Reversal of PCNA Ubiquitylation by Ubp10 in *Saccharomyces cerevisiae*

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Abstract

Regulation of PCNA ubiquitylation plays a key role in the tolerance to DNA damage in eukaryotes. Although the evolutionary conserved mechanism of PCNA ubiquitylation is well understood, the deubiquitylation of ubPCNA remains poorly characterized. Here, we show that the histone H2B ubiquitin protease Ubp10 also deubiquitylates ubPCNA in *Saccharomyces cerevisiae*. Our results sustain that Ubp10-dependent deubiquitylation of the sliding clamp PCNA normally takes place during S phase, likely in response to the simple presence of ubPCNA. In agreement with this, we show that Ubp10 alters in different ways the interaction of PCNA with DNA polymerase (–)–associated protein Rev1 and with accessory subunit Rev7. While deletion of *Ubp10* enhances PCNA–Rev1 interaction, it decreases significantly Rev7 binding to the sliding clamp. Finally, we report that Ubp10 counteracts Rad18 E3-ubiquitin ligase activity on PCNA at lysine 164 in such a manner that deregulation of Ubp10 expression causes tolerance impairment and MMS hypersensitivity.

Introduction

In living cells, tolerance mechanisms ensure that DNA can be replicated when it is damaged. These mechanisms prevent irreversible DNA replication fork collapse when the replisome encounters bulky lesions at damaged sites that block progression of replicative DNA polymerases [1,2]. DNA lesions are bypassed either by a mechanism involving low stringency DNA polymerases called translesion synthesis (TLS) polymerases or by promoting template-switching between nascent chains within the same replication fork [2–6]. It is thought that both mechanisms efficiently prevent replisome stalling at damaged sites. The use of TLS polymerases may be mutagenic because they induce an error-prone process that causes damaged-dependent mutations. However, it has been shown that in yeast ultraviolet-radiation-induced DNA lesions are predominantly bypassed via translesion synthesis [7].

Eukaryotes ubiquitylate proliferating-cell nuclear antigen (PCNA) to signal damaged DNA and regulate the choice of alternative pathways to bypass DNA lesions during S-phase, therefore, to tolerate DNA damage [2–6]. The sliding clamp PCNA is monoubiquitylated at Lys164 by the Rad6–Rad18 (E2–E3) ubiquitin ligase complex in response to endogenous or exogenous damage causing disruptive covalent modifications of DNA interfering with high-fidelity replicative polymerases during S phase. Mono-ubiquitylated PCNA (ubPCNA) enhances the affinity of error-prone TLS DNA polymerases which facilitate translesion synthesis bypass. Then, the Mms2-Ubc13-Rad5 ubiquitin ligase complex may further ubiquitylate Lys164-mono-ubiquitylated PCNA to promote template switching, the error-free component of the bypass that involves sister-strand pairing [5,8] and references therein). This regulatory mechanism based on covalent modifications of the Lys164 of the sliding clamp PCNA is a solidly established model conserved in all eukaryotes [2–5,9].

Ubiquitylation of Lys164-PCNA (ubPCNA) greatly enhances binding of the sliding clamp with TLS polymerases [10]. In contrast with replicative enzymes, TLS polymerases are low fidelity DNA polymerases, non-processive enzymes that lack any proofreading activity but capable of replicating over DNA lesions [11] (and references there in). Indeed, TLS polymerases are DNA damage-tolerant enzymes but also mutagenic because they may incorporate mispaired deoxynucleotides opposite to lesions (damaged template) in an error-prone process [12,13] (and references there in). Because of their low fidelity and low processivity when incorporating deoxynucleotides across from damaged and undamaged base pairs [12,14–17], cells need to keep TLS DNA polymerases from sampling replicative DNA more than strictly required and/or to prevent them from extended interaction with replication forks. Therefore, cells may need a control mechanism to deubiquitylate ubPCNA as soon as TLS DNA polymerases have been able to replicate over the damaged site.

Human Usp1 has been identified as a protease that deubiquitylates mono-ubPCNA [18]. Upon UV-light induced DNA damage, Usp1 is degraded so that PCNA becomes ubiquitylated [18,19], suggesting that Usp1 deubiquitylates PCNA continuously in the absence of DNA damage [18]. However, accumulation of...
ubPCNA does not correlate with Usp1 proteolysis when the progression of replication forks is stalled with HU [20], suggesting either a complex regulation of Usp1 activity (or its subcellular localization) when cells are exposed to other DNA damaging agents or the existence of at least one other PCNA deubiquitylating enzyme in mammals acting in response to other DNA damaging agents. Despite the identification of Usp1, little is known about the deubiquitylation of ubPCNA in any other organism.

In \textit{Saccharomyces cerevisiae}, the protease (or proteases) that deubiquitylates ubPCNA remains unknown. Potential candidates in budding yeast are 17 genes that code for different ubiquitin-specific proteases. Few of them have been extensively studied while others remain poorly characterized [21–23]. These genes are named \textit{UBP}s (from \textit{UBP}1 to \textit{UBP}17), where \textit{UBP} stands for ubiquitin protease. Among the ubiquitin-specific proteases characterized, Ubp10/Dot4 is remarkable; this is a deubiquitylating enzyme in mammals acting in response to other DNA damaging agents. Despite the identification of Usp1, little is known about the deubiquitylation of ubPCNA in any other organism.

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both mono- and di-ubiquitylated PCNA forms rapidly disappeared in cells overexpressing UBP10, suggesting that Ubp10 also deubiquitylates di-ubPCNA forms. These deubiquitylation events depended on the protease activity of Ubp10 as a catalytically inactive Ubp10C371S mutant form was unable to deubiquitylate PCNA in vivo in similar conditions (Figure 2B). We have also observed that Ubp10 overproduction reverts ubiquitylation of PCNA induced by treatments with HU, 4-NQO and UV radiation. In summary, these experiments indicate that overexpression of catalytically active Ubp10 can deubiquitylate ubPCNA in vivo. Importantly, this in vivo reaction did not require any other UBP gene, as active Ubp10 did deubiquitylate ubPCNA in any single UBP1-17 deletion (Figure S3).

Yeast PCNA mutants lacking the ubiquitin/SUMO-conjugation site K164 or mutated in the PCNA K164-E3 ubiquitin ligase Rad18 are hypersensitive to MMS (and other DNA damaging agents) because the ubiquitylation of this K164 amino acid residue is critical to tolerate DNA damage [29]. It is then reasonable to predict that the overexpression of the K164-ubPCNA ubiquitin-specific protease will counteract Rad18 activity and induce MMS hypersensitivity. Therefore, we exposed UBP10-overexpressing cells to the chronic presence of the alkylating chemical and found,
as predicted, that high levels of expression of the catalytically active form of this ubiquitin protease (but not the inactive Ubp10C371S form) induced hypersensitivity to MMS (Figure 2C). Significantly, this effect was specifically related to high levels of expression of Ubp10 because overexpression of any other UBP gene neither sensitize cells to MMS nor induce PCNA deubiquitylation \textit{in vivo} (Figure S4). Regarding \textit{UBP10} overexpression, two additional and testable predictions can be made, first, the hypersensitivity to MMS should depend on the PCNA lysine 164 modification. To test this prediction we used a simple epistasis analysis to determine the order of function of the \textit{POL30} and \textit{GAL1,10:UBP10} (Figure S5). We have indeed found that \textit{POL30} is epistatic to \textit{GAL1,10:UBP10} indicating that the MMS-sensitivity of Ubp10 overproduction depends on the PCNA lysine 164 modification. Second, given that mono-ubiquitylation of the K164 residue of PCNA is in principle important to enhance its interaction with mutagenic TLS polymerases, it is plausible to predict that the mutagenesis frequency of cells overexpressing \textit{UBP10} should be reduced as compared to wild-type cells. We have found that this is the case (Figure S6).

Catalytically active Ubp10 deubiquitylates PCNA \textit{in vivo} independently from histone H2B deubiquitylation

The above observations correlated the enzymatic activity of Ubp10 with PCNA deubiquitylation \textit{in vivo}. However, these effects may depend on deubiquitylation of histone H2B, as Ubp10

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Figure 2. \textit{GAL1}-driven overproduction of \textit{UBP10} reverts PCNA ubiquitylation in response to DNA damage. (A) Time-course analysis of active GST-Ubp10 induction. An asynchronously growing culture of \textit{GAL1,10:GST-UBP10}, incubated in raffinose as unique carbon source, was incubated 30 minutes in the presence of 0.02% MMS. Expression of GST-Ubp10 was either repressed by adding glucose (GAL OFF) or induced with galactose (GAL ON) in the continuous presence of the alkylating chemical (as described). Samples were taken at indicated intervals and processed for immunodetection of modified PCNA forms, PCNA, GST-Ubp10 and Rad53. Ponceau staining of the blotted protein extracts is shown. Mono-ubiquitylated PCNA was quantitated, normalized and plotted. (B) Catalytically active Ubp10 reverts PCNA ubiquitylation \textit{in vivo}. Immunodetection of ubiquitylated and di-ubiquitylated PCNA forms in wild-type cells and in cells repressed (GAL OFF) or induced (GAL ON) for GST-Ubp10 or GST-Ubp10C371S expression, after a 90 minutes treatment with 0.020% MMS. TCA-obtained cells extracts were processed for immunoblotting with \textalpha-PCNA and \textalpha-GST antibodies. Ponceau staining of the blotted protein extracts is shown for loading control. (C) Ectopic expression of a catalytically active Ubp10 ubiquitin protease hypersensitizes cells to MMS-induced DNA damage. Ten-fold dilutions of equal numbers of cells of wild-type, \textit{GAL1,10:UBP10} and \textit{GAL1,10:ubp10C371S} were incubated at 25 °C in the absence or the presence of indicated percentages of MMS for 72 hours and photographed.

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deubiquitylates K123 ubH2B [24,25]. In order to understand whether deubiquitylation of H2B and PCNA were independent from each other, we repeated our overexpression analysis in a bre1A mutant background. Bre1 is the E3 ubiquitin ligase that ubiquitylates histone H2B in yeast cells, thus, deletion of the BRE1 gene impedes H2B ubiquitylation [30,31]. Importantly, BRE1 deleted cells are viable, providing a tool to answer the question. As shown in Figure S7, overproduction of catalytically active Ub10 reverts PCNA ubiquitylation and hypersensitize cells to MMS similarly in wild-type and bre1 mutant cells. These results indicate that Ub10-dependent PCNA deubiquitylation is functionally separable from ubiquitylation of histone H2B.

Ub10 is required for rapid PCNA deubiquitylation after MMS–induced DNA damage

MMS modifies guanines and adenines to methyl derivatives causing DNA base mispairing, inducing DNA damage and slowing down progression of DNA replication forks during S-phase [32–35]. MMS also induces ubiquitylation of PCNA in all model organisms tested to-date (reviewed in [4]). To further study the role of Ub10 in the modulation of PCNA ubiquitylation in yeast, we analyzed by Western blot samples taken at regular intervals from wild-type cells treated for 60 minutes with the alkylating chemical and compare them to samples taken from UB10 mutant cells in similar conditions (Figure S8). As observed in the Figure S8, wild-type cells ubiquitylate PCNA after the MMS treatment and then actively deubiquitylate the sliding clamp in such way that 45 minutes after the release from the drug treatment ubiquitylated PCNA was barely detectable. In contrast UB10 deleted cells maintained steady state levels of ubiquitylated PCNA throughout the experiment, suggesting that these cells lack the appropriate enzyme involved in the deubiquitylation of the modified clamp.

PCNA interacts in vivo with Ub10

Having observed that deletion and overexpression phenotypes of Ub10 were consistent with the hypothesis that this ubiquitin-specific protease deubiquitylates PCNA in yeast, we next addressed whether Ub10 and PCNA interact in vivo, as expected for an enzyme-substrate complex.

Addition of single ubiquitin residue to Lys164 of PCNA in yeast is controlled by the E2–E3 complex Rad6–Rad18 during S-phase [7,28]. Accordingly, the Rad6–Rad18 enzyme complex and its substrate PCNA interact in vivo, as has been observed by yeast two-hybrid analyses [29]. We speculated that Ub10 could form a complex with PCNA in a Rad18 dependent manner, as it has been described previously for other E3-ubiquitin ligases [36–38]. If this were true, it could be predicted that these interactions might be detected by co-immunoprecipitation analysis. In particular, we were interested in determining a possible in vivo PCNA-Ub10 interaction at endogenous levels of both proteins. Since we used a C-terminally myc-tagged Ub10 strain we carefully checked growth rate, gene expression levels, PCNA and histone H2B deubiquitylation and found no differences with untagged wild-type controls, as shown for PCNA (Figure S9). By Western and co-immunoprecipitation assays, we found that Ub10–myc is stable upon exposure to DNA damage and that Ub10 binds PCNA throughout the cell cycle and in response to MMS-induced DNA damaged (Figure S9). We then studied Ub10-PCNA interaction in wild-type and rad18A mutant cells and observed that Ub10 and PCNA interact in vivo in a Rad18 semi-dependent manner (Figure 3A and 3B). We then tested Ubp10 and Rad18 interaction and found that Rad18 can associate in vivo with Ub10 both in undamaged and exogenously DNA-damaged cells (Figure S10). These results suggest that in yeast cells Ub10, PCNA and Rad18 could form a complex. These findings, particularly those related to PCNA and Ubp10 interaction, strongly support the hypothesis that Ub10 is an ubiquitin-specific protease that deubiquitylates PCNA in yeast cells.

Deletion of UB10 results in a net increase in the interaction of Rev1 with PCNA

In S.cerevisiae, REV1 encodes a deoxycytidyltransferase required for the bypass of abasic sites in damaged DNA. Rev1p forms a complex with the subunits of DNA polymerase ζ Rev3 and Rev7, which are involved in error-prone lesion bypass as yeast TLS DNA polymerases [14,39]. Furthermore, it has been shown that yeast Rev1 interacts with, and its activity is stimulated by, PCNA [40,41]. Therefore, we reasoned that the accumulation of mono-ubiquitylated PCNA observed in UB10 mutant cells could lead to an increased interaction between PCNA and TLS DNA polymerases, including the TLS-interacting Rev1 protein. We tested this possibility by co-immunoprecipitation assays in vivo using strains carrying myc-tagged Rev1 and either wild-type or C-terminal FLAG-tagged PCNA. We detected the reported interaction between the sliding clamp PCNA and the deoxycytidyltransferase Rev1 in the wild-type strain and, importantly, it was increased in cells lacking a functional Ubp10, as predicted (Figure 4A and 4B). We also found that this increase observed in ubp10D mutant cells was dependent on the PCNA lysine 164 modification (Figure S11). Interestingly, we found that the sliding clamp co-immunoprecipitated Rev1 from asynchronous or MMS-damaged cell cultures (Figure 4C and Figure S12). If this enhancement (in Rev1-PCNA interaction) observed in ubp10D mutant cells is due to an increase in the ubiquitylation of PCNA, it would be expectable to detect ubiquitylated PCNA in undamaged cells. To our knowledge, detection of ubPCNA in undamaged budding yeast cells remains elusive. However, by immunoprecipitating the sliding clamp from POL30-FLAG tagged cells, although weakly, we detected ubiquitylated PCNA in asynchronous cultures of exponentially growing wild-type and UB10 mutant cells and indeed found that the mutant accumulated ubiquitylated PCNA (Figure S13). This observation supports the correlation between the increase in ubPCNA and the enhancement of Rev1-PCNA interaction in undamaged cells. Finally, we did not observe PCNA-Rev1 interaction in G1 synchronized cells, even though the Rev1 protein was present in the cell extracts (Figure 4B, 4C and Figure S12).

We next analyzed chromatin-associated Rev1 foci and found that, in agreement with the co-immunoprecipitation results, ubp10D mutant cells had increased numbers of Rev1 foci (mean ± s.d.: wild type, 16.64 ± 0.42; ubp10D, 20.47 ± 10.24). Remarkably, a detailed analysis revealed a significant increment in nuclei with high numbers of Rev1 foci in UB10 mutant cells (Figure 4D). In theory, the observed increased interaction between PCNA and Rev1 in UB10 deleted cells could be suggestive of a greater TLS activity on replicating chromatin that would result in increased mutagenic rate. Therefore, we next monitored the forward mutation rate to canavanine resistance [42] in undamaged or MMS-damaged ubp10D mutant cells. However, we found no statistically-significant differences in the mutagenic rate when compared to that of wild-type cells (Figure S14), indicating that increasing levels of PCNA ubiquitylation has no observable impact in the frequency of mutation.

Analysis of the interaction of Rev3 and Rev7 with PCNA in cells deleted for UB10

The Rev1-Rev3/Rev7 complex formation has been successfully tested in yeast [43,44]. However, having shown that mutation of UB10 enhances Rev1 interaction with PCNA but does not
Figure 3. PCNA interacts in vivo with Ubp10. (A) The sliding clamp PCNA and Ubp10 specific-ubiquitin protease interact physically in vivo. Co-immunoprecipitation assay showing physical interaction between Ubp10-myc and FLAG tagged PCNA. PCNA-FLAG was immunoprecipitated from formaldehyde-crosslinked protein extracts (see methods) both from untreated or 0.020% MMS-treated cells, blots were incubated with α-myc (to detect Ubp10) or α-FLAG (to detect PCNA). The immunoblots shown are those from untreated cells (a similar result was obtained with MMS-treated cells). As indicated the strains used in this assays were UBP10-myc POL30-FLAG and UBP10-myc POL30-FLAG rad18Δ. Immunoprecipitated Ubp10-myc was quantitated, normalized and plotted. Each immunoprecipitation experiment was repeated three times to gain an estimate of error. (B) Co-immunoprecipitation assay showing physical interaction between Ubp10-myc and PCNA. PCNA was immunoprecipitated both from untreated or 0.020% MMS-treated cells, blots were incubated with α-myc (to detect Ubp10) or α-PCNA. The immunoblots shown are those from MMS-treated cells (a similar result was obtained with untreated cells). As indicated the strains used in this assays were UBP10-myc and UBP10-myc rad18Δ. Immunoprecipitated Ubp10-myc was quantitated, normalized and plotted. Note that in our experiments we detect Ubp10 interacting with unmodified PCNA (or unmodified PCNA-FLAG).

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increase mutation frequency (and in order to explain this discrepancy), we wondered whether the Rev3/Rev7 (DNA polymerase \( f \)) interaction with PCNA was regulated in a different way than the observed for Rev1 in \( ubp10 \) yeast mutant cells. In order to test this hypothesis, we first analysed Rev3-PCNA interaction in wild-type and \( ubp10 \) mutant strains. By co-immunoprecipitation assays, we found that Rev3, the catalytic subunit of pol zeta, interacts with PCNA in wild-type and \( ubp10 \) mutant strains. We also observed that the amount of Rev3 co-immunoprecipitated with PCNA was similar in both strains either in asynchronous cultures or when cells were treated with MMS.

We next studied the interaction of PCNA with the accessory subunit of DNA polymerase \( f \), Rev7 (Figure 5B and 5C). Rev7 stimulates the activity of Rev3 [14] and is required for mutagenesis induced after DNA damage in such a manner that deletion of REV7 decreases mutagenesis frequency in yeast [45]. Significantly, in our co-immunoprecipitation assays we did observe that the interaction of PCNA with Rev7 was greatly reduced in cells deleted for \( UBP10 \) supporting an explanation for the wild-type-like mutagenesis frequency observed in them.

Cells lacking Ubp10 accumulate mono- and di-ubiquitylated forms of PCNA in response to HU-induced DNA replication blocks

The evidence presented up to here indicate that the activity of Ubp10 is required for reverting PCNA ubiquitylation but does not
A

WCE MMS As MMS 1 2 3 4 ip FLAG mock ip ip FLAG mock ip ip FLAG mock ip ip FLAG mock ip

α-myc

α-FLAG

short exposure

1: REV3myc POL30FLAG
2: REV3myc POL30FLAG ubp10Δ
3: POL30FLAG
4: REV3myc

B

MMS

WCE 1 2 As As MMS 1 2 ip FLAG mock ip ip FLAG mock ip ip FLAG mock ip

α-myc

α-FLAG

1: REV7myc POL30FLAG
2: REV7myc POL30FLAG ubp10Δ
3: REV7myc
4: POL30FLAG

C

WCE

α-myc

α-FLAG

Asynchronous

Rev7myc

PCNA-FLAG
address when Ubp10-mediated PCNA deubiquitylation takes place during the cell cycle. Therefore, we were next interested in understanding whether deubiquitylation of PCNA occurs during S-phase. Through the depletion of nucleosides, the drug hydroxuracil (HU), an effective ribonuclease reductase inhibitor, causes an early S-phase arrest in S. cerevisiae cells [46] and induces ubiquitylation of PCNA [27], thus, providing a way to study the regulation of PCNA ubiquitylation in the presence of stalled DNA replication forks. In this scenario, we compared PCNA ubiquitylation in wild-type and ubp10Δ mutant cells (Figure 6). Cells in logarithmic growth at 30°C were synchronized with α-factor and then released in 0.2M HU at the same temperature and samples (taken at regular intervals) processed for Western analysis of PCNA. We used as S-phase markers PCNA SUMOylation [29], Rad53 activation [35] and Cbi5 accumulation [47–50]. As recently described [7,27,28], we detected PCNA ubiquitylation as soon as cells entered S-phase, coincident with the appearance of PCNA SUMOylation, Rad53 activation (in response to HU) and Cbi5 accumulation (Figure 6C). Under the chronic presence of HU, in wild-type cells PCNA ubiquitylation reached a maximum 40 minutes after the release from the pheromone arrest and then started to decline with stalled DNA replication forks as judged from all markers, including DNA content analysis by FACS. The timing of PCNA ubiquitylation observed here correlates well with the recently described timing of association of Rad18 with replicating chromatin in HU-treated cells [27]. The decrease in ubPCNA observed in wild-type cells was somewhat surprising; however, it does indicate that yeast cells down-regulate the modification of the clamp during S-phase. In contrast, cells lacking Ubp10 activity, even though they progressed into S-phase later or more slowly than controls (Figure 6B and 6C), accumulated increased amounts of mono and di-ubiquitylated forms of the clamp that remained high all throughout the synchronous experiment (see bar plot for ubPCNA in Figure 6C). The analysis of ubp10Δ mutant cells is consistent with the idea that this ubiquitin-specific protease down-regulates PCNA ubiquitylation during S-phase and suggest that Ubp10 is a major deubiquitylating enzyme for ubPCNA in budding yeast cells (see model in Figure 7).

Discussion

In this work we present clear evidence indicating that Ubp10 controls PCNA deubiquitylation in S. cerevisiae. Ubp10 has a well established role as an ubiquitin-specific protease of ubH2B, a role related to gene-silencing (at telomeres, rDNA and cribetic mating type loci), together with Ubp8, the SAGA-associated ubH2B deubiquitylase involved in gene expression [24,25]. Thus, in combination Ubp8 and Ubp10 regulate the global balance of ubH2B [24,25]. In addition to this role, here we present results supporting that Ubp10 is an important ubiquitin-specific protease also in removing ubiquitin from ubPCNA in budding yeast. Our observations that wild-type cells deubiquitylate ubPCNA in response to the alkylating chemical MMS or under the chronic presence of HU show that there exists an active control to revert PCNA ubiquitylation in S. cerevisiae yeast cells. Moreover, our experiments with ubp10Δ mutant strains indicate that such control depends on the catalytic activity of Ubp10.D048.

Ubp10 deleted cells or cells carrying a catalytically inactive form of Ubp10 accumulate ubPCNA, a phenotype consistent with the idea that in vivo Ubp10 is the protease that removes ubiquitin from ubiquitylated PCNA. In agreement with this role, overexpression of active Ubp10 reverts PCNA ubiquitylation and hypersensitizes cells to MMS. Moreover, Ubp10 and the sliding clamp PCNA interact in vivo as expected from the formation of and enzyme-substrate complex. Importantly, the function of Ubp10 as ubPCNA ubiquitin-specific protease is separable from histone H2B ubiquitylation, as Ubp10 deubiquitylates ubPCNA in cells lacking Bre1, the E3 ubiquitin ligase that in complex with Rad6 monoubiquitylates histone H2B [31,51]. However, the ubPCNA and ubH2B deubiquitylation roles of Ubp10 might be functionally related. One interesting hypothesis is that Ubp10-dependent deubiquitylation of ubPCNA and ubH2B are inseparable functions. It is arguable that Ubp10 might modulate both replication bypass and histone modification in order to leave the epigenetic marks unaltered during DNA replication. In fact, it has been inferred from DT40 chicken cells defective in Rev1 that this TLS-associated deoxycytidyl transferase is involved in replication of G4-structured DNA regions and, as a consequence of it, in leaving intact their histone methylation epigenetic marks [52]. Since here we report a functional link between Rev1, PCNA, Rad18 and Ubp10, it is reasonable to surmise that Ubp10 would modulate PCNA ubiquitylation and (the maintenance of) histone imprinting during replication. These modulatory roles are also consistent with the fact that the modulator (Ubp10) might form part of the complexes (PCNA, Rad6-Rad18, Rad6-Bre1) involved in both actions.

An important observation presented in this work is that Ubp10 is able to remove mono-ubiquitin as well as di-ubiquitin from PCNA in vivo, suggesting that this ubiquitin protease enzyme may be crucial for keeping TLS polymerases in check as well as for down-regulating the error-free bypass. Thus, a single deubiquitylating enzyme might downregulate both branches of the tolerance pathway to DNA damage in budding yeast. Where does PCNA deubiquitylation take place? The answer to this simple question is not necessarily trivial, since the localization of Ubp10 might be a point of interest for future analysis. Initial studies in formaldehyde-fixed cells suggested that Ubp10 localizes primarily at the nucleus [53]; however, using in vivo studies of
Ubp10-GFP as well as immunofluorescence analysis of Ubp10-myc on nuclear spreads, we have found that Ubp10 localizes mainly in the rDNA-containing nucleolar region (our own unpublished observations). Thus, does Ubp10 localize permanently to the nucleolus? ChIP evidence has confirmed rDNA loci, telomeres and cryptic mating type loci localization [24,25,54] so that Ubp10-dependent deubiquitylation of ubH2B should take place there. Deubiquitylation of ubPCNA may follow a more dynamic pattern (as DNA replication forks move during ongoing replication). Alternatively, and more simply, an undetected fraction of Ubp10 might be permanently located out of the nucleolus or might be released from this nuclear compartment to control the deubiquitylating processes during S-phase and postreplication repair. Future studies will address these alternatives.

As in yeast cells, PCNA ubiquitylation is required for mammalian cell survival after UV irradiation, HU or MMS treatment [55]. In human cells Usp1 deubiquitylates PCNA as well as the Fanconi’s anaemia protein FANCD2 [19,56–58]. It has been shown that human Usp1 incessantly deubiquitylates ubPCNA in the absence of DNA damage [18]. Upon UV light-induced DNA damage, Usp1 is (auto)proteolysed, such that PCNA becomes ubiquitylated [18,19]. Our work has uncovered several differences in the regulation of PCNA deubiquitylation between yeast and human cells. First, we observed that UBP10 deleted yeast cells accumulate ubiquitylated PCNA forms in response to MMS, HU, UV-light and 4-NQO, suggesting that a single DUB (Ubp10) may control PCNA deubiquitylation in budding yeast. Second, Ubp10 appears to deubiquitylate PCNA during S-phase (when the sliding clamp is modified). Finally, Ubp10 protein levels remained constant when cells are exposed to DNA damage. Thus, it is unlikely that a similar Usp1-like autoregulatory mechanism on yeast Ubp10 ubiquitin protease would exist.

The evidence presented here supported the hypothesis that Ubp10 deubiquitylates PCNA to limit the residence time of TLS polymerases on DNA replication forks during S-phase. We tested this hypothesis directly by studying Rev1-PCNA interaction because Rev1 serves as a scaffold for the polymerase θ, encoded by REV3 and REV7, for efficient bypass of DNA lesions [59–61]. In agreement with this hypothesis, we found that deletion of UBP10 resulted in an increased interaction between PCNA and Rev1 in undamaged and DNA-damaged cells, and that, in turn, this enhanced interaction resulted in a net increase in Rev1 foci in chromatin. However, in contradiction with an increased number of Rev1 foci, we have also found that deletion of UBP10 does not increase the mutagenic frequency. A conceivable explanation for

![Figure 6. Cells lacking Ubp10 accumulate ubiquitylated PCNA forms early during S-phase in response to HU-induced DNA replication blocks.](image-url)
this contradiction would be that an additional level of control on TLS polymerases may exist to regulate their activity. In this context, one simple possibility is that DNA polymerase ε interaction with replicating chromatin may be hindered in UBP10 deleted cells. Therefore, to explain the observed discrepancy we studied the interaction of DNA polymerase ε subunits Rev3 and Rev7 with PCNA. Significantly, we have found that DNA polymerase ε accessory subunit Rev7 requires Ubp10 to fully interact with the sliding clamp PCNA. This observation explains why ubp10Δ mutant cells have a wild-type-like mutagenic frequency and, more importantly, it opens the unexpected possibility that Rev1 and DNA polymerase ε subunits may be regulated in quite distinct ways regarding their interaction with PCNA and, thus, follows the classical regulatory model. Here, we propose that Ubp10 participates in this modulation through the deubiquitylation of ubPCNA. However, from the observations presented here we also deduced that Ubp10 may play a direct or indirect role in regulating Rev7 interaction with the sliding clamp apparently in a PCNA ubiquitylation independent manner.

It is proper to mention here that the activity TLS-DNA polymerases activity may be regulated by checkpoint kinases. For example, it has been shown in budding yeast that Rev1 is regulated during the cell cycle [62], and that it is phosphorylated by the Mec1-Ddc2 kinase in response to various types of DNA damages [63–65]. Thus, in response to DNA damage, yeast cells would have two different levels of control: first, in modulating the interaction of PCNA and TLS polymerases, and second, in regulating TLS polymerases activity and/or stability. A control mechanism that may be conserved as ATR-mediated phosphorylation of DNA polymerase η is involved in the proper response to UV-mediated DNA damage in human cells [66].
What might be the biological significance of Ubp10-mediated ubPCNA deubiquitylation in budding yeast? It is tempting to say that our results suggest that the biological significance of the control of PCNA deubiquitylation in *S. cerevisiae* is to prevent extended residence time of Rev1 in replicating chromatin. However, there is no unfavorable outcome for yeast cells deleted for *UBP10* as they fail to support a full interaction of (DNA polymerase ζ subunit) Rev7 with PCNA and, consequently, they show a wild-type-like mutagenic frequency. It is true that these opposite effects on Rev1 and Rev7 suggest the hypothesis that Ubp10 has a complex role in modulating TLS subunits interaction with PCNA (and perhaps with replicating chromatin). However, additional studies will be required to test this hypothesis. Significantly, it has been reported the functionality in tolerance extended residence time of Rev1 in replicating chromatin. It is tempting to say, as they fail to support a full interaction of (DNA polymerase ζ subunit) Rev7 with PCNA and, consequently, they show a wild-type-like mutagenic frequency. Thus, an alternative interpretation of our results is that Ubp10-driven deubiquitylation of ubPCNA may not be that important to tolerate DNA damage in yeast as deletion of *UBP10* has no impact in MMS sensitivity nor leads to a mutator phenotype.

**Materials and Methods**

General experimental procedures of yeast Molecular and Cellular Biology were used as described previously [68–71].

**Yeast strains, cell culture, and flow cytometry**

All the budding yeast used in our studies are listed in Table S1. Yeast strains were grown in rich YPA medium (1% yeast extract, 2% peptone, 50 μg/ml adenine) containing 2% glucose. For block-and-release experiments, cells were grown in YPA with 2% glucose (except where indicated) at 25°C and synchronised with α-factor pheromone in G1 by adding 40 ng/ml (final concentration, 2.5 hours). Cells were then collected by centrifugation and released in fresh media in the absence or in the presence of MMS (or other drugs as indicated). Overexpression experiments with cells grown in YPA medium with 2% raffinose at 25°C were conducted by adding to the medium 2.5% galactose (to induce) or 2% glucose (to repress) and further incubating with/without MMS. For flow cytometry, 10^6 cells were collected by centrifugation, washed once with water, and fixed in 70% ethanol and processed as described previously [68,72]. The DNA content of individual cells was measured using a Becton Dickinson FACScan. Cells were prepared for flow cytometry as described [72,73].

**MMS and drugs sensitivity assays**

Exponentially growing or stationary cells were counted and serially diluted in YPA media. Tenfold dilutions of equal numbers of cells were used. 10 μl of each dilution were spotted onto YPAD (2% glucose) or YPAGal (2.5% galactose) plates (always supplemented with 50 μg/ml adenine), YPAD or YPAGal plates containing different concentrations of MMS (Sigma), or HU (Sigma), incubated at 25°C and scanned. MMS plates were always freshly made.

**Mutagenesis assay**

Forward mutation analysis at the CAN1 locus was performed essentially as described previously [74]. Cells were grown in rich medium (YPAD or YPAGal) to log phase and MMS (at indicated concentrations) was added to the half of each culture, which were further incubated until the saturation point was reached (24 hours for wild-type, *ubp10Δ* and *ubp10Δ rev3Δ* strains in Figure S14 to 48 hours for wild-type, *gal11*, *ubp10*, *rev3Δ* and *gal11*, *ubp10* rev3Δ strains in Figure S6). Then, cells were plated on solid medium without arginine but containing 60 μg/ml canavanine (Sigma) and also in control YPAD plates (for reference). After 4 days, colonies were counted and the mutagenesis frequency (canavanine resistant cells versus total population) was calculated for each culture. The frequencies provided are mean values of six or more independent cultures of each indicated genotype, in at least three independent experiments.

**Tagging yeast proteins and gene deletion**

Tagged alleles were constructed using the single step PCR-based gene modification strategy [75]. A similar strategy was used to generate specific gene deletions. The selection markers used were *KanMX6*, which allows selection with geneticin, *HphMX4*, which allows selection with hygromycin or *NatMX4*, which allows selection with nourseothricin. We used also *LEU2* and *HIS3* markers (as indicated in Table S1). The resulting genomic constructs were confirmed by PCR and sequencing. In the case of tagged alleles, the presence of tagged proteins was confirmed by Western blot.

**Immunoprecipitation, Western blot analysis, and antibodies**

**Protein extract preparation for Western analysis.** TCA cell extracts were prepared and analyzed as described previously [70,76]. SDS-PAGE gels at 15%, 12%, 10% and 7.5% were used for detection of histone H2B, PCNA (12% and 10%) and Rad53, respectively.

**Protein extract preparation for immunoprecipitations.** Soluble protein extracts were prepared basically as described previously [77]. Cells were collected, washed, and broken in HB2T buffer using glass beads. The HB2T buffer contained 60 mM β-glycerophosphate, 15 mM β-nitrophenylphosphate, 25 mM 4-morpholinepropanesulfonic acid (pH 7.2), 15 mM MgCl2, 15 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 20 mg/ml leupeptin and aprotinin. The glass beads were washed with 500 ml of HB2T, and the supernatant was recovered. Protein concentrations were measured using the BCA assay kit (Pierce). We repeated the immunoprecipitation of PCNA or PCNA-FLAG experiments in the presence of the crosslinking agent formaldehyde (as indicated in Figure legends), and cell extracts were prepared and then processed as for ChIP [69,78]. After immunoprecipitation of PCNA or PCNA-FLAG, tagged proteins were detected by immunoblotting with specific monoclonal antibodies (the IPs were washed as for ChIP assays, however, it was mixed with Laemmli buffer before incubation at 95°C for 30 min to reverse the crosslinking and denature the eluted proteins). The *in vivo* interactions described in the Results section (in particular PCNA with Ubp10 and PCNA with Rev1) were quantitated from Western analysis of co-immunoprecipitates. In every case, the experiments were repeated three times to gain an estimate of error.

**Western blotting.** Protein extracts and immunoprecipitates were electrophoresed using SDS-polyacrylamide gels ranging from 7.5 to 15%. For Western blots, 40–80 μg of total protein extracts from each sample were blotted onto nitrocellulose, and proteins were detected using a characterized anti-PCNA affinity-purified polyclonal antibody (1:1500; a generous gift from Dr. Paul Kaufmann). We also used Cib5, Sic1 and Rad53 antibodies from Santa Cruz Biotechnology (used as indicated by the supplier), as well as the 12CA5 monoclonal antibody (Roche Molecular Biochemicals; 1:500), or the anti-FLAG monoclonal antibody (1:3000), or the anti-Myc monoclonal antibody (1:3000). Polyclonal anti-GST antibody (1:3000) was also used. Horseradish
peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse antibodies (as required) and the ECL kit (GE Healthcare) were used. The antibodies required for immunoblots were used at the indicated dilutions for Western analysis.

**Imaging of cells and citology**

Immunofluorescence of nuclear spreads was performed essentially as described previously [71,79]. The anti-myc tag antibody (clone 4A6, 05-724; Millipore) was used at 1:500 dilution and the Alexa Fluor 594-conjugated anti-mouse secondary antibody (A11032; Molecular Probes) was used at 1:200 dilution. Images were captured using a Nikon Eclipse Ti fluorescence microscope equipped with an Orca-AG (Hamamatsu) CCD camera and a PlanApo VC 100 x/1.4 objective. Images were processed and analyzed with the MetaMorph software (Molecular Devices).

**Supporting Information**

**Figure S1** Immunodetection of ubiquitylated forms of PCNA in yeast TCA-cell extracts to show that UBP10 mutant cells accumulate K164 but not K127 modified PCNA forms. Immunoblot analysis with α-PCNA antibody of TCA-protein extracts from pol30K164R (unable to ubiquitylate or SUMOylate PCNA at K164), wild-type (wt), pol30K127R (unable to ubiquitylate or SUMOylate PCNA at K127), ubp10Δ, ubp10Δ pol30K127R, ubp10A, G1 wild-type (wt α-factor), sig1Δ (unable to SUMOylate PCNA), mms2A (unable to di-ubiquitylate PCNA), rad18Δ (unable to ubiquitylate PCNA), and ubp10A pol30K127R cells treated 90 minutes with 0.020% MMS and resolved in a 12% polyacrylamide gel, get the presence of a sample from untreated wild-type cells (8th lane).

**Figure S2** ubp10 but not ubp8 mutant cells accumulate ubiquitylated forms of PCNA in response to MMS-induced DNA damage. Immunodetection of mono-ubiquitylated (ubPCNA) and di-ubiquitylated PCNA (ub2PCNA) in wild-type, ubp8Δ, ubp10Δ and ubp8Δ ubp10Δ cells treated with 0.020% MMS (as indicated). Ubiquitylated PCNA (ubPCNA) samples were quantified, normalized to loading controls and plotted. Rad53 phosphorylation is used for testing checkpoint activation upon MMS-treatment.

**Figure S3** GAL1-driven overproduction of UBP10 reverts PCNA ubiquitylation in any UBP1-17 deletion. Catalytically active Ubp10 reverts PCNA ubiquitylation in vivo in ubp1Δ (1A) to ubp17A (17A) single mutant. Immunodetection of K164 mono-ubiquitylated PCNA forms in wild-type cells (wt), GAL1-regulated overexpressing UBP10 cells (wt*), and GAL1-regulated overexpressing UBP10 ubp1Δ (1A to ubp17A (17A) single mutant cells either repressed (OFF) or induced (ON) for Ubp10 overexpression, after a 90 minutes treatment with 0.020% MMS. TCA-obtained cells extracts were processed for immunoblotting with α-PCNA antibody.

**Figure S4** Analysis of MMS sensitivity and PCNA ubiquitylation in GAL1-regulated overexpressing UBP1, UBP2, UBP3, UBP4, UBP5, UBP6, UBP7, UBP8, UBP9, UBP10, UBP11, UBP12, UBP13, UBP14, UBP15, UBP16 and UBP17 yeast cells. (A) Tenfold dilutions of equal numbers of wild-type and GAL1,10-expressing UBP1, UBP2, UBP3, UBP4, UBP5, UBP6, UBP7, UBP8, UBP9, UBP10, UBP11, UBP12, UBP13, UBP14, UBP15, UBP16 and UBP17 cells were incubated at 25°C in the absence or in the chronic presence of MMS (as indicated) for 72 hours and photographed.

**Figure S5** Epistasis analysis of pol30K164R and UBP10 mutant alleles. (A) Tenfold serial dilutions of wild-type, pol30K164R ubp10A and ubp10A pol30K164R cells incubated at 25°C on YPAD plates with or without the indicated percentages of MMS for 72 hours and photographed. (B) Tenfold dilutions of equal numbers of (otherwise isogenic) wild-type, pol30K164R, GAL1,10:UBP10 and GAL1,10:UBP10 pol30K164R cells incubated at 25°C on YAPD plates (GAL OFF) to repress GAL1,10-driven UBP10 expression or YAPGal plates (GAL ON) to induce GAL1-driven UBP10 expression (with or without MMS, as indicated).

**Figure S6** Forward mutation analysis in wild-type and GAL1,10:UBP10 strains. Canavanine resistance was assayed in wild-type, GAL1,10:UBP10, rev3Δ, and GAL1,10:UBP10 rev3Δ cells either incubated in galactose to induce UBP10 overexpression (GAL ON) or in glucose to repress it (UBP10 expression) and treated with 0.0005% MMS. Note that a low concentration of MMS was used in this assay because of the hypersensitivity of UBP10 overexpressing cells (as shown in Figure S4B) to the DNA alkylating chemical. For the same reason, in these experiments a 56 hours exposure to the chemical was required for cultures to reach saturation (before plating onto canavanine Petri dishes). Plots of the resulting forward mutation frequencies are shown.

**Figure S7** Catalytically active Ubp10 deubiquitylates PCNA in vivo independently from histone H2B deubiquitylation. (A) Tenfold dilutions of equal numbers of wild-type, ubp10A, GAL1,10:GST-UBP10, GAL1,10:GST-ubp10C371S, GAL1,10:GST-UBP10 bre1A and GAL1,10:GST-ubp10C371S bre1A cells were incubated at 25°C in the absence or the presence of indicated percentages of MMS for 72 hours and photographed. (B) Catalytically active Ubp10 reverts PCNA ubiquitylation in vivo independently from BRE1. Immunodetection of ubiquitylated PCNA forms in wild-type cells and in cells repressed (GAL OFF) or induced (GAL ON) for GST-Ubp10 or GST-Ubp10C8 expression, after a 90 minutes treatment with 0.020% MMS. Protein extracts were processed for immunoblotting with polyclonal α-PCNA antibody. Ponceau staining of the blotted protein extracts is shown for loading control.

**Figure S8** Ubp10 is required for rapid deubiquitylation after MMS-induced DNA damage. Asynchronously growing cultures of wild-type and (otherwise isogenic) ubp10A strains were incubated 60 minutes in the presence of 0.02% MMS, washed twice in fresh (pre-warmed) media and release in YAPD (in the absence of the alkylating chemical). Samples were taken at indicated intervals and processed for immunodetection of PCNA forms and Rad53 phosphorylation with α-PCNA and α-Rad53 antibodies. ubPCNA was quantitated, normalized and plotted.

**Figure S9** Analysis of ubp10-myc and ubp10C371S-myc strains. (A) Asynchronously growing Ubp10-myc cells were blocked in G1
with α-factor and then released in fresh medium to analyze the quantity of Ubp10 through the cell cycle; additionally, Ubp10-myc asynchronous cells were treated with 0.02% MMS 90 minutes, 0.2 M HU 90 minutes or 150 Jm\(^{-2}\) UV light. TCA-extracted protein samples were collected for detection of Ubp10-myc, PCNA and Rad33. (B) The lack of deubiquitinating activity of Ubp10\(^{C371S}\) does not alter the level of the protein, but it causes an accumulation of ubiquitinated PCNA forms in a similar way than the deletion of the UBP10 gene. Wild-type (wt), ubp10A, ubp10\(^{C371S}\)-myc (two different clones) and UBP10-myc cells were treated with 0.02% MMS during 90 minutes and TCA-extracted protein samples were processed for Western analysis (to detect Ubp10-myc, PCNA and Rad33), all along with an untreated wt sample (as indicated). Note that, while UBP10 mutants (ubp10A and the two ubp10\(^{C371S}\)-myc clones) accumulate more mono- and di-UbPCNA, the ubp10-myc strain has wild-type levels. (C) Ubp10 interacts in vivo with PCNA throughout the cell cycle. Co-immunoprecipitation assay showing physical interaction between Ubp10-myc and PCNA. PCNA was immunoprecipitated from untreated asynchronous (As), α-factor synchronized (G1), 30 minutes released S-phase (S) or 75 minutes released G2 (G2) cells. Blots were incubated with α-myc (to detect Ubp10-myc) or α-PCNA. Appropriate input (WCE) and mock-Ip controls are shown. (D) Ubp10 interacts in vivo with PCNA in undamaged and MMS-damaged cells. PCNA was immunoprecipitated from untreated asynchronous (As) or 0.02% MMS-treated cells. Blots were incubated either with α-myc (to detect Ubp10-myc) or α-PCNA. Input (WCE) and mock-Ip controls are shown. (JPG)

**Figure S10** The E3-ubiquitin ligase Rad18 and Ubp10 ubiquitin-specific protease interact physically in vivo. Co-immunoprecipitation assay showing physical interaction between Ubp10-myc and Rad18-Ha. Ubp10-myc was immunoprecipitated either from untreated (Asyn) or 0.02% MMS-treated cells (MMS), blots were incubated with α-myc (to detect Ubp10) or α-Ha (to detect Rad18-Ha) as indicated. Appropriate single tagged, input (WCE) and mock-Ip controls are shown. (JPG)

**Figure S11** Analysis of Rev1-PCNA interaction in pol30K164R cells in wild-type and ubp10A strains. Co-immunoprecipitation assay showing physical interaction between Rev1-myc and PCNA in pol30K164R cells. PCNA was immunoprecipitated either from untreated or from 0.02% MMS-treated cells, blots were incubated with α-myc (to detect Rev1) or α-FLAG (to detect PCNA). As indicated the strains used in this assays were REV1-myc pol30K164R FLAG and REV1-myc pol30K164R FLAG ubp10A. Note that the relative amount of immunoprecipitated Rev1-myc was similar in UBP10 or ubp10A cells indicating that Rev1 interacts with unmodified PCNA (pol30K164R) and that this interaction is not enhanced in ubp10A mutants. (JPG)

**Figure S12** Co-immunoprecipitation assay showing physical interaction between Rev1-myc and PCNA-FLAG in MMS-treated cells. Cell extracts were prepared as for ChIPs (in the presence of the crosslinking agent formaldehyde, see methods). PCNA-FLAG was immunoprecipitated from 0.02% MMS-treated cells (45’ or 90’ samples) or α-factor blocked cells (as indicated), blots were incubated with α-myc (to detect Rev1) or α-FLAG (to detect PCNA). As indicated, the strains used in this assays were REV1-myc POL30-FLAG and REV1-myc POL30-FLAG ubp10A. Note that this is a representative Western blot of the experiments plotted in Figure 4C. (JPG)

**Figure S13** Detection of ubiquitylated PCNA forms in asynchronous cultures of wild-type and ubp10A cells by immunoprecipitation. Immunoprecipitation of FLAG-tagged PCNA from asynchronous (Asyn) or 0.02% MMS-treated (MMS) cultures. Samples were taken from exponentially growing cultures or 90 minutes MMS-treated cultures of POL30-FLAG (wild-type) and POL30-FLAG ubp10A (ubp10A) strains and processed for immunoprecipitation with α-FLAG. Immunoblots were incubated with α-PCNA (to detect unmodified and modified PCNA). Note the detection of ubiquitylated PCNA in untreated wild-type and ubp10A cells, and the accumulation of ubiquitylated forms of PCNA in untreated and MMS-treated ubp10A cells (compared to wild-type samples). (JPG)

**Figure S14** Forward mutation analysis in wild-type and ubp10A strains. (A) Canavanine resistance was assayed in ubp10A, ubp10A riv3Δ and wild-type control cells either untreated or treated with 0.002% or 0.005% MMS (as indicated). Plots of the resulting forward mutation frequencies are shown. (B) Viability analysis in wild-type, riv3Δ and ubp10A strains. Exponentially growing wild-type, riv3Δ and ubp10A strains were exposed the indicated times to 0.05% or 0.2% MMS and test for colony formation. Plots of the resulting viability test are shown. (JPG)

**Table S1** Yeast strains used in this study. (DOC)

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**Author Contributions**

Conceived and designed the experiments: AG-S PAS-S AB. Performed the experiments: AG-S SA FC PAS-S. Analyzed the data: AG-S PAS-S AB. Performed the experiments: AG-S SA FC PAS-S. Wrote the paper: AB. Performed immunoprecipitation experiments: AG-S SA FC PAS-S. Analyzed the data: AG-S PAS-S AB. Conceived and designed the experiments: AG-S PAS-S AB. Performed the experiments: AG-S SA FC PAS-S. Analyzed the data: AG-S PAS-S AB. We are grateful to the B05 and B10 laboratories (Cell Cycle Group) at the Centro de Investigación del Cáncer for helpful discussions; we specially thank the help from Sandra Ufano (B05 lab). We also thank S. Moreno and M. Sacristán for critically reading this manuscript. We would like to particularly thank M. D. Vázquez-Novelle for a key suggestion during the revision process. We are in debt with N. Skinner for editing the English text. We are grateful to Paul D. Kaufmann (UMass Medical School) for the anti-PCNA antibody; we are also grateful to T. Hishida (Osaka University), D. E. Gottschling (Fred Hutchinson Cancer Research Center), G. C. Walker (MIT), J. Svejstrup (Cancer Research UK), C. Vázquez de Aldana (IBFG, Salamanca), and S.Moreno (IBMCC, Salamanca) for strains and plasmids.

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