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Understanding the role and regulation of Polη for damage-induced cohesion

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Understanding the role and regulation of Polη for damage-induced cohesion

THESIS FOR DOCTORAL DEGREE (Ph. D.)

By

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ABSTRACT

In response to DSBs, *de novo* sister chromatid cohesion can be established after DNA replication in *Saccharomyces cerevisiae*. The damage-induced cohesion is formed close to the break and genome-wide on undamaged chromosomes. Polymerase eta (Polη), which bypasses ultraviolet-induced DNA lesions, is specifically required for genome-wide damage-induced cohesion. However, its role in this process is unclear. The works in this thesis aimed to understand the mechanistic role of Polη for damage-induced cohesion, and how damage-induced cohesion is generated genome-wide. In the first study, the possibility that Cdc28 and Eco1 modulate activity of Polη was being investigated. Based on *in vitro* kinase assay and structure modeling, Polη appeared to be an attractive substrate for the cyclin-dependent kinase, Cdc28. Abolishing the potential Polη-S14-phosphorylation by a serine to alanine mutation resulted in reduced protein level of Polη and impaired damage-induced cohesion *in vivo*. Although Polη was acetylated by the acetyltransferase Eco1 *in vitro*, absence of Eco1 did not affect protein level or nuclear accumulation of Polη *in vivo*. This contrasted with certain non-acetylatable *Polη-KR* mutants, implicating that other factor could regulate Polη through modifying these lysine residues. In the second study, it was hypothesized that transcriptional activation/regulation facilitates generation of damage-induced cohesion, based on related findings in budding yeast and fission yeast. Absence of Polη or prevention of Polη-S14-phosphorylation perturbed transcription elongation because the mutants were sensitive to transcription elongation inhibitors and showed reduction of RNA polymerase II-binding on chromatin. Therefore, mutants defective in histone exchange or regulation of transcription elongation were created to mimic or suppress the transcriptional deficiency of the Polη null mutant (*rad30Δ*). Interestingly, these mutants mimicked or suppressed the lack of damage-induced cohesion in *rad30Δ* cells. Furthermore, establishment of damage-induced cohesion was compromised by transcription inhibition. Taken together, these studies showed that Polη is potentially phosphorylated by Cdc28 at the S14 residue, which appeared to be functionally related with transcription and formation of damage-induced cohesion. In addition, persistent absence of Polη affects transcriptional regulation. This consequently impaired formation of damage-induced cohesion, implicating a linkage between transcriptional regulation and establishment of genome-wide damage-induced cohesion after DNA replication.

LIST OF SCIENTIFIC PAPERS

I. Post-translational regulation of DNA polymerase η, a connection to damage-induced cohesion in *Saccharomyces cerevisiae*
Pei-Shang Wu, Elin Enervald, Angelica Joelsson, Carina Palmberg, Dorothea Rutishauser, B. Martin Hällberg, and Lena Ström
Genetics. 2020 Dec; 216(4):1009-1022

II. Deficiency of Polη in *Saccharomyces cerevisiae* reveals the impact of transcription on damage-induced cohesion
Pei-Shang Wu, Jan Grosser[¶], Donald P. Cameron[¶], Laura Baranello, and Lena Ström
PLOS Genetics. 2021 Sep; 17(9):e1009763

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LIST OF ABBREVIATIONS

APC/C	anaphase-promoting complex/cyclosome
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CKIs	CDK inhibitors
CPDs	cyclobutane pyrimidine dimers
DAPI	4′,6-diamidino-2-phenylindole
DDR	DNA damage response
DSBs	double strand breaks
FACT complex	facilitates chromatin transactions complex
FN	fragile nucleosome
GFP	green fluorescent protein
GRFs	general regulatory factors
GSEA	gene set enrichment analysis
HIR complex	histone regulatory complex
HO	homothallic switching endonuclease
HR	homologous recombination
MNase	micrococcal nuclease
MPA	mycophenolic acid
NHEJ	non-homologous end-joining
nLC-MS/MS	nano-liquid chromatography coupled tandem mass spectrometry
NLS	nuclear localization sequence
NuA4 complex	nucleosomal acetyltransferase of histone H4 complex
PCNA	proliferating cell nuclear antigen
PFGE	pulsed field gel electrophoresis
PIKKs	phosphatidylinositol 3-kinase-like kinases
PIP	PCNA-interacting protein

PTMs	post-translational modifications
qPCR	quantitative polymerase chain reaction
RNAPII	RNA polymerase II
Rpd3S	Rpb3-Sin3 small (histone deacetylase complex)
SAGA complex	Spt-Ada-Gcn5 acetyltransferase complex
SMC	structural maintenance of chromosomes
SUMO	small ubiquitin-like modifier
SWR1	Swi2/Snf2-related 1
TAD	topological associating domain
TCA	trichloroacetic acid
TetOs/TetR	tetracycline operators/tetracycline repressor
TLS	translesion synthesis
UBZ	ubiquitin-binding/zinc-finger
UV	ultraviolet
XP-V	xeroderma pigmentosum-variant

1 INTRODUCTION

Cohesin is one of the structural maintenance of chromosomes (SMC) complexes. Cohesin tethers sister chromatids together during DNA replication, thereby establishing sister chromatid cohesion. This ensures faithful chromosome segregation at anaphase onset. In *Saccharomyces cerevisiae* (budding yeast), site-specific double strand breaks (DSBs) can trigger *de novo* establishment of sister chromatid cohesion after DNA replication. The damage-induced cohesion is generated both close to the breaks, and globally on undamaged chromosomes. The break-proximal cohesion is known to facilitate DNA repair, while importance of the genome-wide damage-induced cohesion is unclear. Interestingly, polymerase eta (Polη) - a translesion synthesis polymerase that bypasses ultraviolet-induced DNA damage - is specifically required for genome-wide damage-induced cohesion. This was demonstrated in budding yeast that the Polη null mutant (*rad30Δ*) was deficient in establishment of genome-wide damage-induced cohesion after DSBs induction during G_2/M. However, the role of Polη for this biological process remains unknown.

Aims of studies

Based on previous findings, the investigations in this thesis aimed to understand the mechanistic role of Polη for damage-induced cohesion, and how damage-induced cohesion is established genome-wide. These aims were firstly addressed by identifying potential regulators of Polη (paper I), followed by studying the non-canonical role of Polη in transcription since it was hypothesized that transcriptional regulation could be relevant to formation of genome-wide damage-induced cohesion (paper II).

2 LITERATURE REVIEW

2.1 DNA DOUBLE STRAND BREAK (DSB) AND DNA DAMAGE RESPONSE (DDR)

Genome integrity can be challenged by both endogenous and exogenous DNA damages. DSB is one of the deleterious DNA damages as unrepaired DSBs can lead to loss of genetic information, chromosome rearrangements and/or cell death. DSBs can spontaneously occur during DNA replication or externally induced by DNA damaging agent and irradiation. However, formation of DSBs can also be part of programmed cellular processes, such as promotion of homologous recombination between homologs during meiosis or induction of immunoglobulin class-switching [1].

DNA damage is under surveillance of cell cycle- or DNA damage-checkpoint, which is regulated by the phosphatidylinositol 3-kinase-like kinases (PIKKs). The DDR-related PIKKs include ATM, ATR and DNA-PKcs in higher eukaryotes [2]. These correspond to Tel1 and Mec1 in budding yeast, while the DNA-PKcs counterpart is absent [3]. ATM and DNA-PKcs are recruited to the DSB region, depends on NBS1 (subunit of the MRN complex) and Ku80 respectively [4,5]. In contrast, ATR is recruited to the RPA-coated single stranded DNA region through ATRIP [6]. The recruited and activated PIKKs in turn transmit the DNA damage signal to the downstream mediators and effectors through a cascade of phosphorylation (summarized in Table 1) [7]. As a result of checkpoint activation, cell cycle is delayed or arrested. This allows possibility for DNA repair to prevent genetic alterations and/or cell death.

Table 1. Functional orthologs of DDR factors in budding yeast and human cells

	S. cerevisiae	*H. sapiens*
9-1-1 checkpoint clamp (early DSB sensor)	Ddc1–Rad17–Mec3	RAD9–RAD1–HUS1
9-1-1 checkpoint clamp loader	Rad24–RFC	RAD17–RFC
DSB signaling; activator of Tel1 (ATM)	Mre11–Rad50–Xrs2	MRE11–RAD50–NBS1
Checkpoint kinase	Tel1	ATM
Checkpoint kinase	Mec1	ATR
Mec1 (ATR)-interacting protein	Ddc2	ATRIP
Mec1 (ATR)-activator	Dpb11	TopBP1
Mediator	Mrc1	Claspin
Mediator	Rad9	53BP1
Effector	Rad53	CHK2
Effector	Chk1	CHK1

2.1.1 DSB repair

The two main pathways for repairing DSBs are homologous recombination (HR) and non-homologous end-joining (NHEJ). The HR pathway utilizes sister chromatid, homologous chromosome or other homologous sequence presents on another chromosomal region as template for repair. In contrast, the NHEJ pathway ligates two broken DNA ends directly. The choice of DSB repair pathway on the one hand is determined by initiation of end resection, which is mediated by the MRN complex (Mre11-Rad50-Xrs2 in budding yeast; Mre11-Rad50-Nbs1 in mammals). On the other hand, it depends on cell cycle stage for availability of the homologous sequence [8].

In response to a DSB, the MRN complex and the Ku70/80 heterodimer are rapidly recruited to the DSB region. The Ku70/80 heterodimer is functionally related to the NHEJ pathway. As an initial step required for both HR and NHEJ pathways, the DSB ends are tethered by the ATPase domain of Rad50 (subunit of the MRN complex) [9]. Rad50 subsequently triggers conformational changes of the MRN complex by ATP-hydrolysis, and thus the active site of Mre11 can have access to the DNA substrate [10]. The nuclease activity of Mre11 and the subsequent DSB end resection are either induced or promoted by Cdc28-dependent phosphorylation during S and G2 phases, as further described in section 2.4. Since DSB end resection is the initial step of HR, DSB repair is directed to the HR pathway in S and G2 phase. Conversely, due to limited Cdc28 activity and lack of a homologous sequence in G1 phase, NHEJ is the favored pathway [1].

2.1.2 Additional roles of ATM and ATR in relation to DSB repair

In higher eukaryotes, the histone variant H2AX is phosphorylated by ATM in response to DSB, resulting in propagation of γ-H2AX around the DSB region [11]. With the absence of H2AX in budding yeast, the Tel1/Mec1-mediated H2AS129-phosphorylation is referred to as γ-H2A [12]. The γ-H2AX (γ-H2A) can be a platform recruiting DNA repair factors and regulating the chromatin structure around a DSB. For instance, the γ-H2AX signal is recognized by the scaffold protein MDC1, which recruits the RING ubiquitin ligase RNF8 to the DSB region in human cells. The RNF8/RNF168-mediated ubiquitylation cascade subsequently recruits the DNA repair proteins BRCA1 and 53BP1 to the damaged site [13]. In budding yeast, γ-H2A either directly or indirectly recruits the chromatin remodeling complexes SWI/SNF, SWR1 and INO80. Activity of these ATP-dependent chromatin remodelers results in relaxation of the chromatin structure around the DSB, thereby facilitating accessibility for DNA repair factors [14,15]. Besides, an unrepairable chromosome can be relocated to the nuclear periphery in a Mec1-dependent manner [16,17]. Tel1 and Mec1 are also required for cohesin enrichment around DSBs and formation of damage-induced cohesion. This is further described in section 2.3.

2.2 TRANSLESION SYNTHESIS (TLS) POLYMERASES

TLS polymerases refer to DNA polymerases that can use damaged DNA as template, thereby bypassing lesions that may cause stalling of replication forks. Most of the TLS polymerases belong to the Y family DNA polymerases, which have certain structural domains optimized for bypassing DNA lesions. This includes Rev1 and polymerase eta (Polη) in budding yeast [18]. Polymerase zeta (Polζ) is another TLS polymerase in budding yeast, which contains a Rev3 subunit that exceptionally belongs to the B family replicative DNA polymerases, but lack a functional proofreading domain [19,20].

TLS polymerases share most of the structural features with replicative DNA polymerases, with some exceptions (Figure 1). Similar to the replicative DNA polymerases, TLS polymerases use the thumb and finger domains to contact the DNA substrate and the incoming nucleotide respectively. The catalytic residues of TLS polymerases are located in the central palm region and they are required for coordinating the divalent magnesium ions to stabilize incoming dNTPs. However, TLS polymerases have reduced processivity and fidelity on undamaged DNA compared to the replicative DNA polymerases. This is because the relative short thumb and finger domains can only make limited contact with DNA, in addition to the lack of a 3'-to-5' proofreading domain in TLS polymerases [21-23]. Despite that, these polymerases appear to be lesion-specific and bypass the lesion accurately. Such DNA lesion specificity was suggested to depend on their relatively open active site, and the unique little finger domain (also called polymerase-associated domain; PAD) that contacts the lesion-containing region [24,25].

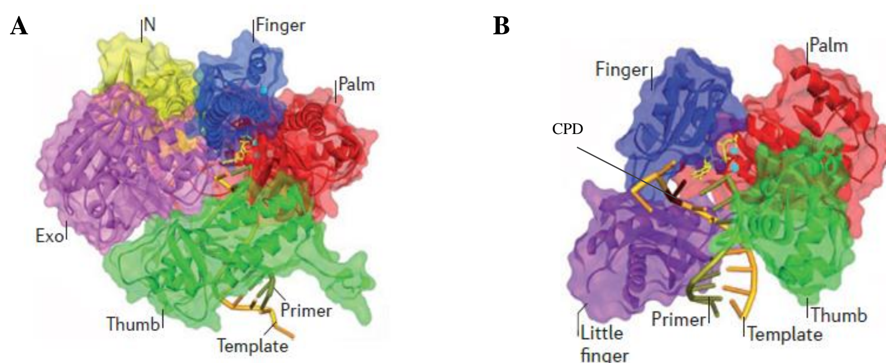

Figure 1. Structure of the B family and Y family DNA polymerases

(A) Structure of the B family DNA polymerase from bacteriophage [26], which is shown as a comparison to the Y family DNA polymerase. Exo, exonuclease; N, N-terminal domain. (B) Structure of the human Polη with a cyclobutane pyrimidine dimer (CPD). Blue spheres in (A) and (B) indicate the metal ions. This figure is adapted from [27].

2.2.1 Models of translesion synthesis

Based on genetic and biochemical studies, two models have been proposed for the action of TLS polymerases to bypass DNA lesions. This includes the polymerase-switching model and the gap-filling model, which are not mutually exclusive [28,29].

The polymerase-switching model suggests a switch between the replicative DNA polymerase and a TLS polymerase during ongoing replication (Figure 2A; Polη is illustrated as an example). In response to a DNA lesion that blocks DNA replication, the Rad18 ubiquitin ligase and the Rad6 ubiquitin-conjugating enzyme consecutively localize to the single-stranded region of the stalled replication fork [30]. The TLS polymerases are then recruited to the site of DNA damage, possibly as a result of Rad6-Rad18 mediated mono-ubiquitination of proliferating cell nuclear antigen (PCNA) [31]. It was proposed that the polymerases are switched to bypass the DNA lesion [32,33], followed by de-ubiquitination of PCNA [34] or destabilization of the TLS polymerase/DNA complex [35] to change the TLS polymerase back to the replicative DNA polymerase. The bypassed lesion will be removed by a suitable DNA repair pathway when the stalled replication fork is resumed.

In contrast to the polymerase switching model, the gap-filling model describes TLS within single-stranded gaps that contain DNA lesions after S phase (Figure 2B; Polη is depicted as an example). These gaps may arise from DNA replication that restarted downstream of a blocking lesion [36,37]. TLS polymerases could be recruited to the gaps by PCNA, the alternative clamp 9-1-1 or Rev1 to bypass the lesion [38,39]. The DNA nicks left after lesion bypass are sealed by DNA ligases, and the bypassed damages will be removed in the subsequent S phase.

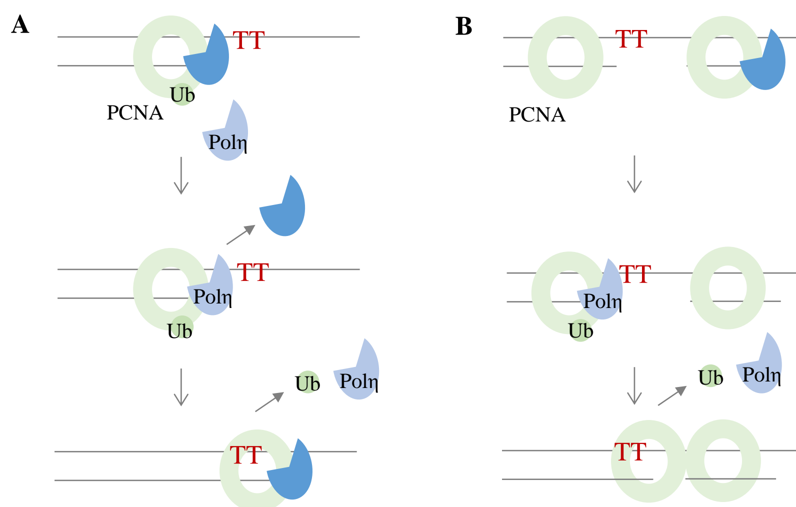

Figure 2. Models of TLS

(A) Polymerase switching model. Polη is recruited to the site of lesion by mono-ubiquitinated PCNA. The replicative DNA polymerase, as shown in dark blue, is temporarily switched to Polη for bypassing the lesion. (B) Gap-filling model. Presence of a single-stranded DNA gap as replication restarted downstream of the DNA lesion. The lesion-containing region is bypassed by Polη, leaving DNA nicks that will be sealed by DNA ligases. Noted that Polη is illustrated here as an example of the TLS polymerases. TT represents the UV-induced cyclobutane pyrimidine dimer, the DNA lesion recognized by Polη.

2.2.2 Polη in TLS

Polη is well characterized for bypassing *cis-syn* cyclobutane pyrimidine dimers (CPDs), the bulky DNA adducts induced by ultraviolet (UV) irradiation [40-42]. In addition to CPDs, Polη can also bypass a broad range of DNA lesions such as those induced by reactive oxygen species [43] and DNA cross-linking agents [44]. In contrast to its ability to bypass various DNA lesions, Polη appeared to have the lowest fidelity on undamaged DNA compared to other TLS polymerases *in vitro* [45]. Consistently, accessibility of Polη to undamaged chromosomes is regulated in human cells (described in section 2.2.4).

2.2.3 Non-canonical roles of Polη

According to epistatic analyses in budding yeast, the *RAD30* gene (encoding Polη) belongs to the *RAD6* epistasis group because *rad6* and *rad18* are epistatic to *rad30*. As described in section 2.2.1, Rad6-Rad18 mediate mono-ubiquitination of PCNA, which was proposed to recruit the TLS polymerases. And yet, there is no epistatic relationship between *rad30* and *rad5*, *rev1*, *rev3/7* (subunits of Polζ), implicating that Polη could have independent functions besides the error-free pathway defined by *RAD5* or the error-prone TLS pathway [46]. Recent studies showed that Polη indeed is involved in other pathways, both in lower and higher eukaryotes. For instance, Polη in vertebrates contributes to variety of immunoglobins by generating mutations in the variable region of immunoglobulin genes [47,48]. Human Polη suppresses common fragile site instability by preventing cells with under-replicated DNA from entering mitosis [49]. Additionally, human Polη is implicated in homologous recombination [50,51] and alternative lengthening of telomeres [52]. In yeast, Polη is required for genome-wide damage-induced cohesion that formed after DSB-induction during G_2/M [53] and potentially plays role in transcription [54]. These non-canonical roles of Polη in yeast are further described in the 'Damage-induced cohesion' section (2.3.6); investigated and discussed in papers I and II.

2.2.4 Regulation of Polη

Activity of Polη can be regulated by post-translational modifications (PTMs), protein-protein interactions, steady-state protein level and depends on the cell cycle phase. These regulations, however, may vary among species.

Accessibility of Polη to replication forks is partly regulated by protein-protein interactions and PTMs. To bypass DNA lesions, Polη can interact with PCNA and Rev1 through its PCNA-interacting protein (PIP) motif (Figure 3) [33,55]. Lately, a second PIP-like motif is discovered in yeast Polη, which mediates its interaction with the Rad6-Rad18 complex [56]. In yeast, an interaction between the Polη-ubiquitin-binding/zinc-finger (UBZ) motif and the mono-ubiquitinated PCNA enhances TLS activity of Polη [57,58]. However, whether this interaction occurs and contributes to TLS activity of Polη in mammals remain inconclusive [59].

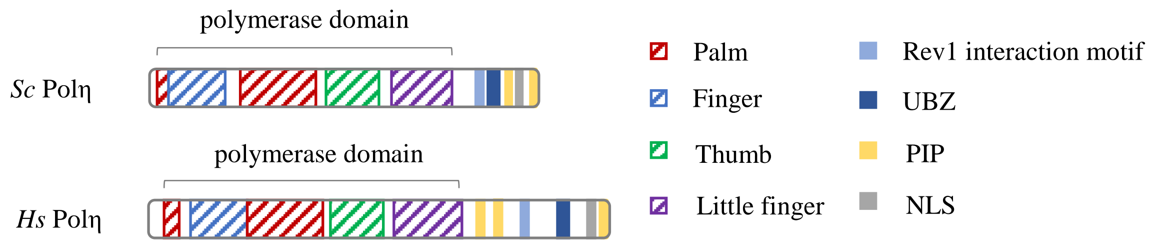

Figure 3. Functional domains and motifs of Polη

UBZ, ubiquitin-binding/zinc-finger motif; PIP, PCNA-interacting protein motif; NLS, nuclear localization sequence; *Sc*, *S. cerevisiae*; *Hs*, *H. sapiens*. The putative NLS in yeast Polη is depicted to show its relative position in the protein sequence.

Nevertheless, activity of Polη is also regulated by other PTMs besides mono-ubiquitination of PCNA, both in the absence and presence of DNA damage. On the one hand, the UBZ domain auto-catalyzes mono-ubiquitination of yeast Polη, independently of Rad6-Rad18 or DNA damage [60]. Studies in human cells further suggested that such ubiquitination hinders PCNA from interacting with Polη, thereby negatively regulating recruitment of Polη to chromatin in the absence of DNA damage [61,62]. However, it should also be noted that only a small portion of Polη is ubiquitinated in unchallenged cells, suggesting that additional regulatory mechanisms may exist to regulate access of Polη to undamaged chromosomes [63]. On the other hand, ubiquitination of Polη is subsequently downregulated when cells are exposed to DNA damages [61,64]. In addition, the UBZ domain of Polη is required for phosphorylation of Polη-S601 after UV-irradiation in human cells, contributing to activation of the DNA damage checkpoint and cell survival [65]. Human Polη is further poly-SUMOylated at sites of UV-induced damage, making it susceptible to dissociate from the damaged sites [66]. This is in consistence with the short residence time of human Polη in subnuclear foci, which is approximately 150 ms [67].

In addition to PTMs, the protein level of Polη appeared to be regulated throughout the cell cycle, particularly in budding yeast. Steady-state protein level and protein stability of yeast Polη increased from G_1 to G_2/M phase. Despite being most abundant and stable during unperturbed G_2/M phase, transcript levels of Polη were not significantly varied between cell cycle phases [68]. In contrast, when asynchronized or G_1 arrested yeast cells were exposed to UV-irradiation, the transcript level of Polη increased [64,69]. The relative protein level, however, was not affected [64]. This indicates that yeast Polη is regulated differently at the transcriptional and translational levels, in the absence or presence of DNA damage. On the contrary, although it is not clear if the protein level of Polη is cell cycle regulated in other organisms, Polη in *Caenorhabditis elegans* [70] and human cells [71] was subsequently targeted for proteasomal degradation through poly-ubiquitination after UV exposure.

These examples show a fine modulation of Polη through different perspectives, but how Polη is regulated by PTMs in the presence of DNA damage is less known in yeast. This was further addressed in paper I, focusing on PTMs of Polη with and without DSB induction during G_2/M.

2.2.5 Polη deficiency and cancer

Mutations in the *POLH* gene (human Polη) cause Xeroderma pigmentosum-variant (XP-V) disease [72,73]. XP-V patients typically show no or mild symptoms at young ages, but tend to develop melanoma and non-melanoma skin cancers in later life [74]. Polη-deficient cells also showed increased sensitivity to common cancer drugs, suggesting that Polη could modulate the cellular response to chemotherapeutic agents [75]. Understanding the roles and regulation of Polη therefore would improve our knowledge about how Polη contributes to genome integrity.

2.3 THE STRUCTURAL MAINTENANCE OF CHROMOSOMES (SMC) COMPLEXES

The SMC complex family consists of cohesin, condensin, and the Smc5/6 complex. In brief, cohesin adheres sister chromatids together from DNA replication until anaphase onset, ensuring faithful chromosome segregation [76-78]. Condensin compacts mitotic chromosomes and resolves catenation between sister chromatids, enabling proper chromosome segregation [79-81]. In addition, cohesin and condensin both contribute to spatial organization of chromosomes by DNA loop extrusion [82-84]. The Smc5/6 complex is implicated in multiple processes such as recombination-mediated DNA repair [85,86], maintenance of ribosomal DNA [87] and DNA topological stress response [88]. Here, as a background of the studies in this thesis, cohesin and its roles in sister chromatid cohesion, damage-induced cohesion and DSB repair will be further described.

2.3.1 Components and structure of cohesin

Cohesin comprises Smc1, Smc3 and the kleisin Scc1 as core subunits, as well as Scc3, Pds5 and Wapl as regulatory subunits. The Smc1 and Smc3 subunits are long coiled-coil proteins, containing a hinge domain in the middle, a 'Walker A' ATP-binding motif at the N-terminus and a 'Walker B' ATP-hydrolysis motif at the C-terminus (Figure 4A). Smc1 and Smc3 fold back on themselves, thereby placing the hinge domain at one end, and forming an ATPase 'head' domain at the other end. The head domains of Smc1 and Smc3 are dimerized upon binding ATP, and bridged by Scc1 [89,90]. For the regulatory subunits, Scc3 and Pds5 interact with cohesin through Scc1 [91-93], while Wapl sub-stoichiometrically interacts with Pds5 (Figure 4B) [94]. Scc3 and Pds5 exist in vertebrates as two isoforms, which are SA1/2 and PDS5A/B respectively [95-97]. In addition, sororin is another regulatory protein that only exists in vertebrates [98], which competes with Wapl in PDS5-binding during S phase [99].

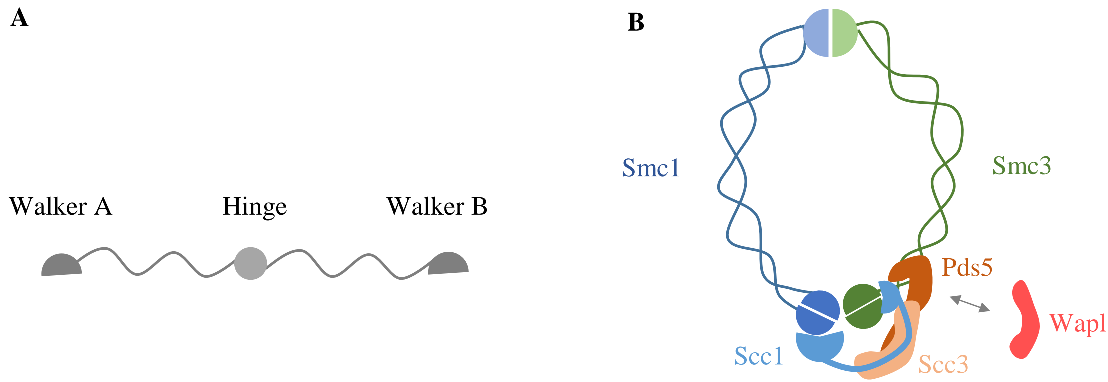

Figure 4. Structure and composition of cohesin

(A) Illustration of an unfolded Smc1/Smc3 subunit. (B) Structure and components of the cohesin complex in budding yeast. The double-headed arrow indicates protein-protein interaction between Pds5 and Wapl.

2.3.2 Loading and chromosomal association of cohesin

Cohesin begins to be loaded onto chromosomes in late G_1 phase in budding yeast [100], or in telophase in human cells [101], and the loading continues through the cell cycle. Since cohesin is assembled before loading, the complex must be transiently opened for DNA entry. It was proposed that cohesin entraps DNA through the hinge domain between Smc1 and Smc3 [102], and releases DNA through the gate between Smc3 and Scc1 [94,103,104]. Alternatively, DNA could enter and exit cohesin through the same Smc3-Scc1 gate via ATP-hydrolysis [105,106]. Cohesin is loaded at centromeres and promoters of highly transcribed genes [107,108] by the Scc2-Scc4 (human NIPBL-MAU2) complex [100]. Cohesin loading at promoters is facilitated by the chromatin remodeler RSC complex, which generates nucleosome free regions [109,110]. Replisome-associated proteins also aid in cohesin loading behind replication forks [111,112]. Cohesin enriched at centromeres is required for biorientation of sister chromatids, and to resist the pulling forces from microtubules [113]. Cohesin associated with promoters subsequently slides from the loading sites to the end of convergent genes, possibly driven by active transcription [108]. In addition to the positions of cohesin loading, chromosomal association of cohesin is dynamic because when cohesin is loaded, meanwhile, Wapl and Pds5 promote its dissociation [114-116].

2.3.3 Sister chromatid cohesion

During replication, the acetyltransferase Eco1 acetylates the K112 and K113 residues of Smc3 [117-119]. Acetylation of Smc3 counteracts cohesin-releasing activity of Wapl [115], and potentially blocks dissociation of Scc1 from Smc3 [94], thus sister chromatid cohesion is established. Cohesin complexes loaded before DNA replication and those *de novo* loaded on replication forks both can be converted into cohesive state [111,112]. Sister chromatid cohesion is further promoted and maintained by the regulatory subunits of cohesin. In contrast to its role during G_1, Pds5 promotes acetylation of Smc3 during S phase and prevents Smc3-deacetylation by the Hos1 deacetylase during G_2/M phase [116]. Scc3 is also required

for maintenance of sister chromatid cohesion after replication [120]. In addition, the specific regulatory subunit in vertebrates – sororin – is recruited by Smc3-acetylation and competes with Wapl for binding with PDS5 [99].

At anaphase onset, sister chromatid cohesion is scheduled for removal. In yeast and higher eukaryotes, the anaphase-promoting complex/cyclosome (APC/C)-Cdc20 complex triggers degradation of Pds1 (Securin), which in turn allows separase-dependent cleavage of Scc1 and Hos1-dependent deacetylation of Smc3 [78,121]. In addition, Scc3 is implicated in promoting deacetylation of Smc3 in budding yeast [120]. In vertebrates, there is a separase-independent pathway called the prophase pathway. Since the interaction between sororin and PDS5 is destabilized during mitosis, the interaction between Wapl and PDS5 becomes possible. This promotes Wapl-mediated cohesin dissociation from chromosome arms, while cohesin enriched around centromeres is not affected [122]. Absence of the prophase pathway in budding yeast causes cohesin re-loading occurs in late G_1 phase instead of telophase, as all the Scc1 subunits are cleaved in anaphase and need to be re-synthesized in G_1 phase.

2.3.4 Cohesin recruitment to DSBs

In response to DSB induction, cohesin is recruited and enriched around the DSB region. In budding yeast and human cells, the cohesin loader is required for this cohesin enrichment, indicating that cohesin is *de novo* loaded around the breaks [123-125]. In post-replicative budding yeast cells, cohesin accumulation around DSBs is dependent on several DNA damage response (DDR) proteins. These include Mre11, Mec1, Tel1, γ-H2A, Rad9; the histone methyltransferase Dot1 and partially dependent on Rad53 [123,124,126]. In addition, the RSC complex is required for cohesin loading after DSB induction, likely through sensing the DSBs and recruiting the Mec1/Tel1 kinases [127]. The recruited cohesin could span approximately 50-100 kb around the DSB, but was not further enriched genome-wide [123,124]. Similarly in human cells, the MRN complex [128] and the ATM-mediated phosphorylation of SMC3 [129] are required for cohesin enrichment at breaks, but the bound region is relatively small (about 5 kb) [130].

In addition to these DDR factors, cohesin recruitment to the site of DSBs in human cells is promoted by the Smc5/6 complex [131], while recruitment of the cohesin loader NIPBL depends on the heterochromatin binding protein HP1γ and ubiquitin ligases RNF8/RNF168 [125,132]. It was further demonstrated that the SUMO ligase Mms21, a subunit of Smc5/6, mediates SUMOylation of Scc1 for cohesin enrichment around DSBs in budding yeast [133]. Further studies would be needed to know if localization of the cohesin loader to the break is promoted by other factor(s) in yeast, as in the case of human cells.

2.3.5 Roles of cohesin in DNA damage response and DNA repair

It was demonstrated that in human cells, cohesin is required for activation of S phase and G2 checkpoints in response to DNA damage. During replication, SMC1 and SMC3 are phosphorylated by ATM after irradiation [134-136]. Despite phosphorylation of SMC1 being dispensable for cohesin enrichment at the breaks [135], non-phosphorylatable SMC1 or SMC3 compromised inhibition of DNA synthesis after irradiation [134-136]. Depletion of SCC1 during G2 also impaired cell cycle arrest, recruitment of the DDR protein 53BP1 and activation of CHK2 in response to irradiation [137]. It was further shown that cohesin enriched at the DSB region mediates formation of topological associating domains (TADs) through loop extrusion. The TADs enable spreading of γ-H2AX, the early DDR signal, and recruitment of DDR factors such as 53BP1 [138]. Whether cohesin plays a direct role in checkpoint activation in budding yeast is not reported.

Inactivation of cohesin subunits resulted in reduced cell survival after irradiation and/or decreased DNA repair product in various organisms [139-142], indicating that cohesin is required for DNA repair. In budding yeast, deficiencies of the *scc2-4* and *eco1-1* mutants in repairing irradiation-induced DNA damage [140] further implied that *de novo* cohesin loading and cohesion establishment both are needed. For replication-born DSBs, cohesin could promote sister chromatid exchange by tethering sister chromatids in proximity [143] and prevent distant DSB-end joining [144]. The movement of broken DNA could be further constrained by sister chromatid cohesion [145]. Intriguingly, in response to DSBs during G_2/M, Scc1 is cleaved by separase in order to remove the pre-existing cohesin [146]. The cohesin removal is required for DNA end resection and/or efficient DNA repair in fission yeast and budding yeast [146,147], implicating that cohesin occupancy around the DSB region could be limited. Nonetheless, the roles of cohesin and cohesion during the steps of DSB repair are not fully depicted.

2.3.6 Damage-induced cohesion

In budding yeast, site-specific DSBs are sufficient to trigger *de novo* establishment of damage-induced cohesion in post-replicative cells. Despite that cohesin is not further enriched genome-wide, damage-induced cohesion is formed both close to the DSBs and globally on undamaged chromosomes [148,149]. The break-proximal cohesion is required for DSB repair [53], while the functional role of genome-wide damage-induced cohesion – the cohesion newly formed on undamaged chromosomes – is not clear. Most of the factors required for cohesin recruitment after DSB-induction are also indispensable for genome-wide damage-induced cohesion in yeast. This includes Mre11, Mec1, Scc2, Smc6 and Mms21 [133,148,149]. In contrast, genome-wide damage-induced cohesion was only partially affected in the absence of Tel1 or when H2A was non-phosphorylatable [148]. In addition to these factors, the acetyltransferase Eco1 is vital for damage-induced cohesion [148,149]. Over-expression of *ECO1* even bypassed the need of DSBs to generate *de novo* cohesion

after replication [149]. This led to further investigations on the regulation of Eco1 after S phase and the potential substrate of Eco1 after DSB-induction.

In the absence of DNA damage, Eco1 is targeted for degradation after S phase. Eco1 degradation relies on sequential phosphorylation events that is initiated by Cdc28 (Cdk1), followed by the Cdc7-Dbf4 and Mck1 kinases, and in turn trigger its ubiquitylation by SCF^{Cdc4}. Conversely, DSB-induced DDR inhibits activity of Cdc7, thereby preventing Eco1 from degradation [150,151]. Therefore, Eco1 is stabilized and available for formation of damage-induced cohesion after replication. It was proposed that in response to DSBs, Eco1 acetylates Scc1, thereby antagonizing the cohesin-releasing activity of Wapl during G_2/M. According to this model, the DSB-induced DDR signal is transmitted through the activated Mec1 to the downstream effector Chk1. Chk1 in turn phosphorylates Scc1-S83 as a signal for the subsequent Scc1-acetylation at K84 and K210 residues by Eco1 [152,153].

In addition to the factors mentioned above, Polη was identified as a novel factor required for damage-induced cohesion. Polη contributes to genome-wide damage-induced cohesion but is dispensable for break-proximal cohesion and DSB repair [53]. This also indicated that damage-induced cohesion at the break and genome-wide on undamaged chromosomes could be regulated differently. Although Polη is the unique TLS polymerase that involved in damage-induced cohesion, its mechanistic role in this process is unclear. By studying point-mutants in generation of genome-wide damage-induced cohesion, it appeared that the polymerase activity of Polη and its interaction with PCNA are not required. Absence of Polη also does not affect cohesin binding, Smc3-acetylation or stabilization of Eco1 in the presence of DNA damage after replication. In addition, over-expression of the Scc1-acetyl-mimic allele suppressed the lack of damage-induced cohesion in the Polη null mutant [53]. The potential role and regulation of Polη in genome-wide damage-induced cohesion therefore were further investigated in papers I and II.

In contrast to budding yeast, studies of damage-induced cohesion are limited in vertebrates because the sister chromatids appear as discrete entities in G2 cells under fluorescence microscope [154]. Despite that, damage-induced cohesion possibly forms in vertebrates, based on the decreased distance between sister chromatids and the induced SMC3-acetylation after DSB-induction [129,155]. It was also shown that in bacteria treated with a genotoxic agent, the frequency of proximity-dependent sister chromatid recombination decreased in the absence of a SMC-like protein [156]. Although the approaches might be less direct, these studies suggested that damage-induced cohesion could be a conserved process among species.

2.4 CDC28 (CDK1) IN BUDDING YEAST

Cdc28 is a cyclin-dependent kinase (CDK), which is essential and sufficient to drive the cell cycle of budding yeast. Viability of a *cdc28* null mutant can be complemented by expression of human CDK1 and CDK2, indicating that the biological activities of these proteins are

closely relevant between species [157]. In budding yeast, the role of Cdc28 in the cell cycle is additionally supported by a nonessential CDK Pho85 under stress condition [158]. Cdc28 is in a two-lobed structure, with β-sheets in the N-lobe and α-helixes in the C-lobe, and the active site is sandwiched in between. A monomeric Cdc28 is inactive because the activation segment in the C-lobe is partially disordered and the substrate binding site is blocked by a 'T-loop' segment [159]. It is fully activated through association of cyclin [160] and the Cak1-mediated phosphorylation of T169 in the T-loop [161-163], since both events induce conformational changes of Cdc28 [164,165].

Activity of Cdc28 is low in G_1 phase because of the low cyclin level and the presence of CDK inhibitors (CKIs) [166,167]. As expression of G_1 cyclins increased and CKIs are degraded in late G_1 phase, Cdc28 associates with the G_1 cyclins (Cln1-3) to regulate cell entry into early S phase. Subsequently, Cdc28 interacts with three different pairs of B type cyclins (Clb1-6) to modulate cell cycle events during S to G_2/M phase (reviewed in [168]). Although Clb1 and Clb2 cyclins both are expressed during G_2/M, Clb2 is the major contributor of the Cdc28 kinase activity in G_2/M phase [169]. Cdc28 remains active until the G_2/M cyclins begin to be degraded and the CKIs are re-expressed at the M/G_1 border [166,170,171].

2.4.1 Targets of Cdc28 in relevance to the cell cycle events

The Cdc28/cyclin complex modulates cell cycle events through phosphorylation. For instance, Cdc28 mediates the choice of DSB repair pathway during S and G_2/M phases by phosphorylating the Sae2 and Dna2 endonucleases, as well as the Fun30 chromatin remodeler [172-174]. These factors either induce or promote DSB-end resection and thus direct the pathway to HR [175-177]. Cdc28 also targets Srs2 to promote its function as a helicase during HR [178,179]. In contrast to higher eukaryotes which inhibit CDK1 upon activation of cell cycle checkpoints, Cdc28 remains active during cell cycle arrest in budding yeast [180,181]. Cdc28 could target the DDR factors Rad9 and Rad53 in response to DNA damage [182,183]. However, the potential requirement of Cdc28 for checkpoint activation might depend on the type of DNA damage and the cell cycle stage [184]. Interestingly, Eco1 and Polη appeared as potential targets of Cdc28 in a proteomic analysis [185]. In addition, Cdc28 was implicated in sister chromatid cohesion as Cdc28 genetically interacts with Eco1 and Scc1 respectively [186,187]; the *cdc28* point mutants showed precocious separation of sister chromatids in G_2/M phase [186,188]. It is not clear if Cdc28 targets Eco1 or Scc1 *in vivo*, but Cdc28 prevents Scc1 cleavage before anaphase by phosphorylating Pds1, which in turn protects Pds1 from degradation by the APC/C-Cdc20 complex [189].

As part of the projects included in this thesis, the potential interaction between Cdc28 and Polη was investigated *in vitro* and supported by structure modelling, as presented and discussed in paper I. In addition, the putative phosphorylation of Polη contributes to formation of genome-wide damage-induced cohesion. Functional implication of this Polη-phosphorylation was further studied in paper II.

2.5 REPLICATION-INDEPENDENT NUCLEOSOME ASSEMBLY/HISTONE EXCHANGE

The main building block of eukaryotic chromatin, the nucleosome, is a dynamic entity. During transcription initiation and elongation, the nucleosomal histones are disassembled and reassembled to facilitate passage of RNA polymerase II (RNAPII). Some of the disassembled histones are exchanged with post-translationally modified histones or histone variants before reassembly, independently of DNA replication. Such histone exchange is assisted by histone chaperones, histone-modifying enzymes and chromatin remodelers. The potential effect of histone exchange on formation of genome-wide damage-induced cohesion was tested in paper II, the related protein factors are described herein.

2.5.1 Replication-independent deposition of histone H3

In metazoans, the canonical histone H3 can be replaced with the H3.3 variant in a replication-independent manner [190]. Conversely, the canonical H3 in budding yeast is similar to the H3.3 variant in metazoans [191]. H3 in budding yeast is exchanged with post-translationally modified H3, mediated by the histone chaperones Asf1 and the HIR complex. The newly synthesized H3-H4 dimer is bound by Asf1 and presented to the histone acetyltransferase Rtt109 for H3K56-acetylation [192,193]. The HIR complex, which physically interacts with Asf1, subsequently deposits the modified H3 during transcription [194]. The H3 exchange mainly takes place at promoters and the 3´-end of active genes, but basal H3 exchange also occurs to poise inactive promoters for optimal transcription [195-197]. Since the H3K56 residue is located at the DNA entry-exit point of the nucleosome core, the interaction between histones and DNA is loosened via acetylation of this residue, and thus improves DNA accessibility [198]. Interestingly, H3K56-acetylation is largely reduced in G_2/M phase, unless the cells are under persistent DNA damage [199].

2.5.2 H2A.Z/H2A exchange

In addition to the H3 exchange, the conserved H2A variant H2A.Z can substitute for H2A at promoters. In budding yeast, the H2A.Z-H2B dimer is shuttled from the cytosol into the nucleus by the Nap1 histone chaperone [200]. The dimer is then handled by another histone chaperone Chz1 or incorporated by the SWR1 complex into the nucleosome in a stepwise manner [200,201]. Since the SWR1 complex recognizes nucleosome free regions [202], H2A.Z is preferentially incorporated at promoters of both active and inactive genes [203,204]. Once deposited, the N-terminal tail of H2A.Z is acetylated by the NuA4 and SAGA histone acetyltransferase complexes [205,206]. The H2A.Z-containing nucleosome was suggested to be unstable, as incorporation of H2A.Z destabilized the interface between the core histones [207], and H2A.Z is susceptible to loss from chromatin *in vitro* [203]. In addition to the unstable nature of H2A.Z-containing nucleosomes, assembly of the transcription preinitiation complex triggers constitutive H2A.Z/H2A exchange at promoters [208]. H2A.Z facilitates

transcription activation and relieves the +1 nucleosome barrier to RNAPII through its eviction from promoters [203,204,209].

2.5.3 Passage of RNAPII during transcription elongation

During transcription elongation, disassembly of a single H2A-H2B dimer is sufficient for RNAPII to passage through a nucleosome within the coding region [210]. Displacement of the H2A-H2B dimer depends on the histone chaperone FACT complex, facilitated by mono-ubiquitination of H2B [211,212]. When the H2A-H2B dimer is disassembled, the remaining histone hexamer is stabilized by the histone chaperone Nap1 *in vitro* [213]. It is of note that H3 exchange also occurs during transcription elongation, but the exchange is less frequent in budding yeast and mainly observed at the 3' end of highly transcribed genes [195,197]. After passage of RNAPII, the FACT complex and H2B-ubiquitination are interdependent for reassembly of the H2A-H2B dimer [214].

In addition to the histone chaperones and H2B-ubiquitination, chromatin dynamics during transcription elongation are regulated by Set2. Set2 is the sole H3K36 methyltransferase in budding yeast [215], which physically associates with elongating RNAPII [216]. After passage of RNAPII, the co-transcriptional H3K36-methylation reduces affinity of histone chaperones to histones [217]. In addition, the chromatin remodeling complex Isw1b is recruited by H3K36-methylation [218]. Isw1b acts in concert with the chromatin remodeler Chd1 to prevent histone exchange within coding regions [219,220]. Isw1b also maintains proper spacing between nucleosomes through its nucleosome sliding activity [221]. This facilitates histone deacetylation by the Rpd3S complex, whose catalytic activity is stimulated by the H3K36-methylation [221,222]. Thus, histone exchange within coding regions is prevented and the histones are converted into a hypoacetylated state after passage of RNAPII.

3 MATERIALS AND METHODS

3.1 MODEL ORGANISM

Saccharomyces cerevisiae was used as the model organism in all papers included in this thesis. *S. cerevisiae*, also known as baker's yeast or budding yeast, is a single-celled eukaryote. The term 'budding yeast' readily describes the feature of cell budding, which begins in the early S phase, with the bud size continuously increasing until mitosis. Budding yeast exists as haploid in nature, either as 'a' or 'α' mating type. An unbudded haploid yeast cell is about 5 μm in diameter, and thus easy to see under a light microscope. The haploid genome size is approximately 12 Mb, with about 6000 protein-coding genes distributed on 16 chromosomes. This reflects a tiny but compact genome (roughly about one gene in 2 kb), with approximately 4% of all genes containing introns [223,224]. Budding yeast is a widely used model organism, with short doubling time (about 90 minutes at 30℃ when cultured in rich media) and the experimental cultures are easy to handle. In addition, gene deletion or integration is not difficult to perform because of its highly efficient homologous recombination. Strains with opposite mating types can also be crossed to generate mutants with desired genotypes. Due to its highly conserved amino acid sequence and protein function, studies in budding yeast can provide insight into biological processes in higher eukaryotes, including humans.

3.2 HO CUT AT MAT LOCUS ON CHROMOSOME III

Budding yeast in the wild undergoes mating type switching from 'a' to 'α', or vice versa, in order to form diploids for sporulation under poor living conditions. To switch mating type, the homothallic switching endonuclease (HO) generates a DSB at the *MAT* locus on chromosome III, thereby inducing gene conversion of *HMR* or *HML* with the *MAT* locus (Figure 5) [225]. The *HO* gene is inactivated in laboratories to maintain strains with stable *MAT* type, while the site-specific HO cleavage is applied for DSB-induction in experiments. This can be achieved by integrating an ectopic *HO* under control of an inducible promoter. The *GAL* promoter driven, P_{GAL}-*HO* allele integrated at the *ADE3* locus was the major approach for DSB-induction in papers I and II.

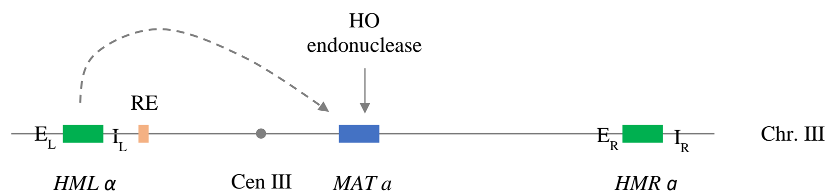

Figure 5. HO-induced mating type switching

The *HML* and *HMR* genes are silenced by interactions between silencing proteins and the cis-acting silencer sequences E and I, while the *MAT* gene is actively expressed. After a HO-induced DSB at the *MAT* a locus, for instance, the recombination enhancer (RE) promotes the use of *HML* α as the donor of gene conversion (indicated with dashed arrow), resulting in switched mating type to MAT α.

3.3 DAMAGE-INDUCED COHESION ASSAY

This assay monitors the percentage of sister chromatid separation after inducing DSBs in G_2/M arrested cells. The experimental procedure is illustrated in paper I, Figure 6A and in paper II, Figures S5A and S6A.

In detail, a microtubule depolymerizing agent - benomyl - was used to arrest cells in G_2/M phase by activation of the cell cycle checkpoint. DSBs were mainly induced by expression of the P_{GAL}-*HO* allele described above, but γ-irradiation was used as an alternative approach if the mutant was not responding to *GAL*-induction (such as the *htz1Δ* cells) or if the *GAL*-induction was incompatible with the experiment purpose (such as the Rpb1-anchor away experiment). To differentiate damage-induced cohesion from sister chromatid cohesion established during replication, strains with the temperature sensitive (*ts*) *smc1-259* allele were used in this assay. An ectopic Myc-tagged, WT Smc1 was integrated under control of an inducible *GAL* promoter. After *GAL*-induction, both of the *smc1-259* and the P_{GAL}-*SMC1-MYC* alleles would be used for generation of damage-induced cohesion at permissive temperature. However, cohesion formed with the *smc1-259 ts* allele, including the S phase cohesion, was subsequently inactivated at the restrictive temperature.

To measure genome-wide damage-induced cohesion, an array of tetracycline-operators (tetOs) was integrated at the *URA3* locus on the undamaged chromosome V. A tetracycline-repressor (tetR) tagged with green fluorescent protein (GFP) was constitutively expressed and bound to the tetO array. The sisters of chromosome V will therefore appear as a single GFP dot if damage-induced cohesion is generated, while observation of a G_2/M arrested cell with 2 GFP dots indicates the lack of damage-induced cohesion. Although this method measures cohesion *in vivo*, one should be aware that the cells might escape from G_2/M arrest especially at the last experimental time-point. To assure the cells determined for sister separation were arrested in G_2/M, the sample aliquots were recommended to supplement with DAPI. Besides, the samples were coded to reduce bias during counting the GFP-dots.

3.4 SPOT ASSAY

This assay monitors cell viability under normal growth or challenged condition; used in papers I and II. Exponentially growing cells were spotted on normal media or drug-containing plates after serial dilutions. By comparing the growth of WT, single mutants and double mutants on plates after incubation, this is a simple assay to investigate potential genetic interaction between the favored genes under certain condition. This is determined by the tested double mutants either mimicking, enhancing or suppressing the phenotype of single mutants, compared to the WT cells and the included controls under a specific treatment.

3.5 TOTAL RNA EXTRACTION AND RNA-SEQUENCING

In paper II, total RNA extraction and RNA-sequencing were performed to analyze transcriptional profiles of the Polη null mutant, both before and after DSB induction. To prepare total RNA extracts, collected cells were pretreated with zymolyase to remove the cell wall before following the manufacture's guidelines to extract total RNA with a column-based purification kit. The possible variation in cell numbers between samples were corrected by determination of genomic DNA content in each sample prior to total RNA extraction, in order to adjust the final volume of total RNA at the step of elution accordingly. The DNase treated total RNA samples were then handled by the Novogene limited company for construction of cDNA libraries and paired-end RNA-sequencing. Prior to making the cDNA libraries, the mRNA was enriched with oligo(dT) beads. Quality of the total RNA samples, concentration of the constructed cDNA libraries and the sizes of cDNA inserts were checked before RNA-sequencing.

3.6 ADDITIONAL TECHNIQUES

Various controls were included for all damage-induced cohesion experiments in papers I and II. To validate G_2/M arrest, DNA content was determined by flow cytometry analysis based on propidium iodide-staining. Pulsed field gel electrophoresis (PFGE) was used to confirm DSB-induction on chromosome III. PFGE enables separation of large DNA molecules (> 20 kb) by periodically switching the voltage at 3 different directions during gel electrophoresis. For irradiated samples, a defined volume of control cells (whose chromosome XVI is artificially split into half) was mixed with the irradiated cells prior to PFGE. Southern blotting was then performed after PFGE with a probe against chromosome XVI to measure the relative amount of chromosome XVI in the irradiated cells. Protein extracts were prepared based on the glass beads disruption method to monitor expression of the ectopic Smc1-Myc with western blots.

Several proteins of interest, such as Polη and the Polη-point mutants in paper I, were added with an epitope tag for detection in western blots or for protein purification. Small-scale protein extracts were prepared with the glass beads disruption method mentioned above, or with TCA- or sodium hydroxide-based methods. The TCA method was used to preserve the phosphorylation signal of Rad53. The rapid sodium hydroxide-based method was used lately because it recovers higher amount of protein than the glass beads disruption method.

In paper I, nano-liquid chromatography coupled tandem mass spectrometry (nLC-MS/MS) was used to detect post-translational modifications (PTMs) of Polη. In brief, peptides were firstly separated on a LC column, followed by ionization and transfer to the first mass spectrometer (MS1). In MS1, the ions are separated according to their mass-to-charge ratio (m/z). The ions with predetermined m/z-ratio will then be fragmented and introduced to the second mass spectrometer (MS2). Mass spectra will finally be produced according to the ion fragments separated in MS2. The tandem MS allows separation of ions with highly similar

m/z-ratio. PTMs of a protein can be detected by MS based on changes of amino acids molecular weights. The identified PTMs were then tested by immunoprecipitation and western blot, or by genetic analysis after mutating the specific residue(s) *in vivo*. Although the MS analyses often provide interesting information on the question of interest, it might not be always reproducible in the following investigations *in vivo*. This might be due to the *in vitro* experimental condition for MS analysis was different from that of *in vivo*, or certain technique/buffer condition was required to detect the PTMs *in vivo*. Such potential drawback has to be taken into consideration before proceeding to the costly MS analyses, and creation of point mutants could take some time.

In paper II, chromatin immunoprecipitation (ChIP) was performed to investigate chromosomal associations of RNA polymerase II (RNAPII) and Htz1 (H2A.Z) in strains of interest. The protein-DNA interactions were firstly crosslinked by addition of formaldehyde. The cells were then lysed, and the chromatin was sheared into 300-500 bp fragments by sonication. Subsequently, the protein of interest was immunoprecipitated by a specific antibody conjugated on beads. The fixed protein-DNA interactions were then reversed, followed by DNA purification. Chromosomal association of the target protein at specific region(s) relative to the input fraction was then measured by quantitative PCR (qPCR). Since unspecific binding might occur, untagged strains or low binding sites for the protein of interests should be included in the ChIP-qPCR analyses as controls. Besides, the qPCR primers should be designed according to certain criteria, and they have to be tested for achieving optimal qPCR conditions. In contrast to ChIP-qPCR analysis that focuses on certain selected sites, ChIP-sequencing is feasible for understanding genome-wide distribution of a protein of interest. Chromosomal association of Polη, for instance, was analyzed by ChIP-sequencing. The subsequent metagenomic analyses were useful for characterizing genomic distribution of Polη. For example, the relative enrichment of Polη at the upstream and downstream of gene bodies, and the types of promoters that Polη prefers to associate with were identified by metagenomic analyses.

4 RESULTS AND DISCUSSION

With the aims described in introduction, the findings of each paper in this thesis are presented and discussed as follows.

4.1 PAPER I

Post-translational regulation of DNA polymerase η, a connection to damage-induced cohesion in *Saccharomyces cerevisiae*

As one of the approaches to decipher the mechanistic role of Polη in genome-wide damage-induced cohesion, we investigated potential regulators of Polη during G_2/M phase. In this paper, we studied if the cyclin-dependent kinase Cdc28 and the acetyltransferase Eco1 modulate the activity of Polη for generation of genome-wide damage-induced cohesion.

In addition to the proteomic analysis mentioned in section 2.4 [185], Polη could be a Cdc28 substrate based on the two full and one partial CDK-consensus motifs found in its coding sequence. This possibility was addressed by *in vitro* kinase assay and structure modeling. We showed that purified recombinant Polη was only phosphorylated in the presence of both Cdc28/Clb2 and radiolabeled γ-^{32}p-ATP *in vitro*. Phosphorylation of Polη was abolished if the three potential phosphorylation sites (Polη-S14, T547 and T612) were simultaneously mutated. The potential interaction was further supported by structure modeling, where the Polη-S14 could be modeled in position for Cdc28-phosphorylation. The interface between Polη and Cdc28 in this model was about 1900 Å^2, which is above average for validated heterodimers that form protein complexes [226]. However, the interaction between Cdc28 and Polη-T547/T612 could not be modeled, as the structure of the Polη C-terminus is not fully available. The structure modeling also suggested that the Polη-K17 could interact with the phosphorylated Cdc28-T169, a modification that is important for its kinase activity.

Further *in vivo* studies on the single non-phosphorylatable (SA/TA) mutants revealed that mutation of Polη-S14, but not Polη-T547 or Polη-T612, affected protein level and formation of damage-induced cohesion. Despite that a constitutive strong *ADH* promoter was integrated to compensate for the reduced *Polη-S14A* protein level, the P_{ADH}-*Polη-S14A* mutant was still deficient in formation of damage-induced cohesion. This showed that the lack of damage-induced cohesion was not due to the reduced protein level, but rather that Polη-S14-phosphorylation is specifically required for formation of damage-induced cohesion. We also noted that constitutively increased expression of WT or mutated Polη did not affect normal cell growth. It is also worth mentioning that neither the single- nor the triple-SA/TA mutants were sensitive to UV irradiation. These indicate that (1) Cdc28 potentially regulates protein stability of Polη in G_2/M phase through phosphorylation of Polη-S14; (2) Polη-S14p is important for damage-induced cohesion, independently of its polymerase activity.

In addition to Cdc28, Eco1 appeared to be a potential regulator of Polη. Over-expression of Eco1 bypassed the need of Polη for formation of damage-induced cohesion. Furthermore,

Eco1 and Polη are expressed as the fusion protein Eso1 in fission yeast [227]. To investigate the possible functional interaction between Polη and Eco1, we began by monitoring UV sensitivity of the Polη null mutant *rad30Δ* and that of *eco1Δrad61Δ* cells. We used the *eco1Δrad61Δ* double mutant rather than an *eco1 ts* mutant because the latter is prone to generate revertants. Deletion of *RAD61*, which encodes for Wapl, omits the need of *ECO1* for cell viability. Based on spot assay, the *rad61Δ* mutant was insensitive to UV irradiation, while the *eco1Δrad61Δ* mutant was more sensitive than the *rad30Δ* mutant compared to the WT cells. Interestingly, deletion of *RAD30* in *eco1Δrad61Δ* cells caused an additive effect on UV sensitivity. This suggested that Polη and Eco1 both are required but act in different pathway in response to UV irradiation. Subsequently, we additionally examined the possible interaction between cohesin and Polη in response to UV irradiation. Similar results were observed in the *ts* mutants of the cohesin loader *scc2-4,* as well as the cohesin subunits *scc1-73* and *smc1-259*, which were all UV sensitive. This sensitivity was further enhanced by simultaneous deletion of *RAD30*. Together with the observations in *eco1Δrad61Δ* cells, this implicated that not only the presence of cohesin, but sister chromatid cohesion is required for cell survival after UV irradiation.

Despite that Polη and Eco1 act in parallel in response to UV irradiation, the possibility that Polη would be a substrate of Eco1 for damage-induced cohesion remained since polymerase activity of Polη is dispensable for establishment of damage-induced cohesion. We showed that Eco1 acetylated Polη in the presence of ^{14}C-CoA, followed by identification of Eco1-acetylation sites *in vitro* by mass spectrometry (MS) analysis. We also determined the DSB-specific acetylation of Polη *in vivo* by additional MS analysis. The potential acetylation sites detected from the two MS were illustrated in paper I, Figure 4B. To know if any of these acetylations contributed to formation of damage-induced cohesion, non-acetylatable (KR) mutants were created according to the MS results. However, none of the single point mutants was defective in damage-induced cohesion. Therefore, *Polη-K17R K546R K603R* and *Polη-K17R K546R K615R* triple KR mutants were generated as these acetylation sites are closed to the potential phosphorylation sites. In addition, Polη-multiple KR mutants were created according to the Eco1-acetylation sites *in vitro* and the DSB-specific acetylation sites *in vivo.* These mutants were referred to as "*Polη-in vitro KR*" and "*Polη-in vivo KR*" respectively.

Considering that the K603 and K615 residues are located within the putative NLS of Polη (602-617 amino acids), nuclear localization and UV sensitivity of the Polη-triple KR and the Polη-multiple KR mutants were analyzed. These mutants, except the *Polη-in vivo KR* that has no mutations at the K603 and K615 residues, showed reduced nuclear localization of Polη and were sensitive to UV irradiation. This indicated that acetylation within the putative NLS does contribute to nuclear localization and TLS activity of Polη. In addition, the protein level of Polη is relatively stable in the *Polη-in vitro KR* mutant compared to the two Polη-triple KR mutants, albeit these mutants contain the same mutations at the K17, K546, K603 and/or K615 residues. This suggested that some of the acetylations identified *in vitro* might counteract each other for stabilization of Polη. To test if any of these acetylations are

mediated by Eco1 *in vivo*, in situ staining and western blotting were performed to test the *eco1Δrad61Δ* mutant, including *rad61Δ* cells as control. However, this possibility was ruled out because deletions of *ECO1* and *RAD61* did not affect the protein level or nuclear localization of Polη. Furthermore, since nuclear localization of Polη is affected by the two Polη-triple KR and the *Polη-in vitro KR* mutations, only the *Polη-in vivo KR* mutant was tested for formation of damage-induced cohesion. Due to reduced protein level of *Polη-in vivo KR*, the *ADH* promoter was integrated to improve expression of this mutated allele. However, as compared to the controls, the *in vivo KR* mutations did not affect formation of damage-induced cohesion.

Summary

Polη is an attractive substrate for Cdc28. Phosphorylation of Polη-S14 not only affects protein level, but also contributes to formation of damage-induced cohesion. Eco1 and cohesin subunits act in parallel with Polη for cell survival after UV irradiation. In addition, Eco1 can acetylate Polη *in vitro*. Several potential acetylation sites identified from MS regulate protein level, nuclear localization and TLS activity of Polη, yet independently of Eco1. These findings improve our understanding of how post-translational modifications modulate the activity of Polη in budding yeast and provide new insight into regulation of Polη for generation of damage-induced cohesion.

Discussion

In this study, however, whether Cdc28 phosphorylates Polη *in vivo* remained to be demonstrated. This could be addressed by conditionally inactivate Cdc28 and monitor Polη-S14p by western blots, as well as monitor damage-induced cohesion in a Polη-S14 phosphorylation mimic mutant after inactivation of Cdc28. Since we did not observe an obvious mobility shift of Polη in western blots, phosphorylation status of Polη cannot be monitored directly with an antibody that detects the epitope tag of Polη. We tried to monitor phosphorylation of Polη by immunoprecipitating Polη and detecting with an α-Ser-p antibody, or vice versa, without succeeding. It might be feasible to use a general phospho-Ser CDKs substrate antibody or design a Polη-S14p-specific antibody, to test if this phosphorylation exists *in vivo*, and if it is mediated by Cdc28 in a cell cycle-dependent manner. An alternative protein extraction method, such as the TCA method, could also be considered to improve the detection of Polη-phosphorylation in western blots. In regard to damage-induced cohesion, we found that inactivation of Cdc28 resulted in precocious separation of sister chromatids in G_2/M phase, in line with previous studies [186,188]. Therefore, the combined effect of a Polη-S14 phosphorylation mimic allele and inactivation of Cdc28 on formation of damage-induced cohesion was not tested.

Among the three potential Cdc28-phosphorylation sites, phosphorylation of Polη-S14 affects its protein abundance in G_2/M phase. In addition, a protein cleavage product was observed in the *Polη-S14A* and *Polη-S14A T612A* mutants, but not in the *Polη-T547A, Polη-T612A* single mutants or the triple mutant in western blots. This implied that phosphorylation of Polη-T547

could promote cleavage of Polη, but possibly prevented by phosphorylation of Polη-S14 (summarized in paper I, Figure S1). Further studies on the proposed sequential phosphorylation of Polη would improve our understanding of how the protein level of Polη is regulated during the cell cycle.

In addition to Cdc28, we demonstrated that Polη can be a substrate of Eco1 *in vitro*. The potential physical interaction between Polη and Eco1 *in vivo* is of interest to study, but it was not detectable in co-IP experiments in our hands. This could be due to the action of Eco1 being too transient to capture. Furthermore, we analyzed Eco1-mediated (*in vitro*) and DSB-specific (*in vivo*) acetylation of Polη by MS. However, the detected acetylation sites between these two experiments hardly overlapped. This could be improved by including the *rad61Δ* and *eco1Δrad61Δ* mutants in the *in vivo* experiment, to clarify which residue(s) were the actual target site(s) of Eco1 on Polη after DSB-induction. However, the reduced protein level of the *Polη-in vivo KR* allele was most likely independent of Eco1 because absence of Eco1 does not affect protein level of Polη. This argues that an alternative enzyme is responsible for the post-translational modifications of these lysine residues. Interestingly, nuclear localization and protein level of Polη both were affected by the Polη-KR mutations within the putative NLS, independently of DSB-induction. Whether nuclear localization affects regulation of Polη protein level is not known. This together with identification of the enzyme that modifies the lysine residues of Polη would be interesting questions for future investigations.

4.2 PAPER II

Deficiency of Polη in *Saccharomyces cerevisiae* reveals the impact of transcription on damage-induced cohesion

In this paper, we hypothesized that transcription activation/regulation could facilitate formation of damage-induced cohesion post-replication. This hypothesis was based on the findings that Polη could be functionally associated with transcription in budding yeast [54], and that *de novo* cohesion was formed at the loci of heat shock genes in response to the raise of temperature in fission yeast [228]. With this idea, we firstly asked if transcription is deregulated in the absence of Polη. Then, we tested if this is relevant to the deficient damage-induced cohesion of *rad30Δ* cells through a genetic approach.

To test the correlation between active transcription and formation of damage-induced cohesion, we began by testing sensitivity of the Polη null and *Polη-S14A* mutants to transcription elongation inhibitors. Interestingly, as compared to WT cells, cell viability of both mutants decreased when growing on the actinomycin D- and mycophenolic acid-containing plates. To know if this reflected a reduced capacity of cells for transcription, chromosomal association of Rpb1 (the largest subunit of RNAPII) was monitored by ChIP-qPCR. Four actively expressed genes in G_2/M arrested WT cells were selected for analyses. The Rpb1-binding at promoters and coding regions of these genes was reduced in the Polη

null and *Polη-S14A* mutants, while the total Rpb1 protein level increased and remained stable compared to WT cells. This suggests that Polη may facilitate chromatin association of Rpb1 during transcription through phosphorylation of the Polη-S14 residue.

To know if transcription is deregulated in the absence of Polη, gene expression profiles of WT and *rad30Δ* cells were analyzed by RNA-sequencing. The difference between WT and *rad30Δ* cells in gene expression was readily observed before DSB induction during G_2/M, with approximately 400 genes were either up- or down-regulated in *rad30Δ* cells. Based on gene set enrichment analysis (GSEA), genes belonging to the chromatin assembly and positive transcription regulation pathways were downregulated in *rad30Δ* during G_2/M compared to WT cells. This was in line with the moderate increase of global nucleosome occupancy in *rad30Δ* cells, determined by a titrated micrococcal nuclease (MNase) digestion assay. The *rad30Δ* cells also appeared to be less responsive to DSB induction compared to WT cells, as indicated by the relatively fewer genes that were differentially expressed in *rad30Δ* cells after DSBs. Even though the up- and down-regulation of gene expression appeared to be trended in the same direction between WT and *rad30Δ* cells after DSBs, the GSEA showed that the changes in some cases were in different magnitude. For example, the genes belonging to nucleotide metabolism and oxidative phosphorylation pathways were upregulated to a greater extent in *rad30Δ* compared to WT cells. Altogether, transcriptional regulation was perturbed in the absence of Polη, both before and after break induction during G_2/M phase.

As an approach to understand the possible role of Polη during transcription, several published datasets were applied to analyze if the differentially expressed genes in *rad30Δ* cells were associated with certain types of promoters; in a similar way as reported [229]. A significant number of downregulated genes in *rad30Δ* cells during G_2/M overlapped with the genes grouped under closed promoters, the promoters without nucleosome-free regions and often regulate stress-related genes [230]. In addition, the up- and down-regulated genes in G_2/M arrested *rad30Δ* cells were dominated by TATA-containing promoters, which are highly regulated and related to stress response. The GSEA also indicated that the genes belonging to stress response were downregulated in G_2/M arrested *rad30Δ* compared to WT cells. Furthermore, through ChIP-sequencing, Polη was found to be enriched at 100 bp upstream of the transcription start sites and 100 bp downstream of the transcription end sites, but not within the coding regions. Polη was frequently associated with the closed and TATA-containing promoters, as well as promoters with fragile -1 nucleosomes (FN promoters). This in principle agreed with the analyses based on the published datasets, except the relative enrichment of Polη at the FN promoters.

To know if the preference of Polη for certain types of promoters directly affects transcription, expression of six Polη-bound or -unbound genes was monitored in *rad30Δ* and Polη-depleted cells. A combination of the auxin-inducible degron and the Tet-off systems was used for temporal depletion of Polη during G_2/M, resulting in a significant reduction of Polη protein

level in 1.5 hour after addition of auxin and doxycycline. Expression of the Polη-bound genes, however, was reduced only in the *rad30Δ* but not in the Polη-depleted cells. This was also true when expression of five additional Polη-bound genes was compared between cells with and without depletion of Polη. This indicates that the role of Polη for transcription is indirect. Despite that, the investigations described above showed that transcription is deregulated under persistent absence of Polη.

Since transcriptional regulation is perturbed in *rad30Δ* cells, we tested if the factors related with replication-independent nucleosome assembly/histone exchange were required for formation of damage-induced cohesion. This was because perturbing histone exchange at promoters will negatively affect transcriptional activation, which may mimic the situation in *rad30Δ* cells. To this end, Hir1−a subunit of the HIR complex involved in histone H3 exchange at promoters−was one of our candidates. Deletion of Hir1 alone is sufficient to disrupt the interaction between the HIR/Asf1 complex [194]. By inducing DSBs with the *P_{GAL}-HO* allele or through γ-irradiation, the *hir1Δ* mutant was partially deficient in damage-induced cohesion formation compared to the WT cells. In addition, preventing the exchange of Htz1 for H2A at promoter regions through *HTZ1* deletion negatively affected formation of damage-induced cohesion after γ-irradiation. In line with this, to know if histone exchange is perturbed in the absence of Polη, occupancy of Htz1 at six selected promoters was monitored by ChIP-qPCR. Interestingly, the *rad30Δ* mutant showed increased Htz1 occupancy at three promoters compared to WT cells, specifically after DSB induction. This indicates that the Htz1/H2A histone exchange at some promoters was affected in *rad30Δ* cells. With the deficiencies of *hir1Δ* and *htz1Δ* cells, this together supports the idea that transcriptional deregulation influenced formation of damage-induced cohesion.

We were also interested in testing if deletion of *SET2* would affect damage-induced cohesion formation, as it might suppress the transcriptional deficiency of *rad30Δ* cells. This speculation was based on the finding that *SET2* deletion suppressed sensitivity of certain transcription elongation factor mutants to 6-azauracil [231]. The 6-azauracil is a mechanistic analog of mycophenolic acid (MPA) that inhibits transcription elongation [232,233]. Therefore, we firstly tested if *SET2* deletion would suppress sensitivity of *rad30Δ* cells to actinomycin D and MPA. Viability of cells on the drug-containing plates was not affected in the absence of Set2, as compared to the WT cells. Interestingly, sensitivity of *rad30Δ* cells to these transcription elongation inhibitors was suppressed by *SET2* deletion. As *SET2* deletion appeared to compensate for the transcriptional deficiency of *rad30Δ* cells, we tested if *SET2* deletion would show a similar suppression effect on damage-induced cohesion. Damage-induced cohesion was formed in the absence of Set2, and deletion of *SET2* did suppress the lack of damage-induced cohesion in *rad30Δ* cells. This again implies that deficient damage-induced cohesion of *rad30Δ* cells is related to its transcriptional deregulation.

Finally, to address the possible connection between regulation of transcription and damage-induced cohesion in a relatively direct manner, we inhibited transcription through anchoring Rpb1 away from nucleus. The Rpb1-anchoring away was based on heterodimerization of the Rpl13A-FKBP12 anchor and the FRB-tagged target (Rpb1), induced by addition of rapamycin [234]. After addition of rapamycin for one-hour, Rpb1 was distributed over the cytoplasm and expression of a number of selected genes reduced approximately two-fold. This one-hour rapamycin treatment did not compromise protein level of the P_{GAL}-driven ectopic Smc1-Myc, or provoke an early DNA damage response. With the created 'Rpb1-anchor away' strain, formation of damage-induced cohesion was determined in the presence and absence of rapamycin, in combination with exposure to γ-irradiation or not. In consistent with our previous experiments, damage-induced cohesion was formed in cells treated with γ-irradiation alone. However, formation of damage-induced cohesion was hampered by transcription inhibition, as shown in the cells treated with rapamycin and γ-irradiation. This supports the notion that transcriptional deregulation driven by persistent absence of Polη leads to deficient damage-induced cohesion.

Summary

To pinpoint the potential connection between transcriptional activation/regulation and formation of damage-induced cohesion, the possible role of Polη in transcription was addressed. Although Polη might play an indirect role for transcription, persistent absence of Polη disrupted regulation of transcription. The mutants that appeared to mimic or suppress transcriptional deficiency of *rad30Δ* cells also mimicked or suppressed the lack of damage-induced cohesion in *rad30Δ* cells. This suggests that the transcriptional defect of *rad30Δ* cells consequently impaired formation of damage-induced cohesion. This idea was further supported by transcription inhibition through the Rpb1-anchor away experiments, which showed negative impact of transcription inhibition on damage-induced cohesion formation.

Discussion

The *rad30Δ* mutant showed sensitivity to transcription elongation inhibitors and reduced chromosomal association of Rpb1. It also showed an altered transcriptional profile compared to WT cells during G_2/M, and reduced Htz1 occupancy at promoters of active genes specifically after break induction. This altogether indicated that absence of Polη affects transcriptional regulation, although how its absence indirectly affects transcription remained unclear. The relatively frequent association of Polη with closed, FN and TATA-containing promoters could indicate that Polη interacts with factors that play roles at these specific promoters. These include the Rsc9/Swr1 chromatin remodelers, the general regulatory factors (GRFs) and the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex [235-237]. Further investigations on these potential interactions might improve our understanding about how absence of Polη affects transcriptional regulation, even though the effect and/or interactions may be indirect.

The *Polη-S14A* mutant interestingly mimicked the Polη null mutant regarding sensitivity to transcription elongation inhibitors and reduction in chromosomal binding of Rpb1. In addition, the *Polη-S14A* mutant was as deficient as the *rad30Δ* mutant in damage-induced cohesion [238]. Since the *Polη-S14A* mutant is UV-insensitive, this indicates that the polymerase activity of Polη could be dispensable for transcription and formation of damage-induced cohesion. Consistently, establishment of damage-induced cohesion was not affected in 'polymerase-dead' *rad30* point mutants ([53] and data not shown). It would be interesting to know if phosphorylation of Polη-S14 contributes to its potential interactions with other factors during transcription, as the *Polη-S14A* mutant phenocopied the *rad30Δ* mutant in several ways.

Among the mutants that were tested in this study, the *set2Δ* mutant suppressed sensitivity of *rad30Δ* cells to transcription elongation inhibitors and deficient damage-induced cohesion. Understanding the reason why *SET2* deletion suppressed the defects of *rad30Δ* cells could provide a mechanistic insight into how transcription affects establishment of damage-induced cohesion. We therefore monitored chromosomal association of Rpb1 in the *set2Δrad30Δ* mutant and found that *SET2* deletion subtly compensated for the reduced Rpb1-binding in *rad30Δ* cells during G_2/M. However, expression of selected genes was similar between *rad30Δ* and *set2Δrad30Δ* cells (data not shown). This might be due to a limited number of genes were analyzed by RT-qPCR, but similar findings were described by Kelby O. Kizer *et al.* [239]. The question of how *SET2* deletion suppressed the *rad30Δ* phenotypes remains to be explored. This could be further addressed with *set2* mutants that contain truncations of each individual functional domain.

Through a combination of meta-genome analyses and genetic approach, our study suggests that the transcriptional deregulation driven by the absence of Polη consequently impaired generation of damage-induced cohesion. This also raised the possibility that transcriptional regulation contributes to formation of damage-induced cohesion post-replication. Based on deficiency of the *hir1Δ* and *htz1Δ* mutants in damage-induced cohesion, as well as downregulation of other genes belonging to the chromatin assembly pathway in *rad30Δ* cells, it is possible that replication-independent nucleosome assembly (histone exchange) facilitates formation of damage-induced cohesion during G_2/M phase. Although Polη may play an indirect role for transcription, we speculated that absence of Polη and Polη-S14-phosphorylation affected formation of a certain chromatin state. Our current study, however, is insufficient to propose how histone exchange could facilitate formation of damage-induced cohesion.

5 CONCLUSIONS AND FUTURE PERSPECTIVE

With the aims to understand the mechanistic role of Polη in damage-induced cohesion and how damage-induced cohesion is established genome-wide, we studied post-translational regulation of Polη and the non-canonical role of Polη in transcription. In paper I, Polη appeared to be an attractive substrate for Cdc28 *in vitro*. Phosphorylation of the Polη-S14 residue, which could be mediated by Cdc28, regulates the steady-state protein level of Polη during G_2/M. Importantly, this specific phosphorylation is required for formation of damage-induced cohesion. In paper II, we showed that absence of Polη or abolishment of Polη-S14A-phosphorylation negatively affects transcription elongation. Through combining meta-genome analyses and genetic approach, our study suggests that transcriptional deficiency of the Polη null mutant consequently impairs formation of damage-induced cohesion. Consistently, establishment of damage-induced cohesion was compromised by transcription inhibition. Although the roles of Polη and its S14-phosphorylation in transcription may be indirect, our results also suggest that transcriptional activation/regulation may facilitate formation of genome-wide damage-induced cohesion.

Several studies showed that yeast Polη is regulated at the steady-state protein level or the transcriptional level (described in section 2.2.4), while information about its regulation through PTMs was limited. Our identification of the potential Polη-S14-phosphorylation provides new insight into regulation of Polη protein level through PTMs in budding yeast. In addition, the protein level of Polη could be regulated by S14- and T547-phosphorylation in a counteractive manner, based on presence and absence of a cleavage product on western blots. Further investigations on these specific phosphorylation, in concern of the potential interaction between Polη and Cdc28 in different cell cycle stages, would improve our understanding of how Polη is being modulated during cell cycles. This might also provide some clues for the potential Polη-Cdc28 interaction during transcription, in connection to damage-induced cohesion formation. Besides of phosphorylation, we also found that certain KR mutations within the NLS affected both protein level and nuclear localization of Polη. This raised a possibility that Polη outside of the nucleus is preferably targeted for degradation, which would also be interesting to explore.

Since the non-canonical role of Polη in transcription is less studied, we asked if transcriptional regulation is perturbed in the absence of Polη prior to addressing the potential linkage between transcriptional activation/regulation and damage-induced cohesion. Our study provided a possible explanation for deficiency of *rad30Δ* cells in formation of genome-wide damage-induced cohesion. However, the underlying mechanism of how histone exchange (or transcription in general) could facilitate generation of damage-induced cohesion post-replication remains unclear. This might be investigated from the perspective of Polη, for instance, studying potential interactors of Polη during transcription as discussed in paper II. How sister chromatid cohesion is established during replication, on the other hand, is also not completely clear. Although the mechanism might be different, future studies

about establishment of sister chromatid cohesion along with progression of the replication fork would also provide new insight into generation of genome-wide damage-induced cohesion.

In addition to the questions described above, we would like to discover the importance of genome-wide damage-induced cohesion. We speculated that damage-induced cohesion formed on undamaged chromosomes could restrain chromosome movements after DSB induction. It was reported that mobility of chromosomes increased in response to DSBs, possibly facilitating homologous recombination [240,241]. The mobility of chromosomes, however, was restricted by sister chromatid cohesion in response to spontaneous DNA damage [145] and less dynamic for the undamaged chromosomes [240,241]. Therefore, it is possible that genome-wide damage-induced cohesion could restrict movements of undamaged chromosomes, to limit unfavorable recombination.

Whether damage-induced cohesion is a conserved biological process is always of concern. There are limitations to study damage-induced cohesion in other organisms besides budding yeast, as described in section 2.3.6. Although formation of damage-induced cohesion in other organisms may not be measured as we did in budding yeast, those studies indicated that damage-induced cohesion could be conserved. We also found that diploid yeast cells were able to establish damage-induced cohesion (data not shown). Formation of damage-induced cohesion might be further tested in fission yeast, possibly with a truncated *eso1* that leaves the Polη counterpart out, and vice versa.

It is worth mentioning that there is limitation for studying damage-induced cohesion in budding yeast as well. Since a strain of interest will undergo prolonged G_2/M arrest in our damage-induced cohesion assay, the strain must show no defect in maintenance of sister chromatid cohesion established during replication. Otherwise, the strain will show high separation of sister chromatids regardless of DSB induction. Understanding if genome-wide damage-induced cohesion is important for a certain biological process in a way could ease this limitation. It is then at least possible to perform a genetic screen based on a certain phenotype caused by deficient damage-induced cohesion, albeit being indirect. Considering that Scc1 is a potential target of Eco1 for damage-induced cohesion [152,153], it may also be plausible to monitor the specific Scc1-acetylation in strains of interest, if an Scc1-acetylation specific antibody is available.

In summary, studying genome-wide damage-induced cohesion through Polη led us to explore novel factors involved in this biological process. Identification of these factors not only provided new perspective on establishment of damage-induced cohesion post-replication, but also raised interesting questions that open for future investigations.

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