ACCELERATED PUBLICATION

The human checkpoint sensor and alternative DNA clamp Rad9–Rad1–Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery

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The human checkpoint sensor and alternative clamp Rad9–Rad1–Hus1 can interact with and specifically stimulate DNA ligase I. The very recently described interactions of Rad9–Rad1–Hus1 with MutY DNA glycosylase, DNA polymerase β and Flap endonuclease 1 now complete our view that the long-patch base excision machinery is an important target of the Rad9–Rad1–Hus1 complex, thus enhancing the quality control of DNA.

Key words: DNA glycosylase, DNA ligase I, DNA polymerase β, Flap endonuclease 1 (Fen1), proliferating-cell nuclear antigen (PCNA), Rad9–Rad1–Hus1 complex (9-1-1 complex).

INTRODUCTION

The mammalian genome undergoes approx. 100000 modifications per day. DNA base damage generated by ionizing radiation and simple alkylating agents, as well as by endogenous hydrolytic and oxidative processes, is corrected by the BER (base excision repair) pathway [1]. Two BER sub-pathways have been characterized using *in vitro* and *in vivo* methods, and have been classified according to the length of the repair patch as either 'short-patch' BER (SP-BER, one nucleotide) or 'long-patch' BER (LP-BER; more than one nucleotide) [2]. The first step in both BER sub-pathways is carried out by DNA glycosylases [examples being OGG1 (8-oxoguanine DNA glycosylase 1), NTH1 (Nth endonuclease III homologue 1) and MYH (MutY human homologue)], which are specific for a particular type of base damage or, more commonly, a group of related types. These enzymes remove the damaged bases, leaving potentially mutagenic AP (apurinic/apyrimidinic) sites. A resulting abasic site is then recognized by APE1 [AP endonuclease 1, also known as HAP1 (human apurinic endonuclease 1)], which incises the damaged strand, leaving a single-nucleotide gap with 3′-OH and 5′-dRP (5′-deoxyribose phosphate) groups flanking the nucleotide gap. Further repair can be accomplished via two pathways that involve different subsets of enzymes and result in replacement of one (SP-BER pathway) or two to ten (LP-BER pathway) nucleotides. In mammalian cells, SP-BER is considered to be the major BER pathway, whereas LP-BER is an important back-up pathway [3].

In the LP-BER pathway, the collaboration of DNA pol (polymerase) δ/ε, PCNA (proliferating-cell nuclear antigen), RF-C (replication factor C) and Fen1 (Flap endonuclease 1) can displace the 5′ nick and synthesize up to ten nucleotides. The flap is cut by Fen1, and the final nick is sealed by DNA ligase I [4,5]. Both pols δ and ε can participate in this reaction [6], and DNA ligase I is likely to be the patch size mediator in LP-BER [7]. The same protein components are also required for *in vitro* reconstitution of the LP-BER pathway for 7′8′-dihydro-8-oxoguanine [8]. On the other hand, the LP-BER pathway can also be carried out by pol β, Fen1 and DNA ligase I. Here, pol β, with its strand displacement synthesis, and Fen1 co-operate with the so-called 'hit-and-run' mechanism [9]. Finally, it was found that the tumour-suppressor protein APC (adenomatous polyposis coli) can block the strand-displacement synthesis by pol β during LP-BER, thus increasing the sensitivity to methylmethane sulphonate [10]. The LP-BER proteins DNA glycosylase, HAP1, pol β, Fen1 and DNA ligase I can all interact physically with the PCNA clamp [11].

On the other hand, checkpoints are activated upon DNA damage in eukaryotic cells in order to stop cell-cycle progression. This activation requires the action of DNA-damage sensors and transducers [12]. Among these, the three human proteins Rad9, Hus1 and Rad1 form a heterotrimeric complex (called the 9-1-1 complex) exhibiting structural similarity with the homotrimeric clamp PCNA [13,14] that can be loaded on to DNA by the Rad17–RF-C_{2-5} clamp loader [15]. Moreover, the 9-1-1 complex, Rad17–RF-C_{2-5} and PCNA co-localize in foci formed upon DNA damage [16,17]. These data suggested a mechanism in which Rad17–RF-C_{2-5} would localize on DNA lesions, allowing the recruitment of the 9-1-1 complex to these sites. Subsequently, the 9-1-1 complex would serve as a recruiting platform for the checkpoint effector kinases such as Chk1 or Chk2, which are subsequently phosphorylated by the ATR [ATM (ataxia telangiectasia mutated) and Rad3-related]/ATM kinases [18]. Additionally, a model has recently been proposed by two different groups, where the 9-1-1 complex and the Rad17–RF-C_{2-5} clamp loader could stabilize stalled replication forks [19,20].

The mechanisms by which DNA repair occurs are now quite well unravelled [1]. Likewise, the way that cells trigger the DNA-damage checkpoints is also starting to be deciphered [18]. However, the link between checkpoint engagement and the recruitment to DNA lesions of repair machineries is far from being understood. Recent studies performed in yeast have shown interaction or co-localization of the alternative checkpoint clamp, the 9-1-1 complex, with proteins involved in various DNA-repair processes upon DNA damage [17,21,22]. In view of these studies,

Abbreviations used: 9-1-1 complex, Rad9–Rad1–Hus1 complex; AP, apurinic/apyrimidinic; ATM, ataxia telangiectasia mutated; BER, base excision repair; DTT, dithiothreitol; Fen1; Flap endonuclease 1; GST, glutathione S-transferase; HAP1, human apurinic endonuclease 1; LP-BER, long-patch BER; PCNA, proliferating-cell nuclear antigen; pol, DNA polymerase; RF-C, replication factor C; SP-BER, short-patch BER; the prefix h denotes human.

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and of its localization on to DNA lesions, the 9-1-1 checkpoint complex could be a potential candidate for the role of 'recruiting platform' for DNA-repair effectors.

Recent investigations searched for a possible link between the human 9-1-1 complex and the BER pathway. First, we reported a physical and functional interaction of the 9-1-1 complex with the main BER pol, pol β [23]. Similar physical and functional interactions with the 9-1-1 complex were subsequently identified for human Fen1 [24,25] and for the MutY homologue of *Schizosaccharomyces pombe* [26]. In the present paper, we document the physical and functional interaction of another LP-BER component, DNA ligase I, with the 9-1-1 complex. We hypothesize that the alternative clamp 9-1-1 might be an important sensor and adapter for the BER machinery in human cells.

Table 1 The clamp PCNA and the alternative clamp and checkpoint sensor 9-1-1 complex can interact with components of the LP-BER machinery

LP-BER component	Interacting partner	Reference
DNA glycosylase	PCNA	[29]
MutY DNA glycosylase	9-1-1 complex	[26]
HAP1	PCNA	[30]
HAP1	9-1-1 complex	Not known
Pol β	PCNA	[31]
Pol β	9-1-1 complex	[23]
Fen1	PCNA	[32]
Fen1	9-1-1 complex	[24,25]
DNA ligase I	PCNA	[33]
DNA ligase I	9-1-1 complex	The present study

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]dATP (3000 Ci/mmol) and unlabelled ATP were from Amersham Biosciences. DNA oligonucleotides were purchased from Microsynth GmbH (Balgach, Switzerland). DNase, RNase and protease-free formamide were from Acros Organics. T4 polynucleotide kinase was from New England Biolabs. All other reagents were from Merck, Fluka or Sigma.

Nucleic acid substrates

The sequences of oligonucleotides used to prepare the substrates for the DNA ligase I assays are the following: 46-mer, 5′-AGATTTTTCATTTGCTGCTGGCTCTCAGCGTGGCACTGTTGCAGGC-3′; 25-mer, 5′-CCTGCAACAGTGCCACGCTGAGAGC-3′; 19-mer, 5′-CAGCAGCAAATGAAAAATC-3′ (for schematic representations see Figure 2A). The 25-mer and 19-mer were labelled at the 5′-end in a buffer containing 70 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT (dithiothreitol), an equimolar amount of [γ-^{32}P]ATP or unlabelled ATP and T4 polynucleotide kinase for 45 min at 37 °C. T4 polynucleotide kinase was heat-inactivated for 10 min at 80 °C, and free ATP was removed on Microspin™ G-25 columns. To generate the substrates for the DNA ligase I assays, the appropriate oligonucleotides were mixed in a 1:1 molar ratio in a buffer containing 50 mM Tris/HCl, pH 7.4 and 150 mM NaCl, heated for 10 min to 75 °C and slowly cooled to room temperature (20 °C).

Enzymes and proteins

Myoglobin was purchased from Serva Feinbiochemica (Heidelberg, Germany). hPCNA (human PCNA) was produced in *Escherichia coli* using the plasmid pT7/hPCNA and was purified to homogeneity as described in [27]. Human DNA ligase I was expressed in *E. coli* and was purified as described in detail by Jónsson et al. [28]. Untagged or histidine-tagged (His-tagged) 9-1-1 complexes were respectively obtained by co-expressing in Sf9 insect cells the three baculoviruses encoding the recombinant hRad1, hRad9 and hHus1 or recombinant hRad1, hRad9 and His-hHus1 (see Figure 2B). The 9-1-1 complex was subsequently purified as described in [23]. GST (glutathione S-transferase) was expressed in *E. coli* strain TG1 and was purified by binding on glutathione–Sepharose beads as described by Toueille et al. [23].

Pull-down assays

His-tagged human DNA ligase I bound to ProBond™ beads (Invitrogen) was incubated with purified 9-1-1 complex or purified GST as a negative control for 2 h at 4 °C in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.1 % (v/v) Nonidet P40, 1 mM 2-mercaptoethanol and 1 mM PMSF. After washing four times in the same buffer, the beads were heated for 5 min at 95 °C in Laemmli buffer, and the co-precipitated proteins were analysed by Western blot using the corresponding antibodies according to established methods.

Activity assays for DNA ligase I

The labelled DNA substrate presented in Figure 2(A) (25 fmol) was incubated for 20 min at 37 °C with recombinant human DNA ligase I in presence or absence of various amounts of the His-tagged 9-1-1 complex or PCNA, or myoglobin as a negative control. The reaction was performed in a final volume of 10 μl containing 40 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP. After boiling for 5 min at 100 °C with formamide containing loading buffer, samples were electrophoresed through a 15 % denaturing polyacrylamide gel containing 7 M urea and 7 M formamide. Labelled oligonucleotides were detected by autoradiography and quantified by PhosphorImager analysis (Molecular Dynamics).

RESULTS AND DISCUSSION

The human checkpoint sensor and alternative DNA clamp 9-1-1 complex can interact with components of the LP-BER machinery

The clamp PCNA and the alternative checkpoint clamp, the 9-1-1 complex, can interact with components of the LP-BER machinery. It is known that PCNA, initially identified as a processivity clamp for pols δ and ε, can interact with more than 25 cellular proteins (reviewed in [11]). Among them there are components of the LP-BER machinery (Table 1). They include DNA glycosylase [29], HAP1 [30], pol β [31], Fen1 [32] and DNA ligase I [33]. We have initially shown that the 9-1-1 complex can interact physically with pol β *in vitro*, and functional analysis revealed that the 9-1-1 complex had a specific stimulatory effect on pol β activity [23]. Pol β stimulation resulted from an increase in its affinity for the primer template, and the interaction with the 9-1-1 complex stimulated deoxyribonucleotide misincorporation by pol β. Finally, the 9-1-1 complex enhanced DNA strand-displacement synthesis by pol β, an activity required for LP-BER, raising the possibility that the 9-1-1 complex might attract pol β to DNA-damage sites, thus connecting checkpoints and DNA repair directly. More recently, it was found that the 9-1-1 complex can interact with and stimulate Fen1 [24,25], and that the 9-1-1 complex of *S. pombe* can interact with DNA glycosylase MutY homologue [26].

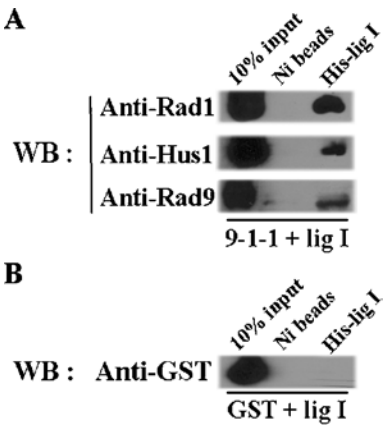

Figure 1 The human 9-1-1 complex interacts with human DNA ligase I

Pull-down of the 9-1-1 complex and DNA ligase I. (**A**) His pull-down experiments were performed in the presence of either His-tagged DNA ligase I (5 μg) or nickel beads alone, and purified 9-1-1 complex (3.6 μg). (**B**) The control His pull-down experiments were performed in the presence of either His-tagged DNA ligase I (5 μg) or nickel beads alone, and purified GST (3.6 μg). The presence of co-precipitated proteins was determined by SDS/PAGE followed by Western blot (WB) analysis. lig I, DNA ligase I.

The human 9-1-1 complex can physically interact with DNA ligase I

Based on these results, we first tested whether the 9-1-1 complex can interact with DNA ligase I. As shown in Figure 1, the 9-1-1 complex can interact physically with DNA ligase I in a His pull-down experiment performed after incubation of the purified 9-1-1 complex with His-tagged DNA ligase I or with nickel beads alone (Figure 1A). Western blot analysis against Rad9, Rad1 and Hus1 showed a specific co-precipitation of the complex with His-tagged DNA ligase I, but not with nickel beads used as a control. To check for the specificity of this interaction, GST was incubated under the same conditions with DNA ligase I or nickel beads. As shown in Figure 1(B), GST was not co-precipitated with either DNA ligase I or the nickel beads, confirming that the 9-1-1 complex interacts specifically with DNA ligase I *in vitro*.

The human 9-1-1 complex can stimulate DNA ligase I

Next, we tested the effect of the 9-1-1 complex on the activity of DNA ligase I *in vitro*. From Figures 2(C) and 2(D), it is evident that the 9-1-1 complex can stimulate DNA ligase I even at a 1:1 ratio (Figure 2C, compare lanes 3 and 4, and see quantification in Figure 2D). Stimulation of DNA ligase I activity up to 12-fold was observed at a 9-1-1 complex/DNA ligase I ratio of 27:1 (Figure 2C, compare lane 3 and 7, and see quantification in Figure 2D). The stimulation is specific, since adding the same amounts of myoglobin gave virtually no stimulation (Figure 2C, lanes 9–13, and see quantification in Figure 2D).

Human PCNA can stimulate human DNA ligase I slightly, but cannot prevent the stimulation of DNA ligase I by the human 9-1-1 complex

Since it is known that PCNA can interact with DNA ligase I [33], we next tested the effect of PCNA on DNA ligase I and compared it with the stimulatory effect of the 9-1-1 complex. Figure 3(A) (lanes 3–7) documents as a positive control the stimulation of DNA ligase I by the 9-1-1 complex. Furthermore, PCNA could, as expected, also stimulate DNA ligase I, but to a much lesser extent than the 9-1-1 complex (up to a 2.8-fold stimulation; Figure 3A, lanes 9–13 and Figure 3C-2). Next, we tested the

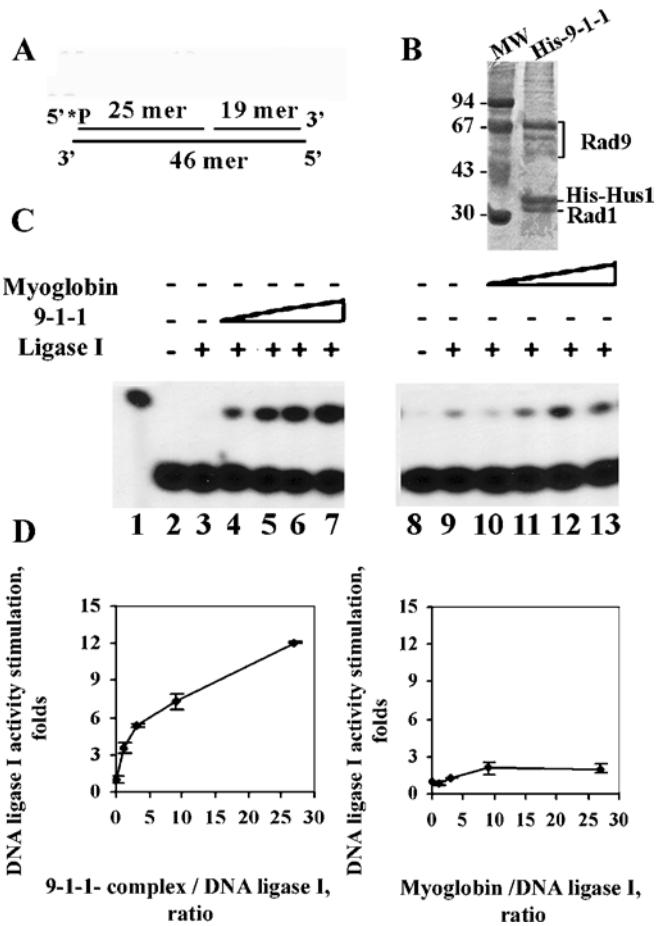

Figure 2 The human 9-1-1 complex can stimulate human DNA ligase I

DNA ligase activity was determined as described in the Materials and methods section. (**A**) Substrate used in DNA ligase assays. (**B**) SDS/PAGE, followed by Coomassie Blue staining of the purified His-9-1-1 complex used in the assays. MW, molecular-mass values. (**C**) Effect of the 9-1-1 complex on DNA ligase I activity. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, 46-mer marker; lane 2, no enzyme control, 25-mer marker; lane 3, DNA ligase I alone; lanes 4–7, 30, 90, 270 or 810 fmol respectively of the purified recombinant human 9-1-1 complex was added; lane 8, no enzyme control; lane 9, DNA ligase I alone; lanes 10–13, 30, 90, 270 or 810 fmol respectively of myoglobin was added. (**D**) Quantification of the effect of the 9-1-1 complex (lanes 3–7 in **C**) and of myoglobin (lanes 9–13 in **C**) on DNA ligase I activity. The data represent the means for three experiments.

effect of PCNA in the presence of DNA ligase I and 9-1-1 complex at a ratio of 9:1 (Figure 3B, lanes 9–12). At this 9-1-1 complex/DNA ligase I ratio, stimulation of the ligase activity by the 9-1-1 complex was clearly visible, but not saturating, which allows detection of either a stimulatory or an inhibitory effect of PCNA. As expected, the 9-1-1 complex stimulated DNA ligase I (compare lanes 8 and 9 in Figure 3B), but upon further addition of PCNA, neither stimulation nor inhibition by PCNA was observed (Figure 3B, lanes 9–12, and Figure 3C-4). This suggests strongly that, once the 9-1-1 complex binds to DNA ligase I, PCNA is unable to interfere. Finally, we tested the effect of the 9-1-1 complex in the presence of a PCNA/DNA ligase I ratio of 9:1 (Figure 3B, lanes 3–6 and Figure 3C-3). The 9-1-1 complex could stimulate DNA ligase I even in the presence of excess PCNA, although the observed stimulation was slightly, but not significantly, weaker. These results suggest that, under the present conditions, PCNA does not prevent the stimulation by the 9-1-1 complex of DNA ligase I activity. Considering that the 9-1-1 complex is recruited on to DNA upon genotoxic stress, the stronger

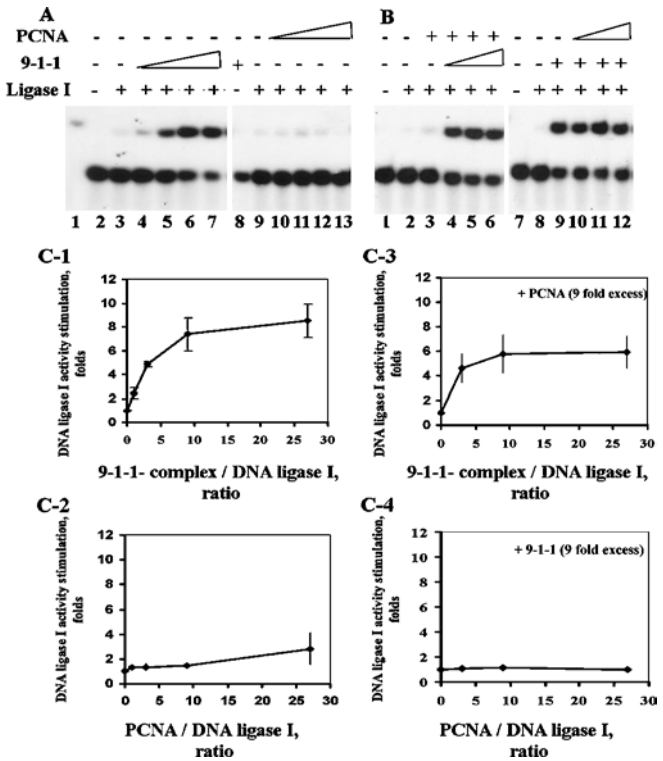

Figure 3 Human PCNA can slightly stimulate human DNA ligase I, but cannot prevent the stimulation of DNA ligase I by the human 9-1-1 complex

DNA ligase activity was determined as described in the Materials and methods section. (**A**) Effect of the 9-1-1 complex and PCNA on the activity of human DNA ligase I. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, 46-mer marker; lane 2, no enzyme control, 25-mer marker; lane 3, DNA ligase I alone; lanes 4–7, 30, 90, 270 or 810 fmol respectively of the purified recombinant human 9-1-1 complex was added; lane 8, 2430 fmol of 9-1-1 complex was added in the absence of DNA ligase I, no ligase control; lane 9, DNA ligase I alone; lanes 10–13, 30, 90, 270 or 810 fmol of the purified recombinant human PCNA was added. (**B**) Effect of PCNA on the stimulation of DNA ligase I by the 9-1-1 complex. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, no enzyme control, 25-mer marker; lane 2, DNA ligase I alone; lanes 3–6, DNA ligase in the presence of 270 fmol of PCNA and 0, 90, 270 or 810 fmol respectively of the 9-1-1 complex; lane 7, no enzyme control, 25-mer marker; lane 8, DNA ligase I alone; lanes 9–12, DNA ligase I and 270 fmol of 9-1-1 complex in the presence of 90, 270 or 810 fmol respectively of PCNA. (**C**) 1, Quantification of the effect of the 9-1-1 complex alone (lanes 3–7 in **A**); 2, quantification of the effect of PCNA alone (lanes 9–13 in **A**); 3, quantification of the stimulation by the 9-1-1 complex in the presence of PCNA (lanes 3–6 in **B**); 4, quantification of the PCNA effect in the presence of the 9-1-1 complex (lanes 9–12 in **B**). The data presented in (**C**) are the means for three experiments.

effect observed compared with PCNA may reflect the fact that the stimulation of DNA ligase I is greater, when its activity is needed as a priority at a place of DNA damage.

Conclusions

Most components of the LP-BER machinery can interact with the clamp PCNA. Moreover, as shown very recently by us and others, the checkpoint alternative clamp 9-1-1 complex can interact with and stimulate at least the four components, DNA glycosylase, pol β, Fen1 and DNA ligase I, of the LP-BER machinery. In this work we compared for the first time the effect of the 9-1-1 complex with that of PCNA on a LP-BER factor. We showed that the stimulation by the checkpoint sensor 9-1-1 complex is stronger than that by the classical DNA clamp PCNA. Moreover, the latter cannot prevent the stimulatory effect of the 9-1-1 complex on DNA ligase I. These findings now open the way to elucidate the *in vitro* and *in vivo* situations under which the clamp PCNA is 'replaced' by the alternative clamp 9-1-1. In our efforts to understand the role of the 9-1-1 complex in the process of BER, we have already characterized, in simple systems, the interaction of the 9-1-1 complex with different BER proteins, namely pol β [23], DNA ligase I (the present study) and Fen1 ([24,25], and E. Friedrich-Heineken, M. Toueille, B. Tännler and U. Hübscher, unpublished work). Hence, we are now in a position to establish a more physiological system including the respective clamp loaders RF-C for PCNA and its alternative clamp loader Rad17–RF-C_{2-5} for the 9-1-1 complex and a DNA template harbouring an AP site. This should allow us to test whether PCNA interferes with the effects of the 9-1-1 complex on different components and at different steps of the LP-BER pathway. Eventually, we will learn more about the possible alternative role of the two clamps in the function of the LP-BER mechanism.

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