or dried on DE81 paper and exposed to a Phosphorimager screen and imaged in Fuji FLA 9000 imager and analyzed with the Multi Gauge software (Fuji).

**DNA damage sensitivity**

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

To calculate relative MMS resistance, all the strains were plated at the same MMS concentration (0.025% for the *POL30 RAD5* background; 0.008% for the *pol30-RR RAD5* background and 0.0012% for the *POL30 rad5Δ* background) such that individual colonies (20–200 per plate) could be obtained and counted after 3 days at 30°C. Three to six individual cultures were tested this way. The Resistance Coefficient of each mutant is the ratio between the average number of colonies in plates with MMS and the average number of colonies obtained in plates without MMS, divided by the results observed in the wt control.

**Chromatin fractionation assay**

Cells from 50 ml cultures (OD₆₀₀ < 1.0) were collected by centrifugation, successively washed with ddH₂O, PSB (20 mM Tris–Cl pH 7.4, 2 mM EDTA, 100 mM NaCl, 10 mM b-ME) and SB (1 M Sorbitol, 20 mM Tris–Cl pH 7.4), and transferred to a 2 ml eppendorf tube. Cells were suspended in 1 ml SB, 30 μl Zymolase 20T (20 mg/ml in SB) was added, and samples were incubated at 30°C with rotation until >85% spheroplasts were observed (60–90 min). Spheroplasts were collected by centrifugation (2K, 5 min, 4°C), washed twice with SB, and suspended in 500 ml EBX (20 mM Tris–Cl pH 7.4, 100 mM NaCl, 0.25% Triton X-100, 15 mM β-ME + protease/phosphatase inhibitors). Triton X-100 was added to a 0.5% final concentration to lyse the outer cell membrane, and the samples kept on ice for 10 min with gentle mixing. An aliquot was taken for western blot analysis (WCE), and the remainder of the lysate was layered over 1 ml NIB (20 mM Tris–Cl pH 7.4, 100 mM NaCl, 1.2 M sucrose, 15 mM β-ME + protease/phosphatase inhibitors) and centrifuged at 12K RPM for 15 min, at 4°C. A sample of the upper cytoplasmic layer fraction was taken for Western blot analysis (cytoplasm) and the rest of the supernatant was discarded. The glassy white nuclear pellet was suspended in 500 μl EBX and Triton X-100 was added to a 1% final concentration to lyse the nuclear membrane. The chromatin and nuclear debris were collected by centrifugation (15K, 10 min, 4°C). Chromatin was suspended in 50 μl Tris pH 8.0 for western blot analysis (Chromatin). To each fraction an equal volume of 2× SDS-PAGE loading buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, 200 mM DTT) was added, samples were incubated at 95°C for 5 min and sonicated at 80%, 5 cycles, 10 s per cycle. Samples were analyzed by SDS-PAGE and western blot analyses.
1) Threonines 386/7 in Elg1 correspond to asparagusin 694 and 695 of the human Rfc1, which were shown to be involved in the interaction between Rfc1 and the C-terminal region of PCNA (40). These two threonine residues were mutated to differently charged amino acids: either aspartic acid (hereby referred to as elg1-386/7DD mutant) or to alanines (elg1-386/7AA mutant). By introducing these mutations we expected to reduce the strength of interaction between Elg1 and PCNA and therefore to disturb Elg1’s clamp unloading activity.

2) An unstructured loop, spanning from aa 290 to aa 319, containing a hydrophobic patch. This loop is conserved throughout all Elg1 orthologs but is absent in other components of the RFC complex and in the other Rfc1-like proteins (Ctf18 and Rad24). An unstructured loop with a hydrophobic patch is a possible protein-protein interaction site and therefore we deleted this region to identify functions unique to the Elg1–RLC, replacing it with a short flexible linker (see Materials and Methods). We hereafter refer to this allele as elg1-linker.

3) Putative SUMO interaction motifs (SIMs) within the N-terminal domain of Elg1 were shown to affect the interaction between Elg1 and SUMOylated PCNA (19). Mutations in these motifs are likely to disrupt the interaction of Elg1 with the SUMO moiety, but not other interactions (19). The point mutations are located at the following positions: SIM1: I28A, SIM2: I93K, SIM3: I121,122AA.

4) Finally, we combined the elg1-sim and TT386/387 alleles; these alleles are referred to as elg1-sim+386/7AA and elg1-sim+386/7DD. In these alleles, we expect the interactions with both PCNA and SUMO to be disrupted. Corresponding isogenic strains carrying MYC tagged versions of wt or mutant elg1 alleles were generated, at the normal genomic loci and under regulation of the endogenous promoter. The Elg1 protein level remains unchanged in different elg1 alleles compared to the wt (Supplementary Figure S1A). Next, we performed co-IP and showed that the interaction between the various Elg1 alleles and the RFC small subunits is also not altered in the mutants (Supplementary Figure S1B). Hence, any phenotypic change caused by these mutations is not a consequence of reduced protein expression or a reduced formation of the Elg1–RLC complex. This conclusion is further supported by the fact that all mutations presented here are recessive and the MMS sensitivity of these strains can be fully complemented by a centromeric plasmid carrying the wt ELG1 allele (Supplementary Figure S1C).

Elg1 mutants accumulate varying levels of PCNA on the chromatin

Cumulative results from several laboratories suggest that the main role of the Elg1–RLC is to unload PCNA and indeed, elg1Δ cells accumulate PCNA on chromatin (18–20,23). As PCNA can be post-translationally modified, various types of PCNA modifications can be distinguished according to their electrophoretic mobility ([18,19,41,42], Supplementary Figure S1D). In elg1Δ, the increased accumulation of SUMOylated PCNA is the most dramatic (~50-fold increase, compared to only 3-fold increase in accumulation of unmodified PCNA (Figure 2). Fractionation experiments revealed large differences in the ability
of the various elg1 mutants to unload PCNA from chromatin: The elg1-386/7 AA mutant accumulates only a small amount of PCNA on the chromatin, suggesting that this mutant retains most of Elg1’s unloading ability. In contrast, the elg1-386/7 DD mutant accumulates a larger proportion of both SUMOylated and unmodified PCNA on the chromatin. When the three SIM motifs in the N-terminal domain of Elg1 are mutated (elg1-sim), a very mild accumulation of both modified and unmodified PCNA is observed on the chromatin (Figure 2). The double mutant elg1-sim + 386/7DD is as defective in PCNA unloading as the elg1-386/7DD allele, which shows that the SIM motifs are not only mediating the interaction of Elg1 with the SUMO moiety attached to PCNA, but are also important for the interaction with PCNA itself, either directly (by changing the affinity of Elg1 to PCNA) or indirectly (by interacting with another SUMOylated protein to stabilize the interaction and promote the unloading process). In contrast to these results, the elg1-linker mutant appears completely proficient in PCNA unloading, and did not accumulate any form of PCNA on the chromatin (Figure 2).

After determining the PCNA unloading activity of each of the point mutants, we investigated whether it correlates with the severity of the mutants’ phenotypes.

Point mutations in Elg1 impinge on its ability to inhibit in vitro DNA synthesis

Since Elg1–RLC preferentially unloads SUMOylated PCNA from the chromatin during DNA replication (18,21), we asked whether a similar biochemical activity is also necessary for DNA repair. Furthermore, we wished to study the activity of Elg1 in vitro using a purified complex. We therefore modified an already existing in vitro repair/replication system [Figure 3A; (33,43)]. In our assay, Pol δ synthesizes DNA using a D-loop as primer, successfully recapitulating the DNA synthesis step required in all homology-dependent repair/tolerance pathways (44,45) (Figure 3A, upper panel). Importantly, we have successfully used this assay to determine that the Srs2 helicase dissociates the complex between SUMO–PCNA and Polymerase (Pol) δ, thereby regulating the length of extension products during PCNA-dependent DNA repair synthesis (33). The known mechanism of action of Srs2 in the assay served as a unique control for our experiments with Elg1–RLC. In this assay, addition of Elg1–RLC together with SUMO–PCNA resulted in a reproducible 5-fold reduction in DNA synthesis (Figure 3A, compare lanes 9 and 12), which did not occur upon addition of unmodified PCNA to the reaction (Figure 3A, compare lanes 5 and 8), suggesting that Elg1–RLC unloads SUMO–PCNA in vitro. To further corroborate the role of SUMO, we tested whether the presence of Ulp1, a SUMO-specific protease that deSUMOylates proteins, together with Elg1–RLC, would reverse the observed inhibition. As shown in Supplementary Figure S2A, addition of Ulp1 resulted in restoration of DNA synthesis, suggesting that Elg1–RLC indeed acted preferentially upon SUMOylated PCNA.

Elg1–RLC also efficiently inhibited DNA synthesis in the well-defined ΦX-174 system (37) in the presence of SUMO–PCNA (Supplementary Figure S2B), suggesting that the Elg1–RLC PCNA unloading activity is independent of the substrate tested, and therefore relevant for both DNA repair and DNA replication.
Figure 3. Elg1 mutants affect DNA synthesis in vitro. (A) Upper panel. Schematic representation of the reaction. (see Materials and Methods). Lower panel. Elg1–RLC inhibits DNA synthesis in vitro, preferentially in the presence of SUMOylated PCNA. (B) elg1-386/7AA mutant complex inhibits DNA synthesis in the presence of SUMOylated PCNA, whereas elg1-386/7DD complex is defective in the inhibition. (C) elg1-sim is proficient in inhibiting DNA synthesis in the presence of unmodified PCNA. (D) elg1-sim is only partially proficient in inhibiting DNA synthesis in the presence of SUMO–PCNA.

Srs2 inhibits DNA synthesis by dissociating the DNA polymerase from the reaction (33). To test the mode of action of Elg1, we carried out order-of-addition experiments (Supplementary Figure S2C and D). Both Srs2 and the Elg1–RLC complex inhibit DNA synthesis in vitro (Supplementary Figure S2C, lane 9 and Supplementary Figure S2D, lane 9). When SUMO–PCNA remains on the DNA, an addition of the de-SUMOylating enzyme Ulp1 after the action of Srs2 restarts DNA synthesis (Supplementary Figure S2B, lane 13), as Srs2 has 7-fold lower affinity to PCNA over SUMO–PCNA (46). In contrast, when an analogous experiment was performed with Elg1–RLC, we observed no restart of DNA synthesis when Ulp1 was added alone (Supplementary Figure S2A, lane 13). Only when increasing concentrations of the RFC complex were added along with Ulp1, did we observe restart of DNA synthesis. These results are in line with the model in which Elg1–RLC unloads SUMO–PCNA from DNA and thus more RFC complex is needed in order to reload it and allow further DNA synthesis in vitro.

Interestingly, at high Elg1–RLC concentrations a small decrease in mobility of the band that represents the reaction product (e.g., Figure 3A, lane 8) can be observed. This observation points to the possibility that Elg1–RLC may interact with the free PCNA in the reaction, reducing the amount of free PCNA that is able to compete for Polymerase δ binding, and resulting in longer DNA synthesis tracks.

Next, we compared the mutants known to accumulate PCNA on chromatin in this in vitro assay. While elg1-386/7AA mutant alleles showed a minor inhibition of in vitro DNA synthesis, the addition of the elg1-386/7DD protein did not inhibit DNA synthesis (Figure 3B), suggesting that this mutant is unable to unload SUMO–PCNA. These results correlate with the PCNA accumulation data and show that the different elg1 mutants inhibit DNA synthesis in vitro in accordance with their PCNA unloading capabilities in vivo.

The elg1-sim mutant had the same inhibitory effect on DNA synthesis in the presence of unmodified PCNA as the wt Elg1 (Figure 3C, lanes 7 and 10), but when SUMO–PCNA was added to the reaction, the inhibition by elg1-sim was not as high as by wt Elg1 (Figure 3D, lanes 7 and 10). This is in accordance with the role of the SIM motives as
mediators of the interaction of Elg1 with the SUMO moiety attached to PCNA.

The sensitivity of elg1 mutants to DNA damage correlates with their PCNA accumulation status

In order to determine the significance of PCNA accumulation in genomic stability, we tested the sensitivity of various elg1 mutants to DNA damage. MMS (Methyl Methanesulfonate) is a DNA damaging agent that causes DNA methylation. When compared to the elg1 Δ allele, the elg1-386/7AA, elg1-sim and elg1-linker mutants are resistant to MMS whereas the elg1-386/7DD mutant exhibits an intermediate sensitivity to MMS. The double elg1-sim+386/7DD mutant is as sensitive to DNA damage as the elg1Δ strain, showing an additive effect for the SIM motifs and the PCNA interaction site mutations regarding DNA damage sensitivity (Figure 4A). In order to better quantify the effect of the various mutations on the sensitivity to DNA damaging agents, we devised a Resistance Coefficient (RC) parameter (see Materials and Methods for details). In brief, all mutants and the wt strain were diluted and plated on plates without or with 0.025% MMS, and the survival on MMS was calculated for each strain, normalized by comparing it to the survival of the wt. When the RC value is plotted against the relative PCNA accumulation value (derived from the fractionation assays followed by a western blot), a clear correlation can be observed. Figure 4C and D show that the MMS resistance of the elg1 mutants is tightly correlated with both the SUMOylated and unmodified PCNA accumulation status of these mutants (R² = 0.81 and 0.90, respectively). Very similar results were obtained with 4-nitroquinoline 1-oxide (4-NQO), a DNA damaging agent that forms bulky adducts (47) (Figure 4B). Taken together, these results support a model in which the DNA damage sensitivity of elg1Δ cells is caused by PCNA accumulation on the chromatin.

The high recombination rates of ELG1 mutants are dependent on the PCNA unloading activity

It has been reported that elg1Δ cells exhibit high rates of homologous recombination [HR; (19,31)]. Using MK166, a strain in which different types of recombination events can be monitored, we measured the rate of direct repeat recombination (DRR) and non-reciprocal recombination between Ty elements (gene conversion) (30) (see Materials and Methods and Figure 5A). As previously described (31), elg1Δ strains exhibit a greatly increased rate of HR (~5-fold increased for DRR, ~18-fold for Ty recombination). The DRR and Ty recombination rates of the elg1-386/7AA and elg1-sim mutants are comparable to those of the wt strain, whereas in the elg1-386/7DD mutant the Ty recombination rate is intermediate and the DRR rate is similar to that of elg1Δ. Combining the SIM mutations with the 386/7 replacements results again in an additive effect, as in the DNA damage sensitivity assay, and the rates of Ty recombination and DRR of the double mutant elg1-sim+386/7DD are close to those seen in the strain harboring a complete ELG1 deletion. The elg1-linker mutation did not affect the levels of homologous recombination (Figure 5B). Thus, we could see a strong correlation between the rates of recombination and the level of unmodified or SUMOylated PCNA accumulation (R² = 0.80 and 0.88, respectively; Figure 5C and D). Our results show that the elg1 mutants that accumulate more PCNA on the chromatin are more sensitive to DNA damage and have higher recombination rates and thus PCNA accumulation correlates with genomic instability.

The MMS sensitivity of elg1Δ is related to unmodified PCNA unloading

PCNA can undergo ubiquitination at lysine 164, and SUMOylation at lysines 127 and 164 (3). pol30-ΔKK164,127RR (hereafter referred to as pol30-RR) is a mutant allele of PCNA in which these two critical lysines are mutated to an arginine, preventing post translational modifications.

We tested various mutant elg1 alleles in the background of the pol30-RR mutation in order to determine the importance of PCNA modifications for the activity of the Elg1–RLC (Figure 6). Significantly, the elg1Δ mutation increases the sensitivity to MMS of a pol30-RR mutant, implying that part of the elg1Δ phenotype is independent of PCNA modifications (Figure 6A).

The MMS sensitivity of the mutants in a pol30-RR background shows the same gradient of sensitivities as in the POL30 background (Figure 4A), with a general shift towards more severe sensitivity: the elg1-386/7DD mutant is as sensitive as elg1Δ, elg1-386/7AA is less severe than elg1-386/7DD but more sensitive than the wt. The elg1-sim and elg1-linker mutations show no MMS sensitivity, and combining the 386/7 mutations with the SIM mutations resulted in an additive phenotype: the pol30-RR elg1-sim+386/7AA mutant shows a more severe phenotype than the single mutants and the pol30-RR elg1-sim+386/7DD combination shows a sensitivity as high as that of the pol30-RR elg1Δ double mutant. The unmodified PCNA accumulation level in the different elg1 mutant strains varies in the pol30-RR background according to the same gradient that was observed in the wt background: elg1-386/7DD and elg1-sim+386/7DD mutants accumulate unmodified PCNA at levels similar to those seen in the elg1Δ pol30-RR double mutant. elg1-386/7AA, elg1-sim and elg1-sim+386/7AA accumulate only a small amount of unmodified PCNA and the elg1-linker mutants do not accumulate unmodified PCNA at all. Thus, the correlation between DNA damage sensitivity and PCNA accumulation is not affected by mutations that preclude PCNA modification (R² = 0.847; Figure 6C).

Taken together, our results show that the phenotypic variation observed among the elg1Δ mutants correlates with the amount of PCNA that is retained on the chromatin, irrespective of its modification status.

Suppression of the DNA damage sensitivity of rad5Δ mutants

Another interesting phenotype of elg1Δ is its suppression of the sensitivity to MMS of the rad5Δ mutant (19). RAD5 is best known as an E3 ubiquitin ligase that poly-ubiquitinates PCNA in response to DNA damage, together with the E2
Mms2/Ubc13 (48,49). Poly-ubiquitination of PCNA occurs sequentially after mono ubiquitination by the SUMO-dependent Ubiquitin ligase Rad18 (50) and is required to direct the cell towards an error-free lesion bypass pathway. In addition, Rad5 has an helicase activity that has been suggested to reverse fork progression (51). rad5Δ cells are very sensitive to MMS and a deletion of ELG1 partially suppresses this high sensitivity. ELG1 deletion was shown to specifically suppress the MMS sensitivity caused by defects in the E3 ubiquitin ligase activity of Rad5 (19). The mechanism by which a deletion of ELG1 suppresses rad5Δ MMS sensitivity and the PCNA modification status that is required to allow this suppression effect is unclear. We decided to approach this question by trying to correlate the PCNA unloading activity and the ability of the different ELG1 mutations to suppress the MMS sensitivity of rad5Δ.

With the exception of the elg1-linker allele, which seems to retain the full activity of wt Elg1, all the other mutations suppress rad5Δ MMS sensitivity to the same extent as the elg1Δ allele (Figure 6A). Strains deleted for RAD5 accumulate suppressors at a very high rate and therefore it was challenging to perform fractionation assays; Figure 6B shows that the accumulation of unmodified PCNA of elg1 mutants in the absence of RAD5 was similar to that seen in the RAD5 background, with the elg1-386/7DD and elg1-sim+386/7DD mutants showing the highest levels. Interestingly, the SUMOylated fraction of PCNA, which is usually much less prominent than the unmodified fraction, was not consistently detected in rad5Δ mutants regardless of the ELG1 allele in the strain (see Discussion). The RC scores for unmodified PCNA accumulation levels in the various mutants in the presence of MMS in the rad5Δ background showed no correlation (R² = 0.052) with MMS sensitivity (Figure 6C). The almost-universal suppression of rad5Δ by elg1 alleles can be interpreted in two ways (see model in Figure 8C): (i) Reduced Elg1–RLC activity leads to both modified and unmodified PCNA accumulation. In the absence of the error free DNA repair pathway the SUMOylated PCNA that is retained on the chromatin recruits the Srs2 helicase (8) and thus causes an inhibition of the HR pathway. However, the unmodified or the mono-ubiquitinated PCNA that is retained on the chromatin is enough to enable a pathway that can partially bypass the DNA damage sensitivity, and this is achieved in all the mutants tested, with the exception of the elg1-linker allele, which seems to retain the full capacity of PCNA unloading of wt Elg1 (Figure 6B and C). (ii) Alternatively, the suppression of the MMS sensitivity phenotype of rad5Δ mutants is a consequence of another activity of Elg1 that is not related to PCNA unloading, and which is abolished by all the mutations except for the elg1-linker mutant. We prefer the first option, which is more parsimonious. In addition, we have recently found that mutations which prevent Elg1 phosphorylation upon DNA damage (S6,8,112A and S,6,8,112E) suppress the MMS sensitivity of rad5Δ, even when, in a RAD5 genetic background, they show no MMS sensitivity (52). The large number of independent mutants with partial or total reduction of Elg1 PCNA unloading activity that are able to suppress the DNA damage sensitivity of rad5Δ mutants suggests that an accu-
Figure 5. Recombination rates of *elg1* mutants are correlated with PCNA accumulation. (A) A schematic representation of the MK166 recombination levels monitoring system. (B) Recombination rates were calculated for various *elg1* mutants by plating the MK166 yeast strain on selective media (see Materials and Methods). (C and D) The recombination rates plotted against unmodified and SUMOylated PCNA accumulation, respectively.

Figure 6. The sensitivity to MMS in the various mutants is not dependent on PCNA modifications. (A) Serial dilutions of yeast cultures on minimal SD-complete plates with or without MMS in the indicated concentrations show different sensitivities of *elg1* mutants to MMS in MK166 *pol30-RR* background. (B) PCNA levels on the chromatin of the same strains. (C) The RC in a *pol30-RR* background correlates with the ability of the different *elg1* mutants to unload PCNA.
elg1 mutantssuppress rad5Δ MMS sensitivity. (A) Serial dilutionsofyeastculturesonminimal SD-completeplateswithormwithout MMS at the indicated concentrations show suppression of MK166 rad5Δ MMS sensitivity by all of the mutants except for elg1-linker. (B) Unmodified PCNA levels on the chromatin in the various elg1 mutants in the rad5Δ background. (C) The RC in rad5Δ background does not correlate with the ability of the different elg1 mutants to unload PCNA.

Suppression of the MMS sensitivity of rad5Δ mutants requires PCNA modification

To further understand the mechanism that suppresses the sensitivity of rad5Δ mutants when ELG1 is deleted, we introduced all the elg1 mutations into a rad5Δ pol30-RR background, in which PCNA cannot be modified, and tested the ability of the various strains to grow in the presence of MMS. The pol30-RR allele suppresses the rad5Δ MMS sensitivity (to the level of resistance observed in the single pol30-RR) showing that the MMS sensitivity of rad5Δ is due to PCNA modification. Importantly, deletion of ELG1 had the same additive effect with the pol30-RR allele in the double mutant pol30-RR rad5Δ background and in the single pol30-RR background, and so did the various elg1 alleles (compare Figures 6A and 8A). This implies that while PCNA modifications are a pre-requisite for rad5Δ MMS sensitivity (there is no sensitivity if PCNA is not modified), the level of PCNA unloading by Elg1–RLC has a different phenotypic effect that depends on the PCNA modification status (in a modifiable PCNA background all elg1 alleles have the same effect, whereas a gradient of phenotypes is seen when PCNA is unmodified).

To directly test the effect of PCNA accumulation on the chromatin and the PCNA modifications required to allow elg1Δ suppression of rad5Δ MMS sensitivity, we overexpressed three PCNA alleles: modifiable wt POL30, the unmodifiable pol30-RR allele, or a PCNA allele that cannot be ubiquitinated (pol30-164R), in wt, elg1Δ, rad5Δ and rad5Δ elg1Δ mutant strains (Figure 8B). It has been shown (22,53), that PCNA overexpression causes an accumulation of PCNA on the chromatin. Overexpression of pol30-RR confers slight MMS sensitivity to both wt and elg1Δ strains. However, whereas overexpression of POL30 has no effect on the wt strain, it leads to increased sensitivity in the elg1Δ strain; this is consistent with the accumulation of PCNA being responsible for the MMS sensitivity: when Elg1–RLC is active, some of the PCNA can be removed from the chromatin; when it is inactive (in elg1Δ strains), PCNA accumulates even more. Interestingly, the slight sensitivity conferred by the overexpression of pol30-RR is similar whether Elg1 is present or absent, showing again a preference of the Elg1–RLC for unloading SUMOylated PCNA (19).

Consistent with the idea that PCNA modifications are required for elg1Δ suppression of the MMS sensitivity of rad5Δ, overexpression of neither pol30-RR nor pol30-164R suppresses the sensitivity of a rad5Δ mutant. Only the overexpression of modifiable POL30 is able to suppress the MMS sensitivity of rad5Δ (Figure 8B). These results suggest that lysine 164, the target of mono-ubiquitination, is essential for the suppression of rad5Δ. The suppression effect of pol30-164R overexpression is epistatic to the effect caused by deletion of ELG1, again supporting the idea that the sup-
elg1Δ suppression of rad5Δ MMS sensitivity depends on PCNA modifications. (A) Serial dilutions of yeast cultures on minimal SD-complete plates with or without MMS at the indicated concentrations show different sensitivities of elg1 mutants to MMS in MK166 pol30-RR rad5Δ background. (B) An overexpression plasmid of POL30, pol30-RR or pol30-164R alleles was introduced into the indicated strains and the MMS sensitivity of these strains was tested. (C) A deletion of REV3 abolishes the suppression effect of elg1Δ on rad5Δ MMS sensitivity. (D) A schematic representation of the model proposed: in wt cells the Elg1 RLC unloads PCNA from the DNA. This may take place during DNA repair or during DNA replication. Polyubiquitination of PCNA by Rad5 allows error-free repair. In the absence of ELG1, PCNA accumulates and becomes modified, mainly by SUMOylation. In rad5Δ cells, the error-free post-replication bypass mechanism triggered by PCNA poly-ubiquitination is eliminated, leading to sensitivity to DNA damage. In the absence of both RAD5 and ELG1 the TLS damage bypass pathway, mediated by PCNA mono ubiquitination and Rev3, can operate.

pression of rad5Δ by deletion of Elg1 is due to the accumulation of mono-ubiquitinated PCNA. Accordingly, overexpression of PCNA that cannot be ubiquitinated (either pol30-164R or pol30-RR) abolishes the suppression effect conferred by elg1Δ in the rad5Δ background (Figure 8B). In contrast to the almost full suppression observed in rad5Δ siz1Δ or rad5Δ srs2Δ double mutants (8, 17, 54), which are explained by the availability of HR as an alternative repair mechanism in the absence of the error-free Rad5-initiated bypass pathway, deletion of ELG1 has a milder effect, which can be explained by the use of the less efficient trans-lesion synthesis (error-prone) pathway upon accumulation of low levels of mono-ubiquitinated PCNA. Mono-ubiquitination of PCNA enables the recruitment of trans-lesion synthesis polymerases, such as Rev3 (PolZeta) [reviewed in (3)].

In order to test that hypothesis we deleted REV3 in the rad5Δ elg1Δ background. As expected, ablation of the trans-lesion synthesis polymerase abolished the suppression effect of elg1Δ on rad5Δ (Figure 8C). We therefore conclude that the suppressive effect of elg1 mutants in the rad5Δ background is caused by accumulation of mono-ubiquitinated PCNA on the chromatin, which partially rescues rad5Δ hyper sensitivity phenotype in a REV3-dependent manner.

**DISCUSSION**

In this paper, we have investigated the correlation between the Elg1’s clamp unloading activity, the accumulation of modified and unmodified PCNA on the chromatin, and genomic instability. In order to do so we generated a set of mutations in designated regions with a differential ability to unload PCNA. Accordingly, overexpression of PCNA that cannot be ubiquitinated (either pol30-164R or pol30-RR) abolishes the suppression effect conferred by elg1Δ in the rad5Δ background (Figure 8B). In contrast to the almost full suppression observed in rad5Δ siz1Δ or rad5Δ srs2Δ double mutants (8, 17, 54), which are explained by the availability of HR as an alternative repair mechanism in the absence of the error-free Rad5-initiated bypass pathway, deletion of ELG1 has a milder effect, which can be explained by the use of the less efficient trans-lesion synthesis (error-prone) pathway upon accumulation of low levels of mono-ubiquitinated PCNA. Mono-ubiquitination of PCNA enables the recruitment of trans-lesion synthesis polymerases, such as Rev3 (PolZeta) [reviewed in (3)].

We have also mutated a unique unstructured loop containing a hydrophobic patch that is conserved among ELG1 orthologs but not in other Rfc1-like proteins. Surprisingly, deletion of this loop had no noticeable phenotype and the
mutants were able to carry out all the known activities of Elg1 at wt levels. The function of this loop thus remains unknown.

All previous in vitro work with Elg1 was carried out with Elg1–RLC immunoprecipitated from yeast cells. Here we show for the first time, using purified proteins, that the Elg1 complex inhibits DNA synthesis, by a mechanism that is different from that shown for the Srs2 helicase (33): Whereas Srs2 interacts with SUMOylated PCNA and dissociates it from the DNA polymerase, the Elg1–RLC unloads the modified PCNA altogether (Figure 3 and Supplementary Figure S2C and D).

We have tested the PCNA accumulation status of the mutants and discovered a gradient of accumulation levels, which reflects the capacity of the various mutants to unload PCNA from chromatin (Figure 2). A striking correlation was found between the PCNA unloading ability and genomic instability phenotypes such as: MMS sensitivity, 4-NQO sensitivity and the recombination frequency of the elg1 mutant strains. This correlation suggests that the sensitivity to DNA damage and the increased recombination rate in elg1 mutants are the consequence of the retention of PCNA on the chromatin, probably through the inappropriate recruitment of interacting partners that are important for choosing the repair mechanism or the processing of recombination intermediates.

ELG1 is not an essential gene; in its absence PCNA is eventually unloaded by alternative mechanisms and therefore either the physical accumulation of the clamps for an extended period of time or the delay in completing DNA replication cause genome instability.

What form of PCNA (modified or unmodified) is responsible for the phenotypes of elg1 mutants? Abolishing PCNA modifications (by using pol30-RR allele) caused a sensitization of all the elg1 mutants in a way that retained these mutant’s graduated differential DNA damage sensitivity phenotype observed in the POL30 background (Figure 4). Thus, PCNA modification is not a pre-requisite for the enhanced MMS sensitivity of elg1 mutants. While this paper was in review, Johnson and co-workers presented additional evidence that the MMS sensitivity of elg1 mutants. While this paper was in review, Johnson and co-workers presented additional evidence that the MMS sensitivity of elg1Δ is a consequence of PCNA retention on the chromatin (22). Taken together, our results fit a model in which PCNA SUMOylation functions as a hallmark of PCNA retention and its levels build up when PCNA is not unloaded from the chromatin by the Elg1 complex. Since PCNA requires proximity or attachment to DNA in order to get SUMOylated (42), it is possible that the accumulation of SUMOylated PCNA on the chromatin is time-dependent, and thus more SUMOylated proteins are seen on the chromatin as a function inversely proportional to the activity of the various elg1 mutants.

Paradoxically, we see that the suppressive effect of elg1 mutations on rad5Δ requires PCNA modification. Except for the elg1-linker mutants, all the other mutants were able to suppress the MMS sensitivity conferred by deletion of RAD5. Previously, several groups have shown that the sensitivity of rad5Δ mutants to genotoxic agents can be suppressed either by preventing PCNA SUMOylation (by using the pol30-RR allele or deleting SIZ1), or by deleting the helicase SRS2 (8,17,54). The current model thus proposes that the MMS sensitivity of rad5Δ strains is caused by PCNA SUMOylation that indirectly prevents the cells from repairing DNA damage by HR. The suppression of rad5Δ by mutations in ELG1 is milder than that seen in the absence of PCNA SUMOylation or Srs2, and it was unclear what is the precise mechanism by which it takes place. As most elg1 mutants tested in this study, and in a recently published one (52) show full suppression, irrespective of the position of the mutation or of the severity of the unloading defect, it appears that a minor accumulation of PCNA provides a threshold above which the suppression can occur. Since the only allele that failed to suppress the sensitivity of a rad5Δ strain is the elg1-linker allele, which displays wild type levels of PCNA unloading ability, the suppression effect seems related to the clamp unloading activity of Elg1. Overexpression experiments (Figure 8B) showed that the suppression requires modifiable, and specifically mono-ubiquitinated, PCNA, which would allow lesion bypass by recruiting a trans-lesion synthesis polymerase (56). Indeed, deletion of REV3, the gene encoding the main trans-lesion synthesis polymerase in yeast, abolished the suppression conferred by deletion of ELG1 (Figure 8C). Previous genetic results indeed showed that rad5Δ mutants exhibit high levels of mutagenesis, which depend both on the TLS polymerase encoded by REV3 and on the mono-ubiquitination activity of the RAD18 gene (57). Interestingly, whereas a single copy of the pol30–RR allele in the genome suppresses the sensitivity of rad5Δ mutants (compare Figures 7A and 8A) and similar results are observed when wt POL30 is overexpressed (Figure 8B), overexpression of pol30–RR and of pol30-164R (in the presence of a wt genomic PCNA copy) failed to show such effect, even in the absence of Elg1–RLC activity. These results imply that the sensitivity of rad5Δ to MMS is due to the presence of SUMOylated PCNA; if the only copy of PCNA present cannot be modified, a reduced sensitivity to genotoxins is seen. This is consistent with our model (Figure 8D): In wt cells, lesions are repaired in an error-free manner by the RAD5 pathway, enabled by poly-ubiquitination of PCNA. In the absence of the Rad5-initiated bypass mechanism, recruitment of Srs2 to SUMOylated PCNA causes sensitivity, as it prevents an alternative repair by HR. Deletion of ELG1 suppresses the high sensitivity of rad5Δ mutants by delaying PCNA unloading, allowing more mono-ubiquitinated PCNA to remain on the chromatin, and thus enabling the recruitment of the Rev3 trans-lesion synthesis polymerase (Figure 8D).

In conclusion, our work shows that modified and unmodified PCNA have a variety of roles in conferring genome stability and these roles are greatly affected by the activity of Elg1-RFC clamp unloader.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank all members of the Kupiec and Krejci labs for ideas and support.
FUNDING
Israel Science Foundation (ISF); Israel Cancer Research Fund (to M.K.); Czech Science Foundation [GACR13-26629S, GACR207/12/2323 to L.K.]; project no. LQ1605 from the National Program of Sustainability II (MEYS CR) [FNUSA-ICRC no. CZ.1.05/1.1.00/02.0123 (OP VaVpI)]; ICRC-ERA-HumanBridge [316345] funded by the 7th Framework Programme of the European Union. Funding for open access charge: ISF.

Conflict of interest statement. None declared.

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