Ribonucleotide Reductase Activity Is Coupled to DNA Synthesis via Proliferating Cell Nuclear Antigen

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Summary

Synthesis of deoxynucleoside triphosphates (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 minimizing the mutation rate [3–7], and this is achieved by tight regulation of RNR [2, 8, 9]. In fission yeast, RNR is regulated in part by a small protein inhibitor, Spd1, which is degraded in S phase and after DNA damage to allow upregulation of dNTP supply [10–12]. Spd1 degradation is mediated by the activity of the CRL4Cd2 ubiquitin ligase complex [5, 13, 14]. This has been reported to be dependent on modulation of Cd2 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 Cd2 level 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 proliferating cell nuclear antigen (PCNA), complexed onto DNA. This mechanism thus provides a direct link between DNA synthesis and RNR regulation.

Results and Discussion

High Cd2 Levels Are Necessary but Not Sufficient to Induce Spd1 Degradation

Spd1 degradation is mediated by the activity of the CRL4Cd2 ubiquitin ligase complex, which consists of a scaffold protein (Cul4), an adaptor protein (Ddb1), and a substrate-recruiting factor (Cd2) [5, 13, 14]. As with Sml1 in Saccharomyces cerevisiae, Spd1 is degraded in S phase and after DNA damage to allow upregulation of deoxynucleoside triphosphate (dNTP) levels. This has been reported to be dependent on modulation of Cd2 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 Cd2 abundance. Furthermore, Moss et al. [7] reported that Rad3-dependent elevation of Cd2 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 Cd2 levels or plays an additional role required for Spd1 proteolysis. To address this possibility, we expressed Cd2 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 cd2. Therefore, deletion of yox1 results in deregulated cd2 expression (Figure 1A, “log yox1Δ`). In a yox1Δ strain, degradation of Spd1 occurred after DNA damage as in a wild-type strain, but this was no longer Rad3 dependent (Figure 1B). We carried out a similar experiment with cells arrested at meiosis using an nda3 block; under these conditions, Cd2 levels were also high (Figure 1A, “mitotic-arrested WT”). Again, degradation of Spd1 following DNA damage was not dependent on Rad3 (Figure 1B, lower panels). These experiments indicate that the only role of the DNA damage checkpoint in Spd1 proteolysis is to allow cd2 expression, and that this requirement can be bypassed when cd2 overexpression is achieved by other pathways.

Interestingly, we noted that although Cd2 levels were high in mitotically arrested cells, Spd1 levels were not lower than those observed in exponentially growing cells (Figure 1C) unless DNA damage was induced (Figure 1B, lower panels). This observation is at odds with the model where Spd1 regulation is driven only by fluctuations in Cd2 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

Given that high Cd2 levels alone do not seem to be sufficient to induce Spd1 degradation while DNA synthesis is required, it seems likely that an event involved in replication itself is necessary for proteolysis. Ubiquitylation of several other substrates of CRL4Cd2, such as Cd1, p21, E2F, DNA pol δ, and Set8, requires interaction of the substrate with the polymerase processivity factor proliferating cell nuclear antigen (PCNA) [16–23]. For Cd1, Set8, and p21 substrates, it has been shown that ubiquitylation occurs on chromatin and that DNA loading of PCNA is required to stimulate substrate ubiquitylation [19, 23]. To determine whether Spd1 turnover is regulated by this mechanism, we examined whether inactivation of replication factor C, which blocks loading of PCNA onto DNA, affects Spd1 degradation. Cells arrested in S phase with hydroxyurea (HU) required active Rfc1 for Spd1 degradation (Figure 2A, left panel). Similarly, Spd1 proteolysis seen after DNA damage was also blocked by Rfc1 inactivation (Figure 2A, right panel), and thus these observations suggest that Spd1 ubiquitylation and subsequent proteolysis are dependent on DNA-associated PCNA. We also observed that after Rfc1 inactivation, Cd2 levels increased notably but Spd1 accumulated, confirming that elevated Cd2 levels are necessary but not sufficient for Spd1 degradation (see Figure S1 available online).

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Report
PCNA Regulates Ribonucleotide Reductase

To test more directly whether PCNA is required for Spd1 degradation, we examined a mutant of PCNA that is defective in CRL4 function. Havens et al. [24] recently found that mutating the surface of PCNA that surrounds the CRL4 interaction (D122A) has no major effect on binding of the PIP degron to PCNA but rather prevents recruitment of CRL4 to PCNA. Strikingly, we found that PCNA D122A blocked Spd1 proteolysis after arresting cells in S phase with HU or exposure to DNA-damaging methyl methanesulfonate (MMS) (Figure 2B). Furthermore, this mutation prevented Spd1 degradation after MMS treatment even in a yox1Δ background or in mitotically arrested cells (Figure 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 ribonucleotide reductase (RNR) inhibition [5, 13, 14]. This is most clearly apparent in the effect of PCNA D122A on premeiotic S phase, which is very sensitive to downregulation of RNR activity [5]. PCNA D122A slows premeiotic S phase, but this was suppressed by deletion of the spd1 gene (Figure 3A), arguing that Spd1 is an important target for S phase execution. We also observed that in the vegetative cell cycle, pcn1 D122A cells were elongated, but this was again suppressed by spd1 deletion (Figure 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 replication checkpoint pathways were inactivated by deletion of the rad3 gene, unless the spd1 gene was deleted as well (Figure 3B). To confirm the synthetic lethality of pcn1 D122A and rad3Δ, we used a temperature-sensitive rad3 allele to construct the double mutant. This pcn1 D122A rad3TS mutant was inviable at the restrictive temperature, and this was partially suppressed by deletion of spd1 (Figure 3C). Finally, we also observed elevated spontaneous minichromosome loss rate in the pcn1 D122A strain, which was largely suppressed by deletion of the spd1 gene, again implying that failure to degrade Spd1 promotes genome instability (Figure 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 tolerated only in checkpoint-proficient cells. This highlights the importance of PCNA function in coordinating RNR activity for DNA replication and genome stability.

It has been previously reported that the DNA damage sensitivity of cdt2Δ mutants is not reversed by spd1 deletion [13]. In concordance with this observation, pcn1 D122A cells were sensitive to DNA-damaging agents, but this sensitivity was not suppressed by deletion of spd1 (Figure S2A). We also found that cdt2Δ phenotypes were not enhanced in a double pcn1 D122A cdt2Δ mutant (Figures S2B and S2C), which argues that both mutations act by blocking the same pathway, i.e., proteolysis of CRL4 targets, and that the pcn1 D122A mutation does not cause other defects in PCNA function. The simplest explanation for these findings is that whereas Spd1 is a key target of CRL4 proteolysis in unperturbed cells, stabilization of other targets, via cdt2Δ 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-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

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**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 exponentially growing yox1Δ mutant cells (2698). (B) Western blot analysis showing Spd1-TAP levels after methyl methanesulfonate (MMS) addition in a wild-type strain (1766) and in a rad3Δ 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 are either wild-type for checkpoint function (2678) or DNA damage checkpoint defective rad3Δ (lower panels). (C) Spd1-TAP levels in exponentially growing (log) or mitotically arrested wild-type cells (2678). Tubulin is shown as a loading control.
found in Spd1 at position 30 that is conserved in the Schizosaccharomyces genus (Figure 4A). A C-terminally truncated Spd1 mutant (Spd1<sup>1–43</sup>) retaining this PIP degron was degraded similar to wild-type Spd1 following HU treatment (Figure 4B). In contrast, when the N terminus of Spd1, including this PIP box, was deleted, proteolysis after HU was significantly reduced (Figure 4B, Spd1<sup>44–124</sup>). Mutating conserved amino acids in the PIP box (Figure 4A, Spd1<sup>MPIP</sup>) also reduced Spd1 proteolysis after HU treatment (Figure 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.

**Figure 2. Chromatin-Bound PCNA Is Required for Spd1 Proteolysis**

(A) Spd1-TAP levels in a wild-type strain (1766) and in an rfc1<sup>−44</sup> thermosensitive mutant (2072), treated with hydroxyurea (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 cdt2<sup>D122A</sup> mutant (2849).

(C) Spd1-TAP levels after HU treatment of cdt2<sup>D122A</sup> yox1<sup>Δ</sup> (2755) cells and mitotically arrested nda3<sup>−</sup> cdt2<sup>D122A</sup> (2664) cells. Tubulin is shown as a loading control. See also Figure S1.

**Spd1 Interaction with PCNA Is Reduced by Mutation of the PIP Box**

We used bimolecular fluorescence complementation (BiFC) [26, 27] to determine whether 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 expressed in a cdt2<sup>Δ</sup> background (Figure 4C). However, when coexpressed in a cdt2<sup>Δ</sup> background to stabilize Spd1, nuclear YFP fluorescence was seen in all cells, irrespective of cell cycle stage (Figure 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 (Figure 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 did not have a significant effect on Spd1 levels in a cdt2<sup>Δ</sup> mutant background (Figure S3B). Spd1<sup>1–43</sup>, retaining the PIP box, showed a BiFC signal similar to that seen with wild-type Spd1 (Figure 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<sup>44–124</sup> showed a reduced BiFC signal, as did the Spd1<sup>MPIP</sup> mutant (Figure 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 that high Cdt2 levels are not sufficient to promote Spd1 degradation. Significantly, we demonstrate that the critical step in Spd1 proteolysis, and therefore in RNR upregulation, is the interaction of Spd1 with DNA-bound PCNA. Because PCNA serves as an essential polymerase processivity factor in S phase, as well as promoting repair synthesis by pol δ and other repair polymerases, this provides a direct mechanism to synchronize DNA synthesis with stimulation of RNR to upregulate dNTP production. This mechanism is distinct from proteolysis of Sml1 in S. cerevisiae. In this organism, which lacks the CRL4<sup>Cdt2</sup> 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 [28, 29].

The type 1a class of RNRs, found in eukaryotes and eubacteria, consists of a heterotrimer composed of two large catalytic R1 subunits and two small R2 subunits that 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, whereas 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 (Figure 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.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Length (µm)</th>
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<tr>
<td>Wild-type</td>
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<tr>
<td>pcn1&lt;sup&gt;D122A&lt;/sup&gt;</td>
<td>19.3 ± 5.7</td>
</tr>
<tr>
<td>spd1Δ</td>
<td>13.5 ± 2.6</td>
</tr>
<tr>
<td>rad3Δ</td>
<td>11.4 ± 2.7</td>
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<td>pcn1&lt;sup&gt;D122A&lt;/sup&gt; spd1Δ</td>
<td>13.8 ± 4.3</td>
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<tr>
<td>pcn1&lt;sup&gt;D122A&lt;/sup&gt; spd1Δ rad3Δ</td>
<td>12.4 ± 2.5</td>
</tr>
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Figure 3. PCNA<sup>D122A</sup>-Induced Defects Are Suppressed by spd1 Deletion

(A) pat1<sup>Δ</sup> strains also containing pcn1<sup>D122A</sup> (2912), pcn1<sup>D122A</sup> spd1<sup>Δ</sup> (2915), or spd1<sup>Δ</sup> (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 premeiotic S phase was followed by flow cytometry.

(B) Upper panels: images of exponentially growing cells from wild-type (137), spd1<sup>Δ</sup> (2671), rad3<sup>Δ</sup> (1811), pcn1<sup>D122A</sup> (2738), pcn1<sup>D122A</sup> spd1<sup>Δ</sup> (2747), and pcn1<sup>D122A</sup> spd1<sup>Δ</sup> rad3<sup>Δ</sup> (2842) cultures. Lower panel: average cell length ± SD (150 cells were measured for each strain).

(C) Viability of wild-type (137), pcn1<sup>D122A</sup> (2738), spd1<sup>Δ</sup> (2671), rad3<sup>Δ</sup> (2839), pcn1<sup>D122A</sup> rad3<sup>Δ</sup> (2887), pcn1<sup>D122A</sup> spd1<sup>Δ</sup> rad3<sup>Δ</sup> (2888), spd1<sup>Δ</sup> rad3<sup>Δ</sup> (2889), and pcn1<sup>D122A</sup> spd1<sup>Δ</sup> rad3<sup>Δ</sup> (2747) strains on YE3S at 26°C and 37°C analyzed by spot tests.

(D) Rate of minichromosome loss in wild-type (2836), pcn1<sup>D122A</sup> (2898), spd1<sup>Δ</sup> (2885), and pcn1<sup>D122A</sup> spd1<sup>Δ</sup> (2900) strains.

See also Figure S2.
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 *S. pombe* Spd1 (gray 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 and SpCdt1_2818), PIP degron and PIP box consensus sequences [25], and the mutated sequence of Spd1MPIP. Green and blue bars at bottom show the Spd11–43 and Spd144–124 deletion mutants. The cyan bar labeled “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 Spd11–43 mutant (2612), the Spd144–124 mutant (2744), and the Spd1MPIP mutant (2673). Tubulin is shown as a loading control.

(C) Bimolecular fluorescence complementation (BiFC) of exponentially growing live cells expressing VN173-pcn1 and spd1-VC155 in a wild-type (2536) and in a cdt2Δ (2546) background.

(D) Comparison of the intensity of BiFC in exponentially growing live cells. A wild-type (2752) and an spd11–43 mutant (2741), a wild-type (2752) and an spd144–124 (2751) strain (left panels), a wild-type (2752) and an spd1MPIP (2748) strain (right panels) were compared. Phase (blue) and BiFC signal (green) channels are shown merged (upper panels). In all combinations, cells carrying the mutation in Spd1 were stained with MitoTracker Red to allow direct comparison between the two strains (lower panels). All strains were in a cdt2Δ background to stabilize Spd1; comparison of Spd1 levels in these strains is shown in Figure 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 with and inhibiting RNR in the cytoplasm and possibly interacting with free PCNA in the nucleus. CRL4ΔCdt2 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.
and the net reduction of Spd1 levels causes RNR activation (Figure 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 to 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 [30].

So far, no Spd1 ortholog has been identified in metazoans, 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<sup>Cdt2</sup> pathway, identification of mammalian protein inhibitors of RNR might be facilitated by inactivation of this mechanism.

Proteolysis of CRL4<sup>Cdt2</sup> targets triggered by PCNA chromatin binding is emerging as an important mechanism in DNA replication control (reviewed in [25]). Degradation of Cdt1 blocks Mcm chromatin binding directly, whereas p21 proteolysis leads to nuclear export of the Cdc6 licensing factor [21], and downregulation of Set8 in S phase also seems to be important for blocking rereplication [31, 32]. Our results here, showing that an inhibitor of the elongation step of replication is degraded simultaneously with destruction of licensing activators, emphasize how PCNA activation of CRL4<sup>Cdt2</sup> is a master switch in the transition from G1 to S phase.

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.02.070.

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References


