Inventory of Supplemental Information

Supplementary information inventory:

- Figure S1, related to figure 2. Protein levels in an *rfc1-44* mutant.
- Figure S2, related to figure 3. *pcn1*^{D122A} strain is DNA damage sensitive and *cdt2*Δ and *pcn1*^{D122A} mutations are epistatic.
- Figure S3, related to figure 4. In vitro binding of recombinant PCNA to Spd1-FLAG and Spd1 levels in BiFC strains.
- Table S1. Strains used in this work.
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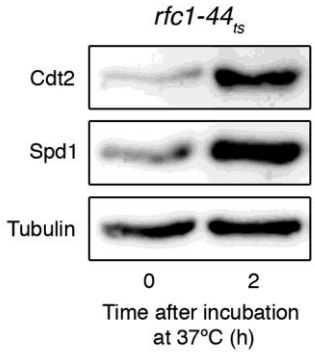

Figure S1 (related to figure 2). *Protein levels in an rfc1-44 mutant.* Cdt2 and Spd1 levels in an *rfc1-44* thermosensitive mutant (2617) growing exponentially and after 2 hours of incubation at the restrictive temperature (37ºC). Tubulin is shown as a loading control.

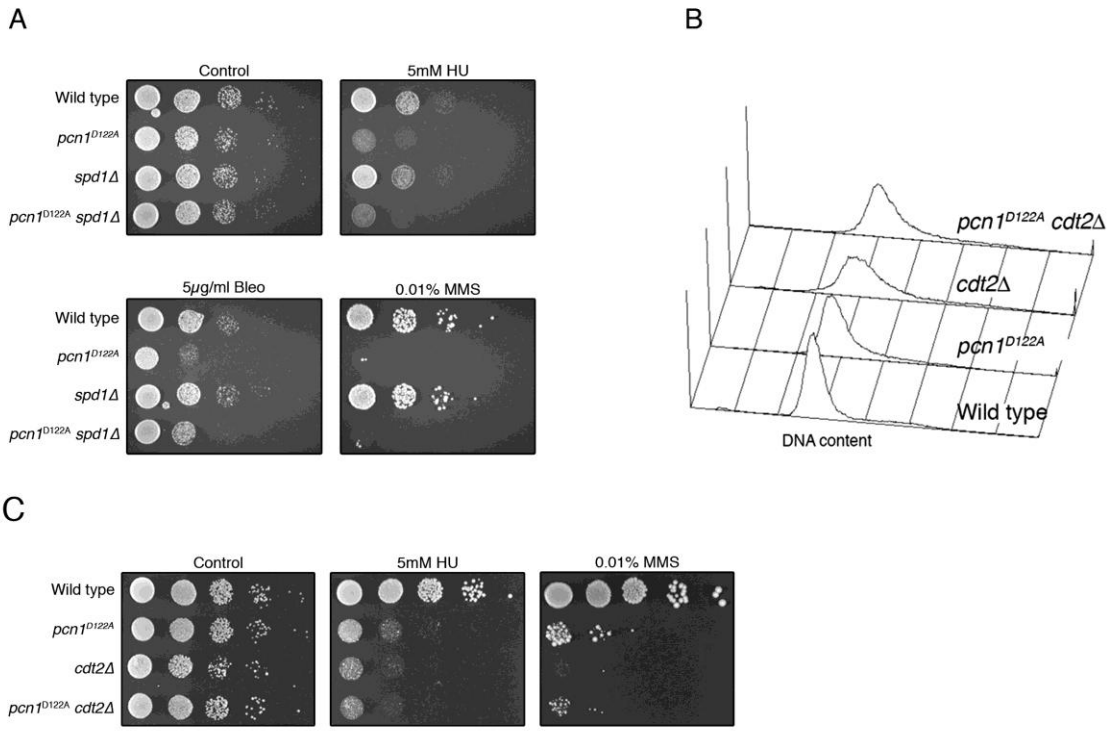

Figure S2 (related to figure 3). *pcn1^{D122A} strain is DNA damage sensitive and cdt2*Δ *and pcn1^{D122A} mutations are epistatic.* (A) Spot tests of wild type (137), *pcn1^{D122A}* (2737), *spd1*Δ (2671) and *pcn1^{D122A} spd1*Δ (2747) strains on YE3S, YE3S + 10mM HU, YE3S + 5μg/ml Bleomycin, and YE3S + 0.01% MMS. (B) Flow cytometric analysis of exponentially growing wild type (137), *pcn1^{D122A}* (2738), *cdt2*Δ (1666) and *pcn1^{D122A} cdt2*Δ (2827) cells. (C) Spot tests of cells shown in *B* on YE3S, YE3S + 10mM HU, and YE3S + 0.01% MMS.

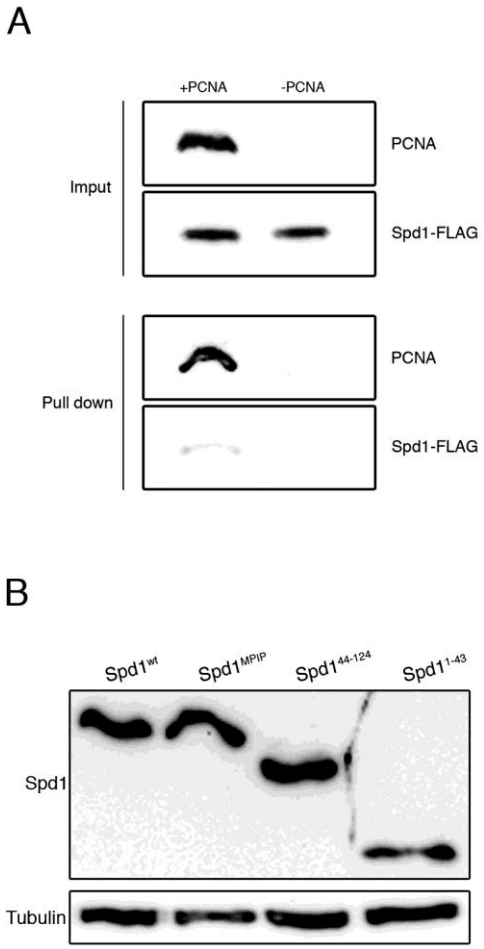

Figure S3 (related to figure 4). *In vitro binding of recombinant PCNA to Spd1-FLAG and Spd1 levels in BiFC strains.* (A) Spd1-FLAG was incubated with and without His-PCNA as indicated, and His-PCNA was pulled down using Ni-NTA beads. The beads were extensively washed and both proteins were detected after SDS-PAGE using anti-PCNA and anti-FLAG antibodies. The figure is representative of three independent experiments. (B) Spd1 levels in exponentially growing cells expressing wild-type *spd1-VC155-NLS* (2752), *spd1^{MPIP}-VC155-NLS* (2748), *spd1$^{44-124}$-VC155-NLS* (2751) or *spd1$^{1-43}$-VC155-NLS* (2750), all in a *cdt2*Δ background.

Table S1. Strains used in this work.

Strain	*Genotype*
137	h+ *ade6-M216 leu1-32 ura4-D18*
1666	h- *ade6-704 leu1-32 ura4-D18 cdt2Δ::ura4+*
1766	h+ *leu1-32 ura4-D18 spd1-TAP::kanMX6*
1811	*h- ura4-D18 rad3Δ::ura4+*
2072	h+ *spd1-TAP::kanMX6 rfc1-44ts*
2536	h- *ade6-M210 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1-VC155-natMX6*
2546	h- *ade6 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1-VC155-natMX6 cdt2Δ::ura4+*
2612	h- *ade6-M210 leu1-32 ura4-D18 spd1$^{1-43}$-YFP-NLS::ura4+*
2617	*leu1-32 ura4-D18 spd1-TAP::kanMX6 cdt2-TAP::kanMX6 rfc1-44ts*
2644	h- *ura4D-18 spd1-TAP::kanMX6 rad3Δ::ura4+*
2649	h+ *ade6-M216 leu1-32 spd1-TAP::kanMX6 pcn1-D122A::Hyg*
2664	h+ *leu1-32 ura4-D18 spd1-TAP::kanMX6 nda3-311 pcn1-D122A::Hyg*
2671	h+ *ade6-M210 leu1-32 ura4-D18 spd1Δ::kanMX6*
2672	h+ *ade6-M216 leu1-32 ura4-D18 spd1–YFP-NLS::ura4+*
2673	h- *ade6-M210 leu1-32 ura4-D18 spd1^{MPIP}-YFP-NLS::ura4+*
2677	h- *leu1-32 cdt1-TAP::kanMX6 spd1-TAP::kanMX6 nda3-311 rad3Δ::ura4+*
2678	h- *leu1-32 ura4-D18 spd1-TAP::kanMX6 nda3-311*
2698	h- *ura4-D18 spd1-TAP::kanMX6 cdt2-TAP::kanMX6 yox1Δ::kanMX6*
2710	*cdt2-TAP::kanMX6 nda3-311*
2711	h+ *ura4-D18 spd1-TAP::kanMX6 yox1Δ::kanMX6*
2713	h+ *ura4-D18 spd1-TAP::kanMX6 yox1Δ::kanMX6 rad3Δ::ura4+*
2737	h- *ade6-M210 leu1-32 ura4-D18 pcn1-D122A::Hyg*
2738	h+ *ade6-M210 leu1-32 ura4-D18 pcn1-D122A::Hyg*
2744	h- *ade6-M210 leu1-32 ura4-D18 spd1$^{44-124}$-YFP-NLS::ura4+*
2747	h+ *ade6-M210 leu1-32 ura4-D18 pcn1-D122A::Hyg spd1Δ::kanMX6*
2748	h+ *ade6-M210 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1^{MPIP}-VC155-NLS::natMX6 cdt2Δ::ura4+*
2750	h+ *ade6-M210 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1$^{1-43}$-VC155-NLS::natMX6 cdt2Δ::ura4+*
2751	h+ *ade6-M210 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1$^{44-124}$-VC155-NLS::natMX6 cdt2Δ::ura4+*
2752	h+ *ade6-M210 leu1-32 ura4-D18 VN173-pcn1::kanMX6 spd1-VC155-NLS::natMX6 cdt2Δ::ura4+*
2755	*ura4-D18 spd1-TAP::kanMX6 yox1Δ::kanMX6 pcn1-D122A::Hyg*
2827	h+ *ade6 leu1-32 ura4-D18 pcn1-D122A::Hyg cdt2Δ::ura4+*
2836	h+ Ch^{16}-RMYAH *ade6-M210 leu1-32 ura4-D18 his3-D1 arg3-D4*
2839	h- *ade6-704 leu1-32 ura4-D18 rad3ts*
2842	*ade6-M210 leu1-32 ura4-D18 pcn1-D122A::Hyg spd1Δ::kanMX6 rad3Δ::ura4+*
2885	h- Ch^{16}-RMYAH *ade6-D1 ura4-D18 spd1Δ::ura4*
2887	*ade6- leu1-32 ura4-D18 pcn1-D122A::Hyg rad3ts*
2888	*ade6- leu1-32 ura4-D18 pcn1-D122A::Hyg spd1Δ::kanMX6 rad3ts*
2889	*ade6- leu1-32 ura4-D18 spd1Δ::kanMX6 rad3ts*
2898	h+ Ch^{16}-RMYAH* *ade6-M210 leu1-32 ura4-D18 D18 pcn1-D122A::Hyg*
2900	h+ Ch^{16}-RMYAH* *ade6-M210 leu1-32 ura4-D18 D18 pcn1-D122A::Hyg spd1Δ::kanMX6*
2912	*pat1-114 pcn1-D122A*
2915	*pat1-114 pcn1-D122A spd1Δ::ura4+ ura4-D18*
2930	*pat1-114 spd1Δ::ura4+ ura4-D18*

*Ch16-RMYAH (Ch16 *yps1::arg3+ ert1::MAT*a*-hph ade6-M216 cid2::his3+*)

Table S2. Primers used in this study.

Primer	*Sequence 5'-3'*
813	AAAGGGCCCCTTGAAAGCAGTTGGCAATAAAAGAGAAAGCG
814	AAACCCGGGATAATCTCTCATTGGTACCCTCCATGACAAC
843	TGCCGCGTCCATTGCGGAGCCGGCAATGCTTTCGGGAAGCTGAGGACGC
844	GCCGGCTCCGCAATGGACGCGGCAATGCGGGTTCGTAAATCAATTTCCA
891	AAACCCGGGTTGATTTACGAACCCGCATTCCCACGT
950	TTTCCCGGGGTACCCGTCCGGCGTGCAAAATC
951	TTTGGATCCCTTGTACAGCTCGTCCATGCCGA
982	CGACTTGTATCCGGTGGAAATCATTTTTTCCAAAATAAATTGTCGACGAACC
983	ATTTCCACCGGATACAAGTCGAAA C
1012	TTTCTCGAGATGCACAGCAGCAAGCGAGTTATG
1013	TTTTCTAGATCATAAATCATAAGAAATTCGCTTATTTAGAAGTGG
1021	CCAATCCTCCTCAAGACTTTGAAGAACCGGAATGGTTGAAGCCGTTTGACGTTGTCATGGAGGGTACCAATGAG AGATTACGGATCCCCGGGTTAATTAA
1022	TAGCCGTCACTCACAAAACCAATAATAAAATGTATTAATGGTATAGGGAATCAACAAAGTCAAAGACTAATGAAC GAAGGGAATTCGAGCTCGTTTAAAC
1023	TTTGGATCCGCAGCTACTTGTCCTTCATGGC
1024	TTTTTAATTAATAATCTCTCATTGGTACCCTCCAT

Materials and methods

Fission Yeast Methods. Strains used in this study are listed in Supplementary Table S1. Standard genetic methods and flow cytometry were used as described previously [1, 2]. Hydroxyurea (HU) was used at a concentration of 18 mM and methyl methanesulfonate (MMS) was used at 0.2%, unless otherwise indicated. Bleomycin (Bleo) was used at 5µg/ml.

Strains construction. The *spd1-YFP-NLS* strain was constructed by amplifying *spd1* with its promoter with the primers 813 and 814, containing ApaI and XmaI restriction sites respectively (Supplementary Table S2). The PCR product was digested with ApaI and XmaI and cloned into pSMUY-NLS. The plasmid was integrated at the *spd1* promoter after linearizing with BglII. The $spd1^{1\text{-}43}$ mutant was constructed by amplifying the *spd1* fragment with the 813-891 primer pair. The product was digested with ApaI and XmaI and cloned into pSMUY-NLS. For $spd1^{44\text{-}124}$, a fragment containing the promoter and the ATG codon was amplified with 813-982 primers, and a partial complementary fragment containing $spd1^{44\text{-}124}$ was amplified with 983-814 primers. Both fragments were annealed and amplified and the purified product was cloned into ApaI-XmaI digested pSMUY-NLS. These plasmids were integrated as described with wild type *spd1*. To construct the $spd1^{MPIP}$ mutant strain, two partial complementary fragments were amplified using primers containing the mutation (813-843 and 844-814); subsequently, the fragments were annealed and amplified. After DNA purification, the fragment was cloned into ApaI and XmaI digested pSMUY-NLS, and integrated as above. *spd1-VC155* was constructed by amplifying the VC155-natMX6 cassette from the pFA6a-VC155-natMX6 plasmid [3]

with the long oligos 1021-1022, and transforming directly with the purified PCR products. The *spd1-VC155-NLS* plasmid and its derivatives were constructed by amplifying the VC155 fragment from the pFA6a-VC155-natMX6 plasmid with the primers 950 and 951 containing XmaI and BamHI sites. After digestion, the fragment was cloned into pSMU-*spd1*-YFP-NLS and the mutant derivatives, substituting YFP by VC155. The *VN173-pcn1* construction is described in [4], and the *pcn1-D122A* construction is described in Havens et al, [5]. For the pTNT-spd1-5xFLAG construction, *spd1* was amplified with oligos 1023-1024, digested with BamHI and PacI and cloned into pFA6a-5FLAG-kanMX6 [6]. *spd1-FLAG* was amplified from pFA6a-spd1-5FLAG-kanMX6 with the oligos 1012-1013, digested with XhoI and XbaI and integrated in pTNT^{TM} (Promega). All plasmids and strains constructed were verified by sequencing.

Cell-cycle synchronization. *nda3-311* strains were grown at 32°C in YES medium and arrested in M phase by incubation for 4 h at 20°C. After MMS addition, cells were kept arrested at 20ºC. For the *rfc1-44* strain, cells were grown at 26°C until mid-log phase and then were shifted to 37°C for 4 hours before drug addition. After HU or MMS addition, cells were incubated at restrictive temperature.

Protein Analysis. Protein extracts were made by TCA extraction and analyzed by western blotting as described previously [7]. TAP-tagged Spd1 and Cdt2 were detected with peroxidase–anti-peroxidase–soluble complex (P1291, Sigma). Spd1-YFP was detected

using Anti-GFP antibody (catalogue number 11814460001, Roche) and α-tubulin was used as loading control and detected with antibody T5168 (Sigma).

In vitro protein–protein interaction assay. Bacterial expression and purification of PCNA fusion protein was performed as described in Reynolds et al. [11]. Spd1-FLAG *in vitro* expression was carried out with the TNT® SP6 High-Yield Wheat Germ Protein Expression kit (Promega), following the manufacturer's recommendations. For the interaction assay, 1 μg of His_6-PCNA was mixed with 10 μl of *in vitro* expressed Spd1-FLAG extract in a total volume of 500 μl of buffer (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100, 20 mM imidazole, 2 μg/ml linear DNA) and incubated for 40 min at room temperature on a rotating wheel. A 30 μl volume of a 50% (v/v) slurry of Ni-NTA beads (Qiagen) was then added and the incubation continued for a further 40 min, at which point the beads were extensively washed with the same buffer. After a final wash, the supernatant was discarded and the beads boiled in an equal volume of 2x SDS-PAGE gel sample buffer. Proteins were visualized by western blotting, using anti-FLAG (Sigma, F3165) and anti-PCNA (Sigma, P8825) antibodies.

Fluorescence microscopy. For analysis by fluorescence microscopy, live cells were mounted in 1.2% low melting temperature agarose. To compare two different strains in the same image, MitoTracker Red CM-H2XRos (Invitrogen) was added to one of the cultures to a final concentration of 0.4 μM, 30 min before collecting the cells. The cells were washed twice with the same medium; resuspended in the original volume; mixed with the same volume of the other culture; and mounted as above. Images were collected

using a Zeiss Axioplan microscope, coupled to a Hamamatsu ORCA ER camera; open source μManager software [8] was used to control the camera and microscope.

Spot test. Cells were grown to mid-log phase and adjusted so that the final concentration was the same in all the cultures. Serial ten-fold cell dilutions were spotted onto YE3S plates or YE3S plates containing 5mM HU, 5μg/ml Bleo or 0.01% MMS. Plates were incubated at 30°C for 3 days and photographed, unless otherwise indicated.

Analysis of pre-meiotic S phase. pat1 strains were arrested in EMM-N medium at 26°C for 16h, then resuspended in EMM+N medium at 34°C and samples were taken for analysis by flow cytometry.

Rate of minichromosome loss per generation. Essentially, this assay was performed as described in Murakami et al., 1995 [9]. Briefly, exponentially growing cultures of the different mutants containing the minichromosome (Ch^{16}-RMYAH) [10] in selective medium (EMM without adenine) were centrifuged and resuspended in rich medium, followed by growth for 10-20 generations. Cells were then plated out on rich medium plates and incubated for two days at 30°C. The colonies were replicated to selective medium plates and the number of red Ade^- colonies (due to loss of the minichromosome) was scored. The rate of minichromosome loss per generation (p) was calculated with the equation $p=(1-e^{(1/n)\ln Rn/R0}) \times 100$, where R0 and Rn are the percentage of Ade^+ cells at the 0 and *n* generation after transfer to the nonselective medium, respectively.

Supplemental references

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3. See reference 25 in the main text.
4. See reference 18 in the main text.
5. See reference 24 in the main text.
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