PCNA ubiquitylation ensures timely completion of unperturbed DNA replication in fission yeast

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Abstract

PCNA ubiquitylation on lysine 164 is required for DNA damage tolerance. In many organisms PCNA is also ubiquitylated in unchallenged S phase but the significance of this has not been established. Using \textit{Schizosaccharomyces pombe}, we demonstrate that lysine 164 ubiquitylation of PCNA contributes to efficient DNA replication in the absence of DNA damage. Loss of PCNA ubiquitylation manifests most strongly at late replicating regions and increases the frequency of replication gaps. We show that PCNA ubiquitylation increases the proportion of chromatin associated PCNA and the co-immunoprecipitation of Polymerase \(\delta\) with PCNA during unperturbed replication and propose that ubiquitylation acts to prolong the chromatin association of these replication proteins to allow the efficient completion of Okazaki fragment synthesis by mediating gap filling.

Author summary

PCNA is a homotrimeric complex that clamps around the DNA to provide a sliding platform for DNA polymerases and other replication and repair enzymes. The covalent modification of PCNA by ubiquitin on lysine residue 164 has been extensively studied in the context of DNA repair: it is required to mediate the bypass of damaged template bases during DNA replication. Previous work has shown that PCNA is modified by ubiquitin during normal S phase in the absence of DNA damage, but the significance of this modification has not been explored. Here we show that, in addition to regulating bypass of damaged bases, lysine 164 ubiquitylation plays a role in ensuring the completion of unperturbed DNA replication.
Introduction

It is well established that the replication machinery encounters a variety of obstacles and is thus designed with a degree of flexibility. This plasticity of DNA replication depends on both alternative components and regulation by post-translational modification. For example, while genetic and physical studies indicate that the leading and lagging strands are primarily replicated by DNA polymerase ε (Polε) and DNA polymerase δ (Polδ), respectively [1–4], this assignment is flexible: Polδ synthesises the leading strands on rare occasions [5–7], synthesises both strands during viral replication [8] and can sustain cell viability in the absence of Polε [9].

Key to orchestrating enzymes for DNA replication is PCNA, which serves as a scaffold for recruiting many of the numerous enzymes involved, including the replicative DNA polymerases. In addition, PCNA ubiquitylation on lysine164 regulates DNA damage tolerance (DTT). When replication is blocked by damaged DNA bases the Rad6-Rad18 E2-E3 ligase complex binds to single stranded DNA coated with RPA and mono-ubiquitylates PCNA to promote translesion DNA synthesis by non-canonical polymerases [10, 11]. Subsequent to mono-ubiquitylation, PCNA can be poly-ubiquitylated by the Ubc13-Mms2-Rad5 complex [12, 13] to initiate damage bypass by HR-dependent template switching [14]. The level and duration of PCNA ubiquitylation is additionally regulated by constitutive deubiquitylation [15–17].

The prevailing view is that PCNA ubiquitylation is a DNA damage-induced phenomena. This is consistent with the budding yeast situation, where PCNA ubiquitylation is barely detectable in unperturbed S phase but robustly induced in response to replication-blocking DNA lesions [10, 12]. However, PCNA is robustly ubiquitylated during unperturbed replication in fission yeast [18] and significant levels of PCNA ubiquitylation are evident during unperturbed replication in frog extracts and metazoan cells [19, 20]. Several observations suggest that PCNA ubiquitylation is linked to DNA replication: PCNA ubiquitylation is upregulated in response to an increase in canonical replication intermediates [21–23] and a recent synthetic genetic array analysis in budding yeast showed that the PCNA ubiquitylation pathway is genetically correlated with the mechanism of lagging strand DNA synthesis [24]. Moreover, in vitro reconstitution of PCNA ubiquitylation demonstrates that efficient mono-ubiquitylation is coupled to DNA synthesis by Polδ [25].

Despite the accumulating evidence that PCNA ubiquitylation is linked to the processes of DNA replication, there have been no reports that examine if the process of unperturbed DNA replication is influenced by the ubiquitylation of PCNA and the role of this modification during unperturbed S phase remain unclear. To address this question experimentally, we investigated how replication dynamics are influenced by PCNA ubiquitylation in fission yeast. We find that, in the absence of PCNA ubiquitylation DNA replication is slower and that there is an increase in single stranded DNA gaps in S phase cells. We also observe that PCNA ubiquitylation increases the amount of chromatin associated PCNA and influences the recruitment of Polymerase δ. We propose that PCNA ubiquitylation facilitates the completion of Okazaki fragment synthesis.

Results

In fission yeast PCNA is ubiquitylated during unperturbed S phase [18] and is not significantly further induced by UV-induced DNA damage during S phase (Fig 1A and S1A and S1B Fig). Cells arrested in early S phase by hydroxyurea maintained high levels of PCNA ubiquitylation (S1C Fig) and after irradiation in S phase PCNA ubiquitylation persisted for longer (Fig 1A), likely due to the slowed S phase progression. Thus, the PCNA ubiquitylation promoted by UV-irradiation of asynchronous fission yeast cultures (S1D Fig) is primarily a consequence of
Fig 1. The impact of PCNA ubiquitylation on genome replication. (A) Time-course of PCNA ubiquitylation during S-phase in fission yeast cells. Top-left, experimental scheme: cells were synchronised at the G1/S boundary, UV-irradiated, released from the arrest by incubation at 25°C and samples were analysed at the indicated time points for cell-cycle profile (top-right) and PCNA ubiquitylation status (bottom). As = asynchronous cells. (B) Experimental scheme to analyse replication dynamics by BrdU incorporation: fission yeast cells are synchronised in G2 and BrdU

Ub-PCNA in unperturbed replication
cells accumulating in S phase. To explore what replication defects result in PCNA ubiquitylation, we examined PCNA-Ub in selected temperature-sensitive replication mutants. We observed that inactivation of enzymes required for lagging strand synthesis (DNA ligase 1, Polδ), but not enzymes associated with replisome progression (the MCM complex, Polε), resulted in elevated ubiquitylation levels at lysine 164 of PCNA (S1E and S1F Fig). Collectively, these results indicate that the accumulation of lagging strand intermediates [21–23], but not fork stalling per se, are a major cause of PCNA ubiquitylation.

PCNA ubiquitylation contributes to the progression of replication forks

If incomplete lagging strand synthesis activates PCNA ubiquitylation, it is possible that PCNA-Ub participates in the completion of Okazaki fragment synthesis. To examine this possibility, we first determined the contribution of PCNA ubiquitylation to the progression of unperturbed S phase by assessing replication dynamics in synchronised populations (Fig 1B–1F). Since S. pombe Pcn1 can be modified on lysine 164 by either ubiquitin or SUMO, we first examined cells defective for the Rhp18 E3-ligase (Rhp18 is the S. pombe homolog of S. cerevisiae Rad18. For clarity, we refer to this E3 ligase as Rad18 through the text). While S phase entry was slightly delayed in rad18Δ cells (Fig 1C), bulk replication progression proceeded with similar kinetics when assessed by total bromodeoxyuridine (BrdU) accumulation (Fig 1C). In contrast, while cells carrying the mutation of the ubiquitylated PCNA residue, pcn1-K164R, also slightly delayed S phase entry, their progress through S phase was also defective (S2A and S2B Fig). Importantly, rad18Δ was epistatic with pcn1-K164R for the slight delay to S phase entry (S2A Fig), confirming that the delay seen in rad18Δ cells is PCNA ubiquitylation dependent. It is unclear why the pcn1-K164R mutation also conferred a ubiquitylation-independent defect in S phase progression (S2A Fig). We observed that replication timing was also perturbed and that Polε DNA association during S phase was reduced (see below) in a manner that was independent of the Pli1 SUMO ligase. As pcn1-K164R is thus clearly acting as a hypomorphic allele, we concentrated our analysis on the rad18 deletion mutant cells.

To establish if PCNA ubiquitylation affected the DNA replication kinetics of specific loci, we examined enrichment of BrdU across the genome during mid to late S phase by BrdU-IP in rad18Δ and rad18Δ cells (Fig 1D). This showed changes to the replication dynamics, with advanced replication close to origins and delayed replication for the inter-origin regions. Because relative BrdU enrichment between two samples does not directly reflect relative replication kinetics (the two samples will not be at exactly the same point in S phase), we performed independent replication time courses for rad18Δ and rad18Δ cells and normalised for replication progression in order to directly compare DNA replication timing across the genome.

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added to the media when cells were released. Samples are taken at 75 and 90 minutes for the experiments in panel D and at the indicated times for experiments in panels C and E. (C) Global BrdU incorporation during synchronous S-phase. Replication was analysed by detecting BrdU in purified total genomic DNA by dot-blot. (D) A representative region showing BrdU enrichment following immunoprecipitation and high throughput sequence analysis at two time points for rad18Δ and rad18Δ cells progressing through S phase. (E) Replication profiles throughout S phase. Local replication extents were determined following BrdU immunoprecipitation and high throughput sequence analysis. The progression of local replication for each 300 bp chromosomal region was plotted for three conditions: when global genomic replication progression was either 25%, 50% or 75% complete. The blue line represents rad18Δ, the red line rad18Δ. Filled shaded area between these lines highlights the regions where replication extents differ between the two strains; light blue—rad18 > rad18Δ, light red—rad18 < rad18Δ. (F) Ensemble analysis of replication timing at origins. The distribution of local replication progress when global genomic replication progress is either 25%, 50% or 75% complete. Left: early replicating regions (origins, indicated by open triangle in E). Right: late-replicating regions distal to the origins (indicated by inverted solid triangle in E). (G) Correlation between late replication and relative change in replication timing between rad18Δ and rad18Δ. The latest replicating regions in rad18Δ cells are shown in pink (see S4 Fig).
Replication progression was calculated at each local region of the genome when the global genome replication level was either 25, 50 or 75%. I.e. we used the global extent of replication to standardise comparisons between rad18+ and rad18Δ strains such that the extent of local replication was compared between strains with equivalent global levels. rad18Δ cells showed delayed replication at regions distal to replication origins which are, relative to origins, late replicating (light blue, Fig 1E). This was compensated for by higher local replication at many origin-associated regions that are relatively early replicating (light red, Fig 1E). Some additional peaks were also observed, for example regions 1770-kb region in Chr. II and 3320-kb in Chr. III, suggesting reduced fork progression rates are partially compensated for by firing cryptic origins [26]. The distribution of BrdU at genomic regions surrounding origins would be expected to become wider as S phase progressed (ultimately it would be flat at the end of S phase). Consistent with our hypothesis that replication fork progression is subtly delayed in rad18Δ cells (Fig 1E), we observed that deletion of rad18 resulted in a narrower distribution of BrdU later in S phase when compared to rad18+ control cells (S3A–S3E Fig). Control experiments where we allowed cells to progress into S phase in the presence of hydroxyurea confirmed that the two strains initiated S phase at the same origins and confirm that our sequencing methodology is reproducible (S3B Fig).

To examine further whether rad18Δ caused delayed replication in regions that replicate late, a meta-analysis was performed by computationally identifying replication origins and analysing the relatively late replicating inter-origin regions. As shown in Fig 1F the local replication extent of the early replicating origins was not perturbed in rad18Δ. In contrast, later replicating regions show a significant decrease in their extent of replication, even when adjusted for the global replication amounts. This effect was particularly striking in the regions that were amongst the last to be replicated (Fig 1G). Analysis of the specific loci that were most under-replicated in rad18Δ cells (S4A and S4B Fig) showed they correspond to those loci that we previously demonstrated to be the last to be replicated in wild type cells [5]. These data demonstrate that the lack of PCNA ubiquitylation delays replication fork progression, with the cumulative effect manifesting most obviously at late replicating regions.

**DNA-loaded PCNA is stabilised by ubiquitylation**

PCNA is loaded during DNA replication, functions as the replicative clamp and remains chromatin associated until the polymerase has finished replication and ligation is complete. We speculated that PCNA ubiquitylation may contribute to PCNA retention on the chromatin. However, in native cell extracts PCNA is progressively deubiquitylated, compromising the ability to measure PCNA ubiquitylation during chromatin association assays. To overcome this limitation, we increased the level of PCNA ubiquitylation by engineering a strain, Purg1−rad18+, where rad18+ is under the control of an inducible promoter (Fig 2A). Fractionation of cell extracts following rad18 induction revealed that ubiquitylated PCNA was preferentially associated with chromatin (Fig 2B) in a manner dependent on K164 ubiquitylation (Fig 2C and 2D). This suggests the modification contributes to the stability of PCNA chromatin association. Consistent with this, shut-off of rad18+ transcription from Purgl when combined with induced Rad18 degradation resulted in rapid PCNA disassociation from chromatin, concomitant with deubiquitylation (S5 Fig). Because it is not practical to assay native fission yeast extracts for endogenous levels of ubiquitylated PCNA on chromatin due to its deubiquitylation by isopeptidase in native extracts we compared the total chromatin-associated PCNA in rad18+ and rad18Δ cells during S phase. In rad18+ cells, PCNA accumulated in S phase and gradually diminished towards the completion of replication. Comparatively, in rad18Δ cells, the amount of chromatin associated PCNA decreased during the late stages of replication.
Fig 2. PCNA ubiquitylation influences PCNA chromatin association. (A) PCNA ubiquitylation levels in log phase cells when Rad18 is absent (Δ), expressed normally (+) or upregulated in purg1-rad18 cells (++).

Ubiquitylation was assessed following protein extraction in denaturing condition (TCA prep). (B) PCNA chromatin association. The cells shown in panel A expressing different levels of Rad18 and thus harbouring distinct level of PCNA ubiquitylation were analysed for PCNA chromatin association. Pgk1 (a soluble cytosolic protein) and histone H3 were used as controls. (C) Dependency of chromatin association on PCNA-K164 and
Ub-PCNA in unperturbed replication

An equivalent experiment as shown in panel B for pcn1-K164R and ubc13 mutant backgrounds. (D) Quantification of the modified and unmodified PCNA from C. (E) Chromatin association of PCNA during S phase progression. Top left, experimental scheme: cells were synchronised at the G1/S boundary by temperature shift and PCNA chromatin association (bottom left panel) and cell cycle profiles (right) monitored at the indicated time points after release into S phase. The relative amounts of chromatin-associated PCNA were quantified (centre) (F) Quantification of DNA-associated PCNA by single molecule PALM imaging. Top left: schematic showing how motion blurring filters out mobile molecules. Bottom left: example of the visualisation of only the DNA-associated molecules. Middle: example of static localised molecule (top) and the typical signal from a diffusing molecule (bottom). Right: box plots of the numbers of visualised immobile PCNA molecules in the S phase nuclei of the indicated strains. Bi-nucleate (S phase) mEos3-pcn1 cells (expressing mEos3-tagged PCNA) were imaged and mEos3-PCNA localisations quantified per nucleus.

We verified the observed effect of Rad18 loss on PCNA chromatin association using a photo-activated localization microscopy (PALM)-based technique that directly visualises DNA-associated PCNA [27]. Briefly, this method exploits motion blurring to selectively eliminate signals arising from rapidly diffusing molecules, allowing visualisation of low mobility signals derived from DNA-associated molecules (Fig 2F). Previously we reported that low mobility PCNA (mEos3.1-Pcn1) is notably enriched during S phase [27]. Deletion of rad18 significantly reduced the fraction of these molecules (Fig 2F, right), thus confirming that PCNA-K164 ubiquitylation results in increased amounts of loaded PCNA during unperturbed S phase.

Polδ association with PCNA is increased by PCNA ubiquitylation

One possible explanation for the increased amount of chromatin-associated PCNA accompanying K164 ubiquitylation is that this contributes to the function of DNA polymerases during DNA replication. In unchallenged cells we could detect the association of Polδ, but not Polε, with PCNA by immunoprecipitation (S6A and S6B Fig). This would be consistent with the higher PCNA-dependency of Polδ function [28–30], but may equally reflect the lower levels of DNA-associated Polε during S phase when compared to Polδ. Increased PCNA ubiquitylation (by Rad18 overexpression via Purg1-rad18) increased Polδ co-immunoprecipitation with anti-PCNA without influencing cell cycle profiles (Fig 3A and 3B). PCNA ubiquitylation and co-immunoprecipitation were also both enhanced by hydroxyurea treatment of rad18" and Purg1" rad18 cells. Thus, Polδ: PCNA co-immunoprecipitation intensity scaled with PCNA ubiquitylation (Fig 3A and 3B). We also noted that the PCNA which co-immunoprecipitated with Polδ was biased toward ubiquitylated forms (Fig 3C) and that the loss of poly-ubiquitylation (ubc13 deletion) showed an intermediate decrease in co-immunoprecipitation of Polδ when compared to loss of all ubiquitylation (pcn1-K164R) (Fig 3D). Using the PALM motion blurring assay (see Fig 2F) we did not detect a decrease in the Polδ immobile fraction in untreated S phase rad18Δ cells (Fig 3E), possibly because our assay is insufficiently sensitive. However, when rad18Δ cells were arrested within S phase by hydroxyurea treatment, the fraction of low mobility Polδ molecules decreased when compared to rad18" controls, providing support for the contention that PCNA ubiquitylation contributes to Polδ function.

PCNA recruits DNA polymerases after it is loaded [31] and the affinity of PCNA: Polδ binding is not influenced by K164 ubiquitylation [32]. Thus, the increased Polδ-PCNA association could be accounted for purely by the increased amount of PCNA on DNA due to ubiquitylation inhibiting clamp unloading. This predicts that increasing PCNA chromatin association independently of its ubiquitylation status would lead to increased Polδ: PCNA co-immunoprecipitation. To address this, we examined Polδ-PCNA association in cells deleted

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(Fig 2E). This is reminiscent of the predominant effect of loss of PCNA ubiquitylation manifesting at late replicating regions (Fig 1F and 1G).

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for elg1, where PCNA chromatin association is enhanced due to inactivation of the Elg1 unloader (Fig 3F) [33]. Loss of Elg1 resulted in an increase in Polδ co-immunoprecipitation with PCNA in both rad18Δ and rad18Δ backgrounds (Fig 3G). This result demonstrated that the amount of loaded PCNA relates to the level of PCNA-polymerase association, although we...
cannot rule out the possibility that additional factors that directly respond to PCNA ubiquitylation can also influence the association.

PCNA ubiquitylation is proposed to help ‘replace’ replicative polymerases with non-canonical polymerases. We therefore examined co-immunoprecipitation of several DNA damage tolerant polymerases, Polη, Polκ and Polζ, with PCNA (S6C Fig). Marginal Polη: PCNA co-immunoprecipitation was observed in parg1-rd18 cells, where PCNA ubiquitylation levels were high, consistent with the ubiquitin-binding zinc-finger domain of Polη directing PCNA association. Co-immunoprecipitation of Polκ or Polζ with PCNA was not detectable, presumably due to sparse protein levels (S6C Fig). Taken together, these data indicated that the non-canonical polymerases do not appreciably outcompete Polδ for association with ubiquitylated PCNA. Consistent with this, neither of Polη, Polκ nor Polζ were responsible for the altered BrdU incorporation observed in rad18Δ cells during unperturbed S phase (S6D Fig).

To establish if PCNA modification influences Polδ and Polε function we examined synthetic genetic interactions between rd18Δ and temperature sensitive (ts) polymerase mutations (Fig 4A). For cdc6-23, (Polδ-ts), concomitant rd18Δ reduced the restrictive temperature, consistent with PCNA ubiquitylation enhancing Polδ activity. Importantly, this synthetic genetic interaction was also observed for pcn1-K164R and combining both rd18Δ and pcn1-K164R showed no additive effect (Fig 4B). For cdc20-m10 (Polε-ts) rd18Δ did not affect the restrictive temperature, suggesting Polε activity is not influenced by PCNA ubiquitylation. Consistent with this, when we examined the fraction of low mobility Polε in S phase cells using PALM motion blurring, we did not detect a significant change in when rd18 was deleted (S6E Fig). Interestingly, when we examined Polε mobility in a pcn1-K164R background, a significantly lower fraction of Polε displayed low mobility in S phase cells (S6E Fig). This phenomenon was not observed in a pli1 deletion mutant (S6F Fig). Thus, the K164R mutation has effects beyond that of PCNA ubiquitylation (c.f. S2 Fig) which are unlikely to be related to modification by small Ub-like molecules.

**PCNA ubiquitylation during unperturbed replication promotes gap filling**

During DDT ubiquitylation of PCNA promotes ssDNA gap filling opposite DNA lesions [22, 34, 35]. We have confirmed (S1E Fig) that PCNA ubiquitylation is induced following dysfunction of Okazaki fragment synthesis and demonstrated that this increases the fraction of Polδ co-immunoprecipitating with PCNA (Fig 3A–3C) and can contribute to the chromatin association of this lagging strand polymerase (Fig 3E). Since Polδ repeatedly disassociates from and re-associates with the template during synthesis [32], relatively long lived ssDNA gaps may occur stochastically between Okazaki fragments. We reasoned that PCNA ubiquitylation could act to supress or repair such events via a DTT-like gap filling mechanism during unperturbed S-phase (Fig 4C). This predicts that the absence of PCNA ubiquitylation would result in ssDNA gap accumulation during DNA replication.

To estimate the extent of ssDNA gaps in vivo, we first utilised an S1 nuclease-based assay [36] previously developed for detecting ssDNA in replicated molecules (Fig 5A). By calculating the distribution of DNA fragment sizes from gel intensities (S7 Fig) we infer that rad18Δ cells displayed increased DNA fragmentation when compared to rad18+ cells, with small (<1 kb) fragments accumulating in rad18Δ throughout S phase (Fig 5B–5D). As an alternative assay, we BrdUTP labelled ssDNA gaps in genomic DNA prepared in agarose plugs. When DNA from rad18Δ cells was compared to rad18+, increased signal was evident in mid to late S phase (Fig 5E–5G). These two experiments support a model where PCNA ubiquitylation occurs between Okazaki fragments (Fig 4C) and prevents the accumulation of ssDNA gap during unperturbed S phase (see Discussion).
Fig 4. PCNA ubiquitylation predominantly affects Polδ function. (A) Spot tests showing the influence of PCNA ubiquitylation on cell growth/viability in genetic backgrounds compromised for Polε or Polδ function. cdc20-m10 and cdc6-23 are temperature sensitive mutations in the catalytic subunits of Polε and Polδ, respectively. (B) The effect of pcn1-K164R mutation and rad18 deletion on the temperature sensitivity of cdc6-23 (Polδ) are epistatic. (C) Model. Left: Schematic of Okazaki fragment synthesis. (i) Following priming by Polo-primase PCNA is loaded by RFC. (ii) Polδ associates with PCNA and synthesis begins. Rad18-Rad6 mono-ubiquitylates PCNA. (iii) Stochastic dissociation of Polδ from PCNA. Ubiquitylation prevents PCNA dissociation/unloading. (iv) Polδ re-associates with PCNA and synthesis resumes. (v) Okazaki fragment synthesis is completed. Right, a potential effect of loss of Rad18: (vi) Following priming by Polo-primase PCNA is loaded by RFC. (vii) Polδ associates with PCNA and synthesis begins. (viii) Stochastic dissociation of Polδ from PCNA. (ix) PCNA is not ubiquitylated and thus can dissociate (possibly unloaded by Elg1). (x) Okazaki fragment synthesis is not completed and a gap remains.

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Discussion

Here we have used the fission yeast model to demonstrate that, in addition to its known role in DNA damage tolerance, PCNA K164-ubiquitylation contributes to the timely completion of unperturbed DNA replication. Our results show that PCNA association with chromatin is stabilised by PCNA-K164 ubiquitylation during S phase. We also observed an increased co-immunoprecipitation of Polα with PCNA when PCNA is ubiquitylated and we provide evidence that the chromatin association of Polα is promoted by PCNA ubiquitylation. In budding yeast, PCNA ubiquitylation is barely detectable in unperturbed S phase [22] and robustly...
induced in response to DNA lesions that block the canonical replicative DNA polymerases [10, 12]. Consequently, PCNA ubiquitylation has been studied almost exclusively in the context of its key role in DNA damage tolerance [37]. In contrast, in fission yeast PCNA is robustly ubiquitylated during unperturbed S phase [18] and this is not significantly further induced if DNA is damaged during S phase. Budding and fission yeast thus represent opposite ends of what appears to be a spectrum. We note that both yeasts have approximately similar genome sizes and there is no evidence to suggest that fission yeast suffers from elevated levels of spontaneous DNA damage. Interestingly, mammalian cells exhibit both S phase-dependent PCNA ubiquitylation and DNA damage induced PCNA ubiquitylation (see S8A and S8B Fig).

It is currently not known what underlies the differences between organisms in terms of PCNA ubiquitylation in unperturbed S phase. However, as Rad18 is activated by regions of single stranded DNA it is possible that PCNA ubiquitylation is reflecting the extent of ssDNA present when DNA replication is active. In support of this, in budding yeast a defect in short-flap Okazaki fragment processing caused by compromising the function of the Fen1 flap endonuclease, which normally processes the 5’ end of Okazaki fragments, induced detectable levels of PCNA ubiquitylation [24]. This is explained by the accumulation of long 5’ ssDNA flaps that bind RPA and activate Rad18 ubiquitylation. However, when Okazaki fragment-processing is proficient, the vast majority of flap structures are cleaved by Fen1 when they are 1 or 2 nucleotide in length [38]. Thus, Okazaki fragment processing is unlikely to be a significant source of ssDNA during unperturbed S phase. We have shown here that the lack of PCNA ubiquitylation leads the accumulation of ssDNA gaps during S phase in fission yeast. We propose that the dynamics of Polδ disassociation from PCNA result in stochastic formation of transient gaps during lagging strand synthesis. These gaps trigger Rad18-dependent ubiquitylation of PCNA, which stabilises PCNA on the DNA, allowing association of Polδ and rapid gap resolution. In the absence of PCNA ubiquitylation, a proportion of these gaps persist and thus gaps are detected in our assays. The generation of transient gaps during lagging stand synthesis likely explains the fact that PCNA is ubiquitylated during S phase in this organism.

In support of fission yeast generating increased regions of ssDNA during unperturbed DNA replication (when compared to budding yeast) we note that the abrogation of recombination pathways in fission yeast (e.g. rad51Δ or rad52Δ mutants) causes a much more severe growth defect than the equivalent loss of recombination pathways in budding yeast and that the combination of rad51 deletion with rad18 or pcn1-K164R results in synthetic lethality (S9 Fig). This suggests that homologous recombination and DTT pathways cooperatively repair ssDNA gaps, which may be abundant compared to S. cerevisiae.

In considering the origin of ssDNA during S phase that we observe in S. pombe and the different PCNA ubiquitylation between S. pombe and S. cerevisiae in unperturbed S phase, it is interesting to consider that the kinetics of Polδ holo-enzyme dissociation from PCNA. It has recently been reported [32] that the S. cerevisiae enzyme is more processive than its human counterpart: human Polδ dissociates more rapidly from PCNA than its budding yeast counterpart and it was estimated that ~14–31% of human Okazaki fragments are completed by two independent Polδ: PCNA association events. Conversely, >99% of budding yeast Okazaki fragments are predicted to be completed by a single Polδ: PCNA interaction. While the kinetics of S. pombe Polδ dissociation from PCNA has not been studied, the fact that PCNA ubiquitylation is strongly influenced by the intermediates of lagging strand DNA synthesis [21–23] (see S1E and S1F Fig) is consistent with the fission yeast PCNA ubiquitylation pathway, in addition to regulating translesion synthesis during DTT, functioning to maintain accurate Okazaki fragment synthesis in the face of frequent Polδ: PCNA dissociation.

Okazaki fragment synthesis is necessarily coupled, either directly or indirectly, to the movement of replication forks. Approximately 10⁵ and 10⁷ Okazaki fragments are synthesised per
cell cycle in fission yeast cells and human cells, respectively. The potential for failure during this process as a consequence of premature Polδ dissociation would therefore need to be minimised by ensuring the re-association of Polδ and completion of Okazaki fragment synthesis. We propose that this is facilitated by PCNA ubiquitylation, which ensures that PCNA is not prematurely unloaded. The fact that we show that the loss of PCNA ubiquitylation results in the accumulation of ssDNA gaps during unperturbed S phase in S. pombe (Fig 5) supports our model. Intriguingly, preliminary analysis (S10 Fig) showed the positive effect of PCNA ubiquitylation on PCNA chromatin association is evident only when the Elg1 unloader complex is active, suggesting that PCNA ubiquitylation may inhibit its unloading by Elg1, a PCNA unloading factor currently characterised only in S. cerevisiae [33].

In S. cerevisiae yeast, SUMOylated PCNA is the predominant modification during unperturbed S phase [12, 39]. Previous work showed that Elg1 preferentially interacts with SUMO-modified PCNA [40]. However, Elg1 unloads both unmodified and SUMOylated forms of PCNA, an event which in budding yeast requires the ligation of Okazaki fragments [41]. However, in fission yeast, and in human cells, SUMOylated PCNA is much harder to detect and the SUMO-interacting motifs identified in S. cerevisiae Elg1 are not conserved. Thus, the influence of PCNA SUMOylation is unlikely to be prominent and we propose that the effect of PCNA ubiquitylation on stabilising PCNA is more predominant in fission yeast cells and potentially higher eukaryotes. It has also been suggested that the unloading of PCNA in response to HU or MMS in S. cerevisiae is dependent on its ubiquitylation and concomitant activation of the DNA damage checkpoint [42]. One interpretation of this apparent contradiction could be that, under extensive replication stress, checkpoint activation changes the response to PCNA ubiquitylation. Alternatively, this may again reflect a difference between the two organisms in the regulation of PCNA unloading.

In fission yeast a significant proportion of PCNA is ubiquitylated during unperturbed S phase. To avoid a global engagement of error prone DNA polymerases, we propose that the replicative polymerases remain the preferred binding partners for ubiquitylated PCNA. However, when a replicative polymerase is stalled at a blocking lesion, the ubiquitin binding domain-containing polymerases are provided an increased opportunity to sample the damaged base. In budding yeast the situation is distinct: PCNA is not significantly ubiquitylated in unperturbed S phase, but is robustly ubiquitylated in response to a replicative polymerase arrested at a lesion. Thus, we would predict that the binding kinetics for the replicative and error-prone DNA polymerases will be different between the two organisms in order to maintain the same biological outcomes: an appropriate balance between unsuitable use of error prone DNA polymerases during unperturbed S phase (to minimise constitutive mutagenesis) and their appropriate use during DNA damage tolerance to maximise cell survival in response to DNA damage [43].

In summary, our analysis shows that PCNA ubiquitylation, in addition to controlling DNA damage tolerance pathway usage, also participates in the timely completion of unperturbed DNA synthesis. We propose that this function is related to the increased association of ubiquitylated PCNA with chromatin. We suggest that, when Polδ stochastically dissociates during Okazaki fragment synthesis, the consequent ssDNA results in PCNA ubiquitylation which ensures it remains DNA-associated to facilitate the recapture of Polδ and completion of Okazaki fragment synthesis.

Materials and methods

Yeast strains and molecular genetics

Standard S. pombe genetic and molecular techniques were employed as described previously [44]. The BrdU-incorporating strains have been already reported [45]. Polδ-GFP cells were
constructed by introducing the sequence encoding GFP into the N-terminal of the cdc6 gene on S. pombe genome based on the Cre-loxP method [46]. Pole-GFP cells were constructed by introducing GFP at the C-terminal of cdc20 gene using PCR-based integration [47]. P_{arg1} rad18 strains were based on rad18Δ cells in which ORF of the rad18 gene fused with the AID degron construct [48] was used to replace the endogenous urg1 ORF [49].

**Cell cycle synchronisation in human cells**

U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum (DMEM-FBS10%) in a 5% CO2 atmosphere. The medium was exchanged with one containing 400 ng/ml of nocodazol. Following 18 hr incubation, mitotic cells were detached by gentle shaking of the culture vessel and passaged in DMEM-FBS10%. Cells were then either UV-irradiated (254 nm peak; 20J/m2), or not, 2 hr prior to sampling. At the indicated time points cells were sampled and then subjected to immunoblotting with anti-PCNA antibody (mouse monoclonal, PC10 clone, Abcam). To determine the S-phase fraction of the synchronised cells, 5uM if EdU was added into an aliquot and EdU positive cells scored 2 hr after EdU addition [50]. 1BR3hTERT cells were cultured in DMEM-FBS10% and the medium was exchanged with DMEM without FBS. Following 15 days, cells were passaged into DMEM-FBS10%. Cells were UV-irradiated and scored for S-phase fraction as described. Cell lines from GDSC collection. Authenticated 2015 by STR profiling.

**BrdU-labelling assay**

cdc25-22 cells harbouring the constructs for BrdU-incorporation were grown to exponential phase (0.2 x10^6 /ml) at 25˚C and synchronised at G2 phase by incubation at 36˚C for 3.5 hr. After adding bromodeoxyuridine (0.5 μM), cells were further incubated at 25˚C. At relevant time points, 1x10^6 cells were pelleted and subjected to genomic DNA extraction. To detect total BrdU incorporation, dot blotting was performed as previously described [34]. The intensity of BrdU-incorporation was established by quantifying the signal using an ImageQuant LAS 4000 imager (GE Healthcare Life Sciences). Global replication rates for each time point after release from G2 phase were estimated by dividing signal intensities at each time-points by that for 150 min, at which genome replication was completed. Local replication rates were established from BrdU-IP-Sequencing.

**Analysis of BrdU-IP sequencing**

Paired-end reads from high throughput sequencing were aligned to the S. pombe genome sequence (ASM294v2.23: chromosomes I, II and III, downloaded from 'PomBase' website) using bowtie2–2.2.2. From the alignment data the position of the centre of each read was calculated and the number of reads in 300bp-bins across genome counted. The Perl program converting alignment data to count data: ‘sam-to-count.pl’ is available on the GitHub website (https://github.com/yasukasu/sam-to-bincount). The counts at the chromosome coordinate x, C_B(t,x)=C_B(t,x)/S_C_B(t,x), C_I(t,x)=C_I(t,x)/S_C_I(t,x). Enrichments for BrdU-incorporated fragments were calculated: E(t,x) = N_B(t,x)/N_I(t,x). As BrdU is an analogue of thymine and its enrichment is thus likely to be biased towards A/T rich regions, the dataset of enrichment was normalised using the A/T-ratio of each 300-bp bin AT(x): E'(t,x) = E(t,x)/AT(x). Moving average of E'(t,x) with 8 bins at both side were calculated and plotted (Fig 1D). To estimate the extent of local replication, enrichments across the genome were multiplied by the global replication amount G(t) determined from the dot-blot assay (Fig 1C): L(t,x) = E'(t,x) ×G(t). These
were then normalised with that of the last time point, at which all the cells had completed genome replication: \( L'(t,x) = L(t,x)/L(160, x) \). To obtain a function of local replication extent, data of multiple time points at each 300 bp \((L'(70, x), L'(75, x), L'(80, x), L'(85, x), L'(90, x))\) were fitted with a cumulative normal distribution function in which the global replication amount is variable, \( F(G, x) \). Using this function, Local replication extent when the global replication was 25%, 50% or 75% completed was determined: \( F(0.25, x) \), \( F(0.5, x) \) and \( F(0.75, x) \). Fig 1E–1G and S4 Fig is derived from these datasets. The custom R scripts used for this computational analysis are available on request.

**Chromatin isolation**

Whole cell extracts were prepared by spheroplast lysis using Zymolyase 100T (Seikagaku) and lysing enzyme (Sigma-Aldrich). Extracts were fractionated into soluble and chromatin-bound fractions by centrifugation through a sucrose cushion [51].

**Immunoprecipitation**

5 x 10^8 exponentially growing cells in 50 ml YE medium were treated with 1% formaldehyde for 15 min at RT under agitation. The crosslinking reaction was quenched by adding 2.5 ml of 2.5 M glycine. Cells were washed with ice-cooled PBS, pelleted and re-suspended in 700 μl of RIPA buffer (50mM HEPES pH7.5, 1mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% (w/v) sodium deoxycholate) supplemented with complete protease inhibitor (Roche), 1 mM AEBSF & 1μg/ml pepstatin (Sigma-Aldrich). After adding zirconia/silica beads (biospec), cells were ribolysed (6 bursts of 30 sec at speed 6.5 in a FastPrep ribolyser (MP-Biomedicals). 300μl of cell lysate was sonicated (7 cycles; 30 sec on, 30 sec off) using a bioruptor pico (diagenode), 1 μl of benzonase (novagen) added and incubated for 20 min on ice. The lysate was then centrifuged (14000 rpm for 30 min in a microfuge) and the supernatant transferred to new tube. 30 μl was kept as the 'input' sample. 2 μg of anti-GFP antibody (rabbit IgG, A11122, Life technologies) or anti-PCNA antibody [18] was added to the isolated cell extract. After a 3 hr incubation at 4˚C with gentle agitation, 20 μl of magnetic G protein dynabeads (Life technologies) was added and incubated for a further 1 hr. Beads were washed twice with RIPA buffer and once with TE. Following addition of 60 μl of elution buffer, beads were incubated at 65˚C for 15 min. Supernatant was isolated as the 'IP' sample. Laemmli buffer was added into both 'IP' and 'input' samples and western blots were interrogated with anti-PCNA or anti-GFP (mouse IgG clones 7.1 and 13.1, Roche).

**S1-endonuclease assay**

1 x 10^8 cells were incubated in YE media containing 50 μg/ml of BrdU and subjected to genomic DNA extraction [44]. 2 μg of extracted DNA was digested with 1 μl of S1-nuclease (Life Technologies) using the manufactures buffer in a 20 μl reaction mixture. The reaction was stopped by the addition 2 μl of 0.5 M EDTA and heating to 70˚C for 10 min. The complete reaction mixture was subjected to agarose (1.5%) electrophoresis. DNA was transferred onto GeneScreen Plus membrane (PerkinElmer) by neutral capillary transfer and the BrdU signal detected by the immunoblotting [34]. Normalisation of the BrdU incorporation intensities to the fraction of S1-digested fragments was performed as previously described for alkaline digested DNA [52].

**BrdU gap-filling assay**

4 x 10^7 cells were harvested and subjected to Zymolyase 100T (0.5 mg/ml, Seikagaku) and lysing enzyme (1mg/ml, Sigma-Aldrich) treatment in 1ml of spheroplasting buffer (20 mM
citrate-phosphate buffer, 50 mM EDTA and 1.2M sorbitol). After spheroplasting, cells were re-suspended in 80 μl of spheroplasting buffer without enzymes, mixed with 80 μl of 2% agarose (SeaPlaque GTG agarose) and then 20 μl volume of agarose plugs were prepared. Plugs were washed with detergents and treated with Protease K as described previously [53]. Three plugs were subjected to treatment with T4 polymerase (6 units, New England Biolabs) and dNTP with BrdUTP (Sigma-Aldrich) instead of dTTP (200 μM each) in 100 μl at 37˚C overnight. DNA was recovered from plugs by phenol/chloroform extraction and applied to dot blots (Scie-Plas Ltd.). BrdU signal was detected as described above.

PALM microscopy

Analysis of DNA binding in vivo by PALM was performed as previously described using a custom-built microscope system [27]. Photoconversion and excitation of mEos3 molecules was controlled by continuous wave illumination with 405nm and 561nm laser light. The intensity of the 405nm laser was modulated during the imaging such that the number of photoconverted molecules for any one frame was kept low to reduce the chances of overlapping static molecules, or the possibility of blurring molecules masking static localisations. Laser intensities at the sample were calculated as 0.1 - 1W/cm² (405nm) and 1kW/cm² (561nm). Camera EM gain was set at 250 and exposure time for each frame was 350ms. Typical data acquisition consisted of 3000–4000 frames and 6000–10000 frames for polymerases and PCNA respectively. Data sets were built from of a minimum of 3 biological repeats. Raw image data were processed using a custom ImageJ 2D-Gaussian fitting routine as previously described. Code available on GitHub: https://github.com/aherb/GDSC-SMLM and as a Fiji update site (GDSC SMLM). Scale bar 1.5 micrometers.

Accession numbers

Data files for BrdU-IP sequence have been deposited in the Gene Expression Omnibus database under accession number GSE70033.

Supporting information

S1 Fig. Observation of PCNA ubiquitylation in S phase cells, HU-treated cells and mutants in which lagging strand DNA synthesis is compromised. (A). A repeat of the experiment shown in Fig 1A with quantification (right). (B) Time-course of PCNA ubiquitylation during S-phase in fission yeast cells. Top-left, experimental scheme: cells were synchronised in G2 phase using cdc25-22 and released from the arrest by incubation at 25˚C. At the onset of S phase (45 min after release), cells were UV-irradiated (T = 0). Samples were analysed at the indicated time points for cell-cycle profile (top-right), ratios of cells during mitosis and separated cells, which is the indicator of being in S-phase (bottom-right) and for PCNA ubiquitylation status (bottom-left). As = asynchronous cells. (C) Observation of PCNA ubiquitylation in HU-treated cells. Following releases of cdc10-m17 cells from the G1/S boundary, cells were incubated with media containing 10mM hydroxyurea. (D) PCNA in UV irradiated asynchronous cultures. Exponentially growing cells were irradiated with the indicated dose of UV and then incubated at 30˚C for 1–3 hr. (E) PCNA ubiquitylation analysed in response to specific defects in DNA replication. The indicated temperature sensitive replication mutants were shifted from the permissive to the restrictive temperature and analysed for cell cycle profile (top) and PCNA ubiquitylation status (bottom). cdc10 encodes the homolog of E2F subunit (G1 arrest negative control), cdc20 encodes the catalytic subunit of Pole (leading strand polymerase), cdc6 encodes the catalytic subunit of Polδ (lagging strand polymerase), cdc17 encodes DNA ligase I, cdc21 encodes the Mcm4 homolog (replicative helicase subunit).
(F) Dependency of induced PCNA ubiquitylation due to dysfunction of Polδ or DNA ligase on Lys164 of PCNA, Rad18, Ubc13 and Mms2. The indicated cells were incubated at 36˚C (restrictive temperature) for 4hr.

(TIF)

S2 Fig. The effect of rad18 deletion and pcn1-K164R mutation on cell cycle progression and DNA replication. (A) BrdU-incorporation into genomic DNA during the subsequent S-phase after G2 arrest and release of cdc25-22 cells (see Fig 1 for details). (B) Fraction of septated cells after release from G2 phase.

(TIFF)

S3 Fig. Genome-wide replication profiles in rad18+ and rad18Δ cells. (A) Experimental scheme: cdc25-22 cells were synchronised at G2 and released. At the indicated time points cells were harvested and subjected to BrdU-IP-seq. (B) Representative view of BrdU-incorporation at a genomic region. The data for untreated time points is reproduced from Fig 1D to provide a comparison for the HU treated time points. The counts of reads at the chromosome coordinate x (300-bp bin), CB(x)—BrdU-IP sample, CI(x)—input sample derived from cells before release from G2, were normalised with the total number of reads: NB(x) = CB(x)/ΣCB(x), NI(x) = CI(x)/ΣCI(x). Enrichment of BrdU-incorporated fragments was calculated and plotted: E(x) = NB(x)/NI(x). (C) The computational detection of replication tracks. Replication origins were detected as peaks (blue dots) in the profiles in HU-treated cells. The regions where more than 15 kb of shoulder was associated both sides of the origin were designated as replication tracks (pink boxes). (D) The Overlay of BrdU enrichment data from the ‘untreated, 75min’ sample for the designated replication tracks. Blue: rad18+, red: rad18Δ. Enrichments for each track were normalised with the value at the peak and plotted: i.e. the maximum value of each plotted track is 1. (E) Averages of normalised BrdU enrichment data of all replication tracks.

(TIF)

S4 Fig. Lack of PCNA ubiquitylation results in a significant delay to DNA replication at the latest replicating latest of the genome. (A) Scatter plot of local replication progress at late replicating regions. The sub-population of loci that deviate substantially from the distribution of replication rates in rad18Δ cells is boxed. (B) The late replicating regions indicated in A are marked (pink lines) on the global replication profile [5] of the three fission yeast chromosomes.

(TIF)

S5 Fig. De-ubiquitylation of PCNA results in its release from chromatin. PCNA loaded onto chromatin was monitored upon the shut-off of Rad18. Following growth under inducing conditions of Parg1-rad18-aid (presence of uracil (+Ura), the expression was shut off by washing out uracil (+Ura to -Ura). Residual auxin-degron tagged Rad18 was degraded (+Auxin) or not (-Auxin) by the addition of auxin.

(TIF)

S6 Fig. Co-immunoprecipitation of the replicative polymerases Pore or Polδ with PCNA and the motion blur assay for Pore. (A) Whole cell extracts were prepared from untagged and Polδ-gfp tagged (cdc6-gfp) cells and immunoprecipitated with anti-GFP antibody following protein-crosslinking. (B) Equivalent immunoprecipitation was performed with extracts derived from Pore-gfp tagged (cdc20-gfp) cells. (C) Co-immunoprecipitation of Pore, Polη and Polk using anti-PCNA antibody in cells that exhibit distinct levels of PCNA ubiquitylation (see panel a). * = GFP-tagged polymerase. h ch. = heavy chain. (Bottom right: Alternative exposure of gel emphasising the GFP tagged polymerase bands. (D) BrdU incorporation in cells which
lack individual translesion polymerase; Polξ (rev3Δ), Polη (eso1-D147N) or Polκ (kap1Δ). Two independent experiments were shown. (E,F) DNA-associated Polε was quantified by single molecule PALM imaging (see Fig 2F for details). ns = not significant.

**S7 Fig. Normalisation of BrdU incorporation intensities to the fraction of S1-digested fragments.** Calculations were performed as previously described [52]. (A) Fluorescence intensity curves derived from BrdU incorporation in Fig 5C. (B) The horizontal axis of the graph in A was converted from the position on the gel (pixel) to size of S1-digested fragment (kb). (C) Fluorescent intensity, i.e. the amount of BrdU incorporation per pixel, was converted to the fraction of fragments along with the axis of fragment length.

**S8 Fig. Analysis of PCNA ubiquitylation in human cells.** (A) PCNA ubiquitylation during cell cycle progression in human U2OS cells. Top-left, experimental scheme: cells were synchronised with nocodazole, released into fresh media and samples either irradiated, or not irradiated before harvesting at the indicated time points. The fraction of S phase cells was determined by EdU staining (top-right) and PCNA ubiquitylation status was monitored by western blot (bottom). (B) Equivalent experiment using 1BR3hTERT immortalized human fibroblasts synchronised by serum starvation.

**S9 Fig. Synthetic lethality of homologous recombination and DDT pathways.** Tetrad analysis of a cross between rad52Δ cells and rad18Δ or pcn1-K164R cells. rad52Δ colonies exhibit a slow growth phenotype whereas rad52Δ rad18Δ or rad52Δ pcn1-K164R double mutants are lethal.

**S10 Fig. Quantification of DNA-associated PCNA in elg1-deleted cells by single molecule PALM imaging.** Motion blur of mEos3-PCNA in S phase cells in rad18Δ and rad18Δ cells (see Fig 2F) and in the rad18Δ elg1Δ and rad18Δ elg1Δ backgrounds.

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**Conceptualization:** YD AMC.

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References
1. Kunkel TA. Balancing eukaryotic replication asymmetry with replication fidelity. Curr Opin Chem Biol. 2011; 15(5):620–6. https://doi.org/10.1016/j.cbpa.2011.07.025 PMID: 21862387
2. Miyabe I, Kunkel TA, Carr AM. The major roles of DNA polymerases epsilon and delta at the eukaryotic replication fork are evolutionarily conserved. PLoS Genet. 2011; 7(12):e1002407. https://doi.org/10.1371/journal.pgen.1002407 PMID: 22144917
3. Clausen AR, Lujan SA, Burkholder AB, Crebaugh CD, Williams JS, Clausen MF, et al. Tracking replication enzyme activity in vivo by genome-wide mapping of ribonucleotide incorporation. Nat Struct Mol Biol. 2015; 22(3):185–91. https://doi.org/10.1038/nsmb.2957 PMID: 25622296
4. Reijns MA, Kemp H, Ding J, de Proce SM, Jackson AP, Taylor MS. Lagging-strand replication shapes the mutational landscape of the genome. Nature. 2015; 518(7540):502–6. https://doi.org/10.1038/nature14183 PMID: 25624100
5. Daigaku Y, Keszthelyi A, Muller CA, Miyabe I, Brooks T, Retkute R, et al. A global profile of replicative polymerase usage. Nat Struct Mol Biol. 2015; 22(3):192–8. https://doi.org/10.1038/nsmb.2962 PMID: 25664722
6. Miyabe I, Mizuno K, Keszthelyi A, Daigaku Y, Skouteri M, Mohebi S, et al. Polymerase delta replicates both strands after homologous recombination-dependent fork restart. Nat Struct Mol Biol. 2015; 22(11):932–8. https://doi.org/10.1038/nosmb.3100 PMID: 26436826
7. Flood CL, Rodriguez GP, Bao G, Shockley AH, Kow YW, Crouse GF. Replicative DNA polymerase delta but not epsilon proofreads errors in cis and in trans. PLoS Genet. 2015; 11(3):e1005049. https://doi.org/10.1371/journal.pgen.1005049 PMID: 25742645
8. Waga S, Stillman B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature. 1994; 369(6477):207–12. https://doi.org/10.1038/369207a0 PMID: 7910375
9. Kesti T, Flick K, Keranen S, Syvaoja JE, Wittenberg C. DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. Mol Cell. 1999; 3(5):679–85. PMID: 10360184
10. Davies AA, Huttner D, Daigaku Y, Chen S, Ulrich HD. Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a. Mol Cell. 2008; 29(5):625–36. https://doi.org/10.1016/j.molcel.2007.12.016 PMID: 18342608
11. Niimi A, Brown S, Sabbioneda S, Kannouche PL, Scott A, Yasui A, et al. Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells. Proceedings of the National Academy of Sciences. 2008; 105:16125–30.
12. Hoege C, Pfander B, Moldovan G-L, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature. 2002; 419:135–41. https://doi.org/10.1038/na00991 PMID: 12226657
13. Stelter P, Ulrich HD. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. Nature. 2003; 425(6954):188–91. https://doi.org/10.1038/nature01965 PMID: 12968183
14. Ulrich HD. Timing and spacing of ubiquitin-dependent DNA damage bypass. FEBS letters. 2011; 585:2861–7. https://doi.org/10.1016/j.febslet.2011.05.028 PMID: 21605556
15. Huang TT, Nijman SM, Mirchandani KD, Galardy PJ, Cohn MA, Haas W, et al. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nat Cell Biol. 2006; 8(4):339–47. https://doi.org/10.1038/ncb1378 PMID: 16531995
16. Gallego-Sanchez A, Andres S, Conde F, San-Segundo PA, Bueno A. Reversal of PCNA ubiquitylation by Ubp10 in Saccharomyces cerevisiae. PLoS Genet. 2012; 8(7):e1002826. https://doi.org/10.1371/journal.pgen.1002826 PMID: 22629782
17. Alvarez V, Vinas L, Gallego-Sanchez A, Andres S, Sacristan MP, Bueno A. Orderly progression through S-phase requires dynamic ubiquitylation and deubiquitylation of PCNA. Sci Rep. 2016; 6:25513. https://doi.org/10.1038/srep25513 PMID: 27151299
18. Frampton J, Irmisch A, Green CM, Neiss A, Trickey M, Ulrich HD, et al. Postreplication repair and PCNA modification in Schizosaccharomyces pombe. Mol Biol Cell. 2006; 17(7):2976–85. https://doi.org/10.1091/mbc.E05-11-1008 PMID: 16641370
19. Simpson LJ, Ross AL, Szuts D, Alviari CA, Oestergaard VH, Patel KJ, et al. RAD18-independent ubiquitylation of proliferating-cell nuclear antigen in the avian cell line DT40. EMBO Rep. 2006; 7(9):927–32. https://doi.org/10.1038/sj.embor.7400777 PMID: 16888649
20. Leach CA, Michael WM. Ubiquitin/SUMO modification of PCNA promotes replication fork progression in Xenopus laevis egg extracts. J Cell Biol. 2005; 171(6):947–54. https://doi.org/10.1083/jcb.200508100 PMID: 16344309

21. Das-Bradoo S, Nguyen HD, Wood JL, Ricke RM, Haworth JC, Bielinsky A-K. Defects in DNA ligase I trigger PCNA ubiquitylation at Lys 107. Nat Cell Biol. 2010; 12:74–9. https://doi.org/10.1038/ncb2007 PMID: 20010813

22. Karras GI, Jentsch S. The RAD6 DNA Damage Tolerance Pathway Operates Uncoupled from the Replication Fork and Is Functional Beyond S Phase. Cell. 2010; 141:255–67. https://doi.org/10.1016/j.cell.2010.02.026 PMID: 20403322

23. Suzuki M, Niimi A, Limshichaikul S, Tomida S, Miao Huang Q, Izuta S, et al. PCNA mono-ubiquitination and activation of translesion DNA polymerases by DNA polymerase {alpha}. J Biochem. 2009; 146(1):13–21. https://doi.org/10.1093/jb/mvp043 PMID: 19279190

24. Becker JR, Pons C, Nguyen HD, Costanzo M, Boone C, Myers CL, et al. Genetic Interactions Implicating Postreplicative Repair in Okazaki Fragment Processing. PLoS Genet. 2015; 11(11):e1005659. https://doi.org/10.1371/journal.pgen.1005659 PMID: 26545110

25. Masuda Y, Piao J, Kamiya K. DNA replication-coupled PCNA mono-ubiquitination and polymerase switching in a human in vitro system. Journal of molecular biology. 2010; 396:487–500. https://doi.org/10.1016/j.jmb.2010.01.003 PMID: 20064529
40. Parnas O, Zipin-Roitman A, Pfander B, Liefshitz B, Mazor Y, Ben-Aroya S, et al. Elg1, an alternative subunit of the RFC clamp loader, preferentially interacts with SUMOylated PCNA. EMBO J. 2010; 29 (15):2611–22. https://doi.org/10.1038/emboj.2010.128 PMID: 20571511

41. Kubota T, Katou Y, Nakato R, Shirahige K, Donaldson AD. Replication-Coupled PCNA Unloading by the Elg1 Complex Occurs Genome-wide and Requires Okazaki Fragment Ligation. Cell Rep. 2015; 12 (5):774–87. https://doi.org/10.1016/j.celrep.2015.06.066 PMID: 26213319

42. Yu C, Gan H, Han J, Zhou ZX, Jia S, Chabes A, et al. Strand-specific analysis shows protein binding at replication forks and PCNA unloading from lagging strands when forks stall. Mol Cell. 2014; 56(4):551–63. https://doi.org/10.1016/j.molcel.2014.09.017 PMID: 25449133

43. Hishida T, Kubota Y, Carr AM, Iwasaki H. RAD6-RAD18-RAD5-pathway-dependent tolerance to chronic low-dose ultraviolet light. Nature. 2009; 457(7229):612–5. https://doi.org/10.1038/nature07580 PMID: 19079240

44. Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 1991; 194:795–823. PMID: 2005825

45. Rhind N. Incorporation of thymidine analogs for studying replication kinetics in fission yeast. Methods Mol Biol. 2009; 521:509–15. https://doi.org/10.1007/978-1-60327-815-7_29 PMID: 19563126

46. Werler PJ, Hartsuiker E, Carr AM. A simple Cre-loxP method for chromosomal N-terminal tagging of essential and non-essential Schizosaccharomyces pombe genes. Gene. 2003; 304:133–41. PMID: 12568722

47. Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A 3rd, Steever AB, et al. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast. 1998; 14 (10):943–51. https://doi.org/10.1002/(SICI)1097-0061(199807)14:10<943::AID-YEA292>3.0.CO;2-Y PMID: 9717240

48. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. Nat Methods. 2009; 6(12):917–22. https://doi.org/10.1038/nmeth.1401 PMID: 19915560

49. Watson AT, Daigaku Y, Mohebi S, Etheridge TJ, Chahwan C, Murray JM, et al. Optimization of the Schizosaccharomyces pombe urg1 expression system. PLoS One. 2013; 8(12):e83800. https://doi.org/10.1371/journal.pone.0083800 PMID: 24376575

50. Ogi T, Limsiritchakul S, Overmeers RM, Volker M, Takenaka K, Cloney R, et al. Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells. Mol Cell. 2010; 37(5):714–27. https://doi.org/10.1016/j.molcel.2010.02.009 PMID: 20227374

51. Liang C, Stillman B. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle incdc6 mutants. Genes Dev. 1997; 11(24):3375–86. PMID: 9407030

52. Lujuan SA, Williams JS, Clausen AR, Clark AB, Kunkel TA. Ribonucleotides are signals for mismatch repair of leading-strand replication errors. Mol Cell. 2013; 50(3):437–43. https://doi.org/10.1016/j.molcel.2013.03.017 PMID: 23603118

53. Smith CL, Matsumoto T, Niwa O, Klico S, Fan JB, Yanagida M, et al. An electrophoretic karyotype for Schizosaccharomyces pombe by pulsed field gel electrophoresis. Nucleic Acids Res. 1987; 15 (11):4481–9. PMID: 3295780