Post-translational Regulation of DNA Polymerase η, a Connection to Damage-Induced Cohesion in Saccharomyces cerevisiae

Pei-Shang Wu,* Elin Enervald,*,1 Angelica Joelsson,* Carina Palmberg,† Dorothea Rutishauser,†,2 B. Martin Hällberg,* and Lena Ström*,3

*Department of Cell and Molecular Biology and †Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm SE-171 77, Sweden

ORCID IDs: 0000-0003-2303-103X (D.R.); 0000-0002-8898-3675 (L.S.)

ABSTRACT Double-strand breaks that are induced postreplication trigger establishment of damage-induced cohesion in Saccharomyces cerevisiae, locally at the break site and genome-wide on undamaged chromosomes. The translesion synthesis polymerase, polymerase η, is required for generation of damage-induced cohesion genome-wide. However, its precise role and regulation in this process is unclear. Here, we investigated the possibility that the cyclin-dependent kinase Cdc28 and the acetyltransferase Eco1 modulate polymerase η activity. Through in vitro phosphorylation and structure modeling, we showed that polymerase η is an attractive substrate for Cdc28. Mutation of the putative Cdc28-phosphorylation site Ser14 to Ala not only affected polymerase η protein level, but also prevented generation of damage-induced cohesion in vivo. We also demonstrated that Eco1 acetylated polymerase η in vitro. Certain nonacetylatable polymerase η mutants showed reduced protein level, deficient nuclear accumulation, and increased ultraviolet irradiation sensitivity. In addition, we found that both Eco1 and subunits of the cohesin network are required for cell survival after ultraviolet irradiation. Our findings support functionally important Cdc28-mediated phosphorylation, as well as post-translational modifications of multiple lysine residues that modulate polymerase η activity, and provide new insights into understanding the regulation of polymerase η for damage-induced cohesion.

KEYWORDS polymerase eta; cohesin; damage-induced cohesion; Cdc28/Cdk1; Eco1

DNA double-strand breaks (DSBs) can potentially be deleterious to cells since inefficiently repaired DSBs affect the physical integrity of chromosomes, and thereby genome stability. As part of the DSB response, cohesins are recruited to the break site (Kim et al. 2002; Ström et al. 2004; Unal et al. 2004), and de novo cohesion established locally at the DSB. This so-called DSB proximal damage-induced cohesion is instrumental for accurate DSB repair. We previously demonstrated that damage-induced cohesion, established genome-wide on undamaged chromosomes in Saccharomyces cerevisiae (Ström et al. 2004, 2007; Unal et al. 2007), depends on polymerase η (Polη) (Enervald et al. 2013). However, our knowledge on its regulation during this process remained sparse.

Polη (encoded by the RAD30 gene) is one of three known translesion synthesis (TLS) polymerases in S. cerevisiae and belongs to the Y-family of specialized DNA polymerases. TLS polymerases are characterized by an open active site, thereby having the capacity to catalyze nucleotide incorporation opposite bulky lesions that cannot be bypassed by high-fidelity replicative DNA polymerases (Waters et al. 2009). Polη is mainly known for incorporation of two adenine bases opposite cyclobutane pyrimidine dimers, the major type of DNA lesions induced by ultraviolet (UV) irradiation, in a principally error-free manner (Johnson et al. 1999b). Accordingly, impaired Polη function leads to severe UV sensitivity, and is the cause of
xeroderma pigmentosum variant syndrome with high incidence of skin cancer (Johnson et al. 1999a; Masutani et al. 1999).

Besides its canonical TLS activity, growing evidence suggest Polh TLS-independent functions (Acharya et al. 2019), such as DNA single-strand gap-filling for immunoglobulin somatic hypermutation, and extension of D-loops during DSB repair via homologous recombination (HR). Polh also has noncatalytic functions, such as recruiting human E3 ubiquitin ligase RAD18 to PCNA (Durando et al. 2013), and being important for the mentioned damage-induced cohesion in yeast (Enervald et al. 2013). Involvement of Polh in these multiple processes implied various interaction partners for distinct functions, and potential regulation through differential post-translational modifications.

In line with this, Polh activity is strictly regulated, presumably to avoid potential misincorporation of nucleotides due to its low fidelity on undamaged templates. Polh ubiquitination, for example, restricts its accessibility to replication forks (Cipolla et al. 2019). In addition, phosphorylation of human Polh is required for its localization to sites of UV-induced DNA lesions (Chen et al. 2008; Bertoletti et al. 2017; Peddu et al. 2018). These findings prompted us to investigate whether Polh would be regulated through post-translational modification(s) also for formation of damage-induced cohesion.

Cohesin is a highly conserved, multisubunit protein complex composed of the structural maintenance of chromosome proteins Smc1 and Smc3, the kleisin subunit Sec1 (or Mcd1), and the HEAT repeat protein Scc1. Two additional HEAT repeat proteins, Pds5 and Wpl1, are associated with the complex at substoichiometric levels (Nasmyth and Haering 2009). Cohesin complexes are loaded on DNA before replication onset through the action of the cohesin loader Scc2-Scc4 (Ciosk et al. 2000). The canonical function of cohesin is to generate sister chromatid cohesion during S phase, which is maintained until anaphase (Nasmyth and Haering 2009; Peters and Nishiyama 2012; Uhmann 2016). Eco1 is the acetyltransferase that determines establishment of sister chromatid cohesion via acetylation of the Smc3 subunit (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; Zhang et al. 2008), which has been suggested to counteract a cohesion antiestablishment activity performed by Wpl1 together with Pds5 and Sec3 (Gerlich et al. 2006; Chan et al. 2012; Lopez-Serra et al. 2013). Interestingly, Eco1 is targeted for degradation at the end of S phase through interdependent actions of the Cdc28, Cdc7, and Mck1 kinases. However, Cdc7 is inactivated in response to DSBs. Consequently, Eco1 is stabilized, thereby enabling formation of damage-induced cohesion even postreplication (Lyons et al. 2013). We previously reported that overexpression of Eco1 rescues the lack of damage-induced cohesion in Polh-deficient cells (Enervald et al. 2013). Therefore, we speculated that Eco1-mediated acetylation regulates Polh for their concerted action during establishment of damage-induced cohesion.

Since Polh protein level is regulated in a cell-cycle-dependent manner (Plachta et al. 2015; Bertoletti et al. 2017), Cdc28 appeared to be another potential regulator of Polh. Cdc28, also known as Cdk1, is the sole cyclin-dependent kinase (CDK) in S. cerevisiae. The temporal regulation of Cdc28 is controlled by association with cell cycle phase-specific cyclins. Thus, Cdc28 is largely inactive in G1 due to low levels of cyclins, while it is active from late G1 until anaphase. Cdc28/cyclin complexes regulate cell cycle progression through phosphorylation of targets involved in cellular processes, such as cell cycle checkpoints (Morgan 1997; Enserink and Kolodner 2010). Besides its essential role for cell cycle regulation, Cdc28 is important for DNA damage checkpoint activation and for DSB repair via HR (Ira et al. 2004). Cdc28 also genetically interacts with Eco1 and Scc1 (Heo et al. 1999; Brands and Skibbens 2008), besides regulating Eco1 stability, as described above.

Here we aimed at improving our understanding of Polh regulation for damage-induced cohesion genome-wide. We investigated the possibilities that Cdc28 and Eco1 act as modifiers of Polh in vitro, and examined the functional importance of identified modifications in vivo. Through in vitro phosphorylation and structure modeling, we showed that Polh is an attractive substrate for Cdc28. We also found that putative Cdc28 mediated Polh-S14 phosphorylation affects Polh protein level, and is important for generation of damage-induced cohesion in vivo. In addition, we demonstrated that Eco1 acetylated Polh in vitro. Furthermore, Eco1 is required for cell survival after UV irradiation but acts in parallel with Polh. Several nonacetylatable Polh mutants showed reduced protein level, deficient nuclear accumulation, and affected the TLS activity of Polh. Taken together, our findings show that Cdc28-mediated phosphorylation and post-translational modifications of lysines modulate Polh activity. Identification of Cdc28 as a potential regulator also provides new insight into understanding the regulation of Polh for damage-induced cohesion.

Materials and Methods

Yeast strains and growth conditions

All S. cerevisiae yeast strains were W303 derivatives (ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 RAD5 GAL ρsi*). Most strains were haploids, and a few diploids (listed in Supplemental Material, Table S1). Gene deletions were performed with one-step replacement, using the kanamycin (kanMX6)-, the hygromycin (hphMX4)-, or the nourseothricin (natMX4)-resistance marker. Transformations were performed with the lithium acetate method. Some strains were crossed to obtain desired genotypes. Yeast extract peptone (YPE) supplemented with 40 μg/ml adenine was used as yeast media.

Mutagenesis and plasmids

To generate Polh single-point mutants, the full-length RAD30 ORF was amplified from genomic DNA with primers where the 5’ ends were flanked with recognition sequences for the restriction enzymes HindIII and SalI. The amplified product
was then cloned into HindIII- and Sall-digested pAG25 (natMX4 marked) or pAG32 (hphMX4 marked) plasmids (Euroscarf). The cloned RAD30-natMX4 or RAD30-hphMX4 vector was used as template for PCR amplifications with Phusion high-fidelity DNA polymerase. Designed forward primers including desired point mutation annealed to the RAD30 internal sequence; while the reverse primer, with RAD30 downstream overhang, annealed after the plasmid selection marker. Amplified PCR fragments were transformed, and NAT+ or HPH+ candidates were analyzed with PCR to confirm correct integrations. Point mutations were verified by DNA sequencing (Toulmay and Schneiter 2006).

To generate Polh “in vivo” and “in vitro” multiple KR mutants, we designed RAD30 alleles containing selected multiple-point mutations with additions of HindIII and Sall recognition sequences at the 5’ and 3’ ends. Designed sequences together with the pAG32 vector were sent to Invitrogen GeneArt Gene synthesis (Thermo Fisher Scientific) for gene synthesis and subsequent cloning according to our design. These plasmids were used as PCR template. A forward primer, with RAD30 upstream overhang, was designed to anneal 14 bp away from the RAD30 start codon; and a reverse primer, with RAD30 downstream overhang, annealed to the plasmid after the hphMX4 cassette. Selection of candidates from transformations was performed as above.

**Spot assay**

Yeast cells cultured in YEPD (glucose, final 2%) were grown to midlog phase. Twenty thousand cells were pelleted according to OD600, resuspended in water and 10-fold serially diluted before spotting on assigned plates. Spotted cells were then exposed to indicated doses of ultraviolet C (UVC; 254 nm) and kept dark for 3 days at room temperature, or 2 days at 30° or 32°. Two technical repeats were included for each treatment and each spot assay was done at least twice.

**Damage-induced cohesion assay**

Cells harboring the smc1 temperature-sensitive (ts) allele (smc1-259) were grown to exponential phase at 23° in YEPR (raffinose, final 2%). Subsequently benomyl (final 80 μg/ml) was added for G2/M phase arrest. Galactose (final 2%) was then added for 1 hr to induce expression of the ectopic Pgal-SCMC1-MYC, and Pgal-HO to generate DSBs at the MAT locus on chromosome III. In response to break induction, both endogenous and ectopic Smc1 are used for damage-induced cohesion. Cohesion established during replication depends on the smc1-259 ts allele alone, and was inactivated by raising the temperature to 35°. The break induction was stopped by switching the media to YEPD with benomyl, and samples were collected up to 90 min at 35°. Collected samples were fixed for 15 min with 3.7% formaldehyde at 23°, pelleted, and resuspended in 100% ethanol. To monitor genome-wide damage-induced cohesion, we used the Tet-operators/Tet-repressor-GFP (“Tet-O/TetR-GFP”) system that utilizes a Tet-operator array inserted at the URA3 locus, 35 kb from the chromosome V centromere, which endogenously expressed GFP tagged Tet-repressors bind to. Separation of two fluorescent foci in a G2/M-arrested cell indicates separated sisters, i.e., deficient damage-induced cohesion. The experimental set up is illustrated in Figure 6A. Each mutant was tested at least twice and at least 200 cells were counted for each time point.

**Purification of GST-tagged recombinant proteins**

The RAD30 and ECO1 ORFs were amplified with PCR and integrated into the pGEX-4T-3 vector to generate glutathione S-transferase (GST)-tagged proteins with a thrombin site. Two times YT medium (yeast extract, tryptone, NaCl, and 0.3 mM isopropyl β-D-1-thiogalactopyranoside; Thermo Fisher Scientific) was used for expression of recombinant Polh in BL21 at 25° for 6–8 hr, or Eco1 in C41 (DE3) at 30° for 3 hr. GST-tagged proteins were purified on glutathione sepharose 4B resin (GE Healthcare), followed by thrombin (Sigma, St. Louis, MO) cleavage for 3 hr at 21°. Eluates were then concentrated through Vivaspin columns (Sartorius Steim), and buffer was changed to kinase buffer (20 mM Tris-Cl pH 7.5, 50 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 20% glycerol, 0.01% Triton X-100, and 2 mM DTT) for the *in vitro* kinase assay; or HAT buffer (50 mM Tris-Cl pH 8.0, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 5% glycerol; supplemented with 1 mM PMSF, 1 mM DTT, 10 mM sodium butyrate, and 5 μM TSA) for *in vitro* acetylation assay. Protein concentrations were measured with a Bradford assay.

**In vitro kinase assay**

Purified recombinant Polh-GST or Polh-3A-GST (0.8 μg) was co-incubated with the Clb2-HA/Cdc28 complex, immunoprecipitated from yeast whole-cell extracts (Lyons and Morgan 2011), in kinase buffer, with or without γ-32P-ATP for 15 or 30 min. The reaction was stopped by adding 4× SDS sample buffer. Recombinant human histone H1 (4 μg per reaction) was used as positive control (New England Biolabs, Beverly, MA). The samples were separated by SDS-PAGE and analyzed with autoradiography.

**In vitro acetylation assay**

Purified recombinant GST-tagged Polh (6 μg) and Eco1 (12 μg), were mixed with 1 μl of 14C-acetyl-coenzyme A ([14C-CoA; 60 mCi/mm01; PerkinElmer, Norwalk, CT] in HAT buffer (final 25 μl), and incubated for 4 hr at 35°. Samples were then boiled in SDS sample buffer (2×) and analyzed by Coomassie staining or autoradiography after separation on SDS-PAGE. Samples for mass spectrometry analysis were prepared as above, except using unlabeled acetyl-CoA (Roche). After gel purification, protein samples were analyzed by nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS).

**Purification of FLAG-tagged Polh to identify acetylation sites in vivo**

To purify FLAG-tagged Polh (Polh-FLAG), 4 liters of yeast cells cultured in YEPR were grown to OD600 1.8. Benomyl (final 80 μg/ml) was added and when cells reached G2/M,
two liters of cells were harvested as the “minus break” sample. Galactose (final 2%) was added to the remainder to induce DSBs by P<sub>GAL</sub>-HO on chromosome III, and chromosome VI with an ectopic integrated HO site. The remaining cells were collected as the “plus break” sample after 90 min. Cell pellets were then frozen with liquid nitrogen and stored at −80°C until preparation of whole-cell extracts. Thawed pellets were resuspended in lysis buffer (25 mM HEPES-NaOH pH 7.9, 400 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, protease inhibitor cocktail, and phosphatase inhibitor cocktail) and lysed in a 6870 freezer mill (SPEX, CertiPrep). Whole-cell extracts were cleared by centrifugation at 17,000 g for 30 min. The soluble fraction was incubated with equilibrated M2-FLAG affinity resin (Sigma) at 4°C, and rotated overnight to immunoprecipitate PolH-FLAG. The M2-FLAG affinity resin was then washed once with lysis buffer and PolH-FLAG was eluted through 3XFLAG peptide (Sigma) competition. Eluates were precipitated with TCA, resuspended in 8 M urea, and run on SDS-PAGE, followed by Coomassie staining before gel purification and nLC-MS/MS analysis.

**In-gel digestion of Coomassie-stained gel bands**

Tryptic digestion was performed by a liquid-handling robot (MultiProbe II; PerkinElmer), including protein reduction in 10 mM DTT and alkylation in 55 mM iodoacetamide. Gel pieces were dehydrated in 100% acetonitrile, and digested for 5 hr at 37°C with trypsin (13 ng/µl).

**Mass spectrometry analysis**

nLC-MS/MS analysis was performed using an Easy-nLC system (Thermo Fisher Scientific) directly coupled to a hybrid LTQ Orbitrap Velos ETD mass spectrometer (Thermo Fisher Scientific). Peptides were separated in a 10-cm fused silica tip column (New Objective, Inc.) that was in-house packed with 3 µm C18-AQ ReproSil-Pur (Dr. Maisch GmbH), using a linear gradient from 3% to 48% acetonitrile in 42 min with flow rate 300 nL/min. The two mass-spectrometry acquisition methods were both comprised of one survey full scan, ranging from m/z 300 to m/z 2000, acquired in the FT-orbitrap with a resolution of R equal to 60,000 at m/z 400, followed by either up to 10 data-dependent collision-induced dissociation MS2 scans in profile mode, or pairs of electron-transfer dissociation/higher-energy collisional dissociation scans of up to five precursor ions with charge state ≥2.

**Mass spectra database search**

Tandem mass spectra from the LTQ Orbitrap Velos were extracted using Raw2MGF (in-house software), and the resulting mascot generic files from each gel lane were searched by Mascot Daemon 2.3.0 search engine (Matrix Science Ltd.). The search engine was set to search the SwissProt protein database (selected for *S. cerevisiae*, version 2013.04), using trypsin and two missed cleavage sites. Peptide mass tolerance was set to 10 ppm, 0.25 Da for the collision-induced dissociation and electron-transfer dissociation fragment ions, and 0.05 Da for higher-energy collisional dissociation fragment ions. Cysteine carbamidomethylation was specified as a fixed modification, whereas methionine oxidation; asparagine and glutamine deamidation; lysine, serine, histidine, tyrosine, and threonine acetylations; as well as serine, threonine, and tyrosine phosphorylation were defined as variable modifications. Post-translational modifications were verified by calculating A-Score and localization probability using Scaffold PTM 1.1.3 (Proteome Software). Peptides with localization score ≥90%, or with Mascot scores >30 were further considered. For identification of Polh acetylation sites *in vivo*, only DSB-specific acetylations were selected.

**In situ immunofluorescence staining**

Wild-type (WT) and Polh mutants were overexpressed by integration of the GAL promoter upstream of respective ORF, except the PolH-S144A mutant, for which the constitutive strong GPD or ADH promoter was utilized. To compare nuclear accumulation with and without break induction, isogenic strains with or without the P<sub>GAL</sub>-HO allele were prepared. Cells were fixed with formaldehyde (final 3.7%) and spheroplasts were prepared through zymolyase treatment. The spheroplasts were subsequently applied on poly-L-lysine precoated slides, and fixed with ice-cold methanol followed by acetone, before blocking (3% BSA in 1× PBS). Cells were then stained with either anti-FLAG or anti-Myc primary antibody at 4°C overnight. After 10 washes with blocking solution, the slides were incubated in the dark for 1 hr with Alexa Fluor 555-conjugated anti-mouse secondary antibody (Invitrogen, Carlsbad, CA). Finally, the slides were washed 10 times with blocking solution, once with 1× PBS, and then mounted with ProLong Gold or ProLong Diamond including DAPI. An untagged strain and secondary antibody alone were included as controls. Image overlays were generated with the Openlab software (Improvision) or with ImageJ. The experiment was repeated at least twice and at least 200 cells of each sample from individual experiments were assessed.

**Flow cytometry**

To confirm G<sub>1</sub> or G<sub>2</sub>/M arrest, cells were pelleted and fixed with 70% ethanol (kept at 4°C overnight) before RNase treatment (final 240 µg/ml; in 50 mM Tris-HCl, pH 7.8 at 37°C overnight). Cells were then pelleted and resuspended in buffer containing 200 mM Tris-HCl, pH 7.5, 211 mM NaCl, and 78 mM MgCl<sub>2</sub> with propidium iodide (final 100 µg/ml). Cells were then sonicated before analysis with Becton Dickinson FACS Calibur, with 10,000 cell counts per sample.

**Pulsed-field gel electrophoresis**

To monitor break induction, samples were collected from damage-induced cohesion experiments before, and 1 hr after galactose addition. DNA plugs were prepared and run on 1% pulsed-field grade agarose gel with Bio-Rad CHEF-DR III as described (Desany *et al.* 1998). Initial switch time was set to
Protein extractions and Western blotting

Protein extracts were prepared through glass bead disruption, with buffer containing 20 mM Tris-Cl, pH 8, 10 mM MgCl₂, 5% glycerol, 0.3 M ammonium sulfate, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail tablet. Bolt 4%-12% Bis-Tris gels (Invitrogen) were used for electrophoresis and the Trans-blot Turbo system (Bio-Rad, Hercules, CA) for protein transfer. Membrane blocking and incubation with antibodies were performed with general procedures.

In silico modeling of Polh in complex with Cdc28

A homology model of S. cerevisiae Cdc28 was generated using SWISSMODEL (Waterhouse et al. 2018) from a cocrystal complex of human CDK2/Cyclin A and a target peptide (1QMZ; Brown et al. 1999). The target peptide in 1QMZ was then used as a reference to approximately orient the CDK consensus motif in a structure of DNA-bound Polh (2R8J; Alt et al. 2007) to have a canonical CDK-substrate interaction mode. The manual modeling was performed in COOT (Emsley and Cowtan 2004). The resulting draft model was thereafter energy minimized using PHENIX (Liebschner et al. 2019). The interface area was calculated as the difference in total accessible surface areas of isolated and interfacing structures divided by two as implemented in the PISA server (Krissinel and Henrick 2007).

Statistical analysis

One-way ANOVA, Scheffe post hoc test was used to assess statistical significance (α = 0.05 or 0.1); analyzed with SPSS statistics software (IBM). Error bars represent SD.

Data availability

All data and methods required to confirm the conclusions of this work are within the article, figures, and supplemental materials. Strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Primer sequences are available upon request. Supplemental material available at figshare: https://doi.org/10.25386/genetics.13055816.

Results

Polh is a substrate of the Cdc28 kinase in vitro

To better understand the regulation of Polh during its non-canonical, polymerase-independent role in formation of genome-wide damage-induced cohesion (Enervald et al. 2013), we sought to identify potential Polh regulators. By protein sequence, Polh appears to be a Cdc28 (Cdk1) substrate, containing two full and one partial CDK consensus motifs (Nigg 1993); where the S14, T612, and T547 residues would be the phosphorylation sites (Figure 1A). To test if Polh can be phosphorylated by Cdc28, we performed an in vitro kinase assay where a Polh-3A mutant, with all the three potential Cdc28 phosphorylation sites mutated to alanine, was compared with WT Polh. Cdc28 was purified through co-immunoprecipitation of the HA-tagged Clb2, the cyclin expressed during G2/M phase. In addition, recombinant human H1 was included as positive control (Lyons and Morgan 2011). The in vitro assay showed that the purified WT Polh was phosphorylated only in the presence of both the Cdc28/Clb2 complex and radiolabeled γ-32p-ATP. Importantly, phosphorylation of Polh was completely abolished when the three putative phosphorylation sites were mutated to alanine, implicating that one or more of these residues are the Cdc28 targets (Figure 1B).

Supporting the kinase assay result, we found when modeling a heterodimer constituted of S. cerevisiae Polh and a homology model of Cdc28, that Cdc28 and Polh could potentially form a complex with an interface area of ~1900 Å² (Figure 1C). The interface is located distant to substrate DNA and the polymerase active site. Furthermore, S14 on Polh could be modeled in position for phosphorylation, situated right by the γ-phosphate in the modeled Cdc28 kinase active site. In addition, the loop containing the basic P+3 residue in the phosphorylation motif, here K17, is relaxed in our minimized protein complex model, compared to the template structure of DNA-bound Polh (PDB: 2R8J; Alt et al. 2007). In this relaxed position, K17 is able to interact with the phospho-T169 in Cdc28 required for kinase activity (Cross et al. 2007). Potential interactions between Cdc28 and Polh-T547 or Polh-T612 could not be modeled since a complete structure for the Polh C terminus is not available (Powers et al. 2018). Taken together, our in vitro and modeling results indicate that Polh is a target for Cdc28 phosphorylation.

Preventing Polh-S14 phosphorylation affects protein level, but has no effect on Polh TLS activity

In both yeast and human cells, Polh protein level is cell cycle regulated, peaking in G2/M phase (Plachta et al. 2015; Bertoletti et al. 2017). Since phosphorylation by Cdc28 regulates protein stability (Hall et al. 2008), we asked whether Cdc28-mediated phosphorylation affects abundance of Polh in G2/M. We found that among the three individually mutated sites, only the Polh-S14A mutation caused reduced protein level in G2/M phase. Interestingly, such reduction was not observed when the other two putative phosphorylation sites were mutated simultaneously with S14 (Polh-3A) (Figure 2A). Furthermore, accompanied with reduced protein levels in the Polh-S14A single and Polh-S14A T612A double mutants, an additional band ~40 kDa was observed, possibly representing a Polh degradation or cleavage product (Figure S1, A and B; see also Discussion).

By affecting abundance of the full-length protein, S14A could potentially act as a null allele if the absolute Polh level is important. Furthermore, preventing phosphorylation of any of the potential phosphorylation residues could independently influence protein functionality. To test this, we first
examined if the single Polh-S14A, T547A or T612A mutations would render cells sensitive to UV irradiation. In contrast to the UV-sensitive Polh null mutant (rad30Δ), the Polh-phosphorylation mutants were as viable as WT cells (Figure 2B), indicating that the cells ability to efficiently bypass UV damages does not depend on phosphorylation of these residues. From this we conclude that Cdc28-mediated S14 phosphorylation is a potential mechanism for Polh stabilization in G2/M phase, but has no effect on Polh TLS function.

**Eco1, cohesin, and Polh are all required for cell survival after UV irradiation**

In addition to Cdc28, we speculated that Eco1, the acetyltransferase required for establishment of cohesion, could interact with or regulate Polh. This was based on several indications. First, Eco1 overexpression rescues cells void of Polh in formation of damage-induced cohesion (Enervald et al. 2013), suggesting a functional or genetic interaction. Second, it is noteworthy that the Eco1 ortholog in *Schizosaccharomyces pombe* (Eso1) is expressed as a fusion protein with Polh (Madril et al. 2001). As a start, we investigated whether Polh and Eco1 would interact genetically for Polh’s canonical role in bypassing UV-induced DNA lesions. Since the eco1Δ null mutant is lethal, and several identified eco1 ts alleles easily gain revertants, we chose to use an eco1D rad61D strain for the epistasis analysis. Deleting Wpl1 (encoded by the RAD61 gene), allows simultaneous Eco1 deletion (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; Rowland et al. 2009). As expected, the rad30Δ cells showed dose-dependent UV sensitivity (Figure 3, A–C). In contrast, deleting the RAD61 gene did not result in any obvious effect on cell survival after UV exposure, although the rad30Δ rad61Δ double mutant showed a subtle decrease in viability at 30 J/m² (Figure 3A). The eco1Δ rad61Δ mutant displayed UV sensitivity already at low-irradiation dose (12 J/m², Figure 3A). Notably, simultaneous RAD30 deletion greatly enhanced UV sensitivity of the eco1Δ rad61Δ mutant at high doses (24 and 30 J/m²), the doses where the effect of UV irradiation on the rad30Δ single mutant can be seen.

Furthermore, we analyzed the possible genetic interactions between Polh and the cohesin loader Scc2, as well as the cohesin subunits Scc1 and Smc1. Since these are all essential,
we used the respective ts alleles (Michaelis et al. 1997) for epistasis analyses. Restrictive temperatures, however, resulted in almost complete loss of viability following UV irradiation (Figure S2). Since genetic interactions were not possible to determine under such conditions, semipermissive temperatures were applied. As can be seen in Figure 3, B and C, scc2-4, scc1-73, and smc1-259 were all sensitive to UV irradiation even at a low dose (12 J/m²). Similar to the eco1Δrad61Δrad30Δ mutant, RAD30 deletion in these cohesin ts mutants resulted in additive effects on their UV sensitivities at higher doses (Figure 3, B and C). Loss of functional Eco1, or any of the cohesin subunits, together with loss of Polη activity, were in all cases additive. These results clearly show that the cohesin network is, like Polη, important for the response to UV damage, albeit acting in a different pathway from that of Polη.

**Polη is a substrate of Eco1 in vitro**

Although Polη and Eco1 appeared to act separately in response to UV irradiation, the possibility that Eco1 acetylates Polη for formation of damage-induced cohesion cannot be ruled out, since the latter does not depend on Polη as a TLS polymerase (Enervald et al. 2013). Thus, we continued with investigating whether Polη can be targeted for acetylation by Eco1 in vitro. To this end, we incubated purified Polη-GST alone or together with purified recombinant Eco1-GST, in the absence or presence of 14C-CoA. This indeed showed efficient acetylation of Polη by Eco1 in the presence of 14C-CoA (Figure 4A), and that Eco1 was autoacetylated as previously reported (Ivanov et al. 2002). To identify the Eco1-mediated Polη acetylation sites, we coupled the in vitro acetylation assay with mass spectrometry analysis. Again, Polη-GST was purified and incubated in vitro with and without recombinant Eco1-GST, as described but with unlabeled acetyl-CoA. Gel-purified Polη samples were then analyzed by mass spectrometry. The identified residues are depicted in Figure 4B in green, and further described below.

Since DNA damage has been suggested to trigger Eco1-mediated acetylation of PCNA (Billon et al. 2017), and the cohesin subunit Scc1 (Heidinger-Pauli et al. 2009), we were inspired to investigate if Polη was acetylated in vivo in response to DNA damage. WT Polη-FLAG was immunoprecipitated from whole-cell extracts, before and after DSB induction (PGAL::H2O) in G2/M-arrested cells, and subsequently analyzed by mass spectrometry to identify modified residues. The DSB-specific acetylations that appeared in vivo are depicted in Figure 4B, in blue. The complete mass spectrometry analysis results are shown in the Supplemental Material (Table S3, Files S1 and S2), and the selection criteria are described in the Materials and Methods.

The acetylation sites were widely distributed across the Polη coding sequence, in different functional domains such as the catalytic domain, the ubiquitin-binding/zinc-finger motif, and the putative bipartite nuclear localization sequence (NLS; 602–617 amino acids) at its C-terminal (Kannouche et al. 2001). The Eco1-mediated (in vitro) and the DSB-specific (in vivo) residues showed very limited overlap. This could suggest that the Eco1-mediated acetylations identified in vitro are not triggered by DNA DSBs. That the DSB-induced Polη acetylations, detected in vivo, were not identified as Eco1 targets in the in vitro acetylation assay could either mean that they are catalyzed by another acetyltransferase, or are Eco1 targets but require other factors and/or modifications present in vivo but not in vitro for acetylation.

**Polη-KR mutations affect Polη nuclear accumulation, protein level, and TLS activity**

To investigate if the Eco1-mediated (in vitro) and DSB-specific (in vivo) acetylation sites are biologically important, single lysine to arginine (KR) point mutants were generated according to the mass spectrometry results (depicted in Figure 4B). We then examined if these acetylation sites contributed to generation of damage-induced cohesion, but found that none of the tested single Polη-KR mutants were deficient (data not shown). We speculated that the effect of single Polη-KR mutations could be masked by compensatory acetylations, and therefore focused on Polη-multiple KR mutants. From the in vitro identified Eco1-mediated acetylation residues, the K17, K546, K603, and K615 residues were selected for further analyses, as they were close to the potential Ddc28-phosphorylation sites (S14, T547, and T612). This resembled Scc1-K84, next to the potential phosphorylation site Scc1-S83, which are both important for damage-induced cohesion (Heidinger-Pauli et al. 2009). Thus, we generated the Polη-K17R K546R K603R and Polη-K17R K546R K615R triple mutants, referred to as “Polη-triple KR” mutants. In addition, in an unbiased approach, we generated a “Polη-in vitro KR” allele based on the identified Eco1-mediated...
acetylations from the in vitro assay, and a “Pol η-in vivo KR” allele containing mutations of the identified DSB-specific acetylations (see Figure 4B, in green and blue, respectively).

Because the K603 and K615 residues are located within the putative NLS motif, and acetylated lysines within an NLS regulate nuclear localization (Cao et al. 2017), we decided to first examine nuclear accumulation of the Pol η-triple KR mutants by in situ immunofluorescence staining. Since the normal Pol η protein level was too low to generate a robust immunofluorescence signal (data not shown), we utilized the inducible GAL promoter for overexpression of endogenous FLAG-tagged WT and various Pol η KR mutants. This improved Pol η visualization and resulted in 70%–90% of cells scoring positive for α-FLAG staining (data not shown). As the Pol η-in vivo KR allele was generated based on modifications found after DSB induction, we were also interested in investigating possible DSB dependent nuclear accumulation. The cells were arrested in G2/M, in the presence of galactose for Pol η overexpression and DSB induction (in the strains harboring the P<sub>GAL</sub>-HO allele). Cells with distinct Pol η staining (anti-FLAG), clearly colocalizing with the DAPI signal (the nucleus) were defined as positive for nuclear accumulation. On the contrary, cells with a fuzzy, evenly distributed Pol η staining throughout the cells were scored negative (Figure 5A). Approximately 90% of the cells expressing WT Pol η displayed nuclear accumulation, with and without break induction. As suspected, the two Pol η-triple KR mutants and the Pol η-in vitro KR mutant showed either largely reduced or abolished nuclear accumulation, while that of the Pol η-in vivo KR mutant was not significantly affected (Figure 5B).

In parallel, we noticed that the Pol η steady-state level was affected by the two Pol η-triple KR and the Pol η-in vivo KR mutations (Figure 5C). In contrast, the Pol η-in vitro KR mutant appeared to be more stable than the two Pol η-triple KR mutants, despite containing the same mutations (K17R, K546R, K603R, and K615R), suggesting that some potential acetylation residues identified from the in vitro assay counteract others for Pol η stabilization.

Reduced protein levels of the two Pol η-triple KR mutants, and compromised nuclear accumulation of the Pol η-triple KR as well as the Pol η-in vitro KR mutants, could indicate that Eco1 is required for Pol η stability and nuclear localization. To test this, we monitored nuclear accumulation and protein level of WT Pol η in the absence of Eco1, in G2/M phase. However, Pol η nuclear accumulation and protein level were not significantly affected in neither the rad61Δ nor the eco1Δrad61Δ mutant (Figure 5, D and E). Instead, these results suggest that the disrupted NLS indeed compromise nuclear accumulation of the Pol η-triple KR and Pol η-in vitro KR mutants, independently of Eco1.

Figure 3 Eco1, cohesin, and Pol η are required for cell survival after UV irradiation. (A–C) Tenfold serial dilutions of midlog phase cells were spotted on YEPD, with or without follow-on UVC exposures. Semipermissive temperatures for the temperature-sensitive cohesin loader and cohesin subunits mutants were used: 30° for scc2-4 and scc1-73, and 32° for smc1-259. Plates were documented on the second or third day. One representative experiment from two independent assays performed is shown. RT, room temperature.
Besides, similar to the Pol\(\eta\)-phosphorylation mutants, we were interested in knowing if any of the lysines identified as acetylation targets were important for the canonical TLS activity of Pol\(\eta\). As expected, the Pol\(\eta\)-multiple KR mutants with affected nuclear accumulation showed increased UV sensitivity (Figure 5F). However, despite being proficient in nuclear accumulation, expression of Pol\(\eta\)-in vivo KR sensitized cells to UV irradiation (Figure 5, A, B, and F). To rule out the possibility that UV sensitivity of Pol\(\eta\)-in vivo KR was due to reduced protein level (Figure 5C), the constitutive strong ADH promoter was integrated upstream of the Pol\(\eta\)-in vivo KR coding sequence. Although the \(P_{ADH}\)Pol\(\eta\)-in vivo KR expression did not reach the level of WT Pol\(\eta\) controlled by the ADH promoter (\(P_{ADH}\)Pol\(\eta\)), it was markedly enhanced compared to its endogenous level (Figure 5G). We then noted that constitutively increased expression of WT or mutated Pol\(\eta\) did not perturb normal cell growth (Figure S3A; control). Elevated expression of the Pol\(\eta\)-in vivo KR allele under the ADH promoter clearly reduced, but did not fully rescue the UV sensitivity of this mutant (Figure S3A). This indicated that the UV sensitivity of the Pol\(\eta\)-in vivo KR mutant predominantly was due to reduced protein level, but partly also as a consequence of one or several of the mutated residues (K279, K287, K399, K436, K491). These results together indicate that post-translational modification of selected lysine residues contribute to proper nuclear accumulation of Pol\(\eta\), regulates its protein steady-state level, and is important for Pol\(\eta\) TLS activity.

Pol\(\eta\)-S14 is important for establishment of damage-induced cohesion

Having characterized the Pol\(\eta\)-SA/TA and Pol\(\eta\)-multiple KR mutants described above, we were ultimately interested in knowing if these post-translational modifications were important for formation of damage-induced cohesion. We monitored generation of damage-induced cohesion as previously described (Ström et al. 2004, 2007; see also Materials and Methods and Figure 6A). Briefly, strains of interest, with \textit{smc1-259} background, were arrested in G\(\_\)M. DSBs (\(P_{GAL^{+}}\text{HO}\)) were subsequently induced by addition of galactose, which simultaneously activated expression of ectopic \(P_{GAL^{+}}\text{SMC1-MYC}\). The TetO/TetR-GFP system was used for determination of sister separation. Proper G\(\_\)M arrest, efficiency of break induction, and protein expression of \(P_{GAL^{+}}\text{Smc1-Myc}\) were also confirmed. Examples of such controls can be found in Figure S4.

Since monitoring damage-induced cohesion includes a prolonged G\(\_\)M arrest, it was possible that the deficient damage-induced cohesion observed in \textit{rad30}\(\Delta\) cells reflected a cohesion maintenance defect. To exclude this possibility, before proceeding, we monitored sister chromatid cohesion maintenance in \textit{rad30}\(\Delta\) cells, and concluded that no defect in cohesion maintenance could be noticed in \textit{rad30}\(\Delta\) cells (Figure S5B). Thus, the deficient damage-induced cohesion in \textit{rad30}\(\Delta\) cells is not due to lack of cohesion maintenance.

We then choose to investigate if the Pol\(\eta\)-in vivo KR mutant could establish damage-induced cohesion, since these acetylations were identified as DSB specific, and that this mutated Pol\(\eta\) in principle was proficient in nuclear accumulation (Figure 4B and Figure 5, A and B). Because of reduced protein level of the Pol\(\eta\)-in vivo KR under control of its endogenous promoter, we again expressed it from the ADH promoter. Compared to WT Pol\(\eta\) (\(P_{ADH}\)Pol\(\eta\)), the \(P_{ADH}\)Pol\(\eta\)-in vivo KR mutant did not show deficiency in damage-induced cohesion, indicating that none of the K279, K287, K399, K436, or K491 residues are important for the same (Figure 6B).

Regarding the putative Cdc28-mediated phosphorylation of Pol\(\eta\), we first tested the abilities of Pol\(\eta\)-SA/TA single mutants for generation of damage-induced cohesion. We found that while the Pol\(\eta\)-TS47A and Pol\(\eta\)-T612A mutants were not deficient, the Pol\(\eta\)-S14A mutant showed similar degree of sister separation as the \textit{rad30}\(\Delta\) null mutant (Figure 6C). Despite our finding that reduced protein level of the Pol\(\eta\)-S14A mutant (Figure 2A) did not impede Pol\(\eta\) TLS activity (Figure 2B), the ability to establish damage-induced cohesion could potentially be more sensitive to reduced Pol\(\eta\) protein level. Thus, to directly test if the Pol\(\eta\)-S14 is required for damage-induced cohesion, regardless of the reduced protein level, we integrated the ADH promoter also in front of the Pol\(\eta\)-S14A coding sequence. The resulting protein level was similar to the WT Pol\(\eta\) under the ADH promoter (\(P_{ADH}\)Pol\(\eta\)) (Figure 6D); however, this was not sufficient for Pol\(\eta\)-S14A to
generate damage-induced cohesion (Figure 6E), indicating that the actual phosphorylation of Polh-S14 residue is indeed required for formation of damage-induced cohesion.

In summary, post-translational modifications of the Polh-in vivo residues (K279, K287, K399, K436, and K491) do regulate protein level and TLS activity of Polh, but are not influencing generation of damage-induced cohesion. In contrast, among the three putative Cdc28 phosphorylation sites, Polh-S14 is clearly required for formation of damage-induced cohesion. These findings depict discrete functions of the
identified Polh post-translational modifications, and provide new insights into regulation of Polh for generation of damage-induced cohesion.

Discussion

Similar to a previous study which identified Polh as a Cdc28 target through proteomic screening (Ubersax et al. 2003), we showed that Polh can be phosphorylated by Cdc28 in vitro. In addition, our structure modeling displayed a high degree of sterical complementarity, and indicated that the Polh-S14 residue is an attractive target for Cdc28. The putative binding mode features an interface area of 1900 Å², which is well above the average for heterodimers of validated protein-protein complexes (Nooren and Thornton 2003). This interface area can also be compared to the 1200 Å² for a Polh homodimer, and the 1600 Å² area for a human CDK2-Cyclin heterodimer (Brown et al. 1999; Alt et al. 2007).

Of the three mutated putative Cdc28 consensus residues, we found that S14 phosphorylation was required for formation of damage-induced cohesion, while Polh TLS activity was independent of this modification. We have so far not been able to directly detect Polh-S14 phosphorylation in vivo. We therefore wanted to investigate if Polh-S14 is a Cdc28 target in vivo through an alternative approach. Assuming that Cdc28 inactivation would cause deficient damage-induced cohesion, we planned to test if a Polh-S14 phospho-mimic allele would be able to rescue such defect. However, this could not be done since Cdc28 inactivation caused premature loss of S phase established cohesion (data not shown), which was also in agreement with previous reports (Kitazono et al. 2003; Brands and Skibbens 2008).

Our results suggest that the Cdc28-mediated phosphorylation, which should be cell cycle regulated, could be important for the reported increased Polh abundance in G2/M, due to stabilization (Plachta et al. 2015; Bertoletti et al. 2017). In addition, we observed a complex pattern of Polh cleavage or degradation, where the cleavage product was observed in Polh-S14A single and Polh-S14A T612A double mutants, but not in the Polh-T547A and Polh-T612A single mutants and the Polh-3A mutants. These results suggest that serial phosphorylation could act in concert to promote or prevent...
PoLy degradation. We suspect that phosphorylation of PoLy-T547 could be a signal for PoLy cleavage, which is antagonized by PoLy-S14 phosphorylation (summarized in Figure S1C). Future investigations will be needed to test this, and to understand the temporal and spatial regulation of possible PoLy cleavage.

In line with our finding that Cdc28-dependent phosphorylation was important for PoLy function in response to damage, phosphorylation of human PoLy after UV irradiation is required for localization to sites of lesions, and contributes to cell survival after UV exposure (Chen et al. 2008; Bertoletti et al. 2017; Peddu et al. 2018). As opposed to the human counterpart, however, nuclear accumulation of yeast PoLy seems to be independent of the DNA damage response, since WT PoLy and the PoLy-S14A mutant both accumulated in the nucleus in the absence of break induction, and were insensitive to UV irradiation (Figures 2B and 5B and Figure S3).

When investigating a possible genetic interaction between PoLy and proteins in the cohesin network, we found that cohesin mutants were highly sensitive to UV irradiation. In line with this, cohesin suppresses interhomolog recombination after UV exposure in budding yeast (Covo et al. 2010). In addition, fission yeast Rad21 (Scc1) is cleaved by separase after UV exposure, while noncleavable Rad21 results in slower repair of UV-induced thymine dimers (Nagao et al. 2004). These studies, together with our data, suggest that cohesin is indeed important for repair of UV-induced DNA damages. The additive sensitivity of eco1Δrad61Δ cells and cohesin ts mutants in combination with rad30Δ, further suggests that not only cohesin, but also sister chromatid cohesion is required for repair of UV-induced lesions.

We showed that PoLy is an Evo1 substrate in vitro, and identified putative PoLy acetylation sites in vitro and in vivo for the first time. However, nuclear accumulation deficiency and reduced protein level observed in several PoLy-multiple KR mutants turned out to be independent of Evo1-mediated acetylation. Although we cannot rule out the possibility that these lysine residues are substrates of alternative post-translational modifications, such as sumoylation or ubiquitination, identification of the authentic enzyme actually modifying these PoLy residues for protein level and UV-repair competence could be an interesting future topic.

Genome-wide damage-induced cohesion on undamaged chromosomes might be argued to be a unique biological process in haploid budding yeast cells. However, studies in Arabidopsis thaliana and chicken DT40 cells propose that generation of damage-induced cohesion could be conserved [reviewed in Dorsett and Strom (2012)]. In addition, in HeLa cells, cohesin binding is reinforced in an ESCo1 (human Evo1)-dependent manner in response to ionizing radiation. This suggests that reactivation of cohesion establishment could also occur in mammalian cells (Kim et al. 2010). This concept is also supported by our observation that diploid yeast cells, which resemble higher eukaryotes with the presence of homologs, do generate damage-induced cohesion (data not shown).

In conclusion, our investigations of PoLy-KR mutants provide better understanding of the PoLy regulation for nuclear accumulation, protein level, and TLS activity. Most importantly, we have revealed that PoLy can be a Cdc28 substrate. PoLy-S14 phosphorylation not only regulates PoLy protein level, but also contributes to formation of damage-induced cohesion. Further studies on PoLy-S14 phosphorylation should in the future improve our understanding about how PoLy contributes to establishment of damage-induced cohesion.

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