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Defects in DNA Ligase I Trigger PCNA Ubiquitination at Lysine 107

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Abstract

In all eukaryotes, the ligation of newly synthesized DNA, also known as Okazaki fragments, is catalyzed by DNA ligase I. An individual with a DNA ligase I deficiency exhibited growth retardation, sunlight sensitivity and severe immunosuppression², likely due to accumulation of DNA damage. Surprisingly, not much is known about the DNA damage response (DDR) in DNA ligase I-deficient cells. Because DNA replication and DDR pathways are highly conserved in eukaryotes, we utilized *Saccharomyces cerevisiae* as a model system to address this question. We uncovered a novel pathway, which facilitates ubiquitination of lysine 107 of proliferating cell nuclear antigen (PCNA). Unlike ubiquitination at lysine 164 of PCNA in response to UV irradiation, which triggers translesion synthesis³, modification of lysine 107 is not dependent on the ubiquitin conjugating enzyme (E2) Rad64 nor the ubiquitin ligase (E3) Rad185, but requires the E2 variant Mms26 in conjunction with Ubc47 and the E3 Rad58,9. Surprisingly, DNA ligase I-deficient *cdc9-1* cells that carry a PCNA^{K107R} mutation are inviable, because they cannot activate a robust DDR. Furthermore, we show that ubiquitination of PCNA in response to DNA ligase I-deficiency is conserved in humans, yet the lysine that mediates this modification remains to be determined. We propose that PCNA ubiquitination provides a “DNA damage code” that allows cells to categorize different types of defects that arise during DNA replication.

Previous studies have suggested that DNA ligase I-deficient *cdc9* mutants arrest in G2 phase after completing DNA synthesis^{10,11} because of the accumulation of nicked DNA¹². These observations implied that S phase proceeds normally despite single-stranded breaks in

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S.D.-B. and H.-D.N. conducted all PCNA ubiquitination experiments in yeast, helped with data analysis and manuscript writing. J.L.W. conducted all experiments with human cells, helped with data analysis and manuscript writing. R.R.M conducted cell cycle arrest and RIP mapping experiments and helped with data analysis. J.C.H helped with the construction of yeast mutants. A.-K.B. planned and supervised the project and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

nascent DNA, suggesting that cells may not be able to efficiently sense this type of DNA damage during S phase. To reinvestigate whether DNA ligase I is required for S phase progression, we have analyzed three different temperature sensitive alleles of *CDC9* (Supplementary Information, Fig. S1a and b online, and Supplementary Information, Fig. S2a and b online). One of these alleles is a thermo labile degron mutant¹³ (*cdc9-td* in Fig. 1a). To ensure that ligase activity was sufficiently inactivated in these cells, we performed replication initiation point mapping and did not detect any measurable ligation of Okazaki fragments over the yeast origin *ARS1* (Supplementary Information, Fig. S2c online) as previously demonstrated for the *cdc9-1* allele¹⁴. Whereas DNA ligase I was not required for entry into S phase (Fig. 1b), we found it to be necessary to release from a hydroxyurea (HU) block (Fig. 1c). This was also true for two additional temperature sensitive alleles, *cdc9-1* and *cdc9-215* (Fig. 1d). This result was surprising as earlier reports suggested that DNA ligase I-deficient cells could complete DNA synthesis without joining Okazaki fragments to each other^{10,11}. As expected, cell cycle delay in S phase was dependent on the DDR gene *RAD9*^{10,11} because *cdc9-1 rad9* mutants progressed farther than *cdc9-1* cells (Supplementary Information, Fig. S3a online). Importantly, however, the mediator of the replication checkpoint (Mrc1) appeared to contribute equally to Rad9 (Supplementary Information, Fig. 3a and b online). Mrc1 has been shown to have two roles, one in DNA replication and one in activating Rad53 after replication fork stalling, which results in exposure of single-stranded DNA¹⁶. Rad53 is a downstream target of the mitotic entry checkpoint gene *MEC1*, a homolog of the ATM/ATR checkpoint kinases in humans¹⁷. Complementation of *cdc9-1 mrc1* double mutants with the S phase checkpoint-deficient *mrc1^{AQ}* allele¹⁶ failed to induce cell cycle arrest (Supplementary Information, Fig. S3c online), suggesting that the S phase checkpoint and not the replication function of Mrc1 is important to delay S phase progression. Furthermore, the finding that both Mrc1 and Rad9 are activated in *cdc9-1* cells at the non-permissive temperature implies that the DNA substrate recognized contains single stranded DNA at stalled replication forks as well as physical damage, which may have arisen from the lack of Okazaki fragment ligation¹². Besides triggering a checkpoint response, certain types of DNA damage at replication forks have also been shown to cause ubiquitination of PCNA¹⁸. PCNA can be either mono-ubiquitinated or poly-ubiquitinated. Mono-ubiquitination of PCNA triggers the error-prone repair pathway through translesion polymerases, whereas PCNA poly-ubiquitination is needed for error-free repair^{3,19,20}. Mono-ubiquitination depends on Rad6 and Rad18 and is a pre-requisite for poly-ubiquitination, which in turn is mediated by the ubiquitin conjugating complex Ubc13/Mms2 and Rad53. Interestingly, ubiquitin is linked through lysine 63 in these poly-ubiquitin chains^{3,21}. To explore whether loss of DNA ligase I leads to PCNA ubiquitination, we examined the status of PCNA in whole cell extracts. We utilized an antibody specific for yeast PCNA²², which displays multiple non-specific bands in undiluted extracts (Supplementary Information, Fig. S4 online), but produces clean immunoblots with diluted extracts (Fig. 2a). Both *cdc9-1* and *cdc9-2* mutants exhibited a modified form of PCNA of approximately 39 kDa when shifted to the non-permissive temperature (Fig. 2a). Co-immunoprecipitation (Co-IP) experiments with strains that expressed Myc-tagged ubiquitin^{23,24} identified this 39 kDa band as ubiquitinated PCNA, which we did not observe when we mixed cell extracts only with beads (Fig. 2b). Curiously, we observed a non-specific band slightly above the 49 kDa marker, which was especially

obvious in extracts from *cdc9-1* cells. Unfortunately, our co-IP studies did not allow us to draw any conclusions about the nature of this band, although we cannot exclude that it represents poly-ubiquitinated PCNA sticking non-specifically to the beads. Therefore, we overexpressed different ubiquitin mutants, including a G75,76A double mutant, specifically designed to interfere with mono-ubiquitination. Another mutant carried substitutions in all seven lysines (Ub-KO) and was thus defective in forming poly-ubiquitin chains. Analysis of trichloroacetic acid-precipitated whole cell extracts confirmed that the 39 kDa form of PCNA represented mono-ubiquitinated protein (Fig. 2c). Furthermore, two forms of PCNA that represented poly-ubiquitinated protein (at ~52- and 76 kDa), because they disappeared in cells overexpressing the Ub-KO mutant, were visible (Fig. 2d). A distinct ladder of ubiquitinated PCNA with similar molecular weight distribution has also been reported by van der Kemp et al.²⁵ Surprisingly, poly-ubiquitin chains on PCNA were not linked through K6321, as they are in response to other forms of replication stress³, but rather through K29 (Fig. 2d). Based on this result, we predicted that at least some of the genes that are known to play a role in K63 linked poly-ubiquitination (namely *RAD6*, *RAD18*, *MMS2*, *UBC13*, *RAD5*)³ are dispensable for the ubiquitination of PCNA in *cdc9-1* mutants. Indeed, whereas *MMS2* and *RAD5* were required for mono- as well as poly-ubiquitination, *UBC13* and *RAD18* were not (Fig. 3a and Supplementary Information, Fig. S5a online). Moreover, deletion of *RAD6* did not have any effect (Supplementary Information, Fig. S5b online), suggesting that PCNA ubiquitination in response to Okazaki fragment ligation defects differs from that triggered by other types of DNA damage. Our results also indicated that Mms2 likely cooperates with other ubiquitin conjugating enzymes besides Ubc1321. To determine Mms2's potential partner in this reaction, we took advantage of the fact that lysine 29 linkages are catalyzed by UbcH5A in mammalian cells²⁶. UbcH5A's homolog in yeast is Ubc4, which is 93% identical to Ubc5⁷. Unlike Ubc4, which is highly expressed in cycling cells, steady state levels of Ubc5 are low and are upregulated in stationary phase⁷. Ubc4 and 5 have previously been implicated in the degradation of unfolded proteins in yeast⁷, but not in PCNA ubiquitination. When we deleted *UBC4* from *cdc9-1* mutants and performed a temperature shift experiment, PCNA ubiquitination was drastically reduced, whereas deletion of *UBC5* had no effect (Supplementary Information, Fig. S5c online). Because we were unable to generate an *ubc4 ubc5* double mutant in the *cdc9-1* background, we attached a nuclear export sequence onto *UBC4* in a *cdc9-1 ubc5* strain. Again, this resulted in a 95% reduction of PCNA mono-ubiquitination (Fig. 3b). These results suggested to us that the nuclear fraction of Ubc4 cooperates with Mms2 and Rad5 to ubiquitinate PCNA in DNA ligase I-deficient cells.

To further discern the function of PCNA ubiquitination in DNA ligase I-deficient yeast, we monitored cell cycle progression upon release from G1. Specifically, we compared *cdc9-1* cells to *cdc9-1 mms2* and *cdc9-1 ubc13* double mutants. Consistent with our finding that *UBC13* is not involved in the ubiquitination of PCNA (Fig. 3a), *cdc9-1 ubc13* cells arrested in S phase similar to the *cdc9-1* strain (Fig. 4a). In contrast, *cdc9-1 mms2* mutants that fail to ubiquitinate PCNA at the non-permissive temperature (Fig. 3a), readily progressed through S phase (Fig. 4a). The observed lack of PCNA ubiquitination and S phase arrest went hand-in-hand with the inability to phosphorylate Rad53 (Fig. 4b). Importantly, this was not due to a general defect in checkpoint activation, as we detected

Rad53 phosphorylation in all three strains after exposure to MMS (Fig. 4c). To address whether mono-ubiquitination is sufficient for Rad53 activation, we disrupted poly-ubiquitination by overexpressing a ubiquitin mutant in which lysine 29 was substituted with arginine. This did not significantly affect Rad53 activation (Supplementary Information, Fig. S6a and b online). These results argue that mono- rather than poly-ubiquitination of PCNA is a prerequisite for checkpoint activation of DNA ligase I-deficient yeast cells. Next, we set out to determine which lysine in PCNA was ubiquitinated. PCNA has a total of 18 lysines, nine of which are exposed and easily accessible²⁷. We mutated these nine lysines at positions 107, 108, 117, 127, 164, 168, 183, 242 and 253. Wild-type or mutated forms of PCNA were introduced on a plasmid before the endogenous copy was deleted. When we monitored mono-ubiquitination of PCNA in *cdc9-1* cells after they were shifted to the non-permissive temperature, the only mutant that lacked the characteristic 39 kDa band was *pol30K107R* (Fig. 5a). However, further examination revealed that this particular mutant had a second site suppressor mutation, which resulted in elevated DNA ligase I protein levels that were comparable to those in wild-type cells (Supplementary Information, Fig. S7a online). Moreover, the strain was no longer temperature sensitive (Supplementary Information, Fig. S7b online). We attempted several times to make the double mutant but these efforts remained unsuccessful, suggesting that the mutations were synthetically lethal. To prove this point, we performed a plasmid shuffle assay. We introduced *pol30K107R* into the chromosomal copy of PCNA in wild-type and *cdc9-1* mutants, and expressed *POL30* from a plasmid. Cells were forced to abandon the plasmid by re-streaking them onto 5-fluoroorotic acid-containing medium. Whereas *CDC9 pol30K107R* cells were viable and formed colonies, *cdc9-1 pol30K107R* mutants did not, indicating that K107 of PCNA becomes essential in this background (Supplementary Information, Fig. S8 online). This was further confirmed by tetrad analysis (data not shown), which seemingly yielded viable double mutants, but all of these strains had elevated DNA ligase I levels due to a second site suppressor mutation (data not shown). However, we were able to isolate a single double mutant in which Cdc9 levels were only slightly increased compared to *cdc9-1* cells (Supplementary Information, Fig. S9a online). We designated this mutant *cdc9-1* pol30K107R*. In fact, the double mutant retained its temperature sensitivity (Supplementary Information, Fig. S9b online) and failed to ubiquitinate PCNA under non-permissive conditions (Fig. 5b). Importantly, PCNA ubiquitination in response to MMS was still functional (Fig. 5c). At this point, it is worthwhile to point out that we detected ubiquitinated and unmodified PCNA at an apparent ratio of approximately 1:1. However, it is not clear that the antibody binds these two forms of PCNA with equal affinity, and therefore it is difficult to make quantitative assessments. In addition, we take the fact that we could readily generate *cdc9-1 mms2* and *cdc9-1 rad5*, but not *cdc9-1 pol30K107R* double mutants as an indication that a redundant ubiquitination pathway might exist that targets K107 with low efficiency, thereby ensuring cell survival. To confirm that K107 is the target of PCNA ubiquitination in DNA ligase I-deficient cells, we introduced the *pol30K107* mutation into *cdc9-td* cells. As expected, *cdc9-td pol30K107R* double mutants retained viability, because *CDC9* is expressed from an inducible copper promoter¹³. Under non-permissive conditions, the ratio of mono-ubiquitinated to unmodified PCNA in *cdc9-td pol30K107R* cells resembled that of the DNA ligase I-proficient control strain, which carries the same mutation in *POL30* (Fig. 5d). The same was true for poly-ubiquitinated PCNA (Fig. 5e).

Furthermore, *cdc9-td pol30K107R* cells failed to exhibit robust Rad53 phosphorylation in the absence of DNA ligase I, whereas they displayed Rad53 activation in response to MMS (Fig. 5f). From these results we conclude that ubiquitination of PCNA at lysine 107 is a prerequisite for checkpoint activation of DNA ligase I-deficient yeast cells. This is in stark contrast to the previously described ubiquitination of PCNA at lysine 164 in response to UV irradiation or DNA alkylating agents³. Although exposure to UV light or DNA alkylating agents triggers both Rad53 phosphorylation and ubiquitination of PCNA at lysine 164, these processes are thought to occur independently and belong to separate genetic pathways²⁸. We demonstrate that this cannot be generalized, but that PCNA ubiquitination in response to DNA ligase I deficiency must occur before Rad53 can be fully activated. It is worth noting that K107 is conserved in *S. pombe* and *C. elegans*, but higher eukaryotes have a conserved lysine residue at position 110, raising the possibility that this PCNA ubiquitination pathway is not only specific for *S. cerevisiae* (Supplementary Information, Fig. S10 online). To test whether human cells depleted for DNA ligase I exhibit PCNA modifications, we generated several U2OS cell lines expressing short hairpin RNAs (shRNAs) that interfere with DNA ligase I expression (Supplementary Information, Fig. S11 online). In the chromatin-bound protein fractions of these cells, we detected a slower migrating form of PCNA with the same mobility of mono-ubiquitinated PCNA induced by UV irradiation²⁹ (Fig. 5g, upper panel and Supplementary Information, Fig. S11 online). In contrast, cells expressing control shRNA behaved like untreated U2OS cells (Fig. 5g, upper panel). Importantly, when we analyzed one representative DNA ligase I-specific shRNA clone side by side with a PCNA- or an ubiquitin-specific antibody, both antibodies recognized bands at the exact same position, consistent with the notion that PCNA ubiquitination in response to DNA ligase I-deficiency is conserved in humans, although the lysine residue of PCNA that is ubiquitinated in these cells is still under investigation (Fig. 5g, upper and middle panel).

In the course of our studies, we also explored *rad27* and *dna2-1* mutants in *S. cerevisiae*, because both Rad27 and Dna2 have been implicated in lagging strand synthesis³⁰. However, these mutants did not ubiquitinate PCNA (Supplementary Information, Fig. S12 online). This suggested that PCNA ubiquitination at K107 might be specific for DNA ligase I-deficiency. In summary, whereas ubiquitination at lysine 164 is responsible for activation of translesion synthesis, and is thus a result of DNA damage in the template strand, ubiquitination of lysine 107 in DNA ligase I-deficient cells might provide a signal for DNA damage residing in freshly synthesized, nascent DNA. Most importantly, our study demonstrates that PCNA provides a DNA damage-specific code via the ubiquitination of different lysines.

Methods

Strains and plasmids

All strains are isogenic derivatives of W303-1a or SSL204. A complete list of strains can be found in Supplementary Information, Table 1 online. The W303-1a strain carries the *rad5-535* mutation (<http://wiki.yeastgenome.org/index.php/CommunityW303.html>), whereas SSL204 derivatives (*CDC9*, *cdc9-1*, *cdc9-2*) harbor wild type *RAD5*, which was confirmed by sequencing. SSL204 exhibits severe temperature sensitivity at 37°C and

therefore the temperature shift experiments were performed at 35°C. To construct the *cdc9-td* strain, the first 510 base pairs of *CDC9* were inserted into pPW66R (a gift from J. F. X. Diffley) using *HindIII* restriction sites¹³. The resulting pPW66R-*CDC9* plasmid was linearized with *BclII* and transformed into YKL83, which contains *UBR1* under the control of the *GALI* promoter at the endogenous *UBR1* locus³¹. To delete various genes (*MRC1*, *RAD9*, *UBC4*, *UBC5*, *RAD6*, *RAD18*, *MMS2*, *UBC13*, *RAD5*) in *SSL204* and *SSL612*, standard one-step PCR gene replacement was used³² and correct integration was confirmed by sequencing. Two independent colonies of each strain were isolated.

The *UCB4*-NES-3HA fragment containing a nuclear export sequence (NES)³³ and three hemagglutinin (HA) tags was generated by PCR. This was inserted into the pRS404 plasmid (a gift from D. Koepf) using *KpnI* and *SacI* restriction sites. The resulting integration plasmid was linearized using *BglIII* and transformed into two independent *cdc9-1 ubc5* strains. Correct integration was confirmed by sequencing and immunoblotting with an anti-HA (16B12, Covance) antibody.

For the *MRC1* complementation experiments, pAO138 and p*MRC1* (gifts from S. J. Elledge) were transformed into ABy287 (*CDC9 mrc1*⁻) and ABy293 (*cdc9-1 mrc1*⁻). pAO138 contains wild-type *MRC1* expressed from its endogenous promoter and p*MRC1* expresses a *mrc1* mutant that has 17 putative SQ or TQ phosphorylation sites switched to AQ (*mrc1*^{AQ} mutant) and is also expressed from its endogenous promoter¹⁶. pRS316 served as the empty vector control.

mrc1 rad9 cells were derived from a strain containing a high copy number plasmid expressing *RNR1* from its endogenous promoter (a gift from D. J. Clarke) to maintain cell viability.

PCNA lysine mutants were generated using plasmid YCplac22-*POL30* (a gift from S. Jentsch)³. YCplac22-*POL30* expresses full length PCNA from its endogenous promoter. Lysine to arginine substitutions in PCNA were introduced at positions 107, 108, 117, 168, 183, 253 by QuikChange mutagenesis (Stratagene). Four other lysine to arginine mutations (K127R, K164R, K127/164R, K242R) used in this study were a gift from the Jentsch laboratory³. As described previously³², the endogenous *POL30* gene was subsequently deleted by replacement with either a *URA3* or *LEU2* marker in *SSL204* and YKL83 strains, respectively.

To determine the cell viability of the double mutant *cdc9-1 pol30-K107R*, a plasmid expressing *POL30* from its endogenous promoter (*pRS316-POL30*, a gift from D. M. Livingston) was first transformed into *SSL204* (*CDC9*) and *cdc9-1*. PCNA lysine K107R mutant was generated using plasmid pCH1572 (a gift from L. Prakash). The resulting Leu2.PCNA-K107R PCR product was subsequently transformed into strains containing *pRS316-POL30* plasmid. PCNA-K107R mutation at the endogenous locus was confirmed by sequencing. Strains were streaked out onto SC-Ura plate and 5'-FOA (2 mg/ml) plates. All plates were incubated at 25°C for 2-3 days.

For immunoprecipitation of ubiquitinated PCNA, YEpl105 plasmid, expressing a synthetic Myc-tagged ubiquitin gene from a copper-inducible promoter (a gift from M.

Hochstrasser)²³ was transformed into SSL204 (*CDC9*), SSL612 (*cdc9-1*), SSL613 (*cdc9-2*). For the expression of ubiquitin mutants carrying lysine to arginine substitutions at positions 6, 11, 27, 29, 33, 48, 63, YEp105 was altered by QuikChange mutagenesis. The C-terminal glycine to arginine mutations in ubiquitin at positions 75 and 76 were constructed in YEp105 by QuikChange mutagenesis.

Yeast culture conditions

Temperature shift experiments were carried out in YPD, unless stated otherwise. For a typical temperature shift experiment, cells were grown to mid-log phase ($OD_{600} = 0.6$) at 25°C and then shifted to the restrictive temperature of 35°C for 3 hours or as indicated.

The degron strain, *cdc9-td*, was grown overnight at 28°C in YP plus 2% raffinose and supplemented with 10 μ M $CuSO_4$ to induce *CDC9* gene expression. Copper sulfate was omitted when cells were shifted to 37°C. To increase the efficiency of Cdc9-td degradation, Ubr1 was overexpressed from a galactose-inducible promoter in the presence of 2% galactose.

For all experiments in which PCNA or ubiquitin were expressed from a plasmid, two independent colonies of each strain were grown in synthetic complete medium lacking tryptophan. In ubiquitin overexpressing strains, ubiquitin was induced over the time period of three generations by addition of 0.1 mM $CuSO_4$ at a low cell density ($OD_{600} = 0.1$) as described earlier²³.

Co-Immunoprecipitation

Whole cell extracts from exponentially growing cells were prepared using glass beads as described earlier³⁴. A cocktail of freshly prepared protease inhibitors (pepstatin, leupeptin, benzamidine and phenylmethyl sulfonyl fluoride) and N-ethylmaleimide were used to preserve ubiquitinated proteins by inhibiting deubiquitinating enzymes²⁴. For immunoprecipitation, 4 μ g of anti-Myc antibody (9E11, Thermo scientific) was added to the extract for 2 hours at 4°C. PCNA was detected by Western blotting with an anti-PCNA antibody (clone S871, a gift from Z. Zhang)²².

Protein Preparation and Western Blot Analysis

Total protein extracts were prepared from cycling yeast cultures using TCA precipitation³⁵ (see Supplementary Information, Fig. S1b online) and proteins were detected by Western blot analysis. Cdc9-td-HA was detected with an anti-HA antibody (16B12, Covance), histone H3 was detected with an anti-histone H3 antibody (Abcam), endogenous Cdc9 was detected with an anti-Cdc9 antibody (gift from A. E. Tomkinson) and endogenous Rad53 was detected with an anti-Rad53 antibody (gift from J. F. X. Diffley). α -tubulin served as a loading control. In some experiments shown in the Supplementary Information, Ponceau S staining of the nitrocellulose membrane prior to blotting served as a loading control.

Detection of PCNA ubiquitination

Unmodified and mono-ubiquitinated PCNA were analyzed by Western blotting with an anti-yeast PCNA antibody (clone S871, a gift from Z. Zhang)²². To detect mono-ubiquitination

with the polyclonal S871 antibody, we diluted WCEs 10-fold. Poly-ubiquitinated PCNA was detected with the same antibody in undiluted, TCA-precipitated whole cell extracts. Please note that this antibody was not able to detect the 76 kDa poly-ubiquitinated form of PCNA in immunoprecipitated samples (Fig. 2b).

Cell Synchrony, FACS Analysis, and Microscopy

To arrest cells in G1, alpha-factor was added to a final concentration of either 50 ng/ml (*bar1* cells) or 15 µg/ml (*BARI* cells). Cells were blocked in S phase with the addition of 200 mM hydroxyurea (HU). For G2/M arrest, nocodazole was added to a final concentration of 10 µg/ml. Cell cycle progression was monitored using flow cytometry as described earlier³⁶. DNA was stained with propidium iodide in case of YKL83 strains, whereas Sytox Green was used for all other strains. All FACS samples were analyzed using a Becton Dickinson FACSCalibur.

MMS sensitivity

Cells were grown to mid-log phase ($OD_{600} = 0.5-0.6$) at 25°C for *cdc9* strains and at 28°C for YKL83 derived strains. Cells were then incubated with 0.02% MMS3 at 30°C for *cdc9-1* strains and at 37°C for YKL83 strains for the indicated time period. For YKL83 strains, 2% galactose was added for 30 minutes prior to shifting the cells to 37°C. After each time point, sodium thiosulfate (10%) was added to the treated cells to inactivate the MMS. Yeast cells were pelleted, washed with distilled water and total protein was TCA-precipitated.

Generation of stable shRNA expressing cell lines

U2OS cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂ (v/v). To generate stable DNA ligase I knockdown cell lines, DNA ligase I specific lentiviral shRNA plasmids and control shRNA were purchased from Open Biosystems. The sequences are shLIG#1 TGCTGTTGACAGTGAGCGCGCTTTACCTGCGAATACAAATAGTGAA GCCACAGATGTATTTGTATTTCGCAGGTGAAAGCTTGCCTACTGCCTCGGA shLIG#2 TGCTGTTGACAGTGAGCGACCTGTTTGTACCGGAAGCAAATAGTGAA GCCACAGATGTATTTGCTTCCGGTACAAACAGGCTGCCTACTGCCTCGGA. We also used a non-silencing control shRNA TGCTGTTGACAGTGAGCGATCTCGCTTG GCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCAAGCGAGA GTGCCTACTGCCTCGGA. These vectors have been described previously³⁷. Stable cell lines were constructed as described³⁸. Briefly, U2OS cells were transfected with 10 µg of shRNA plasmid (control or ligase I) with Fugene (Roche) per manufacturer's instructions. 48 h later cells were trypsinized and plated in media containing 2 µg/ml puromycin at different dilutions for colony formation. 12 days later drug-resistant colonies were picked and expanded and screened by Western blot for DNA ligase I expression.

Chromatin fractionation

To obtain the chromatin fraction, one 10 cm plate for each cell line was harvested and chromatin fractions were prepared exactly as described³⁹. Briefly, cells were harvested, extracted to release soluble proteins and nuclear (insoluble) proteins pelleted. The nuclear

pellet was then sonicated to release chromatin bound proteins. Proteins were fractionated on SDS polyacrylamide gels and analyzed by Western blots. Anti-DNA ligase I- (Santa Cruz), anti-histone H3- (Abcam), anti-PCNA- (Labvision), anti-ubiquitin- (Millipore), and anti-tubulin antibodies (Sigma) were used in this study.

Mammalian Cell Lysate and Western blotting

Cells were lysed with NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40 plus protease inhibitors) on ice and then rocked for 10 min at 4°C. Crude cell lysates were then centrifuged at 14,000 rpm for 10 min and cleared lysates were collected. Samples were boiled in 2X Laemmli buffer and fractionated on SDS-PAGE. Membranes were blocked in 5% milk-TBST and then probed with antibodies as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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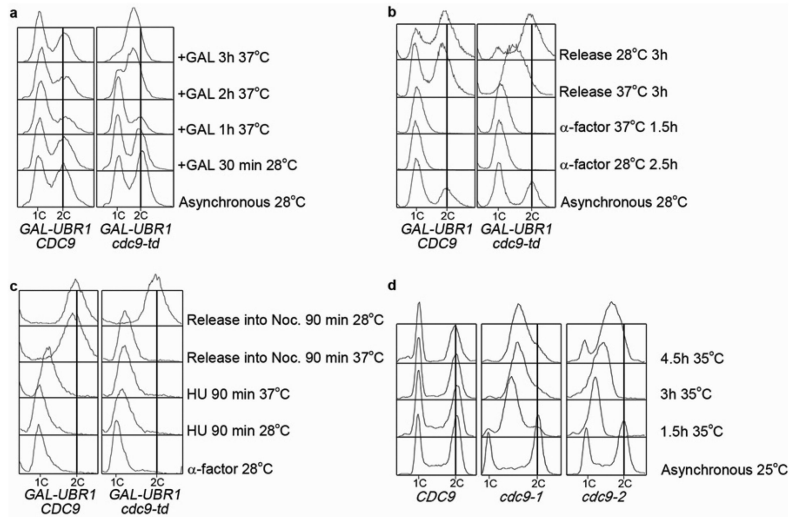


Figure 1. DNA ligase I is required for S phase progression. **(a)** Asynchronous cultures of ABy010 (*GAL-UBR1 CDC9*) and ABy008 (*GAL-UBR1 cdc9-td*) were induced with galactose at 28°C for 30 min and subsequently shifted to 37°C. **(b)** Strains were arrested in G1 phase at 28°C and shifted to 37°C in 2% galactose and α-factor. After 90 min, cells were released from G1 phase either at 37°C in galactose or at 28°C in glucose. **(c)** Strains were arrested in G1 and released into S phase at 28°C in the presence of HU. Once arrested in S phase, cultures were shifted to 37°C in the presence of 2% galactose and HU. After 90 min, cells were transferred into nocodazole either at 37°C or 28°C. **(d)** Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), and SSL613 (*cdc9-2*) were shifted to 35°C. In **a-d**, DNA content was monitored by flow cytometry and the vertical line indicates a 2C DNA content.

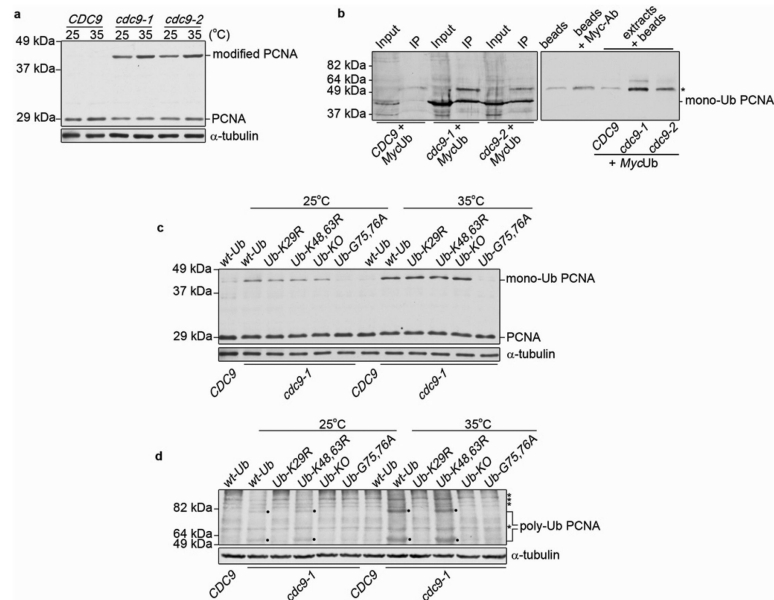


Figure 2.

S. cerevisiae PCNA is mono-ubiquitinated in *cdc9* mutants. **(a)** Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), and SSL613 (*cdc9-2*) were shifted to 35°C for 3 h. Total protein was TCA-precipitated, diluted and probed with a yeast specific PCNA antibody (S871). **(b)** Cells were shifted to 35°C for 3 h in the presence of copper sulfate to induce expression of Myc-tagged ubiquitin. Whole cell extracts were immunoprecipitated with an anti-Myc antibody and PCNA was detected with anti-PCNA antibody (S871). The right panel shows the beads control with or without the cell lysate. 5% of the input was loaded. **(c)** PCNA is mono-ubiquitinated in *cdc9* mutants. Wild-type or mutant ubiquitin expression was induced in asynchronously growing cultures. Total protein was TCA-precipitated and diluted. Unmodified and mono-ubiquitinated PCNA was detected by PCNA antibody (S871). **(d)** PCNA poly-ubiquitination is linked through lysine 29 in *cdc9* mutants. Undiluted TCA-precipitated protein samples from **c** were probed with anti-PCNA antibody (S871) to detect poly-ubiquitinated PCNA. In **b**, **d**, asterisks indicate non-specific bands. In **d**, filled circles (right side of the band) indicate poly-ubiquitinated forms of PCNA. In **a**, **c**, **d**, α -tubulin served as a loading control.

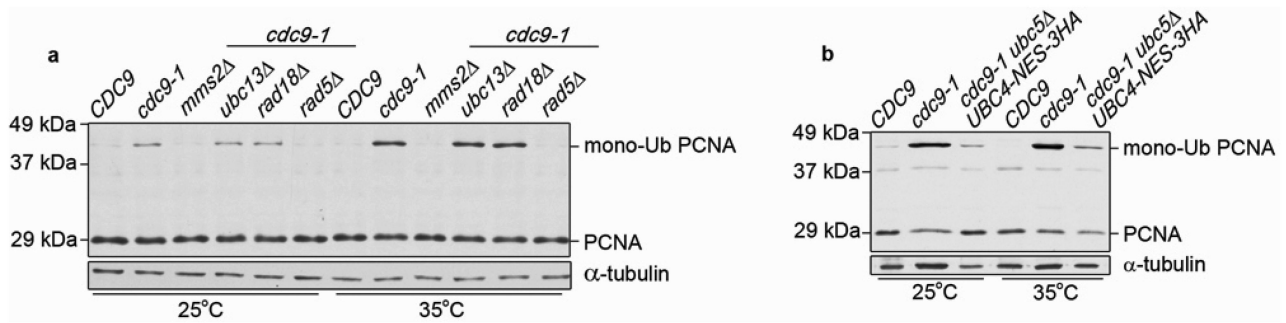


Figure 3.

PCNA mono-ubiquitination in *cdc9* mutants is mediated by Mms2, Rad5 and Ubc4 but not Ubc13. **(a)** Cultures were grown asynchronously and shifted to 35°C for 3 h. **(b)**

Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*) and ABy579 (*cdc9-1 ubc5 UBC4-NES-3HA*) were grown at 25°C and shifted to 35°C for 3 h. In **a-b**, unmodified and mono-ubiquitinated PCNA was detected using PCNA antibody (S871). α-tubulin served as a loading control.

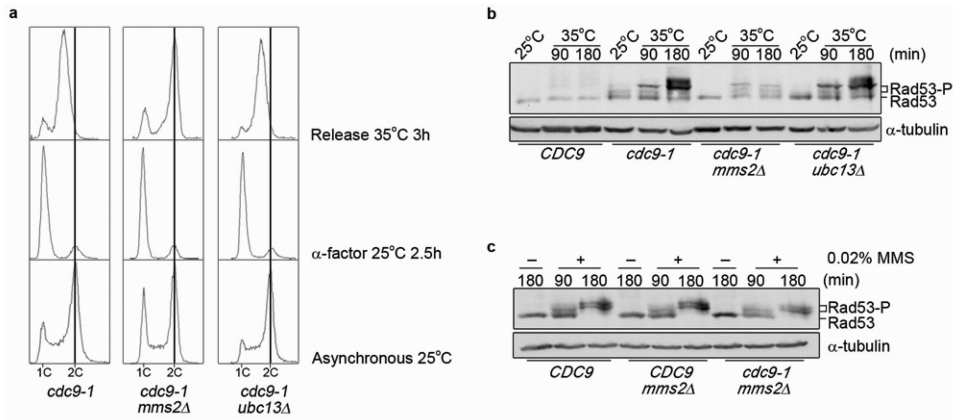


Figure 4. MMS2 but not UBC13 is required for S phase checkpoint activation in *cdc9* mutants. **(a)** Cells were arrested in G1 phase at 25°C and released at 35°C for 3 h. DNA content was monitored by flow cytometry. The vertical line indicates a 2C DNA content. **(b)** Cells were grown asynchronously at 25°C and then shifted to 35°C for the indicated time period. Total protein was TCA-precipitated and Rad53 was detected by an anti-Rad53 antibody. **(c)** Asynchronous cultures were split and shifted to the non-permissive temperature of 35°C in the presence of methyl methane sulfonate (MMS) for 90 and 180 min or left untreated for 180 min. Rad53 was detected as described in **b**. In **b-c**, α-tubulin served as a loading control.

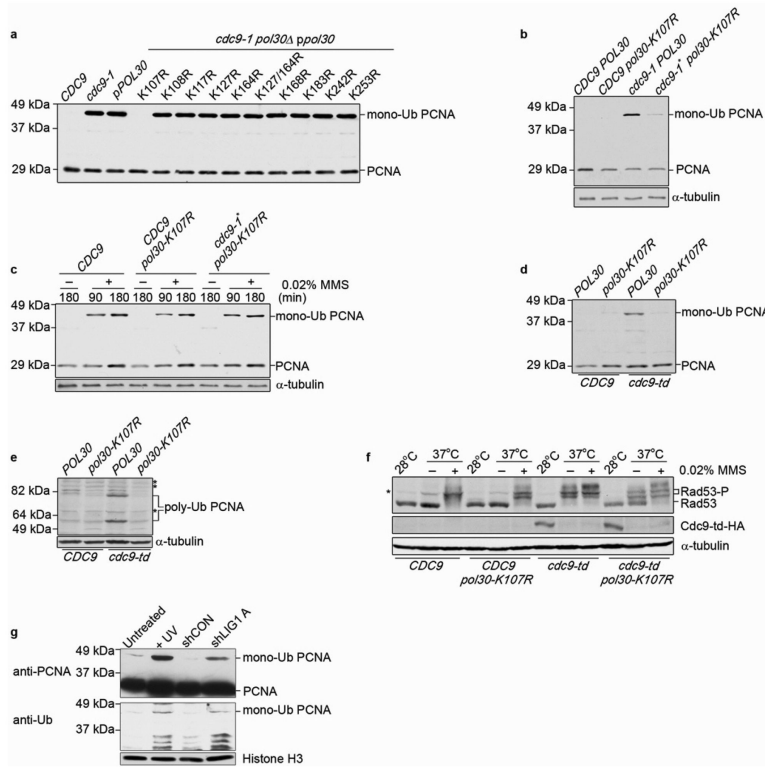


Figure 5. PCNA mono-ubiquitination occurs at lysine 107 in DNA ligase I mutants and is required for Rad53 activation. **(a)** PCNA lysine mutants in DNA ligase I deficient cells (*cdc9-1*) were grown asynchronously and then shifted to 35°C for 3 h. **(b)** Cultures of SSL204 (*CDC9*), ABy685 (*CDC9 pol30-K107R*), SSL612 (*cdc9-1*), ABy782 (*cdc9-1* pol30-K107R*) were grown asynchronously and then shifted to 30°C for 3 h. **(c)** Asynchronous cultures were grown at 25°C and shifted to 30°C in the presence of MMS for the indicated time. Cultures not treated with MMS served as negative controls. **(d)** Cultures were induced with galactose at 28°C for 30 min and subsequently shifted to 37°C for 3 h. In **a-d**, TCA-precipitated protein samples were diluted and PCNA was detected with anti-PCNA antibody (S871). **(e)** Undiluted TCA-precipitated samples from **d** were run on SDS-PAGE and poly-ubiquitinated PCNA was detected with anti-PCNA antibody (S871). **(f)** Cells were grown asynchronously at 28°C and then shifted to 37°C in the presence or absence of MMS for 3 h. Rad53 and Cdc9-tid-HA were detected with anti-Rad53 and anti-HA antibodies, respectively. In **b, c, e, f**, α -tubulin served as a loading control. In **e-f**, the asterisks indicate non-specific bands. **(g)** U2OS cells were either untreated or treated with 60 J/m² UV and harvested 2 h later along with control shRNA (shCON) and DNA Ligase I shRNA (shLIG1 A) cell lines. Chromatin fractions were prepared, fractionated on SDS-PAGE and analyzed with the indicated antibodies. Samples on the PCNA and ubiquitin blots were analyzed side by side. Unmodified PCNA served as a loading control for the PCNA blot and histone H3 served as a loading control for the ubiquitin blot.