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The Role and Molecular Mechanisms of Wss1 in Preserving Genomic Stability

Daniel Kwesi Sam
South Dakota State University

THE ROLE AND MOLECULAR MECHANISMS OF WSS1

IN PRESERVING GENOMIC STABILITY

BY

DANIEL KWESI SAM

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THESIS ACCEPTANCE PAGE

Daniel Sam

This thesis is approved as a creditable and independent investigation by a candidate for

the master's degree and is acceptable for meeting the thesis requirements for this degree.

Acceptance of this does not imply that the conclusions reached by the candidate are

necessarily the conclusions of the major department.

Jaime Lopez
 Advisor   Date

Volker Brozel
Department Head   Date

Dean, Graduate School   Date

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ABBREVIATIONS

ABC- ATP binding cassette

ACRC- acid repeat containing protein

ALT- alternative lengthening of telomeres

ARS- autonomous replication sequence

ATM- ataxia telangiectasia mutated

ATR- ataxia telangiectasia and Rad3 related

ATP- adenosine triphosphate

BER- break excision repair

CDK- cyclin dependent kinase

CPD- cyclobutane-pyrimidine dimers

CPT- camptothecin

DDK- Dbf4 dependent kinase

DDR- DNA damage response

DNA- deoxy ribonucleic acid

DSB- double strand break

DPC- DNA protein crosslink

dNTP- deoxyribonucleotide triphosphate

FA- Fanconi anemia

FACS- fluorescent antibody cell sorting

FHA- fork head associated

FRT- Flp recombinase recognition target

GCNA- germ cell nuclear antigen

GG-NER- global genome wide nucleotide excision repair

HU - hydroxy urea

HR- homologous recombination

ICL- inter-strand crosslinks

MAPK- mitogen activated protein kinase

MCM- mini-chromosome maintenance complex

MMEJ- micro-homology-mediated end-joining

MMS- methyl methane sulfonate

MRX- Mre11/Rad50/Xrs1

NDSM- negatively charged amino acid dependent SUMOylation motif

NHEJ- non-homologous end-joining

NER- nucleotide excision repair

NSE- non-structural element

ORC- origin replicative complexes

ORF- open reading frame

PIAS- protein inhibitor of activated STAT

PI3K- phosphatidyl-inositol 3 kinase

PARP- poly-ADP ribose polymerase

PDSM- phosphorylation dependent SUMOylation motif

6-4PP- pyrimidine -pyrimidone photoproducts

ROS- reactive oxygen species

RNA- ribonucleic acid

SENP- Sentrin-specific protease

SIM- SUMO interacting motif

SMC- structural maintenance of chromosomes

snRNA- small nuclear RNA

SSB- single strand break

STUBL- SUMO targeted ubiquitin ligases

SUMO- small ubiquitin like motif

TC-NER- transcription coupled nucleotide excision repair

TDP- tyrosyl DNA phosphodiesterase

TLS- translesion repair synthesis

TOP- topoisomerases

UV- ultraviolet

Wss1- weak suppressor of smt3

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ABSTRACT

THE ROLE AND MOLECULAR MECHANISMS OF WSS1

IN PRESERVING GENOMIC STABILITY

DANIEL KWESI SAM

2020

Cells are constantly under threat from both exogenous and endogenous sources of DNA damage. Eukaryotic organisms, however, possess conserved mechanisms that accurately and faithfully respond to DNA damage. The inability to effectively remove DNA lesions can lead to an accumulation of mutations which can compromise cellular viability. The DNA damage response is conserved from bacteria to eukaryotic organisms and have been well characterized, however, how covalently crosslinked proteins are removed from DNA remains enigmatic

This thesis provides genetic and biochemical evidence implicating Wss1, a yeast metalloprotease in genome maintenance. We have identified SUMOylation to be an important signal that mediates the removal of as DNA-protein crosslinks. DNA protein crosslinks (DPC) are lethal lesions which are covalently linked to DNA. These lesions can impeding essential DNA transactions including chromosome duplication, chromatin remodeling and gene transcription. We characterized Siz1 and Mms21 E3 SUMO ligases to be important for modifying DPCs. To further expand the role of Mms21 in DPC repair we probed the impact of structural maintenance of chromosome (SMC) mutants in

repairing DPCs.

This thesis shows that Wss1 is involved in cleaving histones in order to prevent their accumulation during hydroxyurea induced replication stress. *In vitro* cleavage assays with purified proteins indicate that Wss1's histone H3 cleavage activity is dependent on its protease activity alone and not its SUMO binding nor p97 domains, unlike in Wss1-mediated removal of DNA-protein crosslinks. Together, we provide molecular evidence suggesting that Wss1 is an important mediator in genome maintenance.

Chapter 1: Introduction: DNA damage response and repair of DNA protein crosslinks

1.1 Overview

This thesis focuses on work I have done to identify how Wss1-mediated repair influences genome stability. This chapter provides an introduction to the topic and mechanisms that contribute to maintenance of genome integrity. Chapter 2 of this thesis focuses on our finding that two SUMO E3 ligases, Mms21 and Siz1 are importance for the repair of DNA protein crosslinks (DPC), thereby suggesting a mechanism by which cells tolerate DPCs. Chapter 3 describes molecular mechanisms by which Wss1 promotes tolerance to hydroxyurea-induced replication stress. Taken together, this thesis provides additional mechanistic insight on how the yeast protease, Wss1 contributes to genome maintenance, which can be extended to eukaryotic cells.

DNA encodes genetic sequences that transcribe genes necessary for all cellular functions. Cells respond to growth cues to initiate DNA replication in order to maintain growth, however faults or replication stress can occur during the process of replication. DNA damage events that occur during replication are sufficient to halt cellular function if not appropriately repaired. Damage can occur through exogenous as well as intrinsic sources. However, metazoan and protozoan organisms have dedicated processes that enable the efficient repair of DNA damage. The DNA damage field is a widely investigated area of research however little information exists on how cells tolerate the covalent attachment of proteins to DNA, which is termed as DNA-protein crosslink (DPC) (Y. Sun et al.). DPCs are amongst the most deleterious forms of DNA damage a cell can encounter and their repair is severely understudied. DPCs are abundant lesions that can form through a number of different means depending on the type of protein that becomes covalently attached to

DNA. As such, DPCs can be broadly categorized into two main groups– enzymatic and non-enzymatic DPCs (Stingele et al., 2017). Enzymatic DPCs occur when enzymes that naturally function by forming a transient covalent intermediate with DNA, become trapped on DNA either by chemical inhibition or through inherent errors in the activity of the enzyme. Non-enzymatic DPCs result when DNA binding proteins become cross-linked to DNA by being in close proximity to aldehyde production sites (Barker et al., 2005). Both enzymatic and non-enzymatic DPCs have been shown to be physical impediments to DNA replication and gene transcription machineries and their removal is important for maintaining genome integrity as well as cellular homeostasis (Balakirev et al., 2015; Stingele et al., 2014).

To date, DNA-protein crosslink repair involves three enzymes - Wss1, Mre11, and Tdp1 (Stingele et al., 2017). However, how these three repair enzymes work in concert and how they recognize DPCs is not entirely understood. Wss1 is a yeast metalloprotease which is recruited to sites of DPCs as well as sites of replication stress. All known DNA-protein crosslinks identified to date are post-translationally modified with SUMO chains. Given that Wss1 possesses SUMO interacting motifs, my hypothesis is that DPCs are modified with poly-SUMO chains and this modification serves as a signal that recruits Wss1 to sites of damage.

To study DPC repair and replication stress, this research seeks to use *Saccharomyces cerevisiae* as a fundamental model system to probe the molecular mechanisms of Wss1-mediated DNA damage repair.

1.2 Budding yeast as a biological model system

Model organisms are used in research investigations to obtain a clear understanding in areas of biology. These models facilitate tentative insight into concepts in biology (Russell et al., 2017).

Early molecular biology work on understanding the central dogma, utilized bacteriophage, bacteria and yeast, while developmental biology made use of flies, Arabidopsis, mice and worms to seek understanding (Russell et al., 2017).

With the advantage of technological instrumentation, these organisms have benefitted from wide-scale genomic projects, which have paved the way for a more comprehensive understanding of the cellular function of yeast, Drosophila, *Caenorhabditis elegans* and their relation to other organisms (Russell et al., 2017). This approach is possible due to the principle of conservation of cellular organization.

Over the past years, *S. cerevisiae* has remained a model system for studying the genetics and cellular biology due to compelling features such as its ability to reproduce quickly (90 minutes), ease of DNA manipulation, ease of replicating plating, non-pathogenicity, and their existence in both haploid and diploid genetic stages. The sequencing of the *S. cerevisiae* genome in 1996 made it easier to obtain more genomic information of the structure and organization of budding yeast genome (Duina et al., 2014). Several scientific discoveries including the secretory pathway (Franzusoff et al., 1991) and cyclin-dependent kinases (Beach et al., 1982) were previously made using yeast and later, homologous variants identified in metazoans. This makes it important to compare yeast cellular research to that of higher organisms because most of the metabolic pathways are highly conserved.

1.2.1 Budding yeast genomic structure and chromosome replication

In 1996 an international collaboration involving 600 scientists spanning across Europe, Japan and North America published the complete sequenced genome for *S. cerevisiae* (Goffeau et al., 1996). The essence of the genome project was to map the genome and to provide adequate genomic data for scientific research. Since then, yeast geneticists have been able to make a library of complete deletions of every open reading frame (ORF) in the yeast genome (Winzeler et al., 1999) as well as curate libraries of essential genes in budding yeast which have further provided wealth of information about the cell and molecular biology of eukaryotes.

S. cerevisiae possesses a haploid set of 16 chromosomes which range from 200-2200 kb (Duina et al., 2014). Analysis of the yeast genome specifies the presence of 6275 ORFs encoding 99 amino acids, however 390 are not likely to be translated into proteins (Goffeau et al., 1996). The yeast genome also reveals the presence of 140 ribosomal RNA genes on chromosome XII and 40 small nuclear RNA (snRNA) in its 16 chromosomes. All these attributes contribute to the unique compactness of the yeast genome (Dujon, 1996) as compared to other multicellular organisms.

Comparing budding yeast genome to that of *S. pombe* shows that there are about 40% introns (Dujon, 1996), non-replicating segments of DNA in the fission yeast genome as compared to 4% in budding yeast (Goffeau et al., 1996).

1.2.2 Growth, life cycle and mating signaling in S. cerevisiae

S. cerevisiae has been described as the *Escherichia coli* of eukaryotic organisms due to its ability to grow rapidly in rich media and its simple life cycle. Yeasts physiologically exist in two forms, haploid and diploid. A haploid cell could also exit as an a or α cells, which can undergo mitotic cell division to yield daughter cells as buds. In the absence of sufficient nutrients, haploid cells arrest at G1 phase of the cell cycle and only assume the cell cycle when nutrients are available (**Figure 1.1**). A mother yeast cell can form approximately 20-30 buds in its lifetime. It is easy to determine the age of a mother cell by visualizing the number of bud scars on the cell wall (Schneiter, 2004).
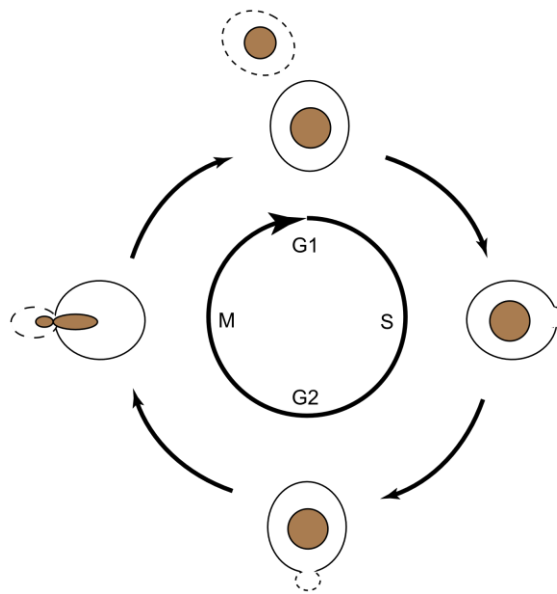
Figure 1.1 *Saccharomyces cerevisiae* **mitotic life cycle**.

The mother cell is represented in solid line whereas the daughter cell is shown in dotted line. The nucleus (shaded circle) is divided and shared between daughter and mother cells. G1, S, G2 and M represent the cell cycle phases.

Under the control of the mating-type locus, a and α cells respond to cellular cues to mate and form diploid cells (**Figure 1.2**) (Herskowitz, 1988). Haploid cells are identified by the presence of a radial bud while a diploid cell has an axial bud. Under conditions of nutrient starvation, diploid yeast undergo meiosis to form a meiotic product containing four haploid spores (**Figure 1.2**).

The life cycle of budding yeast is initiated by mating (schmooing) between an a and α cell, initiated by the release of signaling pheromones by the various mating types. Thus, an a cell releases an α pheromone and *vice versa*. The mechanism for the transcription of mating type gene expression is controlled by cell surface heterodimeric G-proteins which initiate the mitogen activated protein kinase (MAPK) cascade (Merlini et al., 2013). a and α pheromones bind to the G-protein cell surface receptors Ste2 and Ste3 respectively and stimulate GDP to GTP exchange on the G-protein (Elion, 2000). The activated G-protein then initiates a mating cascade which is centered around the synergistic roles of p-21-activated protein kinase (PAK), Ste11, Ste7, MAPK Fus3 and MAPK Kss1 (Elion, 2000).
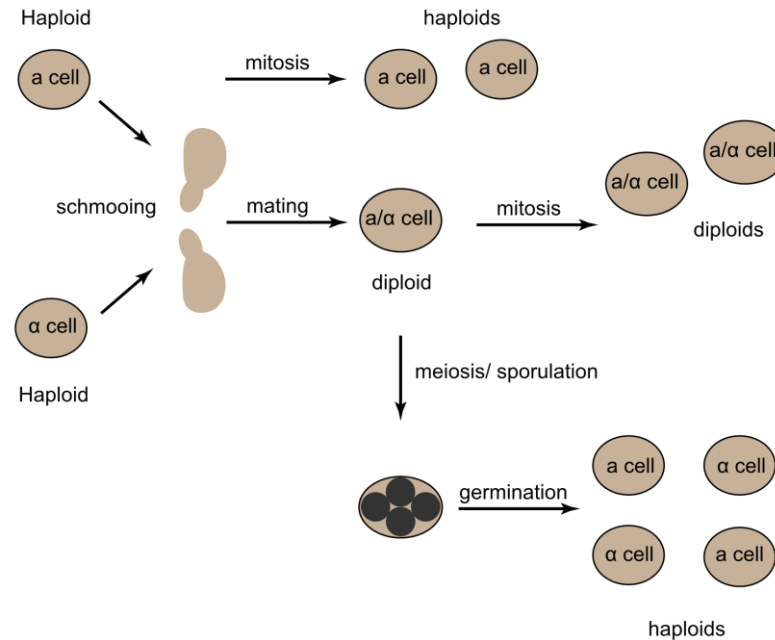
Figure 1.2: Life cycle of budding yeast.

Haploid a and α cells undergo schmooing to form a diploid cell. Haploid and diploid cells can mitotically divide to generate daughter cells. Under nitrogen starvation, diploids undergo meiosis to generate four meiotic products.

1.2.3 Chromosome replication in *Saccharomyces cerevisiae*

Living organisms respond to mitogenic growth factors which makes cells proceed through G1, S, G2 and M cell cycle phases. Proliferating cells replicate their genomic DNA before passing on genetic traits to the progeny through the process of DNA replication. DNA replication is a highly conserved evolutionary process which occurs at the S phase of the cell cycle. Accumulating data shows that the origin and regulation of DNA replication among lower and higher eukaryotes vary significantly (Masai et al., 2010). Eukaryotic chromosomes are large and therefore are replicated by two replication forks that move in opposite directions at cis chromosome locations called replicators. Replication involves two steps, licensing and firing. Replication licensing is a set of initial processes which ensures that all the factors necessary for replication are present but not yet committed to

activate firing, which is a state of active DNA replication. Several layers of regulatory mechanisms achieved by cyclin dependent kinases phosphorylate events occurring at both the licensing and firing steps to ensure accurate duplication of genetic material (Reviewed in (Sacco et al., 2012).

The site of replication is called an Origin (Toone et al., 1997), a DNA sequence which contains A, B1 and B2 DNA elements specifying the binding sequence for origin replicative complexes (ORC) (Rao et al., 1995; Rowley et al., 1995). Origins were first identified to provide autonomous replication to plasmids (Palzkill et al., 1988; Stinchcomb et al., 1979). These origins were then termed as autonomous replication sequence (ARS). The application of 2-dimensional gel electrophoresis techniques to these origins proved their association with the origin of replication (Bonita J. Brewer et al., 1987; Huberman et al., 1987). ARS elements are 11 base pair long and consist of ARS consensus sequences (ACS) (Broach et al., 1983). Mutations in ARS sequence elements result in impaired origin function or proper origin firing (Bell et al., 1992; Rao et al., 1995). ORC is a six multi-subunit scaffold (Orc1-6) that binds replication origins in an ATP dependent manner and recruits more replication dependent factors such as mini-chromosome maintenance complex (MCM 2-7) (Fox et al.), Cdc6 and Cdt1 to the ARS, thus, forming a pre-replicative complex (pre-RC) before DNA replication (Mechali, 2010; Rao et al., 1995; Rowley et al., 1995) (**Figure 1.3**).

At the pre-RC, DNA replication initiator proteins interact with DNA helicase which unwinds double stranded DNA prior to replication (Kawakami et al., 2010). Budding yeast possess about 400 replication origins along its 16 chromosomes (Linskens et al., 1988) as compared to 30,000-50,000 in humans (Mechali, 2010). Origin firing is initiated at early

S-phase through the S-phase cyclin dependent kinase (S-Cdk) and Dbf4 dependent kinase (Ddk)-dependent phosphorylation of Sld3 and Sld2 (**Figure 1.3**).

Phosphorylated Sld3 mediates the loading of Cdc45 and subsequent binding to Dpb11 through its BRCT domains recruits the GINS (go-inchi-ni-san) complex (Sld5, Psf1, Psf2 and Psf3) to chromatin (S. Tanaka et al., 2007; Zegerman et al., 2007). Formation of this multi-subunit complex on chromatin associates with DNA polymerases and commits to unwinding and bi-directional replication of DNA (Aparicio et al., 1997).
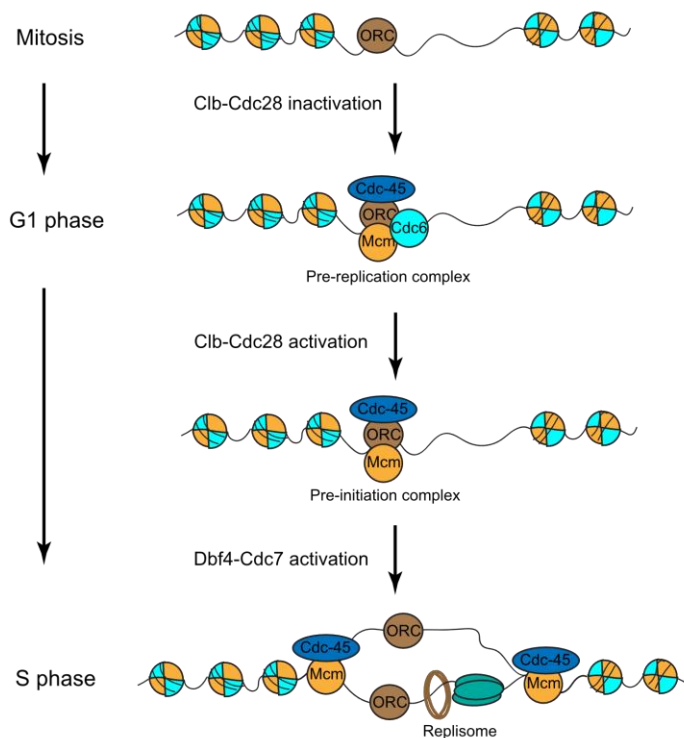
Figure 1.3: Cell cycle dependent activation of replication origins.

ORC remains active on origins throughout the cell cycle. Clb (B type cyclin) and Cdc28 inactivation facilitates the formation of pre-RC at the G1-S boundary. Upon activation of Clb and Cdc28, Cdc6 is degraded leading to the formation of a pre-initiation complex as a result of Cdc45 tight binding to chromatin (L. Zou et al., 1998). Dbf4-Cdc7 activation leads to DNA replication at S-phase.

1.3 The DNA damage response (DDR)

Genomes of organisms are constantly under threat from different forms of insults which when unrepaired have the potential to induce mutations and chromosomal instability. Eukaryotic organisms however possess conserved mechanisms that accurately and faithfully respond to DNA damage. Even though there are many distinct forms of DNA damage and their repair proteins, there is generally a common signaling pathway which when elicited leads to DNA damage checkpoints activation to slow cell cycle progression until the damage has been repaired (Lowndes et al., 2000). It is remarkable that a single chromosomal break in a tightly packed chromatin can elicit global DNA damage response leading to detection and repair (S. E. Lee et al., 1998; A. Pellicioli et al., 2001). The ability for cells to respond to DNA damage involves an interplay of phosphatidyl-inositol 3 kinase (PI3K) cascade at the site of DNA damage. This cascade pathway phosphorylates downstream effector proteins leading to a coordinated cell cycle progression and subsequent recruitment of repair factors (**Figure 1.4**).

At the center of the yeast DDR cascade is Mec1 (ATR in metazoans) and Tel1 (ATM in metazoans) which initially sense DNA damage and phosphorylate a second set of downstream kinases, Chk1 and Rad53 (Chk2 in metazoans). Rad53 and Chk1 are checkpoint kinases, thus upon phosphorylation, activates transcription of DNA damage repair genes as well as proteins that regulate dNTP (deoxyribonucleotide triphosphate) (Chabes et al., 2003; Weinert, 1998) leading to cell cycle arrest until repair has been achieved. Rad9 (53BP1 in metazoans), a BRCT containing protein which gets phosphorylated by Chk1 (Z. Sun et al., 1998) serves as a mediator for activating Rad53 (**Figure 1.4**). Rad9 phosphorylation leads to oligomerization with Rad53 thereby

facilitating an in-trans hyperphosphorylation of Rad53 (Z. Sun et al., 1998). Rad53-Rad9 binding also engages a feedback loop which ensures maintenance of checkpoint signaling by releasing hyperphosphorylated Rad53 from DNA to phosphorylate its downstream targets as well as regulating damage site processing (Usui et al., 2009) (**Figure 1.4**).

At the site of a double strand break for instance, H2AX (H2A in yeast) histone variant gets phosphorylated by DNA damage response kinases to form γH2AX (Stiff et al., 2004), which serves as central docking site for downstream signaling factors to initiate repair (Rothkamm et al., 2015). Although DNA damage response differ for different types of lesions, there is an interplay of signaling molecules and pathways (reviewed in (Rothkamm et al., 2015). Thus a double strand break (DSB) can signal the recruitment of Tel1 whereas Mec1 is recruited by a broad spectrum of lesions (Gobbini et al., 2013).
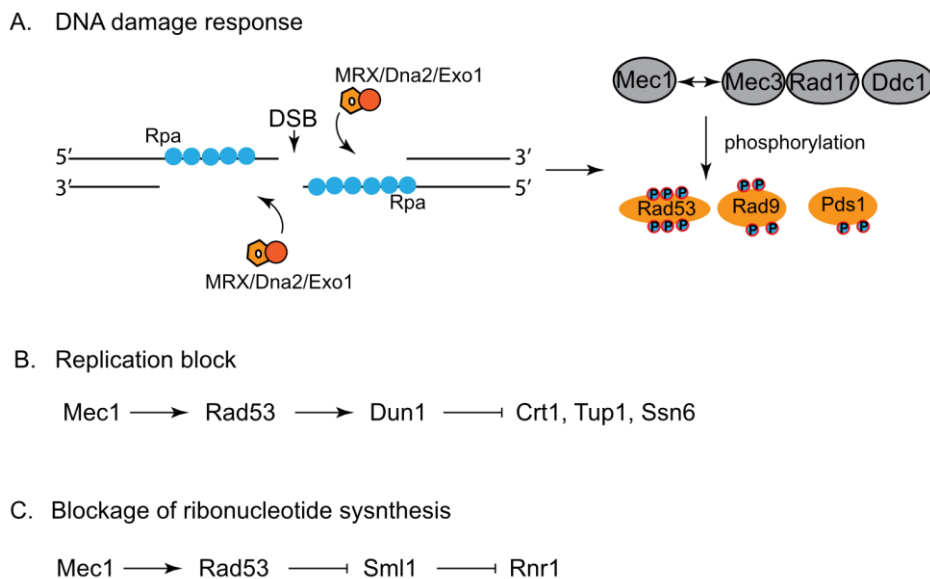
Figure 1.4: Molecular anatomy of budding yeast DNA damage checkpoint pathways.

(A) DNA damage induced G2/M arrest results from binding of Rad24, Mec3, Rad9, Rad17 and Ddc1 to single stranded DNA (ssDNA). Mec3 interacts with Ddc1 through Rad17 and recruits Mec1 leading to phosphorylation of Rad9, Pds1 and Rad53. Phosphorylated Rad53 interacts with phosphorylated Rad9 through its fork head associated (FHA) domain thereby causing a G2/M arrest. (B) Rad53 mediated

phosphorylation of Dun1 blocks DNA replication and activates transcription of DNA repair genes by inhibiting Crt1, Ssn6 and Tup1 which bind to promoters of repair genes to repress their transcription. (C) Rad53 phosphorylation by Mec1 leads to ribonucleotide reductase repression resulting in reduction in the levels of dNTPs.

1.3.1 Single strand break repair

DNA single strand breaks (SSB) usually result from the loss of a single nucleotide at a DNA damage site. Common causes of single strand break include oxidative attack of DNA by reactive oxygen species (Caldecott, 2014a), disintegration of oxidized sugar backbone of DNA and also erroneous or abortive activity of DNA polymerase 1 (J. C. Wang, 2002). Physiological repair response to SSB involves SSB detection, DNA end processing and gap filling and lastly DNA ligation [reviewed in (Caldecott, 2014a)]. SSBs produced from oxidized deoxyribose sugar are detected by poly-ADP ribose polymerase 1 (PARP1) which gets modified by the attachment of several units of poly-ADP ribose sugar (PADPr) (Caldecott, 2014b). After detection by PARP1, poly-ADP ribose glycohydrolase (PARG) degrades the PADPr attached to PARP1 thereby enabling further processing of more SSBs by PARP1 (Davidovic et al., 2001). Following SSB detection, damaged 3' and /or 5' termini of DNA SSBs are reverted to the 3'-hydroxyl and 5'-phosphate moieties prior to gap filling and ligation. This restoration is carried out by polynucleotide kinase/phosphatase (PNKP) and AP endonuclease 1 (APE1) [reviewed in (Caldecott, 2014a)]. Gap filling of missing nucleotides are filled by polymerase B whereas the final step of ligation is catalyzed by Lig1 (for short SSB repair) and Lig3α (for long SSB repair) (Caldecott, 2007).

1.3.2 Double strand break repair

Double strand breaks (DSB) are among the most lethal forms of DNA damage which can occur randomly during the cell cycle (Vilenchik et al., 2003) and are produced when two complimentary DNA strands get broken in a manner that renders chromatin structure incapable to maintain DNA ends together (Stephen P. Jackson, 2002). A DSB however, can serve as an intermediate in homologous recombination and meiotic recombination (Gobbini et al., 2013; S. E. Lee et al., 1998). DSBs are often lethal lesions, which are formed impulsively by exposure to ionizing radiations, chemotherapeutic drugs, and reactive oxygen species (ROS), therefore creating replication stress for cellular survival (S. P. Jackson et al., 2009). DSBs pose serious threat to genome instability because they could recombine within any site of the genome and therefore lead to mutations and cell death (Stephen P. Jackson, 2002). In the event of a DSB, cells mount up a concerted effort to repair, a process known as the DNA damage response (DDR) (Ceccaldi et al., 2016; S. P. Jackson et al., 2009) as discussed earlier.

In response to DSBs cells activate the PI3K-like kinase transduction cascades which involves a damage sensing protein and a protein kinase cascade which activates downstream effector pathways (Stephen P. Jackson, 2002), as discussed in the Section 1.3 above. In yeast, the MRX complex (Mre11/Rad50/Xrs1) and Yku70/80 complex are among the first proteins that localize to a DSB site (Lisby et al., 2004). This localization ensures resection of DNA and allows binding of the replication protein A (Rallabhandi et al.) single stranded DNA binding complex. A 5'-3' resection of the break site leading to RPA coating also commits repair to homologous recombination and also recruits Mec1

mediated damage response (Longhese et al., 2010). Studies in budding yeast have shown that DSB resection by the MRX complex is also initiated by Sae2 (CtIP in mammals or Ctp1 in fission yeast) which gets phosphorylated at Ser297 (Huertas et al., 2008; Ivanov et al., 1994) by CDK.

In mammalian cells a component of DNA DSB cascade involves the protein kinase ATM, which is recruited and activated at sites of DSB (Andegeko et al., 2001). Upon activation, ATM phosphorylates substrates such as p53, Chk2, BRCA1 and NSB1 (Stephen P. Jackson, 2002). In yeast, Mec1 is actively recruited at the S/G2 phase of the cell cycle by binding RPA coated single stranded DNA and its activator, Ddc2 (ATRIP in mammals) (Lee Zou et al., 2003). Mec1 is finally activated through its interaction with the heterotrimeric complex, Ddc1-Rad17-Mec3 (Rad9-Rad1-Hus1 in humans) and RFC clamp loader Rad24-Rfc2-5 (Majka et al., 2006) (**Figure 1.4 and 1.5**).
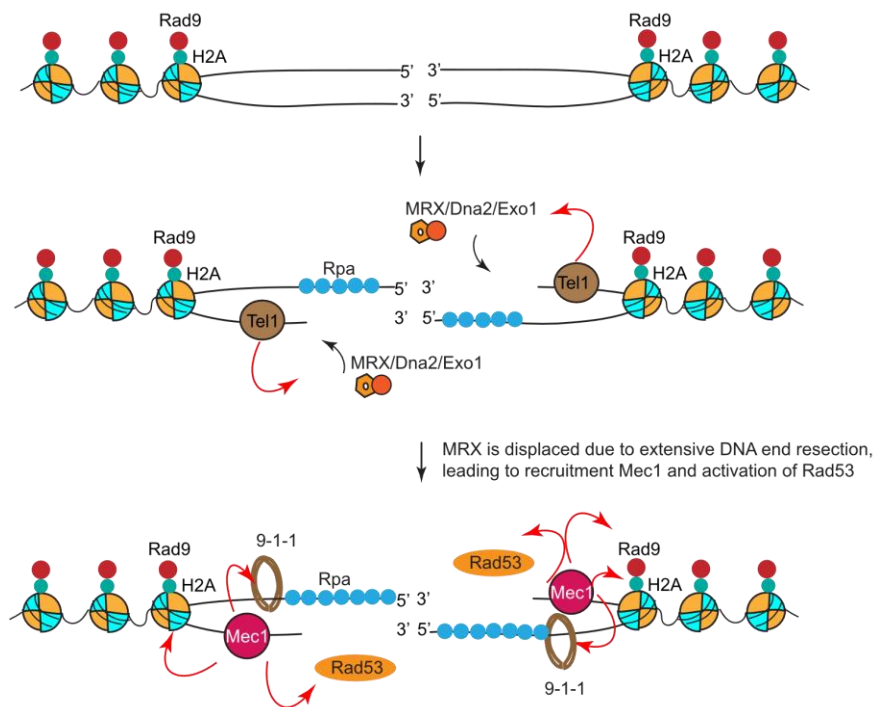
Figure 1.5: Mec1 and Tel1 checkpoint kinase dependent events at DSBs.

The MRX complex recognizes the DSB and recruits Tel1, which phosphorylates Sae2 and histone H2A (γH2A). DSB end resection is initiated through MRX, Sae2, Exo1 and Sgs1, leading to the generation of 3′-ended ssDNA tails that become coated with RPA, allowing the loading of the Mec1-Ddc2 complex. Tel1, possibly by acting on the MRX complex, promotes DSB resection, which activates Mec1 and concomitantly inhibits Tel1. Mec1 activation requires Dpb11, the 9-1-1 complex and possibly the MRX/MRN complex itself. Once recruited to the DSB, Mec1 regulates the generation of 3′-ended ssDNA by phosphorylating Sae2 and histone H2A. Mec1 also activates downstream signaling by phosphorylating Rad53 and Rad9 and Rad53 phosphorylating itself. Phosphorylated Rad9 also promotes activation of Rad53 by allowing its in-trans autophosphorylation. Activated Rad53 is then released from DNA and can regulate both DSB processing by phosphorylating and inhibiting Exo1 and its specific targets in the checkpoint cascade. Red arrows represent phosphorylation.

1.3.3 Homologous recombination

DNA DSB repair pathways include homologous recombination (HR), micro-homology-mediated end-joining (MMEJ) and non-homologous end-joining (NHEJ) which acts complimentarily to each other. The pathway chosen to repair DSBs is dependent on the nature of the break (modified or free DNA ends), the cell cycle phase and the extent of

DNA resection (**Figure 1.6**). HR acts by copying genetic information from an undamaged complimentary DNA strand while NHEJ repairs DSBs by ligating two DSBs without the requirement for homology. The ultimate goal of HR is to repair by using the homologous chromosome as a template and therefore occurs at S or G2/M phases of the cell cycle (Escribano-Díaz et al., 2013). End resection is a major determinant of HR activation. At DSB sites, HR is initiated through immediate 3'-5' single stranded DNA processing (short range) by the MRX complex. Other nucleases recruited to sites of resection include Exo1 and Sgs1, however they are involved in 5'-3' resection (long range resection). Recruitment of MRX depends on Sae2, which is phosphorylated by Cdk1 to initiate resection.
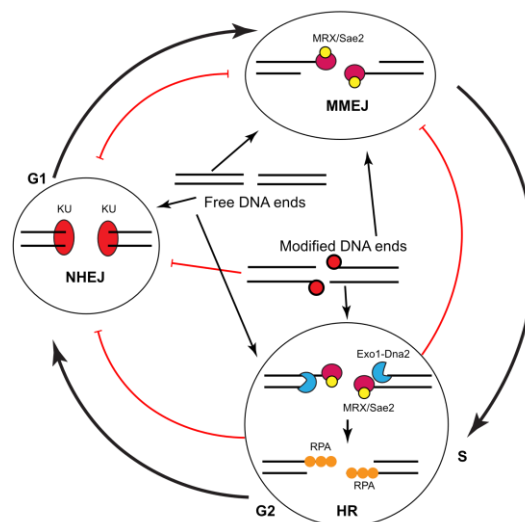
Figure 1.6: DSB repair pathway choice.

The nature of the DNA break site determines the repair pathway. Modified ends (indicated by red star) inhibits NHEJ but commits repair to MMEJ or HR. Free DNA ends commit repair to any of the pathways. NHEJ repair occurs mostly at G1 whereas HR could occur at S and G2/M phases. MMEJ can occur throughout the cell cycle. Negative interactions are shown by red arrows; positive interactions are shown by black arrows; grey arrows represent cell cycle.

1.3.4 Non-homologous end-joining in budding yeast

DSBs arise from insults which compromise the integrity of DNA and can lead to mutations if unrepaired. As previously reported, homologous recombination is the safest mechanism of repairing DSBs when a homologous template is available during S phase or mitosis, however, cells choose to rely on other mechanisms when a template is not available. NHEJ is thus one of the commonly used mechanisms of repairing DSBs by re-ligating two break sites however, other NHEJ events require processing of DSB ends prior to re-ligation (Daley et al., 2005). As described in Figure 6, NHEJ is primarily activated in G1 due to the absence of a sister chromosome, however, it can occur at any stage of the cell cycle (Sonoda et al., 2006; Symington et al., 2011). DSB events that necessitate repair by NHEJ are characterized by blunt ends and are repaired in three steps; synapsis, DNA end processing and ligation of broken DNA ends.

In most DNA break repair, end resection is critical and involves a set of protein complexes (Table 1). Ku70/80 and the MRX complex are recruited to the break site shortly after damage to initiate resection and bridging of the two DNA strands (Daley et al., 2005). Ku70/80 is also thought to be an early sensor at break sites by forming a Ku:DNA complex that serves as a docking platform for polymerases and kinases to associate and repair the break (Lieber, 2008). Ku is arguably the most striking component of the NHEJ core proteins because it is conserved from bacteria to higher organisms (Doherty et al., 2001). It exists as a heterodimer and has a unique β barrel crystal structure that is conserved in yeast and in human (Walker et al., 2001). Ku is able to bind and slip DNA ends through the barrel in only one orientation. Necessary for NHEJ activity is the MRX complex (Mre11:Rad50:Xrs2) which is the only component with defined roles in homology directed

repair. Rad50 belongs to the structural maintenance of chromosome (SMC) family proteins, and thus hydrolyses ATP at its ATPase domain to serve as a tether that bridges two DNA strands together. Together with Xrs2 and Mre11, an endonuclease, the MRX heterodimer forms a complex with DNA to efficiently repair DSBs through the NHEJ pathway by resecting the DNA ends. In vertebrates, Artemis: DNA-PKc complex performs endo-nucleolytic resection on DNA ends (Lieber, 2010). After successful tethering of DNA ends, binding of Dnl4 and Lif1 is initiated to promote ligation of the annealed overhangs. Lastly, Pol4 polymerizes and seals off the DNA ends. (Lieber, 2008).

Table 1.1: Protein components of the NHEJ pathway

Functional component	Prokaryotes	Budding yeast	Multicellular eukaryotes
Protein complex	Ku	Ku70/80	Ku70/80
Polymerase	Pol domain of LigD	Pol4	Pol μ and Pol λ
Nuclease	Uncertain	Mre11:Rad50:Xrs2	Artemis:DNA-PK
Kinase/Phosphatase	Phosphoesterase domain of LigD	Tpp1 and others	PNK and others
Ligase	Ligase domain of LigD	Nej1:Lif1:Dnl4	XLF:XRCC4:DNA ligase IV

Adapted from (*Gu et al., 2008*)

1.3.5 Fanconi anemia pathway

Fanconi anemia (FA) is a rare genetic disease in which patients suffering from this condition are predisposed to extreme sensitivity to agents which induce inter-strand crosslinks (ICL). Most FA patients show developmental abnormalities, early bone marrow failure and acute myeloid leukemia (AML). As the disease progresses, these patients

display an increased risk of developing head, neck, and anogenital region carcinomas (Dong et al., 2015). Genomic instability is therefore common in these patients and has been attributed to mutations in 19 genes, characterized under the FA pathway. The FA pathway genes, FANC A, B, C, D1, D2, E, F, G, H, I, J, L, M, N, O, P, Q, R, S and T, have also been characterized in other organisms (Dong et al., 2015; L. C. Wang et al., 2010).

ICLs are particularly toxic DNA lesions which arise between DNA strands and have the tendency to block DNA transactions such as replication and transcription (L. C. Wang et al., 2010).

ICL repair in higher eukaryotes remains enigmatic, however, there exist some similarities in the repair pathway choice in yeast and higher eukaryotes. DSBs arising from an ICL is repaired by HR and replication-dependent ICL repair (L. C. Wang et al., 2010). However, there is evidence suggesting that ICL repair could also exist in a replication-independent manner which relies on error-prone repair pathways (H. Zheng et al., 2003). The hypersensitivity of FA patients to crosslinking agents directly indicates the importance of the FA pathway genes in sensing and repairing lesions arising from crosslinking agents. Replication-dependent ICL repair makes use of the error-free HR pathway to remove DNA crosslinks whereas NER (nucleotide excision repair) or translesion repair synthesis (TLS) repairs ICL during the G1/G0 phase.

Recognition and repair of ICLs has been attributed to the FA pathway. Elegant work in Xenopus and mammalian cells shows that ICL induces an FA pathway dependent ATR checkpoint signal supporting the role of FA pathway in ICL repair (Ben-Yehoyada et al., 2009). Further, inactivation of the FA pathway reduces the efficiency of ICL repair. Aside from the characterized roles of the FA pathway in ICL repair, there is growing evidence in

higher vertebrates and yeast of their implication in the DNA damage response (Deans et al., 2009; Mathew, 2006). The FA core complex comprising of eight FANC complex proteins and three non-FA proteins are activated during DNA damage (Dong et al., 2015). Their translocation to sites of DNA damage is mediated by UBE2T dependent mono-ubiquitination of the FANCD2/I complex and the interaction with BRCA1/2 and Rad51 proteins. Consistent with this evidence, FANCD deficient cells exhibit defects in activating ATM kinase after doses of ionizing radiation (Castillo et al., 2011). Interestingly, FA patients with deficiencies in BRCA2 or FANCD show severe clinical phenotype than other FA phenotypes (Hirsch et al., 2004; Howlett et al., 2002).

1.3.6 Nucleotide excision repair

Nucleotide excision repair (NER) is committed to removal of DNA adducts that are generated through the chemical reaction of ultraviolet (UV) light or certain carcinogens with DNA. Lesions generating from NER sources could be classified as global genome wide (GG- NER) or transcription coupled (TC-NER) which are typically caused by RNA polymerase stalling at gene transcription sites (Marteijn et al., 2014). In human, mutations in about 40 NER repair genes have been correlated with NER associated diseases (Ferri et al., 2020). NER is a very important pathway that contributes to the removal of bulky DNA lesions including cyclobutane-pyrimidine dimers (CPDs) and 6-4 pyrimidine -pyrimidone photoproducts (6-4PPs) which could also be generated by UV radiation (Marteijn et al., 2014).

The main damage sensor for GG-NER involves XPC (Rad4 in budding yeast), Rad23 (HR23 in vertebrates) and Centrin 2 (CETN2). Based on the crystal structure of yeast Rad4 complexed to ssDNA containing CPD lesions (Min et al.), XPC scans DNA for bulky DNA

lesions by probing with its carboxy-terminal double β-hairpin between the dsDNA and ssDNA interface. Additional repair factors such as DDB1 and DDB2 (makes up the UV-DNA damage binding (DDB) protein complex) binds and recruits more XPC to the lesion (Wakasugi et al). Recognition of CPD or 6-4PP lesions by UV-DDB also requires association with CRL (cullins 4A regulator of cullins 1 E3 ubiquitin ligase) complex (Groisman et al 2003).

After XPC recruitment to the site of lesion, two helicases, XPB and XPD of the TFIIH complex opens DNA to verify to the lesion (Compe et al., 2012). The lesion is excised by site specific endonucleases, XPF and XPG which cleave DNA at the 5' and 3' end respectively, leaving about 20-30 bp overhangs which activate DNA damage response (Fagbemi et al., 2011).

1.4 DNA protein cross-link

DNA-protein crosslink (DPC) is the covalent attachment of a protein to DNA resulting in a covalent intermediate which is capable of affecting the processing of DNA for other physiological activities. DPCs can block processes such as gene transcription and DNA replication (Nakano et al., 2013) and lead to large adducts that are lethal for cells if not repaired. Due to the bulkiness of DPCs they are usually not able to be repaired by canonical repair pathways, thus cells have specialized repair mechanisms responsible for their removal. The architecture of a DPC involves a protein moiety, which is covalently bound to DNA by strong covalent bonds. Repair of DPCs could be achieved by targeting each of the three components of a DPC. DPCs are classified based on their origin and nature and can be grouped as enzymatic, non-enzymatic but covalently trapped proteins (Stingele et

al., 2017; Stingele et al., 2015). Based on the source of metabolites that crosslink proteins to DNA, DPCs could be formed by UV light, ionic radiation as well as platinum-based chemotherapeutic drugs such as cisplatin (Barker et al., 2005; Stingele et al., 2015). The specific mechanistic detail of the various DPC causal agents will be discussed in the text below.

1.4.1 DPC induction by endogenous agents

DPCs are transiently created endogenously through the physiological entrapment of topoisomerases by intercalation of enzyme-DNA boundary. Topoisomerases are DNA binding proteins whose major function is to reduce DNA super-coiling. Topoisomerases perform this function by creating a nick in DNA and religating the nick, however inherent errors in the enzymatic activity of topoisomerases can result in the inability to reduce DNA topological stress, leading to trapping on DNA. Morphological changes within the structure of DNA can also favor covalent linkage of topoisomerases and as a result, creating a stable covalent intermediate as a DPC adduct (Pommier et al., 2014). Endogenous metabolites such as aldehydes produced from alcohol metabolism or histone demethylation (Shi et al., 2004) are able to also trigger crosslinking of proteins to DNA in a manner that creates DPCs [reviewed in (Barker et al., 2005)].

1.4.2 DPC induction by exogenous sources

DPCs can be formed by exogenous compounds such chemotherapeutic drugs, exposure to ionizing radiation, acetaldehyde [reviewed in (Barker et al., 2005; Stingele et al., 2017)]. Most of these agents have the tendency to cause both enzymatic and non-enzymatic DPCs.

Platinum-based chemotherapeutics such as cisplatin orchestrate a DPC through non-specific crosslinking of chromatin interacting proteins to DNA (Chvalova et al., 2007). Other chemotherapeutics such as Camptothecin and Etoposide act by creating an irreversible pseudo-substrate for topoisomerase 1 and topoisomerase 2 respectively, which upon binding leads to their entrapment and a subsequently halt in DNA replication (Nitiss, 2009). In contrast to the activity of topoisomerase poisons such as Camptothecin and Etoposide, 5-aza-2`-deoxycytidine which is used as a drug for myelodysplastic syndrome, acts as an analogue of cytosine and thus serves as a pseudo-substrate resulting in the enzymatic trapping of DNA methyltransferase 1 (Maslov et al., 2012).

Aside from chemotherapeutic drugs, chemicals like formaldehyde have been widely used in chromatin immunoprecipitation due to its ability to crosslink proteins to DNA. Formaldehyde reacts with amino and imino groups of proteins as well as DNA to form a Schiff base which then reacts with other amino groups (McGhee et al., 1975).

1.5 DPC repair mechanisms

Studies in yeast have shown how nucleotide excision repair (NER) removes formaldehyde-induced DPCs. Though there is ongoing research in unraveling how cells tolerate and repair DPCs current research findings suggest factors that are implicated in DPC repair. The current model for DPC repair suggests repair of the protein, DNA and covalent bond components by a protease, DNA endonuclease and phosphodiesterase respectively. However, the remaining protein remnants crosslinked to DNA may require a translesion synthesis polymerase to allow DNA replication to continue, albeit leading to mutations and genomic instability [reviewed in (Stingele et al., 2015)].

1.5.1 Tyrosyl-DNA phosphodiesterase 1

Tyrosyl-DNA phosphodiesterase 1 (Tdp1) is a highly conserved protein that was originally discovered in budding yeast (S. W. Yang et al., 1996). Tdp1 catalyzes the removal of topoisomerase 1 (Top1) bound to the 3'-end of DNA at its active site tyrosine residue. Top1 has the capacity to relieve topological stress in DNA by creating an interval in DNA and religating the break. Top1 forms a reversible phosphodiester linkage between DNA and its specific tyrosine residue, however, in the presence of topoisomerase inhibitors or imperfections in its normal function on DNA (Yeh et al., 1994), the re-ligating activity of topoisomerase 1 is blocked leading to the covalent attachment of the enzyme to DNA. The activity of Tdp1 in phosphodiester linkage repair is modulated by posttranslational modification of its amino-terminus which thereby influences the localization and enzymatic activity of the enzyme (Stingele et al., 2017). Tdp1 is also attracted to sites of DNA damage by sumoylation of its lysine 111 residue (Hudson et al., 2012). Not only is Tdp1 involved in DPC removal, but also the removal of Top1 intermediates from a stalled replication fork during DNA replication (Vance et al., 2002). This discovery makes Tdp1 important for removing damage lesions and has been described as a neuroprotective enzyme, whose absence predisposes to spinocerebellar ataxia with axonal neuropathy (SCAN1) in post-mitotic neuronal cells (Takashima et al., 2002).

1.5.2 Wss1

Wss1 was originally identified as a weak suppressor of Smt3 in yeast (Biggins et al., 2001). Though, not an essential gene, Wss1 has been found to be involved in multicellular

functions including DNA damage response, sumoylation (O'Neill et al., 2004a) as well as providing tolerance to replication stress (Maddi et al., 2020). Wss1 protein is a 30Kd size protein which possesses conserved WLM domain, two putative SUMO interacting motifs (SIM) and two other domains, VCP and SHP that bind to AAA-ATPase and Cdc48 respectively. The WLM domain is highly conserved among proteases of the minigluzincin family including the human DPC repair protease, SPRTN. The WLM domain's zinc binding site follows the conserved HEXXH motif and harbors Wss1's metalloprotease activity. The catalytic activity of Wss1 is mediated by its glutamate (E) amino acid residue at position 116. In exploring the DNA protein crosslink repair capabilities of Wss1, it was reported that single stranded DNA-binding activates the catalytic activity of Wss1 (Stingele et al., 2014) however this function is mediated by a cysteine switch (Balakirev et al., 2015), which seems to be a control mechanism to regulate DPC repair proteases (Stingele et al., 2017). To elucidate the mechanism of DPC repair by Wss1, further experiments revealed that Wss1 forms a complex with Cdc48 and its adaptor protein, Doa1 in removing chromatin-bound SUMOylated proteins (Balakirev et al., 2015). There is recent evidence suggesting the role of SUMOylation in the removal of chromatin bound proteins (Borgermann et al., 2019; K. C. Lee et al., 2018; Schellenberg et al., 2017). Several SUMOylated protein targets were identified after formaldehyde dependent cross-linking of proteins. Most of the identified proteins control important metabolic processes such as DNA replication, DNA damage checkpoint and sister chromatid cohesion (Borgermann et al., 2019). These findings support the hypothesis SUMO-dependent Wss1 protease may possess the ability to cleave chromatin-bound SUMOylated proteins. Even though there is agreement in literature that Wss1 binds to SUMOylated proteins (Balakirev et al., 2015;

Stingele et al., 2014), it still remains unknown how it identifies SUMOylated proteins and what SUMO E3 ligase recruits Wss1 in its role in DNA damage response.

Genetic and biochemical data from our work validates Siz1 and Mms21 SUMO E3 ligases to be important for DPC repair in yeast and corroborates the finding that cells employ the SUMO pathway to respond to cues to remove DPCs from chromatin (unpublished data).

1.5.3 SPRTN

After the discovery of Wss1, three groups identified SPRTN as the dedicated mammalian metalloprotease for DPC degradation (Lopez-Mosqueda et al., 2016; Stingele et al., 2016; B. Vaz et al., 2016). Even though Wss1 is non-essential in yeast, SPRTN, a homolog of Wss1, is an essential gene in vertebrates and is necessary for embryonic development. Hypomorphic mutations of SPRTN in humans leads to Ruij-Aalfs syndrome (RJALS), hypersensitivity to DPC-inducing drugs, genomic instability and hepatocellular carcinoma (Lessel et al., 2014). Furthermore, depletion of SPRTN in mice induces a RJALS-like phenotype (Maskey et al., 2014).

 In vitro characterization of the role of SPRTN in DPC proteolysis indicated that SPRTN is directly activated at stalled replication forks (Larsen et al., 2018). Consistent with this evidence, depletion of SPRTN in mammalian cells resulted in reduced replication fork progression under physiological conditions (Halder et al., 2019) and in the presence of formaldehyde-induced DPCs (Bruno Vaz et al., 2016). Recently, it was reported that SPRTN is recruited to chromatin bound substrates through an ATR-Chk1 phosphorylation loop (Halder et al., 2019). In this model, SPRTN cleaves C-terminal region of Chk1 (inhibitory domain) leading to N-terminal Chk1-mediated phosphorylation of SPRTN and

subsequent recruitment to chromatin. Recent evidence in yeast and mammalian cells also implicated the SUMO pathway in DPC proteolysis (Y. Sun et al., 2019), indicating a mechanism by which SPRTN contributes to genome maintenance, either through a SUMO (Bruno Vaz et al., 2020) or proteasome mediated repair (Larsen et al., 2018). These findings point to the important role SPRTN plays in maintaining genomic stability.

1.5.4 ACRC/GCNA

GCNA (germ cell nuclear antigen), also known as ACRC (acid repeat containing protein), can be detected in pluripotent cells of the earliest eukaryotes (Carmell et al., 2016). The expression of GCNA is particularly prominent in human testes likely because of its role in maintaining genome stability in germ cells. Preliminary analysis suggests GCNA may have a similar function as SPRTN in removing DPCs. In *C. elegans,* GCNA mutants are sensitive to DNA damage and show increased mutational rate, which can carry to the next generations (Davis, 2019). These evidences suggest that GCNA plays an important role in maintaining genomic integrity in early embryos and germline cells.

1.5.5 Ddi1

More recently, Ddi1 was implicated to function in parallel to Wss1 for providing replication stress tolerance (M. Svoboda et al., 2019). Ddi1 is an aspartic protease that serves as a shuttle factor for the proteasome. It was initially discovered as a suppressor to *wss1 tdp1* double mutant in yeast, defective in processing topoisomerase induced DPCs. This study provides evidence that Ddi1 removes covalently trapped DPCs in an S-phase dependent manner (Serbyn et al., 2020). Mass spectrophotometry analysis in yeast also

indicated a fold enrichment of Ddi1 in a wss1 mutant upon hydroxyurea induced replication stress (Maddi et al., 2020).

1.6 The SMC complex

DNA in living organisms are usually longer if stretched across their entire length. Cells are able to pack these long pieces of DNA into a relatively small space in the nucleus. How cells pack DNA and maintain chromosomes is defined by the role of structural protein complexes known as the structural maintenance of chromosomes (SMC) complex. These proteins are conserved from bacteria to human (T. Hirano et al., 1994; Strunnikov et al., 1993) and are known to contribute to chromosome compaction, sister chromatid cohesion, DNA repair/ recombination. SMC proteins were initially identified as a result of defects in chromosomal segregation and condensation, hence their name.

The SMC complex is a large dimeric protein complex distinctly recognized by their characteristic ring shape (Anderson et al., 2002; Haering et al., 2002; Frank Uhlmann, 2016). SMC proteins have a long intermolecular coiled-coil region which dimerize to form a hinge. Each monomeric SMC protein has an N and C-terminal portion, also known as the Walker A and B motifs, respectively. The Walker A and B motifs constitute the head region, which harbors an ATP binding cassette (ABC) family ATPase domain. Structural analysis reveals that ATP hydrolysis by the head region bridges two SMC proteins together and contributes to their structural function on DNA (Lammens et al., 2004).

Except in bacteria which forms only one homodimer SMC complex (Graumann et al., 2009; M. Hirano et al., 2002), eukaryotes possess 6 different SMC proteins which form heterodimer complexes to exert their function. SMC1/3 complex, also known as Cohesin,

is important for chromosome compaction during G1 phase. SMC2/4 complex makes up condensin, which is widely known for its role in chromosome compaction and resolution.

1.6.1 Cohesin

Cohesin is known to form a link between sister chromatids until segregation during cell division. Its evolutionary conserved role is facilitated by its multi-subunit complex (**Figure 1.7**) made up of SMC units (SMC1/3), Kleisin subunit (Scc1- also known as Rad21/Mcd1) and HEAT subunits (Scc3 and Pds5). Together with Wapl and Sororin (only found in vertebrates), cohesin collectively forms a ring-like complex that physically holds sister chromatids in a phenomenon known as topological embrace (Frank Uhlmann, 2016). The physical force exerted on sister chromatids ensures that chromosomes are not duplicated prematurely prior to anaphase.

The coiled-coil domain of Smc1/3 forms a heterodimer which is stabilized at the head region by Scc1 (Haering et al., 2002; F. Uhlmann et al., 1998). Thus, the ring-like complex formed on the sister chromatid generates a force that keeps sister chromatids together. Experiments in yeast revealed that scc1 mutants abrogate alignment of chromatids leading to premature segregation at the metaphase plate (Tóth et al., 1999; F. Uhlmann et al., 1998). In a screen for proteins responsible for proper segregation of chromosomes (Tóth et al., 1999), the SMC1 and 3 and its loading factors, Scc2 and Scc4 were identified to be required for proper loading and establishment of cohesion. Absence of Scc2 and Scc4 leads to loss of cohesion, however mutations in either Scc2 or Scc4 does not affect the assembly of cohesion complex, suggesting that the Scc2 and Scc4 are only needed for the initial loading of cohesin unto chromosomes (Ciosk et al., 2000). In Xenopus egg extracts (Gillespie et

al., 2004; Takahashi et al., 2008), Scc2/Scc4 dependent loading of cohesion requires MCM2 and DDK at pre-RC, however, recent evidence in yeast (Hinshaw et al., 2017) and mammalian cells (G. Zheng et al., 2018) indicate that the phosphorylation of DDK similarly recruits Scc2 and Scc4. This activity also requires MCM2, suggesting that the requirement of replisome components for cohesion is conserved across multiple organisms. Recent evidence suggests that cohesin is loaded in a chromatin remodeling complex dependent manner, thus eviction of histones precedes the successful loading of cohesion (Muñoz et al., 2019).

Due to their role in chromosome maintenance, SMC complexes are known to physically attach at centromeres (D'Ambrosio et al., 2008). Cohesin is known to bind to several other locations on yeast DNA, however they are highly enriched at centromeres (Blat et al., 1999; T. Tanaka et al., 2000), which is topologically advantageous, given that the force exerted by mitotic spindles are strongest here. The presence of cohesin at centromere has been shown to be responsible for sister centromere re-attachment during chromatid splitting (T. Tanaka et al., 2000).

Establishment of cohesion on chromosomes is facilitated by Wap1, whose association favors repeated cohesion loading and unloading during S phase. By depleting cells of Wap1, cohesin assumes a condensin-like role due to the appearance of chromatin condensation in interphase chromosomes (Tedeschi et al., 2013). The maintenance of this configuration on cohesion is facilitated by the acetylation of two lysine residues in Smc3 (K106,105 in human, K112,113 in budding yeast) by Eco1 acetyltransferase (Zhang et al., 2008). Loss of acetylation of Smc3 is consistent with an increase in sister chromatid separation. Acetylation prevents the ATP hydrolysis of the ATPase domain of Smc3, thus

locking the cohesion ring in position on DNA.
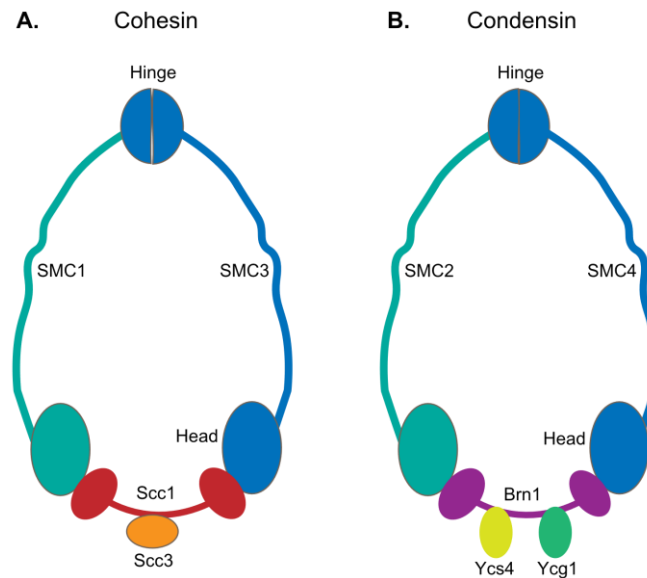
Figure 1.7: Cohesin and condensin ring-like structures.

Cohesin (A) protein complexes involve SMC1 and SMC, which interact at the hinge region as a heterodimer. They form a ring-like structure at their head region by interacting with Rad21 (Scc1 in budding yeast- kleisin subunit) as well as Scc3 (SA in invertebrates) and Pds5 (not shown). Condensin (B) forms a heterodimer at the hinge and interacts with CADH2 (Brn1 in budding yeast), CAPD3 (Ycs4) and CAPG2 (Ycg1). SA-stromal antigen; SMC-structural maintenance of chromosome; CAP- condensin associated protein.

1.6.2 Condensin

Condensin, similar to cohesion is an evolutionarily conserved protein complex from bacteria to humans (T. Hirano et al., 1994). Its main function is to organize and condense chromosomes during meiosis and mitosis by folding high order chromatin fibers into compact chromosomes. In-vitro evidence suggests that condensin purified from *Xenopus* eggs reconfigures DNA in an ATP-dependent manner by remodeling circular DNA into supercoils (Kimura et al., 1997). Inactivation of condensin from bacteria to humans leads to failure in compacting chromosomes as wells as the formation of chromosome bridges due to failure in resolving separating chromosomes (Frank Uhlmann, 2016).

Condensin is a 5-unit protein complex (**Figure 1.7**) made up of heterodimers of SMC2 and SMC4, a kleisin subunit, Brn1, and two HEAT subunits, Ycs4 and Ycg1 (Frank Uhlmann, 2016). There are two forms of condensin, condensin 1 and 2 in multicellular organisms. Data from high resolution maps indicate localization of condensin at promoter sites, 3'-ends of transcribed genes as well as at topologically associated domains (TADs) and transcription factor IIIC binding sites (Sutani et al., 2015; Yuen et al., 2018).

Our current understanding of how condensin contributes to genome organization is explained by two models. Support for the loop extrusion model comes from the discovery that condensin translocates while it extrudes DNA into loops (Terakawa et al., 2017), however real time imaging of yeast condensin provided convincing evidence that condensin compacts DNA through an ATP-mediated loop extrusion in an asymmetrical manner (Ganji et al., 2018).

The second model has gained support from the observation that condensin serves as a crosslinker in the presence of topoisomerases, by forming 10nm fibers at condensin binding sites (Cuylen et al., 2011; Thadani et al., 2012).

Aside its role in chromosome condensation and resolution, condensin is also recruited to sites of DNA damage similar to cohesin and SMC5/6 complex (D'Ambrosio et al., 2008).

1.6.3 SMC5/6 complex

The SMC5/6 complex (**Figure 1.8**) is an essential protein complex found in eukaryotic organisms. Its importance is exemplified in its role in DNA repair. It was initially discovered in fission yeast (Lehmann et al., 1995) on the basis that a rad18 (SMC6) mutant is sensitive to ultraviolet and gamma radiations and that Rad18 forms a heterodimeric

interaction with Spr18 (SMC5), but has since been characterized in humans (Taylor et al., 2001), plants (Watanabe et al., 2009), *X. laevis* (Tsuyama et al., 2006) and budding yeast (Fujioka et al., 2002). Since its initial discovery, other components of the complex known as nonstructural elements (NSEs;1-6) have been characterized (Sergeant et al., 2005; Zhao et al., 2005). Nse1, which interacts with the complex is described to be a ubiquitin ligase (McDonald et al., 2003) whereas Nse2 (Mms21 in budding yeast) is an SP-RING SUMO E3 ligase which is known to SUMOylate SMC5 as well as SMC5/6 substrates in response to DNA damage (Bermúdez-López et al., 2015; Zhao et al., 2005). Nse1 and Nse3 form a winged helix domain that is capable of forming homo and heterodimers with the complex (Palecek et al., 2006). In addition, they bind to Nse4, a kleisin subunit that bridges SMC5 and 6 ATPase heads (Palecek et al., 2006). Nse5 and 6 are heterodimers that contain HEAT repeats which allow them to form scaffolds with other substrates. Nse5 and 6 are known to bind to the head (Palecek et al., 2006) (in budding yeast) and hinge (fission yeast) regions of SMC5/6 (Xinyuan Duan et al., 2009).
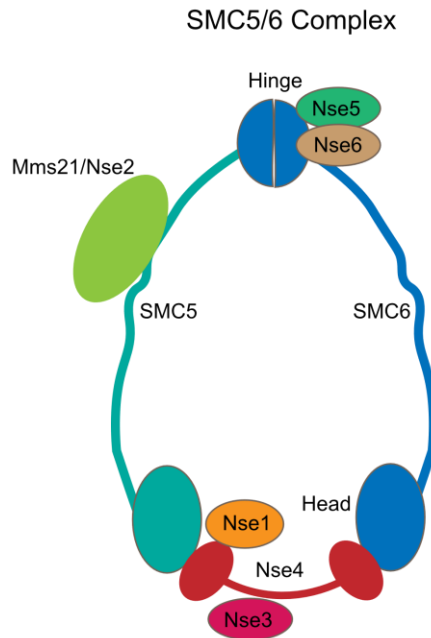
Figure 1.8. Architecture of the SMC5/6 complex.

The SMC5/6 complex is made up of 8 proteins that form three sub complexes; SMC5-SMC6-Nse2, Nse1-Nse3-Nse4 and Nse5-Nse6 subcomplexes. The interaction of Nse5-Nse6 with the SMC heterodimer is species specific.

1.7 The role of SMC5/6 in DNA damage repair

The SMC5/6 complex is widely known to be activated during DNA damage. In experiments that led to its discovery, hypomorphic allele mutants of SMC5 and SMC6 showed gross sensitivity to a wide range of genotoxic agents (Onoda et al., 2004; Sánchez et al., 2015; Watanabe et al., 2009). Besides Nse2 was also identified due to the sensitivity of nse2 mutants to methyl methane sulfonate (MMS) (Prakash et al., 1977). Taken together, the SMC5/6 complex has been associated with the DNA damage repair pathway. Epistatic analysis with Rad51 and Rad52 epistatic group also proved that the SMC5/6 complex is indispensable in homologous recombination (HR) (Lehmann et al., 1995), with defects in sister chromatin HR found in multiple organisms (Potts et al., 2006; Stephan et al., 2011;

Watanabe et al., 2009). The role of SMC5/6 complex in HR has been further supported by chromatin immunoprecipitation based assays. It has been shown that the complex is recruited to sites of HO-induced DSBs in budding yeast as well as to I-SceI induced DSB sites in human (Lindroos et al., 2006; Potts et al., 2006). The localization of SMC5/6 to sites of DSB is not entirely understood but has been determined to be Mre11-dependent (Lindroos et al., 2006).

In undamaged cells, SMC5/6 is known to localize with cohesin (SMC1/3), however this co-localization is ablated in Scc1 (cohesin subunit) and Scc2 (chromatin loader) mutants (Lindroos et al., 2006), therefore, suggesting the possible role in holding sister chromatids together during DNA replication. One possible explanation for the role of the SMC5/6 complex in DSB repair could be the recruitment of SMC1/3 to hold broken chromosome up and facilitate sister chromatid cohesion [Reviewed in (Potts, 2009)].

SUMOylation is thought to be an essential process that mediates DNA damage repair because mutations in E1, E2, Nse2 (Mms21) or SUMO are known to induce genotoxic stress. Hypomorphic alleles of Mms21 defunct in its E3 ligase activity (unpublished data) or binding to SMC5 (X. Duan et al., 2009) have also been shown to induce sensitivity to various DNA damaging agents, suggesting the importance of the SMC5/6 complex in DNA damage repair. A recent report also indicates that SUMOylation of SMC5 during DNA damage activates the by-pass of error-prone translesion synthesis repair (Zapatka et al., 2019). They showed that an smc5-KR hypomorphic allele which cannot be SUMOylated displays high translesion synthesis-induced mutation rate as well as gross chromosomal disjunction.

Aside the dynamic function of the SMC5/6 complex in DNA damage repair, there are

several other roles which have been identified to be dependent on SMC5/6 functional activity [Reviewed in (Aragón, 2018)]. Among these roles include the maintenance of telomere integrity. The SMC5-SMC6-Nse2 subcomplex is known to play a role in telomere biology. SMC5/6 mutants in yeast and human cells show marked defects in telomere maintenance (Zhao et al., 2005) and resort to alternative lengthening of telomeres (ALT) (Potts et al., 2007), respectively. About 10-15 % of human cancers possess ALT activity (Cesare et al., 2010). Nse2 SUMOylates telomere end-protection proteins such as TRF1, TRF2 and RAP1, thereby aiding their recruitment to ALT-associated PML bodies which are also SUMO substrates of Nse2. In ALT cells with ablated SMC5/6, there is increased telomere shortening and cell senescence, indicating their importance in ensuring stable maintenance of telomeric repeats in ALT cells (Potts et al., 2007). In budding yeast, the SMC5/6 complex is however required for the aggregation and clustering of telomere in the nucleus (Zhao et al., 2005).

1.8 The SUMO pathway

SUMOylation is a reversible protein modification that occurs on proteins leading to cellular localization of the modified proteins. SUMO (Smt3 in yeast) was identified in a budding yeast genetic screen in an attempt to obtain suppressors for Mif2 (Meluh et al., 1995). Since its discovery, the consequences of SUMOylation and the molecular mechanisms has been fully characterized however, it is impossible to predict since modification could alter localization, stability or activity (Geiss-Friedlander et al., 2007).

The human genome and other vertebrates have more than one SUMO genes whereas budding yeast and other organisms have a single SUMO gene however, SUMO is conserved in eukaryotic organisms (Geiss-Friedlander et al., 2007). All SUMO proteins

are expressed in an immature form after which a stretch of amino acids in the C-terminal end is cleaved to reveal di-glycine (Gly-Gly) residues which remain on the mature SUMO protein. SUMO is an essential gene in most organisms except in fission yeast (K. Tanaka et al., 1999).

1.8.1 The mechanisms of reversible SUMOylation

Like ubiquitination, sumoylation proceeds in a three-cascade reaction where SUMO is attached through its Gly-Gly residue to the epsilon amino group of the lysine residue in a target protein (**Figure 1.9**). The first step is catalyzed by an E1 activating enzyme leading to a SUMO-E1 thioester linkage, which occurs on a C-terminal cysteine residue (E. S. Johnson et al., 1997). Next, an E2 conjugating enzyme transfers SUMO from the E1 to its C-terminal end through a thioester bond (Erica S. Johnson et al., 1997). Finally, SUMO is put onto a substrate through the facilitation of an E3 ligase. The E3 ligase catalyzes the formation of an isopeptide bond between the Gly-Gly residue of SUMO and an acceptor lysine on the target protein.

The largest class of E3 ligases possess an SP-RING motif, which confers their ligase activity (Hochstrasser, 2001). SP-RING type E3 ligases bind their substrate and E2 directly in order to transfer SUMO, a characteristic feature which is unique to this class of E3 ligases. A sub-class of SP-RING ligases are called PIAS (protein inhibitor of activated STAT), which are characterized by the presence of SAP domain (binds to DNA) and SUMO interacting motif (SIM), in addition to the SP-RING motif (Sharrocks, 2006).
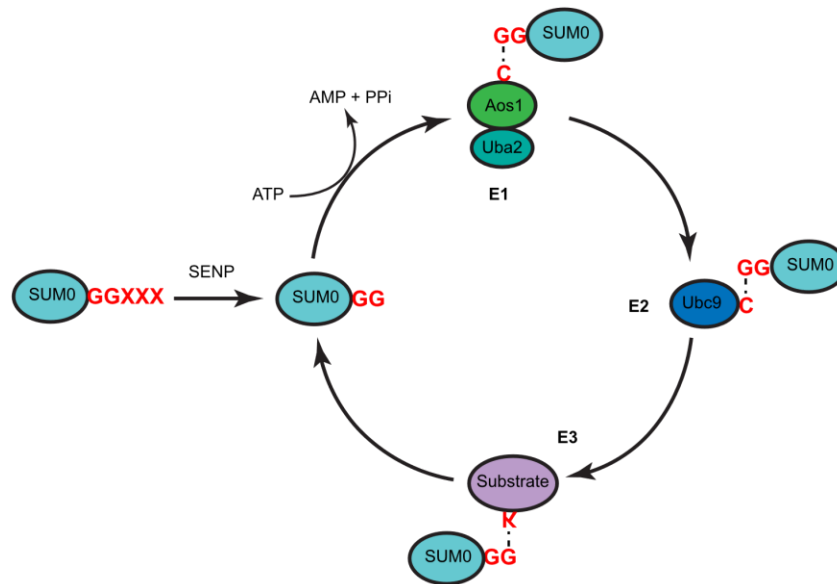
Figure 1.9: The SUMO cycle.

Before a new SUMO modifier can be conjugated to a substrate, it first off requires cleavage by SUMO-specific isopeptidases to remove C-terminal amino acids to reveal di-glycine residues. The mature SUMO is then bound by an activation enzyme (E1) in an ATP-dependent manner through a thioester bind linkage. An E2 (SUMO conjugating enzyme) transfers SUMO from the E1 unto its catalytic cysteine residue. In the presence of an E3 ligase, SUMO is linked to a lysine residue on the target protein through an isopeptide bond.

1.8.2 SUMO acceptor sites/ consensus sequences

Mapping out several modified targets (RanGAP1, PML, Sp100, p53 and c-Jun) revealed a conserved SUMO consensus sequence (Kamitani et al., 1998; Mahajan et al., 1998; Müller et al., 2000; Sternsdorf et al., 1999). A general SUMO consensus sequence exists as ψKxE (in which ψ is an aliphatic amino acid whereas x is any amino acid). The general consensus sequence is therefore classified into two groups: phosphorylation dependent SUMOylation motif (PDSM) (Hietakangas et al., 2006) and negatively charged amino acid dependent SUMOylation motif (NDSM) (S.-H. Yang et al., 2006). PDSM follows ψKxExxpSP, where serine is phosphorylated, followed by a proline. NDSM on the other hand has a negatively charged residue before the proline residue, and has been shown to induce a stronger SUMOylation (S.-H. Yang et al., 2006).

The concept of a consensus sequence for SUMOylation could be in part to the fact that a single E2 is responsible for transfer of SUMO unlike ubiquitin which has more than 20 different E2s with many E3 ligase combinations (Geiss-Friedlander et al., 2007)(Melchior). Even though most SUMOylated targets follow the consensus sequence, few proteins such as PCNA (K164 in budding yeast) and the human E2-25k (K14) do not contain any consensus sequence (Hoege et al., 2002).

1.8.3 Deconjugation of SUMOylation

SUMOylation, like ubiquitination is reversible because there are specific proteases that can remove SUMO from modified targets. SUMO isopeptidases are Cysteine proteases which belong to the Ulp class of proteins (Ulp1 and 2 in budding yeast) or SENP (Sentrin-specific protease in mammalian cells) (Di Bacco et al., 2006; S. J. Li et al., 2000). Aside from their isopeptidase activity they also contribute to maturation of SUMO by cleaving off the C-terminus to reveal di-glycine residues(Di Bacco et al., 2006). There exist differences in expression profile of SUMO isopeptidases. Ulp1 and SENP2 are enriched in the nuclear pore complex (S.-J. Li et al., 2003) while SENP5 exists in the nucleolus (Di Bacco et al., 2006). These differences depict the role of SUMOylation on cellular function.

1.8.4 Molecular consequences of SUMOylation

The molecular role of SUMOylation on specific targets is hard to predict however there are several examples of how SUMO modulates protein-protein interaction, stability and subcellular localization. SUMOylation of protein targets can influence protein-protein interactions include: SUMOylation of RanGAP1 favors an interaction with RanBP2

(Matunis et al., 1996), SUMOylated PCNA (proliferating cell nuclear antigen) recruits yeast Srs2 to sites of replication fork stalling (Papouli et al., 2005). SUMOylation of certain proteins can also abrogate protein-protein interactions; CtBP loses its physical interaction with the PDZ domain of nNos upon SUMOylation (X. Lin et al., 2003).

SUMOylation can also facilitate protein-protein interactions through non-covalent SUMO binding. This model was confirmed by NMR and Xray crystallography studies that showed that SIMs bind in a parallel or antiparallel manner to the alpha helix and beta strands of SUMO (Hannich et al., 2005; Hecker et al., 2006). The SIM is a hydrophobic core of the target protein, flanked by acidic or serine residues which are thought to regulate SUMO interactions through phosphorylation (Hannich et al., 2005). Several proteins including PML, Daxx, SUMO targeted ubiquitin ligases (STUBL), Uba2 and other PIAS E3 ligases possess SIMS that mediate their cellular function and regulation (D.-Y. Lin et al., 2006; Shen et al., 2006; Song et al., 2004; Xie et al., 2010).

Chapter 2: The SUMO E3 ligases, Siz1 and Mms21 are important for DNA protein crosslink repair

2.1 Abstract

Accurate duplication of chromosomes and their faithful transmission to daughter cells is essential to all eukaryotic organisms. This essential process can be interrupted by DNA damage. DNA damage including DNA-protein crosslinks (DPCs), which are defined as the covalent attachment of proteins to DNA are known to be physical impediments to DNA replication and transcription machinery. Failure to repair DPCs results in genomic instability, which is a hallmark of cancer. To date, how DPCs are distinguished and targeted for repair remains unknown. Given that Wss1, the protease that removes DPCs, is endowed with the ability to bind to SUMOylated proteins, we hypothesized that DPCs are recognized and targeted for removal through SUMOylation. However, a dedicated SUMO E3 ligase involved in DPC repair has not been identified. Here, we made use of a mutant Flp recombinase (*Flp-H305L*) expressed under an inducible promoter to model repair of a single site specific DPC in the budding yeast *Saccharomyces cerevisiae*. Using this approach, we screened all yeast SUMO E3 ligases and found that Mms21 (Nse2) and Siz1 are required for providing tolerance to the model Flp-DPC as well as DPCs induced with formaldehyde and Camptothecin, suggesting that Mms21 and Siz1 are required for repairing all DPCs including Topoisomerase1 covalent complexes.

2.2 Introduction

The timely and accurate replication of DNA is important for cell survival and the maintenance of genome integrity. DNA damage, however, poses a serious threat to cells by depriving them the ability to replicate their genome if repair mechanisms are compromised. Fortunately, cells possess faithful mechanisms that allow the timely response to DNA damage.

A form of DNA damage which involves the covalent attachment of proteins to DNA had not received wide scientific attention (Baker et al., 2007; Lopez-Mosqueda et al., 2016; Pommier, 2006; Stingele et al., 2014). DNA protein crosslinks (DPCs) occur when proteins become crosslinked to DNA either through abortive enzymatic intermediates (Pommier, 2006) or by drugs and ionizing radiation (Barker et al., 2005; Ward, 1988). The mechanisms underlying how cells remove these toxic lesions is not entirely understood. However, limited data suggests that DPCs are converted into double strand breaks (DSBs) which elicits direct DNA damage response (DDR) (Nielsen et al., 2009). DDR recruits downstream signaling including the activation of the MRX/MRN complex which depends on the catalytic activity of Mre11 endonuclease to resect DNA (Stracker et al., 2011)

DPC repair is conserved in eukaryotes including humans, yeast as well as in plants (Balakirev et al., 2015; Enderle et al., 2019; Stingele et al., 2016). Previous studies had characterized Mre11, Tdp1 and Tdp2 (Borgermann et al., 2019; Pommier, 2006; Pommier et al., 2014; Stingele et al., 2014) as factors necessary for repairing Topoisomerase1/2 (Top1/2) covalent complexes, however, how DPCs are recognized and targeted for repair remains fairly unknown. Very recently, Wss1 was identified as a dedicated protease important for DPC removal (Stingele et al., 2014) but how Wss1 targets DPCs for

degradation remains unknown. Here, we identified SUMO E3 ligases that SUMOylate DPCs for subsequent removal by Wss1 based on Wss1's ability to bind to SUMOylated proteins through its putative SUMO interacting motifs (SIM). Our working model is that a DPC becomes converted into a double strand break upon a direct collision with a replication. By using an inducible site specific-DPC model in yeast we report that DPCs are SUMOylated by Siz1 and Mms21 SUMO E3 ligases in budding yeast. These results provide a mechanism by which DPCs are processed for repair. Our data also reveals that the SMC5/6 complex which has a role in DNA damage repair is also needed for tolerance to DPCs.

2.3 Results

2.3.1 A mutant Flp recombinase to model a DNA-protein crosslink

To identify factors that are important for providing cells tolerance to DNA-protein crosslinks, we sought to make use of the Flp-nick system to study the repair of a single site-specific DNA-protein crosslink. The Flp-nick system was originally designed to induce a single strand DNA break by covalently attaching a mutant Flp (Flp-*H305L*) to its cognate FRT DNA sequence. We made use of a galactose-inducible Flp-*H305L*) which aborts it enzymatic reaction on DNA (Nielsen et al., 2009; Parsons et al., 1988), leading to a single covalently attached protein to the 3'-DNA end (**Figure 2.1A**) and a resulting single strand break. Unlike camptothecin (CPT) or formaldehyde dependent DPCs which are stochastic, this model induces a single DPC, thereby making it easier to determine factors responsible for repair without the use of crosslinking agents or enzyme poisons. Using this approach, we determined that wildtype yeast cells can tolerate constitutive induction of an

irreparable Flp-DPC (**Figure 2.1B**). Importantly, cells devoid of the DNA repair endonuclease, Mre11, cannot tolerate constitutive induction of the Flp-DPC (**Figure 2.1B**). Expression of a Flp tyrosine mutant (*Flp-Y343F*) whose catalytic cleavage of DNA is dysfunctional, in *mre11Δ* cells significantly restored the growth defect (**Figure 2.1C**), suggesting that the growth phenotype was due to the irreparable Flp-DPC. During chromosomal break events, cells activate DNA damage response through a Mec1/Tel1 mediated mechanism leading to activation of the DNA damage checkpoint kinase, Rad53 (Gobbini et al., 2013).

In a previous report, was Top1 identified as a suppressor which rescues the synthetic lethality between Tdp1 and Wss1 (Stingele et al., 2014). We generated a *top1Δ tdp1Δ wss1Δ* triple mutant to ascertain if Tdp1 and Wss1 are both required to tolerate repair of a Flp-DPC. Our Flp DPC assay did not reveal any growth phenotype for *tdp1Δ* nor *wss1Δ* alone, but showed a phenotype for *top1Δ tdp1Δ wss1Δ* triple mutant (**Figure 2.1B**), suggesting that both Tdp1 and Wss1 are essential to tolerate Flp dependent DPC.
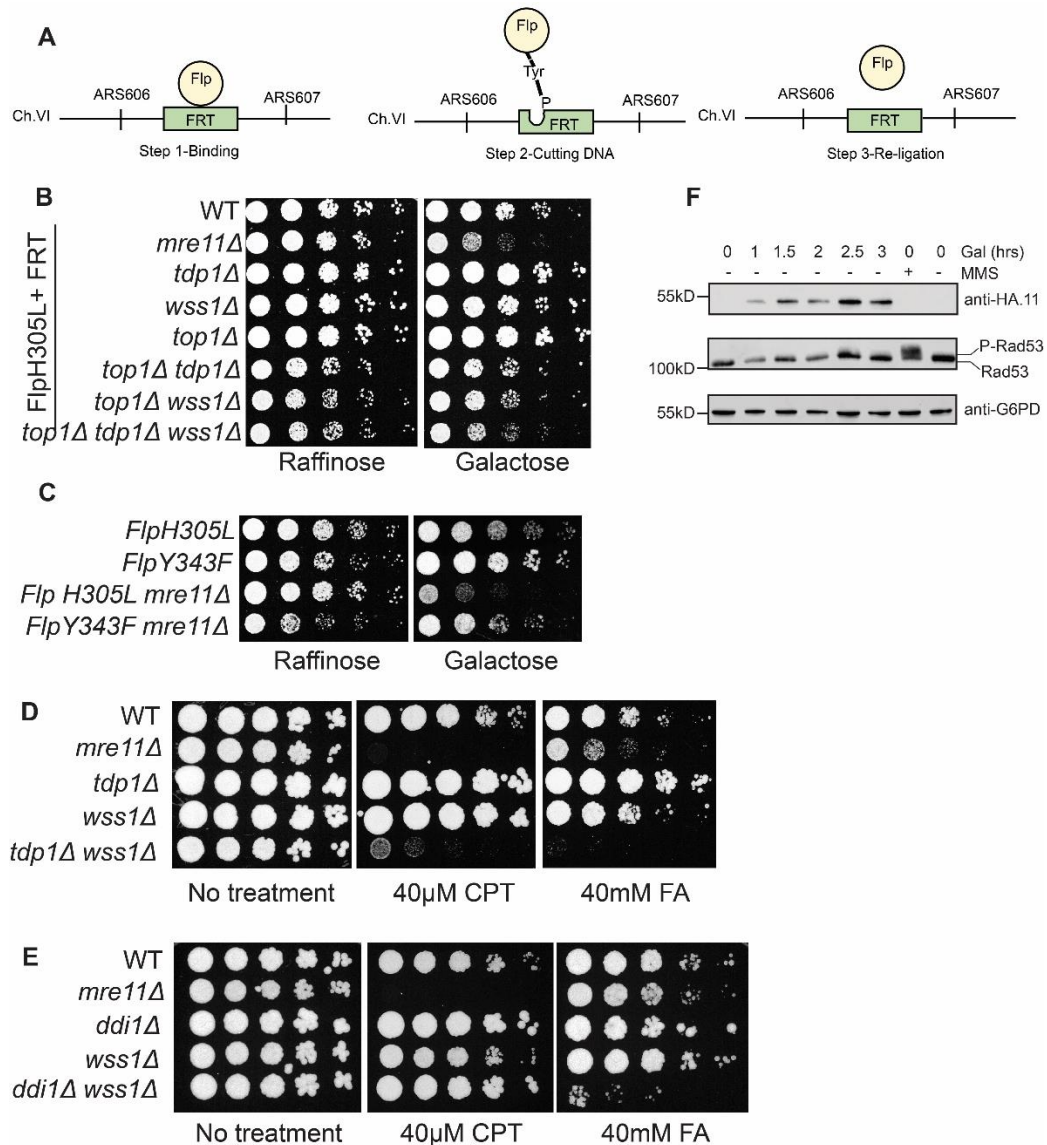
Figure 2.1. The Flp recombinase DPC model.

(**A**) Schematic diagram showing steps involved in Flp recombinase's normal activity. In this model, Flp binds to FRT sequence inserted between ARS 606 and 607 on chromosome VI. Flp recognition target (FRT), Autonomous Replication Sequence (ARS).

 (**B&C**) Five-fold serial dilution of yeast cells expressing Flp*H305L* mutant and lacking known DPC repair factors were spotted on YEP plate containing Raffinose (Raff) or galactose (Gal). Plates were incubated for 2 days at 30°C. or (F) 40µM Camptothecin (CPT) or 40Mm formaldehyde (FA). FRT sequence is present in all yeast strains.

(**D&E**) Five-fold serial dilution of yeast cells were spotted on plates containing (F) 40µM Camptothecin (CPT). Cells were treated 40mM formaldehyde (FA) as described (Stingele et al., 2014)

(**F**) 3HA-tagged Flp*H305L* strain was grown in raffinose overnight. Galactose was added to express 3HA-Flp. 3X-Flag Rad53 was probed with anti-Flag. 0.05% MMS was added to induce Rad53 shift. Anti-G6PD serves as a loading control.

We also observed significant growth sickness in the *tdp1Δ wss1Δ* mutant upon exposure to formaldehyde and camptothecin (**Figure 2.1D**), suggesting that both Tdp1 and Wss1 act in the same pathway, as previously reported (Stingele et al., 2014). Very recently Ddi1 was reported as an aspartic protease capable of removing DPCs (Serbyn et al., 2020; Michal Svoboda et al., 2019) in the absence of Wss1. We performed a pathway analysis that showed that cells are not able to remove genome wide covalent crosslinks in the absence of Ddi1 and Wss1 (**Figure 2.1E**).

To tease out the basic mechanism required for Wss1-dependent repair of DPCs, we first asked what cell-cycle phase is Wss1 expressed. We performed a cell cycle synchronization, which revealed that Wss1 is expressed throughout the cell cycle (**Figure 2.4A**), unlike SPRTN (human ortholog of Wss1) whose expression is known to be elevated at S-phase (S. Maskey et al., 2017). This result indicated that Wss1 is not cell cycle regulated. To further probe this result, we sought to determine the *in vivo* turnover rate for Wss1. Interestingly, Wss1 expression is very stable under physiological conditions (**Figure 2.4D**). We next asked how Wss1 gets recruited to chromatin if its expression is stable. By performing chromatin fractionation on lysates obtained from Wss1-tagged wildtype cells either treated with or without CPT, we observed recruitment to DNA (**Figure 2.4B**), however, in a DNA damage-independent manner.

2.3.2 Siz1 and Mms21 are necessary for tolerance to DPCs

Having modeled a simple assay for studying the repair of a single DPC, we hypothesized that SUMO is a signal that recruits Wss1 to DPC sites based on the presence of SUMO-

interacting motifs in its C-terminal domain. However, E3 SUMO ligases that catalyze DPC substrate SUMOylation have not yet been characterized. We envisaged that lack of important SUMO pathway genes could starkly impair the removal of a DPC. We therefore deleted the three non-essential yeast E3 SUMO ligases, Siz1, Siz2 and Zip3 and used an allele of Mms21 defunct in its ligase activity, in the Flp*H305L* background. Tetrad analysis showed that mms21 siz1 double mutants are synthetically sick but viable (**Figure 2.2E**). Galactose expression of Flp in these mutants revealed a growth defect in *siz1Δ* and *mms21ringΔ* but not in siz2 and zip3 mutants (**Figure 2.2A**).
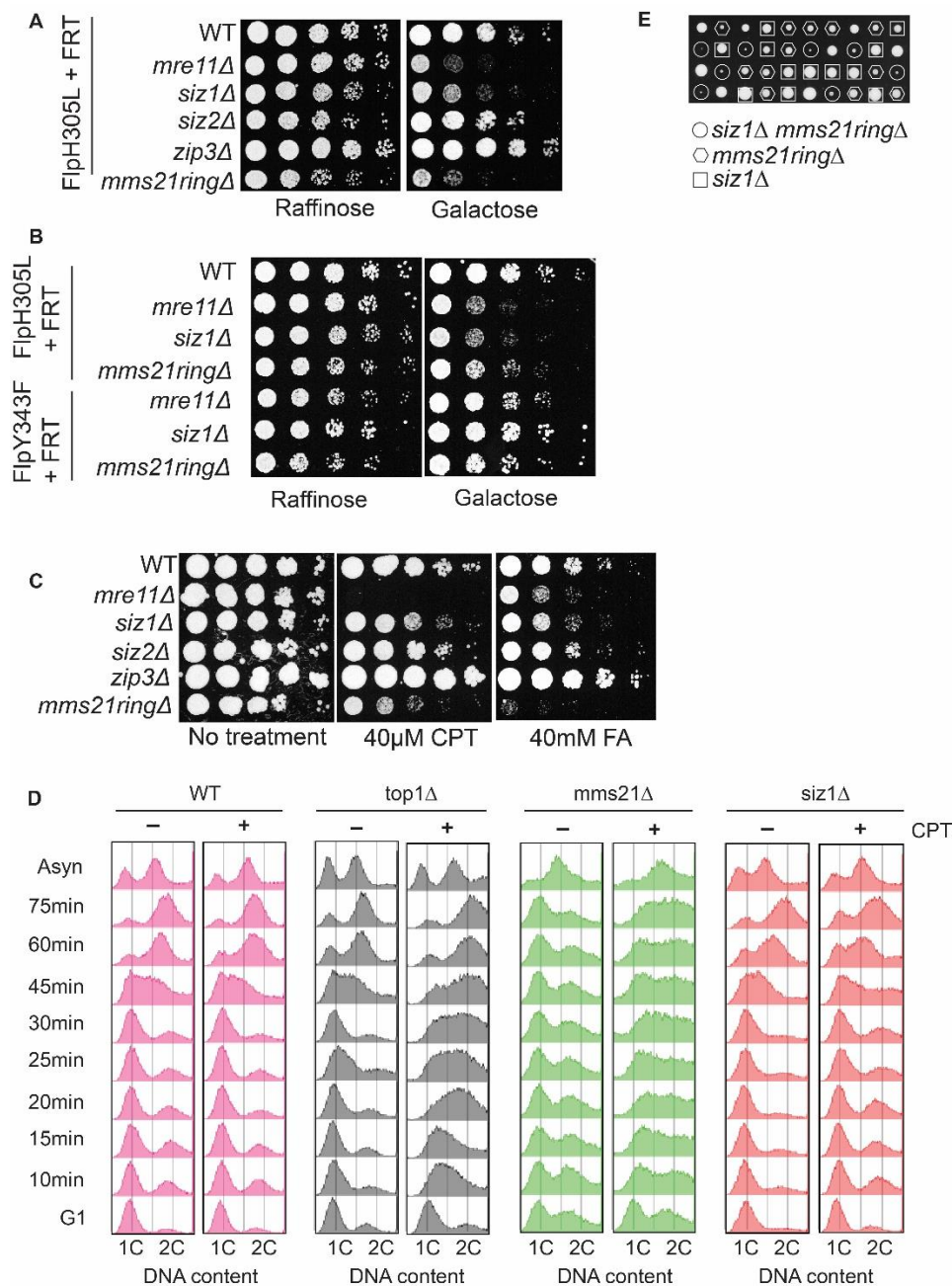
Figure 2.2. Siz1 and Mms21 E3 SUMO ligases are required for DPC repair

(**A-C**) Five-fold serial dilution of SUMO E3 ligase mutants on YEP plates containing raffinose or galactose. *mms21ringΔ* and siz1Δ cells show severe sickness on galactose, CPT and formaldehyde. Cells were incubated for 2 days at 30°C for Raff, Gal and CPT plates or 3 days for formaldehyde plates

(**D**) Exponentially growing cells were synchronized in alpha-factor and released into 20µM CPT. Cells were collected at indicated timepoints and processed for FACS.

(**E**) Spores obtained from heterozygous *mms21ringΔ* and siz1Δ diploids were dissected on a YEPD plate.

We were able to rescue the growth phenotype in *mms21ringΔ* and *siz1Δ* mutants by simply expressing the Flp tyrosine mutant (Flp*Y343F*) (**Figure 2.2B**). We also rescued the growth defect in the *mms21* mutant by complementation with a wildtype copy of MMS21 under all three DNA damage conditions (**Figure 2.5C**). Siz1 and Mms21 were also seen to be necessary for tolerating top1 covalent complexes as well as genome wide formaldehyde-induced DPCs (**Figure 2.2C**).

DNA lesions are also known to be repaired by non-classical mechanisms such as the post-replication and translesion synthesis repair pathway, mediated by the Rad5-Rad6-Rad18 epistaxis group (Torres-Ramos et al., 2002). Since W303 yeast strains are null for Rad5, we reconstituted RAD5 in the *mms21* mutant and measured its growth phenotype in the presence of genotoxic agents. However, RAD5 complementation did not improve the *mms21* mutant phenotype, corroborating our finding that Mms21 is necessary for tolerance to DPCs (**Figure 2.5D**). Mms21 acts in concert with the Smc5/6 complex and is recruited to sites of DNA damage as the sole SUMO E3 ligase responsible for SUMOylating Smc5 and other substrates at DNA damage sites (X. Duan et al., 2009; Zhao et al., 2005). Due to the loss of growth observed in *mms21ringΔ* mutants when challenged with DPC inducing agents (**Figure 2.2A & 2.2C**), we sought to separate the role of Mms21 in complex with SMC5/6 from its role as an independent SUMO E3 ligase. To do this, we used temperature sensitive alleles of the SMC5/6 complex genes (**Figure 2.5A**). When the SMC5/6 complex mutants were challenged with 40μM CPT, we observed significant growth defect in *nse3, nse4, smc5 and smc6* mutants at 37°C, compared to cells incubated at 30°C (**Figure 2.5B**).

2.3.3 Flp and Top1 are SUMOylated by Mms21 and Siz1

Based on the genetic evidence that Siz1 and Mms21 are required for tolerance to DPCs (**Figure 2.2A**), we asked if Mms21 and Siz1 are solely responsible for the SUMOylation of Flp and Top1 when they are covalently crosslinked to DNA. We performed a SUMO pulldown in an HA-tagged Flp strain and probed for Flp in the pulldown (**Figure 2.3A**). We were able to immunoprecipate Flp in the wildtype strain but not the *mms21ringΔ* mutant (**Figure 2.3B**), indicating that Flp is SUMOylated in an Mms21-dependent manner. Based on this finding, we anticipated that Mms21 may be similarly required for SUMOylating Top1. To ascertain this claim, we performed FLAG pulldown on Top1 and blotted for SUMO. Interestingly, we detected more Top1 SUMO species in the *mms21ringΔ* mutant and not in the *siz1* mutant (**Figure 2.3C & 2.3D**). To validate this result, we treated the immunoprecipitated samples with Senp2, a SUMO specific isopeptidase. Senp2 treatment removed high molecular weight SUMO species and weight not ubiquitin (**Figure 2.3E**).

Since mms21 and siz1 mutants show growth defects under genotoxic stress, we were curious to know if unrepaired DPCs may have a functional effect on progression through S-phase. To determine this, cells were synchronized in G1 with alpha factor and released into 20μM CPT with samples taken at indicated timepoints for FACS analysis. We found *siz1Δ* cells to slowly progress through S-phase (60 minute timepoint), in contrast to top*1Δ* and WT cells which progress quickly through S-phase (**Figure 2.2D**). This suggests that Siz1 is required for timely progression through S-phase during a DPC insult. We could not compare the FACS profile of the *mms21ringΔ* mutant because it was abnormally different from the FACS profile of all the yeast strains used.
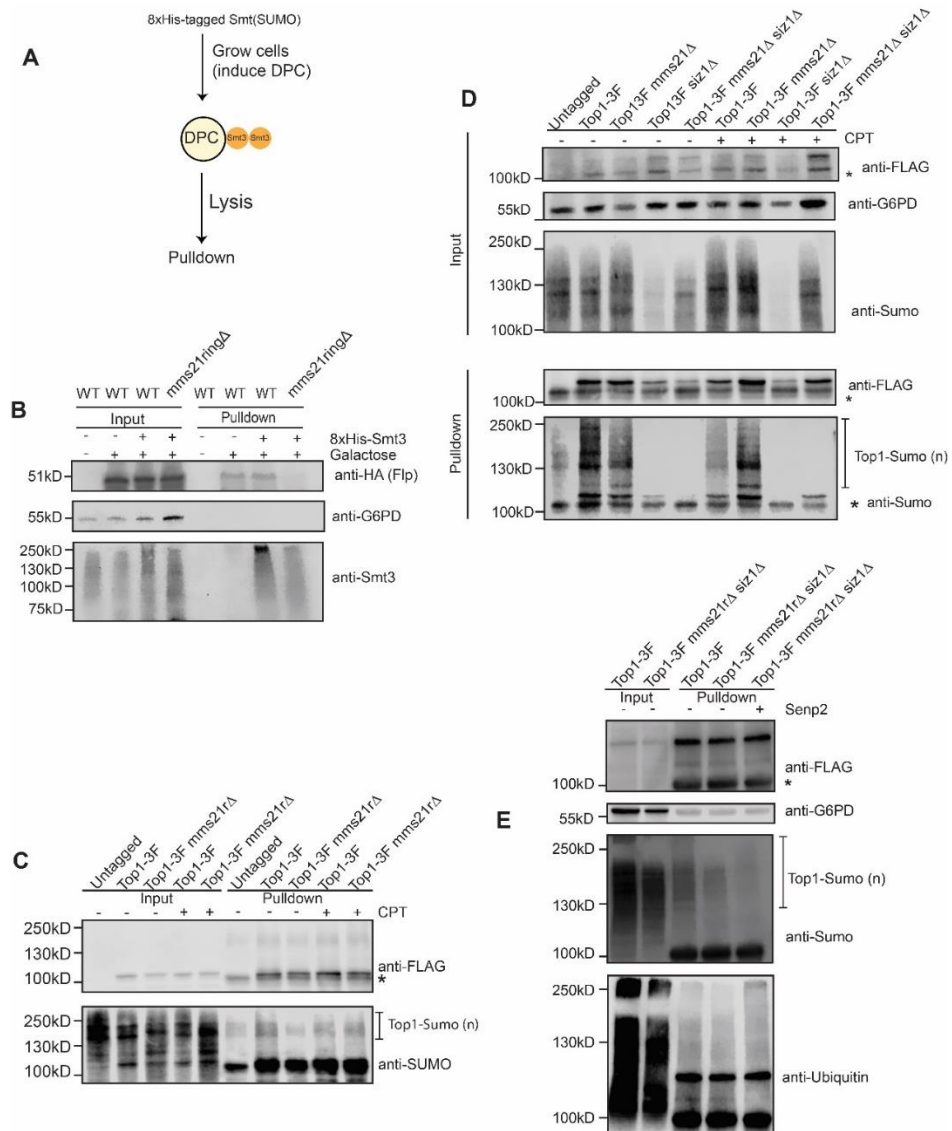
Figure 2.3. Top1 and Flp are SUMOylated by Siz1 and Mms21

(**A**)Schematic diagram describing experimental design. 8xHis-Smt3 expressing WT or mms21 mutant were grown and lysed, after which lysates were incubated on Ni-NTA beads.

(**B**) 8XHis-Smt3 pulldown in either a wildtype or mms21 mutant followed by blotting with anti-HA.11, anti-G6PD (loading control) and anti-Smt3 (SUMO).

(**C-E**) Cells were denatured and Top1-Flag pulldown performed, followed by blotting with anti-Smt3, anti-Flag, anti- G6PD and anti-Ubiquitin. Indicated lanes show treatment with 40µM CPT. In D) Senp2 was added in the indicated lane.

2.3.4 Flp recombinase undergoes minimal resection

To further characterize how cells respond to a Flp-DPC, we tagged Rad53 in a wildtype Flp expressing strain and used Rad53 as a proxy for double strand break processing, based on the hypothesis that a single strand break generated by the Flp-DPC will be converted into a double strand break during an counter with the replisome. Surprisingly, expression of Flp-DPC did not lead to a Rad53 mobility shift, however a Rad53 mobility shift was observed when the yeast strain was exposed to the alkylating agent, methyl methanesulfonate (MMS) which induces genome wide DNA damage (**Figure 2.1F**), suggesting that DNA damage processing was not compromised in the Flp*H305L* strain. Again, we compared Rad53 signal between the galactose inducible Flp-DPC strain and galactose-inducible HO endonuclease in a strain deficient of the mating type locus (**Figure 2.4C**) (Achille Pellicioli et al., 2001). Interestingly, Rad53 is phosphorylated in the HO strain but not in the Flp-DPC strain, suggesting that a Flp-DPC leads to minimal DNA processing, as recently reported (Jakobsen et al., 2019). To characterize DNA damage processing in siz1 and mms21 mutants we tagged Rad53 in these mutants as previously demonstrated. Interestingly, siz1 mutants do not show a Rad53 mobility shift upon Flp-DPC expression, however, the mms21 mutant shows constitutive Rad53 shift (**Figure 2.5E**).
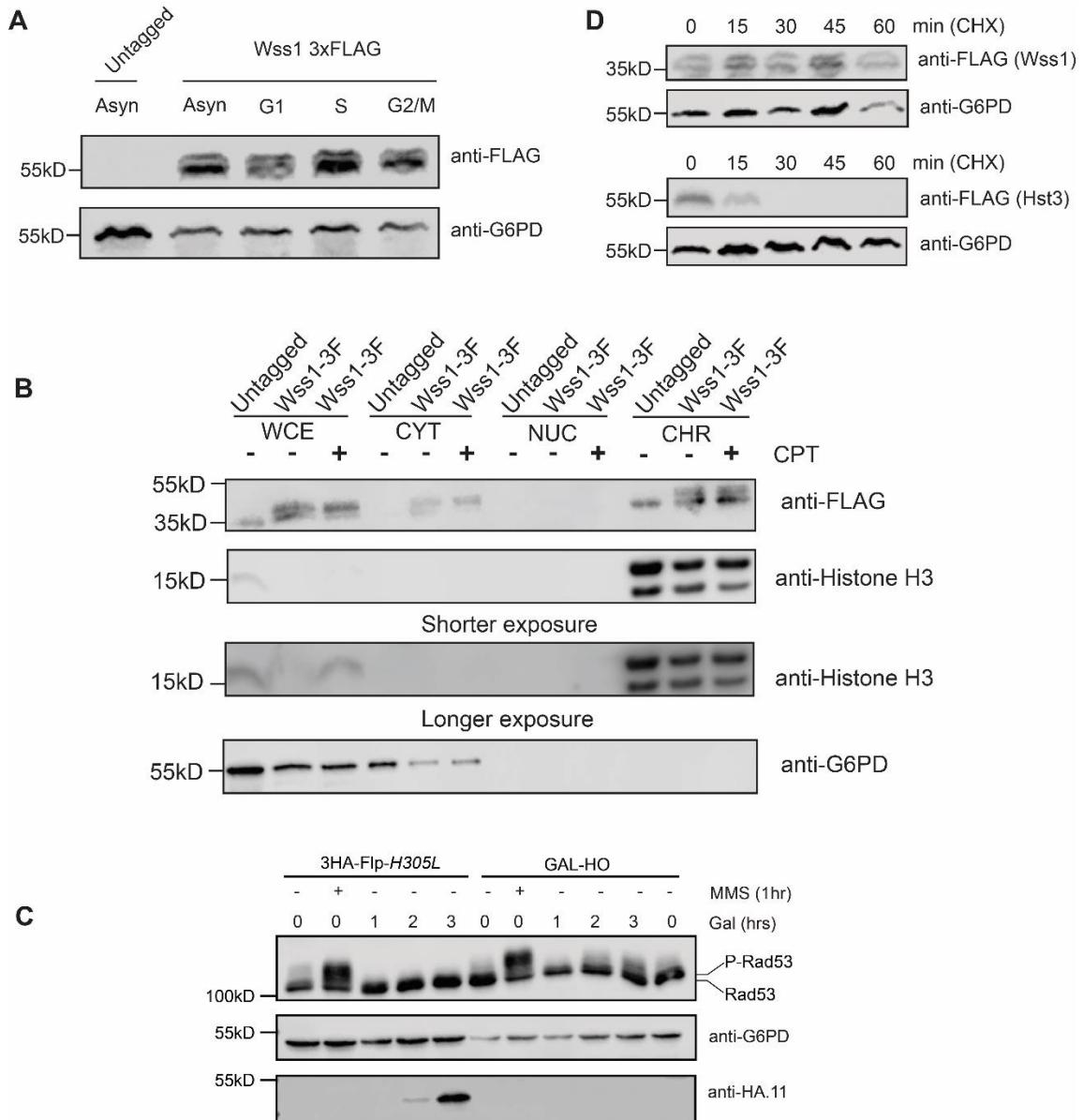
Figure 2.4. Expression of Wss1 is not cell cycle regulated

(**A**) Exponentially growing Wss1 Flag-tagged cells were synchronized in 100 µM alpha-factor, 100mM hydroxyurea and 15ug/ml nocodazole. 3 OD_{600} of cells were collected from each synchronized culture for western blot. Anti-G6PD is serves as a loading control. (**B**) Chromatin fraction (CHR), whole cell (WCE), cytoplasmic (CYT) and nuclear (NUC) extracts obtained from untagged and Wss1 tagged yeast strains were run on an SDS-page gel and probed with the respective antibodies. CPT was added at a concentration of 20µM.

(**C**) 3HA-FlpH305L was expressed in exponentially growing WT cells. HO-endonuclease was expressed in a strain deficient of the mating type loci. 3 OD_{600} of cells were collected for western blot. Rad53 was tagged with 3 tandem copies of Flag epitope. Anti-G6PD served as a loading control. MMS was added at 0.05%.

(**D**) Exponentially growing Wss1 and Hst3 3xFlag tagged yeast strains were treated with cycloheximide and samples taken at indicated timepoints for western blot.
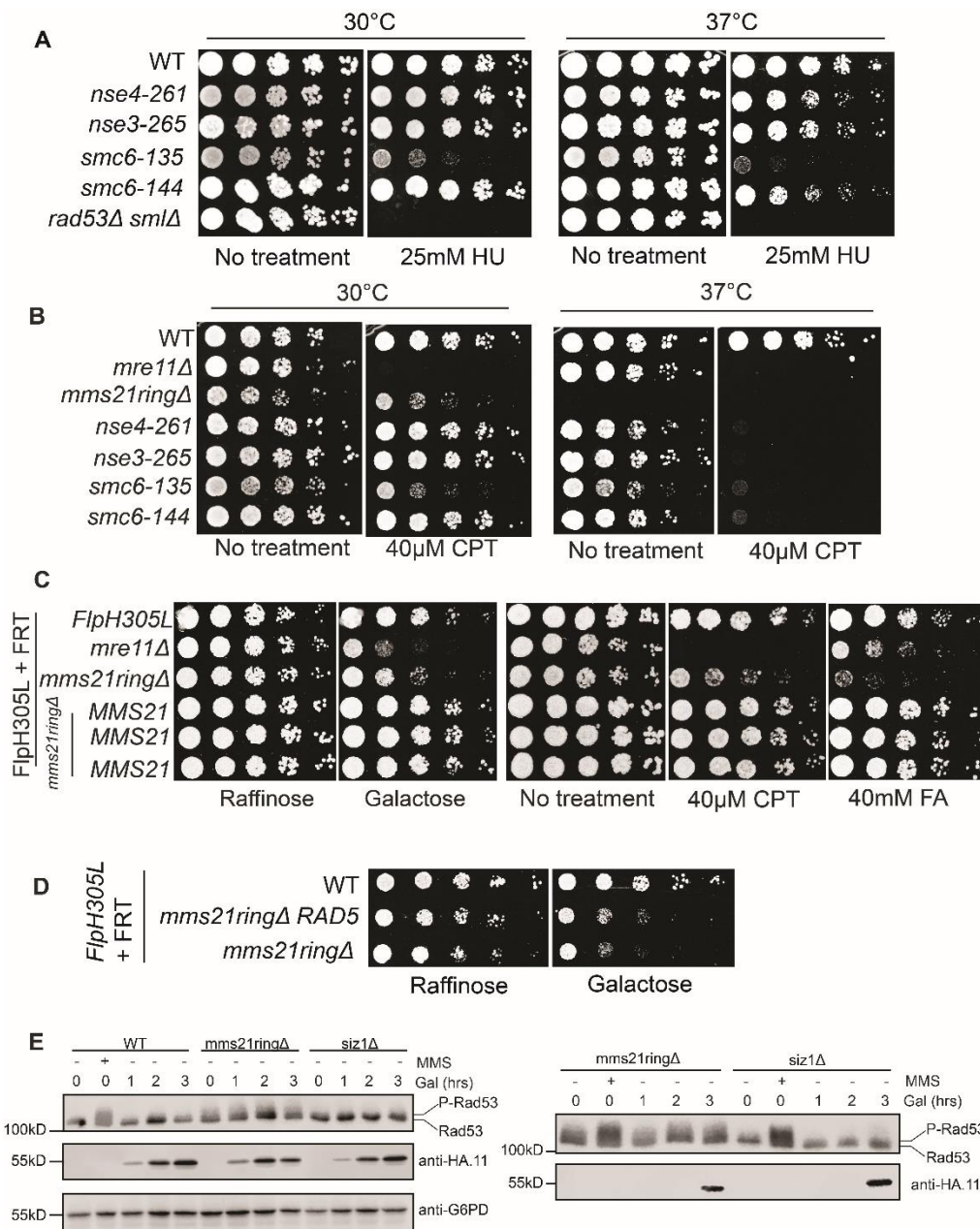
Figure 2.5 The SMC5/6 complex has a role in DPC repair

(**A&B**) Five-fold serial dilution of temperature sensitive SMC5/6 complex mutants were spotted on YEPD plates containing 25mM hydroxyurea (A) or 20µM CPT (B) and incubated at 30°C and 37°C.

(**C**) *mms21ringΔ* cells were complemented with a wildtype (WT) copy of Mms21. Five-fold serial dilution on raffinose and galactose (A) or CPT and formaldehyde (B) shows rescued phenotype in three independent strains.

(**D**) W303 yeast strains lacking RAD5 were reconstituted with wildtype copy of RAD5. Five-fold serial dilution of *mms21ringΔ* complemented with RAD5 does not rescue sickness on galactose.

(**E**) 3HA-FlpH305L was expressed in exponentially growing WT, siz1 and mms21 mutants. 3 OD_{600} of cells were collected for western blot. Rad53 was tagged with 3 tandem copies of Flag epitope. Anti-G6PD served as a loading control. MMS was added at 0.05%.

2.4 Discussion

The accumulation of DPCs pose lethal threats to cells if they are not immediately removed. While DPCs are mostly tolerated in wildtype cells, we hypothesized that SUMO pathway mutants will be challenged in removing DPCs. Our hypothesis stemmed from our proposal that Wss1 binds to SUMOylated proteins, however SUMO E3 ligases that SUMOylate DPCs remain unknown. By utilizing a system where a single DPC insult is monitored for repair, we have been able to identify SUMO E3 ligases that are important for tolerance to DPCs. Previous studies had identified the SUMO pathway to be important for modifying topoisomerase covalent complexes (Chen et al., 2007; Esteras et al., 2017; K. C. Lee et al., 2018; Schellenberg et al., 2017) but no study had implicated SUMO E3 ligases in the context of DPC repair. The identification of Siz1 and Mms21 therefore reveals a mechanism that enables cells to remove covalently crosslinked adducts. This is consistent with how the mammalian E3 SUMO ligase, ZATT451 contributes to removal of Top2 covalent complexes (Schellenberg et al., 2017). Our result supports our working model that SUMO serves as a molecular signal that recruits DPC repair proteases such as Wss1 to DPC substrates. Even though there is global reduction of SUMOylation in siz1 mutants (**Figure 2.3C**)(Chen et al., 2007; Johnson et al., 2001), the fact that siz1 deletes are viable suggests that the global SUMOylation deficiency does not lead to any lethal consequence except during essential SUMO dependent cellular roles as evidenced by the slow progression of siz1 mutants during S-phase. While writing this manuscript, a preprint released by Yves Pommier's group also identified Siz1 to be responsible for SUMOylating Top1 covalent complexes and further validated our observations (Y. Sun et al., 2019).

Because the timely replication of DNA is needed to ensure accurate cell division, removal of DPCs during replication remains essential to cell survival. Our FACS analysis of siz1 mutants indicate late progression due to slow processing and delayed removal of chromatin-bound Top1-ccs. This result explicitly shows that DPC repair is orchestrated in a E3 SUMO ligase dependent manner.

The importance of Mms21 had previously being correlated with its activity with the SMC5/6 complex at sites of DNA damage (Bermúdez-López et al., 2015; Potts et al., 2005; Zhao et al., 2005). Mms21 is known to SUMOylate proteins at damage sites including the components of the SMC5/6 complex (Zhao et al., 2005). Two models have been hypothesized for Mms21 dependent SUMOylation; either in complex with the SMC5/6 complex or independently (Bermúdez-López et al., 2015). Our data supports the later model and further suggests that the role of the SMC5/6 complex in mediating DPC repair is mutually exclusive of Mms21's E3 ligase activity. This claim is supported by our observation that *nse3* (E265R), *nse4* (D261A) and *smc6* (R135E and R144E) single mutants alone are not able to tolerate CPT and formaldehyde induced DPCs. This suggests that mutating Mms21 alone and not the entire SMC5/6 complex is enough to disrupt its unique function during DPC lesions. There is further support for this observation because *smc6-1* mutants are SUMOylated and maintain interaction with SMC5 as well as Mms21.as previously shown (Bermúdez-López et al., 2015). Therefore, these observations suggest the need to identify the molecular tuning of the SMC5/6 complex in DPC removal.

Since the discovery of Wss1 and its importance for DPC, several groups have tried to elucidate its basic function at the genetic and molecular level (Balakirev et al., 2015; Maddi et al., 2020; O'Neill et al., 2004a; Stingele et al., 2014; van Heusden et al., 2008;

Wawrzycka et al., 2012) .

 Wss1 is synthetic lethal with Tdp1, however little has been added to how Wss1's protease activity is controlled at the cellular level. To study the kinetics of this protein we determined that Wss1 is activated at all stages of the cell cycle indicating its constitutive activity at any stage of the cell cycle. We speculate that this observation supports the extensive role of Wss1 both in DPC repair and in genome maintenance, consistent with our recent finding that Wss1 removes histones during replication fork stalling (Maddi et al., 2020).

Taken together, our genetic and biochemical data validates Siz1 and Mms21 to be important for DPC repair in yeast and suggests that cells employ the SUMO pathway to respond to cues that facilitate the removal of DPCs from chromatin.

2.5 Experimental procedures

2.5.1 Yeast strains and growth conditions

All yeast strains used in this study were derived using standard yeast genetic procedures (Guthrie et al., 1991). Genetic deletion and tagging of genes of interest were done on the parental yeast strain (YJLO0). Yeast were always grown in yeast extract peptone (YEP) media + 2% dextrose at 30°C unless in galactose expression assays, where yeast were grown overnight in YEP + 2% Raffinose at 30°C, prior to induction with either 2% dextrose or 2% galactose.

2.5.2 Yeast spot assays

Spot assays were performed by making a 1:5 dilution of yeast cells and spotted 3µL on appropriate agar plate. For spot assays, plates were incubated at 30°C for 2 day, unless otherwise stated. CPT was used at a concentration of 40µM in solid agar or 20µM in liquid cultures. Cells were treated with 40mM FA as previously described (Stingele et al., 2014).

2.5.3 DNA amplification and cloning

All DNA polymerase chain reactions (PCR) performed for confirmation of genetic manipulation in strains followed a 95°C denaturation for 2 minutes, 95°C for 30 seconds, 55°C annealing for 45 seconds, 72°C elongation protocol based on PCR product for a total of 35 PCR cycles. PCR was performed on a ProFlex PCR system (ThermoFisher). 3xHA tag insertion into pFV17 strain (Flp-H305L) was performed by initially digesting 1µg of plasmid with Sal1 restriction enzyme (ThermoFisher) following the manufacturer's instructions. 2µg each of 3HA Tag forward and reverse oligonucleotides, OJLO76 and OJLO77 respectively, were annealed in annealing buffer (10Mm Tris pH 7.5-8.0, 50mM NaCl, 1mM EDTA) in equimolar concentrations to a total volume of 50µL. Mixed oligonucleotides were placed in a thermocycler at 95°C for 2 minutes and allowed to gradually cool to 25°C in a span of 45 minutes. The annealed oligonucleotides and digested vector were ligated using T4 DNA ligase in a ratio of 1:3 using the manufacturer's instructions. 2-3µL of the product was transformed into Top10 competent bacteria and cultured in LB + Ampicillin media. Transformed colonies were grown in LB broth overnight, while plasmid DNA was extracted using ThermoFisher Miniprep kit and

positive clone confirmed by DNA sequencing.

All other bacteria vector-based cloning were performed using NEB HIFI assembly master mix by following manufacturer's instructions.

2.5.4 Western blot analysis

Yeast cells were grown overnight in YEP + 2% dextrose at 30°C, unless otherwise indicated. Cells were diluted to 0.2 OD600 and 3OD of cells collected. Washed cells were suspended in 20% Trichloroacetic acid (TCA) and bead-beated for two pulses of 1 minutes, with 1 minute incubation on ice after each pulse. Extracts were pipetted and beads washed with 5% TCA. Protein extracts were washed in ice-cold acetone and centrifuged in a Savant SpeedVac concentrator to dryness. Dry protein pellets were resuspended in 100μL of 1X Laemmli buffer cocktail (125mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% beta-Mercaptoethanol) and boiled for 5 minutes. Lysates were run on an SDS-PAGE gel, transferred unto a nitrocellulose membrane and immunoblotted with antibodies specific to the protein of interest.

2.5.5 Yeast cell cycle arrest and FACS analysis

Prior to cell cycle arrest, cells were grown in 2% dextrose at 30°C overnight. Yeast cells were arrested at G1 with 10μM alpha factor for 2 hours. Cells were synchronized at S phase with 100mM HU for whereas G2/M synchronization was carried out with nocodazole at a concentration of 15ug/ml.

For flow cytometry analysis of yeast cells, yeast cells were collected at respective time points and fixed immediately in 70% Ethanol in flow cytometry tubes. Next, cells were

sonicated (20% amplitude, 5 sec) to separate clumped cells. Cells were centrifuged at 2000 rpm for 5 min and ethanol was aspirated out. Later, cells were re-suspended in 1mL of RNAse buffer (50mM Tris pH8.0 and 15mM NaCl) with RNAse (0.25 mg/mL) and incubated at 50°C for 1 hour. Further, proteinase K (0.125 mg/mL) was added to the buffer and incubated for additional 1 hour at 50°C. Cells were spun down at 2000RPM to remove the supernatant. Cells were resuspended in 1ml PBS. Propidium iodide was added at a concentration of 10µg/ml and vortexed to have final sample preparation. Readings were recorded on BD FACS Accuri and FACS curves were analyzed using FlowJo software.

2.5.6 SUMO and FLAG pulldown

SUMO pulldowns were performed as previously described by (Sacher et al., 2005) with minor modifications**.** An overnight culture of cells were diluted to 0.2 OD_{600}, allowed to grow further and a 200OD_{600} of cells collected per each pulldown. Cells were washed and the cell pellet frozen at -80°C. The pellet was resuspended in 500µL Buffer A (8M Urea, 100mM KH_2PO4, 10mM Tris-HCL (pH 8), 0.05% Tween 20) together with 500µL of glass beads and bead-beat at maximum pulse on a Minibeadbeater machine for 1 min (2 times). Cells were kept on ice for a minute after each pulse. Cell lysate was clarified at 15000RPM for 10 minutes at 4°C. 5 µL of lysate was saved as input for western blot whereas the remaining lysate was transferred to a 15ml falcon tube and diluted to 5ml with Buffer A (containing 15mM Imidazole to reduce non-specific binding). 50µL Ni-NTA beads were added to the protein extract and incubated at 4°C overnight. Wash beads 3 times in Buffer A (supplemented with 2mM Imidazole) followed by 5 washes in Buffer B (8M Urea, 100mM KH_2PO4, 10mM Tris-HCL (pH 6.3), 0.05% Tween 20). Bound proteins

were eluted with 25μL 2X Laemmli buffer and boiled for 2 minutes.

Top1 FLAG pulldowns were carried out as performed for SUMO pulldowns except that cells were lysed in 500μL Buffer and sonicated at 12% amplitude 10sec on/10sec off for 2min. Lysates were clarified at 15000RPM for 10minutes. Supernatant was diluted in 5ml 100mM Tris.HCL ph7.5 buffer. 50μL FLAG M2 affinity beads were added to lysates and incubated at 4°C for 3 hours.

2.6 Acknowledgements

Chapter 3: Wss1 promotes tolerance to replication stress by degrading histones

3.1 Abstract

Timely completion of DNA replication is central to accurate cell division and to the maintenance of genomic stability. However, certain DNA-protein interactions can physically impede DNA replication fork progression. Cells remove or bypass these physical impediments by different mechanisms to preserve DNA macromolecule integrity and genome stability. In *Saccharomyces cerevisiae*, Wss1, the DNA-protein crosslink repair protease, allows cells to tolerate hydroxyurea-induced replication stress but the underlying mechanism by which Wss1 promotes this function has remained unknown. Here we report that Wss1 provides cells tolerance to replication stress by directly degrading core histone subunits that non-specifically and non-covalently bind to single-stranded DNA. Unlike Wss1-dependent proteolysis of covalent DNA-protein crosslinks, proteolysis of histones does not require Cdc48 nor SUMO-binding activities. Wss1 acts as a multi-functional protease capable of targeting a broad range of covalent and non-covalent DNA-binding proteins to preserve genome stability during adverse conditions.

3.2 Introduction

DNA-bound proteins can be physical impediments to replication forks and associated protein complexes. DNA-protein crosslinks (DPCs) in particular, constitute physical obstacles to DNA and RNA polymerases (Edenberg et al., 2014) and are genotoxic to cells because direct collisions between replication forks and DPCs, convert DPCs into DNA double-strand breaks. In budding yeast, Wss1 was identified as the first DNA-dependent protease important for DPC repair. Wss1-dependent DPC repair requires physical interactions with the ATPase Cdc48 (p97/VCP in mammals), and binding to the small ubiquitin-like modifier, Smt3 (SUMO in higher eukaryotes). Wss1 mutants deficient in binding to either Cdc48 or Smt3 fail to provide tolerance to DPC-inducing agents. In higher eukaryotes, SPRTN is the DNA-dependent protease that removes proteins that become covalently attached to DNA (Lopez-Mosqueda et al., 2016; Stingele et al., 2016; B. Vaz et al., 2016). However, unlike Wss1, SPRTN can repair DPCs independently of its ability to bind to p97 (Cdc48) or Ubiquitin (Lopez-Mosqueda et al., 2016).

Replication fork barriers, such as DPCs, can be bypassed by several distinct mechanisms (Duxin et al., 2014; Sparks et al., 2019; Wickramaratne et al., 2016). Wss1, however, can directly and irreversibly remove the DPC protein component by proteolysis (Balakirev et al., 2015; Stingele et al., 2014). Wss1 is not essential for viability in budding yeast cells nor is it required for surviving chronic exposure to DPC-inducing agents including Camptothecin.

Mass-spectrometry based analysis of isolated DPCs identified DNA-binding proteins including replication proteins, transcription factors, and histones - histones constituting the most abundant protein components of DPCs (B. Vaz et al., 2016). Histones are abundant

proteins that have a high affinity for DNA and their levels are carefully regulated at the transcriptional, translational and post-translational level. Owing to their positive charge, histones can form protein-DNA aggregates (Shintomi et al., 2005*)* that have been previously reported to impede gene transcription (Karsten, 2008). Proteins do not necessarily need to be covalently attached to DNA to constitute a replication fork barrier. To exemplify, Fob1 in yeast (Kobayashi et al., 1996) and the viral protein Epstein-Barr nuclear antigen 1 (EBNA-1) in mammalian cells (Dhar et al., 1991) bind to DNA with high affinity and their binding effectively block replication fork progression. Here we show that Wss1 is important for replication stress tolerance. Wss1 directly elicits proteolytic degradation of histones that are non-specifically bound to single-stranded DNA. Wss1-dependent replication stress tolerance, as well as histone degradation, does not require Cdc48 or Smt3 binding activities. Our results suggest that Wss1 is a multi-functional protease that irreversibly removes covalently and non-covalently bound proteins from DNA to provide replication stress tolerance.

3.3 Results

3.3.1 Wss1 proteolytic activity is necessary for replication stress tolerance

Wss1 is dispensable for yeast viability due, in part, to the redundant pathways that mediate DPC repair (Stingele et al., 2017; B. Vaz et al., 2017). However, cells deficient in Wss1 function display a strong negative genetic interaction with Tdp1, a tyrosyl-DNA phosphodiesterase that assists in the removal of DNA-protein crosslinks, such that cells lacking both, Wss1 and Tdp1, are synthetic lethal (Lopez-Mosqueda et al., 2016; Sharma et al., 2017; Stingele et al., 2014). Wss1 becomes essential for viability when DNA repair

by homologous recombination is compromised, underscoring the notion that multiple pathways coalesce to repair DPCs (Stingele et al., 2014). To identify conditions that require Wss1 function, we screened various genotoxic agents in Wss1-deficient yeast cells (*wss1Δ*) and searched for a slow-growing phenotype (**Figure 3.1A and Figure 3.6A**). We observed that *wss1Δ* cells are sensitive to chronic exposure to the DNA replication inhibitor hydroxyurea (**Figure 3.1A**), which depletes deoxyribonucleotide pools by inhibiting ribonucleotide reductase (O'Neill et al., 2004b) and are sensitive to hydrogen peroxide which produces reactive oxygen species (**Figure 3.6A**). Similarly, when *wss1Δ* cells are only transiently exposed to hydroxyurea, we observed a modest but reproducible viability loss compared to wildtype cells exposed to the same treatment (**Figure 3.1B**). While, hydroxyurea is not known to directly cause DNA-protein crosslinks, Wss1 plays an important role in providing cells tolerance to hydroxyurea.

To account for the hydroxyurea sensitivity observed in *wss1Δ* cells, we first asked whether *wss1Δ* cells have defects traversing S-phase. Wildtype (*WSS1*) and *wss1Δ* cells were arrested in the G1 phase of the cell cycle with alpha-factor and released from the G1 block into media without alpha-factor to allow cells to enter synchronously into the S-phase. We monitored DNA replication dynamics using flow cytometry. Under these conditions, we did not observe an appreciable defect in bulk DNA replication in *wss1Δ* cells. *wss1Δ* cells entered and progressed through S-phase with similar kinetics as wildtype cells (**Figure 3.1C**). We next asked if Wss1 is important for recovery from replication fork stalling. To achieve replication fork stalling, we first blocked cells in G1, then released cells into media containing hydroxyurea and held them for 1 hour. Finally, cells were released from the

hydroxyurea treatment and we monitored replication dynamics using flow cytometry as before. The DNA damage checkpoint kinase, Rad53, is critical for maintaining replication fork stability in hydroxyurea (Desany et al., 1998). As such, *rad53Δ* cells are hypersensitive to hydroxyurea and do not recover from a hydroxyurea treatment. In contrast to *rad53Δ* cells, *wss1Δ* cells recovered from hydroxyurea similarly to wildtype cells (**Figure 3.1D**). We tested whether Wss1 is important for Rad53 activation, as failure to activate Rad53 would result in hydroxyurea sensitivity. We assayed for Rad53 auto-phosphorylation as a measure of Rad53 activation in wildtype and *wss1Δ* cells and did not observe a marked defect in Rad53 activation in *wss1Δ* cells exposed to hydroxyurea (**Figure 3.1E**). Taken together, the hydroxyurea sensitivity observed in *wss1Δ* cells is not due to major defects in completing DNA replication or a general failure to stably maintain replication forks.

To better understand the hydroxyurea induced toxicity observed in *wss1Δ* cells, we next investigated which Wss1 activity is needed to mediate hydroxyurea tolerance. Wss1 is endowed with the ability to physically interact with Cdc48 and Smt3 owing to its SHP/VIM and tandem SIM motifs, respectively. Wss1 is also able to degrade Top1 covalent complexes via its protease domain (SprT) (Balakirev et al., 2015; Stingele et al., 2014). We reconstituted *wss1Δ* cells with either wildtype Wss1 or mutant alleles deficient in protease activity (E116Q), Cdc48 binding (SHP & VIM) or Smt3 binding (SIM1, SIM2) and confirmed expression of wildtype and mutant Wss1 alleles by Western blot analysis (**Figure 3.6C**). As expected, reconstitution of *wss1Δ* cells with wildtype Wss1 restored hydroxyurea resistance, whereas reconstitution with the catalytically inactive protease mutant did not (**Figure 3.1F**). Interestingly, reconstitution of *wss1Δ* cells with SHP/VIM

and SIM mutant alleles also restores hydroxyurea resistance, suggesting that Cdc48 and Smt3 binding to Wss1 are dispensable to mediate hydroxyurea resistance (**Figure 3.1F**). Over-expressing *wss1-E116Q* mutant confers a lethal phenotype in hydroxyurea treated cells compared to cells where Wss1 is absent. This observation suggests that protein complexes that include Wss1 are accumulating in the presence of hydroxyurea. Taken together, these results would suggest that Wss1 targets proteins for degradation to mediate hydroxyurea tolerance and this activity is independent of Cdc48 and Smt3 binding.
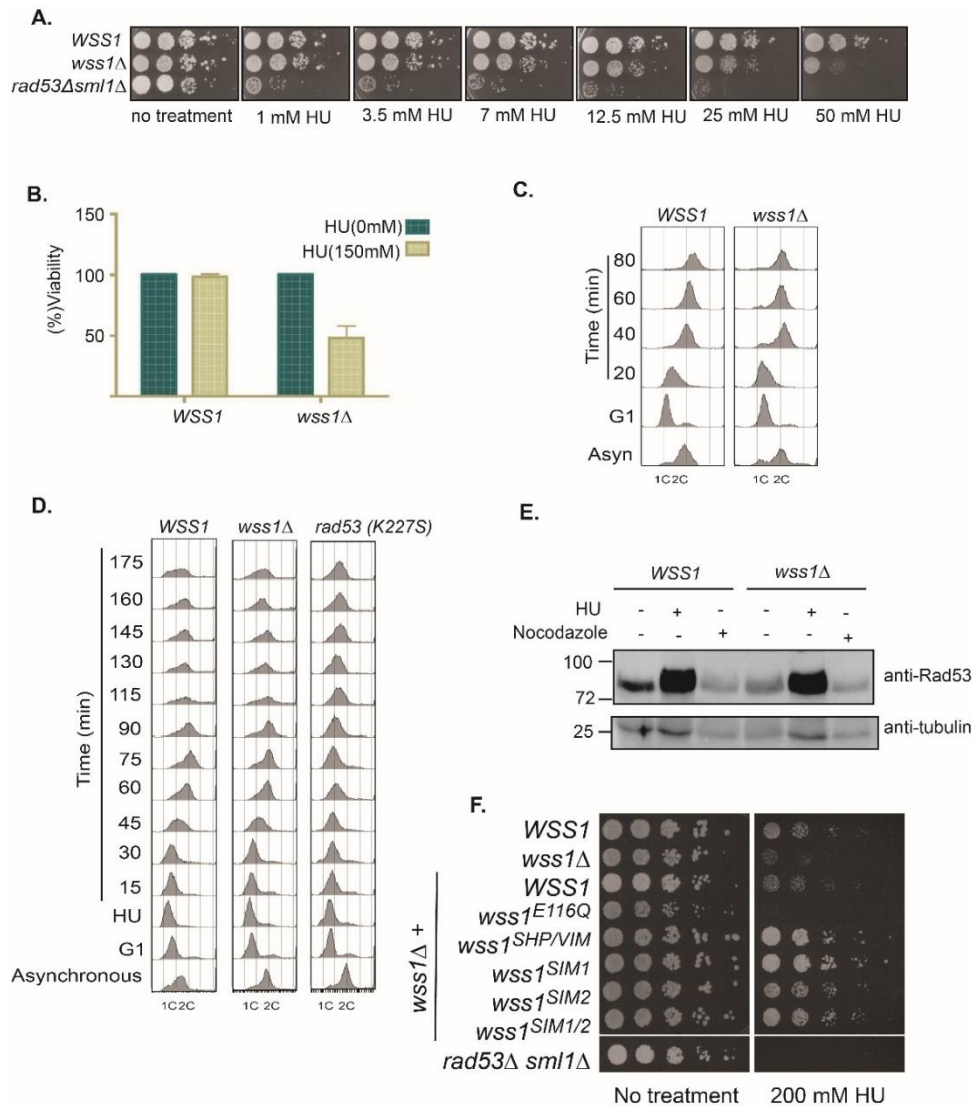
Figure 3.1: Wss1 Proteolytic Activity Is Necessary for Replication Stress Tolerance

(A) wss1Δ cells are sensitive to chronic exposure to hydroxyurea. Ten-fold dilutions of wild-type, wss1Δ, or rad53Δ cells were spotted on YPD plates in the absence or presence of hydroxyurea (1, 3.5, 7, 12.5, 25, and 50 mM) and incubated at 30°C. Plates were imaged after 3 days.

(B) wss1Δ cells are sensitive to transient exposure to hydroxyurea. Exponentially growing wild-type or wss1Δ cells were either untreated or treated with hydroxyurea (150 mM) for 1h and washed with YPD media to remove residual hydroxyurea. Approximately 2×10^3 cells were plated on YPD plates and incubated at 30°C for 2 days. Colonies were counted using ImageJ, and bars in the histogram represent the percentage of viable cells after hydroxyurea treatment. Error bars represent the standard error of the mean (SEM).

(C) Wss1 is not required for DNA replication fork progression. Flow cytometric (fluorescence-activated cell sorting [FACS]) analysis of wild-type or wss1Δ cells synchronized in G1 with alpha factor and released from the G1 arrest in the absence of hydroxyurea.

(D) Wss1 is not required for recovery after hydroxyurea. FACS analysis of wild-type, wss1Δ, or rad53Δ cells synchronously released from a G1 arrest into hydroxyurea (200 mM for 1 h). Cells were washed and released into YPD media without hydroxyurea, and samples were collected at indicated time points.

(E) wss1Δ cells have an intact checkpoint system. Exponentially growing wild-type or wss1Δ cells were treated with hydroxyurea (200 mM) for 1 h and whole-cell extracts were prepared by trichloroacetic acid

(TCA) precipitation method and immunoblotted using a Rad53-specific antibody or a-tubulin for loading control.

(F) Wss1 domain requirements for hydroxyurea tolerance. Five-fold dilutions of wss1Δ cells reconstituted with wild-type or wss1 mutants and cells were spotted on plates with or without hydroxyurea (50, 100, and 200 mM) and incubated at 30°C. Plates with 50 and 100mM hydroxyurea were imaged after 2 days, whereas plates with 200 mM hydroxyurea were imaged after 4 days.

3.3.2 Proteolytic targets of Wss1 upon hydroxyurea stress

Having established that the proteolytic activity of Wss1 is essential to overcome the hydroxyurea stress, we next sought to identify Wss1 target proteins, whose degradation would be important in mediating tolerance to hydroxyurea. We took a comparative proteomics approach to unbiasedly assay the proteome of wildtype and *wss1Δ* cells in the presence and absence of hydroxyurea. We reasoned that putative Wss1 substrates would be more abundant in *wss1Δ* cells than in wildtype cells exposed to hydroxyurea. To quantitatively determine differences in protein abundance we labeled cells with stable isotope labeling by amino acids in cell culture (SILAC). Wildtype and *wss1Δ* were labeled in light and heavy Lysine, respectively. Whole cell extracts from SILAC-labeled wildtype and *wss1Δ* cells, under basal conditions and after hydroxyurea treatment, were generated from three biological replicates (**Figure 3.2A**) and processed for mass spectrometry. We identified 3,793 unique proteins representing ~ 84 of the expressed yeast proteome in the untreated and hydroxyurea-treated cells. Of the 3,793 proteins identified, 1,077 were up-regulated specifically in *wss1Δ* cells treated with hydroxyurea compared to 317 up-regulated proteins in wildtype cells treated with hydroxyurea (**Figure 3.2B**), which is consistent with our hypothesis that proteins would accumulate in *wss1Δ* cells. Using GO enrichment analysis, we observed an enrichment of proteins involved in RNA metabolism and proteins that have nuclear function amongst the up-regulated proteins in hydroxyurea-

treated *wss1Δ* cells (**Figure 3.2C**). Of the proteins that were found to be more abundant in hydroxyurea-treated *wss1Δ* cells, we focused our attention on three candidates - Rad51, Ddi1 and histone H3 (**Figure 3.2D and Figure 3.7A**). To validate our mass spectrometry based results, we tagged Rad51 and Ddi1 at their carboxyl-terminal ends with three tandem copies of the FLAG epitope in wildtype and *wss1Δ* cells. Rad51 is a single-stranded DNA binding protein that is important for initiating recombination, while Ddi1 is an aspartic protease that functions as a shuttle factor for the proteasome. Rad51 accumulation on single-stranded DNA is toxic to cells and its accumulation is counteracted by Srs2 and BLM helicases. *wss1Δ* cells have previously been reported to have a hyper-recombination phenotype (Munoz-Galvan et al., 2013; Stingele et al., 2016). Western blot analysis of Rad51 protein levels in wildtype and *wss1Δ* cells reveals that Rad51 is more abundant in *wss1Δ* cells (**Figure 3.3A**). We reasoned that if Rad51 were to accumulate in *wss1Δ* cells during a hydroxyurea treatment and this Rad51 accumulation is the underlying reason for the hydroxyurea sensitivity, then the genetic ablation of Rad51 should suppress the hydroxyurea sensitivity observed in *wss1Δ* cells. To directly test this hypothesis, we generated *rad51Δ* and *wss1Δ rad51Δ* cells and found that *wss1Δ rad51Δ* cells were more sensitive to hydroxyurea than single mutants alone (**Figure 3.3B**). Similarly, Ddi1 was found to be upregulated in cells devoid of Wss1, as well as in cells treated with hydroxyurea (**Figure 3.7A** and **3.7B**). We generated *ddi1Δ* and *wss1Δ ddi1Δ* cells and tested their ability to tolerate hydroxyurea. We found that *ddi1Δ* cells alone were more sensitive to hydroxyurea than *wss1Δ* cells and a synergistic effect is observed in *wss1Δ ddi1Δ* cells (**Figure 3.7C**). Although deleting Rad51 or Ddi1 did not suppress the hydroxyurea sickness of *wss1Δ* cells, we were able to confirm our mass spectrometry based results to

confirm that Rad51 and Ddi1 are more abundant in *wss1Δ* cells.

An additional protein found in our mass spectrometry experiments, histone H3, has been previously shown to be toxic when it is expressed to high levels. We found that histone H3 and to a lesser extent linker histone H1, were enriched in hydroxyurea treated Δ*wss1* cells compared to treated wildtype cells (**Figure 3.2D**). We first confirmed, by western blot analysis, that histone H3 levels were regulated in a Wss1-dependent manner. We observe an appreciable decrease in histone H3 protein levels when wildtype cells are
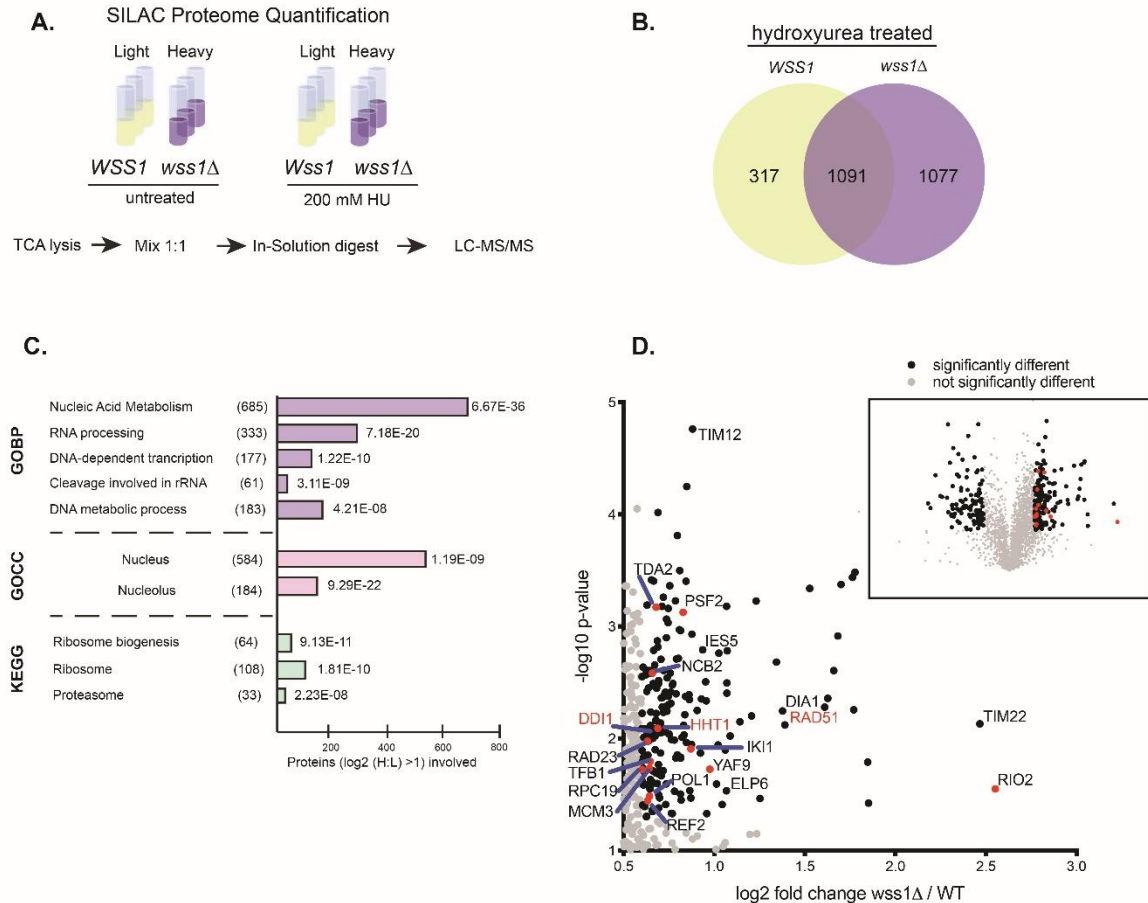
Figure 3.2: Excess Histones Are Toxic to Wss1 Mutant Cells
(A) Mass spectrometry (MS)-based proteome quantification. Experimental scheme used for quantifying protein abundance in wild-type or wss1Δ cells treated with or without hydroxyurea (200 mM). Cells were SILAC labeled with light or heavy lysine. TCA extracts were generated after hydroxyurea treatment and mixed 1:1 before analysis with liquid chromatography-tandem MS (LC-MS/MS).

(B) Venn diagram of regulated proteins identified in wild-type or wss1Δ cells treated with hydroxyurea.

(C) GO enrichment analysis. Proteins found to be statistically significantly enriched in wss1Δ cells to predict the molecular function of Wss1 in the presence of hydroxyurea.

(D) Volcano plots representing SILAC-based quantification of peptides from wild-type and wss1Δ cells treated with hydroxyurea. Inset represents the results of three independent MS-based quantification of proteome changes in wild-type (left) and wss1Δ cells (right) treated with hydroxyurea. Gray circles represent proteins that were not statistically enriched. Black circles represent proteins that were significantly enriched t test p < 0.05. Proteins labeled in red were confirmed by western blot analysis.

treated with hydroxyurea. This decrease in histone H3 protein levels was not observed in similarly treated *wss1Δ* cells (**Figure 3.3C**). Furthermore, the decrease in histone H3 protein levels depends on the proteolytic activity of Wss1 as we do not observe a decrease in histone H3 protein levels when a Wss1 catalytic-inactive mutant is over-expressed (**Figure 3.3D**).

Previous reports indicate that over-expression of histones is toxic in *rad53Δ* cells (Gunjan et al., 2003; Singh et al., 2009; Singh et al., 2010). We hypothesized that in the presence of hydroxyurea, excess histones accumulate in *wss1Δ* cells and this accumulation leads to cytotoxicity. To investigate if excess histones are causal for hydroxyurea-induced cytotoxicity in *wss1Δ* cells we sought to delete histone H3 from cells. However, the fact that histones are essential for yeast viability precluded us from deleting histone H3 from cells alone and in combination with Wss1. To circumvent this technical limitation, we reduced histone levels in *wss1Δ* and wildtype cells by deleting the *HHT2-HHF2* (H3-H4) gene pair that was shown to produce approximately seven-fold more H3-H4 transcripts than the other gene pair, *HHT1-HHF1* (Holmes and Mitchell Smith, 2001). We next generated *wss1Δ hht2-hhf2Δ* cells and tested four independent isolates for hydroxyurea sensitivity. Importantly and in agreement with previous reports, we found that *hht2-hhf2Δ* cells can tolerate hydroxyurea treatment. Remarkably, deletion of the *HHT2-HHF2* gene pair suppressed the sensitivity of *wss1Δ* yeast cells to hydroxyurea suggesting that the

cytotoxicity of *wss1Δ* yeast cells in the presence of hydroxyurea is due, in part, to accumulation of excess histones (**Figure 3.3E**). If reducing histones levels alleviates the hydroxyurea sensitivity of *wss1Δ* cells, then we would expect to find that the over-expression of histones adversely effects *wss1Δ* in the absence of hydroxyurea. To test this hypothesis, we introduced a plasmid into cells bearing histone H3 under control of the inducible galactose promoter. We introduced this plasmid in wildtype cells, *wss1Δ* and *rad53Δ* cells and spotted the resulting yeast strains and assessed the phenotype. In line with our hypothesis, we observed a slight growth disadvantage in *wss1Δ* and *rad53Δ* cells compared to the wildtype cells only when histone H3 is over-expressed with galactose (**Figure 3.3F**). Taken together, our results suggest that Wss1 regulates core histone levels during replication stress.

Interestingly, wildtype Wss1 fails to cleave histone H3 within nucleosomes suggesting that Wss1 preferentially targets histone H3 when it is bound non-specifically to DNA (**Figure 3.4C**). Wss1 can act on histone H3 and this effect is not specific for histone H3 alone, as wildtype Wss1 could also cleave histones H2A and H4 in the presence of ssDNA (**Figure 3.4D**, lanes 6 and 7). Notably, we could not observe cleavage products for histone H2A by western blot, most likely due to the loss of epitope that is recognized by the H2A antibody after Wss1 treatment. Taken together, Wss1 can directly act on core histones (H2A, H3 and H4) bound to ssDNA but not when they are incorporated into nucleosomes which is consistent with a model wherein Wss1 cleaves histones that are non-specifically bound to single-stranded DNA created with hydroxyurea treatment.
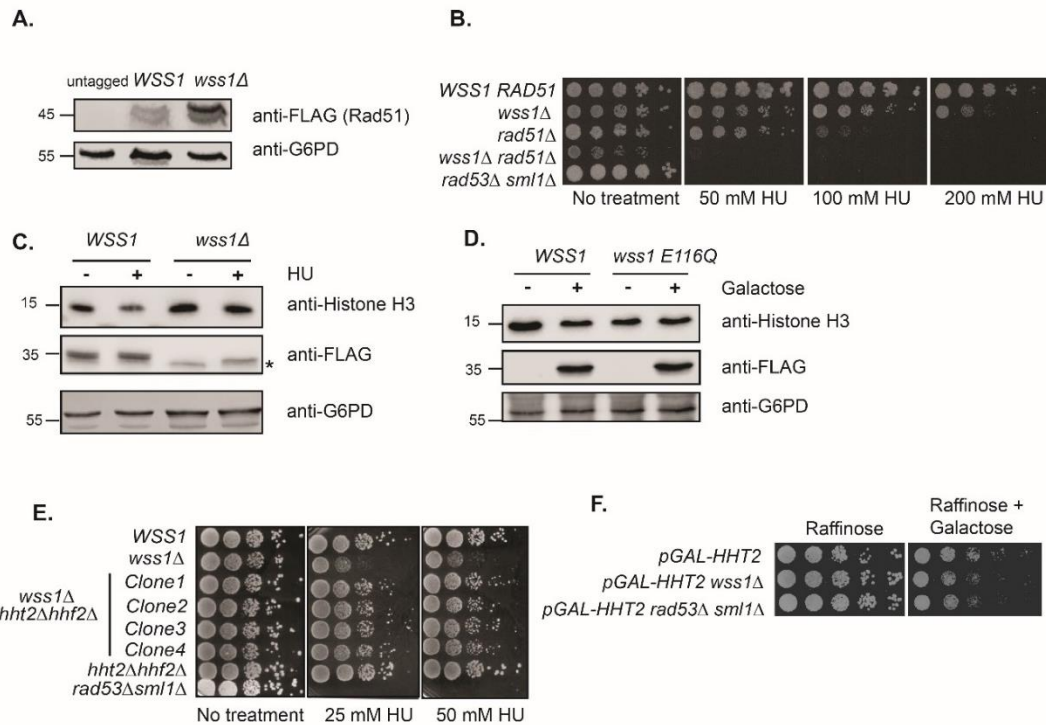
Figure 3.3. Wss1 Targets Histones for Proteolysis

(A) wss1Δ cells express increased Rad51 protein levels compared to wild-type cells. The endogenous Rad51 gene was tagged with 3xFLAG in wild-type or wss1Δ cells. Glucose-6-phosphate dehydrogenase (G6PD) serves as a loading control.

(B) Wss1 and Rad51 double mutants are hypersensitive to hydroxyurea. Five-fold dilutions of wild-type, wss1Δ, rad51Δ, wss1Δ rad51Δ, and rad53Δ were spotted on YPD plates with or without hydroxyurea and incubated at 30°C for 3 days.

(C) Histone H3 levels decrease in hydroxyurea-treated cells in a Wss1-dependent manner. Western blot analysis of endogenous histone H3 protein levels from wild-type and wss1Δ cells treated with or without hydroxyurea. Wss1 was 3xFLAG tagged. The asterisk indicates a non-specific band cross-reacting with the anti-FLAG antibody. G6PD serves as a loading control.

(D) Decreased histone H3 protein levels depend on Wss1 catalytic activity. A western blot analysis of endogenous histone H3 protein levels from cells overexpressing wild-type or catalytic inactive Wss1 (wss1-E116Q) was conducted. The expression of FLAG-tagged Wss1 and wss1-E116Q were placed under the control of the galactose promoter. Cells were grown in the presence of raffinose (-) or raffinose plus galactose (+) to induce expression. G6PD serves as a loading control.

(E) Reducing histone levels suppresses hydroxyurea sensitivity of wss1Δ cells. Five-fold dilutions of wild-type, wss1Δ, rad53D, and htt2Δ hhf2Δ, and four independent clones of wss1Δ htt2Δ hhf2Δ cells are shown. Cells were spotted on YPD plates with and without hydroxyurea. Plates were incubated for 3 days at 30°C.

(F) Overexpression of histone adversely affects wss1Δ cells. Wild-type, wss1Δ, or rad53Δ cells were transformed with a plasmid containing histone H3 under the control of the galactose promoter. Five-fold dilutions of transformed strains were spotted on plates containing raffinose or raffinose + galactose. Plates were incubated for 3 days at 30°C.

3.3.3 Histones inhibit Wss1 self-cleavage activity

Wss1, like SPRTN, elicits self-cleavage activity in the presence of DNA (Balakirev et al., 2015; Lopez-Mosqueda et al., 2016; Stingele et al., 2014; B. Vaz et al., 2016). Whether Wss1 self-cleavage is a pre-requisite for substrate targeting has not been addressed. As such, we next investigated the relationship between Wss1 self-cleavage and Wss1-mediated histone cleavage. *In vitro,* Wss1 self-cleavage was readily observed in the presence of DNA (**Figure 4A**, lanes 3 and 4 and **Figure 5**, lanes 1 and 2) as previously reported (Balakirev et al., 2015; Stingele et al., 2014). However, Wss1 self-cleavage is inhibited in the presence of histone H3 (**Figure 4A**, compare lanes 8 and 9 to lanes 3 and 4). In addition, this inhibitory effect on Wss1 self-cleavage, increases concomitantly with an increasing concentration of histone H3 substrate (**Figure 5A**, lanes 4-6). Wss1, although not self-cleaved, still cleaves histone H3 into distinct products (**Figure 5A**, lanes 3-6) suggesting that Wss1 self-cleavage is not a prerequisite for Wss1 activity. To gain more mechanistic insight into this inhibitory effect of histone H3 on Wss1 self-cleavage, we analyzed the kinetics of Wss1 self-cleavage in the presence or absence of histone H3. Complete Wss1 self-cleavage is achieved in forty minutes in the absence of histone H3 whereas in the presence of histone H3, Wss1 self-cleavage is significantly delayed (**Figure 5B, lane 6**). This raises an interesting possibility that Wss1 self-cleavage is a protease inactivating mechanism and substrates such as histones stabilize Wss1 - preventing its premature inactivation in cells. To test this hypothesis further, we created a Wss1 mutant (wss1-RKR) that does not elicit self-cleavage in the presence of DNA. To obtain this Wss1 mutant, we first mapped the cleavage site and substituted the amino acids at the cleavage

site (**Supplemental Figure 3A**). The Wss1-RKR mutant has attenuated self-cleavage activity although it can still bind to DNA (**Supplemental Figure 3B and 3C**). wss1-RKR was able to cleave histone H3 in the presence of ssDNA indicating that Wss1 self-cleavage activity is not a requirement to cleave substrates (**Figure 5C**). We next asked if the Wss1-RKR mutant would provide *wss1Δ* cells the ability to tolerate hydroxyurea. Indeed, *wss1Δ* cells reconstituted with the *wss1-RKR* mutant allele can tolerate hydroxyurea as wildtype cells (**Figure 5D**). Taken together, our results would suggest that Wss1 substrates can regulate Wss1 self-cleavage.
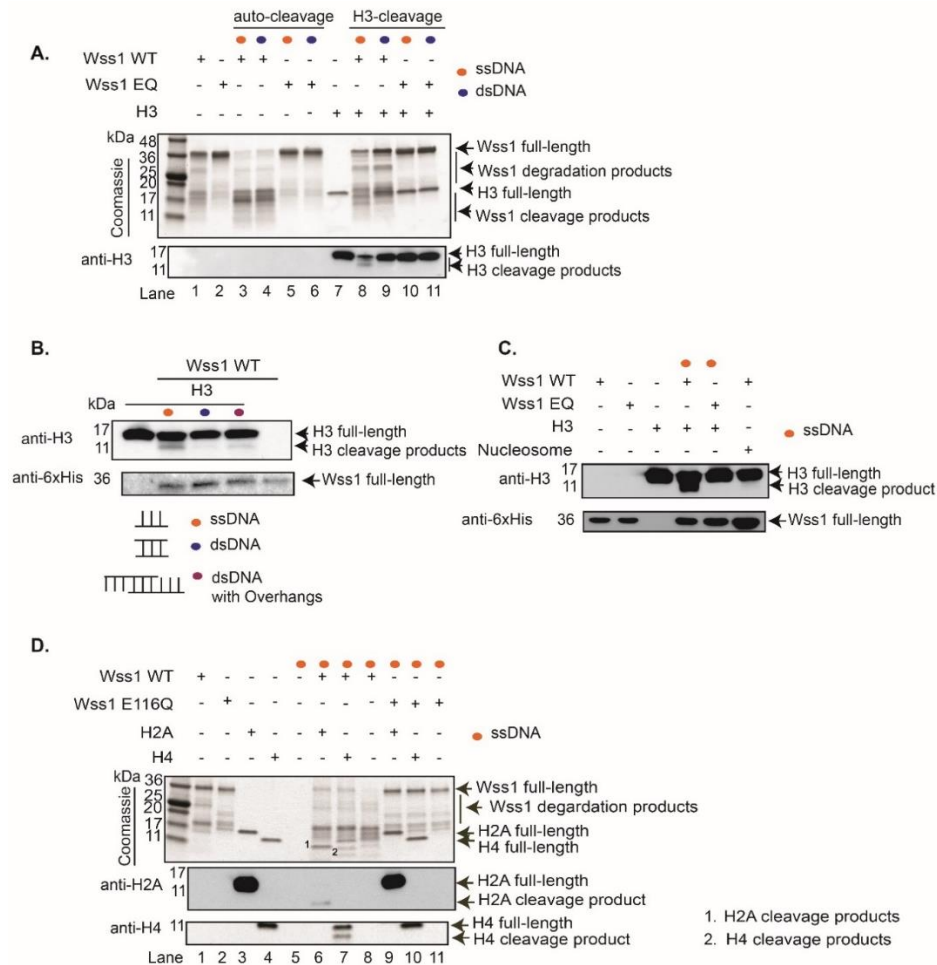
Figure 3.4. Wss1 Directly Targets Histones for Proteolysis

(A) Wss1 cleaves histone H3 in the presence of ssDNA but not dsDNA. Purified wild-type (3.1 mM) or catalytic inactive Wss1 (E116Q) (3.1 mM) mutant proteins were incubated in the absence or presence of histone H3 (3.3 mM) and in the absence or presence of a 73-mer ssDNA or dsDNA for 2 h at 30°C. Reactions were stopped by the addition of 13 Laemmli sample buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue to monitor Wss1 self-cleavage, and histone H3 cleavage was monitored by immunoblotting with antibodies against histone H3.

(B) In vitro Wss1 cleaves histone H3 in the presence of dsDNA with ssDNA overhangs. Purified wild-type Wss1 (3.1 mM) was incubated with histone H3 in the absence or presence of a 73-mer ssDNA or dsDNA or dsDNA with ssDNA overhangs for 2 h at 30°C. Reactions were stopped by the addition of 13 Laemmli sample buffer. Proteins were separated by SDS-PAGE and were further used in the immunoblotting procedure to monitor histone H3 cleavage using antibodies against histone H3.

(C) Wss1 cleaves free histone H3 but not those incorporated into nucleosomes. Purified wild-type Wss1 (3.1 mM) was incubated with histone H3 in the presence of a 73-mer ssDNA or mononucleosomes (0.42 mM) for 2 h at 30°C. Reactions were stopped by the addition of 13Laemmli buffer. Proteins were separated by SDSPAGE and used in immunoblotting to monitor histone H3 cleavage using antibodies against histone H3.

(D) Wss1 cleaves histone H2A and H4. Purified wild-type (3.1 mM) or catalytic inactive Wss1 (E116Q) (3.1 mM) mutant proteins were incubated in the absence or presence of histone H2A (3.3 mM) or H4 (3.3 mM) and in the absence or presence of 73-mer ssDNA for 2 h at 30°C. Reactions were stopped by the addition of 1X Laemmli sample buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue to monitor Wss1 self-cleavage, and histone H2A and H4 cleavage was monitored by immunoblotting with antibodies against histone H2A and H4, respectively.
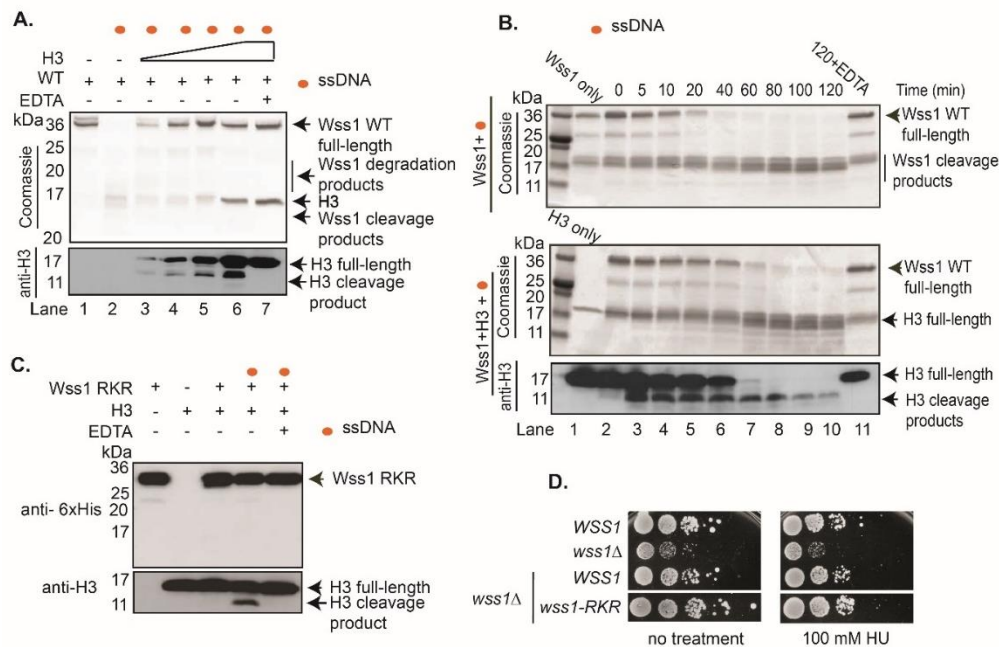
Figure 3.5. Wss1 Self-Cleavage Is Not a Requirement for Proteolytic Activity

(A) Titration of histone H3 in in vitro Wss1 auto-cleavage reactions. Purified wild-type Wss1 (3.1 mM) and varying concentrations of histone H3 (3.3, 6.6, 13.2, and 26.4 mM) were incubated in the presence of a 39-mer ssDNA (10 mM) for 2 h at 30°C. A control reaction without histone H3 or ssDNA was used in the experiment. Reactions were stopped by the addition of 1X Laemmli sample buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue to monitor Wss1 self-cleavage, and histone H3 cleavage was monitored by immunoblotting with antibodies against histone H3.

(B) The presence of H3 delays Wss1 self-cleavage. Purified wild-type Wss1 (3.1 mM) was incubated with or without histone H3 (3.3 mM) in the absence or presence of ssDNA (10 mM) for 2 h at 30°C, and samples were taken at indicated time points. Proteins were resolved by SDS-PAGE and stained with Coomassie blue to monitor the self-cleavage of Wss1, and H3 cleavage was monitored by immunoblotting with antibodies against histone H3.

(C) A self-cleavage Wss1 mutant displays proteolytic activity. Purified Wss1 RKR (3.1 mM) was incubated with histone H3 (3.3 mM) in the absence or presence of ssDNA (10 mM) for 2 h at 30°C. Reactions were stopped by the addition of 1X Laemmli sample buffer. Proteins were resolved by SDS-PAGE and used for immunoblotting to monitor the presence of Wss1-RKR or histone H3 cleavage using antibodies against 6xHis-tagged Wss1 RKR and H3 antibodies, respectively.

(D) wss1-RKR mutant can rescue the hydroxyurea sensitivity of wss1D cells. Five-fold dilutions of wild-type or wss1D cells reconstituted with either wild-type or wss1 RKR mutant alleles were spotted on plates in the absence or presence of hydroxyurea (100 mM) and incubated at 30°C. Plates were imaged after 3 days.

3.4 Discussion

We have utilized the sensitivity of *wss1*Δ cells to hydroxyurea to identify histones as a novel class of Wss1 substrates, which are distinct from covalent DPC substrates. Histones are known to form insoluble protein-DNA aggregates when expressed in excess (Clark et al., 1990). In cells histone abundance is carefully regulated at many levels (Osley, 1991). During S-phase, histone chaperones sequester histones and deposit them into nascent DNA. Wss1 cannot degrade histones assembled in nucleosomes but can degrade histones that are binding to single-stranded DNA. When cells are exposed to hydroxyurea, large tracks of single stranded DNA are exposed at DNA replication forks and newly synthesized histones cannot be assembled into nucleosomes. As such, the histones will bind non-specifically to any nucleic acid and such high affinity/avidity binding of histones to DNA could interfere with DNA metabolism. Fob1 is one notable example of a protein that strongly binds to DNA in budding yeast. Fob1 binding to DNA is sufficient for blocking DNA replication forks at the rDNA locus (B. J. Brewer et al., 1988; Kobayashi et al., 1996). It has been previously shown that histone abundance is cytotoxic by saturating binding to histone modifying enzymes as well as non-specific binding to DNA and RNA (Singh et al., 2010).

Hydroxyurea treatment is more toxic to *wss1*Δ cells overexpressing Wss1 catalytic dead mutants (E116Q) than to *wss1*Δ cells (**Figure 3.1F**). This toxicity could be explained by Wss1-E116Q binding to protein substrates but not cleave them and thereby form higher complexity enzyme-substrate complexes on DNA, but other models are also possible. Reducing histone dosage by deleting the H3-H4 gene pair (*HHT2-HHF2*) suppressed the hydroxyurea sensitivity in *wss1*Δ cells, suggesting that cytotoxicity of *wss1*Δ yeast cells in

the presence of hydroxyurea is due, in part, to non-specific binding of histones to DNA. It is possible that deleting *HHT2-HHF2* from cells strongly confers resistance to hydroxyurea irrespective of Wss1 function. However, deletion of histones does not suppress hydroxyurea sensitivity. For instance, the exoribonuclease Xrn1, when deleted, renders cells hypersensitive to hydroxyurea and this sensitivity is not suppressed when histones are deleted in an *xrn1* mutant background(Lao et al., 2018).

To obtain a direct link between histone proteolysis and Wss1 during hydroxyurea treatment, we have utilized *in vitro* cleavage assays to show that Wss1 could degrade histone H2A, H3 and H4. Importantly, Wss1 could target histones only in the presence of ssDNA, a finding that is consistent with vast ssDNA exposed with hydroxyurea treatment.

Excess histones non-specifically binding to nucleic acids is likely to have adverse effects in cells due to interference with polymerases important for DNA replication, gene transcription and translation, as all these cellular processes have DNA as an initial common template (Edenberg et al., 2014). In the case of *wss1Δ* cells during replication stress, we speculate that histones pose a problem to gene transcription machinery rather than to DNA replication *per se*, as we did not detect problems with global DNA replication under our experimental conditions. It is possible that in *wss1Δ* cells, other pathways cooperate to preserve DNA replication fork progression. Indeed, the histone H3 chaperone, Asf1, was shown to buffer excess histones during replication stress (Groth et al., 2005). In addition, Rad53 mediates a phosphorylation-dependent degradation of excess histones and this mechanism is also likely to function during replication stress (Gunjan et al., 2003; Singh et al., 2009). More recently, Ddi1 was implicated to function in parallel to Wss1 for providing replication stress tolerance (M. Svoboda et al., 2019). Taken together, cells

employ multiple pathways to counteract excess histones during replication stress. Wss1 dependent-degradation of histones is one way cells are able to tolerate replication stress in order to maintain the integrity of the genome.
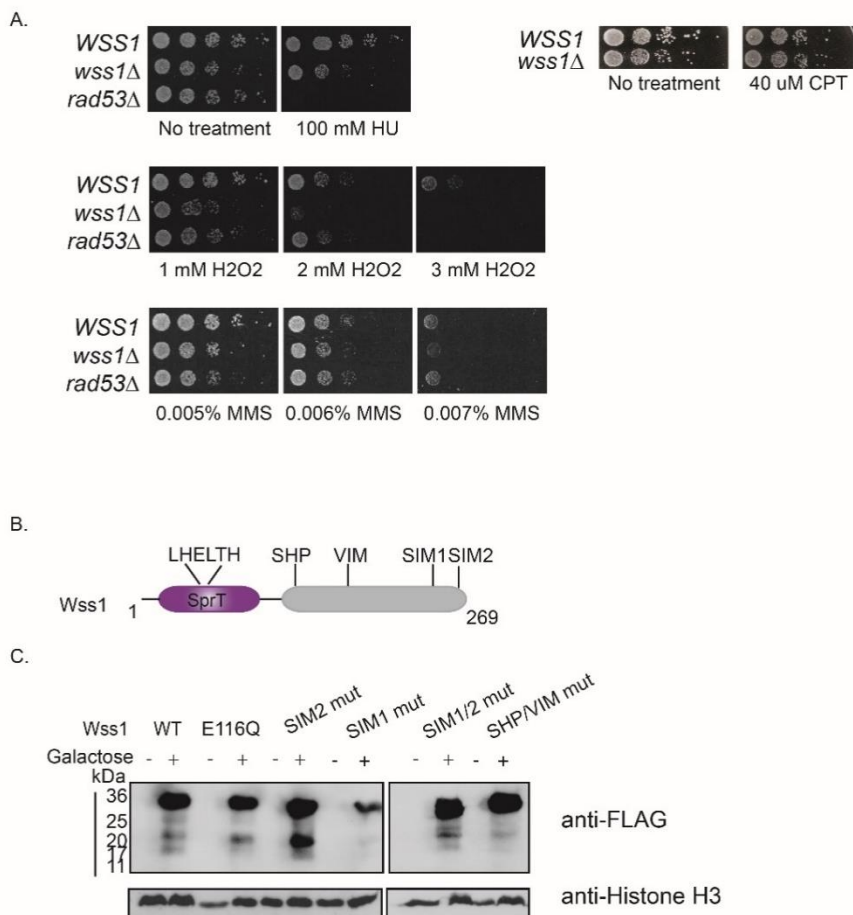
Figure 3.6. Wss1 deficient cells are sensitive to hydroxyurea and hydrogen peroxide

A) Assessment of sensitivity of wss1Δ cells towards various genotoxic agents. Ten-fold dilutions of wild type or wss1Δ yeast cells were spotted on plates in the absence or presence of H2O2 (2 or 3mM), Hydroxyurea (100mM), CPT (40μM) or MMS (0.005-0.007%). The plates were incubated at 30°C and imaged after 3 days. B) Domain organization of Wss1. C) Expression analysis of FLAG-tagged Wss1 wildtype and various mutant alleles in wss1Δ cells. Cell extracts were prepared by TCA precipitation and immunoblotted using anti-FLAG antibody (Sigma) or histone H3 antibody (Abcam).
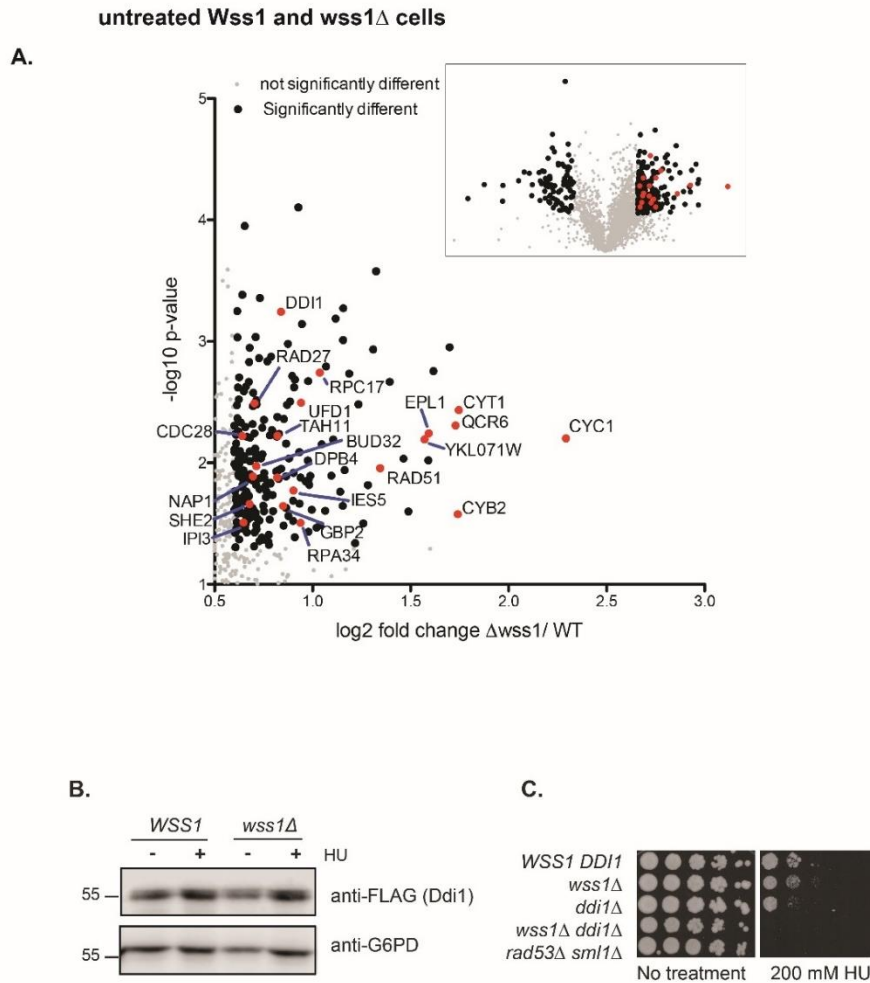
Figure 3.7: Mass spectrometry-based proteome quantification in untreated cells

A) Volcano plots representing SILAC based quantification of peptides from untreated wildtype and *wss1Δ* cells. Inset represents the results of three independent mass spectrometry quantifications of proteome changes in wildtype (left) and *wss1Δ* cells (right). Gray circles represent proteins that were not statistically enriched. Black circles represent proteins that were significantly enriched *t-test p<0.05. Red circles represent the proteins with their respective protein names.* **B**) Hydroxyurea treated *wss1Δ* cells express increased Ddi1 protein levels compared to untreated cells. The endogenous Ddi1 gene was 3xFLAG tagged at its C-terminus in wildtype or *wss1Δ* cells. G6PD serves as a loading control. B) Wss1 and Ddi1 double mutants are hypersensitive to hydroxyurea. Five-fold dilutions of wildtype, *wss1Δ*, *ddi1Δ*, *wss1Δ ddi1Δ*, and *rad53Δ* were spotted on YPD plates with or without hydroxyurea and incubated at 30°C for 3 days.
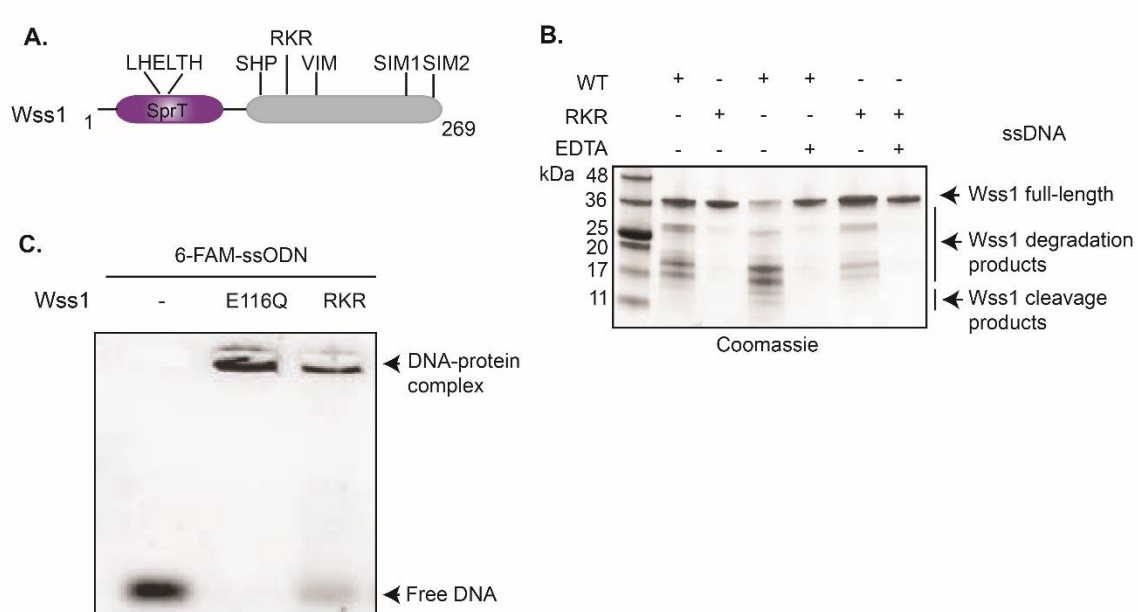
Figure 3.8: Characterization of Wss1-RKR mutant

A) Depiction of RKR site in the domain organization of Wss1. **B**) Wss1-RKR mutant is self-cleavage deficient. Purified wild type (3.1µM) or Wss1-RKR (3.1µM) mutant proteins were incubated in the absence or presence of a 73mer ssDNA and in the absence or presence of EDTA (2mM). Reactions were stopped by addition of 1x Laemmli buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue. **C**) Wss1-RKR binds to ssDNA. EMSA analysis of Wss1 full-length (E116Q) and Wss1-RKR in the presence of a fluorescently labelled ssODN (6'FAM-ssODN).

3.5 Experimental procedures

3.5.1 Yeast Strains

W303a yeast cells were used to generated *wss1Δ* yeast strain by targeted gene deletion using standard yeast methods (yeast gene deletion plasmids: pAG32-hphMx, pAG25-CloNAT, pFA6a-TRP1). To generate *wss1Δ* yeast strains expressing 2xFLAG-Wss1 wildtype or various mutant alleles or 2xFLAG-SPRTN wildtype or catalytic mutant allele under the control of the galactose inducible promoter, Wss1 was cloned into pDONR223 (Invitrogen) using the Gateway cloning system and subsequently cloned into pAG-306GAL-ccdB (pAG-306GAL-ccdB was a gift from Susan Lindquist (Addgene plasmid #14139). The plasmid was linearized at the URA3 loci with Stu1 and transformed into yeast strains.

3.5.2 Yeast spot assays

Yeast strains were grown overnight at 30°C with shaking for yeast spot dilution assays. Next day, 0.2 OD (approximately 2×10^5 cells) of cells including ten- or five-fold dilutions prepared in sterile ddH$_2$O. Cells were platted as spots in either YPD (Yeast extract, peptone and Dextrose) or YPG (Yeast extract, peptone and galactose) or YPD or YPG plates containing drugs hydroxyurea or CPT or H$_2$O$_2$. Plates were incubated at 30°C and photographs of the plates were taken as indicated in the figure legends.

3.5.3 Mass Spectrometry based analysis of yeast whole cell extracts

Protein from yeast whole cell extracts were separated by 1D PAGE, each gel-lane was cut in 12 pieces and subjected to in-gel trypsin digestion. Tryptic peptides were desalted by StageTip cleanup and analyzed by LC-MS/MS. In brief, the peptides were separated by C18 reversed phase chromatography with an Easy nLC 1200 (ThermoFisher) coupled to a Q Exactive HF mass spectrometer (ThermoFisher). For each protein identification and label-free quantification, spectra were extracted and searched against the Uniprot *Saccharomyces cerevisiae* database with Maxquant.

3.5.4 Expression and Purification of Wss1 wildtype and mutant alleles

Wss1 wild type or mutant alleles was cloned in to pET15 b vector with a N-terminal 6x Histidine tag and a 3C-protease (in-house produced) site using restriction sites (Nde1/Xho1) by DNA restriction method. Wss1 was expressed in Rosetta *E. coli* cells. Expression was induced with 0.25mM IPTG overnight at 18°C. Cells were harvested by centrifugation and lysed in lysis buffer (50mM Tris pH 7.5, 1M NaCl, 10% glycerol, 10mM imidazole) and performed Affinity based batch purification using TALON affinity resin. The protein was eluted using elution buffer containing Imidazole (50mM Tris pH7.5, 200mM NaCl, 200mM Imidazole). A final round of size-exclusion chromatography was performed using Superdex 75 16/60 column in the buffer 20mM Tris pH 7.5, 300mM NaCl. Fractions containing monomeric form of wild type or mutant proteins were pooled and were further concentrated using centrifugal filter (10kD cutoff) to a final concentration of 2 mg/ml and flash frozen at -80°C for further use.

3.5.5 Wss1 auto-cleavage assays

Purified Wss1 wildtype (3.1 μM) or various mutant alleles (3 μM) were incubated with ssDNA(Sigma)(5'GCGCGCCCATTGATACTAAATTCAAGGATGACTTATTTC3') (10μM) at 30°C for 2 hrs. EDTA (2mM) was used to inhibit the reactions. Reactions were stopped by adding 1x Laemmli buffer and samples were boiled at 95°C for 10 min. Further, proteins were resolved using SDS-PAGE and further transferred to a nitro-cellulose membrane for Western blot analysis.

3.5.6 Histone titration in the presence of Wss1 and ssDNA

Purified Wss1 wild type (3.1 μM) was mixed with purified histone H3 (Dong et al.) (3.3 μM, 6.6 μM, 13.2 μM and 26.4 μM) in the presence of ssDNA (10μM). Reactions were incubated at 30°C for 2hrs and stopped by addition of 1x Laemmli buffer and boiled at 95°C for 10 min. Further, samples were analyzed using SDS-PAGE analysis. Auto-cleavage of Wss1 was monitored by staining the protein using Coomassie. Histone H3 cleavage was monitored using anti-H3 antibody using western blot analysis.

3.5.7 Kinetics of Wss1 auto-cleavage and histone H3 cleavage

Purified Wss1 wild type (3.1 μM) was mixed with ssDNA (10μM) in the presence or absence of purified histone H3 (3.3 μM). Reactions were incubated at 30°C for 2 hrs. Samples were collected at indicated time points and stopped by addition of 1x Laemmli buffer and boiled at 95°C for 10 min. Further, samples were analyzed using SDS-PAGE analysis. Auto-cleavage of Wss1 was monitored by staining the protein using Coomassie.

Histone H3 cleavage was monitored using anti-H3 antibody using western blot analysis.

3.5.8 EMSA (Electrophoretic mobility shift assay)

Purified proteins in indicated concentrations were incubated with fluorescently labelled ssODN(Sigma)(0.25μM-6-FAM-39mer-5'-GCGCGCCCATTGATACTAAATTCAAG GATGACTTATC-3') in the reaction buffer (10mM Tris 7.5, 0.2mM DTT, 5μM ZnSo4) and incubated at 20°C for 30 min. Protein-DNA complexes were separated on 1.5% Agarose gel and visualized by FUSION-SL imager (Vilber).

3.5.9 Flow cytometry (FACS)

For flow cytometry analysis of yeast cells, yeast cells were collected at respective indicated time points and fixed them immediately with 70% EtOH in flow cytometry tubes. Next, cells were sonicated (20% amplitude, 3 sec) to separate cell clumps. Cells were centrifuged at 300 rpm for 5 min and ethanol was aspirated out. Later, cells were re-suspended in 1ml of RNAse buffer (50mM Tris pH8.0 and 15mM NaCl) with RNAse (0.25 mg/mL) and incubated at 50°C for 1 hour. Further, proteinase K (0.125 mg/mL) was added to the buffer and incubated for additional 1 hour at 50°C. Finally, propidium Iodide at a dilution of 1:1000 was added and vortexed to have final sample preparation. Readings were recorded on BD FACS[TM]CONTO II and FACS curves were analyzed using FlowJo software.

3.5.10 Preparation of yeast whole cell extracts for western blot analysis

TCA precipitation method was employed to obtain the protein extracts used in western blot analysis. For yeast protein extracts, 5-10 O.D cells were harvested by centrifugation were

re-suspended in 500μl of TCA lysis buffer (10mM Tris pH 8.0, 20% TCA, 25mM NH_4CH_3CO2) to precipitate the proteins and centrifuged to remove the supernatant. Precipitated protein pellets were washed with 70% Acetone and re-suspended in 75 μl buffer containing 10mM Tris pH 11.0 and 3% SDS and 75 μl of 1x Laemmli buffer was added to make a final volume of 150 μl and samples were boiled at 95°C for 10 min.

3.6 Acknowledgements

Chapter 4: Future Directions

4.1 Conclusion and future research directions

In summary, our work has provided additional molecular insight into how Wss1 contributes to the maintenance of genome integrity.

We identified the role of SUMOylation as a potential marker that recruits DPC repair proteins such as Wss1 to sites of DNA damage. We identified that SUMO E3 ligases Mms21 and Siz1 may serve important roles in DPC repair. It is intriguing that SUMOylation seems to control the fine molecular tuning of certain DNA damage response proteins, however we lack evidence about SUMO specific sites in Wss1 or DPC substrates that contributes its molecular action. It would be interesting to map SUMO-specific sites in Top1 or other substrates whose lack of SUMOylation would lead to lethal consequences in cells.

Our mass spectrometry data comparing wildtype and wss1 deleted mutants revealed an up-regulation of transcription related proteins in a hydroxy-urea dependent manner. We therefore speculate that wss1 may have a role in transcriptional regulation. To fully understand how Wss1 contributes to histone remodeling and transcriptional regulation, it will be worth proposing new experiments that provide molecular insights in this role. Other unknown functions of Wss1 or interacting partners of Wss1 can also be derived by by using a mass spectrometry-based approach both in the absence or presence of DNA damage.

Finally, it remains enigmatic how Wss1 gets recruited to sites of DNA damage. Understanding the molecular cues that recruit Wss1 will be a step closer to fully understanding the role of Wss1 in DNA damage response.

References

Andegeko, Y., Moyal, L., Mittelman, L., Tsarfaty, I., Shiloh, Y., & Rotman, G. (2001). Nuclear retention of ATM at sites of DNA double strand breaks. *J Biol Chem, 276*(41), 38224-38230.

Anderson, D. E., Losada, A., Erickson, H. P., & Hirano, T. (2002). Condensin and cohesin display different arm conformations with characteristic hinge angles. *J Cell Biol, 156*(3), 419.

Aparicio, O. M., Weinstein, D. M., & Bell, S. P. (1997). Components and Dynamics of DNA Replication Complexes in S. cerevisiae: Redistribution of MCM Proteins and Cdc45p during S Phase. *Cell, 91*(1), 59-69.

Aragón, L. (2018). The Smc5/6 Complex: New and Old Functions of the Enigmatic Long-Distance Relative. *Annual Review of Genetics, 52*, 89-107.

Baker, D. J., Wuenschell, G., Xia, L., Termini, J., Bates, S. E., Riggs, A. D., & O'Connor, T. R. (2007). Nucleotide excision repair eliminates unique DNA-protein cross-links from mammalian cells. *J Biol Chem, 282*(31), 22592-22604.

Balakirev, M. Y., Mullally, J. E., Favier, A., Assard, N., Sulpice, E., Lindsey, D. F., . . . Wilkinson, K. D. (2015). Wss1 metalloprotease partners with Cdc48/Doa1 in processing genotoxic SUMO conjugates. *Elife, 4*.

Barker, S., Weinfeld, M., & Murray, D. (2005). DNA-protein crosslinks: their induction, repair, and biological consequences. *Mutat Res, 589*(2), 111-135.

Beach, D., Durkacz, B., & Nurse, P. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. *Nature, 300*(5894), 706-709.

Bell, S. P., & Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature, 357*(6374), 128-134.

Ben-Yehoyada, M., Wang, L. C., Kozekov, I. D., Rizzo, C. J., Gottesman, M. E., & Gautier, J. (2009). Checkpoint Signaling from a Single DNA Interstrand Crosslink. *Molecular Cell, 35*(5), 704-715.

Bermúdez-López, M., Pociño-Merino, I., Sánchez, H., Bueno, A., Guasch, C., Almedawar, S., . . . Torres-Rosell, J. (2015). ATPase-Dependent Control of the Mms21 SUMO Ligase during DNA Repair. *PLOS Biology, 13*(3), e1002089.

Biggins, S., Bhalla, N., Chang, A., Smith, D. L., & Murray, A. W. (2001). Genes Involved in Sister Chromatid Separation and Segregation in the Budding Yeast Saccharomyces cerevisiae. *Genetics, 159*(2), 453-470.

Blat, Y., & Kleckner, N. (1999). Cohesins Bind to Preferential Sites along Yeast Chromosome III, with Differential Regulation along Arms versus the Centric Region. *Cell, 98*(2), 249-259.

Borgermann, N., Ackermann, L., Schwertman, P., Hendriks, I. A., Thijssen, K., Liu, J. C., . . . Mailand, N. (2019). SUMOylation promotes protective responses to DNA-protein crosslinks. *The EMBO Journal*, e101496.

Brewer, B. J., & Fangman, W. L. (1987). The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell, 51*(3), 463-471.

Brewer, B. J., & Fangman, W. L. (1988). A replication fork barrier at the 3' end of yeast ribosomal RNA genes. *Cell, 55*(4), 637-643.

Broach, J. R., Li, Y. Y., Feldman, J., Jayaram, M., Abraham, J., Nasmyth, K. A., & Hicks, J. B. (1983). Localization and sequence analysis of yeast origins of DNA replication. *Cold Spring Harb Symp Quant Biol, 47 Pt 2*, 1165-1173.

Caldecott, K. W. (2007). Mammalian single-strand break repair: mechanisms and links with chromatin. *DNA Repair (Amst), 6*(4), 443-453.

Caldecott, K. W. (2014a). DNA single-strand break repair. *Exp Cell Res, 329*(1), 2-8.

Caldecott, K. W. (2014b). Protein ADP-ribosylation and the cellular response to DNA strand breaks. *DNA Repair (Amst), 19*, 108-113.

Carmell, M. A., Dokshin, G. A., Skaletsky, H., Hu, Y. C., van Wolfswinkel, J. C., Igarashi, K. J., . . . Page, D. C. (2016). A widely employed germ cell marker is an ancient disordered protein with reproductive functions in diverse eukaryotes. *Elife, 5*.

Castillo, P., Bogliolo, M., & Surralles, J. (2011). Coordinated action of the Fanconi anemia and ataxia telangiectasia pathways in response to oxidative damage. *DNA Repair (Amst), 10*(5), 518-525.

Ceccaldi, R., Sarangi, P., & D'Andrea, A. D. (2016). The Fanconi anaemia pathway: new players and new functions. *Nat Rev Mol Cell Biol, 17*(6), 337-349.

Cesare, A. J., & Reddel, R. R. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. *Nature reviews. Genetics, 11*(5), 319-330.

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., & Thelander, L. (2003). Survival of DNA Damage in Yeast Directly Depends on Increased dNTP Levels Allowed by Relaxed Feedback Inhibition of Ribonucleotide Reductase. *Cell, 112*(3), 391-401.

Chen, X. L., Silver, H. R., Xiong, L., Belichenko, I., Adegite, C., & Johnson, E. S. (2007). Topoisomerase I-Dependent Viability Loss in Saccharomyces cerevisiae Mutants Defective in Both SUMO Conjugation and DNA Repair. *Genetics, 177*(1), 17-30.

Chvalova, K., Brabec, V., & Kasparkova, J. (2007). Mechanism of the formation of DNA-protein cross-links by antitumor cisplatin. *Nucleic Acids Research, 35*(6), 1812-1821.

Ciosk, R., Shirayama, M., Shevchenko, A., Tanaka, T., Toth, A., Shevchenko, A., & Nasmyth, K. (2000). Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. *Molecular Cell, 5*(2), 243-254.

Clark, D. J., & Kimura, T. (1990). Electrostatic mechanism of chromatin folding. *J Mol Biol, 211*(4), 883-896.

Compe, E., & Egly, J.-M. (2012). TFIIH: when transcription met DNA repair. *Nature reviews. Molecular cell biology, 13*(6), 343-354.

Cuylen, S., Metz, J., & Haering, C. H. (2011). Condensin structures chromosomal DNA through topological links. *Nat Struct Mol Biol, 18*(8), 894-901.

D'Ambrosio, C., Schmidt, C. K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., & Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. *Genes Dev, 22*(16), 2215-2227.

Daley, J. M., Palmbos, P. L., Wu, D., & Wilson, T. E. (2005). Nonhomologous End Joining in Yeast. *Annual Review of Genetics, 39*(1), 431-451.

Davidovic, L., Vodenicharov, M., Affar, E. B., & Poirier, G. G. (2001). Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism.

Exp Cell Res, 268(1), 7-13.

Davis, G. M., Dokshin, G.A., Sawle, A.D., Eldridge, M.D., Romer, K.A., Gourley, T.E., Molesworth, L.W., Tatnell, H.R., Ozturk, A.R., de Rooij, D.G., et al. (2019). GCNA interacts with Spartan and Topoisomerase II to regulate genome stability. *bioRxiv*.

Deans, A. J., & West, S. C. (2009). FANCM connects the genome instability disorders Bloom's Syndrome and Fanconi Anemia. *Molecular Cell, 36*(6), 943-953.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., & Elledge, S. J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes Dev, 12*(18), 2956-2970.

Dhar, V., & Schildkraut, C. L. (1991). Role of EBNA-1 in arresting replication forks at the Epstein-Barr virus oriP family of tandem repeats. *Mol Cell Biol, 11*(12), 6268-6278.

Di Bacco, A., Ouyang, J., Lee, H.-Y., Catic, A., Ploegh, H., & Gill, G. (2006). The SUMO-specific protease SENP5 is required for cell division. *Molecular and Cellular Biology, 26*(12), 4489-4498.

Doherty, A. J., Jackson, S. P., & Weller, G. R. (2001). Identification of bacterial homologues of the Ku DNA repair proteins. *FEBS Letters, 500*(3), 186-188.

Dong, H., Nebert, D. W., Bruford, E. A., Thompson, D. C., Joenje, H., & Vasiliou, V. (2015). Update of the human and mouse Fanconi anemia genes. *Human genomics, 9*, 32-32.

Duan, X., Sarangi, P., Liu, X., Rangi, G. K., Zhao, X., & Ye, H. (2009). Structural and functional insights into the roles of the Mms21 subunit of the Smc5/6 complex. *Molecular Cell, 35*(5), 657-668.

Duan, X., Yang, Y., Chen, Y.-H., Arenz, J., Rangi, G. K., Zhao, X., & Ye, H. (2009). Architecture of the Smc5/6 Complex of Saccharomyces cerevisiae Reveals a Unique Interaction between the Nse5-6 Subcomplex and the Hinge Regions of Smc5 and Smc6. *The Journal of biological chemistry, 284*(13), 8507-8515.

Duina, A. A., Miller, M. E., & Keeney, J. B. (2014). Budding Yeast for Budding Geneticists: A Primer on the
Saccharomyces cerevisiae
Model System. *Genetics, 197*(1), 33-48.

Dujon, B. (1996). The yeast genome project: what did we learn? *Trends in Genetics, 12*(7), 263-270.

Duxin, J. P., Dewar, J. M., Yardimci, H., & Walter, J. C. (2014). Repair of a DNA-protein crosslink by replication-coupled proteolysis. *Cell, 159*(2), 346-357.

Edenberg, E. R., Downey, M., & Toczyski, D. (2014). Polymerase stalling during replication, transcription and translation. *Curr Biol, 24*(10), R445-452.

Elion, E. A. (2000). Pheromone response, mating and cell biology. *Curr Opin Microbiol, 3*(6), 573-581.

Enderle, J., Dorn, A., Beying, N., Trapp, O., & Puchta, H. (2019). The Protease WSS1A, the Endonuclease MUS81, and the Phosphodiesterase TDP1 Are Involved in Independent Pathways of DNA-protein Crosslink Repair in Plants. *Plant Cell, 31*(4), 775-790.

Escribano-Díaz, C., Orthwein, A., Fradet-Turcotte, A., Xing, M., Young, Jordan T. F., Tkáč, J., . . . Durocher, D. (2013). A Cell Cycle-Dependent Regulatory Circuit

Composed of 53BP1-RIF1 and BRCA1-CtIP Controls DNA Repair Pathway Choice. *Molecular Cell, 49*(5), 872-883.

Esteras, M., Liu, I. C., Snijders, A. P., Jarmuz, A., & Aragon, L. (2017). Identification of SUMO conjugation sites in the budding yeast proteome. *Microbial cell (Graz, Austria), 4*(10), 331-341.

Fagbemi, A. F., Orelli, B., & Schärer, O. D. (2011). Regulation of endonuclease activity in human nucleotide excision repair. *DNA Repair (Amst), 10*(7), 722-729.

Ferri, D., Orioli, D., & Botta, E. (2020). Heterogeneity and overlaps in nucleotide excision repair disorders. *Clinical Genetics, 97*(1), 12-24.

Fox, T. D., Folley, L. S., Mulero, J. J., McMullin, T. W., Thorsness, P. E., Hedin, L. O., & Costanzo, M. C. (1991). [10] Analysis and manipulation of yeast mitochondrial genes. In *Methods Enzymol* (Vol. 194, pp. 149-165): Academic Press.

Franzusoff, A., Rothblatt, J., & Schekman, R. (1991). [45] Analysis of polypeptide transit through yeast secretory pathway. In *Methods Enzymol* (Vol. 194, pp. 662-674): Academic Press.

Fujioka, Y., Kimata, Y., Nomaguchi, K., Watanabe, K., & Kohno, K. (2002). Identification of a novel non-structural maintenance of chromosomes (SMC) component of the SMC5-SMC6 complex involved in DNA repair. *The Journal of biological chemistry, 277*(24), 21585-21591.

Ganji, M., Shaltiel, I. A., Bisht, S., Kim, E., Kalichava, A., Haering, C. H., & Dekker, C. (2018). Real-time imaging of DNA loop extrusion by condensin. *Science, 360*(6384), 102.

Geiss-Friedlander, R., & Melchior, F. (2007). Concepts in sumoylation: a decade on. *Nature Reviews Molecular Cell Biology, 8*, 947.

Gillespie, P. J., & Hirano, T. (2004). Scc2 couples replication licensing to sister chromatid cohesion in Xenopus egg extracts. *Curr Biol, 14*(17), 1598-1603.

Gobbini, E., Cesena, D., Galbiati, A., Lockhart, A., & Longhese, M. P. (2013). Interplays between ATM/Tel1 and ATR/Mec1 in sensing and signaling DNA double-strand breaks. *DNA Repair (Amst), 12*(10), 791-799.

Goffeau, A., Barrell, B., Bussey, H., & Davis, R. (1996). Life with 6000 genes. *Science, 274*(5287), 546-567.

Graumann, P. L., & Knust, T. (2009). Dynamics of the bacterial SMC complex and SMC-like proteins involved in DNA repair. *Chromosome Research, 17*(2), 265-275.

Groth, A., Ray-Gallet, D., Quivy, J. P., Lukas, J., Bartek, J., & Almouzni, G. (2005). Human Asf1 regulates the flow of S phase histones during replicational stress. *Mol Cell, 17*(2), 301-311.

Gu, J., & Lieber, M. R. (2008). Mechanistic flexibility as a conserved theme across 3 billion years of nonhomologous DNA end-joining. *Genes Dev, 22*(4), 411-415.

Gunjan, A., & Verreault, A. (2003). A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. *Cell, 115*(5), 537-549.

Guthrie, C., & Fink, G. R. (1991). Preface. In *Methods Enzymol* (Vol. 194, pp. xvii): Academic Press.

Haering, C. H., Lowe, J., Hochwagen, A., & Nasmyth, K. (2002). Molecular architecture of SMC proteins and the yeast cohesin complex. *Molecular Cell, 9*(4), 773-788.

Halder, S., Torrecilla, I., Burkhalter, M. D., Popović, M., Fielden, J., Vaz, B., . . . Ramadan,

K. (2019). SPRTN protease and checkpoint kinase 1 cross-activation loop safeguards DNA replication. *Nature Communications, 10*(1), 3142-3142.

Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A., & Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *J Biol Chem, 280*(6), 4102-4110.

Hecker, C.-M., Rabiller, M., Haglund, K., Bayer, P., & Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. *The Journal of biological chemistry, 281*(23), 16117-16127.

Herskowitz, I. (1988). Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiological Reviews, 52*(4), 536-553.

Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., & Sistonen, L. (2006). PDSM, a motif for phosphorylation-dependent SUMO modification. *Proceedings of the National Academy of Sciences of the United States of America, 103*(1), 45-50.

Hinshaw, S. M., Makrantoni, V., Harrison, S. C., & Marston, A. L. (2017). The Kinetochore Receptor for the Cohesin Loading Complex. *Cell, 171*(1), 72-84.e13.

Hirano, M., & Hirano, T. (2002). Hinge-mediated dimerization of SMC protein is essential for its dynamic interaction with DNA. *Embo j, 21*(21), 5733-5744.

Hirano, T., & Mitchison, T. J. (1994). A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. *Cell, 79*(3), 449-458.

Hirsch, B., Shimamura, A., Moreau, L., Baldinger, S., Hag-alshiekh, M., Bostrom, B., . . . D'Andrea, A. D. (2004). Association of biallelic BRCA2/FANCD1 mutations with spontaneous chromosomal instability and solid tumors of childhood. *Blood, 103*(7), 2554-2559.

Hochstrasser, M. (2001). SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell, 107*(1), 5-8.

Hoege, C., Pfander, B., Moldovan, G.-L., Pyrowolakis, G., & Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature, 419*(6903), 135-141.

Howlett, N. G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., de Die-Smulders, C., . . . Andrea, A. D. (2002). Biallelic Inactivation of BRCA2 in Fanconi Anemia. *Science, 297*(5581), 606.

Huberman, J. A., Spotila, L. D., Nawotka, K. A., El-Assouli, S. M., & Davis, L. R. (1987). The in vivo replication origin of the yeast 2μm plasmid. *Cell, 51*(3), 473-481.

Hudson, J. J., Chiang, S. C., Wells, O. S., Rookyard, C., & El-Khamisy, S. F. (2012). SUMO modification of the neuroprotective protein TDP1 facilitates chromosomal single-strand break repair. *Nat Commun, 3*, 733.

Huertas, P., Cortés-Ledesma, F., Sartori, A. A., Aguilera, A., & Jackson, S. P. (2008). CDK targets Sae2 to control DNA-end resection and homologous recombination. *Nature, 455*(7213), 689-692.

Ivanov, E. L., Sugawara, N., White, C. I., Fabre, F., & Haber, J. E. (1994). Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae. *Molecular and Cellular Biology, 14*(5), 3414-3425.

Jackson, S. P. (2002). Sensing and repairing DNA double-strand breaks. *Carcinogenesis, 23*(5), 687-696.

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature, 461*(7267), 1071-1078.

Jakobsen, K. P., Nielsen, K. O., Løvschal, K. V., Rødgaard, M., Andersen, A. H., & Bjergbæk, L. (2019). Minimal Resection Takes Place during Break-Induced Replication Repair of Collapsed Replication Forks and Is Controlled by Strand Invasion. *Cell Rep, 26*(4), 836-844.e833.

Johnson, E. S., & Blobel, G. (1997). Ubc9p Is the Conjugating Enzyme for the Ubiquitin-like Protein Smt3p. *Journal of Biological Chemistry, 272*(43), 26799-26802.

Johnson, E. S., & Gupta, A. A. (2001). An E3-like Factor that Promotes SUMO Conjugation to the Yeast Septins. *Cell, 106*(6), 735-744.

Johnson, E. S., Schwienhorst, I., Dohmen, R. J., & Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *The EMBO Journal, 16*(18), 5509-5519.

Kamitani, T., Kito, K., Nguyen, H. P., Wada, H., Fukuda-Kamitani, T., & Yeh, E. T. H. (1998). Identification of Three Major Sentrinization Sites in PML. *Journal of Biological Chemistry, 273*(41), 26675-26682.

Karsten, R. M., J.; Kepper, N. . (2008). Interactions of histones with DNA: nucleosome assembly, stability, dynamics, and higher order structures. In R. S. a. L. Dias, B (Ed.), *DNA interactions with polymers and surfactants* (pp. 135-172). London: Wiley.

Kawakami, H., & Katayama, T. (2010). DnaA, ORC, and Cdc6: similarity beyond the domains of life and diversity This paper is one of a selection of papers published in this special issue entitled 8th International Conference on AAA Proteins and has undergone the Journal's usual peer review process. *Biochemistry and Cell Biology, 88*(1), 49-62.

Kimura, K., & Hirano, T. (1997). ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. *Cell, 90*(4), 625-634.

Kobayashi, T., & Horiuchi, T. (1996). A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells, 1*(5), 465-474.

Lammens, A., Schele, A., & Hopfner, K. P. (2004). Structural biochemistry of ATP-driven dimerization and DNA-stimulated activation of SMC ATPases. *Curr Biol, 14*(19), 1778-1782.

Lao, J. P., Ulrich, K. M., Johnson, J. R., Newton, B. W., Vashisht, A. A., Wohlschlegel, J. A., . . . Toczyski, D. P. (2018). The Yeast DNA Damage Checkpoint Kinase Rad53 Targets the Exoribonuclease, Xrn1. *G3 (Bethesda), 8*(12), 3931-3944.

Larsen, N. B., Gao, A. O., Sparks, J. L., Gallina, I., Wu, R. A., Mann, M., . . . Duxin, J. P. (2018). Replication-Coupled DNA-Protein Crosslink Repair by SPRTN and the Proteasome in Xenopus Egg Extracts. *Molecular Cell*.

Lee, K. C., Swan, R. L., Sondka, Z., Padget, K., Cowell, I. G., & Austin, C. A. (2018). Effect of TDP2 on the Level of TOP2-DNA Complexes and SUMOylated TOP2-DNA Complexes. *Int J Mol Sci, 19*(7).

Lee, S. E., Moore, J. K., Holmes, A., Umezu, K., Kolodner, R. D., & Haber, J. E. (1998). Saccharomyces Ku70, Mre11/Rad50, and RPA Proteins Regulate Adaptation to

G2/M Arrest after DNA Damage. *Cell, 94*(3), 399-409.

Lehmann, A. R., Walicka, M., Griffiths, D. J., Murray, J. M., Watts, F. Z., McCready, S., & Carr, A. M. (1995). The rad18 gene of Schizosaccharomyces pombe defines a new subgroup of the SMC superfamily involved in DNA repair. *Molecular and Cellular Biology, 15*(12), 7067-7080.

Lessel, D., Vaz, B., Halder, S., Lockhart, P. J., Marinovic-Terzic, I., Lopez-Mosqueda, J., . . . Kubisch, C. (2014). Mutations in SPRTN cause early onset hepatocellular carcinoma, genomic instability and progeroid features. *Nat Genet, 46*(11), 1239-1244.

Li, S.-J., & Hochstrasser, M. (2003). The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. *J Cell Biol, 160*(7), 1069-1081.

Li, S. J., & Hochstrasser, M. (2000). The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. *Molecular and Cellular Biology, 20*(7), 2367-2377.

Lieber, M. R. (2008). The mechanism of human nonhomologous DNA end joining. *The Journal of biological chemistry, 283*(1), 1-5.

Lieber, M. R. (2010). The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annu Rev Biochem, 79*(1), 181-211.

Lin, D.-Y., Huang, Y.-S., Jeng, J.-C., Kuo, H.-Y., Chang, C.-C., Chao, T.-T., . . . Shih, H.-M. (2006). Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Molecular Cell, 24*(3), 341-354.

Lin, X., Sun, B., Liang, M., Liang, Y.-Y., Gast, A., Hildebrand, J., . . . Feng, X.-H. (2003). Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Molecular Cell, 11*(5), 1389-1396.

Lindroos, H. B., Ström, L., Itoh, T., Katou, Y., Shirahige, K., & Sjögren, C. (2006). Chromosomal association of the Smc5/6 complex reveals that it functions in differently regulated pathways. *Molecular Cell, 22*(6), 755-767.

Linskens, M. H., & Huberman, J. A. (1988). Organization of replication of ribosomal DNA in Saccharomyces cerevisiae. *Molecular and Cellular Biology, 8*(11), 4927-4935.

Lisby, M., Barlow, J. H., Burgess, R. C., & Rothstein, R. (2004). Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins. *Cell, 118*(6), 699-713.

Longhese, M. P., Bonetti, D., Manfrini, N., & Clerici, M. (2010). Mechanisms and regulation of DNA end resection. *The EMBO Journal, 29*(17), 2864-2874.

Lopez-Mosqueda, J., Maddi, K., Prgomet, S., Kalayil, S., Marinovic-Terzic, I., Terzic, J., & Dikic, I. (2016). SPRTN is a mammalian DNA-binding metalloprotease that resolves DNA-protein crosslinks. *Elife, 5*.

Lowndes, N. F., & Murguia, J. R. (2000). Sensing and responding to DNA damage. *Current Opinion in Genetics & Development, 10*(1), 17-25.

Maddi, K., Sam, D. K., Bonn, F., Prgomet, S., Tulowetzke, E., Akutsu, M., . . . Dikic, I. (2020). Wss1 Promotes Replication Stress Tolerance by Degrading Histones. *Cell Reports, 30*(9), 3117-3126.e3114.

Mahajan, R., Gerace, L., & Melchior, F. (1998). Molecular characterization of the SUMO-

1 modification of RanGAP1 and its role in nuclear envelope association. *J Cell Biol, 140*(2), 259-270.

Majka, J., Niedziela-Majka, A., & Burgers, P. M. J. (2006). The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint. *Molecular Cell, 24*(6), 891-901.

Marteijn, J. A., Lans, H., Vermeulen, W., & Hoeijmakers, J. H. J. (2014). Understanding nucleotide excision repair and its roles in cancer and ageing. *Nature reviews. Molecular cell biology, 15*(7), 465-481.

Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., & Oda, M. (2010). Eukaryotic Chromosome DNA Replication: Where, When, and How? *Annu. Rev. Biochem., 79*, 89-130.

Maskey, R. S., Kim, M. S., Baker, D. J., Childs, B., Malureanu, L. A., Jeganathan, K. B., . . . Machida, Y. J. (2014). Spartan deficiency causes genomic instability and progeroid phenotypes. *Nat Commun, 5*, 5744.

Maslov, A. Y., Lee, M., Gundry, M., Gravina, S., Strogonova, N., Tazearslan, C., . . . Vijg, J. (2012). 5-aza-2'-deoxycytidine-induced genome rearrangements are mediated by DNMT1. *Oncogene, 31*(50), 5172-5179.

Mathew, C. G. (2006). Fanconi anaemia genes and susceptibility to cancer. *Oncogene, 25*(43), 5875-5884.

Matunis, M. J., Coutavas, E., & Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol, 135*(6 Pt 1), 1457-1470.

McDonald, W. H., Pavlova, Y., Yates, J. R., & Boddy, M. N. (2003). Novel Essential DNA Repair Proteins Nse1 and Nse2 Are Subunits of the Fission Yeast Smc5-Smc6 Complex. *Journal of Biological Chemistry, 278*(46), 45460-45467.

McGhee, J. D., & von Hippel, P. H. (1975). Formaldehyde as a probe of DNA structure. II. Reaction with endocyclic imino groups of DNA bases. *Biochemistry, 14*(6), 1297-1303.

Mechali, M. (2010). Eukaryotic DNA replication origins: many choices for appropriate answers. In *Nat. Rev. Mol. Cell Biol.* (Vol. 11, pp. 728-738).

Meluh, P. B., & Koshland, D. (1995). Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Molecular biology of the cell, 6*(7), 793-807.

Merlini, L., Dudin, O., & Martin, S. G. (2013). Mate and fuse: how yeast cells do it. *Open Biology, 3*(3), 130008.

Min, J.-H., & Pavletich, N. P. (2007). Recognition of DNA damage by the Rad4 nucleotide excision repair protein. *Nature, 449*(7162), 570-575.

Müller, S., Berger, M., Lehembre, F., Seeler, J.-S., Haupt, Y., & Dejean, A. (2000). c-Jun and p53 Activity Is Modulated by SUMO-1 Modification. *Journal of Biological Chemistry, 275*(18), 13321-13329.

Munoz-Galvan, S., Jimeno, S., Rothstein, R., & Aguilera, A. (2013). Histone H3K56 acetylation, Rad52, and non-DNA repair factors control double-strand break repair choice with the sister chromatid. *PLoS Genet, 9*(1), e1003237.

Muñoz, S., Minamino, M., Casas-Delucchi, C. S., Patel, H., & Uhlmann, F. (2019). A Role

for Chromatin Remodeling in Cohesin Loading onto Chromosomes. *Molecular Cell, 74*(4), 664-673.e665.

Nakano, T., Miyamoto-Matsubara, M., Shoulkamy, M. I., Salem, A. M., Pack, S. P., Ishimi, Y., & Ide, H. (2013). Translocation and stability of replicative DNA helicases upon encountering DNA-protein cross-links. *J Biol Chem, 288*(7), 4649-4658.

Nielsen, I., Bentsen, I. B., Lisby, M., Hansen, S., Mundbjerg, K., Andersen, A. H., & Bjergbaek, L. (2009). A Flp-nick system to study repair of a single protein-bound nick in vivo. *Nature Methods, 6*(10), 753-758.

Nitiss, J. L. (2009). Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer, 9*(5), 338-350.

O'Neill, B. M., Hanway, D., Winzeler, E. A., & Romesberg, F. E. (2004a). Coordinated functions of WSS1, PSY2 and TOF1 in the DNA damage response. *Nucleic Acids Research, 32*(22), 6519-6530.

O'Neill, B. M., Hanway, D., Winzeler, E. A., & Romesberg, F. E. (2004b). Coordinated functions of WSS1, PSY2 and TOF1 in the DNA damage response. *Nucleic Acids Res, 32*(22), 6519-6530.

Onoda, F., Takeda, M., Seki, M., Maeda, D., Tajima, J.-i., Ui, A., . . . Enomoto, T. (2004). SMC6 is required for MMS-induced interchromosomal and sister chromatid recombinations in Saccharomyces cerevisiae. *DNA Repair (Amst), 3*(4), 429-439.

Osley, M. A. (1991). The regulation of histone synthesis in the cell cycle. *Annu Rev Biochem, 60*, 827-861.

Palecek, J., Vidot, S., Feng, M., Doherty, A. J., & Lehmann, A. R. (2006). The Smc5-Smc6 DNA repair complex. bridging of the Smc5-Smc6 heads by the KLEISIN, Nse4, and non-Kleisin subunits. *The Journal of biological chemistry, 281*(48), 36952-36959.

Palzkill, T. G., & Newlon, C. S. (1988). A yeast replication origin consists of multiple copies of a small conserved sequence. *Cell, 53*(3), 441-450.

Papouli, E., Chen, S., Davies, A. A., Huttner, D., Krejci, L., Sung, P., & Ulrich, H. D. (2005). Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Molecular Cell, 19*(1), 123-133.

Parsons, R. L., Prasad, P. V., Harshey, R. M., & Jayaram, M. (1988). Step-arrest mutants of FLP recombinase: implications for the catalytic mechanism of DNA recombination. *Molecular and Cellular Biology, 8*(8), 3303-3310.

Pellicioli, A., Lee, S. E., Lucca, C., Foiani, M., & Haber, J. E. (2001). Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. *Molecular Cell, 7*(2), 293-300.

Pellicioli, A., Lee, S. E., Lucca, C., Foiani, M., & Haber, J. E. (2001). Regulation of Saccharomyces Rad53 Checkpoint Kinase during Adaptation from DNA Damage Induced G2/M Arrest. *Molecular Cell, 7*(2), 293-300.

Pommier, Y. (2006). Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer, 6*(10), 789-802.

Pommier, Y., Huang, S. Y., Gao, R., Das, B. B., Murai, J., & Marchand, C. (2014). Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair (Amst), 19*, 114-129.

Potts, P. R. (2009). The Yin and Yang of the MMS21-SMC5/6 SUMO ligase complex in

homologous recombination. *DNA Repair (Amst), 8*(4), 499-506.

Potts, P. R., Porteus, M. H., & Yu, H. (2006). Human SMC5/6 complex promotes sister chromatid homologous recombination by recruiting the SMC1/3 cohesin complex to double-strand breaks. *The EMBO Journal, 25*(14), 3377-3388.

Potts, P. R., & Yu, H. (2005). Human MMS21/NSE2 Is a SUMO Ligase Required for DNA Repair. *Molecular and Cellular Biology, 25*(16), 7021.

Potts, P. R., & Yu, H. (2007). The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. *Nat Struct Mol Biol, 14*(7), 581-590.

Prakash, S., & Prakash, L. (1977). Increased spontaneous mitotic segregation in MMS-sensitive mutants of Saccharomyces cerevisiae. *Genetics, 87*(2), 229-236.

Rallabhandi, P., Hashimoto, K., Mo, Y. Y., Beck, W. T., Moitra, P. K., & D'Arpa, P. (2002). Sumoylation of topoisomerase I is involved in its partitioning between nucleoli and nucleoplasm and its clearing from nucleoli in response to camptothecin. *J Biol Chem, 277*(42), 40020-40026.

Rao, H., & Stillman, B. (1995). The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proceedings of the National Academy of Sciences, 92*(6), 2224.

Rothkamm, K., Barnard, S., Moquet, J., Ellender, M., Rana, Z., & Burdak-Rothkamm, S. (2015). DNA damage foci: Meaning and significance. *Environ Mol Mutagen, 56*(6), 491-504.

Rowley, A., Cocker, J. H., Harwood, J., & Diffley, J. F. (1995). Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. *Embo j, 14*(11), 2631-2641.

Russell, J. J., Theriot, J. A., Sood, P., Marshall, W. F., Landweber, L. F., Fritz-Laylin, L., . . . Brunet, A. (2017). Non-model model organisms. *BMC Biol, 15*(1), 55.

S. Maskey, R., Flatten, K., J. Sieben, C., L. Peterson, K., J. Baker, D., Nam, H.-J., . . . J. Machida, Y. (2017). *Spartan deficiency causes accumulation of Topoisomerase 1 cleavage complexes and tumorigenesis* (Vol. 45).

Sacco, E., Hasan, M. M., Alberghina, L., & Vanoni, M. (2012). Comparative analysis of the molecular mechanisms controlling the initiation of chromosomal DNA replication in yeast and in mammalian cells. *Biotechnology Advances, 30*(1), 73-98.

Sacher, M., Pfander, B., & Jentsch, S. (2005). Identification of SUMO-protein conjugates. *Methods Enzymol, 399*, 392-404.

Sánchez, A., & Russell, P. (2015). Ku stabilizes replication forks in the absence of Brc1. *PLoS One, 10*(5), e0126598-e0126598.

Schellenberg, M. J., Lieberman, J. A., Herrero-Ruiz, A., Butler, L. R., Williams, J. G., Muñoz-Cabello, A. M., . . . Williams, R. S. (2017). ZATT (ZNF451)–mediated resolution of topoisomerase 2 DNA-protein cross-links. *Science, 357*(6358), 1412.

Schneiter, R. (2004). Genetics, Molecular and Cell Biology of Yeast.

Serbyn, N., Noireterre, A., Bagdiul, I., Plank, M., Michel, A. H., Loewith, R., . . . Stutz, F. (2020). The Aspartic Protease Ddi1 Contributes to DNA-Protein Crosslink Repair in Yeast. *Molecular Cell, 77*(5), 1066-1079.e1069.

Sergeant, J., Taylor, E., Palecek, J., Fousteri, M., Andrews, E. A., Sweeney, S., . . . Lehmann, A. R. (2005). Composition and architecture of the Schizosaccharomyces pombe Rad18 (Smc5-6) complex. *Molecular and Cellular Biology, 25*(1), 172-184.

Sharma, P., Mullen, J. R., Li, M., Zaratiegui, M., Bunting, S. F., & Brill, S. J. (2017). A Lysine Desert Protects a Novel Domain in the Slx5-Slx8 SUMO Targeted Ub Ligase To Maintain Sumoylation Levels in Saccharomyces cerevisiae. *Genetics, 206*(4), 1807-1821.

Sharrocks, A. D. (2006). PIAS proteins and transcriptional regulation—more than just SUMO E3 ligases? *Genes Dev, 20*(7), 754-758.

Shen, T. H., Lin, H.-K., Scaglioni, P. P., Yung, T. M., & Pandolfi, P. P. (2006). The mechanisms of PML-nuclear body formation. *Molecular Cell, 24*(3), 331-339.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., . . . Shi, Y. (2004). Histone Demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1. *Cell, 119*(7), 941-953.

Shintomi, K., Iwabuchi, M., Saeki, H., Ura, K., Kishimoto, T., & Ohsumi, K. (2005). Nucleosome assembly protein-1 is a linker histone chaperone in Xenopus eggs. *Proc Natl Acad Sci U S A, 102*(23), 8210-8215.

Singh, R. K., Kabbaj, M. H., Paik, J., & Gunjan, A. (2009). Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. *Nat Cell Biol, 11*(8), 925-933.

Singh, R. K., Liang, D., Gajjalaiahvari, U. R., Kabbaj, M. H., Paik, J., & Gunjan, A. (2010). Excess histone levels mediate cytotoxicity via multiple mechanisms. *Cell Cycle, 9*(20), 4236-4244.

Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., & Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proceedings of the National Academy of Sciences of the United States of America, 101*(40), 14373-14378.

Sonoda, E., Hochegger, H., Saberi, A., Taniguchi, Y., & Takeda, S. (2006). Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. *DNA Repair (Amst), 5*(9), 1021-1029.

Sparks, J. L., Chistol, G., Gao, A. O., Raschle, M., Larsen, N. B., Mann, M., . . . Walter, J. C. (2019). The CMG Helicase Bypasses DNA-Protein Cross-Links to Facilitate Their Repair. *Cell, 176*(1-2), 167-181 e121.

Stephan, A. K., Kliszczak, M., Dodson, H., Cooley, C., & Morrison, C. G. (2011). Roles of vertebrate Smc5 in sister chromatid cohesion and homologous recombinational repair. *Molecular and Cellular Biology, 31*(7), 1369-1381.

Sternsdorf, T., Jensen, K., Reich, B., & Will, H. (1999). The Nuclear Dot Protein Sp100, Characterization of Domains Necessary for Dimerization, Subcellular Localization, and Modification by Small Ubiquitin-like Modifiers. *Journal of Biological Chemistry, 274*(18), 12555-12566.

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Lobrich, M., & Jeggo, P. A. (2004). ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res, 64*(7), 2390-2396.

Stinchcomb, D. T., Struhl, K., & Davis, R. W. (1979). Isolation and characterisation of a yeast chromosomal replicator. *Nature, 282*(5734), 39-43.

Stingele, J., Bellelli, R., Alte, F., Hewitt, G., Sarek, G., Maslen, S. L., . . . Boulton, S. J. (2016). Mechanism and Regulation of DNA-Protein Crosslink Repair by the DNA-Dependent Metalloprotease SPRTN. *Molecular Cell, 64*(4), 688-703.

Stingele, J., Bellelli, R., & Boulton, S. J. (2017). Mechanisms of DNA-protein crosslink repair. *Nat Rev Mol Cell Biol, 18*(9), 563-573.

Stingele, J., Habermann, B., & Jentsch, S. (2015). DNA-protein crosslink repair: proteases as DNA repair enzymes. *Trends Biochem Sci, 40*(2), 67-71.

Stingele, J., Schwarz, M. S., Bloemeke, N., Wolf, P. G., & Jentsch, S. (2014). A DNA-dependent protease involved in DNA-protein crosslink repair. *Cell, 158*(2), 327-338.

Stracker, T. H., & Petrini, J. H. (2011). The MRE11 complex: starting from the ends. *Nat Rev Mol Cell Biol, 12*(2), 90-103.

Strunnikov, A. V., Larionov, V. L., & Koshland, D. (1993). SMC1: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family. *J Cell Biol, 123*(6 Pt 2), 1635-1648.

Sun, Y., Jenkins, L. M. M., Su, Y. P., Nitiss, K. C., Nitiss, J. L., & Pommier, Y. (2019). A conserved SUMO-Ubiquitin pathway directed by RNF4/SLX5-SLX8 and PIAS4/SIZ1 drives proteasomal degradation of topoisomerase DNA-protein crosslinks. *bioRxiv*, 707661.

Sun, Z., Hsiao, J., Fay, D. S., & Stern, D. F. (1998). Rad53 FHA Domain Associated with Phosphorylated Rad9 in the DNA Damage Checkpoint. *Science, 281*(5374), 272.

Sutani, T., Sakata, T., Nakato, R., Masuda, K., Ishibashi, M., Yamashita, D., . . . Shirahige, K. (2015). Condensin targets and reduces unwound DNA structures associated with transcription in mitotic chromosome condensation. *Nature Communications, 6*, 7815-7815.

Svoboda, M., Konvalinka, J., Trempe, J.-F., & Grantz Saskova, K. (2019). The yeast proteases Ddi1 and Wss1 are both involved in the DNA replication stress response. *DNA Repair (Amst), 80*, 45-51.

Svoboda, M., Konvalinka, J., Trempe, J. F., & Grantz Saskova, K. (2019). The yeast proteases Ddi1 and Wss1 are both involved in the DNA replication stress response. *DNA Repair (Amst), 80*, 45-51.

Symington, L. S., & Gautier, J. (2011). Double-Strand Break End Resection and Repair Pathway Choice. *Annual Review of Genetics, 45*(1), 247-271.

Takahashi, T. S., Basu, A., Bermudez, V., Hurwitz, J., & Walter, J. C. (2008). Cdc7-Drf1 kinase links chromosome cohesion to the initiation of DNA replication in Xenopus egg extracts. *Genes Dev, 22*(14), 1894-1905.

Takashima, H., Boerkoel, C. F., John, J., Saifi, G. M., Salih, M. A., Armstrong, D., . . . Lupski, J. R. (2002). Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet, 32*(2), 267-272.

Tanaka, K., Nishide, J., Okazaki, K., Kato, H., Niwa, O., Nakagawa, T., . . . Murakami, Y. (1999). Characterization of a Fission Yeast SUMO-1 Homologue, Pmt3p, Required for Multiple Nuclear Events, Including the Control of Telomere Length and Chromosome Segregation. *Molecular and Cellular Biology, 19*(12), 8660.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., & Araki, H. (2007).

CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature, 445*(7125), 328-332.

Tanaka, T., Fuchs, J., Loidl, J., & Nasmyth, K. (2000). Cohesin ensures bipolar attachment of microtubules to sister centromeres and resists their precocious separation. *Nature Cell Biology, 2*(8), 492-499.

Taylor, E. M., Moghraby, J. S., Lees, J. H., Smit, B., Moens, P. B., & Lehmann, A. R. (2001). Characterization of a novel human SMC heterodimer homologous to the Schizosaccharomyces pombe Rad18/Spr18 complex. *Molecular biology of the cell, 12*(6), 1583-1594.

Tedeschi, A., Wutz, G., Huet, S., Jaritz, M., Wuensche, A., Schirghuber, E., . . . Peters, J. M. (2013). Wapl is an essential regulator of chromatin structure and chromosome segregation. *Nature, 501*(7468), 564-568.

Terakawa, T., Bisht, S., Eeftens, J. M., Dekker, C., Haering, C. H., & Greene, E. C. (2017). The condensin complex is a mechanochemical motor that translocates along DNA. *Science, 358*(6363), 672.

Thadani, R., Uhlmann, F., & Heeger, S. (2012). Condensin, chromatin crossbarring and chromosome condensation. *Curr Biol, 22*(23), R1012-R1021.

Toone, W., Aerne, B., Morgan, B., & Johnston, L. (1997). Getting Started: Regulating the initiation of DNA replication in yeast. *Annual Review of Microbiology, 51*, 125-149.

Torres-Ramos, C. A., Prakash, S., & Prakash, L. (2002). Requirement of RAD5 and MMS2 for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae. *Molecular and Cellular Biology, 22*(7), 2419-2426.

Tóth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., & Nasmyth, K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes Dev, 13*(3), 320-333.

Tsuyama, T., Inou, K., Seki, M., Seki, T., Kumata, Y., Kobayashi, T., . . . Tada, S. (2006). Chromatin loading of Smc5/6 is induced by DNA replication but not by DNA double-strand breaks. *Biochemical and biophysical research communications, 351*(4), 935-939.

Uhlmann, F. (2016). SMC complexes: from DNA to chromosomes. *Nature Reviews Molecular Cell Biology, 17*, 399.

Uhlmann, F., & Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. *Curr Biol, 8*(20), 1095-1101.

Usui, T., Foster, S. S., & Petrini, J. H. J. (2009). Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization. *Molecular Cell, 33*(2), 147-159.

van Heusden, G. P. H., & Steensma, H. Y. (2008). The Saccharomyces cerevisiae Wss1 protein is only present in mother cells. *Fems Microbiology Letters, 282*(1), 100-104.

Vance, J. R., & Wilson, T. E. (2002). Yeast Tdp1 and Rad1-Rad10 function as redundant pathways for repairing Top1 replicative damage. *Proceedings of the National Academy of Sciences, 99*(21), 13669.

Vaz, B., Popovic, M., Newman, J. A., Fielden, J., Aitkenhead, H., Halder, S., . . . Ramadan, K. (2016). Metalloprotease SPRTN/DVC1 Orchestrates Replication-Coupled

DNA-Protein Crosslink Repair. *Mol Cell, 64*(4), 704-719.

Vaz, B., Popovic, M., Newman, J. A., Fielden, J., Aitkenhead, H., Halder, S., . . . Ramadan, K. (2016). Metalloprotease SPRTN/DVC1 Orchestrates Replication-Coupled DNA-Protein Crosslink Repair. *Molecular Cell, 64*(4), 704-719.

Vaz, B., Popovic, M., & Ramadan, K. (2017). DNA-Protein Crosslink Proteolysis Repair. *Trends Biochem Sci, 42*(6), 483-495.

Vaz, B., Ruggiano, A., Popovic, M., Rodriguez-Berriguete, G., Kilgas, S., Singh, A. N., . . . Ramadan, K. (2020). SPRTN protease and SUMOylation coordinate DNA-protein crosslink repair to prevent genome instability. *bioRxiv*, 2020.2002.2014.949289.

Vilenchik, M. M., & Knudson, A. G. (2003). Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. *Proc Natl Acad Sci U S A, 100*(22), 12871-12876.

Walker, J. R., Corpina, R. A., & Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature, 412*(6847), 607-614.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Reviews Molecular Cell Biology, 3*, 430.

Wang, L. C., & Gautier, J. (2010). The Fanconi anemia pathway and ICL repair: implications for cancer therapy. *Critical Reviews in Biochemistry and Molecular Biology, 45*(5), 424-439.

Ward, J. F. (1988). DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucleic Acid Res Mol Biol, 35*, 95-125.

Watanabe, K., Pacher, M., Dukowic, S., Schubert, V., Puchta, H., & Schubert, I. (2009). The STRUCTURAL MAINTENANCE OF CHROMOSOMES 5/6 complex promotes sister chromatid alignment and homologous recombination after DNA damage in Arabidopsis thaliana. *Plant Cell, 21*(9), 2688-2699.

Wawrzycka, D., Guerriat, B., Beben, K., Wysocki, R., & Ghislain, M. (2012). The Wss1 Zn-dependent metalloprotease interacts genetically with the Ulp2 poly-SUMO specific isopeptidase. *Febs Journal, 279*, 476-476.

Weinert, T. (1998). DNA Damage and Checkpoint Pathways: Molecular Anatomy and Interactions with Repair. *Cell, 94*(5), 555-558.

Wickramaratne, S., Ji, S., Mukherjee, S., Su, Y., Pence, M. G., Lior-Hoffmann, L., . . . Tretyakova, N. (2016). Bypass of DNA-Protein Cross-links Conjugated to the 7-Deazaguanine Position of DNA by Translesion Synthesis Polymerases. *J Biol Chem, 291*(45), 23589-23603.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., . . . Davis, R. W. (1999). Functional Characterization of the S. cerevisiae Genome by Gene Deletion and Parallel Analysis. *Science, 285*(5429), 901-906.

Xie, Y., Rubenstein, E. M., Matt, T., & Hochstrasser, M. (2010). SUMO-independent in vivo activity of a SUMO-targeted ubiquitin ligase toward a short-lived transcription factor. *Genes Dev, 24*(9), 893-903.

Yang, S.-H., Galanis, A., Witty, J., & Sharrocks, A. D. (2006). An extended consensus motif enhances the specificity of substrate modification by SUMO. *The EMBO*

Journal, 25(21), 5083-5093.

Yang, S. W., Burgin, A. B., Jr., Huizenga, B. N., Robertson, C. A., Yao, K. C., & Nash, H. A. (1996). A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. *Proc Natl Acad Sci U S A, 93*(21), 11534-11539.

Yeh, Y. C., Liu, H. F., Ellis, C. A., & Lu, A. L. (1994). Mammalian topoisomerase I has base mismatch nicking activity. *J Biol Chem, 269*(22), 15498-15504.

Yuen, K. C., & Gerton, J. L. (2018). Taking cohesin and condensin in context. *PLoS Genetics, 14*(1), e1007118.

Zapatka, M., Pociño-Merino, I., Heluani-Gahete, H., Bermúdez-López, M., Tarrés, M., Ibars, E., . . . Torres-Rosell, J. (2019). Sumoylation of Smc5 Promotes Error-free Bypass at Damaged Replication Forks. *Cell Reports, 29*(10), 3160-3172.e3164.

Zegerman, P., & Diffley, J. F. X. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. *Nature, 445*(7125), 281-285.

Zhang, J., Shi, X., Li, Y., Kim, B. J., Jia, J., Huang, Z., . . . Qin, J. (2008). Acetylation of Smc3 by Eco1 is required for S phase sister chromatid cohesion in both human and yeast. *Molecular Cell, 31*(1), 143-151.

Zhao, X., & Blobel, G. (2005). A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proceedings of the National Academy of Sciences of the United States of America, 102*(13), 4777-4782.

Zheng, G., Kanchwala, M., Xing, C., & Yu, H. (2018). MCM2–7-dependent cohesin loading during S phase promotes sister-chromatid cohesion. *Elife, 7*, e33920.

Zheng, H., Wang, X., Warren, A. J., Legerski, R. J., Nairn, R. S., Hamilton, J. W., & Li, L. (2003). Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. *Molecular and Cellular Biology, 23*(2), 754-761.

Zou, L., & Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science (New York, N.Y.), 300*(5625), 1542-1548.

Zou, L., & Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science, 280*(5363), 593-596.