Ribonucleotide reductase activity is regulated by proliferating cell nuclear antigen (PCNA)

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Summary

Synthesis of dNTPs is required for both DNA replication and DNA repair and is catalyzed by ribonucleotide reductases (RNR), which convert ribonucleotides to their deoxy forms [1, 2]. Maintaining the correct levels of dNTPs for DNA synthesis is important for minimising the mutation rate [3-7], and this is achieved by tight regulation of ribonucleotide reductase [2, 8, 9]. In fission yeast, ribonucleotide reductase is regulated in part by a small protein inhibitor, Spd1, which is degraded in S phase and after DNA damage to allow up-regulation of dNTP supply [10-12]. Spd1 degradation is mediated by the activity of the CRL4^{Cdt2} ubiquitin ligase complex [5, 13, 14]. This has been reported to be dependent on modulation of Cdt2 levels which are cell cycle regulated, peaking in S phase, and which also increase after DNA damage in a checkpoint-dependent manner [7, 13]. We show here that Cdt2 levels fluctuations are not sufficient to regulate Spd1 proteolysis and that the key step in this event is the interaction of Spd1 with the polymerase processivity factor PCNA, complexed onto DNA. This mechanism thus provides a direct link between DNA synthesis and ribonucleotide reductase regulation.

Keywords

Spd1; Cdt2; PCNA; Cul4; Ddb1; CRL4; degron; PIP box; DNA replication; DNA repair; ribonucleotide reductase; Cdc22; Suc22; fission yeast

Results and discussion

High Cdt2 levels are necessary but not sufficient to induce Spd1 degradation

Spd1 degradation is mediated by the activity of the CRL4^{Cdt2} ubiquitin ligase complex, which consists of a scaffold protein (Cul4), an adaptor protein (Ddb1) and a substrate-recruiting factor (Cdt2) [5, 13, 14]. As with Sml1 in *S. cerevisiae*, Spd1 is degraded in S

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phase and after DNA damage to allow up-regulation of dNTP levels. This has been reported to be dependent on modulation of Cdt2 levels which are cell cycle regulated, peaking in S phase, and which also increase after DNA damage in a checkpoint-dependent manner [7, 13]. Liu et al. [13, 14] reported that Spd1 degradation in S phase is independent of DNA damage checkpoint activation but requires both Rad3 and Chk1 in G2 phase and is driven by increased Cdt2 abundance. Furthermore, Moss et al. [7] reported that Rad3-dependent elevation of Cdt2 levels following double-strand break induction is responsible for Spd1 proteolysis and elevated dNTP levels, facilitating DNA repair.

From previous work it is not clear whether checkpoint activation simply serves to elevate Cdt2 levels or plays an additional role required for Spd1 proteolysis. To address this possibility, we expressed Cdt2 at a high level in a manner that was not dependent on checkpoint activation. Yox1 is an inhibitor of the Mlu1 binding factor (MBF) [15], which is an activator of the transcription of several genes including *cdt2*. Therefore, deletion of *yox1* results in deregulated *cdt2* expression (Fig. 1A, 'log *yox1* '). In a *yox1* strain, degradation of Spd1 occurs after DNA damage as in a wild-type strain, but this is no longer Rad3 dependent (Fig. 1 B). We carried out a similar experiment with cells arrested in mitosis using an *nda3* block; under these conditions Cdt2 levels are also high (Fig. 1A; 'mitotic-arrested wt'). Again, degradation of Spd1 following DNA damage is not dependent on Rad3 (Fig. 1 B lower panels). These experiments indicate that the only role of the DNA damage checkpoint in Spd1 proteolysis is to allow *cdt2* expression, and that this requirement can be bypassed when *cdt2* over-expression is achieved by other pathways.

Interestingly, we noted that although Cdt2 levels were high in mitotically-arrested cells, Spd1 levels were not lower than those observed in exponentially growing cells (Fig. 1 C) unless DNA damage was induced (Fig. 1B, lower panels). This observation is at odds with the model where Spd1 regulation is only driven by fluctuations in Cdt2 levels, and suggests that there must be another process induced by DNA damage and S phase that is rate-limiting for Spd1 proteolysis.

Spd1 proteolysis requires chromatin-bound PCNA

Since high Cdt2 levels alone does not seem to be sufficient to induce Spd1 degradation while DNA synthesis is required, it seems likely that an event involved in the replication itself is necessary for proteolysis. Ubiquitylation of several other substrates of CRL4[Cdt2], such as Cdt1, p21, E2F, DNA pol η, and Set8 requires interaction of the substrate with the polymerase processivity factor PCNA [16-23]. For Cdt1, Set8, and p21 substrates, it has been shown that ubiquitylation occurs on chromatin and DNA loading of PCNA is required to stimulate substrate ubiquitylation [19, 23]. To determine if Spd1 turnover is regulated by this mechanism, we examined whether inactivation of replication factor C, that blocks loading of PCNA onto DNA, affected Spd1 degradation. Cells arrested in S phase with HU required active Rfc1 for Spd1 degradation (Fig. 2A, left panel). Similarly, Spd1 proteolysis seen after DNA damage is also blocked by Rfc1 inactivation (Fig. 2A, right panel), and thus these observations suggest that Spd1 ubiquitylation and subsequent proteolysis is dependent on DNA-associated PCNA. We also observed that after Rfc1 inactivation Cdt2 levels

increased notably but Spd1 was accumulated, confirming that elevated Cdt2 levels are necessary but not sufficient for Spd1 degradation (Fig. S1).

To test more directly whether PCNA is required for Spd1 degradation we examined a mutant of PCNA that is defective for CRL4^{Cdt2}-mediated ubiquitylation. Havens et al. [24] have recently found that mutating the surface of PCNA that surrounds the PCNA-interacting protein (PIP) binding site prevents CRL4^{Cdt2}-mediated proteolysis. This mutation (D122A) has no major effect on binding of the PIP degron to PCNA, but rather prevents recruitment of CRL4^{Cdt2} to PCNA. Strikingly, we find that PCNA^{D122A} blocks Spd1 proteolysis after arresting cells in S phase with HU or exposure to DNA-damaging MMS (Fig. 2B). Furthermore, this mutation prevented Spd1 degradation after MMS treatment even in a *yox1* background or in mitotically-arrested cells (Fig. 2C), where Cdt2 is highly abundant, implying that PCNA has a critical role in this process.

From previous work it is expected that stabilization of Spd1 would impair genome stability and S phase execution owing to RNR inhibition [5, 13, 14]. This is most clearly apparent in the effect of PCNA^{D122A} on pre-meiotic S phase, which is very sensitive to down regulation of RNR activity [5]. PCNA^{D122A} slows pre-meiotic S phase, but this is suppressed by deletion of the *spd1* gene (Fig. 3A), arguing that Spd1 is an important target for S phase execution. We also observed that in the vegetative cell cycle, *pcn1^{D122A}* cells are elongated but this is again suppressed by *spd1* deletion (Fig. 3B). A plausible explanation is that failure to degrade Spd1 leads to a reduced dNTP supply for S phase and consequent impaired replication or DNA damage which causes a checkpoint delay to mitotic entry. Consistent with this interpretation, we were unable to construct a *pcn1^{D122A}* strain where the repair and checkpoint pathways are inactivated by deletion of the *rad3* gene, unless the *spd1* gene was deleted as well (Fig. 3B). To confirm the synthetic lethality of *pcn1^{D122A}* and *rad3*, we used a temperature-sensitive *rad3* allele to construct the double mutant. This *pcn1^{D122A} rad3^{ts}* mutant is inviable at the restrictive temperature, and this is partially suppressed by deletion of *spd1* (Fig. 3C). Finally, we also observe elevated spontaneous minichromosome loss rate in the *pcn1^{D122A}* strain, which is largely suppressed by deletion of the *spd1* gene, again implying failure to degrade Spd1 promotes genome instability (Fig. 3D). Taken together, these observations indicate that in unperturbed cells, defects in Spd1 proteolysis caused by the *pcn1^{D122A}* mutation, and consequent effects on dNTP supply, lead to defects in DNA replication or DNA damage that are only tolerated in checkpoint-proficient cells. This highlights the importance of the PCNA function in coordinating RNR activity for DNA replication and genome stability.

It has been previously reported that the DNA damage sensitivity of *cdt2* mutants is not reversed by *spd1* deletion [13]. In concordance with this observation, *pcn1^{D122A}* cells are sensitive to DNA damaging agents, but this sensitivity is not suppressed by deletion of *spd1* (Fig. S2A). We also find that *cdt2* phenotypes are not enhanced in a double *pcn1^{D122A} cdt2* mutant (Fig. S2B and C), which argues that both mutations act by just blocking the same pathway, i.e. proteolysis of CRL4^{Cdt2} targets, and that the *pcn1^{D122A}* mutation does not cause other defects in PCNA function. The simplest explanation for these findings is that while Spd1 is a key target of CRL4^{Cdt2} proteolysis in unperturbed cells, stabilization of other targets, via *cdt2* or *pcn1^{D122A}*, impairs the ability of cells to survive DNA damaging

agents. This interpretation is also broadly consistent with the findings of Holmberg et al. [5], who concluded that insufficient RNR activity contributes to 50% of observed mutations in strains defective in CRL4 function.

Spd1 contains a PIP degron that is important for its proteolysis

Most targets of CRL4^{Cdt2}-mediated ubiquitylation contain a PIP degron consisting of a 'classical' PIP consensus sequence with TD before the aromatic residues and a positively-charged amino acid downstream [19]. Spd1 does not contain a clear match to this consensus and no Spd1-PCNA interaction has been detected so far. In addition, extensive mutagenesis of Spd1 did not identify a degron sequence [11]. Nevertheless, a weak match to this consensus is in fact found in Spd1 at position 30 that is conserved in the *Schizosaccharomyces* genus (Fig. 4A). A C-terminally truncated Spd1 mutant (Spd1$^{1-43}$) retaining this PIP degron, is degraded similar to wild-type Spd1 following HU treatment (Fig.4B). In contrast, when the N-terminus of Spd1 including this PIP box, is deleted, proteolysis after HU is significantly reduced (Fig. 4B, Spd1$^{44-124}$). Mutating conserved amino acids in the PIP box (Fig. 4A, Spd1^{MPIP}) also reduced Spd1 proteolysis after HU treatment (Fig. 4B). All of these Spd1 derivatives included a nuclear localization sequence (NLS) as, unlike wild type Spd1, they showed a pancellular distribution (data not shown), and we wanted to ensure that effects on proteolysis were not due to a secondary effect of inefficient nuclear accumulation, which would preclude interaction with PCNA.

Spd1 interaction with PCNA is reduced by mutation of the PIP box

We used bimolecular fluorescence complementation (BiFC) [25, 26] to determine if interaction between PCNA and Spd1 can be detected in live cells. PCNA and Spd1 were tagged with the N and C-terminal domains of Venus-YFP respectively and expressed from their native promoters. No fluorescence was observed when the tagged proteins were in a *cdt2$^+$* background (Fig. 4C). However, when co-expressed in a *cdt2$^-$* background to stabilize Spd1, nuclear YFP fluorescence was seen in all cells, irrespective of cell-cycle stage (Fig. 4C). This indicates that Spd1-PCNA interaction occurs *in vivo* and can be detected provided that the turnover of Spd1 is blocked by lack of Cdt2. An interaction between Spd1 and PCNA was also detectable by immunoprecipitation, using purified PCNA and in vitro expressed Spd1 (Fig. S3A).

We tested the Spd1 mutants to see how removal of the PIP box affected interaction with PCNA. As with the proteolysis experiments, these all included a C-terminal NLS and western blotting was used to show that the mutations do not have a significant effect on Spd1 levels in a *cdt2$^-$* mutant background (Fig. S3B). Spd1$^{1-43}$, retaining the PIP box, showed a BiFC signal similar to that seen with wild-type Spd1 (Fig. 4D; note that in these experiments we marked one of the strains with a mitochondrial stain to allow direct comparison of two strains in the same image). However, Spd1$^{44-124}$ showed a reduced BiFC signal as did the Spd1^{MPIP} mutant (Fig. 4D). We note that deleting or mutating the PIP box only reduces and does not eliminate the BiFC signal and Spd1 proteolysis, possibly indicating that there are other PCNA-interacting regions in Spd1.

Taken together, the results described here indicate that the role of the DNA damage checkpoint activation in Spd1 degradation is simply to provide an adequate level of Cdt2 at times of the cell cycle when levels are low, and high Cdt2 levels are not sufficient to promote Spd1 degradation. Significantly, we demonstrate that the critical step in Spd1 proteolysis, and therefore in RNR up-regulation, is the interaction of Spd1 with DNA-bound PCNA. Since PCNA serves as an essential polymerase processivity factor in S phase, as well promoting repair synthesis by pol δ and other repair polymerases, this provides a direct mechanism to synchronize DNA synthesis with stimulation of RNR to up-regulate dNTP production. This mechanism is distinct from proteolysis of Sml1 in *S. cerevisiae*. In this organism, which lacks the CRL4[Cdt2] pathway, checkpoint activation in S phase and after DNA damage leads to Sml1 phosphorylation and this modified version of the protein is ubiquitylated by the Rad6-Ubr2-Mub1 E2/E3 ubiquitin ligase complex [27, 28].

The type 1a class of RNRs, found in eukaryotes and eubacteria, consists of a heterotetramer composed of two large catalytic R1 subunits, and two small R2 subunits which generate the tyrosyl free radical required for catalysis (for reviews see [1, 2]). Spd1 inhibits RNR activity most probably by binding to the R1/Cdc22 subunit [10], although it has also been implicated in promoting the nuclear import of the R2/Suc22 subunit, facilitating R1-R2 interactions and binding to the R2 subunit [11]. One implication of our findings is that Spd1 is degraded via interaction with a nuclear protein, while its mode of inhibition appears to require interaction with R1/Cdc22, which is predominantly cytoplasmic. RNR is thought to be active in the cytoplasm, with the R2/Suc22 subunit translocating from the nucleus to the cytoplasm to increase RNR activity. In a model to reconcile these observations, Spd1 may shuttle between nucleus and cytoplasm and, in the absence of DNA synthesis, Spd1 is stable during nuclear transit (Fig. 4E, left panel). During S phase or DNA synthesis associated with repair, nuclear Spd1 interacts with chromatin-associated PCNA, leading to its ubiquitylation and proteolysis, and the net reduction of Spd1 levels causes RNR activation (Fig. 4E, right panel). Spd1 proteolysis may also contribute to RNR activation by reducing nuclear import of the R2/Suc22 subunit, thus increasing cytoplasmic R2/Suc22 levels, although the mechanism of this is unclear [11]. Although we note that the active form of the enzyme is thought to be cytoplasmic, the PCNA-Spd1 interaction potentially can target RNR to sites of DNA synthesis. In mammalian cells, Tip60 has been reported to localize RNR to sites of DNA damage, which may be important for providing adequate dNTP at repair sites at times of the cell cycle when dNTP pools are low [29].

So far, no Spd1 orthologue has been identified in Metazoa, but the small size of this protein and its low sequence conservation through evolution makes detection of any related proteins difficult. In addition, the intrinsically disordered nature of Spd1 [11] could mean that proteins without any sequence similarity could perform similar roles in higher organisms. However, given the conservation of the CRL4[Cdt2] pathway, identification of mammalian protein inhibitors of RNR might be facilitated by inactivation of this mechanism.

Proteolysis of CRL4[Cdt2] targets, triggered by PCNA chromatin binding is emerging as an important mechanism in DNA replication control (reviewed in [30]). Degradation of Cdt1 blocks Mcm chromatin binding directly, while p21 proteolysis leads to nuclear export of the Cdc6 licensing factor [21], and down-regulation of Set8 in S phase also seems to be

important for blocking re-replication [31, 32]. Our results here, showing that an inhibitor of the elongation step of replication is degraded simultaneously with destruction of licensing activators, emphasizes how PCNA activation of CRL4^{Cdt2} is a master switch in the transition from G1 to S phase.

Methods

Methods, strains and oligos used are given in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- High Cdt2 levels are necessary but not sufficient to induce Spd1 degradation.

- Spd1 interacts with PCNA *in vivo*.

- Chromatin binding of PCNA synchronizes Spd1 proteolysis with DNA synthesis.
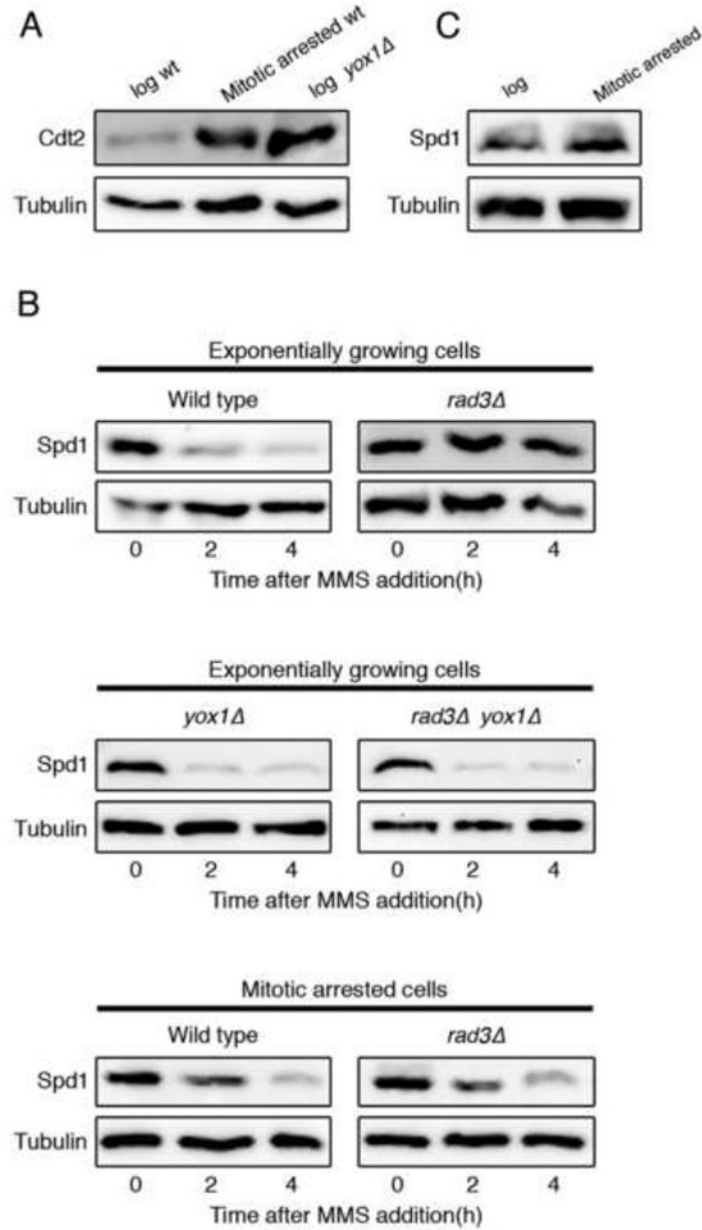
Figure 1. Increased expression of cdt2 is necessary but not sufficient to induce Spd1 proteolysis
(A) Cdt2-TAP levels in exponentially growing (log) or mitotically-arrested wild type cells (2710) and in exponentially growing *yox1Δ* mutant cells (2698). (B) Western blot analysis showing Spd1-TAP levels after MMS addition in a wild type strain (1766) and in a *rad3Δ* (2644) mutant (upper panels); spd1-TAP levels after MMS treatment of *yox1Δ* (2711) and *rad3Δ yox1Δ* (2713) cells (middle panels); and Spd1-TAP levels after MMS treatment of mitotically-arrested cells that were either wild-type for checkpoint function (2678) or DNA damage checkpoint defective (*rad3Δ*) mutant (2677) (lower panels). (C) Spd1-TAP levels in exponentially growing (log) or mitotically-arrested wild type cells (2678). Tubulin is shown as a loading control.
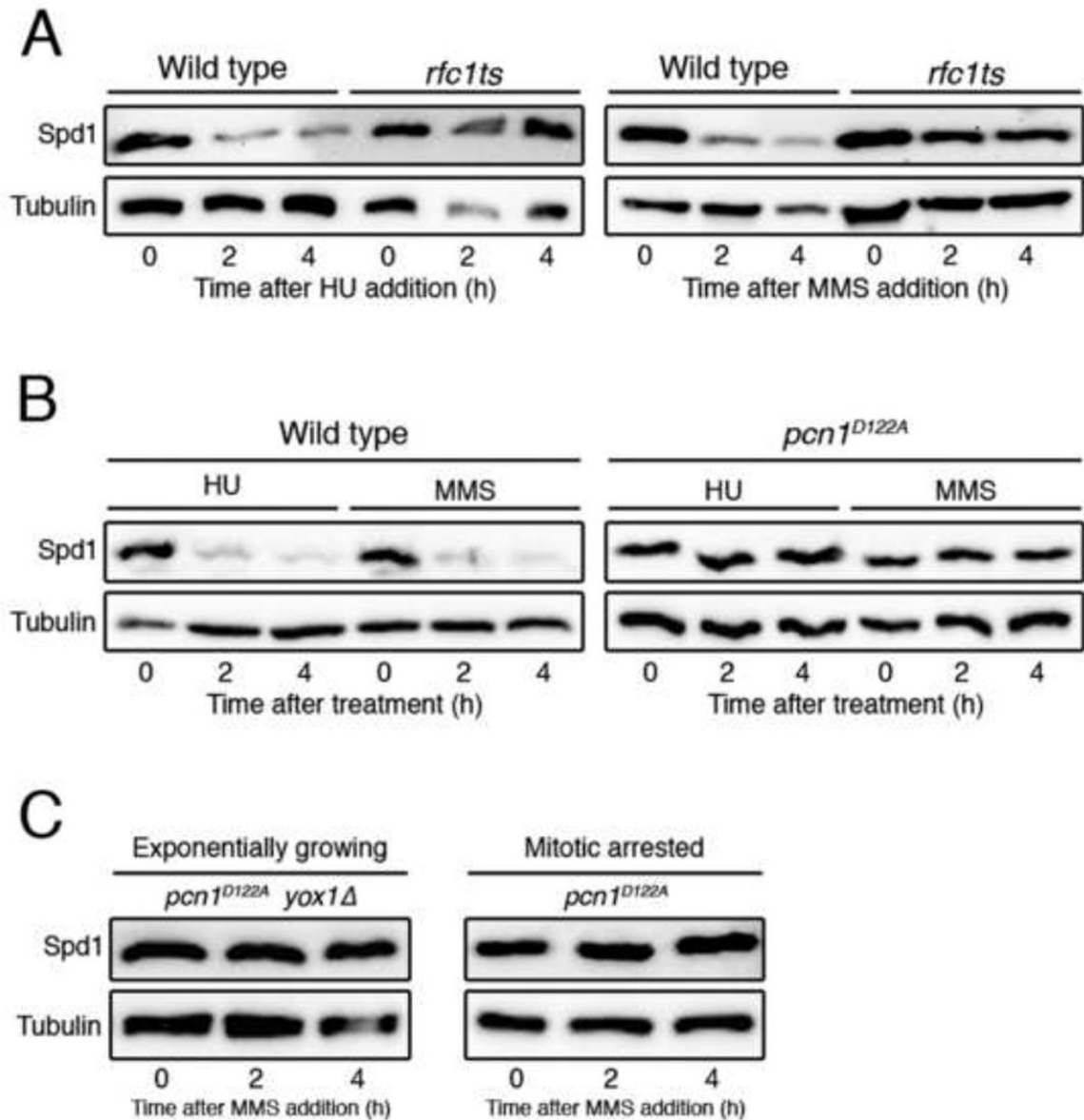
Figure 2. Chromatin-bound PCNA is required for Spd1 proteolysis

(A) Spd1-TAP levels in a wild type strain (1766) and in an *rfc1-44* thermosensitive mutant (2072), treated with HU or MMS at the restrictive temperature (37°C). (B) Western blot analysis showing Spd1-TAP levels after HU or MMS addition in a wild type strain (1766) and a *pcn1^{D122A}* mutant (2649). (C) Spd1-TAP levels after MMS treatment of *pcn1^{D122A} yox1Δ* (2755) cells and mitotically-arrested (*nda3*) *pcn1^{D122A}* cells (2664). Tubulin is shown as a loading control.
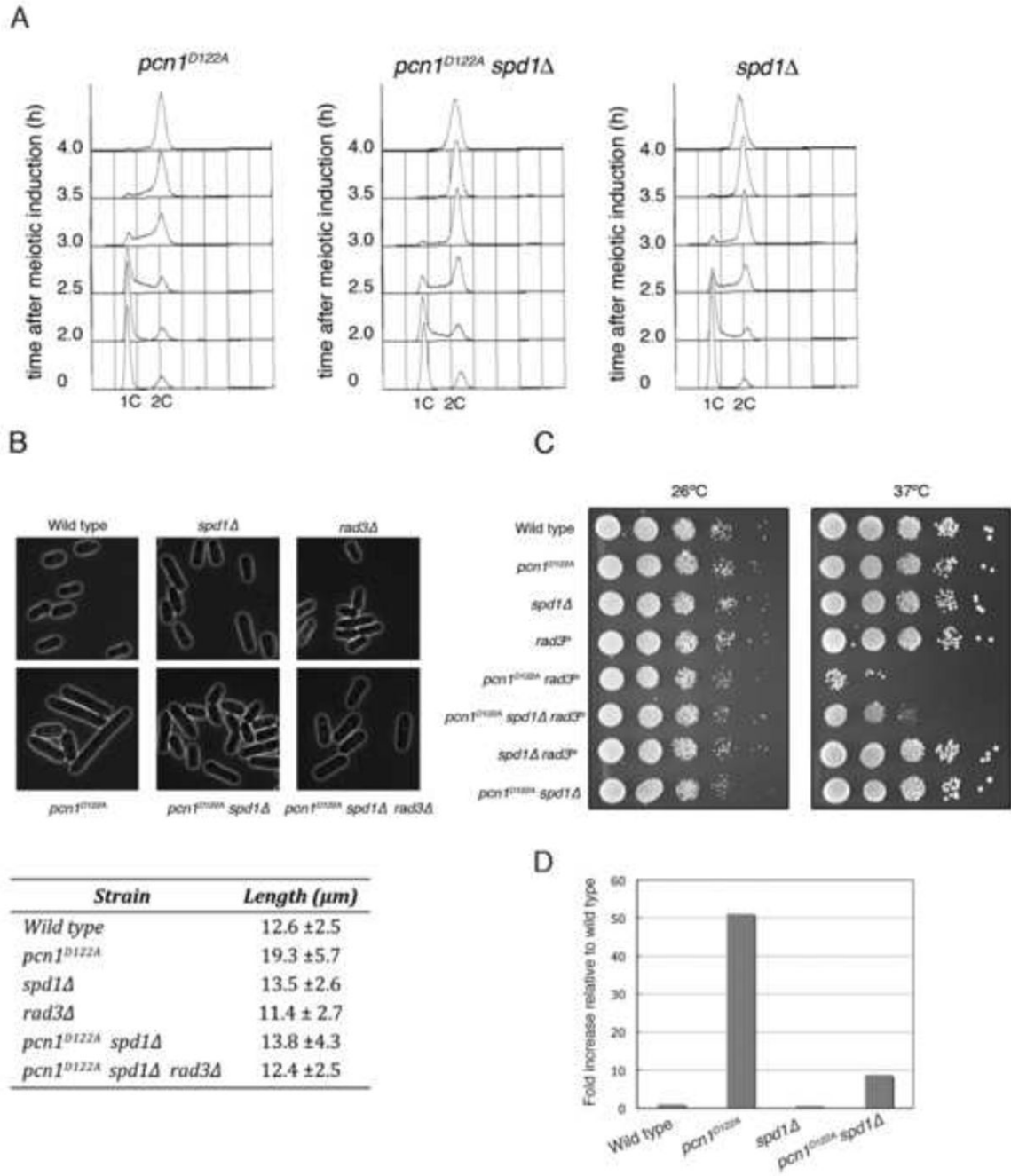
Figure 3. PCNA^{D122A}-induced defects are suppressed by spd1 deletion

(A) *pat1* strains also containing *pcn1^{D122A}* (2912), *pcn1^{D122A} spd1Δ* (2915), or *spd1Δ* (2930) mutations were arrested in G1 by nitrogen starvation, and then released from the block at 34°C to inactivate Pat1 and induce meiosis. The progress of pre-meiotic S phase was followed by flow cytometry. (B) Upper panels show images of exponentially growing cells from wild-type (137), *spd1Δ* (2671), *rad3Δ* (1811), *pcn1^{D122A}* (2738), *pcn1^{D122A} spd1Δ* (2747) and *pcn1^{D122A} spd1Δ rad3Δ* (2842) cultures; average cell lengths (± standard deviation) are shown in the lower panel (150 cells were measured for each strain). (C) Viability of wild-type (137), *pcn1^{D122A}* (2738), *spd1Δ* (2671), *rad3^{ts}* (2839), *pcn1^{D122A} rad3^{ts}* (2887), *pcn1^{D122A} spd1Δ rad3^{ts}* (2888), *spd1Δ rad3^{ts}* (2889) and *pcn1^{D122A} spd1Δ* (2747) strains on YE3S at 26°C and 37°C analyzed by spot test. (D) Rate of minichromosome loss in wild-type (2836), *pcn1^{D122A}* (2898), *spd1Δ* (2885) and *pcn1^{D122A} spd1Δ* (2900) strains.

Figure 4. Spd1 and PCNA interact in vivo and a PIP box in Spd1 is important for this interaction
(A) Identification of a sequence (orange bar and box) in Spd1 (grey bar) that partially matches the PIP degron consensus sequence. Other sequences shown are (bottom to top): corresponding region of *S. japonicus* Spd1 (SjSpd1); PIP degrons in *S. pombe* Cdt1 (SpCdt1_301, SpCdt1_28 [18]): PIP degron and PIP consensus sequences [30]; and the mutated sequence of Spd1^{MPIP}. Lower green and blue bars show the Spd1$^{1-43}$ and Spd1$^{44-124}$ deletion mutants. The cyan bar labelled "R1' represents the location of sequence similarity with a region of Sm11 known to interact with the R1 subunit of RNR [11]. (B) Western blot analysis of Spd1-YFP-NLS levels after HU addition in a wild-type strain (2672), the Spd1$^{1-43}$ mutant (2612), the Spd1$^{44-124}$ mutant (2744) and the Spd1^{MPIP} mutant (2673). Tubulin is shown as a loading control. (C) BiFC of exponentially growing live cells expressing *VN173-pcn1* and *spd1-VC155* in a wild type (2536) and in a *cdt2* background (2546). (D) Comparison of the intensity of BiFC in exponentially growing live cells; a wild type (2752) and an *spd1$^{1-43}$* (2750) strain (left panels), a wild type (2752) and an *spd1$^{44-124}$* (2751) strain (middle panels), and a wild type (2752) and an *spd1^{MPIP}* (2748) (right panels) strain were compared. Phase (blue) and BiFC signal (green) channels are merged (upper panels). In all the combinations, cells carrying the mutation in Spd1 were stained with MitoTracker® Red to allow direct comparison between the two strains (lower panels). All

the strains were in a *cdt2* background to stabilize Spd1 and comparison of Spd1 levels in these strains is shown in Fig. S3B. (E) Model for RNR activity control by PCNA: left panel represents an unperturbed cell in G1, G2 or M phase that is not subject to DNA damage. Spd1 shuttles between nucleus and cytoplasm, interacting and inhibiting RNR in the cytoplasm and possibly interacting with free PCNA in the nucleus. CRL4^{Cdt2} does not ubiquitylate nuclear Spd1 as PCNA is not DNA bound. Right panel represents the situation when S phase starts or DNA synthesis associated with DNA damage repair is occurring. Spd1 shuttles from cytoplasm to nucleus but in the nucleus, following interaction with chromatin-bound PCNA, it is ubiquitylated and proteolyzed. The net reduction in Spd1 levels leads to RNR activation.