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Genome integrity, repair and replication

Ubiquitin protease Ubp1 cooperates with Ubp10 and Ubp12 to revert lysine-164 PCNA ubiquitylation at replication forks

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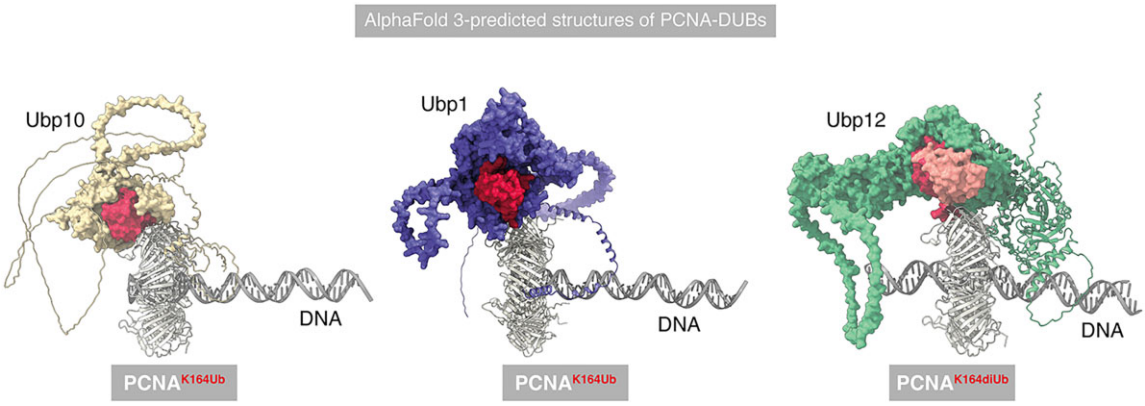
Abstract

Proliferating cell nuclear antigen (PCNA) is essential for the faithful duplication of eukaryotic genomes. PCNA also orchestrates events necessary to address threats to genomic integrity, such as the DNA damage tolerance (DDT) response, a mechanism by which eukaryotic cells bypass replication-blocking lesions to maintain replisome stability. DDT is regulated by the ubiquitylation of PCNA and the consequent recruitment of specialized polymerases that ensure replication continuity. We have recently described that the deubiquitylases Ubp10 and Ubp12 modulate DDT events by reverting the ubiquitylation of PCNA in *Saccharomyces cerevisiae*. This study identifies Ubp1 as a novel PCNA deubiquitylase that cooperates with Ubp10 and Ubp12 in the regulation of DDT during DNA replication. Ubp1, previously known as a cytoplasmic protein, also localizes to the nucleus, where it associates with DNA replication forks. Additionally, Ubp1 interacts with and deubiquitylates PCNA. Here, we provide evidence that Ubp1 collaborates with Ubp10 and Ubp12 to facilitate DNA replication by efficiently reverting PCNA^{K164} ubiquitylation at replication forks under conditions free from exogenous perturbations. Consequently, the deletion of *UBP1*, *UBP10*, and *UBP12* leads to persistent ubiquitylation of PCNA^{K164} and a marked delay in S phase progression.

Graphical abstract



Introduction

DNA replication is a fundamental biological process essential for living organisms, ensuring faithfully genome duplication to propagate genetic material across generations. To this end, replisomes, complex molecular machines assembled at specific replication initiation sites, initiate and execute DNA synthesis under rigorous and overlapping regulatory mechanisms that overcome replication obstacles, preserving genetic stability and cell viability. These obstacles include both endogenous and exogenous DNA damage, to which cells are continuously exposed. During the S phase, cells are particularly vulnerable to DNA lesions, as replicative DNA polymerases are highly accurate but unable to accommodate damaged nucleotides, leading to replication fork stalling. Persistent replication stalling can have severe consequences for genomic stability and cell viability.

To cope with DNA lesions during replication, organisms have evolved DNA damage tolerance (DDT) mechanisms that

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allow the replication machinery to bypass damage, ensuring timely coordination between replication fork progression and DNA repair [1–3]. DDT is exerted through two major pathways: error-prone translesion synthesis (TLS) and error-free template switching (TS), which utilize different molecular mechanisms [3]. TLS involves specialized, evolutionary conserved DNA polymerases capable of bypassing damaged bases, albeit with a higher risk of introducing mutations [4]. In contrast, TS-based DDT mechanisms involve the pairing of a blocked nascent strand with its sister chromatid to copy an intact base, providing an error-free solution (reviewed by Branzei [5]).

Proliferating cell nuclear antigen (PCNA) plays a key role in both DNA replication and DDT [6–8]. It constitutes a moving platform that accurately recruits different factors essential for replication and DDT. Structurally, PCNA forms a ring-shaped homotrimer that encircles DNA and recruits replicative DNA polymerases to accomplish high-fidelity DNA synthesis [9]. Since PCNA lacks enzymatic activity, it exerts its functions through numerous protein–protein interactions, which are regulated by post-translational modifications [7, 10, 11]. An enhancing mechanism in the regulation of DDT is the ubiquitylation of PCNA [7]. Upon encountering DNA lesions, PCNA is monoubiquitylated at lysine 164 (K164) by the evolutionary conserved RAD6/RAD18 (E2/E3) ubiquitin ligase complex [7, 12, 13]. This modification shifts PCNA association from high-fidelity polymerases to low-fidelity polymerases [14–16], promoting the error-prone TLS pathway [17–21], which is essential to prevent replication gaps that constitute a high risk of tumorigenesis [22]. Furthermore, the addition of Lys^{63}-linked ubiquitin chains to monoubiquitylated PCNA (mono-ub$PCNA^{K164}$) by the Rad5/Mms2/Ubc13 PCNA-ubiquitin ligase complex [2, 23] results in polyubiquitylation, which promotes the error-free TS pathway, mitigating genomic instability and tumorigenesis [24, 25].

Although TLS prevents persistent replication stalling, it increases the risk of introducing mutations opposite to DNA lesions, potentially leading to tumorigenesis [7, 13]. On the other hand, TS bypasses lesions without introducing errors, but it also implies certain risks for cells due to the formation of structures between sister chromatids that hinder chromosome segregation [5]. Thus, both TLS and TS pathways must be tightly regulated to minimize deleterious side effects. PCNA deubiquitylation has emerged as a critical regulatory mechanism, limiting DDT activity to maintain normal replication rates and minimize mutagenesis [26, 27].

It has been proposed that specialized PCNA-deubiquitylating enzymes (DUBs), capable of removing ubiquitin residues conjugated to PCNA-K^{164}, suppress excessive DDT events and prevent genomic instability. The mammalian deubiquitylating enzymes Usp1, Usp7, and Usp10 revert PCNA ubiquitylation triggered by DNA damage [26, 28–31]. For instance, USP1 loss leads to aberrant PCNA monoubiquitylation, resulting in enhanced recruitment of error-prone TLS polymerases and destabilized replication forks in cells lacking the homologous recombination factor BRCA1 [31]. Similarly, USP1 knockdown increases mutagenesis levels in 293T human cells [28, 31]. In the fission yeast *Schizosaccharomyces pombe,* the DUBs Ubp2, Ubp12, Ubp15, and Ubp16 cooperate to revert $PCNA^{K164}$ ubiquitylation [32]. In the budding yeast *Saccharomyces cerevisiae*, the ubiquitin protease Ubp10 was initially identified as a PCNA-DUB that removes PCNA ubiquitylation induced by DNA damage or replicative stress [33]. More recently, Ubp10, together with Ubp12, was shown to regulate DDT processes during the progression of exogenously unperturbed S phase [27] or hydroxyurea (HU)-induced replication stress [34]. Moreover, dynamic PCNA ubiquitylation and deubiquitylation occur throughout S phase, with Ubp10 playing a role in Okazaki fragment maturation by promoting $PCNA^{K164}$ deubiquitylation-mediated dissociation of PCNA from chromatin [35].

This study focuses on Ubp1, one of the 17 ubiquitin-specific proteases in the USP family in *S. cerevisiae* [36]. The *UBP1* gene encodes two Ubp1 isoforms: a longer, membrane-anchored form and a shorter, soluble form [37]. The membrane-anchored Ubp1 form has a well-studied role in the regulation of the endoplasmic reticulum-associated protein degradation pathway as a ubiquitin-specific protease of the Hrd1 protein [38], while the shorter form is involved in endocytosis [37]. Here, we show that Ubp1 also functions as a PCNA-DUB in *S. cerevisiae*. A fraction of Ubp1 localizes to the nucleus, where it interacts with PCNA and associates with replication forks. The simultaneous deletion of *UBP1*, *UBP10*, and *UBP12* leads to elevated and stabilized PCNA ubiquitylation throughout unperturbed S phase (defined as an S phase not subjected to any exogenous insult or damage), resulting in severe S phase progression defect. Notably, retention of Ubp1 in the nucleus rescues this phenotype. Consistently, the non-ubiquitylable PCNA variant ($pol30^{K164R}$) mitigated to a notable extent the replication delay observed in the triple DUB mutant. Additionally, by 2D gel analysis of replication intermediates, we found that Ubp1 contributes to resolving transient TS-dependent replication structures generated under replication stress. Altogether, these findings suggest that Ubp1 contributes to proper S phase progression by regulating PCNA deubiquitylation. This study brings a new piece of knowledge about the still enigmatic processes of the regulation of PCNA and contributes to a better understanding of the complex regulation of DNA replication to preserve genomic integrity and cell viability.

Materials and methods

Yeast strains, growth conditions, and media

All the budding yeast used in these studies originate from a *MATa* W303 *RAD5 bar1::LEU2* strain [33] and are listed in Supplementary Table S1. For the *in vitro* analysis of Ubp1 activity, a fission yeast strain listed in the Supplementary Table S1 was used as a source of ubiquitylated PCNA. Budding yeast strains were grown in YPAD medium (1% yeast extract, 2% peptone supplemented with 50 μg/ml adenine) containing 2% glucose. For block-and-release experiments, cells were grown in YPAD with 2% glucose at 25°C and synchronized in G1 with α-factor pheromone (40 ng/ml, 2.5 h). Cells were then collected by centrifugation (3000 rpm, 3 min) and released into fresh media (supplemented with 50 μg/ml of pronase) in the absence or in the presence of HU (0.2 M, Formedium). Overexpression experiments with cells grown in YPAD medium with 2% raffinose at 25°C were conducted by adding to the medium 2.5% galactose (to induce) or 2% glucose (to repress).

For plate survival assays, stationary cells were counted and serially diluted in YPAD media. Ten-fold dilutions of equal

numbers of cells were plated onto YPAD (2% glucose) media (always supplemented with 50 μg/ml adenine) or YPAD containing 0.02% methyl methanesulfonate (MMS), incubated at 25°C for 24, 48, 72, or 120 h, and then scanned.

General experimental procedures

General experimental procedures of yeast molecular and cellular biology were used as described previously [39–41]. A list of the plasmids used for strain generation is shown in Supplementary Table S2. Transformation was performed by the lithium acetate protocol, and transformants were selected by growing in selective medium.

Flow cytometry analysis

For flow cytometry analyses, 10^7 cells were collected by centrifugation, washed once with water, fixed in 70% ethanol, and processed as described previously [42]. Cells were prepared using a modification of the method, by using SYTOX Green (Molecular Probes) for DNA staining [43, 44]. The DNA content of individual cells was measured using a Becton Dickinson Accuri C6 plus FACScan.

Protein methods

Protein extract preparation and western blot analysis

Whole cell extracts (WCEs) were prepared by precipitation with trichloroacetic acid (TCA). Yeast strains were grown in YPAD medium to OD_{600} of 0.8–1.0, and cells (5 ml) were collected by centrifugation just after the addition of 100% TCA to a final concentration of 10% TCA and washed with 20% TCA. Cell disruption was performed with glass beads in a FastPrep and 12.5% TCA. Cell lysates were pelleted by centrifugation at 3000 rpm and resuspended in 1× LB loading buffer and Tris base.

For chromatin-enriched fractions, around 6×10^7 exponentially growing cells were harvested by centrifugation and resuspended in 1 ml of Buffer 1 [containing 150 mM Tris, pH 8.8, 10 mM dithiothreitol (DTT), and 0.1% sodium azide], and incubated at room temperature for 10 min. Cells were pelleted, washed with 1 ml of Buffer 2 (50 mM KH_2PO_4/K_2HPO_4, pH 7.4, 0.6 M sorbitol, and 10 mM DTT), resuspended in 200 μl of Buffer 2 supplemented with 40 μg Zymolyase-100T, and incubated at 37°C for 10 min with intermittent mixing. The resulting spheroplasts were washed with 1 ml of ice-cold Buffer 3 (50 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, and 0.4 M sorbitol), followed by resuspension and a 5-min incubation in 100 μl of EBX buffer [50 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 0.25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor tablets (Ethylenediaminetetraacetic acid-free, EDTA-free, Roche), leupeptin 1 μg/ml, pepstatin 2.5 μg/ml, and RNase 10 μg/ml], with occasional mixing. Aliquots of 30 μl of these disrupted cell suspensions were collected as WCE samples. Remaining volume was layered onto 70 μl of cold EBX-S buffer (EBX buffer supplemented with 30% sucrose) and subjected to centrifugation at 12000 rpm for 10 min at 4°C. Aliquots of 30 μl of the resulting supernatant layer (chromatin-free fraction) were also collected. After discarding supernatant, chromatin pellets were washed with 200 μl of EBX-S buffer, resuspended in 70 μl of EBX buffer supplemented with 0.5 μl of Benzonase, and incubated on ice for 15 min (chromatin fraction). A 5× loading buffer was added to each fraction.

Protein extracts were resolved by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8%, 10%, 12%, or 15%) and transferred to nitrocellulose membranes using a Bio-Rad transfer unit. Blots were then probed against the antibodies indicated. A list of the antibodies used in this study is shown in Supplementary Table S3. Secondary horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse antibodies (as required) were also used and the ECL kit (Amersham Pharmacia Biotech) for detection.

Co-immunoprecipitation

Immunoprecipitation of Flag-tagged PCNA protein was performed from chromatin extracts of strains expressing PCNA-Flag and/or Ubp1-RFP fusion proteins. Cells were grown in YPAD medium at 25°C to an OD_{600} of 0.8–1.0 (25 ml), synchronized with α-factor, and released in the presence of HU (0.2 M) for 90 min. Chromatin extracts were prepared as indicated in the "ChIP-qPCR analysis" section of the "Materials and methods" section. Extracts were incubated with Dynabeads™ protein G (Invitrogen) bound to monoclonal anti-Flag antibody (Agilent Technologies) for 5 h at 4°C. Beads were washed four times with lysis buffer and resuspended in loading buffer. Immunoprecipitates were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and analyzed with anti-RFP (Chromotek) and anti-Flag-HRP conjugated (Sigma) antibodies.

In vitro deubiquitylation assays

Immunoprecipitation of PCNA-Flag was performed from a *ubp12*:NES *ubp15*:NES *Δubp16 pcn1-Flag S. pombe* strain (see Supplementary Table S1), synchronized in S phase by treatment with 20 mM HU for 2 h. *Schizosaccharomyces pombe* PCNA is a reliable and abundant source of ubiquitylated PCNA lacking SUMO-PCNA, which would otherwise hamper our *in vitro* assay [32]. Immunoprecipitation of Myc-tagged Ubp1 proteins was performed from asynchronously growing cells. HA-tagged Ubp8 and Myc-tagged Ubp10 proteins were also immunoprecipitated in the same way to be used as negative and positive controls, respectively. Both wild-type and the catalytically inactive form of Ubp1 were purified from soluble protein extracts prepared as described previously [45]. Briefly, cells were collected, washed, and broken in HB2T buffer (60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM 4-morpholinepropanesulfonic acid (pH 7.2), 15 mM $MgCl_2$, 15 mM ethyleneglycol-bis(β-aminoethyl)-N,N,N,N-tetraacetic acid (EGTA), 1 mM DTT, 0.1 mM sodium orthovanadate, 2% Triton X-100, 1 mM PMSF, and 20 mg/ml leupeptin and aprotinin) using glass beads. Glass beads were washed with 500 μl of HB2T, and supernatant was recovered. Protein concentrations were measured using the BCA assay kit (Pierce), and immunoprecipitations (from 4 mg of protein extracts) were carried out by incubation with anti-Myc or anti-Flag antibody-bound magnetic beads during 5 h at 4°C. Immunoprecipitation assays were confirmed by immunoblotting. Immunoprecipitates were washed twice with lysis buffer and then twice with DUB buffer (60 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 4% glycerol). Beads were incubated 1 h at 30°C. As negative controls, catalytically inactive Ubp1 ($Ubp1^{C110S}$) and Ubp8 were used. Ubiqui-

tin vinyl sulfone (Ub-VS) (Enzo Life Sciences) covalently captures active DUB enzymes and therefore acts as a potent and irreversible inhibitor of DUBs through the covalent modification of their active sites [46]. Reactions were stopped by adding loading buffer and boiling the samples for 5 min at 95°C. Proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and analyzed with anti-Flag-, anti-Myc-, or anti-HA-HRP conjugated antibodies.

ChIP-qPCR analysis

We adapted a described protocol [47] for the analysis of Myc-tagged Ubp1 *ARS305*, *ARS306*, *ARS603*, or *ARS607* binding in *S. cerevisiae* cells. In brief, Ubp1-Myc or wild-type untagged cells (used as control) were synchronized with α-factor and released in the presence of HU (0.2 M). After 1 h, samples (50 ml cultures) were taken and subjected to 30 min of crosslinking with 1% formaldehyde. Then, cells were collected by centrifugation and washed three times with ice-cold Tris Buffer Saline (TBS). Cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) supplemented with antiproteolytic cocktail and broken using glass beads.

Recovered cell lysates were centrifugated at 12000 rpm, supernatants (soluble protein fractions) were discarded, and chromatin pellets were sheared by sonication. Extracts were clarified, and soluble chromatin fractions were used for immunoprecipitation with anti-Myc antibodies (5 h at 4°C). Antibody-bound magnetic beads were washed as for CoIPs assays, and chromatin was eluted in elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) by incubating for 10 min at 65°C. Samples were incubated overnight at 65°C in TE (+1% SDS) for de-crosslinking, treated with proteinase K, DNA extracted by phenol/chlorophorm/isoamyl alcohol (pH 8.0), and treated with 0.3 μg/ml RNase A in Tris-EDTA buffer (TE). Finally, DNA was purified with QIAquick® PCR purification kit and 1–10 ng of immunoprecipitated or input DNA was amplified with iQ™ SYBR Green Supermix (Bio-Rad) using a real-time PCR machine (Bio-Rad IQ™ 5). A list of the specific primers used is shown in Supplementary Table S4. All data in the bar graphs are presented as an average of $n \geq 3$ replicates ± standard deviation (SD), where n represents the number of biological replicates.

Two-dimensional DNA gels (2D gel analysis)

DNA samples for neutral–neutral two-dimensional gel electrophoresis were prepared and analyzed as described previously [39, 48]. DNA was cut with the NcoI restriction enzyme, transferred to Hybond-XL (GE Healthcare) nitrocellulose membrane, and hybridized to probes spanning the *ARS305* and *ARS306* origins of DNA replication. For each origin of replication tested, the specific probe corresponds to the following coordinates (retrieved from SGD): *ARS305* (39073–40557, Chr III) and *ARS306* (73001–73958, Chr III). Images were acquired using a Molecular Imager FX (Bio-Rad) and different replication-associated DNA molecules were quantified using Quantity One 4.6 software (Bio-Rad).

Microscopy

Green Fluorescent Protein (GFP)- and Red Fluorescent Protein (RFP)-tagged strains were grown in YPAD or YPA + 2.5% galactose medium until exponential phase. Expression of the different chimeric Ubp1 forms was either repressed by adding glucose or induced with galactose in the medium. 4′,6-Diamidino-2-fenilindol (DAPI) staining was used to visualize DNA and the presence of specific fluorescence was detected by fluorescence microscopy using a Thunder Imager 3D Tissue (camera, DFC9000; Leica) microscope.

Results

Ubp1 cooperates with Ubp10 and Ubp12 in PCNA deubiquitylation to regulate S phase progression

We have previously reported that in the budding yeast *S. cerevisiae*, the ubiquitin proteases Ubp10 and Ubp12 deubiquitylate PCNA during the S phase to regulate the DDT response, allowing a processive DNA replication in unperturbed cycling cells. The lack of Ubp10 alone already causes a significant slow S phase progression, although it is the lack of both Ubp10 and Ubp12 proteases that is necessary to detect by immunoblotting the accumulation of ubiquitylated PCNA forms in asynchronous cell cultures [27].

To understand the participation of these two ubiquitin proteases in the dynamic ubiquitylation/deubiquitylation-dependent regulation of PCNA during DNA replication, the ubiquitylation state of PCNA in *ubp10Δ ubp12Δ* double mutant cells during S phase progression was analyzed by immunoblot. Cells were synchronized in G1 by treatment with α-factor and then released into fresh medium to allow progression through the S phase (Fig. 1A). Samples were taken every 10 min for 2 h, and genome replication was followed by fluorescence-activated cell sorting (FACS). As expected, no ubiquitylated forms of PCNA were detected in wild-type cells. However, PCNA appeared ubiquitylated between the 50- and 90-min time points after α-factor release in the *ubp10Δ ubp12Δ* double mutant (Fig. 1B). The accumulation of ubiquitylated PCNA forms correlated with a significant delay in the S phase progression of these cells (Fig. 1C), previously observed in cells lacking Ubp10 alone [27], suggesting an important role of PCNA deubiquitylation in supporting normal replication rates. However, ablation of the two known PCNA-DUBs, Ubp10 and Ubp12, did not fully prevent deubiquitylation of PCNA, and the ubiquitylated forms disappear at later replication time points (Fig. 1B), implying that additional PCNA-DUBs may be involved in this process.

Based on our previous biochemical screenings performed to identify the ubiquitin proteases involved in PCNA regulation [33], we analyzed cells lacking each of the different known DUBs in combination with the deletion of *UBP10* and *UBP12*, looking for increased levels of ubiquitylated PCNA in each triple mutant generated. We found that only the deletion of *UBP1*, one of the 17 known ubiquitin-specific protease genes in *S. cerevisiae*, together with the absence of *UBP10* and *UBP12*, resulted in a significant increase in ubiquitylated PCNA forms compared to that observed in *ubp10Δ ubp12Δ* double mutant cells asynchronously growing (Supplementary Fig. S1A), strongly suggesting that Ubp1 was also involved in the deubiquitylation of PCNA.

We next analyzed the pattern of PCNA ubiquitylation during S phase progression in the triple mutant *ubp1Δ ubp10Δ ubp12Δ*. As shown in Fig. 1B, the lack of *UBP1* alone did not affect the ubiquitylation state of PCNA. However, in combination with the absence of *UBP10* and *UBP12*, it caused the stabilization of ubiquitylated PCNA forms from 50 min af-

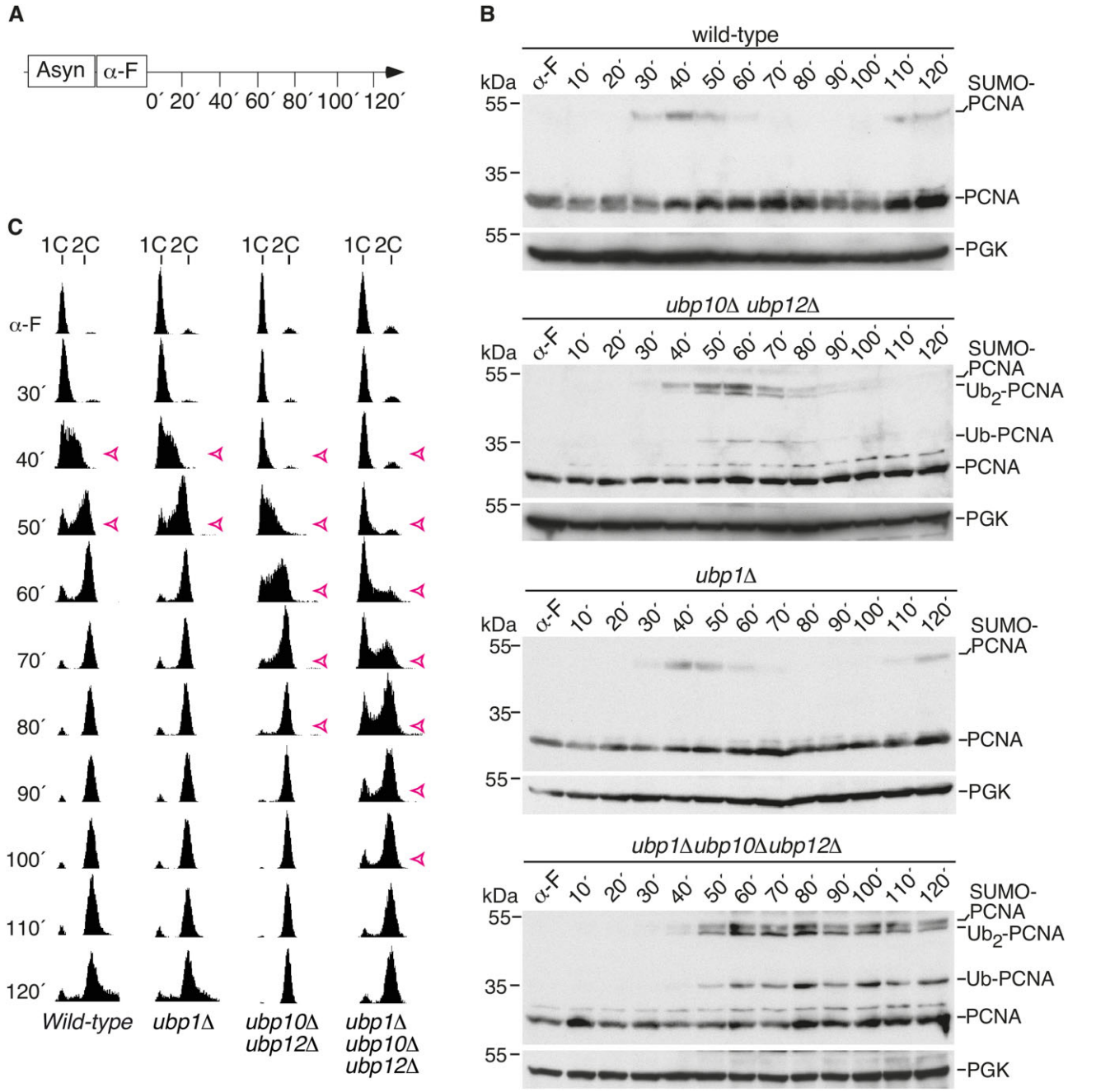

Figure 1. Depletion of Ubp1 in combination with *ubp10Δ ubp12Δ* leads to a marked synergistic delay in S phase progression and to the accumulation of ubiquitylated PCNA forms beyond S phase. (**A**) Experimental design. Exponentially growing cultures of wild-type, *ubp1Δ*, *ubp10Δ ubp12Δ*, and *ubp1Δ ubp10Δ ubp12Δ* strains expressing PCNA-Flag fusion protein were synchronized at G1 by incubation with α-factor and then released into fresh yeast complex medium (YPAD). Samples were taken at indicated intervals and processed for FACS and immunoblot analysis. (**B**) Protein extracts were processed for immunoblotting with anti-Flag antibodies. 3-Phosphoglycerate kinase (PGK) was used as a loading control. (**C**) DNA content analysis by FACS shows the progression of genome replication from α-factor synchronization to the 120 min time point after release. Arrows indicate the replication time points. Two biological replicates were performed, and a representative experiment is shown.

ter α-factor release until the end of the time course analysis. Interestingly, the lack of deubiquitylation of PCNA in the triple mutant correlated with a remarkably extended S phase, longer lasting than the delay observed in Ubp10/Ubp12-ablated cells (Fig. 1C). In the case of the single *ubp1Δ* mutant, progression through the S phase showed wild-type kinetics (Fig. 1C). These data point to Ubp1 as a novel PCNA deubiquitylase collaborating with Ubp10 and Ubp12 in the regulation of S phase progression.

A nuclear soluble form of Ubp1 has a role in PCNA deubiquitylation during DNA replication of unperturbed cell cycle

Ubp1 is a ubiquitin protease involved in the regulation of endoplasmic reticulum-associated protein degradation and vesicle trafficking pathways [37, 38]. Ubp1 has been described as a cytoplasmic protein with two different isoforms originated from two transcription initiation sites (methionine residues 01 and 67) [37] (Fig. 2A). The two isoforms correspond to the

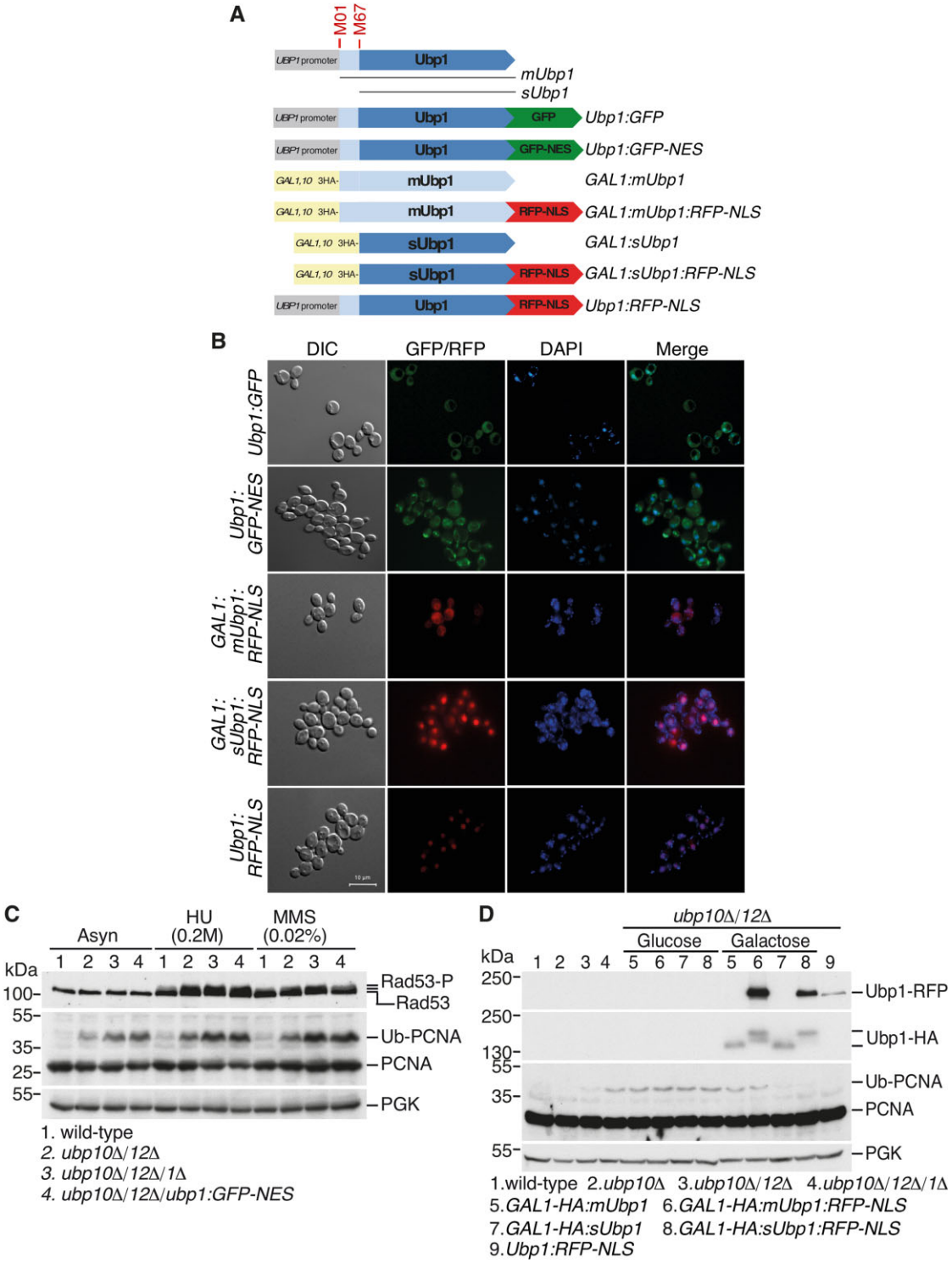

Figure 2. A soluble nuclear population of ubiquitin protease Ubp1 reverts the ubiquitylation state of PCNA. (**A**) Scheme of the chimeric Ubp1 enzyme constructs used in panels (B), (C), and (D). The *UBP1* gene has two transcription initiation sites (M01 and M67), which give rise to two distinct isoforms (*mUbp1* and *sUbp1*). M, methionine residue; NES, nuclear exclusion signal; and NLS, nuclear localization signal. (**B**) Fluorescence microscopy analyses of Ubp1 constructs fused to GFP or RFP fluorescence proteins, as shown in panel (A). Cells were stained with DAPI to visualize DNA. Bar, 10 mm. (**C**) Analysis of ubiquitylated PCNA levels in the indicated strains under both untreated and treated conditions. Asynchronously growing cells (Asyn) were treated with 0.2 M HU or 0.02% MMS for 1 h. Total protein extracts were resolved by 10% SDS–PAGE and immunoblotted with polyclonal anti-PCNA antibody. Rad53 phosphorylation was analyzed to verify checkpoint activation upon treatments. PGK immunoblotting served as a loading control. (**D**) Analysis of ubiquitylated PCNA levels of the indicated strains grown under exogenously unperturbed conditions. The *ubp10Δ ubp12Δ* strain, also containing *GAL1:mUbp1*, *GAL1:mUbp1:RFP-NLS*, *GAL1:sUbp1*, and *GAL1:sUbp1:RFP-NLS* constructs, was incubated in media with glucose or galactose as the sole carbon source. Expression of the different chimeric Ubp1 forms was repressed by glucose or induced by galactose in the medium. Notably, in the presence of glucose, the *GAL1:mUbp1*, *GAL1:mUbp1:RFP-NLS*, *GAL1:sUbp1*, and *GAL1:sUbp1:RFP-NLS* constructs displayed a phenotype similar to that of a *ubp1Δ* mutant. Total protein extracts were resolved by 10% SDS–PAGE, followed by immunoblotting with anti-PCNA, anti-RFP, and anti-HA antibodies. PGK immunoblotting served as a loading control.

full-length isoform, which is membrane-anchored (mUbp1) through an N-terminal transmembrane (TM) segment and localized to the endoplasmic reticulum, and a shorter isoform that lacks the TM domain and is soluble (sUbp1) [37]. The above results, according to which the lack of Ubp1 can increase the PCNA ubiquitylation state, led us to hypothesize the existence of a not-yet-described nuclear population of Ubp1 that might collaborate in the regulation of PCNA ubiquitylation. To address this point, we focused on the localization of Ubp1 by generating two constructs in which Ubp1 was tagged with the fluorescent GFP epitope, either with or without a nuclear exclusion signal (NES) to efficiently prevent its potential nuclear location [32] (Fig. 2A). As expected, the Ubp1-GFP fusion protein was distributed all over the cell, a pattern unable to confirm a clear nuclear location. However, Ubp1-GFP-NES protein showed a specific cytoplasmic location, clearly excluded from the DAPI signal (Fig. 2B), suggesting that Ubp1 is also a nuclear protein. Next, using a rabbit polyclonal antibody that detects PCNA and its monoubiquitylated forms (Supplementary Fig. S1B), we checked the accumulation of PCNA ubiquitylation in Ubp1:GFP-NES mutant in combination with the ablation of Ubp10 and Ubp12 under asynchronous growing conditions. As shown in Fig. 2C, the exclusion of Ubp1 from the nucleus recapitulates the *UBP1* ablation phenotype. In a wild-type background, PCNA is widely ubiquitylated in response to DNA damage or replication stress. Therefore, we tested the ability of *ubp10Δ ubp12Δ Ubp1:GFP-NES* triple mutant to accumulate ubiquitylated PCNA forms upon the induction of replicative stress by treatment with the alkylating agent MMS or the ribonucleotide reductase inhibitor HU. We confirmed that *ubp10Δ ubp12Δ* double mutant cells accumulate higher levels of PCNA ubiquitylation than the wild-type cells ([27] and Fig. 2C). Moreover, similar to what was observed for unperturbed cycling cells, the combination of *ubp1Δ* and *ubp10Δ ubp12Δ* determined a synergistic increase in ubiquitylated PCNA levels in both HU- and MMS-treated cells, which was also observed in the *ubp10Δ ubp12Δ ubp1:GFP-NES* mutant (Fig. 2C; compare lanes 2–4). These data indicate the existence of a nuclear population of Ubp1 that plays a role in PCNA deubiquitylation.

As mentioned earlier, Ubp1 has two different isoforms, a membrane-anchored and a soluble form [37]. We aimed to discriminate between these two forms in terms of their potential ability to regulate PCNA ubiquitylation. To this end, we replaced the endogenous *UBP1* promoter with the conditional *GAL1-10* promoter and engineered cells to express only one of the two isoforms by deleting or not the 66 N-terminal amino acids (*GAL1:mUbp1* or *GAL1:sUbp1* strains, Fig. 2A). In addition, these two Ubp1 forms were tagged with the fluorescent RFP epitope linked to a nuclear localization signal (NLS) to visualize their cellular localization. As a control, Ubp1 was also tagged with the RFP epitope linked to the NLS motif (Fig. 2A). By fluorescence microscopy analysis, we confirmed that while the larger Ubp1 variant (*GAL1:mUbp1:RFP-NLS* strain) surrounds the nucleus, probably co-localizing with the nuclear membrane, soluble Ubp1 (*GAL1:sUbp1:RFP-NLS* strain) shows a clear nuclear localization (Fig. 2B). We then analyzed the effect of overexpressing these isoforms on the characteristic accumulation of ubiquitylated PCNA forms in *ubp10Δ ubp12Δ* mutant cells. As shown in Fig. 2D, only the overexpression of the soluble isoform of Ubp1 counteracted the accumulation of Ub-PCNA (Fig. 2D, compare lanes 7 and 8 in glucose versus galactose). Overexpression of Ubp8, a nuclear DUB unrelated to PCNA, was used as a negative control. As shown in Supplementary Fig. S2, inducing high levels of Ubp8 expression had no impact on the ubiquitylation state of PCNA.

Interestingly, we also found that the mere retention of endogenous Ubp1 in the nucleus (*Ubp1:RFP-NLS* strain), without protein overproduction, was able to restore wild-type levels of PCNA ubiquitylation in *ubp10Δ ubp12Δ* mutant cells (Fig. 2D, lane 9). The same was observed in cells treated with MMS (0.02%) for 90 min to induce DNA damage-mediated ubiquitylation of PCNA. Retention of Ubp1 in the nucleus abolished the higher accumulation of ubiquitylated PCNA forms caused by the lack of both Ubp10 and Ubp12 PCNA-DUBs, reaching the same levels of wild-type cells (Supplementary Fig. S3A). Moreover, the growth defects, as well as the MMS sensitivity of *ubp10Δ ubp12Δ* mutant cells, were also rescued in *ubp10Δ ubp12Δ ubp1:NLS* mutant cells (Supplementary Fig. S3B).

PCNA ubiquitylation dynamics in *ubp10Δ ubp12Δ ubp1:RFP-NLS* cells during the S phase were also analyzed (Fig. 3A). As shown in Fig. 3B and Supplementary Fig. S4, Ubp1-mediated PCNA deubiquitylation was effective, and consequently, no ubiquitylated forms of PCNA were detected during S phase progression, as occurs in wild-type cells. Interestingly, the delay in S phase progression observed in *ubp10Δ ubp12Δ* cells was abolished by Ubp1 activity, restoring a wild-type replication rate (Fig. 3C), suggesting a correlation between both phenotypes. Finally, we investigated whether the slow progression through S phase observed in the *ubp1Δ ubp10Δ ubp12Δ* mutant cells is caused by defects in PCNA deubiquitylation. To address this, we analyzed S phase progression in $pol30^{K164R}$ *ubp1Δ ubp10Δ ubp12Δ* mutant cells and observed that the non-ubiquitylable PCNA variant $pol30^{K164R}$ partially suppresses the S phase progression phenotype that characterizes the *ubp1Δ ubp10Δ ubp12Δ* triple mutant (Fig. 4). These findings support the hypothesis that a soluble nuclear population of Ubp1 is involved in the regulation of PCNA deubiquitylation during DNA replication.

Ubp1 is a PCNA-DUB that associates with replication forks

The above observations correlated Ubp1 with PCNA deubiquitylation *in vivo*. Both the deletion and overexpression phenotypes of Ubp1 were consistent with the hypothesis that Ubp1 has a role as a PCNA ubiquitin protease inside the nucleus. To further address this issue, we first tested whether nuclear Ubp1 is present in chromatin during DNA replication. Cells expressing Myc-tagged Ubp1 were synchronized with α-factor and then released into fresh YPAD medium to allow cells to progress through the S phase. Samples were taken at different time points and processed in order to obtain specific cellular fractions. Genome replication was followed by FACS. As shown in Fig. 5A, although the vast majority of Ubp1 was detected in whole-cell extracts and chromatin-free fractions, it also appeared to be linked to chromatin at all time points analyzed. These results correlate with those obtained by fluorescence microscopy (Fig. 2B) and indicate that a nuclear population of Ubp1 is bound to chromatin.

We next studied whether Ubp1 and PCNA interact *in vivo*. Since C-terminally RFP-tagged Ubp1 fusion proteins were found to be functional by fluorescence microscopy, we used these constructs in combination with Flag-PCNA tagged pro-

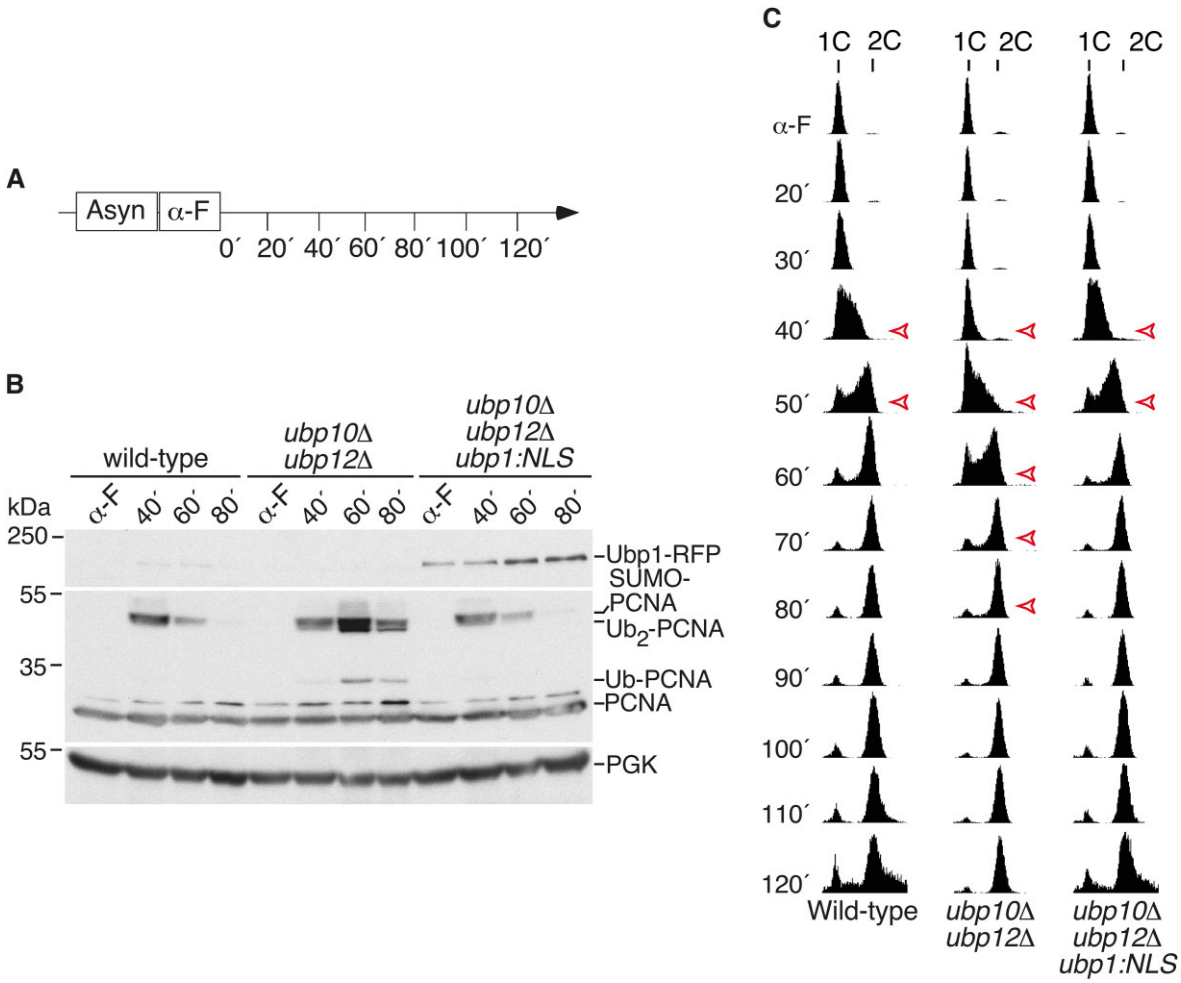

Figure 3. Retention of Ubp1 in the nucleus suppresses the S phase progression delay of PCNA-DUBs Ubp10 and Ubp12 depleted cells. (**A**) Experimental design. Exponentially growing cultures of wild-type, *ubp10Δ ubp12Δ*, and *ubp10Δ ubp12Δ ubp1:NLS* strains expressing PCNA-Flag fusion protein were synchronized with α-factor and released into fresh medium (YPAD). Samples were taken at the indicated time points and processed for FACS and immunoblot analysis. (**B**) *ubp1:NLS* reverts the accumulation of ubiquitylated forms of PCNA in *ubp10Δ ubp12Δ* cells. Protein extracts were analyzed by immunoblotting with an anti-Flag antibody. PGK protein was used as a loading control. (**C**) DNA content analysis by FACS illustrates genome replication progression from α-factor synchronization to the 120 min post-release. Arrows indicate the replication time points. Two biological replicates were performed, and a representative experiment is shown.

tein. Thus, cultures of *pol30:Flag ubp1:RFP* and *pol30:Flag ubp1:RFP-NLS* strains were synchronized with α-factor and released for 1 h in 0.2 M HU to slow S phase progression. Chromatin extracts were obtained, and PCNA was immunoprecipitated with anti-Flag antibodies. We found that Ubp1 binds PCNA and that this binding increased somewhat when Ubp1 was retained in the nucleus (Fig. 5B). In order to check whether or not this interaction depends on the activity of Ubp1, we also analyzed the potential binding with the non-ubiquitylable PCNA $pol30^{K164R}$ variant and observed that there is still some interaction between both proteins, although to a lesser degree than the one attained with wild-type PCNA (Supplementary Fig. S5).

We then performed *in vitro* deubiquitylation assays to assess whether Ubp1 was able to directly deubiquitylate PCNA. Mono- and di-ubiquitylated PCNA was obtained by immunoprecipitation with anti-Flag antibodies from an *S. pombe* strain lacking the $ubp12^{+}$, $ubp15^{+}$, and $ubp16^{+}$ genes, which encode three of the four known PCNA-ubiquitin proteases in this organism, and also expressing PCNA-Flag fusion protein (see [32] and the "Materials and methods" section). Ubp1-myc and $Ubp1^{C110S}$-Myc, a catalytically inactive form of Ubp1, were immunoprecipitated from *S. cerevisiae* strains endogenously expressing each of these fusion proteins. We used Ubp10-myc as a positive control [27, 32], and Ubp8-HA, a nuclear DUB whose expression did not affect the ubiquitylation state of PCNA *in vivo* (Supplementary Fig. S2), as a negative control.

As shown in Fig. 5C and Supplementary Fig. S6, Ubp1-myc exhibits activity on both Ub- and Ub_2-PCNA forms, although more prominently on the monoubiquitylated form. This activity was blocked by the irreversible DUB inhibitor Ub-VS and was dependent on its catalytic residue, cytosine 110. Under these experimental conditions, Ubp10 appears to be a little bit more efficient than Ubp1. Altogether, these data indicate that Ubp1 is a newly identified PCNA-DUB capable of removing ubiquitin moieties from both Ub- and Ub_2-PCNA forms.

Ubp10 and Ubp12 PCNA-DUBs associate with replication forks while carrying out their function on PCNA [27]. To understand whether this is also the case for Ubp1 or, on the contrary, whether Ubp1 can work apart from ongoing replication forks, the association of Ubp1 with different early replication

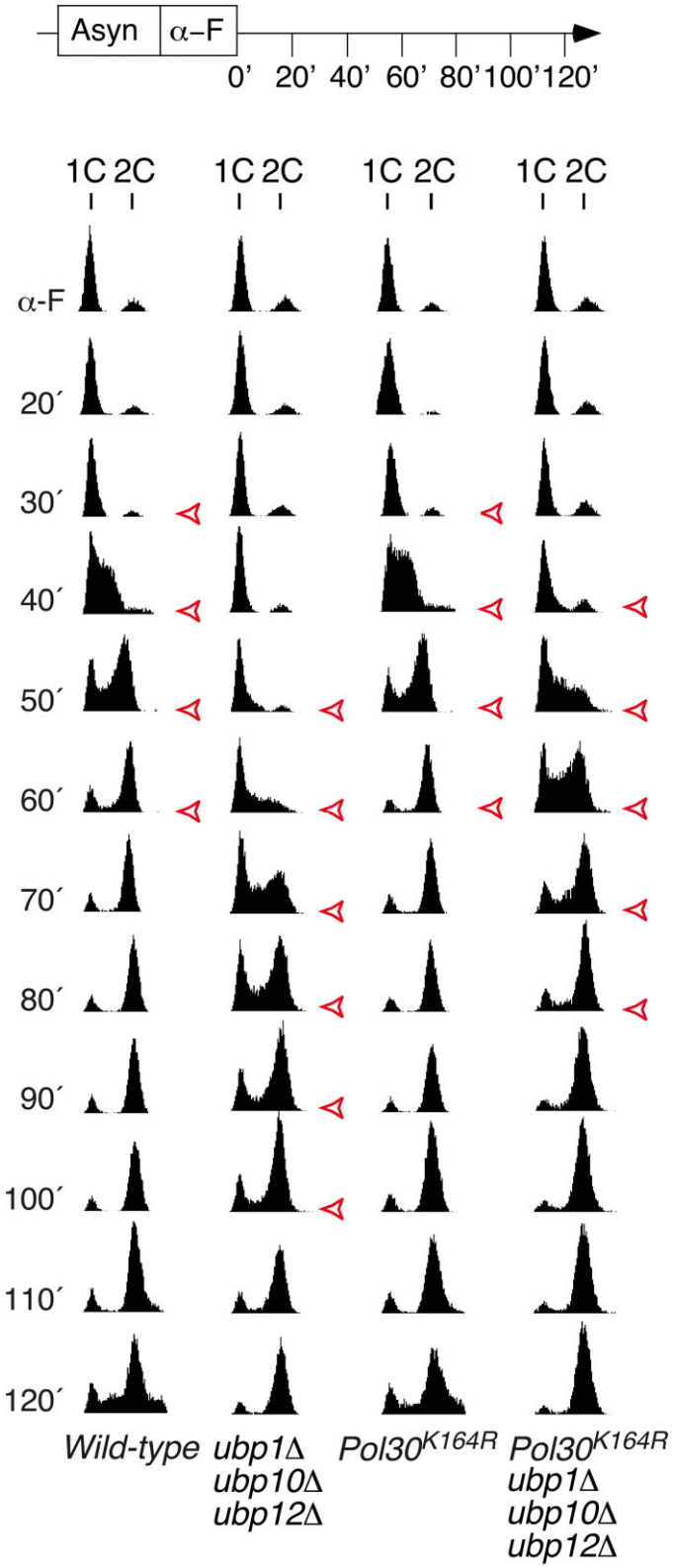

Figure 4. Non-ubiquitylable PCNA variant *pol30*^{K164R} suppression of the slow DNA replication progression of *ubp1Δ ubp10Δ ubp12Δ* triple mutant. Exponentially growing cultures of the indicated strains were synchronized with α-factor and released into fresh medium (YPAD). Samples were collected at the indicated time points and analyzed for DNA content by FACS, covering the period from α-factor synchronization to 120 min post-release. Arrows mark the replication time points. Two biological replicates were performed, and a representative experiment is shown.

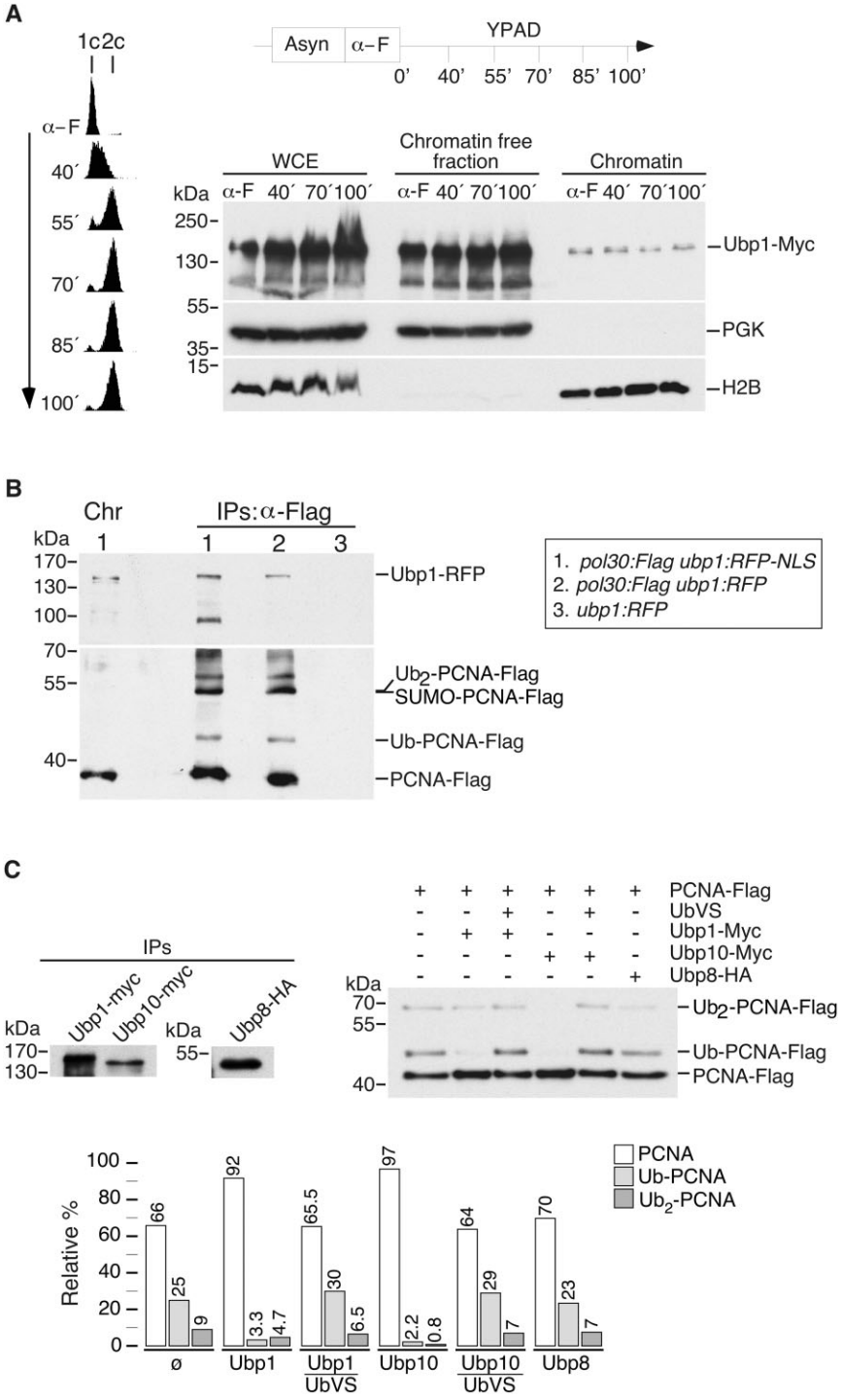

Figure 5. Ubp1 interacts *in vivo* with and deubiquitylates PCNA^{K164}. (**A**) Ubp1 is associated with chromatin. Wild-type cells expressing Ubp1-Myc tagged protein were synchronized with α-factor and released into fresh yeast complex medium (YPAD) to allow progression through the S phase. Samples were taken at the indicated time points and analyzed by FACS and immunoblotting. Soluble and chromatin-bound protein fractions were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and incubated with an anti-Myc antibody. Histone H2B was used as a chromatin marker. WCE: whole cell extract. (**B**) Co-immunoprecipitation assay showing the *in vivo* interaction between PCNA-Flag and Ubp1-RFP fusion proteins. Cultures from the indicated strains were synchronized with α-factor and released in the presence of HU (0.2 mM) for 1 h. Samples were processed for ChIP, and an anti-Flag antibody was used to immunoprecipitate PCNA-Flag. The immunoprecipitated samples were resolved by SDS–PAGE and analyzed by immunoblotting. Cut blots were probed with anti-Flag or anti-RFP antibodies. Chromatin extracts from the *pol30:Flag Ubp1:RFP-NLS* strain were used as a reference for Ubp1-RFP detection, and the *ubp1:RFP* strain not expressing PCNA-Flag was used as a negative control. Chr, chromatin; IP, immunoprecipitates. (**C**) PCNA *in vitro* deubiquitylation assay. Ubiquitylated PCNA was obtained by immunoprecipitation with an anti-Flag antibody from an *S. pombe* strain expressing PCNA-Flag fusion protein and lacking *ubp12*$^{+}$, *ubp15*$^{+}$, and *ubp16*$^{+}$ genes. Ubp1-myc, Ubp10-myc, and Ubp8-HA were also obtained by immunoprecipitation (left panel) from cells expressing each of these proteins. PCNA immunoprecipitates were incubated with Ubp1-myc or Ubp10-Myc in the absence or presence of Ub-VS, a ubiquitin protease inhibitor, for 14 h at 30°C. Incubation with Ubp8-HA served as a negative control. Samples were resolved by SDS–PAGE and analyzed by immunoblotting with an anti-Flag antibody. Quantification of the relative abundance of each PCNA form is shown. Two replicates were performed, and a representative experiment is shown.

origins was analyzed by ChIP-qPCR. Cells were synchronized by treatment with α-factor and released in the presence of HU (0.2 mM) for 1 h to stall replication forks early in S phase, and processed for Ubp1 ChIP, followed by qPCR analysis. Purified DNA samples were subjected to qPCRs using primers close to specific activated autonomous replicating sequences (*ARS305*, *ARS306*, *ARS603*, and *ARS607*). We found that Ubp1 associates with all the active replication origins analyzed (Fig. 6). We reasoned that the lack of Ubp10 and Ubp12 could favor this association. The binding of Ubp1 to replication origins in *ubp10Δ ubp12Δ* mutant cells was also analyzed. As shown in Fig. 6, the recruitment of Ubp1 to all replication origins examined was independent of the presence or absence of Ubp10 and Ubp12 proteins. Not surprisingly, however, and consistent with its association with chromatin (Fig. 5A), when analyzing the association of Ubp1 with regions distant from replication origins, we found Ubp1 to be associated as well, although at lower levels (Supplementary Fig. S7). All these data support the notion that Ubp1 reverts PCNA ubiquitylation at replication forks independently from Ubp10 and Ubp12.

Ubp1 cooperates with Ubp10 and Ubp12 in the regulation of DDT processes

Ubp10 and Ubp12 PCNA-DUBs play a key role in the modulation of DDT during DNA replication by counteracting the engagement of nascent DNA strands in TS events upon replication fork stalling. Therefore, the lack of these two DUBs exacerbates the accumulation of small Y-shaped intermediates to the detriment of the large Y-shaped ones, observed upon dNTP shortage induced by HU treatment in a Rad52-dependent manner [27]. We reasoned that if Ubp1 cooperates on PCNA deubiquitylation during S phase, it should also be involved in modulating the DDT response. To answer this question, we examined through neutral/neutral 2D gel electrophoresis (2D gels) the impact of *UBP1* depletion in combination with the double Ubp10/Ubp12 ablation on the replication intermediates pattern generated upon HU-induced fork stalling. As shown in Fig. 7, the absence of Ubp1 resulted in a decrease in the levels of replication intermediates corresponding to the analyzed origins, *ARS305* and *ARS306*, indicating a reduction in firing of at least these two replication origins. Moreover, the lack of Ubp1 further increased the ratio of small/large Y-shaped intermediates observed in cells lacking Ubp10 and Ubp12 enzymes [27]. It has been suggested that these small non-canonical Y-shaped replication structures most likely correspond to transitional structures in which the nascent DNA strands rearrange under these fork-stalling conditions. Moreover, when analyzing whether these structures corresponded to TS events, it was found that, as expected, they did. Thus, the accumulation of small Y-shaped molecules in the *ubp1Δ ubp10Δ ubp12Δ* mutant was suppressed by the depletion of Rad52 (Fig. 7B–D). The increased accumulation of these Rad52-dependent replication intermediates in the *ubp1Δ ubp10Δ ubp12Δ* mutant compared to the double mutant *ubp10Δ ubp12Δ* following HU-induced replication fork stalling indicates that Ubp1 participates, along with Ubp10 and Ubp12, in the PCNA-DUB-driven TS branch of the DDT response at replication forks. We also checked whether the accumulation of small Y-shaped molecules in the *ubp1Δ ubp10Δ ubp12Δ* mutant was a direct consequence of the increased PCNA ubiquitylation. Thus, we examined the replication intermediates pattern in a *ubp1Δ ubp10Δ ubp12Δ* $pol30^{K164R}$ mutant. This experiment was carried out in parallel with the *ubp1Δ ubp10Δ ubp12Δ* rad52Δ mutant in order to compare the potential suppression effect. As shown in Supplementary Fig. S8D, the non-ubiquitylable PCNA variant, $pol30^{K164R}$, suppressed in some measures the increased ratio of small/large Y-shaped intermediates observed in the *ubp1Δ ubp10Δ ubp12Δ* triple mutant. This, however, was not tantamount to the suppression achieved by means of Rad52 depletion.

Discussion

In this study, we provide a deep characterization of the *S. cerevisiae* ubiquitin protease Ubp1 in the context of DNA replication. Here we show that Ubp1, hitherto only known as a cytoplasmic DUB, has a role as a nuclear protein in the deubiquitylation of PCNA at replication forks. In previous work, we revealed the functional impact of PCNA deubiquitylation in the control of DDT, and identified two specific ubiquitin proteases, Ubp10 and Ubp12, downregulating tolerance events at replication forks [27]. In this research, by identifying a new PCNA-DUB, we confirm that a few deubiquitylating enzymes revert PCNA ubiquitylation during S phase in *S. cerevisiae* [27], as previously observed in *S. pombe* [32], suggesting that PCNA deubiquitylation is an important cellular process in which cells use partially redundant enzymes to ensure the deubiquitylation of the sliding clamp PCNA. Underlining in particular the importance of the removal of ubiquitin moieties from PCNA, the depletion of PCNA-DUBs generates a strong delay in S phase progression in both fission and budding yeasts (see [27, 32] and this work), which is indicative of a role in the maintenance of processive DNA synthesis. One of the main activities of PCNA is to promote tolerance to DNA damage during DNA replication to prevent the formation of single-stranded DNA gaps, a potential cause of genomic instability and tumorigenesis ([7, 14–16, 49–51] for a review). Spontaneous DNA lesions, likely caused by endogenous cellular metabolic damage, may account for those dynamic PCNA ubiquitylation-mediated DDT mechanisms that prevent chromosome replication defects.

This study shows that the S phase progression defect observed in cells lacking Ubp10 and Ubp12 PCNA-DUBs [27] correlates with a transient accumulation of ubiquitylated PCNA during exogenously unperturbed DNA replication, not detected in wild-type cells. The transient nature of this pattern of PCNA ubiquitylation in the *ubp10Δ ubp12Δ* double mutant, which disappears at the end of the S phase, suggested that at least one additional ubiquitin protease was also involved in the process. By generating triple mutants in which the lack of Ubp10 and Ubp12 was added to the loss of one of the 15 remaining specific ubiquitin proteases of the USP family known in *S. cerevisiae*, a third enzyme (and not any other), Ubp1, participating in PCNA deubiquitylation was found. Consequently, ablation of Ubp1 in combination with Ubp10 and Ubp12 results in a much more pronounced delay in S phase progression and a long-lasting accumulation of ubiquitylated PCNA throughout DNA replication and beyond. Hence, the PCNA ubiquitylated levels conferred by MMS-mediated DNA damage were also higher in the *ubp1Δ ubp10Δ ubp12Δ* triple mutant compared to cells lacking PCNA-DUBs Ubp10 and Ubp12. In contrast, overexpression of Ubp1 in the nucleus provides a full rescue of both S phase progression and PCNA

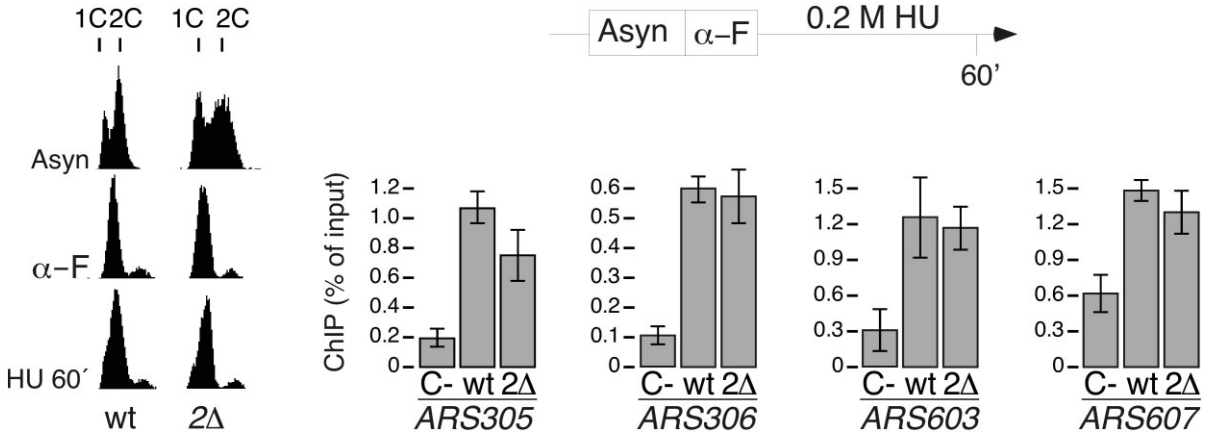

Figure 6. Ubp1 associates with replication forks in early S phase. Both wild-type and *ubp10Δ ubp12Δ* (*2Δ*) strains expressing Ubp1-Myc, as well as wild-type untagged cells (C-), were synchronized with α-factor and released in the presence of HU (0.2 M). After 1 h, samples were collected and processed for ChIP-qPCR assays. Synchronization was confirmed by FACS. DNA content analysis from a representative experiment is shown on the left. qPCR reactions were performed using specific primers to amplify DNA fragments proximal to *ARS305*-, *ARS306*-, *ARS603*-, and *ARS607*-. Means and SDs for three independent experiments are shown.

ubiquitylation defects observed in the DUB mutant, strongly suggesting that Ubp1 is a PCNA-DUB.

Single *ubp1*Δ, *ubp10*Δ, or *ubp12*Δ mutants do not accumulate detectable levels of ubiquitylated PCNA in exogenously unperturbed cells. The fact that mono- and di-ubiquitylated $PCNA^{K164}$ forms are observed during the S phase of unperturbed cell cycle only in *ubp10*Δ *ubp12*Δ [33] or *ubp1*Δ *ubp10*Δ *ubp12*Δ mutants (this work) suggests that all three enzymes collaborate in the deubiquitylation of PCNA *in vivo*. We propose that Ubp10 plays a major role, while Ubp12 and Ubp1 contribute to a lesser extent. In the absence of Ubp10, Ubp12 and Ubp1 partially take over its functions. It has been observed that Ubp10 and Ubp12 act differentially on PCNA, as Ubp10 efficiently removes all ubiquitin residues linked to $PCNA^{K164}$, single ubiquitin monomers, or K^{63}-linked polyubiquitin chains, whereas Ubp12 preferentially removes K63-linked ubiquitin moieties from polyubiquitylated molecules [27]. Further, we have found that Ubp1 shows a moderate preference for monoubiquitylated $PCNA^{K164}$, although it is capable of removing ubiquitin from both mono- and di-ubiquitylated $PCNA^{K164}$. These different preferences for PCNA ubiquitin-chain removal suggest that Ubp10, Ubp12, and Ubp1 might cooperate in PCNA regulation by playing different roles in the DDT pathway or in other processes related with DNA synthesis (see model in Fig. 8). Our findings reveal new levels of complexity in the PCNA-deubiquitylation-mediated DDT mechanisms, which still need further study.

Unexpectedly, we found that Ubp1 is a ubiquitin protease with a broader presence in cellular compartments beyond those known so far, the endoplasmic reticulum and cytoplasm [37]. A subpopulation of Ubp1 located in the nucleus, where it associates with chromatin, was observed by fluorescence microscopy and biochemical analyses. Nuclear Ubp1 constitutes a small proportion of the total protein expressed in the cell, in particular of the soluble Ubp1 population. In addition, we show that nuclear Ubp1 interacts *in vivo* with PCNA. This interaction was observed exclusively in co-immunoprecipitation assays performed with chromatin extracts and not with total protein extracts treated with benzonase, suggesting that the Ubp1–PCNA interaction may be DNA-dependent. However, the absence of interaction under these latter conditions might be explained by the inherently low levels of both Ubp1 and PCNA associated with chromatin, despite their high cellular abundance (PCNA in the chromatin-free fractions of the nucleoplasm and Ubp1 predominantly outside the nucleus), making the interaction detectable only in chromatin-enriched extracts. These experimental limitations leave uncertainty as to whether the interaction between Ubp1 and PCNA is direct or mediated by DNA.

We have demonstrated that Ubp1 can deubiquitylate both Ub- and Ub_2-PCNA forms *in vitro*, showing a more pronounced effect on the monoubiquitylated form. This activity is also observed *in vivo* when driving Ubp1 to be fully expressed in the nucleus by adding an NLS to its C-terminal domain. The association of Ubp1 with replication forks is of particular interest, suggesting that, as in the case of Ubp10 and Ubp12 [27], the function of Ubp1 as a PCNA-DUB is likely carried out, at least in part, during replication fork progression.

Ubiquitylated PCNA accumulates when forks need to read through damaged templates or deal with replicative stress conditions through DDT mechanisms [2, 7, 12, 13, 23]. The fact that mono- and di-ubiquitylated $PCNA^{K164}$ accumulation is much higher in PCNA-DUB-deficient cells [27] (Fig. 2C) shows that deubiquitylation events are also involved in preventing excessive accumulation of ubiquitylated PCNA. It has been shown that PCNA ubiquitin proteases downregulate DDT events acting at replication forks [27]. Transient Rad52-dependent replication intermediates, which are quickly resolved in wild-type cells, accumulate in PCNA-DUB-deficient cells [27]. During replication of alkylated DNA, X-shaped TS intermediates accumulate due to nascent strand exchange events, leading to the formation of joint molecules that eventually dissolve in wild-type cells [25, 52, 53]. In Ubp10- and Ubp12-deficient cells, these Rad52-dependent molecules are not efficiently resolved and accumulate over longer periods, probably due to increased PCNA ubiquitylation [27]. Similarly, under replicative stress conditions generated by dNTPs shortage, the accumulation of small non-canonical Y-shaped Rad52-dependent structures was also observed in Ubp10- and Ubp12-deficient cells but not in the wild-type cells. These non-canonical replicative intermediates accumulate at the expense

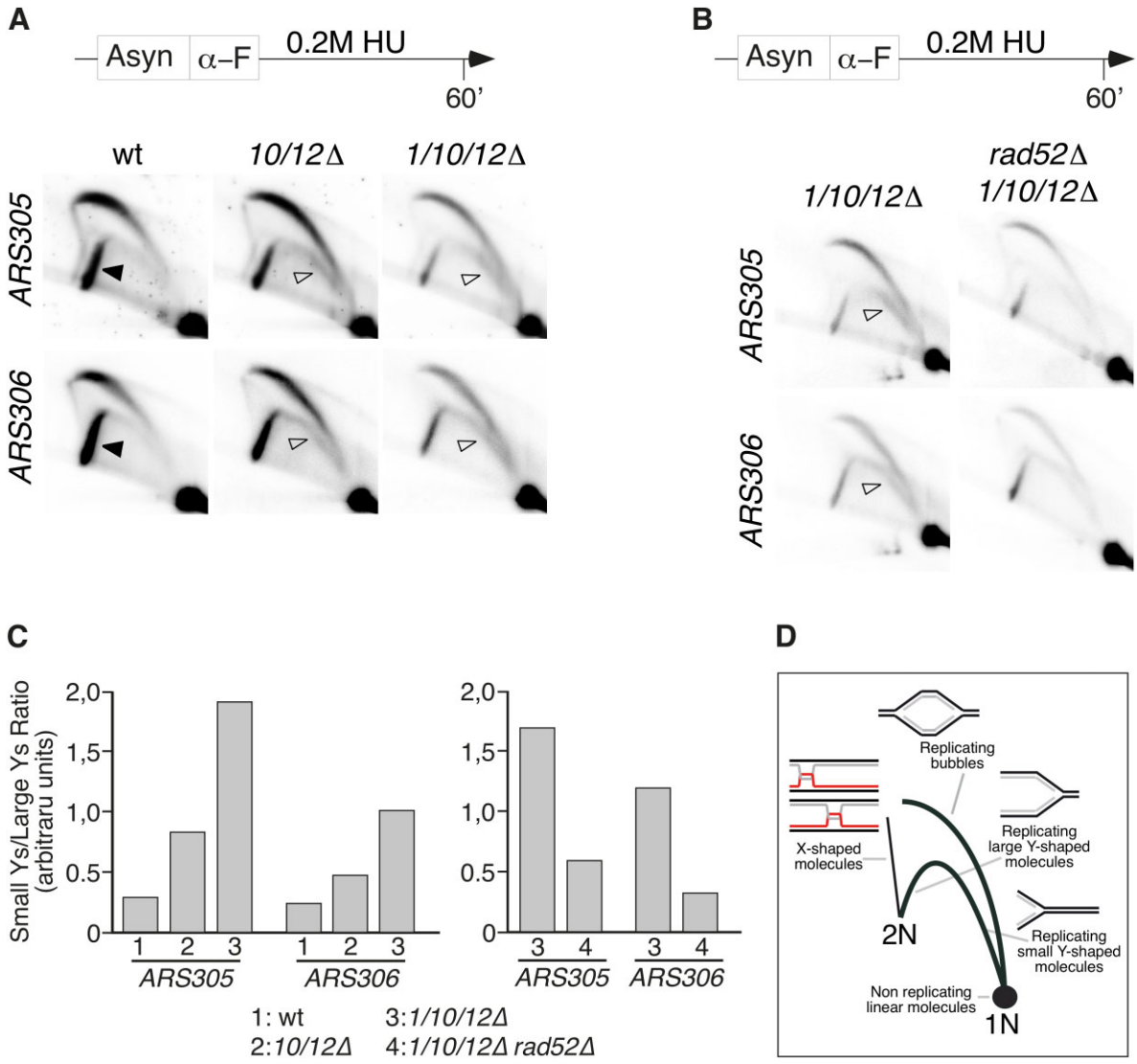

Figure 7. Accumulation of small Y-shaped TS intermediates at stalled forks in *ubp1Δ ubp10Δ ubp12Δ* mutant. (A, B) Wild-type, *ubp10Δ ubp12Δ* (*10/12Δ*), and *ubp1Δ ubp10Δ ubp12Δ* (*1/10/12Δ*) cells (**A**) or *ubp1Δ ubp10Δ ubp12Δ* and *ubp1Δ ubp10Δ ubp12Δ rad52Δ* strains (**B**) were synchronized with α-factor and released in the presence of 0.2 M HU for 60 min. Samples were processed for FACS analysis of DNA content (Supplementary Fig. S8A and B) and 2D gel analysis of replication intermediates. Genomic DNA was digested with *NcoI* endonuclease, resolved by 2D electrophoresis, transferred to nylon membranes, and hybridized with probes spanning the early replication origins *ARS305* and *ARS306*. Open and closed arrows indicate small and large Y-shaped structures, respectively, as detected by 2D gel analysis. (**C**) Ratios of small to large Y-shaped intermediates of experiments shown in panels (A) and (B) are displayed as histogram plots. The experiments were replicated two times, and a representative experiment is shown. (**D**) A schematic representation of canonical replication intermediates and fully replicated joint molecules detected by 2D gel analysis is shown.

of the large canonical Y-shaped ones, probably due to an incomplete synthesis of the nascent strands [27]. Here, we show that in cells lacking Ubp1, combined with Ubp10 and Ubp12 ablation, the ratio of small-to-large Y-shaped intermediates generated after HU treatment is higher than that observed in *ubp10Δ ubp12Δ* mutant cells. This phenotype depends on Rad52 and, at least in part, on $PCNA^{K164}$ ubiquitylation, strongly suggesting that Ubp1 also plays a role in TS-mediated DDT modulation mechanisms at replication forks.

Since the presence of Ubp1 at replication forks is not affected by the deletion of Ubp10 and Ubp12, we can hypothesize that there is not regulation between them and that they could act on PCNA independently. How these three enzymes work together at replication forks, what the function of each enzyme is, where and when each enzyme carries out its work, and how they are regulated are very interesting open questions to address in future studies. While we favor the hypothesis that PCNA-DUBs preferentially revert PCNA ubiquitylation at or near forks, we cannot rule out that Ubp1, Ubp10, and/or Ubp12 also regulate post-replicative DDT events. Evidence shows that PCNA ubiquitylation can function independently of genome replication [54, 55], and MMS-induced lesion processing via DDT and salvage HR can occur away from replication forks [56].

Many factors involved in replication and the response to replicative stress are known to be regulated by ubiquitylation [6, 47, 57, 58]. Since Ubp1 associates with chromatin not only during early S phase but in a permanent manner throughout the cell cycle, we cannot rule out the possibility that its contribution to DNA replication could be also carried out through additional substrates other than PCNA. Ubp7, initially characterized as an endocytic factor in *S. cerevisiae*, has

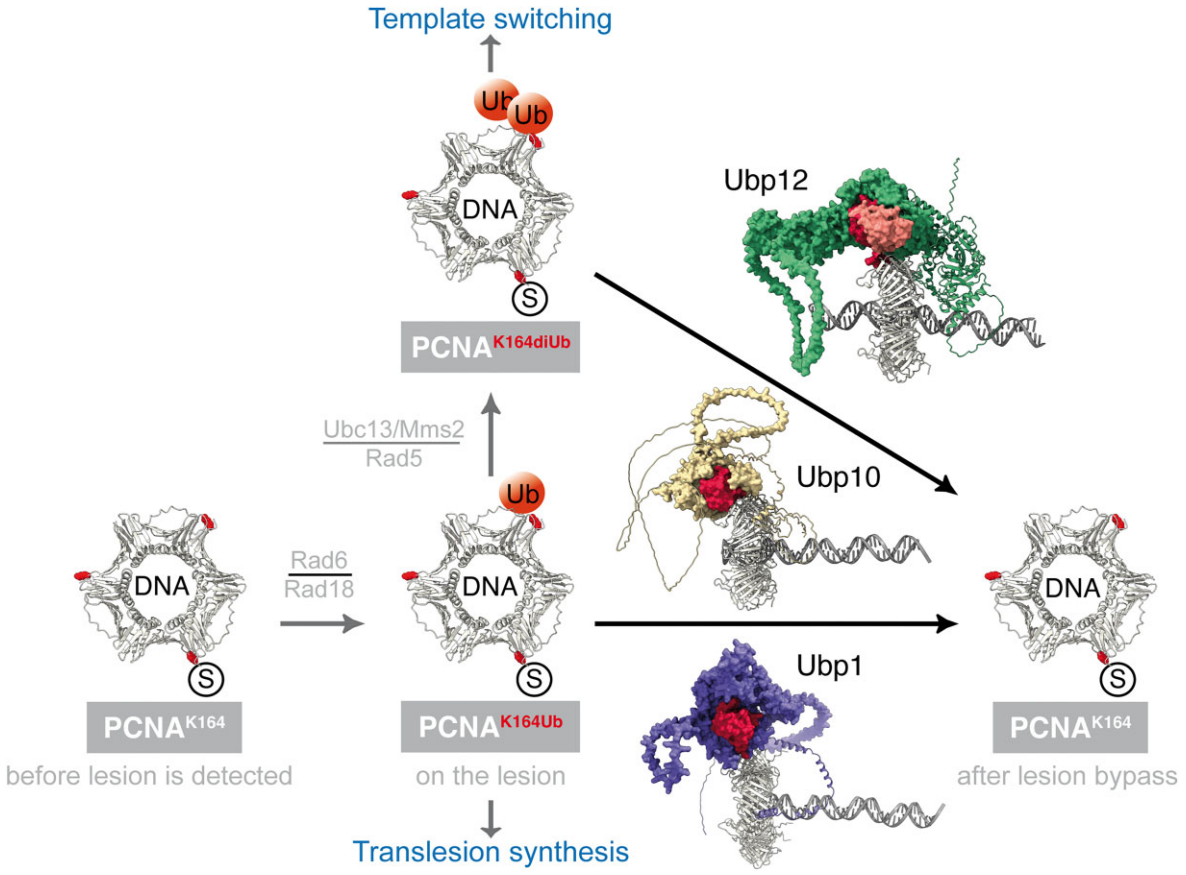

Figure 8. A working model for the role of Ubp1, Ubp10, and Ubp12 in reverting PCNA ubiquitylation in *S. cerevisiae* cells during S phase. SUMO-PCNA^{K164}, loaded onto replicating chromatin, progresses with the replisome at replication forks. The detection of bulky DNA lesions stalls fork progression and triggers Rad6/Rad18-mediated ubiquitylation of PCNA^{K164}. This modification enhances the interaction between monoubiquitylated PCNA^{K164} (Ub-PCNA^{K164}) and TLS DNA polymerases or serves as a platform for further polyubiquitylation of PCNA by the Ubc13/Mms2/Rad5 ubiquitin ligase complex, promoting the error-free TS DDT pathway. Following lesion bypass, Ubp1 and Ubp10 deubiquitylate mono-Ub-PCNA^{K164}, and/or Ubp10 and Ubp12 deubiquitylate di-Ub-PCNA^{K164}, facilitating replisome remodeling and the transition back to replicative DNA polymerases. This switch enables the resumption of rapid and processive DNA replication fork progression.

also been proposed as a component of the regulatory network of S phase progression under conditions of DNA damage, probably working on the chromatin state through a so far unknown substrate [59]. Additionally, Ubp1 could be involved in additional nuclear functions other than DDT and DNA replication.

All this evidence indicates that deubiquitylation-dependent regulation of DNA replication is a complex network involving several ubiquitin-specific proteases. How the different PCNA-DUBs work and are regulated remains unknown. Additional molecular mechanistic studies will shed light on PCNA deubiquitylation-dependent processes.

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Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

None declared.

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Data availability

All data associated with this study are presented in the paper and the Supplementary data.

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