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Supplemental Data

Activation of Ubiquitin-Dependent DNA Damage Bypass Is Mediated by Replication Protein A

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Supplemental Experimental Procedures

Chromatin Immunoprecipitation (ChIP) assays at HO-induced DSBs

Cells of the JKM179 background (Lee et al., 1998) allow the creation of an unrepairable DSB at the *MAT* locus by galactose-induced expression of the HO endonuclease. The DSB was induced by addition of galactose (2%) to yeast cultures exponentially growing in lactate medium. Samples of 10^9 cells were removed at the indicated times, and formaldehyde was added to 1%. After incubation at room temperature for 10 min, the crosslinking reaction was terminated by addition of glycine to 125 mM. Cells were harvested, washed once in phosphate-buffered saline, and total cell extracts were prepared by glass bead lysis in 700 µl RIPA buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) containing 5 mM Benzamidine and Complete™ protease inhibitors (Roche). All steps were performed at 4°C. DNA was sheared by sonication, and the lysate was cleared by centrifugation. To reduce non-specific background, the lysates were pre-treated with Sepharose CL4B for 30 min. Thereafter, 1 µg anti-myc antibody (rabbit polyclonal, Santa Cruz Biotechnology) was added to each sample. After incubation on a rotating wheel for 3 h the extracts were added to 20 µl aliquots of protein G agarose beads. After further incubation for 1 h the beads were washed for 20 min each with RIPA buffer, RIPA buffer containing 500 mM NaCl, wash buffer (10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) and TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Bound material was eluted with 100 µl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) for 15 min at 65°C. Crosslinks were reversed by overnight incubation at 65°C, and poteins were degraded by proteinase K treatment for 1 h at 37°C. LiCl was added to 0.5 M. After extraction with phenol:chloroform the DNA was precipitated with ethanol and resuspended in 100 µl TE.

Aliquots of 6 µl were analyzed by real-time PCR in an ABI prism 700 thermocycler, using ABsolute™ QPCR SYBR Green Mix (ABgene). The *MAT* locus DNA was quantified using primers 5‘-TTATAGAGTGTGGTCGTGGC-3’ and 5‘-CCCGTATAGCCAATTCGTTC-3’. As a reference locus, a sequence from the *ACT1* gene was quantified using primers 5‘-CCAGCCTTCTACGTTTCCATCCA-3’ and 5‘-TCACCGGAATCCAAAACAATACCA-3’. Reactions (25 µl) were set up in triplicate and analyzed by a denaturation step of 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 55°C, 30 s at 72°C. A melting curve was obtained to ensure the presence of the expected products in all samples. Standard curves were obtained with purified genomic DNA of known concentration, and the ratios of the absolute amounts of *MAT* to *ACT1* DNA were calculated for each time point. These values were then divided by the value at time zero. Standard deviations were calculated by error propagation, taking into account the standard deviations obtained for the values of *MAT* and *ACT1* DNA.

Supplemental Figures

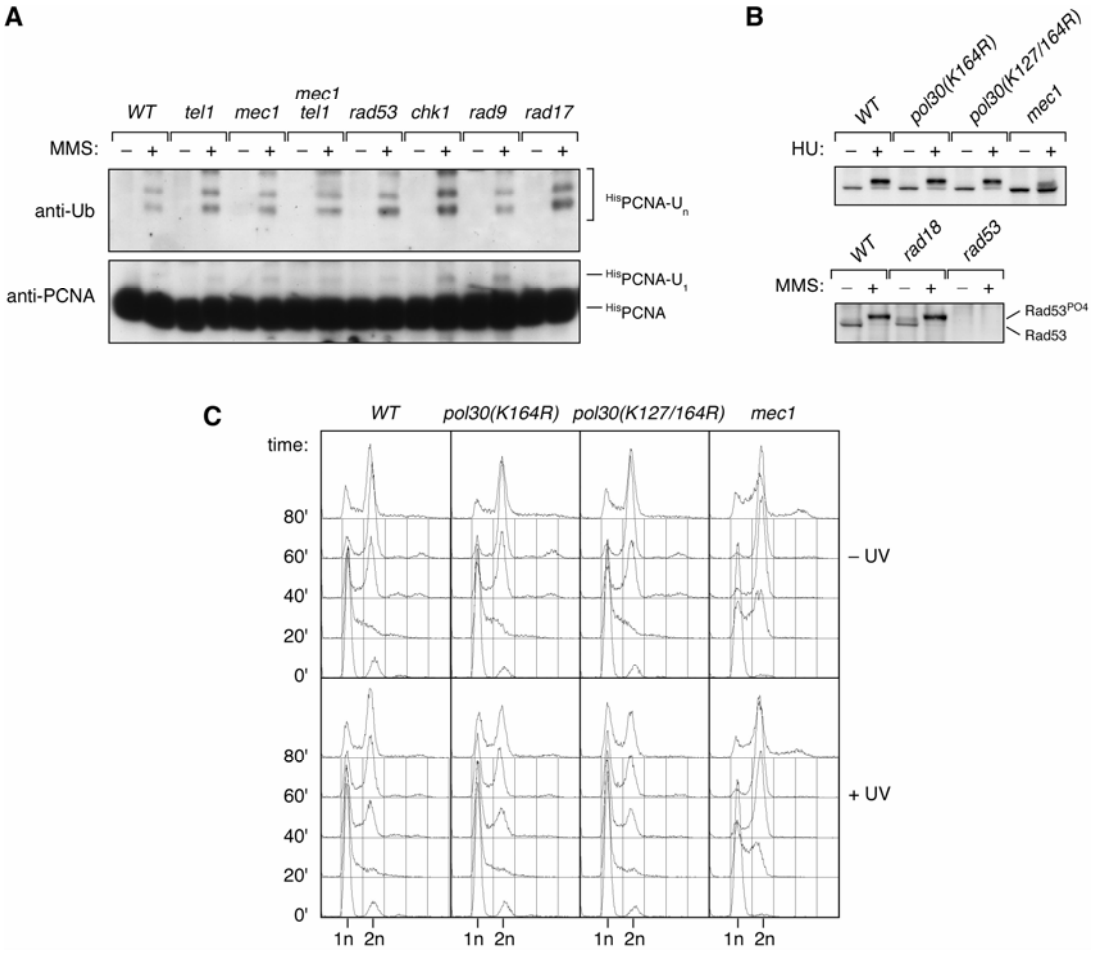

Figure S1. Checkpoint signaling is independent of PCNA modifications.

(A) Damage-induced PCNA ubiquitylation is unaffected in checkpoint mutants. The indicated strains bearing the *HisPOL30* allele were treated with 0.02% methyl methanesulfonate (MMS) for 90 min where indicated, followed by isolation of HisPCNA from total cell extracts under denaturing conditions and detection of ubiquitylated forms by Western blot. Checkpoint genes were disrupted with a *KanMX* or *URA3* cassette. Deletions in *MEC1*, *TEL1* and *RAD53* were generated in the background of an *sml1::hisG-URA3-hisG* deletion.

(B) The replication-dependent intra-S-phase checkpoint is activated normally in strains deficient in PCNA modifications. Replication fork stalling was induced by HU or MMS treatment, and Rad53 and its phosphorylated forms were detected in total cell lysates by Western blotting. The *pol30* mutants have been described previously (Stelter and Ulrich, 2003).

(C) The G1/S checkpoint, measured by delay of entry into S phase after ultraviolet (UV) irradiation in G1, is unaffected by defects in PCNA ubiquitylation. The indicated strains were synchronized in G1, irradiated with a UV dose of 100 J/m^2 where indicated, and released into the cell cycle. Entry into S phase was monitored by flow cytometry.

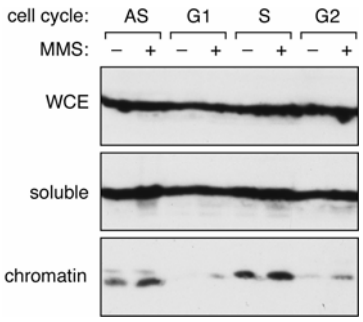

Figure S2. Chromatin association of PCNA in damaged and undamaged cells.

Yeast cultures (*WT*) were treated as described in the legend to Fig. 1 to achieve arrest in G1, S or G2 phase, and 0.02% MMS was added where indicated. After 90 min incubation, whole cell extracts (WCE) were prepared by spheroplast lysis and fractionated by centrifugation through a sucrose cushion into soluble and chromatin-associated (insoluble) material (Liang and Stillman, 1997). PCNA was detected in the individual fractions by Western blot.

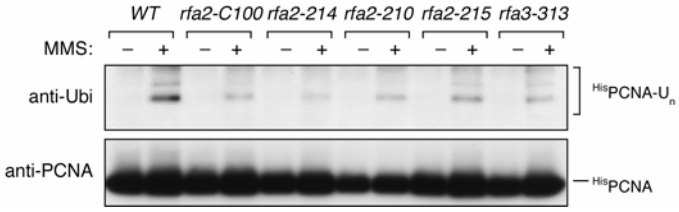

Figure S3. Analysis of PCNA ubiquitylation in *rfa2* and *rfa3* mutants.

PCNA ubiquitylation was assayed in isogenic strains in the W303 background, bearing the ^{His}*POL30* allele integrated into the *LEU2* marker as described in the legend to Figure 1. The alleles [Maniar et al. (1997) Genetics *145*, 891-902] carry the following mutations: *rfa2-C100*: C-terminal truncation by H174Stop; *rfa2-214*: T3S/V74A/Q95L/I154V/K182R/Q183P/F197S/L255W/T264A; *rfa2-210*: L39S/L164V/C173G/K225R; *rfa2-215*: W101R/Y151F/L168S/C251S/L255W/F265S; *rfa3-313*: P31S/E74G/R113G.

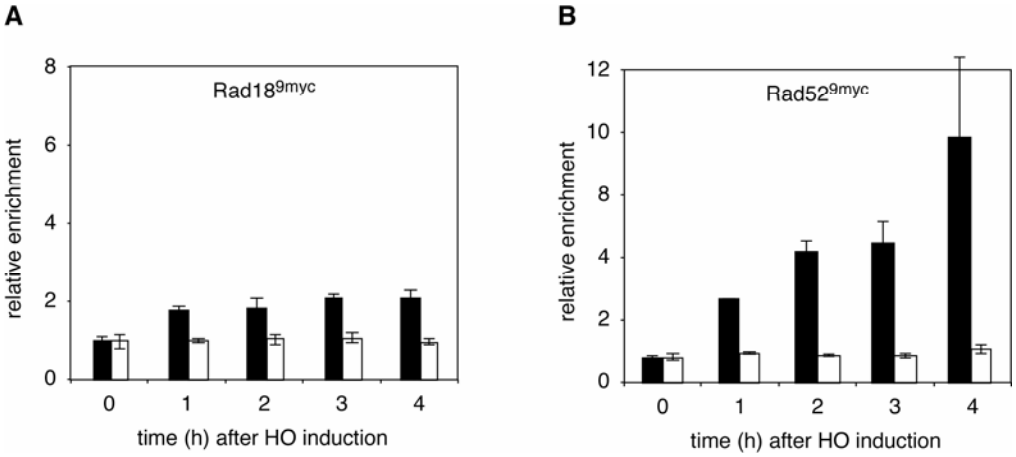

Figure S4. Deletion of *MRE11* prevents access of Rad18 and Rad52 to ssDNA.

(A) Association of Rad18^{9myc} with sequences adjacent to an HO-induced DSB is prevented when resection of the break ends is delayed by deletion of *MRE11*. The presence of Rad18^{9myc} was examined by ChIP as described in the legend to Fig. 5 in *WT* (black) and *mre11* (white) cells.

(B) Rad52^{9myc} was analyzed as a control protein that is known to bind stretches of ssDNA. Its presence at the break ends is likewise reduced in *mre11* (white) compared to *WT* (black) cells, suggesting that the twofold enrichment observed with Rad18^{9myc} is genuine.

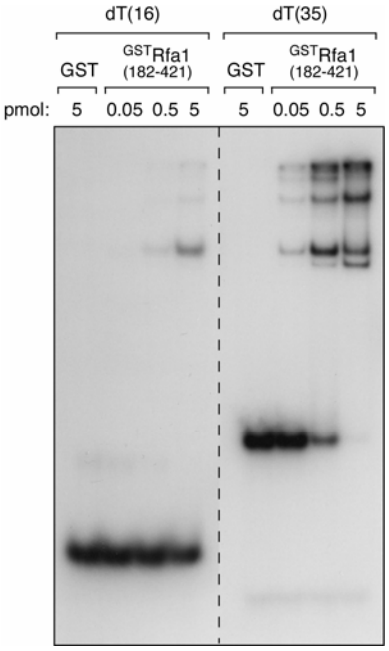

Figure S5. Interaction of ^{GST}Rfa1(182-421) with ssDNA.

Electrophoretic mobility shift assays were performed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA and 0.05% Triton X-100 with 0.5 pmol oligo-dT of 16 or 35 nt length. Oligonucleotides were incubated with the indicated amounts of protein on ice for 20 min, and gels were run at 4°C. A 10mer oligonucleotide does not bind with high enough affinity to be detected by gel shift analysis, but is known to interact with the ssDNA-binding domain of human Rfa1 in the co-crystal [Bochkarev et al. (1997) Nature *385*, 176-181].