Exploring DNA Damage Induced Foci and Their Role in Coordinating the DNA Damage Response

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Department of Molecular Genetics

University of Toronto

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Abstract

DNA damage represents a major challenge to the faithful replication and transmission of genetic information from one generation to the next. Cells utilize a highly integrated network of pathways to detect and accurately repair DNA damage. Mutations arise when DNA damage persists undetected, unrepaired, or repaired improperly. Mutations are a driving force of carcinogenesis and therefore many of the DNA damage surveillance and repair mechanisms guard against the transformation of normal cells into cancer cells. Central to the detection and repair of DNA damage is the relocalization of DNA damage surveillance proteins to DNA damage where they assemble into subnuclear foci and are capable to producing a signal that the cell interprets to induce cellular modifications such as cycle arrest and DNA repair which are important DNA damage tolerance. In this work, I describe my quest to understand the mechanisms underlying the assembly, maintenance, and disassembly of these DNA damage-induced foci and how they affect DNA damage signaling in Saccharomyces cerevisiae. First, I describe phenotypic characterization of a novel mutation that impairs assembly of the 9-1-1 checkpoint clamp complex into foci.
Second, I describe my work to further understand the roles of the histone phosphatase Pph3 and phosphorylated histone H2A in modulating DNA damage signaling. Third, I include my work to uncover the possible mechanism by which the helicase Srs2 works to enable termination of DNA damage signaling. In summary, this thesis documents my efforts to understand the cellular and molecular nature of DNA damage signaling and how signaling is turned off in coordination with DNA damage repair.
Acknowledgments

I would like to thank my supervisor Dr. Daniel Durocher for his guidance and mentorship throughout my time in the lab. Dan, your passion for science has always been an inspiration for me. I would also like to thank my committee members, Dr. Charlie Boone and Dr. Brigitte Lavoie, for their guidance throughout my graduate training. Thank you to my friends who stopped asking me when I was going to graduate in 2009. And finally, I would like to thank my parents who have been unconditionally loving and supportive all these years in my endeavors.
LIFE HERE BEGAN OUT THERE
## List of Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>9-1-1</td>
<td>Checkpoint clamp (Ddc1-Rad17-Mec3)</td>
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<tr>
<td>BIR</td>
<td>Break-Induced Replication</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>CPT</td>
<td>Camptothecin</td>
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<tr>
<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>DDT</td>
<td>DNA damage tolerance</td>
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<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>GC</td>
<td>Gene conversion</td>
</tr>
<tr>
<td>GCR</td>
<td>Gross Chromosome Arrangement</td>
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<td>HR</td>
<td>Homologous recombination</td>
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<tr>
<td>HU</td>
<td>Hydroxy urea</td>
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<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methansulfonate</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PRR</td>
<td>Post-Repliation Repair</td>
</tr>
<tr>
<td>SSA</td>
<td>Single-Strand Annealing</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TLS</td>
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Chapter I

Introduction

1.1 DNA damage, Genome stability, and Cancer

Faithful transmission of genetic information from one generation to the next requires the efficient recognition and accurate repair of DNA damage. Incomplete or inaccurate repair of DNA damage results in mutations or cell death. An extensive cellular network called the DNA damage response (DDR) reacts to DNA damage and coordinates cell cycle arrest, global changes in transcription, chromatin remodeling, DNA repair, and—in metazoans—apoptosis (Jackson and Bartek, 2009). Central to the DDR is an evolutionarily conserved signal transduction cascade called the DNA damage checkpoint. The signaling cascade prevents cell cycle progression until DNA damage is repaired. Mutations in the genes that regulate the checkpoint and the DDR can result in genome instability, a hallmark of cancer (Kolodner et al., 2002; Harper and Elledge, 2007). Therefore, understanding the genetic and molecular mechanisms that control the DNA damage response is important for understanding how cancer develops and how cancer cells thrive.

The DNA damage checkpoint is well-conserved across all organisms. While most of the initial understanding of the mechanisms responsible for detection, signaling, and repair of DNA damage originated from studies in single-celled organisms such as bacteria, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*, research efforts in the last 10-15 years have yielded great progress in elucidating the DNA damage response in complex metazoans including humans.
A conserved phenomenon of the DNA damage response seen from yeast to mammals is the reorganization of checkpoint and repair proteins into punctate subnuclear foci that are detectable by fluorescence microscopy (Lisby and Rothstein, 2005). Foci were first observed with DNA repair proteins in budding yeast and mammalian cells (Bishop, 1994; Haaf et al., 1995). DNA damage foci are important for the proper execution of the DDR, as they allow a rapid and local increase in protein concentration around DNA damage without the need for energetically costly and mechanistically slow up-regulation of gene expression. Furthermore, foci have been widely used as markers of DNA damage and the discovery of the genetic requirements for foci assembly have greatly advanced our understanding of the DDR (Melo et al., 2001; Lisby et al., 2004; Kolas et al., 2007). In contrast, how foci are subsequently disassembled was virtually unexplored when I began in the lab. Indeed, it was unknown if focus disassembly was even a requirement for termination of checkpoint signaling. In this thesis, I will describe my efforts to understand the molecular mechanisms underlying foci assembly and disassembly in coordination with DNA damage checkpoint signaling. This chapter will explore the highly conserved DNA damage response in *Saccharomyces cerevisiae* with reference to the homologs in humans and other species.

1.2 Types of DNA damage

DNA molecules are constantly subjected to lesions that can impede replication, transcription, or chromosome segregation. DNA can be damaged by exogenous agents such as ultraviolet (UV) radiation, ionizing radiation (IR), X-rays, and DNA-damaging
chemicals. Endogenous sources of DNA damage arise from normal cellular metabolism which generates reactive oxygen species that react with the DNA molecule.

Types of DNA damage include single base pair modifications, single-strand breaks, interstrand crosslinks, and double-strand breaks (DSBs). Many different agents are used in the laboratory to study the cellular response to different types of DNA damage. Covalent modifications to nucleotides that result from methyl methane-sulfonoate (MMS), mitomycin C, or UV radiation will impede DNA replication and transcription (Cadet et al., 1997; Lundin et al., 2005). The chemical bleomycin and IR are routinely used to generate DSBs. DSBs are the most toxic DNA lesions, as a single DSB is lethal to a cell (Sandell and Zakian, 1993). Left unrepaired or repaired improperly, the result can be aneuploidy, loss of the acentric portion of the chromosome and gross chromosome rearrangements including translocations and inversions (Kolodner et al., 2002). More generally, induction of DNA damage with DNA damaging compounds such as ethyl methane sulfonoate (EMS) are used for mutagenesis in genetic screens that address a diverse set of biological questions. Much of the work presented in this thesis focuses on the cellular response to DSBs, the most toxic of DNA lesions (Sandell and Zakian, 1993).

DNA replication creates major challenges to genome stability (Branzei and Foiani, 2010). Imperfect replication by DNA polymerases can result in base-pair mismatches or strand slippage in highly repetitive regions of the genome, changing the number of repeats at a locus. Changes in sequence copy number are associated with severe disorders including Huntington’s disease (Price et al., 1998). DNA damage creates barriers to replication and when a replisome encounters DNA damage, it can collapse into
a DSB (Branzei and Foiani, 2005). DNA damage to nucleotides, ssDNA breaks, and interstrand crosslinks are barriers to replisome progression. In addition, DNA-protein complexes at highly transcribed genes, centromeres, and telomeres create natural barriers to replication (Ivessa et al., 2003). These domains are prone to replication fork collapse and DSBs (Szilard et al., 2010). Hydroxyurea (HU) is a chemical frequently used to induce replication stress by limiting the availability of dNTPs for DNA synthesis. Within S-phase, there is a replication checkpoint (or S-phase checkpoint) that shares many common components with the DNA damage checkpoint and stabilizes the replication fork to prevent fork collapse into a DSB (Lopes et al., 2001).

While DNA damage poses a major risk to the faithful transmission of genetic information, cells utilize controlled DNA damage to accomplish a variety of biological functions. During meiosis, DSBs are created to initiate homologous recombination between homologous chromosomes. In budding yeast, a DSB is induced in the MAT locus by the homothallic (HO) endonuclease which is repaired by homologous donor sequences *HMRα* or *HMLα* to accomplish mating type switching (Haber, 1998b). In vertebrates, the generation of antibodies requires the induction of a DSB in the V(D)J locus to promote class-switch recombination, a critical process that generates diverse antigen recognition by the immune system (Dudley et al., 2005). DDR pathways and programmed DSB pathways are intimately related. Often, mutations in DDR genes have pathological consequences in programmed DSB pathways as well, including immunodeficiency or sterility in animals.
1.3 The DNA damage checkpoint

The DNA damage checkpoint is a signaling transduction cascade

The DNA damage checkpoint is activated in the presence of DNA damage and arrests the cell cycle (Zhou and Elledge, 2000). In yeast, a single DSB is sufficient to activate the DNA damage checkpoint and arrest the cell in metaphase just prior to chromosome segregation (Sandell and Zakian, 1993). By convention, this arrest is called “G2/M arrest” (Sandell and Zakian, 1993). The first DNA damage checkpoint gene identified in budding yeast was RAD9 and was found to be essential for cell cycle arrest at G2/M following DNA damage (Weinert and Hartwell, 1988). Later discoveries revealed that the DNA damage checkpoint can control cell cycle progression in other phases of the cell cycle (Painter and Young, 1980) and many components of the checkpoint work to stabilize a replication fork in S-phase (Paulovich and Hartwell, 1995).

DNA damage checkpoint proteins can be classified by their specific functions within the signaling cascade: sensors, transducers, mediators, and effectors (Table 1.3.1) (Melo and Toczyski, 2002). Many of these key players are conserved through evolution, although many important differences between organisms have also been discovered. The checkpoint is a tightly choreographed response that coordinates many cellular changes.

Much like other signal transduction cascades, the DNA damage checkpoint is activated when a stimulus signal—DNA damage—is detected and signaling is amplified when signaling proteins reorganize and concentrate in the proximity of the DNA lesion (Lisby and Rothstein, 2005). In addition to protein redistribution, post-translational modifications—in particular—protein phosphorylation, have a profound role in propagating the signal transduction cascade (Melo and Toczyski, 2002). As a result,
Table 1.3.1. DNA damage checkpoint genes in *Saccharomyces cerevisiae* and their mammalian homologs.

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<th><em>S. cerevisiae</em></th>
<th>Mammalian</th>
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<td><strong>Signal Modifiers</strong></td>
<td>MRE11, RAD50, XRS2</td>
<td>MRE11, RAD50, NBS1</td>
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<td><strong>Sensors</strong></td>
<td>TEL1</td>
<td>ATM</td>
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<td></td>
<td>MEC1, DDC2</td>
<td>ATR, ATRIP</td>
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<td></td>
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<td>DNA-PK</td>
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<td></td>
<td>DDC1-RAD17-MEC3</td>
<td>RAD9-RAD1-HUS1</td>
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<td><strong>Mediators</strong></td>
<td>RAD9</td>
<td>53BP1 (BRCA1?)</td>
</tr>
<tr>
<td></td>
<td>MRC1</td>
<td>Claspin</td>
</tr>
<tr>
<td><strong>Effectors</strong></td>
<td>CHK1</td>
<td>CHK1</td>
</tr>
<tr>
<td></td>
<td>RAD53</td>
<td>CHK2</td>
</tr>
</tbody>
</table>
experimental monitoring of checkpoint activity has often relied on observing changes in phosphorylation-dependent changes in electrophoretic mobility of proteins, kinase activity assays, and more recently fluorescence microscopy (Pellicioli et al., 1999; Lisby et al., 2004). When I started my graduate studies, much of the genetic regulation of checkpoint signaling was known. Figure 1.3.1 illustrates the DNA damage checkpoint as a hierarchical, linear signaling pathway. The use of fluorescence microscopy was becoming a powerful technique to understand the importance of DNA damage protein assembly at sites of DNA damage. Moreover, the DNA damage signals that direct focus formation and the general architecture of foci were beginning to become clear (Melo et al., 2001; Lisby et al., 2004). However, the mechanisms that maintained and dissolved foci following DNA repair were poorly understood.

*The DNA damage signals are ssDNA and γH2A(X)*

In light of the structural heterogeneity of DNA damage, it was not immediately clear how the DNA damage signal was channeled into a single, common pathway. For instance, UV-induced DNA damage and DSBs are structurally different forms of DNA damage; yet, both types of damage can activate the DNA damage checkpoint (Sandell and Zakian, 1993; Siede et al., 1993; Siede et al., 1994). It was possible that there were many different sensors that detected different types of damage. Alternatively, the predominant view that has emerged is that structurally different types of DNA damage are converted into a common intermediate that is detected by a small group of sensor proteins.
**Figure 1.3.1.** The DNA damage checkpoint at a double-strand break (DSB) in *Saccharomyces cerevisiae*. An intact chromosome (i) suffers a DSB (ii). DSB ends are recognized by the MRX complex (Mre11-Rad51-Xrs2) (iii). Xrs2-mediated recruitment of the Tel1 kinase leads to phosphorylation of histone H2A to γH2A (iv). Initiation of resection involves the MRX-dependent recruitment of Exo11 and Dna2-Sgs1 and the Sae2-mediated eviction of MRX (v). Single-stranded DNA (ssDNA) revealed by resection is coated by the replication A (RPA) complex (vi). The Mec1 kinase and its cofactor Ddc2 are recruited to ssDNA by a Ddc2-RPA interaction (vi). The 9-1-1 checkpoint clamp (Ddc1-Rad17-Mec3) is loaded on to heteroduplex junctions by the alternative clamp loader complex (Rad24-Rfc2-5) (vi). Dpb11 is recruited to the DSB by a direct interaction with Ddc1 (vi). Ddc1 and Dpb11 both can activate Mec1. Mec1 can further phosphorylated H2A and other substrates including Rad9, which might interact with γH2A, and Rad53, which binds to Rad9 in a phospho-dependent manner (vii). Phosphorylated and activated Rad53 is a key effector kinase in promoting cell cycle arrest, transcriptional changes, and DNA repair (viii).
(i) Histones

Intact chromosome

(ii) DSB is generated

(iii) DSB ends are bound by the MRX complex

(iv) Tel1 recruitment is mediated by MRX; Tel1 phosphorylates H2A

(v) Resection by exoribonucleases proceeds after MRX eviction

(vi) Recruitment of checkpoint complexes to RPA-coated ssDNA

(vii) Propagation and amplification of the checkpoint signal

(viii) Cell cycle arrest

DNA repair

Transcription

Figure 1.3.1
ssDNA is a central DNA damage signal (Garvik et al., 1995; Zou and Elledge, 2003). In vivo, ssDNA is bound by the heterotrimeric replication protein A complex (RPA) consisting of Rfa1, Rfa2, and Rfa3 in budding yeast. The presence of ssDNA bound by RPA is a potent activator of the checkpoint (Zou and Elledge, 2003). Many different types of DNA damage are modified to become ssDNA. ssDNA is generated by 5’→3’ exonuclease-driven resection of a DSB end, at stalled replication forks, or by nucleotide and base excision repair machinery (Harrison and Haber, 2006). Checkpoint protein localization and foci formation at DNA damage is heavily stimulated by ssDNA-RPA (Melo et al., 2001; Zou and Elledge, 2003; Lisby et al., 2004; Kanoh et al., 2006; Majka et al., 2006a). Therefore, heterogeneous DNA damage can be converted into a common ssDNA intermediate that can be recognized by a small group of DNA damage sensors.

The DNA damage signal also has a chromatin component. In mammals, the histone H2A variant H2AX is phosphorylated on its C-terminal serine 139 to create γH2AX in response to DSBs (Rogakou et al., 1998). γH2AX formation extends several megabases around damage and is essential for the recruitment and focus formation of downstream checkpoint and repair factors such as MDC1 and BRCA1 at DSBs (Goldberg et al., 2003; Stewart et al., 2003; Stucki and Jackson, 2006; Krum et al., 2010). Mouse mutants that cannot phosphorylate H2AX have several genome instability phenotypes: cancer predisposition, immunodeficiency and sterile males (Celeste et al., 2003). The budding yeast genome does not encode an H2AX variant; however, the H2A histone C-terminus is similar to mammalian H2AX and is phosphorylated on serine 129 creating a 50 kb chromatin domain (Shroff et al., 2004). Early work implicated γH2A in
DNA repair while evidence for a checkpoint role was unclear (Downs et al., 2000; Redon et al., 2003; Downs et al., 2004). $h2a$ mutants that cannot be phosphorylated are moderately sensitive to MMS and even more sensitive to camptothecin (Downs et al., 2000; Redon et al., 2003). $\gamma$H2A is recognized by checkpoint proteins (Nakamura et al., 2004; Hammet et al., 2007), chromatin remodelers (Downs et al., 2004), and sister chromatid cohesion proteins (Strom et al., 2004; Unal et al., 2004). Unlike mammalian $\gamma$H2AX, the role of yeast $\gamma$H2A in DNA damage checkpoint signaling is much less clear since signaling defects in $h2a$ mutants have not be reported.

The generation of ssDNA is important for checkpoint signaling in yeast and mammals. Specifically, ssDNA is a signal for replication stress in S-phase in yeast and mammals. $\gamma$H2AX appears to be much more important in DSB signaling in mammals than yeast (Downs et al., 2000; Redon et al., 2003; Stucki and Jackson, 2006). However, recent evidence in yeast suggested that $\gamma$H2A is an important modulator of the duration of DNA damage signaling at DSBs (Keogh et al., 2006).

The MRX complex modulates DSB end resection to produce ssDNA

A DSB is initially recognized by the MRX complex, consisting of Mre11, Rad50, and Xrs2 (MRN in mammals: MRE11, RAD50, NBS1) (Haber, 1998a; Grenon et al., 2001; Lisby et al., 2004). The genes were originally identified as an early component of homologous recombination in meiosis and DNA repair (Game and Mortimer, 1974; Ajimura et al., 1993). Deletion of any of the MRX genes results in DNA damage sensitivity to hydroxyurea and IR (Game and Mortimer, 1974). Fluorescence microscopy experiments confirmed that MRX is a “first responder” to DSBs (Lisby et al., 2004).
Mre11 is a nuclease with 3’→5’ polarity (Paull and Gellert, 1998). Rad50 is a 150 kDa protein belonging to the structural maintenance of chromosomes (SMC) family of proteins (Alani et al., 1989). Xrs2 is the least conserved protein of the complex (NBS1 homolog) and is required to target the MRX complex to DSBs (Trujillo and Sung, 2001).

The initiation of resection is an important step in activating the DNA damage checkpoint. MRX modulates DSB end resection in the 5’→3’ direction to reveal long tracts of ssDNA 3’ overhangs. Paradoxically, in vitro evidence indicated that the Mre11 nuclease worked in a 3’→5’ polarity (Paull and Gellert, 1998). Moreover, mre11Δ cells are still able to carry out resection (Moreau et al., 2001). Therefore, other nucleases were postulated to directly carry out resection. Several exonucleases were identified as contributors to ssDNA formation: Sae2, Exo1, and Dna2 (Mimitou and Symington, 2008; Zhu et al., 2008; Mimitou and Symington, 2009a). Dna2 and Exo1 work in parallel pathways to resect DSBs. Dna2 requires the helicase Sgs1 for its function. Once resection is initiated, ssDNA formation accumulates at an estimated rate of 4.4 kb/h (Fishman-Lobell and Haber, 1992; Zhu et al., 2008) and RPA, visible as foci, rapidly accumulates (Lisby et al., 2004).

Tel1 and Mec1 initiate checkpoint signaling by sensing DNA damage

Two kinases with homology to the phosphoinositide 3-kinase (PI3K) family of serine/threonine protein kinases initiate checkpoint signaling upon detecting DNA damage: Tel1 and Mec1 (ATM and ATR in vertebrates, respectively). Mutations in ATM result in Ataxia Telangiectasia, a condition in humans characterized by radiosensitivity, genome instability, progressive neurodegeneration, and cancer predisposition (Perry and
A third member, DNA-PK, exists in vertebrates but a homolog in budding yeast has not been identified (Smith and Jackson, 1999). Tel1 signaling is restricted to unresected DSB ends (Usui et al., 2001). Instead, as a result of the tendency for yeast cells to resect DSB ends, Mec1 signaling is predominant; however, deletion of either TEL1 or MEC1 results in only minor defects in checkpoint signaling in response to DSBs suggesting that they are partially redundant to this type of DNA damage (Sanchez et al., 1996; Emili, 1998). In contrast, in vertebrates, ATM signaling appears to be the predominant response to DSBs and the ATR pathway is restricted to replication stress (Rouse and Jackson, 2002a; Shechter et al., 2004).

Tel1 and Mec1 both localize to DSBs. Tel1 is recruited directly by an interaction with Xrs2 of the MRX complex (Nakada et al., 2003). Mec1/ATR recruitment is directed by ssDNA-RPA filaments and requires an interacting protein Ddc2 (ATRIP in mammals) that directly interacts with the large subunit of RPA, Rfa1 (RPA70 in mammals) (Rouse and Jackson, 2002b; Zou and Elledge, 2003; Ball et al., 2005; Falck et al., 2005). For this reason, Mec1, but not Tel1, is also central to initiating the replication checkpoint in response to stalled and collapsed replication forks under replication stress where ssDNA arises (Alcasabas et al., 2001; Osborn and Elledge, 2003; Kanoh et al., 2006). Mec1 and Tel1 initiate DNA damage signaling at sites of DNA damage.

Fluorescence microscopy techniques have been instrumental in developing our understanding of Mec1 biology. C-terminally-tagged Ddc2 recombinant proteins are most commonly used as markers for Mec1 localization (Melo et al., 2001; Lisby et al., 2004). The genetic requirements for Ddc2 foci formation corroborate the model that the Mec1-Ddc2 kinase complex senses ssDNA-RPA filaments as the DNA damage signal. RPA is
required for Ddc2 foci formation, as demonstrated in heat-labile degron experiments with a degron-tagged Rfa1 (Lisby et al., 2004). Furthermore, mutations that disrupt the Ddc2-Rfa1 interaction, such as \textit{rfa1-t11}, prevent Mec1 recruitment to DNA damage (Zou and Elledge, 2003).

Mec1 and Tel1 also phosphorylate downstream mediator and effector proteins in the signal transduction cascade (Rogakou et al., 1998; Downs et al., 2000). One of the earliest targets of Mec1 and Tel1 is histone H2A on the PI3K consensus motif SQE on the C-terminal tail. Both kinases transduce signals from damage sites to initiate the checkpoint; for this reason Tel1 and Mec1 are considered sensors of DNA damage (Melo and Toczyski, 2002).

\textit{A PCNA-like DNA clamp senses DSBs and activates Mec1}

Another DNA damage sensor includes a heterotrimeric ring complex consisting of Ddc1, Rad17, and Mec3 which is structurally similar to the proliferating cell nuclear antigen (PCNA), a processivity factor for DNA replication (Kondo et al., 1999; Venclovas and Thelen, 2000; Sohn and Cho, 2009). The Ddc1-Rad17-Mec3 complex is often referred to as the checkpoint clamp or the 9-1-1 complex, after the \textit{S. pombe} and vertebrate homologs: RAD9, RAD1, and HUS1. Whereas PCNA is loaded onto DNA by the Rfc1-5 complex, 9-1-1 is loaded by an alternate clamp loader with Rad24 replacing the large Rfc1 subunit (Majka and Burgers, 2004). 9-1-1 loading is directed by RPA at ssDNA/dsDNA heteroduplex junctions with 3’ overhangs (Lisby et al., 2004; Majka et al., 2006a). Deletions of \textit{RAD24}, or any component of 9-1-1, results in a severe checkpoint defect and DNA damage sensitivity (Kondo et al., 1999).
Early genetic and *in vivo* experiments suggested that *RAD24*, along with the 9-1-1 genes, operated in one epistasis group cooperatively with a *MEC1-DDC2* pathway to activate the checkpoint (Paciotti et al., 1998). Indeed, localization of Ddc1 to chromatin near a DSB is dependent on *RAD24, RAD17*, and, *MEC3*, whereas Mec1 localized independently of the same genes (Kondo et al., 2001). Fluorescence microscopy experiments supported the independent colocalization of Mec1-Ddc2 and 9-1-1 complexes by observing fluorescently tagged Ddc1 and Ddc2 proteins (Melo et al., 2001; Lisby et al., 2004). The independent colocalization of the Mec1-Ddc2 and 9-1-1 checkpoint complexes is essential for Mec1 activation. Biochemical experiments revealed that the unstructured C-terminal tail of Ddc1 stimulates Mec1 kinase activity (Majka et al., 2006b). Notably, the artificial colocalization of Ddc1 and Ddc2 on undamaged chromatin is sufficient to activate the checkpoint even in the absence of DNA damage (Bonilla et al., 2008), which emphasizes the importance of the chromatin component of DNA damage checkpoint activation (Yeung and Durocher, 2008).

More recently, it has become clear that Ddc1 can activate Mec1 by a second mechanism. Mec1 phosphorylates Ddc1 on T602 to recruit Dpb11 (TopBP1 in mammals) which can also activate Mec1 (Puddu et al., 2008; Navadgi-Patil and Burgers, 2009b). The biochemical activation of Mec1 by Ddc1 is unique to budding yeast as experiments in fission yeast and mammalian cell lines have not identified ATR activation potential in 9-1-1. Instead, TopBP1-dependent activation of ATR appears to be the conserved mode of activation (Mordes et al., 2008).
Mediators and effectors of the DNA damage checkpoint

The DNA damage response results in a multitude of cellular responses including cell cycle arrest, genome-wide transcriptional changes, and DNA repair. Effector proteins carry out these important functions. Mec1 and Tel1 modulate the activities of effector proteins through phosphorylation. The Rad53 (human Chk2) and Chk1 (human Chk1) are S/T protein kinases that act as checkpoint effectors. The effector kinases target components of the cell cycle to execute cell cycle arrest (Sanchez et al., 1999).

Rad53 and Chk1 operate on different components of the cell cycle to achieve arrest (Figure 1.3.2). Chk1 prevents degradation of the anaphase inhibitor Pds1 and therefore inhibits the onset of chromosome segregation (Sanchez et al., 1999). Rad53 activates a closely-related kinase, Dun1, in a cascade that targets several components of the cell cycle. Rad53 and Dun1 phosphorylate Sml1, an inhibitor of ribonucleotide reductase, leading to Sml1 degradation and an increase in available dNTPs for DNA synthesis (Zhao and Rothstein, 2002). Rad53-dependent upregulation of dNTPs appears to be the essential role of Rad53, as \textit{rad53Δ} mutant lethality is rescued by mutations such as \textit{sml1Δ} that increase dNTP pools (Desany et al., 1998). Cdc5, a polo-like kinase, is another target of Rad53 and is involved in promoting cell cycle arrest by preventing cyclin degradation and several steps in mitosis (Charles et al., 1998; Sanchez et al., 1999; Smits et al., 2000; Hu et al., 2001b). Several proteomic screens with phospho-specific antibodies have revealed hundreds of peptides that are possibly modified in a ATM-/Tel1- or ATR-/Mec1-dependent manner in response to DNA damage (Ziv et al., 2006; Matsuoka et al., 2007; Smolka et al., 2007; Stokes et al., 2007). Thus, identifying effectors and determining their mechanisms of action remains an ongoing effort.
**Figure 1.3.2.** Rad53 and Chk1 cooperatively inhibit cell cycle progression. Mec1 activation after detection of DNA damage leads to activation of the S/T protein kinases Rad53 and Chk1. Rad53 phosphorylates the replication initiation factor Sld3 to prevent late origin firing. Simultaneously, Rad53 acts through the Dun1 kinase and phosphorylates the ribonucleotide reductase (RNR) inhibitor Sml1, leading to Sml1 degradation, and up-regulation of dNTP pools available for DNA synthesis for DNA repair. Rad53 also promotes Cdc5 kinase activity which prevents cylin degradation and continued CDK activity and prevents exit from mitosis. In parallel, Chk1 prevents degradation of the anaphase inhibitor Pds1, preventing the onset of chromosome segregation.
DNA damage
  ↓
  Mec1
  ↓
  Rad9
  ↓
  Rad53
  ↓
  Sld3
  ↓
  Late origin firing
  ↓
  Sml1
  ↓
  RNR
  ↓
  dNTPs
  ↓
  Anaphase Mitotic Exit
  ↓
  Cdc5
  ↓
  Clb/CDK
  ↓
  Chk1
  ↓
  Pds1
  ↓

Figure 1.3.2
Mediator proteins are adapter proteins that act as recruiters or scaffolds to direct Mec1- and Tel1-dependent activation of effector proteins. Rad9 is the central mediator protein in the budding yeast DNA damage checkpoint. The C-terminus of Rad9 contains tandem BRCT (BRCA1 C-terminal) domains which are important for protein-protein interactions (Soulier and Lowndes, 1999; Rodriguez et al., 2003). Rad9 also possesses tandem tudor domains that have been implicated in chromatin binding of methylated histone H3 (Giannattasio et al., 2005; Toh et al., 2006; Grenon et al., 2007; Lancelot et al., 2007). Outside of the BRCT and tudor domains, Rad9 shares little homology with mediator proteins in other organisms. Possible homologs of Rad9 are 53BP1 in mammals and Crb2 in fission yeast because of the structural similarities of the BRCT and tudor domains (Botuyan et al., 2006; Lancelot et al., 2007).

Rad9 becomes hyperphosphorylated by Mec1 and Tel1 in response to DSBs (Emili, 1998; Gilbert et al., 2001). The phosphorylation enables Rad9 oligomerization through its BRCT domains allowing Rad9 to become a platform for Rad53 activation (Schwartz et al., 2002; Sweeney et al., 2005). Gilbert et al (2001) proposed that once Rad9 was phosphorylated by Mec1 and Tel1, Rad53 was activated by binding to Rad9 to promote autophosphorylation in a Mec1/Tel1-independent manner. In contrast, Sweeney et al (2005) identified several Mec1-targetted residues on Rad53 that become phosphorylated upon binding to Rad9. Strong support for this model comes from evidence that a Ddc2-Rad53 fusion protein bypasses the requirement for Rad9 during Rad53 activation (Lee et al., 2004).

Whereas Rad9 mediates Rad53 phosphorylation in response to DSBs, rad9Δ mutants are largely tolerant to replication stress after treatments of hydroxyurea. Mrc1
Claspin in vertebrates) is another adapter protein that mediates Mec1-dependent Rad53 activation during DNA replication stress (Alcasabas et al., 2001; Osborn and Elledge, 2003). \textit{rad9A mrc1A} double mutants are synthetic lethal and upregulating dNTP pools by \textit{RNR1} over-expression or \textit{SML1} deletion suppresses the lethality, indicating that the two proteins constitute parallel pathways in Rad53 activation (Alcasabas et al., 2001).

\textit{Sensing Replication Stress}

DNA replication presents a unique set of challenges to a cell when faced with DNA damage. Many different lesions can stall or impede replication fork progression, which can result in fork collapse and DSB formation. Lesions that are barriers to replication forks include ssDNA nicks, bulky lesions, inter-strand crosslinks, and DSBs. Non-histone DNA-protein complexes at highly transcribed genes, silent replication origins, and transcriptionally repressed loci are natural barriers to replication (Ivessa et al., 2003). Hydroxyurea (HU) is a chemical that is commonly used in the laboratory to induce replication stress. HU inhibites the ribonucleotide reductase and limits the availability of dNTPs for new DNA synthesis. The replication checkpoint serves to stabilize stalled replication forks to prevent them from collapsing into DSBs and to slow the progression of DNA replication (Santocanale and Diffley, 1998; Lopes et al., 2001; Tercero and Diffley, 2001). The replication checkpoint shares many common players with the DNA damage checkpoint (Paulovich and Hartwell, 1995).

DNA replication is initiated in two phases (Diffley, 2004). First, licensing of replication origins begins with loading of the MCM (minichromosome maintenance) replicative helicases in pre-replicative complexes (Aparicio et al., 1997). CDK activity
regulates the association of MCMs at origins, limiting MCM association with chromatin only in G1 to prevent re-replication during the cell cycle (Diffley, 2004). The second phase of DNA replication initiation is the firing of replication origins which is initiated by CDK and Cdc7 phosphorylation of Sld2 and Sld3 (Bousset and Diffley, 1998; Donaldson et al., 1998; Zegerman and Diffley, 2007). The BRCT domain of Dpb11 interacts with phosphorylated Sld3, an interaction that is essential for DNA replication (Zegerman and Diffley, 2007).

Under replication stress, the replicative MCM helicase can uncouple from the rest of the replisome and continue to unwind DNA, generating ssDNA (Sogo et al., 2002; Nedelcheva et al., 2005; MacDougall et al., 2007). RPA coats the ssDNA and can be recognized by Mec1-Ddc2 and 9-1-1 (You et al., 2002; Zou and Elledge, 2003; Zou et al., 2003). While Rad9 is dispensable for Rad53 activation during replication stress, Mec1 activation of Rad53 is mediated through Mrc1 (Sanchez et al., 1999; Alcasabas et al., 2001; Osborn and Elledge, 2003). Mrc1 forms a heterotrimeric complex with Tof1 and Csm3 that acts as a replication-pausing complex and travels with the replication fork (Foss, 2001; Katou et al., 2003). Activated Rad53 suppresses the initiation of late-firing origins by phosphorylating Sld3, effectively delaying the completion of DNA replication, and prevents stalled replication forks from collapsing into DSBs (Paciotti et al., 2001; Tercero and Diffley, 2001; Lopez-Mosqueda et al., 2010) (Figure 1.3.2).

1.4 DNA Repair

A central role of the DNA damage response is to delay the cell cycle until DNA damage is repaired. There are two principle evolutionarily conserved pathways that repair
DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ involves the direct ligation of two broken ends, a mode of repair that is considered imprecise, since any degradation of the DSB ends result in loss of genetic information after ligation. HR is the more faithful mode of DSB repair, as it involves the direct exchange of genetic information. However, if recombination is not properly regulated, it can result in loss-of-heterozygosity, non-reciprocal translocations, large deletions or duplications all which are examples of chromosome rearrangements (Kolodner et al., 2002; Symington, 2002). HR requires the formation of ssDNA for homology search and the presence of a homologous donor sequence which acts as a template for DSB repair. NHEJ and HR can be viewed as competing pathways of repair. While inhibiting HR has no effect on NHEJ, abrogating NHEJ increases the frequency of HR indicating that NHEJ precedes HR in repair pathway choice (Frank-Vaillant and Marcand, 2002). Initiation of resection irreversibly commits the cell to HR repair (Paull and Gellert, 1998; Symington, 2002).

**Repair by Non-Homologous End-Joining**

Three requirements must be satisfied for NHEJ (Daley et al., 2005). First, DSBs must be protected from resection so that repair is not channeled into the HR pathway. The DSB-end binding protein Ku plays an important role in end protection. Second, the DSB ends must be held in close proximity to each other for ligation. The MRX complex plays an important structural role in holding DSB ends together to prevent translocations. Third, the ligation machinery, namely DNA ligase IV, must be recruited to perform their enzymatic functions.
The Yku70-Yku80 heterodimer is a highly conserved protein complex with strong affinity to DSB ends and telomeres (Feldmann and Winnacker, 1993; Porter et al., 1996). yku70Δ and yku80Δ deletions are synthetic sick with hypomorphic alleles of telomere-binding proteins such as Cdc13, suggesting that the Ku heterodimer functions to prevent chromosome ends from being illegitimately recognized as DSBs (Nugent et al., 1998). Furthermore, yku70/80 mutants have short telomeres, suggesting that Ku would normally shield telomeres from nucleolytic degradation and recombination (Porter et al., 1996; Polotnianka et al., 1998). In the same manner, Ku provides transient stability to DSBs to promote NHEJ.

The MRX complex is proposed to play a structural role in NHEJ (D'Amours and Jackson, 2002; Lobachev et al., 2004). The large Rad50 subunit belongs to the structural maintenance of chromosome (SMC) family of proteins (Hirano, 2002). It contains Walker A and B ATPase motifs at the N- and C-termini separated by 2 coiled-coil domains and 1 globular domain which create a hinge to bring the N- and C-terminal domains together (Anderson et al., 2001; D'Amours and Jackson, 2002). The structure of Rad50 suggests that it creates a scaffold with Mre11 and Xrs2 to tether the broken chromosome together, keeping broken ends in close proximity for ligation (Kaye et al., 2004; Lobachev et al., 2004; Mimitou and Symington, 2009a).

In addition to holding DSB ends together, the MRX complex is proposed to modify DSB ends for ligation. The nucleolytic activity of Mre11 can remove nucleotides at single-strand overhangs to reveal regions of microhomologies of 1-5 base pairs that can enhance the efficiency of chromosome ligation (Paull and Gellert, 1998).
DNA ligase IV in budding yeast is encoded by *DNL4/LIG4* and catalyzes end joining (Wilson et al., 1997). Dnl4 is recruited to a DSB by a physical interaction with the FHA domain of Xrs2 (Palmbos et al., 2008). The activity of Lig4 is regulated by two proteins: Lif1 (XRCC4 in mammals) and Nej1 (XLF in mammals). Nej1 regulates the nuclear localization of Lif1 (Valencia et al., 2001) and in turn, Lif1 promotes Dnl4 ligase activity (Teo and Jackson, 2000).

*The MRX complex: A molecular switch for homologous recombination*

The MRX complex is one of the earliest protein complexes to localize to a DSB (Lisby et al., 2004). It controls the critical junction between NHEJ and HR by regulating DNA end resection, the committal step for HR (Shim et al., 2010). Mre11 is structurally and functionally conserved from prokaryotes to mammals and carries out both exonuclease and endonuclease activities (Paull and Gellert, 1998; Trujillo and Sung, 2001). *mre11Δ* mutants are sensitive to a wide range of DNA damaging agents (Game and Mortimer, 1974) and have elevated levels of gross chromosome rearrangements (Chen and Kolodner, 1999; Kanellis et al., 2007).

Disruption of MRX by deleting any of the components greatly impairs DSB repair by HR (Haber, 1998a; Tsubouchi and Ogawa, 1998); however, the nuclease-dead *mre11-H125N* mutant is still capable of resection, indicating that Mre11 is not the nuclease that performs end resection (Usui et al., 1998). Furthermore, *in vitro* biochemical studies that demonstrated 3’→5’ Mre11 nucleolytic activity while resection occurs in the 5’→3’ direction. Rather, mutations that disrupt the integrity of MRX greatly inhibit resection (Usui et al., 1998), suggesting that the MRX complex plays a structural role in regulating
ssDNA formation and that there are other nucleases that carry out more processive resection (Moreau et al., 1999). Mre11 nuclease activity is suggested to process heterogeneous DSB ends resulting from IR exposure that might lack 5’-PO₄ or 3’-OH for ligation or so that more processive exonucleases can continue resection (Mimitou and Symington, 2009a). Consistent with this model, mre11Δ and mre11 nuclease-dead cells are twice as slow in initiating resection at IR-induced DSBs compared to “clean” endonuclease-induced DSBs (Moreau et al., 1999; Lisby et al., 2004).

Several nucleases have been identified that work downstream of MRX. Sae2 is an endonuclease with affinity to hairpin-capped DSB ends (Lengsfeld et al., 2007). The vertebrate homolog of Sae2, CtIP, has been shown to be required for ssDNA generation at DSBs (Sartori et al., 2007). Experiments with fluorescently-tagged Mre11 and Sae2 proteins revealed that Sae2 localization to DSBs follows the removal of Mre11 but precedes homologous recombination; additionally, Sae2-deficient cells are slower to remove Mre11 foci, suggesting that Sae2 promotes the eviction of MRX from chromatin at the committal step to HR (Lisby et al., 2004).

Exo1 and Dna2 were identified as nucleases critical for the generation of ssDNA (Moreau et al., 2001; Mimitou and Symington, 2008; Zhu et al., 2008; Shim et al., 2010). Specifically, over-expression of EXO1 partially rescues DNA repair defects of mrx mutants (Moreau et al., 2001; Lewis et al., 2002). Moreover, mre11Δ exo1Δ double mutants are synthetically slow-growing and are more sensitive to DNA damage than the single mutants (Moreau et al., 2001). Sgs1-Dna2, which forms a helicase-nuclease complex, is proposed to be involved in end resection in parallel with Exo1 (Mimitou and Symington, 2008; Zhu et al., 2008). In single-strand annealing (SSA) assays which
require homologous sequences to be revealed by resection before DSB repair can occur, 
$sgs1\Delta$ mutants were able to repair repeats 5-7 kb away. However, $sgs1\Delta$ mutants were 
SSA-impaired when the same repeats were placed 25 kb apart, indicating that the 
efficiency of resection was impaired in the absence of Sgs1 (Mimitou and Symington, 
2008; Zhu et al., 2008). Exo1 and Sgs1-Dna2 appear to also function redundantly, as 
$sgs1\Delta\ exo1\Delta$ mutants are completely resection- and SSA-defective (Zhu et al., 2008). 
Taken together, a current model describes resection as a 2-step processing mechanism 
whereby MRX participates in the initial processing of a DSB and Sae2 removes MRX to 
allow Exo1 and Sgs1-Dna2 to continue in a second phase of processive resection 
(Mimitou and Symington, 2009a).

Recently, in chromatin immunoprecipitation experiments (ChIP), MRX was found 
to regulate the association of Exo1 and Dna2 with DSBs while excluding NHEJ proteins 
Yku70 and Yku80 (Shim et al., 2010). Therefore, the decision to utilize HR as a repair 
mechanism is a tightly regulated process and the MRX complex acts as a switch between 
NHEJ and commitment to HR.

*Cell cycle regulation of HR requires CDK phosphorylation of Sae2*

HR repair requires the presence of a homologous donor sequence. At unique 
regions of the genome, a second copy is only available on the homologous chromosome 
in diploids or on the sister chromatid after S-phase. Since the onset of resection commits 
the cell to homologous recombination, DSB resection is tightly regulated with the cell 
cycle such that HR is preferentially utilized outside of G1. The cyclin-dependent kinase 
Cdc28 in *S. cerevisiae* is central to the control of resection during the cell cycle (Ira et al.,
Sae2 is a substrate of Cdc28, which must be phosphorylated on serine 267 to become nucleolytically active (Huertas et al., 2008). sae2-S267A mutants are defective in resection and sensitive to DNA damaging agents such as camptothecin. Sae2 is required for the removal of Mre11 foci (Lisby et al., 2004); however, Mre11 focus retention has not been reported in sae2-S267A mutants. Sae2 phosphorylation by Cdc28 provides a mechanism for cell cycle control of resection.

**DSB Repair by Homologous Recombination**

HR repair of DSBs is often considered to be an error-free process, as it utilizes homologous donor sequences to repair DNA damage. Nonetheless, HR can be either conservative by preserving overall chromosome structure or non-conservative by resulting in gross chromosome rearrangements (GCRs). When HR is improperly regulated, the result can be GCRs such as non-reciprocal translocations, deletions and duplications (Kolodner et al., 2002).

Initiation of HR requires resection of DSB ends to reveal 3’ ssDNA. Following end resection, the 3’ ssDNA strand can engage duplex DNA by invading the double helix to pair or “synapse” with a homologous sequence. Synapsis, the hallmark event of HR, is important for the eventual pairing of homologous DNA sequences and occurs in most HR exchanges (Paques and Haber, 1999). The triple-strand structure of ssDNA invading duplex DNA is called a D-loop (Register and Griffith, 1988). There are many different HR pathways resulting in distinct repair products; however, in general, the homologous donor sequence is copied on to the damaged locus (Figure 1.4.1).
**Figure 1.4.1.** Homologous recombination pathways for double-strand break (DSB) repair. (A) Gene conversion involves the non-reciprocal transfer of genetic information from the donor locus to the DSB. Strand invasion is followed by short stretches of new DNA synthesis until homologies in both DSB ends are sufficient for re-annealing completion of repair. (B) Single-strand annealing repair of DSBs requires single-strand complementarities to be revealed by resection. Direct and parallel repeats must flank the position of the DSB. Annealing is followed by removal of non-homologous flaps and deletion of interstitial DNA between the repeats. (C) Break-induced replication involves strand invasion followed by extensive DNA synthesis. Strand invasion involves one strand of the DSB that arises from asymmetric DSB ends without common homologies to any single donor locus. The invading strand establishes a replication fork and new DNA synthesis can proceed along the entire length of the chromosome.
Figure 1.4.1
Repair of DSBs can be accomplished by one of three mechanistically distinct HR pathways: gene conversion (GC), break-induced replication (BIR), and single-strand annealing (SSA; Figure 1.4.1) (Paques and Haber, 1999). GC is characterized by the non-reciprocal exchange of genetic information from a donor locus to the DSB-containing recipient locus. Typically, GC occurs when both DSB ends have homologies to a common donor locus. BIR can occur when only one end of a DSB has homology to a donor sequence. Strand invasion is followed by extensive new DNA synthesis that can proceed until homology with the second DSB end is synthesized or continue to the end of the chromosome. The final class of HR repair is SSA which involves the direct annealing of complementary ssDNA sequences that are revealed by resection. Therefore, SSA bypasses the requirement for strand invasion.

Much of the understanding of DSB repair mechanisms by HR stem from studies investigating mating type switching in budding yeast, which is a GC event (Strathern et al., 1982; Haber, 1998b). The process is initiated by a DSB induced by the homothallic endonuclease (HO) in the MAT locus (Strathern et al., 1982). Subsequently, the DSB is repaired when one of two donor sequences, \( HMLa \) or \( HMLa \), is copied into the MAT locus without affecting the donor sequences. By placing HO under the control of the galactose promoter, a DSB can be experimentally induced. Importantly, the 24-bp consensus sequence for HO is unique in the yeast genome and therefore, a single DSB is created in a defined location. Later model systems engineered to study HR also utilized the HO-inducible system but placed the HO cut site ectopically to induce other HR mechanisms such as BIR and SSA.
The double-strand break repair model was proposed to explain GC repair of DSBs (Figure 1.4.2) (Szostak et al., 1983). The model postulates that one 3’ strand of the DSB invades the donor locus to act as a primer for DNA synthesis. The displaced strand can anneal with the opposite 3’ ssDNA end and new DNA synthesis can also occur, resulting in a double Holliday junction structure mostly commonly associated with meiotic recombination. Random resolution of the double Holiday junction can create crossover products.

While meiotic HR produces crossover products that can be followed as non-Mendelian inheritance of alleles, crossovers are rare (10-20% estimated frequency) following mitotic gene conversion (Paques and Haber, 1999). To account for this, the synthesis-dependent strand-annealing (SDSA) model was proposed (Figure 1.4.2) (Strathern et al., 1982; Nassif et al., 1994). Similarly to the DSBR model, the invading 3’ strand primes DNA synthesis. When homology to the other 3’ ssDNA end is synthesized, the nascent strand is displaced and re-anneals with the opposite 3’ strand. DNA synthesis in the opposite direction completes repair. A double Holliday junction is avoided; therefore, the donor sequence is left unchanged with no chance of crossover.

In some instances, replication can continue to the end of the chromosome as in BIR (Figure 1.4.2). The invading 3’ strand establishes a replication fork with both leading and lagging strands. This process is important for telomere maintenance in the absence of telomerase (Le et al., 1999; Teng et al., 2000). However, with BIR, there is the possibility of loss of heterozygosity, a potentially deleterious GCR.
Figure 1.4.2. Models of DSB repair initiated by strand invasion. The double-strand break repair (DSBR) model: Strand invasion is followed by DNA synthesis to restore complementarity to the opposite DSB end. Re-annealing of both DSB ends creates a double Holliday junction. Resolution of the Holliday junction can create non-crossover (NCO) or crossover (CO) products. The synthesis-dependent strand annealing (SDSA) model: The invading strand primes DNA synthesis and the nascent strand is displaced to reanneal with the opposite DSB end. The model avoids creation of a double Holliday junction and cross-over products. The break-induced replication (BIR) model: The invading strand primes DNA synthesis and establishes a nascent replication fork that can replace the entire length of the broken chromosome, resulting in non-reciprical exchange of genetic information from the template sequence to the broken chromosome and loss of heterozygosity (LOH).
The RAD52 Epistasis Group

Genetic screens for genes required for IR resistance have identified RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, RFA1, and MRE11 in a common epistasis group (Game and Mortimer, 1974). The RAD gene products execute the steps of HR: resection, homology search, synapsis, and exchange (Table 1.4.1) (Symington, 2002). Mutations in many of these genes lead to defects in meiotic and mitotic recombination, mating type switching, and telomere maintenance (Paques and Haber, 1999).

Rad51 is a well-conserved protein from yeast to mammals and is highly homologous to bacteria RecA, a protein that promotes homologous pairing and strand exchange (Kowalczykowski et al., 1994). The yeast Rad51 protein is essential for all mitotic strand-invasion reactions. rad51Δ cells are viable because there are multiple DSB repair pathways, some which are Rad51-independent (Paques and Haber, 1999; Symington, 2002). For example, rad51Δ cells are defective for GC but are still proficient for SSA (Vaze et al., 2002). In contrast, RAD51 is essential in vertebrates; rad51−/− null mice suffer early embryonic death (Lim and Hasty, 1996; Tsuzuki et al., 1996).

Rad51 homology among species is highest in the Walker A and B motifs that bind nucleotides. Rad51 ATPase activity is activated by DNA-binding and ATP hydrolysis enables the strand exchange reaction (Sung, 1994). Additionally in yeast, ATP binding is required for Rad51 association with DNA (De Zutter and Knight, 1999). Rad51 forms a helical filament on DNA (Ogawa et al., 1993). While Rad51 has affinity to both dsDNA and ssDNA, it has highest affinity with dsDNA with ssDNA tails (Mazin et al., 2000). Rad51 filament formation on ssDNA is strongly stimulated by the presence of RPA.
Table 1.4.1. Homologous recombination genes and their functions. SSA: single strand annealing. GC: gene conversion. BIR: break-induced replication.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in Homologous Recombination</th>
<th>SSA</th>
<th>GC</th>
<th>BIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE11/RAD50/XRS2</td>
<td>Modulation of DSB end resection to generate 3' ssDNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RFA1-3</td>
<td>ssDNA binding protein; eliminates secondary structures from ssDNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAD51</td>
<td>Recombinase that catalyzes homology search and strand invasion</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAD52</td>
<td>Anneals homologous sequences; Replaces RPA with Rad51 on ssDNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAD59</td>
<td>RAD52 paralog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAD55/57</td>
<td>Heterodimer that promotes Rad51 nucleofilament stability; facilitates RPA displacement</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RAD54</td>
<td>SWI/SNF dsDNA-dependent ATPase helicase</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RDH54</td>
<td>SWI/SNF dsDNA-dependent ATPase helicase; Diploid specific recombination</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAD1/10</td>
<td>Flap endonuclease complex; removes non-homologous ends following SDSA or SSA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
(McIlwraith et al., 2000). One prominent model suggests that RPA eliminates secondary structures in ssDNA for Rad51 loading (Symington, 2002).

The Rad51-DNA nucleofilament is proficient for homology search, synapsis, and strand exchange in vitro (Baumann et al., 1996); however, the precise mechanism by which a DSB end identifies a homologous partner is one of the least understood events in HR. Biochemical experiments demonstrate that the addition of RPA to Rad51 nucleofilaments greatly stimulates synapsis (Sung, 1997). In the same study, Sung observed that if both RPA and Rad51 are introduced to DNA simultaneously, strand exchange is inhibited. The simultaneous addition of RPA and Rad51 more closely resembles the in vivo situation where RPA and Rad51 would be expected to compete for ssDNA. Three Rad51 paralogs have been identified that alleviate the RPA-dependent inhibition of Rad51 strand exchange: Rad52, Rad55, and Rad57.

In yeast, Rad52 is essential for all HR exchanges. Consequently, rad52Δ mutants are severely sensitive to DNA damaging agents (Game and Mortimer, 1974). Rad52 fulfills two critical steps of HR: strand annealing of homologous sequences and promotion of Rad51 synapsis (Symington, 2002). Biochemical studies of Rad52 demonstrate that the protein has high affinity to ssDNA and catalyzes annealing of homologous DNA sequences (Mortensen et al., 1996). The annealing reaction is enhanced in the presence of RPA (Sugiyama et al., 1998). In vivo evidence indicates that this reaction can take place independently of Rad51 in the SSA pathway (Bai and Symington, 1996; Ivanov et al., 1996; Vaze et al., 2002). As a mediator of Rad51 filament formation, Rad52 physically interacts with RPA and Rad51 (Shinohara et al., 1992; Hays et al., 1998). Rad52 facilitates RPA exchange for Rad51 to promote synapsis
(Song and Sung, 2000). In support of this model, Rad52 binds to RPA filaments *in vivo* but does not displace RPA until Rad51 interacts with Rad52 (Sugiyama and Kowalczykowski, 2002). Rather than forming helical filaments like RPA and Rad51, scanning electron microscopy of purified Rad52 indicated that the protein assembles into heptameric ring complexes (Shinohara et al., 1998; Stasiak et al., 2000; Ranatunga et al., 2001). The ring complex has a positively charged groove created by the assembled N-termini of Rad52 which creates a DNA-binding surface (Kagawa et al., 2002).

A closely related protein to Rad52 is Rad59. Rad59 has significant homology to the N-terminus of Rad52 (Bai and Symington, 1996). *RAD52* overexpression was found to suppress the DNA damage sensitivity of *rad59Δ* mutants, indicating that the two proteins play overlapping roles; however, Rad59 cannot replace Rad52 *in vivo*. Rad59 interacts with Rad52 and Rad59 can be co-immunoprecipitated with Rad51 in a Rad52-dependent manner (Davis and Symington, 2003). Because the biochemical properties of Rad59 are so similar to Rad52, the specific role of Rad59 in HR remains enigmatic.

The Rad51 paralogs Rad55 and Rad57 form a heterodimer that interacts with Rad51 (Hays et al., 1995; Johnson and Symington, 1995; Sung, 1997). Like *rad51Δ* mutants, *rad55Δ* and *rad57Δ* mutants are sensitive to IR (Game and Mortimer, 1974). However, the DNA damage sensitivity is rescued by over-expressing Rad51, consistent with the idea that Rad55-Rad57 stabilizes Rad51 filaments following Rad52-mediated exchange with RPA (Hays et al., 1995).

Rad54 and Rdh54 are members of the chromatin remodeling SWI/SNF family (Eisen et al., 1995). Both proteins contain characteristic DNA-dependent ATPase and DNA helicase motifs. Much of the early genetic experiments with *RAD54* and *RDH54*
indicated that they are more important recombination in diploid cells (Klein, 1997; Shinohara et al., 1997). Rad54 mutants are viable and are able to complete SSA but are severely impaired in GC, suggesting that it influences Rad51 synapsis (Ivanov et al., 1996). Rad54 and Rdh54 stimulation of Rad51-mediated strand invasion is supported by biochemical evidence (Petukhova et al., 1998; Petukhova et al., 2000). As chromatin remodelers, Rad54 and Rdh54 promote DNA supercoiling in an ATP-dependent manner (Eisen et al., 1995). It is predicted that Rad54 and Rdh54 eases chromatin compaction to facilitate Rad51 D-loop formation and synapsis (Van Komen et al., 2000). Therefore, alteration in the chromatin environment surrounding a DSB is also important for execution of strand invasion (Sinha and Peterson, 2009).

HR is a tightly orchestrated process of genetic exchange that is executed by the RAD52 epistasis group of genes (Table 1.4.1). Chromatin immunoprecipitation (ChIP) experiments exploring the recruitment of HR proteins to DSBs have provided important insight to their temporal and spatial regulation in vivo. Rad51 enrichment at a DSB requires Rad52 and is enhanced by Rad55-Rad57 (Sugawara et al., 2003; Wolner et al., 2003). In addition, rad54Δ mutants did not delay the recruitment of Rad51, suggesting that Rad54 acts in a downstream step of HR after Rad51 loading (Sugawara et al., 2003). Taken together, HR requires the efficient replacement of RPA with Rad51 which is catalyzed by Rad52 and stabilized by Rad55-Rad57. Strand invasion into duplex DNA by Rad51-ssDNA filaments is facilitated by local chromatin relaxation, catalyzed by the Rad54 and Rdh54 chromatin remodelers. The mechanisms of HR repair were deduced from genetic assays that examined repair products and biochemical assays with purified
proteins. An outstanding question in HR repair is how DSB ends find homologous sequences.

Multiple nucleases and helicases resolve recombination intermediates

HR is a multistep process which produces intermediates that are toxic to the cell if not resolved properly. Multiple pathways contribute to the removal of undesirable HR intermediates. Several nucleases and helicases have been identified as enzymes that participate in completing HR repair (Symington, 2002; Mimitou and Symington, 2009b; Marini and Krejci, 2010).

Rad1 and Rad10 form a heterodimeric flap endonuclease that removes non-homologous ends following direct repeat repair (Bardwell et al., 1994). \textit{RAD1} and \textit{RAD10} were initially identified as genes in the nucleotide excision repair pathway (de Laat et al., 1999). Following strand annealing such as in an SSA mechanism, the nuclease complex is directed to the recombination intermediate by Saw1 where it cleaves 3’ flaps to allow strand ligation (Ivanov and Haber, 1995; Li et al., 2008). The resulting repair product includes a deletion of interstitial DNA between direct repeats as illustrated in SSA repair in Figure 1.4.1.

The helicase Srs2 has been identified as the principal anti-recombinase in budding yeast. \textit{SRS2} was initially identified in a \textit{rad6} UV-sensitivity suppressor screen (Suppressor of Rad Six) (Lawrence and Christensen, 1979). \textit{rad6} mutants are defective in post-replication repair and translesions bypass repair pathways (Prakash, 1981). The inactivation of Srs2 is thought to channel UV DNA damage into HR repair pathways (Schiestl et al., 1990). Furthermore, inactivation of Srs2 results in a hyper-recombination
phenotype (Rong et al., 1991). Biochemical analysis of Srs2 indicates that it can directly evict Rad51 from chromatin, implicating the helicase as an antirecombinase (Veauet et al., 2003). The helicase activity of Srs2 is important for preventing illegitimate HR events (Krejci et al., 2003). Srs2 has been implicated in a wide range of roles including minimizing crossover products after DSB repair, promoting NHEJ, and preventing HR at the replication fork (Marini and Krejci, 2010; Leon Ortiz et al., 2011).

Recent work has provided insight into the regulation of Srs2 activity to regulate HR. The Shu complex consists of four proteins: Csm2, Psy3, Shu1, and Shu2 (Shor et al., 2005). Deletions of any of the Shu genes partially rescues the DNA damage sensitivity, slow growth, and hyperrecombination of the topoisomerase mutant top3Δ (Shor et al., 2005). _SHU1_ and _PSY3_ are paralogs of _RAD51_, bearing sequence similarity to the human protein RAD51D (Pittman et al., 1998). The Shu complex positively modulates HR at a replication fork for error-free bypass of DNA damage (Ball et al., 2009). Specifically, the Shu complex promotes Rad51 assembly by preventing Srs2 localization to DSBs (Bernstein et al., 2011).

Other identified resolvases include, the Mus81-Mms4 complex, Yen1, and Sgs1. Mus81-Mms4 and Yen1 are structure-specific endonucleases that resolve Holliday junctions (Haber and Heyer, 2001; Ip et al., 2008). _sgs1Δ srs2Δ_ mutants are synthetic lethal, suggesting that the two helicases have partially redundant roles in HR (Lee et al., 1999). Sgs1 is similar to the human helicase BLM and WRN which when mutated, are implicated in premature aging and cancer predisposition.
**Repair Pathway Choice**

The decision between NHEJ and HR is regulated by the cell cycle. The roles of MRX, CDK, and Sae2 in regulating the decision between NHEJ and HR were discussed earlier. HR is typically favoured in S, G2 and M phases of the cell cycle, although recombination has been observed occasionally occur in G1 (Fabre, 1978). In *S. cerevisiae*, HR is the predominant repair mechanism for DSBs.

Given the alternative repair pathways of GC, SSA, and BIR, the cellular decision to undertake a particular mechanism is expected to be tightly controlled. The mechanisms that determine which HR pathway is used in the cell are not entirely understood. The relative location and orientation of homologous sequences has great influence over which pathway is chosen (Haber, 1999; Jain et al., 2009). GC occurs when both DSB ends have homology to common sequence and engage the homologous sequence together (Ira et al., 2003; Ira et al., 2006). BIR occurs when DSBs have only one homologous end, which can arise from collapsed replication forks, unprotected telomeres, or non-repetitive sequences throughout the genome (Davis and Symington, 2004). However, it is not clear how only one strand is chosen to initiate BIR. If direct parallel repeats are revealed by resection, the ssDNA can directly anneal in SSA to repair the break (Lin et al., 1984; Vaze et al., 2002). The amount of sequence homology also influences repair pathway choice. GC requires a minimum of 100 bp of homology whereas efficient SSA requires at least 400 bp (Sugawara and Haber, 1992; Inbar et al., 2000).

An active mechanism of HR pathway choice was recently proposed (Jain et al., 2009). Jain and others observed that the GC and SSA pathways are initiated prior to attempts at BIR. The switch between to BIR from GC or SSA occurred when the DSB
had persisted longer than 6 hours in the system of study, which utilized an intrachromosomal recombination assay. The rate limiting step of BIR is initiation of new DNA synthesis.

The Sgs1 helicase regulates the decision to switch from GC or SSA to BIR (Jain et al., 2009). Sgs1 reduces the likelihood of BIR by limiting D-loop stability. In the absence of Sgs1, BIR is favoured over GC events. Synapsis efficiency is unaffected in sgs1Δ mutants; however, new DNA synthesis is initiated sooner, indicating that a more stable D-loop encourages initiation of DNA synthesis. GC and SSA mechanisms are generally more conservative than BIR. BIR can re-replicate large portions of a chromosome, leading to loss of heterozygosity (Figure 1.4.2). The early preference for GC and SSA over BIR might indicate that the cell will try to utilize more conservative HR pathways prior to alternative repair mechanisms that result in increased genetic instability such as loss of heterozygosity and non-reciprocal translocations (Llorente et al., 2008).

**Chromosome Dynamics during DNA repair**

DNA repair proteins can be tagged with fluorophores and visualized by fluorescence microscopy. Rad52 forms foci spontaneously during DNA replication and after exposure to IR (Lisby et al., 2001). Notably, Lisby et al (2001) found that the number of Rad52 foci in yeast did not strictly correlate with the amount of damage that was incurred in the genome, suggesting that damage is handled in just a few “repair centres” in the nucleus. It is not clear how DSBs are moved to these repair centres. The
spontaneous foci observed in S-phase might indicate that DNA replication also occurs in defined locations within the nucleus.

Recent publications investigated the dynamics of persistent and slowly repaired DSB. Persistent or slowly repaired DSBs migrated toward the nuclear periphery. The movement is dependent on SUMOylation of the histone variant Htz1 (Kalocsay et al., 2009) and on the nuclear pore proteins Mps3 and Nup84 (Nagai et al., 2008; Oza et al., 2009). The sequestered DSBs at the nuclear periphery are thought to be repaired by telomere healing, a rare type of gross chromosome rearrangement that adds a telomere to DSB ends. Telomere healing results in the loss of the acentric portion of the chromosome. Therefore, the relocalization of DSBs promotes alternative repair mechanisms at the expense of genome stability.

Protein-chromatin interactions observed by conventional ChIP-qPCR (quantitative PCR) methodology are limited to a handful of selected query loci. The recent development of ChIP-chip technology has allowed investigators to probe protein-chromatin interactions at a genome-wide level, providing new insight into the nature of HR. The ChIP-chip method hybridizes immunoprecipitated DNA onto microarrays to determine chromatin enrichment of a protein of interest. Using Chip-chip, Kalocsay et al (2009) observed Rad51 enrichment on a chromosome that received a single HO-induced DSB. The recruitment of Rad51 was rapid (within 60 minutes of DSB induction) and extensive, extending to over half of the broken chromosome (Kalocsay et al., 2009). Remarkably, Rad51 enrichment extended well beyond the expected ssDNA domain based on estimates of DSB resection rates (Vaze et al., 2002; Zhu et al., 2008), indicating that synapsis is initiated soon after Rad51 filament formation and is limited just to the
damaged chromosome. A mechanism that restricts synapsis to the damage chromosome has not been described.

**DNA damage tolerance during DNA replication**

DNA damage during S-phase stalls DNA replication and activates the replication checkpoint (see Section 1.3). To cope with damage, cells utilize a mechanism called post-replication repair that allows the replication fork to avoid damaged template DNA (Broomfield et al., 2001). The pathway does not correct the damage, but it allows the replisome to proceed beyond the DNA damage so that repair can take place following replication. The proliferating cell nuclear antigen (PCNA) is the central coordinator of DNA damage tolerance at the replication fork (Moldovan et al., 2007).

In budding yeast, PCNA is a homotrimeric ring complex made of Pol30 subunits. It is loaded on to DNA by the replication factor C complex (RFC), consisting of proteins Rfc1-5 (Majka and Burgers, 2004). PCNA is an essential cofactor in DNA elongation by tethering DNA polymerases to DNA and the replisome (Moldovan et al., 2007). Polymerases involved in DNA damage tolerance recruited by PCNA to bypass DNA damage so that it can be repaired after DNA replication (Waters et al., 2009).

Post-replication repair can occur in an error-prone translesion synthesis pathway or in an error-free mechanism using template switching. For translesion synthesis, the replisome merely incorporates nucleotides irrespective of the template strand to bypass the lesion; therefore, this mechanism is error-prone. Translesion polymerases include Polη, Polζ, Polτ, and Rev1 (Waters et al., 2009). Error-free bypass utilizes a template switching mechanism where the nascent strand that encounters the lesion can switch to
the sister strand as a new template. The precise mechanism of template switching is still unclear, although it is suspected to share many similarities with HR (Barbour and Xiao, 2003; Allen et al., 2011).

Covalent modification of PCNA by the small polypeptide ubiquitin coordinates post-replication repair function (Andersen et al., 2008). The Rad6-Rad18 complex was identified as a key ubiquitin ligase that acts on PCNA (Hoege et al., 2002). After ubiquitin activation by E1, Rad6 and Rad18 function as ubiquitin E2 conjugating and E3 ligase enzymes, respectively. Rad18 is a RING-finger containing protein that can bind both PCNA and ssDNA, providing a possible mechanism for recruiting ubiquitin machinery to chromatin. PCNA is mono-ubiquitylated on K164 by Rad6-Rad18. PCNA can be additionally polyubiquitylated by the Ubc13-Mms2 E2 heterodimer and the Rad5 RING-finger E3 ligase forming noncanonical polyubiquitin conjugates through K63 (Hoege et al., 2002). K63 chains are modifications typically found in signaling pathways whereas K48 linkages promote proteasome-mediated degradation (Panier and Durocher, 2009). Monoubiquitylation promotes error-prone translesion bypass while polyubiquitylation promotes the error-free pathway (Hoege et al., 2002).

PCNA can also be modified by SUMO on the same K164 residue in an Ubc9- and Siz1-dependent manner (Hoege et al., 2002). SUMO can also be conjugated on to K127 but the modification is not involved in post-replication repair; instead, it participates in sister chromatid cohesion during replication (Moldovan et al., 2006). SUMOylation and ubiquitylation compete for K164. SUMO modification occurs in an unperturbed S-phase and prevents unscheduled HR at the replication fork by recruiting the antirecombinase helicase Srs2 (Papouli et al., 2005; Pfander et al., 2005). While HR can be used to restart
replication forks, the use of this pathway can be deleterious to genome stability, as it can produce gross chromosome rearrangements (Lambert et al., 2005).

*Possible role for Rad18 in DSB repair*

Evidence that implicates Rad18 in NHEJ repair of DSBs has recently emerged. In yeast, the translesion polymerases Polζ and Rev1 are recruited to DSBs in a Mec1-dependent manner to perform DNA synthesis to complete NHEJ (Hirano and Sugimoto, 2006). During replication stress, PCNA ubiquitylation by Rad6-Rad18 recruits Polζ and Rev1 (Waters et al., 2009). Rad18 also localizes to DSB, but it is not dependent on Mec1 or Tel1; instead, Rad6 directs Rad18 recruitment (Hirano et al., 2009). PCNA ubiquitylation by Rad6-Rad18 is required for the recruitment of translesion synthesis pathways to bypass DNA damage during DNA replication (Moldovan et al., 2007). However, Rad18 was not required for Polζ or Rev1 localization to DSBs (Hirano and Sugimoto, 2006). Instead, it is possible that Polζ and Rev1 localize to DSBs first, directed by Mec1, and subsequently interacts with PCNA to stimulate DNA synthesis. It is not clear how PCNA is recruited to DSBs and the temporal relationship of Rad18-directed PCNA ubiquitylation is unknown. Mammalian RAD18 has also been observed at DSBs and appears to promote homologous recombination through an interaction with RAD51C (Huang et al., 2009). Additionally, RAD18 is required for retention of 53BP1, a critical factor in DSB signaling (Stewart et al., 2003; Watanabe et al., 2009). RAD18 has also been implicated in the recruitment of FANCA and FANCD2 to damaged chromatin after treatment with the Topoisomerase I toxin, camptothecin (Palle and Vaziri, 2011). The precise mechanism of action of Rad18 (and RAD18) at DSBs is still under investigation;
however, evidence suggests that Rad18 has functions in DSB repair that is separate from its role in PCNA ubiquitylation and post-replication repair.

1.5 Checkpoint Termination: Recovery and Adaptation

The coordination of cell cycle arrest with DNA repair is essential for DNA damage survival. Equally important is the ability down-regulate the DNA damage checkpoint following DNA repair to resume cell cycling. When the checkpoint is terminated following DNA repair, the process is called “recovery” (Bartek and Lukas, 2007). Alternatively, if DNA damage persists, cells can down-regulate checkpoint activity independently of DNA repair in a process called “adaptation.” Similar to other signal transduction cascades, termination of DNA damage checkpoint signaling coincides with the elimination or repression of the stimulus—the DNA damage.

Recovery following successful repair allows the cell to reestablish homeostasis by reversing the cellular changes that were induced by the DNA damage checkpoint. In recent years, only a few genes such as \textit{PPH3}, \textit{SRS2} and \textit{ASF1} have been identified as regulators (Vaze et al., 2002; Keogh et al., 2006; Chen et al., 2008). Indeed, genes that control recovery remain elusive because of the intrinsic difficulty in identifying mutations that impair checkpoint recovery while not resulting in DNA damage repair defects.

Adaptation refers to a more general phenomenon of signal transduction pathways whereby sensing and signaling components become desensitized to the stimulus signal in a prolonged stress response. The phenomenon has been described in various systems including chemotaxis and hormone signaling. In the presence of irreparable DNA
damage, cells can override the checkpoint and complete one or two subsequent divisions before dying from loss of essential genetic material (Sandell and Zakian, 1993). DNA damage checkpoint adaptation has also been observed in *Xenopus* egg extracts (Yoo et al., 2004).

*Systems to study adaptation and recovery*

Many of the model systems used to study checkpoint adaptation and recovery were adapted from systems used to study DSB repair in yeast. As in DSB repair strains, the model systems typically utilize one or more HO consensus sites where DSB formation, resection and repair can be monitored simultaneously with checkpoint activity.

Adaptation has been studied in strains lacking the donor sequences *HMRa* and *HMLa* to prevent the DSB in the *MAT* locus from being repaired by HR (Figure 1.5.1, A). NHEJ can still repair the DSB, but a perfectly re-ligated chromosome is cut again by HO and initiation of resection eliminates the possibility of re-ligation. Checkpoint and resection dynamics have been studied extensively using *MAT* locus DSBs (Haber, 1998b; Lee et al., 1998; Lee et al., 2001; Lee et al., 2003). Alternatively, the HO consensus sequence can be ectopically inserted elsewhere in the genome (Figure 1.5.1, B). Such a scheme was employed to study checkpoint protein foci during adaptation where the HO cut site was inserted in the *ADH4* locus near the telomere of *Chr VII* (Melo et al., 2001).

To study recovery, a system that repairs the DSB is required. Strains that utilize GC to repair DSBs have been used to study checkpoint recovery in a number of studies (Vaze et al., 2002; Keogh et al., 2006; Chen et al., 2008). Alternatively, direct repeats can
Figure 1.5.1. *S. cerevisiae* strains for studying homologous recombination and checkpoint signaling. In these strains, the HO endonuclease is under the control of a galactose promoter. When HO is expressed, it cuts in the indicated locus to produce a DSB. (A) JKM179: The HO endonuclease cuts in its endogenous *MAT* locus. The *HML* and *HMR* donor loci are deleted to prevent homologous recombination repair of the DSB. (Lee et al, 1998) (B) yJM20: The HO cut site in the *MAT* locus is deleted and ectopically inserted near the *ADH4* locus. The absence of significant homology with ADH4 elsewhere in the genome prevents repair of the DSB by homologous recombination. (Melo et al, 2001) (C) YMV2/YMV80: As in (B), the HO cut site in the *MAT* locus is deleted, but ectopically inserted in the *LEU2* locus. Repair of the DSB occurs by SSA or BIR with a partial *LEU2* duplication inserted either 25 kb (YMV80) or 30 kb (YMV2) away from the cut site. (Vaze et al, 2002).
A

\[ \text{ChrIII} \quad \square \quad \text{JKM179} \]

\[ \triangle \quad \text{hml} \Delta \quad \triangle \quad \text{hmr} \Delta \]

B

\[ \text{ChrVII} \quad \text{adhl4::HOcs} \quad \text{yJM20} \]

\[ \text{HO} \quad \downarrow \]

C

\[ \text{ChrIII} \quad \text{u2} \quad \text{leu2-cs} \quad \text{YMV2/80} \]

\[ \text{25/30 kb} \]

\[ \text{HO} \quad \downarrow \]

5'→3' resection

Single-strand annealing

Repaired chromosome

Figure 1.5.1
be placed on opposite sides of the HO cut site to promote intrachromosomal HR repair by SSA or BIR, such as the YMV2 and YMV80 systems engineered by Vaze et al (2002) (Figure 1.5.1, C). In these strains, the HO consensus site is inserted in the LEU2 locus while MAT, HMRa, and HMLa are deleted. A partial leu2 duplication is inserted in the HIS4 locus 30 kb away (YMV2) or 25 kb away (YMV80) to provide a donor locus. DSB formation, resection and repair can be monitored by southern blot analysis simultaneously with checkpoint activity. In these strains, DSB repair is completed via BIR or SSA.

Adaptation to the DNA damage checkpoint

A single DSB is sufficient to activate the DNA damage checkpoint robustly (Sandell and Zakian, 1993). Checkpoint signaling is mostly commonly monitored by Rad53 phosphorylation or kinase activity. Adaptation occurs 15 hours after the induction of a DSB as measured by dephosphorylation of Rad53 (Pellicioli et al., 2001; Lee et al., 2003). The two checkpoint sensor complexes 9-1-1 and Mec1-Ddc2 localize to DSBs and can be monitored by observing Ddc1-GFP and Ddc2-GFP foci, respectively. When cells adapt, Ddc2-GFP foci become dimmer (Melo et al., 2001). On the other hand, Ddc1-GFP foci increase in intensity over time, irrespective of adaptation (Melo et al., 2001). Ddc2 interacts with RPA-ssDNA filaments (Zou and Elledge, 2003) and the decreased intensity of Ddc2-GFP foci at adaptation might represent signal repression of RPA-ssDNA (Melo et al., 2001). The 9-1-1 checkpoint clamp continues to accumulate at a DSB presumably because the presence of ssDNA-dsDNA junctions allows Rad24-Rfc2-5 to continuously load 9-1-1 on to chromatin. These models have never been directly tested.
Figure 1.5.2. Known regulatory genes for checkpoint adaptation and recovery. To terminate DNA damage checkpoint signaling, cells can overcome persistent DNA damage by adaptation. The decision to adapt is not fully understood; however, it involves the monitoring of DNA damage by DNA repair and DNA damage signaling proteins (Rad51, Rad52, Rdh54, Rfa1, and Rad9) as well as the reversal of post-translational modifications imparted by the DNA damage checkpoint (Cdc5, Ptc2, and Ptc3). Following DNA repair, cells can recover from checkpoint-mediated cell cycle arrest. Only a few genes have been implicated in recovery. Pph3, Ptc2 and Ptc3 are protein phosphates that remove DNA damage-induced phosphorylation. Asf1 is a histone chaperone that participates in the restoration of the chromatin environment in the vicinity of DNA damage.
Suppression of checkpoint signal: Rad51, Rad52, Rdh54, Rfa1, Rad9

Reversal of post-translational modifications: Ptc2/3, Cdc5

? ↓

Adaptation

Checkpoint ON

Recovery

? ↑

Checkpoint OFF

Reversal of post-translational modifications: Pph3, Ptc2/3

Restoration of chromatin: Asf1

Elimination of checkpoint signal

DNA Repair

Figure 1.5.2
There is a strong correlation between the amount of ssDNA and adaptation (Lee et al., 1998). For example, cells that receive 2 or more DSBs fail to adapt. Additionally, Lee et al (1998) found that cells lacking the DSB end-binding proteins Yku70 and Yku80 have accelerated resection rates; the faster generation of ssDNA is associated an adaptation defect. Finally, adaptation is restored in yku mutants when resection is slowed by disrupting the MRX complex. Taken together, the amount of ssDNA has a profound influence on the ability for a cell to adapt.

The Rad51, Rad52, and Rdh54 recombination proteins are suggested to monitor the extent of DNA damage to regulate adaptation (Lee et al., 2001; Lee et al., 2003). rad51Δ and rdh54Δ mutants are adaptation-defective and are thought to act in parallel pathways in the decision to adapt (Figure 1.5.2). For instance, deletion of RAD52 restores adaptation in rad51Δ but not rdh54Δ mutants. rad51 and rad52 mutations that disrupt the Rad51-Rad52 interaction impair adaptation; however, a rad51-K191A allele that does not bind ssDNA or carry out recombinase activity is still proficient for adaptation. Therefore, Rad52 might act as a negative regulator of adaptation by associating with ssDNA and monitoring the extent of DNA damage and the ability of Rad52 to antagonize adaptation depends on Rad51. Separately, the rdh54Δ adaptation defect is not restored by deletion of RAD51. Instead, Rdh54-mediated adaptation appears to rely on Rad9 and Rfa1. rad9Δ and rfa1-t11 mutations restore adaptation in rdh54Δ. The rfa1-t11 allele is replication proficient but HR impaired. The interpretation of these results is complicated by the fact that rfa1-t11 cells do not activate Mec1-dependent checkpoint signaling and rad9Δ cells are severely defective in Rad53 activation in response to DSBs. One of the most puzzling observations in Lee et al (2003) was the synthetic adaptation defect of rfa1-t11 rad52Δ
double mutants, yet neither mutation alone is adaptation-defective. For adaptation, it is still unclear what structures are being monitored, although ssDNA is a likely candidate.

Checkpoint signaling and cell cycle machinery are also targeted during adaptation (Figure 1.5.2). The phosphatases PP2A-type Ptc2 and Ptc3 interact with Rad53 through FHA domains to dephosphorylate and deactivate Rad53 (Leroy et al., 2003). The \textit{cdc5-ad} (adaptation-dead) allele of the polo-like kinase Cdc5 impairs Rad53 inactivation (Pellicioli et al., 2001). Furthermore, Cdc5 over-expression leads to premature checkpoint down-regulation with little effect on the early stages of checkpoint activation such as Ddc1 and Ddc2 foci assembly and Rad53-Rad9 interaction (Vidanes et al., 2010). Cdc5 interacts with Rad53, suggesting that Cdc5 might act at the level of Rad53 dephosphorylation. It is not clear how Cdc5 is inhibited early in the checkpoint; however, human Plk1 is a target of the DNA damage checkpoint (Smits et al., 2000; van Vugt et al., 2001), raising the possibility of ATM/ATR mechanism of suppression. The mechanisms that lift repression on the inhibitor activities of Ptc2/3 and Cdc5 are still not fully understood.

\textit{Recovery from the DNA damage checkpoint}

Protein phosphorylation is an important post-translational modification in amplifying the DNA damage checkpoint (Figure 1.5.2). \textit{PPH3}, \textit{PTC2}, and \textit{PTC3} have been identified as the principle protein phosphatases that reverse this modification to promote recovery (Leroy et al., 2003; Keogh et al., 2006; Kim et al., 2011). One of the earliest events following formation of a DSB is the phosphorylation of H2A to γH2A by Tel1 and Mec1 (Redon et al., 2003; Shroff et al., 2004). Conversely, reversal of H2A
phosphorylation is correlated with checkpoint recovery in yeast and mammalian cells (Keogh et al., 2006; Nakada et al., 2008). The yeast PP2A-type protein phosphatase Pph3, in a complex with its regulatory subunits Psy2 and Psy4, targets γH2A for dephosphorylation and deletion of PPH3 results in delayed Rad53 inactivation and cell cycle resumption after repair of a DSB (Keogh et al., 2006). Importantly, pph3Δ did not affect repair of DSBs and a non-phosphorylatable mutant of H2A, h2a-S129A, rescued the delayed recovery phenotype. The observation that γH2A influences checkpoint maintenance contrasted earlier studies that had implicated the histone modification solely in DNA repair (Downs et al., 2000; Redon et al., 2003). Precisely how γH2A enables prolonged checkpoint activity is not understood; however, the large chromatin domain of γH2A might play a role in maintaining certain checkpoint signaling proteins on DNA.

ChIP and immunofluorescence analysis of γH2A indicated that Pph3 dephosphorylates displaced γH2A and the histone eviction from DNA correlates with the initiation of new DNA synthesis after strand invasion (Keogh et al., 2006). DNA-associated γH2A might therefore act as a signal for incomplete repair, prolonging checkpoint arrest until DNA repair displaces it for dephosphorylation.

The Ptc2 and Ptc3 phosphatases have been implicated in dephosphorylating Rad53 following DNA repair (Leroy et al., 2003). Pph3 has also been implicated in Rad53 dephosphorylation following MMS damage (Travesa et al., 2008). Given the large number of Mec1/Tel1 targets that are known (Matsuoka et al., 2007), very little is known about how dephosphorylation is regulated and the physiological consequences of uncoordinated phosphate removal.
DNA damage and repair is a major disruption to the chromatin environment of a chromosome (Figure 1.5.2). The histone chaperone Asf1 is required for chromatin reassembly following DNA repair, a process that is required for checkpoint recovery (Chen et al., 2008). Asf1 is important for chromatin reassembly after DNA replication, transcription and repair (Tyler et al., 1999) and physically interacts with Rad53 (Emili et al., 2001; Hu et al., 2001a). *asf1*Δ mutants are severely sensitive to DNA damaging agents and cannot resume dividing following DNA repair. The recovery function of Asf1 depends on its ability to promote Rtt109 acetylation of histone H3 on lysine 56. The H3-K56Q amino acid substitution mimics acetylation and bypasses the requirement for Asf1 in recovery. Taken together, H3-K56 acetylation might act as a signal for the completion of repair. While it is not clear how the cell interprets the acetylation signal, it is apparent that chromatin structure plays an important role in influencing the progression of the checkpoint.

The antirecombinase helicase *SRS2* is also required for checkpoint recovery (Vaze et al., 2002). Vaze et al investigated the role of Srs2 in recovery with systems where the HO cut site is ectopically inserted into the *LEU2* locus. A DSB is repaired by SSA or BIR when the DSB ends pair with a partial *leu2* duplication (*u2*) inserted on the same chromosome at different distances away from *leu2*-cs (Figure 1.5.1, C). *srs2Δ* mutants failed to resume cell cycling following DSB induction despite completion of repair SSA or BIR. The recovery impairment depended on the activation of the checkpoint, as *mec1Δ* mutants and strains with a small distance between *leu2*-cs and *u2* that repaired the DSB before checkpoint activation did not arrest irreversibly. In addition, Srs2 is required for recovery following DSB repair by gene conversion, although *srs2Δ* cells are somewhat
defective in completing gene conversion. Importantly, the helicase-dead allele of *SRS2* is also recovery-defective, indicating that its translocating activity is involved in checkpoint recovery. Finally, the recovery defect of *srs2Δ* can be rescued by *rad51Δ*. Taken together, this suggests that the recovery role of Srs2 is tied to its antirecombinogenic activity; however, how this is accomplished remained enigmatic. Srs2 is also required for adaptation, and its role in the process is also poorly understood.

The reestablishment of cell cycle homeostasis during recovery includes reversal of post-translational modifications imparted by the checkpoint and the restoration of the chromatin environment proximal to the site of DNA damage. It is not yet clear if recovery is an active process that requires the inhibition or degradation of recovery inhibitors or a passive process where cell cycle homeostasis regains dominance once the DNA damage signal is eliminated. In the case of Asf1-mediated recovery, it is not known how H3-K56 acetylation is recognized. If eventually characterized, it will be a possible candidate to determine if the histone modification triggers a cellular program similar to a signal transduction cascade to terminate checkpoint signaling. Pph3 dephosphorylation of γH2A appears to be at least partially spatially regulated, as γH2A is displaced from chromatin by DNA repair before it is dephosphorylated (Keogh et al., 2006). Other mechanisms that regulate Pph3 activity in checkpoint recovery have not been explored. Srs2-mediated recovery remains enigmatic, although the helicase activity has been proposed to remove checkpoint proteins from chromatin (Vaze et al., 2002). Thus, our knowledge of checkpoint recovery remains quite limited.
1.6 Summary and Thesis Rationale

The DNA damage response is a highly integrated cellular network that reacts to DNA damage. The detection and subsequent activation of signaling is required for the accurate and efficient repair of DNA damage. The redistribution of checkpoint proteins into subnuclear foci is an essential step in executing the DNA damage response. Many genetic and molecular factors have been implicated in focus assembly; however, the specific components of foci and their chromatin architecture are still being investigated. Therefore, my graduate work addresses questions in DNA damage protein focus assembly, architecture and disassembly. In the following chapters, I address these biological questions.

Extensive research has revealed many of the genetic and molecular requirements for focus assembly. The generation of ssDNA greatly affects DNA damage protein accumulation on chromatin and into foci. Post-translational modifications—in particular, phosphorylation—have a profound impact on the amplification of the checkpoint signal by creating DNA damage inducible protein-protein interaction interfaces. The role of another post-translation modification—ubiquitin—in regulating genome stability has received much attention in recent years. Like phosphopeptides, ubiquitin conjugates form a binding surface for recognition by other proteins (Harper and Schulman, 2006). In mammals, ubiquitin modifications imparted by the RNF8 and RNF168 ubiquitin ligases are critical for the assembly of foci in the ATM signaling pathway (Huen et al., 2007; Kolas et al., 2007; Wang and Elledge, 2007; Stewart et al., 2009). A similar role of ubiquitin in DSB signaling has not been identified in yeast. However, in the case of DNA damage tolerance, ubiquitin modifications on PCNA recruit translesion polymerases for
post-replication repair mechanisms and Rad6-Rad18 plays an evolutionarily conserved role (Andersen et al., 2008). Given the structural similarity within the β-clamp family of protein complexes that include PCNA and the 9-1-1 checkpoint clamp (Majka and Burgers, 2004; Davies et al., 2010), 9-1-1 might also be modified by ubiquitin in a similar manner to PCNA. In Chapter II, I investigate a possible role of Rad6-Rad18-dependent ubiquitin modification on a putative conserved lysine residue of Rad17 in 9-1-1 assembly at DSBs.

The checkpoint complexes Mec1-Ddc2 and 9-1-1 must colocalize in foci to activate the checkpoint (Melo et al., 2001). In contrast, very little is known about the biological impact of foci disassembly on checkpoint signaling. The lack of known genes involved in checkpoint recovery limited the understanding of the process. The discoveries of the checkpoint recovery mutants pph3Δ and srs2Δ provided new genetic systems to study the genetic and molecular basis of checkpoint recovery (Vaze et al., 2002; Keogh et al., 2006). The helicase activity of Srs2 was predicted to remove checkpoint proteins from repaired chromatin to allow checkpoint recovery by Vaze et al (2002). Similarly, γH2A might maintain checkpoint proteins in on chromatin where they continue to actively signal for checkpoint arrest. Indeed, if localization of checkpoint proteins at DNA damage is essential for checkpoint activation (Melo et al., 2001; Bonilla et al., 2008), removing the same proteins after repair might be an important step during recovery. To address this hypothesis, it is possible to examine chromatin enrichment of proteins by chromatin immunoprecipitation (ChIP) methods. However, chromatin enrichment determined by ChIP is restricted to population averages in a culture of cells. Fluorescence microscopy of checkpoint proteins provides an alternative approach to
examine checkpoint dynamics at the single-cell level. I utilize both approaches to examine checkpoint protein retention in checkpoint recovery mutants.
Chapter II

Phenotypic characterization of a 9-1-1 mutant, rad17-K197R

2.0 Abstract

The relocalization of checkpoint proteins into distinct sub-nuclear foci around DNA damage is an evolutionarily conserved phenomenon from yeast to humans and is an essential step for the activation and amplification of the DNA damage checkpoint. The 9-1-1 (Ddc1-Rad17-Mec3) checkpoint clamp structurally resembles proliferating nuclear cell antigen (PCNA) which is post-translationally modified by ubiquitin and SUMO. Rad17-K197 is predicted to be the conserved residue of Pol30-K164 which is modified by Rad6-Rad18 during post-replication repair. Here, I characterize the rad17-K197R mutation that disrupts a putative Rad6-Rad18 ubiquitin receptor site. I find that the mutation greatly impairs Ddc1-GFP focus formation and recruitment after induction of a single DSB. The rad17-K197R and rad18Δ mutants are also slightly defective in Rad53 phosphorylation and mildly sensitive to the DSB-inducing agent bleomycin. These findings implicate a role for Rad18 in DNA damage signaling and repair separate from its well-established role in post replication repair and PCNA modification.
2.1 Introduction

In response to DNA damage, DNA damage proteins can undergo major redistribution to sites of DNA damage seen as punctate subnuclear foci. The relocalization of checkpoint and DNA repair proteins into foci is part of the activation and amplification of checkpoint signaling and the execution of DNA repair (Lisby et al., 2004). Specifically, when I began my graduate studies, two checkpoint complexes were known to colocalize at DSBs: Ddc2-Mec1 and the 9-1-1 checkpoint clamp consisting of Ddc1-Rad17-Mec3 (Melo et al., 2001). The Mec1-Ddc2 complex is directed to DSBs by a direct interaction between Ddc2 and Rfa1 (Rouse and Jackson, 2002b). 9-1-1 is loaded at ssDNA/dsDNA junctions by the alternate clamp loading complex Rad24-Rfc2-5 (Melo and Toczyski, 2002; Rouse and Jackson, 2002a; Majka et al., 2006a). The colocalization of the two complexes is essential to activate the Mec1 kinase (Navadgi-Patil and Burgers, 2009b).

To identify novel transcriptional regulators in the DNA damage response, Fu and colleagues performed a screen for mutations that affected transcription of the LacZ gene fused to the promoter of \textit{DDI1} or \textit{MAG1}, two DNA damage-inducible genes (Liu and Xiao, 1997; Fu et al., 2008). \textit{rad6}\Delta and \textit{rad18}\Delta were the only mutations that were identified to prevent LacZ expression after MMS exposure. Contrary to the canonical DNA damage tolerance pathway of Rad6 and Rad18, \textit{DDI1} and \textit{MAG1} expression occurred in a manner independent of PCNA ubiquitylation, as \textit{pol30-K164R} mutants are still proficient in inducing \textit{DDI1} and \textit{MAG1} transcription. Instead, a \textit{rad24}\Delta mutant reduces \textit{MAG1} transcription to the same level as \textit{rad18}\Delta, suggesting that Rad6-Rad18 works in the \textit{RAD24} pathway which loads the 9-1-1 clamp on to DSBs.
PCNA and 9-1-1 belong to a structurally conserved β-clamp family of DNA sliding clamps (Majka and Burgers, 2004). Since post-translational modifications are so crucial for PCNA function in DNA damage tolerance and 9-1-1 bears striking structural resemblance to PCNA (Andersen et al., 2008), it raises the possibility that 9-1-1 is modified in a similar manner. In collaboration with Wei Xiao, we presented a model where Rad17 is a substrate of the Rad6-Rad18 ubiquitin ligase (Fu et al., 2008). Figures 2.2.1A and Figure 2.3.3B appear in Fu et al (2008). Here, I further examine that model with additional genetic experiments. I characterize the phenotype of the mutation rad17-K197R which abolishes the putative ubiquitin receptor residue. The results lead me to re-examine the model that Rad17 is a Rad6-Rad18 substrate.

2.2 Rad17 is required for Ddc1-GFP focus formation and checkpoint activation

In response to DSBs, Ddc1 forms subnuclear foci detectable by fluorescence microscopy (Melo et al., 2001; Lisby et al., 2004). A strain with the HO consensus sequence ectopically inserted into the ADH4 locus and the endogenous MAT loci deleted was engineered to express Ddc1-GFP (yJM20, Figure 1.5.1, B) (Melo et al., 2001). The HO endonuclease is under the inducible galactose promoter and upon addition of galactose, cells receive a DSB that cannot be repaired by HR. Ddc1-GFP foci become detectable soon after galactose induction and the checkpoint is activated, as observed by Rad53 phosphorylation (Figure 2.2.1). To disrupt the clamp, I deleted RAD17 in yJM20 and induced a DSB with galactose. rad17Δ cells failed to form Ddc1-GFP foci or
Figure 2.2.1. Ddc1-GFP focus formation and checkpoint activation. (A) Micrographs of yJM20 and isogenic rad17Δ cells 8 hours following induction of a single DSB. DIC: Differential interference contrast. (B) Rad53 immunoblotting of protein extracts derived from cells grown in galactose for the indicated times for the same strains as in (A).
Figure 2.2.1
phosphorylate Rad53 (Figure 2.2.1). Therefore, Rad17 and an intact ring structure of the 9-1-1 clamp are required for Ddc1 focus formation at a DSB.

2.3 Rad17-K197 Ddc1-GFP focus formation and localization to damaged chromatin

PCNA and 9-1-1 are structurally similar complexes (Venclovas and Thelen, 2000; Moldovan et al., 2007). Recently, the crystal structure of the human 9-1-1 clamp was solved and indeed, it bears striking resemblance to PCNA (Dore et al., 2009). While there is little primary sequence homology conserved amongst any of the β clamps, the 3D structures are nearly entirely super-imposable (Krishna et al., 1994; Dore et al., 2009). Hypothetical threading of *S. cerevisiae* Rad17 amino acid sequence into the human RAD1 3-dimensional structure predicts a high degree of structural conservation in the yeast 9-1-1 (Figure 2.3.1, A). Although sequence similarity between Pol30 and Rad17 is limited, a sequence alignment between Pol30 and Rad17 revealed that Rad17-K197 lies in a similar motif to Pol30-K164, a residue that is modified by the Rad6-Rad18 ubiquitin ligase complex (Hoege et al., 2002; Fu et al., 2008) (Figure 2.3.1, B). Notably, Pol30 alignments against Mec3 and Ddc1 did not reveal any motifs that aligned to Pol30-K164 (Figure 2.3.1, B). Rad17-K197 is predicted to protrude outward from 9-1-1 at the interface between Ddc1 and Rad17 (Figure 2.3.1, A). Therefore, Rad17-K197 is possibly available for modification. Furthermore, mutating residues in the interface would be a logical approach to disrupt the integrity of the checkpoint clamp. The interface is predicted to be opened and closed by the Rad24-Rfc2-5 clamp loader for 9-1-1 loading on to DNA (Rauen et al., 2000; Majka and Burgers, 2004). Another residue of interest is
Figure 2.3.1. Structure and sequence alignment of the 9-1-1 complex. (A) *S. cerevisiae* 3-dimensional modelling on H. sapiens Rad1. ScRad17 (pink) is superimposed over hRad1 (grey). The overall structure of human 9-1-1 is shown in grey. ScRad17-K197 is shown in green. (B) Alignment of Pol30 with Rad17, Ddc1, and Mec1. The putative conserved lysine motif between Pol30 and Rad17 is boxed.
Figure 2.3.1
Rad17-S198, which lies in a putative SQ motif, a target of PI3K kinases Mec1 and Tel1 (Kim et al., 1999).

To test if Rad17-K197 plays a role in the DNA damage checkpoint, the residue was mutated to an arginine to abolish putative ubiquitylation (rad17-K197R). In parallel, since PCNA is modified by Rad6-Rad18, a rad18Δ mutant was also tested to determine if Rad17-K197 is a substrate of the ubiquitin ligase complex. Cells were spotted on solid media containing different concentrations of MMS to determine DNA damage sensitivity (Figure 2.3.2). Compared to the wild type, rad17-K197R cells were only mildly sensitive to 0.05% MMS. rad17Δ cells were more sensitive to MMS compared to the point mutation. rad18Δ cells were severely sensitive to all concentrations of MMS. Finally, the rad18Δ rad17-K197R double mutation appeared to be as sensitive to MMS as the rad18Δ mutant. Therefore, the Rad17-K197 residue plays a very minor role in MMS tolerance.

MMS creates damage that can be bypassed by the post-replication repair pathway during DNA replication, which is why rad18Δ cells are so sensitive to the drug (Prakash, 1981; Moldovan et al., 2007). MMS damage stalls replication fork progression which can collapse into DSBs; however, the DNA damage created by MMS is heterogeneous not necessarily limited to only DSBs (Lundin et al., 2005). In order to gain further insight into the function the Rad17-K197, the rad17-K197R allele was engineered into the yJM20 strain (Figure 2.3.3, A) where the response to a single DSB could be monitored. A single DSB was induced with galactose and Ddc1-GFP focus formation was observed (Figure 2.3.3, B). Prior to DSB induction, 3.3% of wild type cells and 1.9% of rad17-K197R cells had detectable Ddc1-GFP foci, respectively. After 8 hours, 37.0% of wild type cells had Ddc1-GFP focus and 7.0% of rad17-K197R cells had foci (Figure 2.3.3,
Figure 2.3.2. MMS sensitivity of 9-1-1 and RAD18 mutants. Strains isogenic to yJM20 of the indicated genotypes were spotted in serial dilution onto XY plates containing 2% glucose and the indicated concentrations of MMS.
Figure 2.3.2
B). Therefore, the point mutation was significantly impaired in its ability to form Ddc1-GFP foci. The rad17-K197R impairment in Ddc1-GFP focus formation could be the result of either poor accumulation or inefficient retention of 9-1-1. To try to distinguish from the two possibilities, I performed a time course experiment to examine Ddc1-GFP focus formation over 24 hours (Figure 2.3.3, C). rad17-K197R cells accumulated foci to a much lesser extent than wild type cells. Taken together, I concluded that the rad17-K197R mutation prevents efficient 9-1-1 loading on to DSBs.

If Rad6-Rad18 is indeed the ubiquitin ligase complex that modifies Rad17-K197, then deleting RAD18 should result in similar phenotypes to rad17-K179R mutants. When RAD18 was deleted in yJM20 and Ddc1-GFP focus formation was observed 8 hours following induction of a DSB, I found that 28.6% of rad18Δ cells had a detectable Ddc1-GFP, significantly less than the wild type, but not as severe as rad17-K197R (Figure 2.3.3, B). The rad17-K197R mutation was combined with rad18Δ and Ddc1-GFP focus formation was also assessed. rad17-K197R rad18Δ cells resembled that of the rad17-K197R single mutant with 9.9% of cells having a Ddc1-GFP focus 8 hours after galactose induction (Figure 2.3.3, B). The less severe Ddc1-GFP focus formation phenotype of rad18Δ cells suggests that either Rad17-K197 is not a substrate of Rad18 or there are redundant ubiquitin ligases that ubiquitylate Rad17.

To determine if Rad17-S198 is also a residue that is required for Ddc1-GFP focus formation, rad17-S198A was expressed from a centromeric plasmid in yJM20 rad17Δ cells and I observed Ddc1-GFP focus formation following DSB induction. The serine residue was additionally interesting because it resides in a putative PI3 kinase SQ motif, a classical phosphorylation motif for Mec1 and Tel1 (Kim et al., 1999). Analysis of Ddc1-
**Figure 2.3.3.** Ddc1-GFP focus formation in 9-1-1 mutants. (A) Schematic of the yJM20 strain. (B) Strains isogenic to yJM20 were scored for Ddc1-GFP foci after 0 and 8 h growth in YM-1 containing galactose. Error bars are 95% C.I. (C) Ddc1-GFP focus formation in yJM20 and an isogenic strain containing *rad17-K197R*. Representative of 3 experiments. Approximately 100 cells were scored per time point for each strain.
Figure 2.3.3
GFP focus formation indicated that rad17-S198A had no effect on focus formation relative to expression of wild type RAD17 from the same plasmid (Figure 2.3.4).

To more directly test 9-1-1 localization at a DSB in rad17-K197R cells, I performed chromatin immunoprecipitation experiments to determine Ddc1-GFP chromatin enrichment. To detect 9-1-1, Ddc1-GFP was immunoprecipitated with an anti-GFP antibody from yJM20 cells that were either grown in glucose or had been induced for 8 hours in galactose. Amplicons were designed at 1 and 32 kb away from the DSB (Figure 2.3.5, A). Wild type, rad17-K197R and rad1Δ cells were analyzed for Ddc1-GFP enrichment. Prior to DSB induction, Ddc1-GFP was not enriched at the 1 kb and 32 kb amplicons relative to the control (Figure 2.3.5, B). 8 hours following DSB induction, Ddc1-GFP enrichment was detected at the 0 kb and 32 kb amplicons at similar levels in the wild type strain (15- and 12-fold, respectively). Deleting RAD17 significantly diminished, but did not completely abolish, Ddc1-GFP enrichment (5- and 2.5-fold at the 1 kb and 32 kb amplicons, respectively). Ddc1-GFP enrichment in the rad17-K197R strain was found to be significantly less than the wild type strain only at the 32 kb amplicon (7.5-fold enrichment) but not the 1 kb amplicon (10-fold enrichment). 8 hours following the initial formation of the DSB, the progression of resection is expected to have progressed 32 kb away assuming a 4 kb/hr resection rate (Zhu et al., 2008). This suggests that rad17-K197R affects the initial loading of 9-1-1 and not the subsequent maintenance of the clamp on DNA, consistent with the foci time course data (Figure 2.3.3, B).

Resection of DSB ends generates ssDNA with 3’ ends (Mimitou and Symington, 2009a). The ssDNA greatly enhances checkpoint activation and homologous
Figure 2.3.4. Ddc1-GFP focus formation in *rad17-S129A* cells. yJM20 *rad17Δ* cells carrying either yCPT*<RAD17>* or yCPT*<rad17-S198A>* were grown in galactose for 8 hours to induce a DSB and then fixed and imaged for Ddc1-GFP foci. Data are presented as mean ± 95% C.I.; n = 3.
Figure 2.3.5. Chromatin enrichment of Ddc1-GFP at a DSB. (A) Schematic of ChrVII in yJM20. Amplicons were designed 1 and 32 kb away from the HO cleave site in ADH4. Diagram is not to scale. (B) yJM20 and derivative strains were grown in media containing galactose to induce HO expression. Chromatin derived from the indicated time points were subjected to ChIP with an anti-GFP antibody followed by qPCR. The x-axis represents the relative distance from the HO cut site in kilobases (kb). Enrichment of Ddc1-GFP was determined with the ΔΔCt method using TSC11 as a reference locus. Data are presented as mean ± SEM; n = 3. (*, p = 0.014; †, p < 0.0001)
Figure 2.3.5
recombination (Symington, 2002; Zou and Elledge, 2003). Compared to the 5’ strand, which is rapidly degraded by nucleases, the 3’ strand is assumed to be relatively stable while it is used as a substrate for homology search for recombination repair; however, the dynamics of ssDNA during homology search have been difficult to observe. To examine the stability of 3’ ssDNA, I determined relative DNA levels of amplicons 1 kb and 32 kb away from a DSB (Figure 2.3.6). I found that prior to DSB induction, DNA content at both of the tested loci were equal to the control locus on another chromosome. However, 8 hours following DSB induction, there was significant depletion of the 1 kb amplicon, but not the 32 kb amplicon, indicating that there is substantial degradation of the 3’ ssDNA strand proximal to the DSB. In light of the observation that the 3’ strand is also degraded, models of homologous recombination that assume ssDNA is stable (Jain et al., 2009) must be re-examined.

2.4 rad17-K197R and rad18Δ moderately impair checkpoint signaling at DSBs

The poor accumulation of Ddc1 at DSBs in rad17-K197R cells is expected to affect checkpoint activation. To measure checkpoint activation, I examined Rad53 phosphorylation after DSB induction and found that rad17-K197R cells are slightly impaired in their ability to fully phosphorylate Rad53 (Figure 2.4.1). rad18Δ cells are also slightly checkpoint defective, displaying the same reduction in Rad53 phosphorylation as rad17-K197R cells following DSB induction (Figure 2.4.1). Unexpectedly, when rad18Δ and rad17-K197R mutations were combined, Rad53 phosphorylation was more impaired than the single mutations alone.
Figure 2.3.6. Degradation of 3’ ssDNA. Relative DNA levels were determined for loci 1 kb and 32 kb away from a DSB induced near the \textit{ADH4} locus in yJM20. Analysis was performed on DNA derived from cells harvested at the indicated time points. Relative DNA content of each locus was compared to a control locus, \textit{TSC11}, on another chromosome. Data is presented as fold depletion relative to TSC11 (n = 3; mean ± SEM).
Figure 2.4.1. Checkpoint activation in *rad17* and *rad18Δ* mutants. Rad53 was immunoblotted on protein extracts derived from yJM20 derivatives grown in galactose to induce HO expression.
To test the effect of *rad17-K197R* and *rad18Δ* mutations on DSB repair, cells were spotted onto media containing the DSB-inducing agent bleomycin (Figure 2.4.2). *rad17-K197R* cells were not any more sensitive to 10.0 µg/mL bleomycin than wild type cells. *rad18Δ* cells showed a greater sensitivity to the same concentration of bleomycin compared to *rad17-K197R*. The *rad17-K197R* *rad18Δ* double mutant showed the same sensitivity to bleomycin as the *rad18Δ* single mutant. Therefore, Rad18 appears to be involved in bleomycin tolerance whereas the Rad17-K197 residue is dispensible.

### 2.5 Discussion

*Rad17-K197R affects 9-1-1 loading on to DSBs*

The 9-1-1 clamp structurally resembles PCNA, another DNA clamp that is modified by the Rad6-Rad18 ubiquitin ligase complex (Hoege et al., 2002; Majka and Burgers, 2004). Alignment of the primary sequences of Pol30 and Rad17 suggested that the K197 residue of Rad17 might be homologous to the K164 residue of Pol30 which is modified by Rad6-Rad18 (Fu et al., 2008). Threading of the ScRad17 protein sequence into the predicted 3D structure of human Rad1 suggested that ScRad17-K197 was positioned near the interface between ScDde1 and ScRad17 (Figure 2.3.1), a position that has been proposed to be the point where the clamp is opened for loading onto DNA (Rauen et al., 2000; Majka and Burgers, 2004). The K197 residue is in a position where it is available to be modified and ubiquitylated isoforms have been detected *in vivo* and generated *in vitro* (Fu et al., 2008; Davies et al., 2010).
Figure 2.4.2. Bleomycin sensitivity of rad17 and rad18Δ mutants. Strains isogenic to yJM20 with the indicated mutations were spotted in serial dilution on rich media containing 0, 5.0 or 10.0 µg/mL bleomycin.
Rad17 ubiquitylation has been detected independently by two different groups (Fu et al., 2008; Davies et al., 2010); however, the Rad18 substrate model and a checkpoint function for Rad17 modification are controversial (Davies et al., 2010). An alternative hypothesis for 9-1-1 ubiquitylation offered by Davies et al (2010) proposed that the ubiquitin modification occurred independently of DNA damage and was a signal for proteolytic degradation, not DNA damage signaling (Figure 2.5.1, B).

To address this outstanding controversy, I examined the rad17-K197R mutant for checkpoint defects in response to a single DSB in the yJM20 background strain. I found that Ddc1-GFP focus formation is greatly impaired in rad17-K197R cells in response to a single DSB, but not as severe as a rad17Δ mutant (Figure 2.3.3). rad18Δ cells also had a mild defect in accumulating Ddc1-GFP foci, but was milder than the rad17-K197R mutation (Figure 2.3.3). If Rad17 is a substrate of Rad6-Rad18, the genetic evidence that rad17-K197R has a more severe Ddc1-GFP focus formation impairment than rad18Δ suggests that Rad18 is not the only E3 ligase of Rad17 (Figure 2.2.1). Furthermore, the fluorescence microscopy experiments suggested that rad17-K197R is important for the assembly of the 9-1-1 checkpoint clamp at a DSB.

However, when I examined 9-1-1 chromatin association around a DSB by chromatin immunoprecipitation analysis of Ddc1-GFP, I found that Ddc1-GFP enrichment was locus specific. 8 hours after induction of a DSB, Ddc1-GFP enrichment was significantly less in the rad17-K197R mutant than wild type at an amplicon 32 kb away from the DSB, but not at an amplicon 1 kb away (Figure 2.3.5). Unexpectedly, Ddc1-GFP enrichment was also detected at 1 and 32 kb from the DSB in rad17Δ cells, albeit significantly diminished compared to the wild type. This indicates that an intact
ring structure is required for robust accumulation of 9-1-1 near a DSB while Ddc1 can accumulate at levels below the threshold of detection by fluorescence microscopy even if the 9-1-1 ring structure is incomplete (Figure 2.2.1, A). Resection proceeds at an estimated rate of 4 kb/hr (Zhu et al., 2008). Accordingly, 8 hours after formation of a DSB, the junction between ssDNA and dsDNA is expected to be near the 32 kb amplicon. There is consistently greater enrichment of Ddc1-GFP proximal to the DSB compared to the more distal locus, suggesting that the 9-1-1 clamp is loaded and then slides toward the DSB where it can accumulate. It is not clear what the purpose of clamp sliding is for the 9-1-1 clamp, if it has one at all. The ChIP data suggests that rad17-K197R affects the loading of 9-1-1 on to damage chromatin but not the maintenance of 9-1-1 near the DSB. I observed a slower rate of Ddc1-GFP focus accumulation in rad17-K197R cells compared to wild type cells. If rad17-K197R impairs 9-1-1 loading, the model may explain the apparent discrepancy between the fluorescence microscopy and ChIP experiments. Furthermore, ChIP analysis provides a more detailed observation of protein localization along the damaged chromosome compared to examination of foci by fluorescence microscopy. If Rad17-K197 is required for efficient 9-1-1 loading, then perhaps rad17-K197R is disrupting the stability or dynamics of the Ddc1-Rad17 interface. Indeed, this is the interface that is predicted to be opened and closed by Rad24 when the clamp is loaded. Further examination of the Ddc1-Rad17 interaction in rad17-K197R might provide important insight into the mechanism of 9-1-1 clamp loading at DSBs.
The  rad17-K197R allele affects checkpoint signaling but not DNA damage tolerance

Since the 9-1-1 checkpoint clamp is required to activate the Mec1 kinase (Navadgi-Patil and Burgers, 2009b), poor loading of 9-1-1 might have a deleterious effect on checkpoint signaling. I found that Rad53 phosphorylation is slightly impeded in rad17-K197R cells compared to wild type cells following induction of a DSB (Figure 2.4.1). rad18Δ also resulted in a similar Rad53 phosphorylation defect (Figure 2.4.1). Finally, the combined rad17-K197R rad18Δ mutation has a much more severe Rad53 phosphorylation defect. Taken together, the observations suggest that Rad18 and 9-1-1 work in cooperative pathways to promote Rad53 phosphorylation.

Checkpoint signaling defects can result in DNA damage sensitivity. rad17-K197R cells are mildly sensitive to high concentrations of MMS whereas rad18Δ cells are severely sensitive to even low concentrations of MMS (Figure 2.3.2). Rad18 modifies PCNA to promote DNA damage tolerance by post-replication repair, a central pathway in handling MMS-induced DNA damage (Hoege et al., 2002). The high MMS tolerance of rad17-K197R cells indicate that 9-1-1 is likely not participating in the post-replication repair pathway. rad17-K197R cells were not sensitive to the DSB-inducing agent bleomycin and were indistinguishable to the wild type in a spotting assay (Figure 2.4.2). Surprisingly, rad18Δ cells were much more sensitive to bleomycin than rad17Δ cells. The reason for this is not immediately clear, however it indicates that Rad18 is also important for DSB tolerance. Recent evidence demonstrated that Rad18 localizes to DSBs and may be involved in DSB repair (Hirano et al., 2009; Watanabe et al., 2009). Precisely how Rad18 modulates Rad53 phosphorylation and coordinates DSB repair remains an open question.
Figure 2.5.1. Models for Rad18 involvement in the DSB response. (A) Rad17 is a substrate of Rad6-Rad18 in response to DNA damage. Ubiquitylated Rad17 participates in the activation of the checkpoint (Fu et al, 2008) (B) Rad17 ubiquitylation is non-specific and occurs independently of DNA damage (Davies et al, 2010). (C) An unknown E3 ubiquitin ligase (Ligase X) ubiquitylates Rad17. Ub-Rad17 is bound by Rad18 and cooperatively activates the checkpoint.
Figure 2.5.1
A possible ligase-independent checkpoint role for Rad18

In Fu et al, we presented the Rad18 substrate model where Rad18 was proposed to ubiquitylate Rad17 (Figure 2.5.1, A). Two key pieces of evidence generated by our collaborators were consistent with the Rad18 substrate model: detection of Rad17 ubiquitin modification in cells over-expressing $RAD18$ and a Rad18-Rad17 interaction in a yeast-two-hybrid experiment. If Rad18 does indeed modify 9-1-1, it represents a novel substrate for the ubiquitin ligase. Previously, PCNA was the only known substrate of Rad6-Rad18 (Hoege et al., 2002).

Rad18 interacts with Rad17 in a yeast-2-hybrid assay (Fu et al., 2008) although the nature of the interaction is unknown. The human ortholog of Rad18 is known to bind ubiquitin both covalently and non-covalently via its RING domain (Notenboom et al., 2007), raising the possibility that yeast Rad18 could interact with ubiquitin-conjugated Rad17. This “Rad18 recruitment model” is also consistent with the observation that $RAD18$ over-expression increases the ubiquitin-modified Rad17 high-mobility isotype observed in anti-Rad17 immunoblots (Fu et al., 2008). Rad18 may directly modify Rad17, but it may also protect Rad17 from deubiquitylation or recognition by other ubiquitin ligases that may polyubiquitylate the peptide for proteolytic degradation. It raises possibilities that a Rad18-Rad17 interaction may be important for a robust checkpoint response, or that Rad17-K197-ub participates in Rad18 recruitment to DSBs independently of the Rad18 SAP domain that is required for DNA interaction (Notenboom et al., 2007).

Evidence is mounting to assign a DSB repair role for Rad18. Recent work in budding yeast found that Rad18 associates with DSBs (Hirano et al., 2009). Experiments
performed in mouse and chicken cells revealed, that in those animals, RAD18 also localizes to DSBs as foci after IR in all phases of the cell cycle (Watanabe et al., 2009). In Watanabe et al (2009), they showed that RAD18 is required for cell survival following IR treatment, suggesting that RAD18 participates in DNA repair. Specifically, RAD18 is recruited to DSBs through an interaction with 53BP1. In vitro experiments suggest that RAD18 monoubiquitylates 53BP1. In vivo, the ubiquitylated residue of 53BP1 and the RAD18 RING finger domain are important for RAD18-53BP1 interaction. While the RAD18-53BP1 interaction appears to be specific for G1, RAD18 also forms foci in the rest of the cell cycle. The specific mechanism of recruitment outside of G1 is unclear; nonetheless, it is becoming clear that RAD18 has roles in DNA repair outside of post-replication repair.

The 3' strand is degraded

DSB end resection proceeds in the 5’→3’ direction and is mediated by the MRX complex, and Sae2, Dna2, and Exo1 nuclease (Mimitou and Symington, 2009a). RPA nucleofilaments are a potent activator of the DNA damage checkpoint (Zou and Elledge, 2003). The remaining 3’ ssDNA can be primed for repair by HR with Rad51 and Rad52 (Symington, 2002). In many experimental systems such as the yJM20 strain utilized in this chapter, there is no suitable homologous donor in the genome; therefore, the DSB persists unrepaired. In models of resection, checkpoint activation, and homologous recombination, the 3’ end is assumed to be static and stable. However, qPCR analysis of relative DNA quantities at a locus proximal to a DSB indicated that the 3’ strand 1 kb away from the HO cut site was significantly degraded (Figure 2.3.6). 3’ end degradation
contracts the model of 3’ ssDNA stability suggested by Haber and colleagues (Jain et al., 2009); however, in their experimental system, there is significant homology for DSB repair by recombination. 3’ end degradation might indicate that the 3’ strand is more dynamic during homology search and recombination than previously assumed.

Summary and Concluding Remarks

rad17-K197R mutants were examined and found to be mildly MMS sensitive, impaired in Ddc1-GFP focus assembly on chromatin, and unable to fully phosphorylate Rad53 in response to DSBs. rad18Δ cells were also slightly impaired in Ddc1-GFP focus formation and Rad53 phosphorylation after induction of a DSB. Therefore, I conclude that the K197 residue of Rad17 is playing a previously uncharacterized role to mount a robust checkpoint response. Furthermore, rad18Δ cells were mildly impaired for Ddc1-GFP focus formation, but not to the extreme of the rad17-K197R mutant. The evidence for Rad18-dependent Rad17 ubiquitin modification is not conclusive; however, there may be additional undiscovered ubiquitin ligases that carry out a Rad17 modification. Finally, rad18Δ mutants are sensitive to bleomycin, a DSB-inducing agent, suggesting a previously uncharacterized role in DSB repair and signaling.

2.6 Methods and Materials

Strains and Plasmids. All strains used were derived from yJM20 (D. Toczyski) using standard yeast genetics or high efficiency lithium acetate transformation. Cells were maintained and grown at 30°C unless otherwise indicated. Strains used in this chapter are listed in Table 2.6.1. The rad17-K197R mutation was generated in laboratory of W. Xiao.
A centromeric plasmid containing RAD17 was also a gift from the Xiao lab (yCPT<\textit{RAD17}>). The \textit{rad17-S198A} allele was generated by mutating \textit{RAD17} in yCPT<\textit{RAD17}> with the Stratagene QuikChange kit using the oligos rad17-S198A-f and rad17-S198A-r. The mutation eliminates an EcoRV restriction site. The mutation was confirmed by restriction enzyme digest and sequencing. To generate the \textit{rad17-S198A} strain, the mutated plasmid was transformed into yJM20 \textit{rad17Δ} (DD1838).

**Hypothetical protein threading.** The ScRad17 3D structure was modelled from the predicted human Rad9 3D structure using threading tools available at [http://swissmodel.expasy.org/](http://swissmodel.expasy.org/) (3g65G as the target from [www.pdb.org](http://www.pdb.org) in alignment mode without user intervention). Results were visualized in COOT (Emsley and Cowtan, 2004) and Pymol software ([http://www.pymol.org/](http://www.pymol.org/))

**Fixation and storage of cells for microscopy.** 4% PFA was prepared by dissolving PFA in a solution containing 0.1 M sucrose made basic with 5 M NaOH (14 μL NaOH/12.5mL H2O). Cells were fixed in 4% PFA for 15 minutes then washed, stored, and imaged in a solution containing of 0.1 M potassium phosphate buffer pH 7.5 and 1.2 M sorbitol.

**Microscopy.** Cells stored in 1.2M sorbitol, 0.1M sodium phosphate (pH 7.5) buffer were mounted on Fisherbrand microscope slides and coverslips (12-550A; 12-542B). For GFP, images were captured images at room temperature with a Nikon E600 FN microscope ([http://www.nikonusa.com](http://www.nikonusa.com)) equipped with an ORCA ER2 camera ([http://www.hamamatsu.com](http://www.hamamatsu.com)), Chroma filters (GFP: 41025 PSTN GFP, dichroic Q495LP, batch C37368, excitation = 470 ± 40 nm, emission = 515 ± 30 nm; [http://www.chroma.com/](http://www.chroma.com/)) and a 100X/1.4 oil emersion Nikon Plan Apo objective.
Approximately 100 cells from each time point were scored for foci. Images were processed and deconvolved with Velocity v5.3.0.

**Rad53 phosphorylation assay.** Protein extracts were prepared by mechanical lysis of cells in 20% TCA. Protein extracts were boiled in Laemmli solution and resolved in an 7% polyacrylamide gel (Acrylamide/Bis 37.5:1) by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% milk in TBS-T for at least 1 hour (Carnation Skim Milk Powder). The membrane was incubated with the primary antibody (anti-Rad53) at a dilution of 1:1000 in 1% milk TBS-T for 30-45 minutes. Next, the membrane was washed 3X in TBS-T. The membrane was incubated with the secondary antibody (HRP-conjugated goat anti-rabbit, Jackson ImmunoResearch Laboratories, Inc) at a dilution of 1:10,000 for 30-45 minutes. Following, the membrane was washed 3X in TBS-T. Detection was carried out by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

**Cell viability spotting assay.** 1 mL of a culture with density OD$_{600}$ = 1.0 was spotted (5 µL) on to solid media in 1/5 serial dilutions with increasing concentration of MMS. Plates were incubated at 30°C for 2-3 days prior to imaging.

**Anti-GFP Chromatin Immunoprecipitation.** Chromatin preparation and immunoprecipitation were preformed as described in (Szilard et al., 2010). Cells were harvested at specified time points and fixed in 1% formaldehyde for 30 minutes at room temperature. 250 mM glycine was used to quench the formaldehyde prior to lysis. Lysis was performed by mechanical disruption with glass beads and a lysis buffer containing 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitors (Roche) in a bead beater (FastPrep FP120;
20 seconds at power level 4.5, 8 cycles). The chromatin fraction was purified from the lysate by centrifugation at 1000 x g for 2 minutes. The chromatin was sheared by sonication to generate DNA fragments of 300-500 bp (Misonix 3000; power 6, 15-1 second pulses, 5 cycles) and cleared by spinning in a microcentrifuge for 10 minutes at maximum speed. A small portion of the sheared chromatin fraction was collected to purify total DNA. Immunoprecipitations were performed with a mouse anti-GFP antibody overnight at 4°C. Following immunoprecipitation, beads were washed 2 times with lysis buffer, 2 times with a solution containing containing 50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate wash, two times with a solution containing 10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP40, 0.5% sodium deoxycholate, and 1 mM EDTA, and 2 times with TE. Chromatin was eluted in a solution of TE and 0.1% SDS. Formaldehyde crosslinks were reversed by incubation at 65°C overnight. Samples were then treated with RNase and Proteinase K. DNA was purified by phenol-chloroform extraction followed by ethanol precipitation using glycogen as a carrier. Purified DNA was subjected to qPCR analysis for protein enrichment.

**Quantitative Real-Time PCR (qPCR).** 20 µL qPCR reactions were performed in 96-well plates using Power SYBR Green PCR Master Mix (Applied Biosystems) with 1.5 nM (final) oligos and 8 µL of template DNA. The thermocycling was performed as follows: Step 1: 95°C 10 minutes; Step 2: 95°C 30 s; Step 3: 58°C 30 s; Step 4: Repeat Steps 2 and 3 40 times. Total and IP samples were first normalized to *TSC11* to roughly ensure equal amounts of template DNA. The 1 kb amplicon was amplified with oligos ADD4-G and ADH4-G and the 32 kb amplicon was amplified with oligos CSE1-A and
CSE1-B (see Appendix 1). Fold-enrichment at a query locus was determined by comparison to a control locus, \( TSC11 \), between total DNA and IP DNA and calculated with the \( \Delta \Delta C_t \) method. The oligos used are listed in Appendix.
**Table 2.6.1.** Strains used in Chapter II.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Background</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD1658 (yJM20)</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2</em></td>
<td>yJM20</td>
<td>Melo et al, 2001</td>
</tr>
<tr>
<td>DD1850</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad17-K197R-URA3</em></td>
<td>yJM20</td>
<td>Fu et al, 2008</td>
</tr>
<tr>
<td>DD1838</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad17::URA3</em></td>
<td>yJM20</td>
<td>Fu et al, 2008</td>
</tr>
<tr>
<td>DD3111</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad18::KANMX6</em></td>
<td>yJM20</td>
<td>This study</td>
</tr>
<tr>
<td>DD3113</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad18::KANMX6 rad17-K197R-URA3</em></td>
<td>yJM20</td>
<td>This study</td>
</tr>
<tr>
<td>DD3176</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad17::URA3 yCPT&lt;RAD17&gt;</em></td>
<td>yJM20</td>
<td>This study</td>
</tr>
<tr>
<td>DD3177</td>
<td><em>Matde1 can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3::GalHO tel::HOcute::ura3::HIS3 DDC1-GFP-LEU2 rad17::URA3 yCPT&lt;rad17-S198A&gt;</em></td>
<td>yJM20</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2.6.1**
Chapter III

The Protein Phosphatase Pph3 and Histone H2A modulate the DNA damage checkpoint

3.0 Abstract

The DNA damage checkpoint arrests cells at G2/M in response to DNA damage. Following DNA repair, cells recover to resume cell cycling. The Pph3 histone phosphatase complex is required for timely recovery from the DNA damage checkpoint. Pph3 is the catalytic subunit of a histone phosphatase complex that dephosphorylates γH2A, an early marker of the DNA damage response. Cells lacking Pph3 are severely delayed in their ability to deactivate Rad53 and exit G2/M arrest in a γH2A-dependent manner. I examined the fate of two checkpoint complexes during checkpoint recovery with fluorescence microscopy and found that Ddc2, but not Ddc1, focus disassembly correlated with checkpoint recovery. In h2a-S129A mutants that cannot form γH2A, Rad53 kinase activity and Ddc2 foci are poorly sustained and G2/M arrest is less pronounced in response to a single DSB indicating that γH2A is involved in mounting and sustaining a robust checkpoint response. Moreover, γH2A is an important regulator of genome stability. As an early marker of DNA damage, natural domains of γH2A enrichment mark fragile sites in the yeast genome. h2a-S129A mutants have higher rates of spontaneous damage and are defective in DNA repair and replication stress tolerance. Taken together, these results indicate that γH2A carries out roles in DNA damage signaling, which is antagonized by Pph3, and maintenance of genome stability.
Figures 3.6.1 and 3.6.2 appear in Szilard et al (2010) with Figure 3.6.2 contributed by Dr. Szilard. Portions of Figures 3.2.5 and 3.3.1 appear in Yeung and Durocher (2011).
3.1 Introduction

One of the earliest markers of DSBs or stalled replication forks is the phosphorylation of histone H2A on serine 129 by Tel1 or Mec1 in budding yeast or histone H2AX on serine 139 by ATM, ATR, and DNA-PK in mammals (see Chapter I) (Rogakou et al., 1998; Downs et al., 2000; Fernandez-Capetillo et al., 2004b; Foster and Downs, 2005). In this work, the phosphorylated form of yeast H2A will be referred as γH2A and the mammalian homolog will be referred to as γH2AX. Formation of γH2A or γH2AX can be abolished by a point mutation of the acceptor serine residue (H2AX-S139A in mammals or h2a-S129A in yeast). Early studies of h2a-S129A mutants indicated that γH2A is important for MMS and camptothecin tolerance (Downs et al., 2000; Redon et al., 2003). These studies reported that failure to form γH2A had no impact on cell cycle arrest in response to DNA damage. Notably however, close examination of the role of γH2A in response to DSBs was lacking. In fission yeast, abolishing H2A phosphorylation resulted in DNA damage sensitivity and checkpoint maintenance defects (Nakamura et al., 2004). Furthermore, H2AX(-/-) null mice have several phenotypes that indicate γH2AX is a regulator of genome stability: elevated frequency of spontaneous tumours, immunodeficiency, and sterile males (Celeste et al., 2003). In mammals, γH2AX plays a critical role in establishing a platform for the recruitment of checkpoint proteins such as MDC1 to amplify the DNA damage signal (Fernandez-Capetillo et al., 2004b; Lee et al., 2005; Stucki and Jackson, 2006). Similarly, Crb2, the Rad9 homolog in fission yeast, is also recruited to DSBs by a physical interaction with γH2A (Nakamura et al., 2004). An analogous role for budding
yeast γH2A was just beginning to emerge involving a physical interaction between Rad9 and γH2A in the G1 checkpoint (Hammet et al., 2007).

If γH2A is important for the establishment of the DDR, then dephosphorylation by a protein phosphatase may be equally important for the down-regulation of the checkpoint. Indeed, previous work in the Durocher lab in collaboration with the Greenblatt and Haber groups identified the PP2A-type phosphatase Pph3 as the protein phosphatase that dephosphorylates γH2A (Keogh et al., 2006). Pph3 is the catalytic subunit in a protein complex that also includes Psy2 and Psy4. The dephosphorylation of γH2A is required for timely recovery from the DNA damage checkpoint. This marked delay in checkpoint recovery was rescued by the h2a-S129A mutation.

γH2A is an early marker of DNA damage (Rogakou et al., 1998); therefore, domains of the genome that are naturally enriched for γH2A may represent zones or “fragile sites” that are prone to DNA damage. Work in the Durocher lab identified regions of γH2A enrichment which were proposed to be sites of fork pausing (Szilard et al., 2010). Many of these sites coincided with the same regions where Rrm3 is required for disruption of protein-DNA complexes such as Ty retrotransposons, tRNA genes, rDNA, replication origins and telomeres. These sites of spontaneous γH2A enrichment (γ-sites) represented candidates for fragile sites in the yeast genome.

When I began my graduate work, it was unclear how γH2A participated in the delayed recovery from the checkpoint pph3Δ cells. Indeed, previous work examining γH2A in DNA damage tolerance had reported that h2a-S129A mutants were proficient in establishing cell cycle arrest (Downs et al., 2000; Redon et al., 2003). Therefore, the elevation of γH2A levels in the pph3Δ mutant provided a new system to study the role of
γH2A in checkpoint signaling in response to DSBs. Checkpoint protein redistribution into foci at sites of DNA damage is essential for the proper execution of the checkpoint (Melo et al., 2001; Lisby et al., 2004). Therefore, one possibility was that the γH2A maintained checkpoint proteins on the chromatin which can continue signaling. In this chapter, I first examine the fate of the checkpoint protein complexes Ddc2-Mec and 9-1-1 in pph3Δ and h2a mutants following DSB induction and repair. I employed a genetic approach utilizing synthetic genetic array (SGA) technology and a fluorescent microscopy approach to understand the role of γH2A dephosphorylation in checkpoint recovery. I discovered that γH2A greatly influences the duration of Ddc2 foci but not Ddc1 foci. Finally, to address the role of γH2A in maintaining genome stability at putative fragile sites, I examined the formation of Ddc2 and Rad52 foci in h2a-S129A cells elevated level of spontaneous damage. Therefore, γH2A has dual roles in checkpoint signaling in response to DSBs and regulating genome stability.

3.2 Elevation of γH2A delays recovery from the DNA damage checkpoint

PPH3 encodes a phosphatase that dephosphorylates γH2A (Keogh et al., 2006). Accordingly, a pph3Δ mutant has elevated levels of γH2A even in the absence of DNA-damaging agents (Figure 3.2.1). PP2A family phosphatases must utilize auxiliary proteins for substrate specificity (Sontag, 2001; Cho and Xu, 2007). In the case of Pph3, it forms a complex with Psy2 and Psy4 (Keogh et al., 2006). PSY2 and PSY4 deletions also result in elevated level of γH2A, indicating that the 3 proteins work in concert to regulate γH2A levels (Figure 3.2.1).
Figure 3.2.1. Detection of γH2A by western blot. TCA protein extracts derived from indicated strains were resolved by SDS-PAGE and blotted with an antibody that specifically recognizes γH2A. Pgk1 is presented as a loading control.
Down-regulation of the checkpoint following repair of a DSB is severely delayed in \( pph3\Delta \) mutants and this is dependent on \( \gamma H2A \) (Keogh et al., 2006). In order to understand the role of \( \gamma H2A \) and Pph3 in recovery, I utilized a system adapted from the Haber laboratory, YMV2 (Figure 1.5.1C) (Vaze et al., 2002). In this strain, a single DSB which is repaired by intrachromosomal homologous recombination is experimentally inducible with galactose. Upon addition of galactose, wild type cells activated the DNA damage checkpoint as seen by an upward mobility shift in Rad53 due to phosphorylation and increased Rad53 kinase activity as measured in an \textit{in situ} kinase assay (Figure 3.2.2). Rad53 activity peaked by 3 hours and was sustained through 6 hours post-galactose induction. After 6 hours, Rad53 phosphorylation and kinase activity began to decrease. By 9-12 hours, Rad53 had returned to pre-induction levels indicating that cells had recovered. Another indication that cells had recovered was the proportion of G2/M arrested cells in the population. Cells were stained with DAPI and scored for the proportion of cells in G2/M (Figure 3.2.3). Upon addition of galactose, the proportion of cells in G2/M increased to a maximum of 80% at 3-6 hours. Soon after, the proportion of G2/M cells began to decrease as cells recovered, entered anaphase and resumed cell cycling.

To examine the role of Pph3 in recovery, I performed similar Rad53 and G2/M analyses in the YMV2 strain harbouring \( pph3\Delta \) (Figure 3.2.2 and Figure 3.2.3). Upon addition of galactose, Rad53 is phosphorylated and activated in a similar fashion to the wild type strain. However, \( pph3\Delta \) delays Rad53 down-regulation and cell cycle re-entry by an additional 3-6 hours. Importantly, the delay was not the result of a DSB repair defect, as repair products were detected by PCR at identical times in wild type and \( pph3\Delta \)
**Figure 3.2.2.** Checkpoint recovery of YMV2 wild type and *pph3Δ* strains. TCA protein extracts derived from cells taken at the indicated time points following HO induction by galactose were resolved by SDS-PAGE. Blotted proteins were treated as follows. Top panels: *In situ* Rad53 kinase assay. Rad53 kinase activity is measured by $^{32}$P incorporation. Middle panel: Rad53 western blot detecting native Rad53 and a phosphorylation-depended mobility shift. Bottom panel: Pkg1 western blot is presented as a loading control.
Figure 3.2.2
Figure 3.2.3. G2/M arrest in YMV2 wild type and pph3Δ strains. Cells taken at the indicated time points following HO induction by galactose were stained with DAPI and G2/M cells were scored under the microscope by eye. At least 100 cells were scored per time point.
cells (Figure 3.2.4). This observation is consistent with the delay in Rad53 deactivation in pph3Δ cells.

γH2A is a substrate of Pph3 and previous work revealed that abolishing phosphorylation with a h2a-S129A mutation rescued the recovery delay in pph3Δ cells (Keogh et al., 2006). H2A is encoded by two genes in S. cerevisiae: HTA1 and HTA2. To study the effects of the h2a-S129A mutation on pph3Δ, HTA1 was mutated to hta1-S129A and the duplicated gene, HTA2, was deleted. To simplify the genotype annotation, hta1-S129A hta2Δ will be abbreviated as h2a-S129A unless otherwise noted. When the YMV2 pph3Δ strain was combined with the h2a-S129A mutation, timely recovery was restored. In pph3Δ h2a-S129A cells, Rad53 kinase activity began to decrease 6 hours following galactose addition (Figure 3.2.5). Moreover, pph3Δ h2a-S129A cells begin to exit G2/M at the same time, concurrent with wild type cells but sooner than pph3Δ cells (Figure 3.2.3 and Figure 3.2.6). Therefore, the h2a-S129A allele is capable of restoring timely recovery in pph3Δ cells.

To be sure that deleting HTA2 did not affect checkpoint signaling, wild type and hta2Δ strains were compared directly in the Rad53 and G2/M assays. 3 h following induction of a DSB, Rad53 kinase activity increased in wild type and hta2Δ cells to similar levels and 6 h following DSB induction Rad53 activity in hta2Δ cells was slightly attenuated relative to wild type cells (Figure 3.2.5). However, the G2/M arrest profiles of wild type and hta2Δ cells were indistinguishable (Figure 3.2.6). When the recovery assay was performed on HTA1 hta2Δ and hta1-S129A hta2Δ strains, Rad53 kinase activity is markedly attenuated in the hta1-S129A hta2Δ strain compared to the HTA1 hta2Δ strain (Figure 3.2.5). Moreover, a smaller proportion of hta1-S129A hta2Δ cells arrested at
**Figure 3.2.4.** Single-strand annealing repair PCR assay. Primers P1 and P4 were used to monitor the repair of a DSB induced by HO in YMV2. Prior to DSB formation, P1 and P4 are >30 kb apart. Following DSB repair, P1 and P4 brought within 2 kb of each other and a product can be amplified by PCR (top band). A control locus was amplified in a multiplex PCR reaction to control for template loading (bottom band).
**Figure 3.2.4**

A

Before DSB

\[ \text{u2} \]

\[ \text{le} \text{u2-cs} \]

\[ \text{P1} \]

\[ \text{P4} \]

Repaired chromosome

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Figure 3.2.5. Checkpoint recovery of YMV2 derivative cells. Protein extracts derived from cells taken at the indicated time points following HO induction by galactose were solved by SDS-PAGE. The Rad53 kinase assay was performed on blotted proteins. Pgk1 is presented as a loading control.
Figure 3.2.5
**Figure 3.2.6.** G2/M arrest in YMV2 h2a-S129A cells. Cells taken at the indicated time points following HO induction by galactose were stained with DAPI and G2/M cells were scored under the microscope by eye. At least 100 cells were scored per time point.
Figure 3.2.6
G2/M compared to HTA1 hta2Δ (Figure 3.2.6). In conclusion, a robust checkpoint response to a DSB is dependent on the formation of γH2A.

### 3.3 Ddc2-Mec1 focus disassembly correlates with checkpoint recovery

In response to a DSB, checkpoint and repair proteins reorganize in a tightly choreographed manner within the nucleus (see Introduction) (Melo et al., 2001; Lisby et al., 2004). The assembly of these proteins into domains—or “foci”—can be observed by fluorescence microscopy and is an essential step to establish DNA damage checkpoint signaling. If assembly of foci is required to activate checkpoint signaling, then perhaps disassembly of these protein complexes is required for checkpoint recovery. To examine the fate of the two checkpoint complexes that are essential for the initiation of checkpoint signaling, Mec1-Ddc2 and 9-1-1, YMV2 and derivative strains were engineered to express either Ddc2-GFP or Ddc1-GFP as a marker for each complex.

The YMV2 and YMV2 pph3Δ strains were engineered to express a Ddc2-GFP fusion protein. In one representative experiment, prior to DSB induction in log phase cells, 13.0% of wild type cells had Ddc2-GFP foci. pph3Δ cells had a similar level of foci, at 7.5% cells with a focus. Following addition of galactose, the proportion of wild type cells harbouring a Ddc2-GFP focus reached a maximum of 91.2% after 3 hours. Similarly, pph3Δ cells accumulated foci in 78.4% of cells after three hours and increased to 94.2% 6 hours after. Cells were monitored up to 24 hours post-DSB induction. While wild type cells appeared to completely disassemble Ddc2-GFP foci by around 12 hours, pph3Δ cells showed a 3-hour delay in removal of Ddc2-GFP foci (Figure 3.3.1). Taken
Figure 3.3.1. Ddc2-GFP focus kinetics in YMV2 isogenic mutant cells. At the indicated time points, cells were fixed then imaged by fluorescence microscopy. At least 100 cells were scored per time point. Plots are representative of 3 independent experiments.
Figure 3.3.1
together with the checkpoint kinetics described in Section 3.2, this indicates that removal of Ddc2-Mec1 from the site of damage correlated with checkpoint recovery.

The non-phosphorylatable h2a-S129A mutant rescues the recovery delay of pph3Δ cells (Figure 3.2.5 and Figure 3.2.6). Ddc2-GFP foci were scored over the course of checkpoint activation and recovery in wild type, hta2Δ, hta1-S129A hta2Δ, and pph3Δ hta1-S129A hta2Δ strains (Figure 3.3.2). When pph3Δ and pph3Δ hta1-S129A hta2Δ cells are compared directly, the pph3Δ hta1-S129A hta2Δ strain was found to disassemble Ddc2-GFP foci markedly sooner than the pph3Δ strain, similar to wild type cells (Figure 3.3.1). To be sure that hta2Δ did not affect Ddc2-GFP focus accumulation and disassembly, wild type and hta2Δ cells were compared directly but there was no discernable difference in the kinetics of Ddc2-GFP accumulation and removal between these two strains. Therefore, the abolition of γH2A by the h2a-S129A mutation confers the phenotypic differences. In line with the previous observation that h2a-S129A alone attenuates checkpoint signaling (Figure 3.2.4), the accumulation of Ddc2-GFP foci in of hta1-S129A hta2Δ cells was greatly attenuated compared to wild type, hta2Δ and pph3Δ cells (Figure 3.3.1). These observations further indicate that Ddc2-GFP focus disassembly correlates with recovery and γH2A also plays a role in sustaining Ddc2-GFP foci.

The lower level of accumulation of Ddc2-GFP foci in h2a-S129A cells correlated well with the attenuated Rad53 kinase activity (Figure 3.2.4). It is possible that γH2A participates in the recruitment of Ddc2-Mec1 to chromatin; however, another possibility might be that downstream checkpoint signaling may be required for Ddc2 focal maintenance. Indeed, it is well established that Mec1 is recruited to ssDNA via a physical interaction between Ddc2 and RPA (Zou and Elledge, 2003; Nakada et al., 2005) and
Ddc2-Mec1 maintenance is assumed to also rely exclusively on RPA-ssDNA filaments. To examine the possibility that downstream checkpoint signaling might influence Ddc2-GFP focus accumulation, YMV2 sml1Δ and YMV2 sml1Δ rad53Δ cells were engineered to express Ddc2-GFP (Figure 3.3.2). SML1 was deleted to suppress the lethality of rad53Δ cells by increasing dNTP pools. Prior to induction of a DSB with galactose, 3.0% YMV2 sml1Δ cells had Ddc2-GFP foci, a similar level to wild type cells. YMV2 sml1Δ rad53Δ cells had a higher level of spontaneous Ddc2-GFP foci, at 20.7% of cells. Following addition of galactose, 66.4% of YMV2 sml1Δ cells had accumulated Ddc2-GFP foci by 6 hours whereas only 35.4% of YMV2 sml1Δ rad53Δ cells had accumulated Ddc2-GFP foci. Whereas assembly of DNA damage foci is generally understood to occur in a hierarchical fashion (Lisby et al., 2004), Rad53, a downstream checkpoint effector kinase, is also important for the accumulation and maintenance of Ddc2-GFP foci.

In addition to Ddc2-Mec1, the 9-1-1 checkpoint clamp is recruited to DSBs (Melo et al., 2001; Majka and Burgers, 2003). I engineered the YMV2 and YMV2 pph3Δ strains to express Ddc1-GFP and performed similar fluorescence microscopy analysis to track the fate of the 9-1-1 complex. I found that Ddc1-GFP were much dimmer than Ddc2-GFP foci. However, Ddc1-GFP foci did begin to accumulate soon after DSB induction with galactose in both wild type and pph3Δ cells, as expected (Figure 3.3.3). I observed a maximum of 20.0% percent of wild type cells 17.3% of pph3Δ cells with Ddc1-GFP foci. Unlike Ddc2-GFP, I did not observe a great delay in Ddc1-GFP foci removal in pph3Δ cells. Taken together, Ddc2-GFP foci disassembly correlates with checkpoint recovery; whereas, Ddc1-GFP disassembly does not.
Figure 3.3.2. Ddc2-GFP focus accumulation in YMV2 rad53Δ cells. At the indicated time points, cells were fixed then imaged by fluorescence microscopy. At least 100 cells were scored per time point. Plots are representative of 3 independent experiments.
Figure 3.3.3. Ddc1-GFP focus kinetics in YMV2 wild type and pph3Δ mutant cells. At the indicated time points, cells were fixed then imaged by fluorescence microscopy. At least 100 cells were scored per time point. Plots are representative of 3 independent experiments.
3.4 Pph3-mediated recovery at uncapped telomeres

Cdc13 is an essential ssDNA-binding protein that participates in silencing the DNA damage checkpoint at telomeres. cdc13-1 is a temperature sensitive allele (Garvik et al., 1995), which results in telomeres being recognized by the DNA damage checkpoint machinery as DSBs at the restrictive temperature. Therefore, cdc13-1 strains can be used to study checkpoint recovery in response to multiple DSBs.

When cdc13-1 cells are exposed to the restrictive temperature, telomeres are extensively resected in the 5’ to 3’ direction leading to activation of the checkpoint (Lydall and Weinert, 1995, 1997). Prolonged exposure to the restrictive temperature is catastrophic for cdc13-1 cells (Figure 3.4.1); however, cells can recover from acute exposure, as demonstrated by observing Rad53 dephosphorylation (Figure 3.4.2). To examine the role of Pph3 in recovery from damaged telomeres, Rad53 phosphorylation was monitored in a pph3Δ strain containing cdc13-1 (Figure 3.4.2). Following 2 hours incubation at 34°C, cdc13-1 and pph3Δ cdc13-1 cells were allowed to recover at 23°C and Rad53 phosphorylation was monitored over the course of the experiment. After 2 hours exposure to the restrictive temperature, Rad53 was phosphorylated in both cdc13-1 and pph3Δ cdc13-1; however, the phosphorylation-induced mobility shift of Rad53 in pph3Δ cdc13-1 was greater than cdc13-1 alone. Rad53 was soon dephosphorylated when cdc13-1 cells were shifted back to the permissive temperature. By contrast, pph3Δ cdc13-1 cells were greatly impaired in dephosphorylating Rad53, indicating a recovery delay.
Figure 3.4.1. Temperature sensitivity of *cdc13-1* cells. Wild type and *cdc13-1* cells were spotted in 1/5 serial dilutions on to XY and incubated at the indicated temperatures.
Figure 3.4.2. Rad53 phosphorylation in *cdc13-1* cells following acute exposure to the restrictive temperature. TCA protein extracts derived from cells taken from the indicated time points and temperatures were resolved by SDS-PAGE and blotted for Rad53. Pgk1 is presented as a loading control.
Figure 3.4.3. Ddc2-GFP focus dynamics during recovery from acute restrictive temperature exposure in *cdc13-1* cells. At the indicated time points and temperatures, cells were fixed and imaged by fluorescence microscopy. At least 100 cells were scored per time point.
Next, I engineered *cdc13-1* and *pph3Δ cdc13-1* to express Ddc2-GFP and observed Ddc2-GFP foci formation and disassembly following acute exposure to the restrictive temperature. In contrast to the YMV2 system, Ddc2-GFP foci in both *cdc13-1* and *pph3Δ cdc13-1* strains were removed at identical rates during recovery from telomere exposure, independently of Rad53 dephosphorylation (Figure 3.4.3). This was a striking difference between DSB repair and telomere repair and the results revealed significant differences in how recovery might be achieved in response to different types of DNA damage or DNA damage that is repaired by different mechanisms. Taken together, during recovery from uncapped telomeres, Pph3 is required for Rad53 dephosphorylation but not Ddc2-GFP foci disassembly.

### 3.5 Genetic interactions with PPH3

To gain insight into the role of Pph3 in regulation of the checkpoint, I performed a screen to identify genetic interactions with *PPH3*. Since the DNA damage checkpoint slows or arrests the cell cycle in the presence of DNA damage, it might be expected that elevated levels of DNA damage in chronically delayed checkpoint recovery *pph3Δ* cells could potentially be harmful to cell survival. To further investigate the function of Pph3 in the DNA damage checkpoint, I sought to identify novel genetic interactions using Synthetic Genetic Array (SGA) technology, a well-established screening method to interrogate the non-essential genes of the yeast genome (Tong et al., 2001).

A *pph3Δ* query strain was mated to a collection of ~4700 non-essential yeast gene deletions and double mutant haploid cells were selected essentially as described in Tong et al (2001). For comparison and statistical analysis, a wild type screen where a *PPH3*
strains was mated to the deletion collection was performed in parallel. Hits were confirmed with tetrad analysis (Table 3.5.1). Genes involved in the DNA damage checkpoint and DNA repair such as \textit{MRE11} and \textit{MRC1} were identified as interactors with \textit{PPH3}, strongly suggesting that it is involved in DNA damage signaling. The majority of identified interactions were mild, resulting in only minor synthetic sick phenotypes. Some of these sick phenotypes were previously shown to be enhanced when double mutants were grown on media containing bleomycin, a radiomimetic drug that induces DSBs (Keogh et al., 2006). Genes that suppress spontaneous DNA damage such as \textit{RRM3} and \textit{DIA2} were identified as interactors, as well. In addition, \textit{PPH3} interactions were identified with known chromatin remodelers (\textit{CDC73} and \textit{LEO1}) and microtubule motor proteins (\textit{CIK1} and \textit{KAR3}). Notably, there were no interactions identified that resulted in synthetic lethality. The strongest synthetic interaction was with \textit{DIA2}, although \textit{dia2}\Delta single mutants are also slow-growing (Blake et al., 2006).

Since abolishing H2A phosphorylation with the \textit{h2a-S129A} allele rescues the recovery defect of \textit{pph3}\Delta cells (Section 3.2 and Section 3.3), I performed crosses between the deletion mutants and a strain harbouring \textit{hta1-S129A hta2-S129A} to determine if the synthetic sick phenotype was dependent on γH2A. \textit{MRE11} and \textit{DIA2} interactions were tested in these crosses. Of the resulting progeny from a \textit{mre11::KAN x pph3::NAT hta1-S129A hta2-S129A} cross, all the recovered double deletion mutants were synthetic sick (Figure 3.5.1). In the absence of selectable markers for the \textit{hta1-S129A} and \textit{hta2-S129A} alleles, G418R NAT\textsuperscript{R} colonies were screened for the histone point mutations by restriction enzyme analysis. The nucleotide substitutions that generate the \textit{h2a-S129A}
Table 3.5.1. Genetic interactions with $PPH3$ identified in a high-throughput SGA screen.

Interactions identified by SGA were confirmed by tetrad analysis.

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<td>LEO1</td>
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Figure 3.5.1. Tetrad analysis of `pph3::NAT hta1-S129A hta2-S129A x mre11Δ`.

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**Figure 3.5.2.** Tetrad analysis of \( pph3::\text{NAT} \times \text{dia2::KAN} \) and \( pph3::\text{NAT} \ hta1\text{-S129A} \ hta2\text{-S129A} \ x \ \text{dia2::KAN} \ hta1\text{-S129A} \ hta2\text{-S129A} \).
mutation create a unique FspI restriction site. 10 mre11Δ pph3Δ mutants were screened and 5 were identified as hta1-S129A hta2-S129A, none of which showed any suppression of the slow-growth phenotype. A similar analysis was completed by performing a cross between dia2::KAN hta1-S129A hta2-S129A and pph3::NAT hta1-S129A hta2-S129A. The h2a-S129A mutation did not rescue the sick phenotype of pph3Δ dia2Δ mutants (Figure 3.5.2).

I was unable to identify genetic interactions with synthetic sick that were more severe than the pph3Δ dia2Δ interaction or synthetic lethal interactions. Confronted without obvious genetic clues to the role of Pph3 in recovery, there was no further investigation into the screen hits.

3.6 γH2A marks fragile sites in the yeast genome

γH2A is an early marker of DSBs (Rogakou et al., 1998). Endogenous regions of γH2A enrichment might correlate with loci that are prone to breakage (Szilard et al., 2010). To examine the possibility that natural sites of γH2A enrichment might represent fragile sites that are prone to DNA damage, I compared wild type and h2a-S129A cells engineered to express either Ddc2-GFP or Rad52-YFP to monitor DSB formation. Ddc2-GFP foci mark DSBs and Rad53-YFP foci mark ongoing homologous recombination repair (Lisby et al., 2001; Melo et al., 2001). Log phase cells were harvested and budded cells (S/G2/M phase) were examined for Rad52-YFP or Ddc2-GFP foci (Figure 3.6.1). For exponentially growing wild type cells, 6.7% and 13.0% of budded cells contain a Rad52-YFP focus or Ddc2-GFP focus, respectively. h2a-S129A cells contained roughly twice the number of foci with 16.3% of budded cells with Rad52-YFP foci and 26.2%
**Figure 3.6.1.** *h2a-S129A* elevates levels of spontaneous Ddc2-GFP and Rad52-YFP foci. Wild type and *h2a-S129A* cells growing exponentially were harvested and scored for either Ddc2-GFP or Rad52-YFP foci. As a control, Rad52-YFP foci were scored in *esc2Δ* cells. Data are represented as the mean ± s.d. (n number of samples are indicate in each bar). Statistical significance (*p*-value) was determined with an unpaired *t*-test. Approximately 100 cells were scored per sample.
with Ddc2-GFP foci. As a comparison, Rad52-YFP foci were examined in an *esc2Δ* background. Esc2 participates in stabilizing replication forks and its deletion results in a moderate level of genome instability (Choi et al.; Kanellis et al., 2007; Sollier et al., 2009). 17.1% of *esc1Δ* cells had Rad52-YFP foci, a significantly elevated proportion relative to wild type cells, but similar to *h2a-S129A* cells. In conclusion, the *h2a-S129A* allele increases the spontaneous formation of Rad52-YFP and Ddc2-GFP foci indicating an elevated frequency of spontaneous DNA damage.

One predicted consequence of elevated levels of spontaneous DNA damage is an increased frequency of GCRs. To test this, a reporter strain that detects GCR events was subjected to fluctuation analysis to measure GCR frequency in *h2a-S129A* (Figure 3.6.2, A). In this strain, the left arm of *ChrXV* is marked by *CAN1* and *URA3* which confers canavanine and 5-FOA sensitivity. When this strain is plated on media containing canavanine and 5-FOA, a rate of spontaneous GCR formation can be calculated by the frequency of *CAN^R* 5-FOA^R^ colonies. However, in comparison to the wild type, the *h2a-S129A* mutation does not appear to increase the GCR rate (Figure 3.6.2, B) (Szilard et al., 2010). The reporter strain is biased toward detecting particular classes of GCRs such as telomere addition (Myung et al., 2003) so a different experimental context which favours BIR was utilized. Cells were treated with MMS to induce damage that is primarily repaired by BIR. After treatment with MMS, *h2a-S129A* cells exhibited a 3-fold increase in gross chromosome rearrangement frequency compared to wild type (Figure 3.6.2, B). Therefore, γH2A suppresses GCR formation in response to MMS.
Figure 3.6.2. Fluctuation analysis of gross chromosome rearrangement frequency. (A) Schematic of the reporter strain *ChrXV*. The assay monitors the loss of CAN1 and URA3 genes inserted ~10 kb from the telomere of chromosome XV-L. This chromosome arm contains two regions of homology (HRI, centered on the *PAU20* gene, and HRII, centered on the HXT11 gene) located 12 kb and 25 kb from the telomere that share a high degree of sequence identity with 21 regions in the genome. Consequently, DNA lesions formed at loci telomeric to HRI or HRII are predominantly repaired by BIR, thereby converting a canavanine- and 5-fluoroorotic acid (5-FOA)-sensitive strain (canS FOAS) into a canavanine and 5-FOA–resistant strain (canR 5-FOAR). (B) The *h2a-S129A* mutation results in an increased frequency of drug-induced GCR events. Cells carrying the chromosome XV-L GCR reporter chromosome were grown in the presence or absence of MMS and assayed for survival on medium containing 5-FOA and canavanine. Data are represented as the frequency of GCR events (mean ± SEM; n = 6).
Figure 3.6.2
3.7 Discussion

*Pph3 and γH2A modulate the DNA damage checkpoint*

In the absence of Pph3, cells are profoundly delayed in their ability to exit the DNA damage checkpoint, a delay which is dependent on elevated levels of γH2A (Figure 3.2.2, Figure 3.2.3, and Figure 3.2.5). These observations are consistent with a previous study from Keogh *et al* (2006) which originally identified Pph3 as the catalytic subunit of a phosphatase complex that dephosphorylates γH2A. To explore the role of γH2A in maintaining an active checkpoint, I observed Ddc2-GFP focus assembly and disassembly in wild type and *pph3Δ* cells in response to a repaired DSB and found that Ddc2-GFP foci disassembly correlates with recovery (Figure 3.3.1). By contrast, Ddc1-GFP focus disassembly occurs independently of recovery (Figure 3.3.3). The function of Pph3 in dephosphorylating γH2A and the role of γH2A in maintaining checkpoint signaling seem to be evolutionarily conserved in the mammalian homolog PP4 (Nakada *et al*., 2008).

Notably, the *h2a-S129A* mutation was more severely checkpoint impaired than *pph3Δ h2a-S129A* double mutants in response to a DSB (Figure 3.2.5 and Figure 3.3.1). Therefore, γH2A is not entirely epistatic to *PPH3* and suggests there are other substrates of Pph3 that influence checkpoint arrest. Indeed, evidence indicates that Rad53 is a substrate of Ptc2, Ptc3 and Pph3 phosphatases (Leroy *et al*., 2003; Guillemain *et al*., 2007; Travesa *et al*., 2008). Moreover, *pph3Δ cdc13-1* cells are unable to quickly down-regulate Rad53 phosphorylation after exposure to the restrictive temperature (Figure 3.4.2), but are proficient in disassembling Ddc2-GFP foci (Figure 3.4.3), strongly supporting a mechanism by which Pph3 regulates Rad53 dephosphorylation after telomere uncapping.
In response to DSBs, γH2A is clearly required for robust and sustained checkpoint signaling and arrest (Figure 3.2.5, Figure 3.2.6, and Figure 3.3.1). Precisely how γH2A participates in the maintenance of the checkpoint remains unclear but the maintenance of checkpoint signaling complexes in the vicinity of the DSB remains a possible explanation (Figure 3.3.1). These observations are in direct contrast to previous reports that could not detect checkpoint defects in h2a-S129A mutants (Downs et al., 2000; Redon et al., 2003). The different phenotypes are likely the result of the different type of DNA damage h2a-S129A cells are recognizing. Specifically, MMS and camptothecin create damage that activates the S-phase checkpoint, slowing replication and activating Rad53 in a Mrc1-dependent manner (Alcasabas et al., 2001; Osborn and Elledge, 2003). However, DSBs activate the DNA damage checkpoint, arresting cells at G2/M and activating Rad53 in a Rad9-dependent manner (Weinert and Hartwell, 1988; Schwartz et al., 2002; Sweeney et al., 2005). Importantly, a C-terminal truncation allele h2a-S129*, which also abolishes γH2A formation, showed a mild Rad53 phosphorylation defect in response to the DSB-inducing agent bleomycin (Hammet et al., 2007). Hammet et al showed a distinct dosage effect of Rad53 phosphorylation in response to increasing concentrations of bleomycin. Taken together, yeast γH2A is a positive regulator of DNA damage checkpoint signaling and Pph3 antagonizes γH2A.

γH2A as a regulator of genome stability

In previous studies, h2a-S129A cells were sensitive to treatments of MMS or camptothecin, agents that generate DNA damage and impede DNA replication (Downs et al., 2000; Redon et al., 2003). DNA replication also presents the cell with a unique set of
challenges which stand in the way of faithful replication of the genome (Labib and Hodgson, 2007). Endogenous barriers such as heterochromatin or repetitive sequences can slow or stop the progression of a replication fork. To successfully replicate the genome, cells rely on proteins that remove or bypass endogenous obstacles. For example, the helicase Rrm3 is required to disrupt non-histone protein-DNA complexes which would otherwise impede the progression of the replisome (Ivessa et al., 2003). Over 1400 sites in the genome require Rrm3 for efficient DNA replication. These sites include rDNA, tRNAs, centromeres, and telomeres. If replisome progression is slowed at endogenous barriers, the replication fork may be prone to collapse in those regions, resulting in a deleterious DSB (Labib and Hodgson, 2007). In line with these observations, γH2A marks fragile sites that closely overlap with chromosome domains that require Rrm3 for efficient replication (Szilard et al., 2010). The elevated levels of Ddc2-GFP and Rad52-YFP foci in *h2a-S129A* cells indicate that γH2A is a regulator of genome stability (Figure 3.6.1). Furthermore, it is likely that γH2A regulates replication fork stability through fragile sites in the genome.

*Recovery from “uncapped” telomeres*

When I examined Ddc2-GFP focus dynamics in *pph3Δ* cells in response to *cdc13-1*-dependent DNA damage, I observed that Ddc2-GFP focus disassembly was not affected even though there was a clear delay in Rad53 dephosphorylation (Figure 3.4.2 and Figure 3.4.3). The timely disassembly of Ddc2-GFP foci in *pph3Δ cdc13-1* cells may be the result of the different repair mechanisms that are used to repair resected telomeres compared to DSBs. To repair resected telomeres, cells can use telomerase or a
telomerase-independent break-induced replication mechanism called alternate lengthening of telomeres (ALT) (McEachern and Haber, 2006). Perhaps these repair mechanisms are sufficient to evict the Ddc2-Mec1 complex from the ssDNA. Alternatively, but not mutually exclusively, if DNA replication machinery is involved, then Mrc1 is expected to be involved as well, which can function in a parallel pathway to Rad9 to activate Rad53 (Alcasabas et al., 2001). γH2A-mediated checkpoint activity may depend exclusively on the Mec1-Rad9-Rad53 DNA damage checkpoint; hence, the effect of γH2A on a prolonged DNA damage checkpoint would be bypassed. Thus, recovery from resected telomeres might represent a transition from Rad9-dependent signaling to Mrc1-dependent signaling. Eviction of Ddc2-GFP foci from chromatin might also involve the reactivation of Cdc13-1 at the permissive temperature since Cdc13 is also a ssDNA-binding protein at telomeres and would compete with RPA and Ddc2 for ssDNA (Nugent et al., 1996). Furthermore, evidence presented here indicates that Pph3 targets several substrates in the DNA damage checkpoint other than γH2A, consistent with previous reports that Rad53 is also a substrate of Pph3 (O'Neill et al., 2007).

**Maintenance of an active DNA damage checkpoint**

The DNA damage checkpoint might be viewed as a three stage response. First, in the presence of DNA damage, the checkpoint machinery must recognize and transduce the signal to achieve cell cycle arrest. Next, checkpoint signaling must be maintained for as long as DNA damage is present. Finally, when repair is completed, the checkpoint must be terminated to allow resumption of the cell cycle.
While it is uncertain if checkpoint recovery is a signaling cascade by itself, certainly, there are components of the DNA damage checkpoint that are actively regulated including the dephosphorylation of \( \gamma \)H2A and Rad53. It is not known how the Pph3 phosphatase is temporally regulated. If a maintenance phase exists, perhaps one function of the DNA damage checkpoint is to inhibit Pph3 until repair is completed.

The components of checkpoint maintenance are just beginning to emerge. The drastically attenuated checkpoint in \( h2a-S129A \) mutants suggests that \( \gamma \)H2A plays a role in maintaining an active checkpoint (Figure 3.2.4). The strict correlation of Ddc2-GFP foci with checkpoint activity in response to a single DSB makes it tempting to speculate that the checkpoint is sustained by maintaining the localization of the Ddc2-Mec1 complex on chromatin (Figure 3.3.1 and Figure 3.3.2). Consequently, the removal of the checkpoint complex from chromatin might be important for checkpoint recovery. Indeed, other groups investigating checkpoint recovery have speculated that proteins such as the helicase Srs2 may be required for stripping checkpoint and repair proteins from chromatin to promote repair (Vaze et al., 2002). This possibility is further explored in Chapter IV.

Some clues to the role of \( \gamma \)H2A in checkpoint maintenance come from studies in mammalian systems. Specifically, \( \gamma \)H2AX recruits MDC1 through the MDC1 BRCT domain (Lee et al., 2005; Stucki and Jackson, 2006). The recruitment of MDC1 directs the relocalization of RNF8, 53BP1 and BRCA1 to DSBs (Goldberg et al., 2003; Stewart et al., 2003; Kolas et al., 2007). Therefore, there is strong evidence to support a model where \( \gamma \)H2A participates in the maintenance of active checkpoint complexes on chromatin.
ssDNA-RPA filaments which are generated by resection are potent activators of the checkpoint (Zou and Elledge, 2003); however, downstream checkpoint signaling appears to also greatly influence the accumulation and maintenance of Ddc2-Mec1 in foci (Figure 3.3.3). This might indicate a previously unknown positive feedback signal in the DNA damage checkpoint. The observation that Rad53 is required to maintain Ddc2-GFP foci (Figure 3.3.3) is unexpected from a pathway that is assumed to be initiated by a linear, step-wise accumulation of checkpoint proteins (Lisby et al., 2004). Several factors can influence Ddc2 accumulation and maintenance. Cutting and resection efficiency might be altered in the absence of Rad53. Slower resection would result in slower accumulation of ssDNA and subsequently, recruitment of Ddc2-Mec1. Downstream checkpoint components are known to affect resection rate. For instance, Rad9 is a negative regulator of resection (Lazzaro et al., 2008). Alternatively, the poor accumulation of Ddc2-GFP foci in rad53Δ cells might indicate that downstream signaling components participate in the retention of upstream proteins. Rad53 might participate in the establishment of the maintenance phase by inhibiting proteins that positively regulate checkpoint recovery until repair is completely.

Concluding Remarks

In this Chapter, I have described my efforts to characterize the role of Pph3 and γH2A in the DNA damage checkpoint and repair. A central theme has been the regulation of the duration of the checkpoint. In response to a single DSB that is repaired by intrachromosomal recombination (SSA), Pph3 is required for the dephosphorylation of γH2A to allow timely recovery. The endurance of checkpoint signaling (observed
through Rad53 activity) and checkpoint arrest correlates with the retention of the apical checkpoint kinase complex Ddc2-Mec1, but not the 9-1-1 complex, in subnuclear foci. In addition to H2A, Pph3 targets other DNA damage checkpoint substrates for dephosphorylation. Consistent with this, Rad53 dephosphorylation is delayed in pph3Δ cells recovering from cdc13-1 telomere resection. Finally, γH2A appears to play an important role in maintaining genome stability. h2a-S129A cells have higher levels of spontaneous damage in S-phase, as observed by the formation of Rad52-YFP and Ddc2-GFP foci. DNA damage that generates obstacles to DNA replication, such as treatments with MMS, result in elevated levels of gross chromosome rearrangements. Thus, Pph3 and γH2A are modulators of the DNA damage response.

3.8 Experimental Procedures

Strains and Plasmids. All strains were derivatives of YMV2, W303, S288C or A364a as indicated in Table 3.8.1. Yeast strains were constructed using standard genetic procedures either by genetic crosses or by high efficiency lithium acetate transformation. Standard yeast media and manipulations were used unless otherwise indicated. To reduce background fluorescence, cells harvested for microscopy were grown in YM-1 rich medium (Appendix) or supplemented minimal media. DDC1-GFP and DDC2-GFP constructs were obtained from the GFP-tagged ORF collection (Huh et al., 2003). YFP tagging of DDC2 generated from a PCR-amplified cassette from pDH6 with oligos DDC2-YFP-F (5’ATCTAACCACACTAGAGGAGGCCGATTCATTATATATCTCAA-TGGGACTGGGTCGACGGATCCCCGGG) and DDC2-YFP-R (5’ TAAATTATATAT-AGTTAATATTAAGCATTACAAGGTTTCTATAAAGCGTTATCGATGAATTGCAG
CTCG). W303 and W303 h2a-S129A were transformed with pRAD52-YFP (gift of Grant Brown) to assess Rad52-YFP focus formation.

**γH2A analysis.** Western blotting for γH2A was performed with an anti-yeast phospho-H2A antibody (Upstate). Protein extracts were resolved in a 15% polyacrylamide gel by SDS-PAGE and transferred to a PVDF membrane with a standard transfer protocol. The membrane was blocked in TBS-T plus 3% BSA. The blot was incubated with the primary antibody in a 1:10,000 dilution in TBS-T plus 3% BSA at 4°C overnight. The next day, the anti-body was removed and the blot was washed three times in TBS-T. The blot was incubated with a HRP-conjugated goat-anti-rabbit secondary antibody at a dilution of 1:10,000 in TBS-T plus 3% BSA for 1 hour at room temperature. After the secondary antibody was removed, the blot was washed three times. Detection was carried out by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

**Galactose Induction.** Cells were grown in media containing 2% raffinose overnight. Following, galactose was added to each culture to a total of 2% galactose to induce HO expression.

**Rad53 activity and phosphorylation assays.** Protein extracts were prepared by mechanical lysis of cells in 20% TCA. Protein extracts were boiled in Laemmli solution and resolved in an 7% polyacrylamide gel (Acrylamide/Bis 37.5:1) by SDS-PAGE. Rad53 kinase activity was assayed as previously described (Pellicioli et al., 1999). For the kinase assay, proteins were transferred to a PVDF membrane in a methanol-free solution containing 25 mM Tris-Cl and 192 mM glycine. Following transfer of proteins, the membrane was treated in a buffer containing 7 M guanidine hydrochloride, 50 mM DTT, 2 mM EDTA, and 50 mM Tris-HCl pH 8.0 for 1 hour. The membrane was next
washed 2X in TBS, 10 minutes each. Proteins on the membrane were renatured overnight in a renaturation buffer containing 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.04% Tween-20, and 1% BSA at 4°C. Prior to the in situ kinase reaction, the membrane was incubated in 30 mM Tris-HCl pH 7.5 for an hour at room temperature. The kinase reaction was performed in a buffer containing 40 mM HEPES-NaOH pH 7.5, 20 mM MgCl2, 20 mM MnCl2, 0.1 EGTA, 1 mM DTT, and 10 µCi/mL [γ-32P]ATP. The radiolabelled membrane was washed twice in 30 mM Tris-HCl pH 7.5, once in Tris-HCl pH 7.5 0.1% NP-40, once in Tris-HCl pH 7.5, once in 1 M KOH, once, in ddH2O, once in 10% TCA, and once in ddH2O. Each wash was 10 minutes long. The membrane was then exposed to a phosphorscreen and analyzed with a Molecular Dynamics PhosphorImager. Rad53 immunoblotting was performed as described in Chapter II.

**G2/M Arrest Profile.** Cells were collected at the indicated time points following induction of a DSB and fixed in 70% ethanol. Cells were washed once with ddH2O and stained with a solution of PBS containing 2.5 µg/mL DAPI and then imaged directly.

**Fixation and storage of cells for microscopy.** 4% PFA was prepared by dissolving PFA in a solution containing 0.1 M sucrose made basic with 5 M NaOH (14 µL NaOH/12.5mL H2O). Cells were fixed in 4% PFA for 15 minutes then washed, stored, and imaged in a solution containing of 0.1 M potassium phosphate buffer pH 7.5 and 1.2 M sorbitol.

**Microscopy.** For cells expressing Ddc1-GFP, Ddc2-GFP, or Rad52-YFP constructs, microscopy was performed with a Nikon E600 FN microscope (http://www.nikonusa.com) equipped with an ORCA ER2 camera (http://www.hamamatsu.com), Chroma filters (YFP: JP4 C/YFP, dichroic 86002v2, batch
C37370, excitation = 500 ± 20 nm, emission 535 ± 30 nm; GFP: 41025 PSTN GFP, dichroic Q495LP, batch C37368, excitation = 470 ± 40 nm, emission = 515 ± 30 nm; http://www.chroma.com/) and a 100X/1.4 oil emersion Nikon Plan Apo objective. Approximately 100 cells from each time point were scored for foci. Data was visualized with MetaMorph and images were processed and deconvolved with Velocity v5.3.0.

**Genomic DNA preparation from yeast.** A 200-500 µL pellet of yeast cells was mechanically lysed in a buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, and 2% Triton X-100 plus 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1, Sigma) and 1 volume of glass beads. 200 µL of TE was added prior to separation of aqueous and organic phases in a microcentrifuge. Nucleic acid was ethanol precipitated, then resuspended in TE with 100 µg/mL RNase A. DNA was ethanol precipitated and resuspended in Tris-HCl pH 8.0.

**SSA PCR Assay.** The assay was performed as described in Keogh et al (2006). The oligos SSA-P1 and SSA-P4 were used to observe the formation of repair products in YMV2. The sequences are listed in Table X. As a template concentration control, oligos CDC13-f and CDC13-r were used. PCR products were resolved on a 1% agarose gel in TBE, stained with 0.5 µg/mL EtBr and imaged on a UV gel box.

**Synthetic Genetic Screen.** A screen to identify potential genetic interactors with *PPH3* was performed essentially as described in Tong et al (Tong et al., 2001). An S288C MATα strain harbouring *pph3::NATMX* was mated to a collection of ~4800 deletion strains (*xxx::KANMX*). See Appendix for the media used. Unless otherwise indicated, all incubations were performed at 30ºC. Strains were pinned in a 384-spot format on solid media in OmniTrays (Nunc, 242811). Mating was accomplished on XY media over 1
day. Diploids were selected on XY supplemented with 300 mg/L G418 and 100 mg/L NAT for 2 days and sporulated on SPO media for 5 days at 25°C. MATa haploids were selected with two iterative pinnings on drop-out media lacking HIS and ARG supplemented with 100 mg/L canavinine. G418\textsuperscript{R} cells were selected on drop-out media lacking HIS and ARG supplemented with 100 mg/L canavinine and 300 mg/L G418. Finally, double mutant cells were selected on drop-out media lacking HIS and ARG supplemented with 100 mg/L canavinine and 300 mg/L G418 and 100 mg/L NAT. Plates containing double mutant haploids were imaged and analyzed to identify putative genetic interactors. Several synthetic sick and lethal candidates were selected and confirmed in a secondary screen with tetrad analysis.
Table 3.8.1. Strains used in Chapter III.

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<td>M. Tyers</td>
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<td>YMV2</td>
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<td>M. Lisby &amp;</td>
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Chapter IV

Srs2 enables checkpoint recovery by promoting disassembly of DNA damage foci from chromatin

4.0 Abstract

Following DNA repair, checkpoint signaling must be abated to resume cell cycling in a phenomenon known as checkpoint recovery. Although a number of genes have been implicated in the recovery process, it is still unknown whether checkpoint recovery is caused by a signaling network activated by DNA repair or whether it is the result of the loss of DNA structures that elicit the checkpoint. Here I show that checkpoint recovery can be uncoupled from bulk chromosome DNA repair if single-stranded (ss) DNA persists. This situation occurs in cells that are deficient in the Srs2 helicase, a protein that antagonizes Rad51. srs2Δ cells fail to eliminate Ddc2 and RPA subnuclear foci following bulk chromosome repair due to the persistence of ssDNA. In contrast to cells with DNA double-strand breaks that remain unrepaired, srs2Δ cells remove the 9-1-1 checkpoint clamp from chromatin after repair. However, despite the loss of the 9-1-1 clamp, Dpb11 remains associated with chromatin to promote checkpoint activity. My work indicates that Srs2 promotes checkpoint recovery by removing Rad51 after DNA repair. A failure to remove Rad51 causes persistence of ssDNA and the checkpoint signal. Therefore, I conclude that cells initiate recovery when the DNA structures that elicit the checkpoint are eliminated.
4.1 Introduction

The DNA damage checkpoint in budding yeast is under the control of the kinase Mec1, the ATR homolog (Harper and Elledge, 2007). For Mec1 to be activated, DSBs must be nucleolytically processed to expose tracts of ssDNA, which act as a potent checkpoint signal (Zou and Elledge, 2003; Zhu et al., 2008). ssDNA bound by the heterotrimeric replication protein complex RPA (composed of Rfa1-3) is independently recognized by the Mec1-Ddc2 and Rad17-Mec3-Ddc1 checkpoint complexes (Kondo et al., 2001; Melo et al., 2001; Zou and Elledge, 2003). Rad17-Mec3-Ddc1 is referred to as 9-1-1, a name based on the human orthologs of the complex (RAD9-HUS1-RAD1), and is loaded onto dsDNA/ssDNA junctions by a specialized replication factor C complex containing Rad24 instead of Rfc1 (Ellison and Stillman, 2003; Majka and Burgers, 2003; Majka et al., 2006b). The 9-1-1 complex plays a critical role in Mec1 activation in response to DSBs, which explains its co-localization with Mec1-Ddc2 at sites of DNA damage. The Ddc1 subunit of 9-1-1 (equivalent to human RAD9) can activate Mec1 via two distinct modes. Ddc1 can activate Mec1 directly in biochemical assays, in the absence of any other proteins (Majka et al., 2006b). In addition, the phosphorylated Ddc1 C-terminal tail recruits the BRCT domain-containing protein Dpb11 to activate Mec1, a mode of activation that is evolutionarily conserved (Navadgi-Patil and Burgers, 2009a, b). Activated Mec1 then phosphorylates Rad9, which acts as a mediator protein linking Mec1 to Rad53, the homolog of human CHK2, which is a central effector kinase in the establishment of the checkpoint arrest (Naiki et al., 2004; Sweeney et al., 2005).

In order to survive a DSB, a cell must not only be able to block cell division for as long as damage is present, but also turn off the checkpoint to re-enter the cell cycle after
repair is completed. A failure to do so results in a permanent cell cycle arrest followed by cell death. Resumption of the cell cycle following DNA damage repair is termed “checkpoint recovery” and must be distinguished from a separate process called “checkpoint adaptation” which refers specifically to the down-regulation of checkpoint signaling despite the persistence of DNA damage (Toczyski et al., 1997; Lee et al., 2003; Guillemain et al., 2007; Clemenson and Marsolier-Kergoat, 2009). The mechanisms responsible for recovery are still unclear but it involves the reversal of the post-translational modifications induced by the DNA damage checkpoint (Keogh et al., 2006; Nakada et al., 2008). More fundamentally, however, it is yet unknown whether checkpoint recovery is an actively regulated process (i.e. whether the proteins involved in checkpoint recovery are activated by DNA repair) or whether it is the consequence of the loss of the DNA lesions monitored by the DNA damage checkpoint.

One of the strongest mutations affecting recovery is the \textit{srs2Δ} mutation (Vaze et al., 2002). Deletion of \textit{SRS2} results in near-total abrogation of checkpoint recovery following repair of an HO endonuclease-induced DSB by either single-strand annealing (SSA) or break-induced replication (BIR) (Vaze et al., 2002; Jain et al., 2009). The best-characterized function of Srs2 is its role as an anti-recombinase that disassembles Rad51 presynaptic filaments during homologous recombination (Krejci et al., 2003; Veaute et al., 2003; Marini and Krejci). While DSB repair by SSA/BIR is normal in the \textit{srs2Δ} strain, deletion of the recombinase \textit{RAD51} rescues the \textit{srs2Δ} checkpoint recovery defect (Vaze et al., 2002). This puzzling observation suggests that Rad51 may play an uncharacterized, positive role in promoting the DNA damage checkpoint.

In this study, I examined the accumulation and disassembly of checkpoint
complexes during the course of checkpoint arrest and recovery. I found that the Ddc2-Mec1 complex remained associated with chromatin after DSB repair in checkpoint recovery mutants, correlating with checkpoint arrest. By contrast, the 9-1-1 complex was disassembled normally in checkpoint recovery mutants, indicating that the accumulation of 9-1-1 at DSB sites was not necessary to maintain the checkpoint. Moreover, I found that the role of Srs2 in promoting checkpoint recovery is linked to its ability to remove Rad51 from DNA in order to eliminate the main source of the checkpoint signal, ssDNA. I propose that the termination of the DNA damage checkpoint is linked to the elimination of ssDNA.

4.2 Ddc2-Mec1 and Dpb11, but not 9-1-1, DNA damage foci correlate with checkpoint recovery

To examine the fate of chromatin-associated checkpoint complexes during checkpoint recovery, I utilized a system described in Vaze et al. (2002) and depicted in Fig. 1A. In the YMV2 system, an HO cleavage site is ectopically inserted within the LEU2 gene on ChrIII (creating the leu2-cs site). In addition, a partial LEU2 duplication (u2) is inserted at the HIS4 locus approximately 30 kb away from the HO site, creating his4-u2 (referred to here as u2). This duplication provides a template for the homology-directed repair of the DSB by SSA (Vaze et al., 2002) or, as recently shown, by BIR (Jain et al., 2009). The HO endonuclease is under the control of the GAL1/10 galactose-inducible promoter. Upon addition of galactose to the media, HO is expressed, creating a DSB that is resected to generate ssDNA, a signal for checkpoint activation. The checkpoint is terminated shortly after DNA repair is completed (Vaze et al., 2002). The
YMV80 strain is highly similar to YMV2 except that the distance between *leu2-cs* and *u2* is 25 kb. In this study, all strains are isogenic to YMV2 unless otherwise indicated.

The YMV2 strain was engineered to express either the Ddc2-GFP or Ddc1-RFP fusion proteins as a means to monitor the accumulation of the Mec1-Ddc2 and 9-1-1 complexes, respectively, at sites of DNA damage. Prior to addition of galactose, 3.7% of cells had a Ddc2-GFP focus. These foci likely correspond to spontaneous DNA lesions (Melo et al., 2001). Following HO induction, the proportion of cells displaying a Ddc2-GFP focus steadily increased to reach a maximum of 71%, 3 h post-induction. After reaching this peak, the proportion of cells containing a focus decreased over the remainder of the experiment, to reach background levels by the 24 h time point (Figure 4.2.1). The onset of Ddc2 focus disassembly correlated well with the inactivation of Rad53 kinase activity (Figure 4.2.2A) and the appearance of the DNA repair product, as seen by Southern blot analysis with the related strain, YMV80 (Figure 4.2.2B).

I next examined Ddc2 foci dynamics in *srs2Δ* cells that exhibit defective checkpoint recovery (Vaze et al., 2002). I observed that *srs2Δ* cells were unable to down-regulate Rad53 during the course of a 24 h-experiment (Figure 4.2.2A) and were also severely hampered in their ability to dismantle Ddc2-GFP foci, with 60% of cells retaining a focus at the 24 h time point (Figure 4.2.1). These results indicated that the maintenance of Ddc2-Mec1 foci correlates with checkpoint signaling.

I also examined Ddc1-RFP foci following DSB induction in the YMV2 wild type and *srs2Δ* strains to understand the fate of the checkpoint clamp during recovery. In both
**Figure 4.2.1.** Ddc2-GFP focus dynamics following DSB induction and repair. (A) YMV2 *DDC2-GFP* wild type or *srs2Δ* cells were grown in the presence of galactose (Gal) to induce HO expression and were collected at the indicated time points. Ddc2-GFP foci were observed by microscopy. Representative plot of 3 independent experiments. Approximately 100 cells were imaged per time point. (B) Representative micrographs of the experiment in (A). DIC: difference interference contrast. Scale bar = 1 μm.
A

![Graph showing the percentage of budded cells with foci over time in Galactose (Gal) for Ddc2-GFP in wild type (DD1413) and srs2Δ (DD1633) strains.]

B

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Figure 4.2.1
Figure 4.2.2. Monitoring recovery from the DNA damage checkpoint as measured by Rad53 kinase activity and DNA repair. (A) \textit{In situ} kinase assay for Rad53 activity in YMV2 and YMV2 \textit{srs2}Δ cells. Pgk1 immunoblotting is used to control for protein loading. (B) Southern blot analysis with wild type, \textit{srs2}Δ cells using a probe directed to \textit{LEU2} to measure HO cutting and DSB repair. Strains are isogenic to YMV80.
strains, Ddc1-RFP foci were disassembled shortly after reaching a maximum of 90% of budded cells with foci, 3 h post-HO induction, in both wild type and srs2Δ cells (Figure 4.2.3A). Since the YMV2 srs2Δ DDC1-RFP strain had a strongly reduced capacity to form colonies on galactose (Figure 4.2.3D) and Rad53 dephosphorylation was severely impaired (Figure 4.2.3E) similar to that of the YMV2 srs2Δ strain, I could exclude the possibility that the near-wild-type disassembly observed in Ddc1-RFP-expressing srs2Δ cells was caused by the RFP fusion (Figure 4.2.3). I also observed similar foci kinetics in strains expressing DDC1-GFP (Figure 4.2.3, B). From these observations, I conclude that the focal persistence of the 9-1-1 complex is not necessary for the maintenance of a checkpoint signaling; rather, its disassembly correlates with DSB repair.

The loss of Ddc1 foci in recovery-deficient cells was surprising given that Ddc1 is critical for checkpoint activation (Melo et al., 2001; Bonilla et al., 2008; Navadgi-Patil and Burgers, 2009b). Since one function of Ddc1 is to recruit Dpb11 to the vicinity of the DSB to activate Mec1 (Navadgi-Patil and Burgers, 2009b), I examined whether Dpb11 focus dynamics mirrored that of Ddc1 or Ddc2. I introduced DPB11-GFP into the YMV2 wild type and srs2Δ strains and monitored focal dynamics following an HO-induced DSB. I found that Dpb11-GFP foci were clearly maintained in the srs2Δ strain (Figure 4.2.4AB). Since recruitment of Dpb11 is dependent on the 9-1-1 complex (Navadgi-Patil and Burgers, 2009b), these observations suggest that the 9-1-1 complex is dispensable once Dpb11 has been loaded on chromatin.
**Figure 4.2.3.** Ddc1-RFP focus dynamics following DSB induction and repair. (A) YMV2

*DDC1-RFP* wild type or *srs2Δ* cells were grown in the presence of galactose (Gal) to
induce HO expression and were collected at the indicated time points. Ddc1-RFP foci
were quantified by microscopy. The quantification is representative of 3 experiments.
Approximately 100 cells were scored for foci. (B) Same as (A) except the experiment
was performed with cells expressing DDC1-GFP. (C) Representative micrographs of the
experiment in (A). BF: bright field. Scale bar = 1 µm. (D) DSB sensitivity of YMV2
strains expressing Ddc1-RFP. Indicated strains isogenic to YMV2 were spotted in 1/5
serial dilutions on media containing either glucose (no HO expression) or galactose (HO
expression). (E) Rad53 phosphorylation detected by anti-Rad53 immunoblotting in
YMV2 and YMV2 *srs2Δ* cells expressing Ddc1-RFP.
Figure 4.2.4. Dpb11-GFP focus dynamics following DSB induction and repair. (A) YMV2 *DPB11-GFP* wild type or *srs2Δ* cells were grown in the presence galactose (Gal) to induce HO expression and were collected at the indicated time points. Ddc1-GFP foci were quantified by microscopy. The quantification is representative of 3 experiments. Approximately 100 cells were scored for foci. (B) Representative micrographs of the experiment in (A). BF: bright field. Scale bar = 1 µm.
Figure 4.2.4
4.3 Ddc2, not Ddc1, is sustained on chromatin in srs2Δ mutants

While the maintenance of the Mec1-Ddc2 complex on chromatin in checkpoint recovery mutants correlates with checkpoint signaling, bulk chromosome repair occurs normally (Figure 4.2.2B). To understand how Ddc2 foci are maintained in srs2Δ cells, I asked whether Ddc2 remained associated with the chromatin that surrounded the initial break, after DNA repair. I carried out chromatin immunoprecipitation (ChIP) with an epitope-tagged Ddc2-13myc and assessed accumulation of Ddc2-13myc on ChrIII by hybridizing immunoprecipitated DNA onto a tiled Saccharomyces cerevisiae genome array (a process known as ChIP-chip) (Figure 4.3.1A). I found that prior to DSB induction, there was little enrichment of Ddc2-13myc on ChrIII. In contrast, at 3 h following DSB induction, I observed a robust accumulation of Ddc2-13myc spanning a ~45 kb domain encompassing the DSB. In wild type cells, at 21 h post-DSB induction, when Rad53 phosphorylation was no longer detectable (Figure 4.3.1B), Ddc2-13myc enrichment on chromatin was largely eliminated. However, in srs2Δ cells at the 21 h time point, Ddc2-13myc remained enriched, although the enrichment had somewhat decreased and the domain had spread to ~100 kb. I confirmed these observations by performing ChIP followed by quantitative real-time PCR (ChIP-qPCR) at five loci surrounding the HO cleavage site (Figure 4.3.1C). For example, at 21 h post-galactose induction, all four probes covering the left arm of ChrIII showed Ddc2-13myc enrichment. Therefore, in recovery-deficient srs2Δ cells, Ddc2 remains associated with a large domain surrounding the initial DSB, even after repair is completed.

Next, I confirmed the observation that Ddc1 is removed from chromatin after bulk
**Figure 4.3.1.** Chromatin association of Ddc2-13myc following DSB induction and repair. (A) Genome browser captures of averaged and smoothed log\(_2\) enrichment ratios for Ddc2-13myc (enrichment was calculated as a signal to background ratio obtained following immunoprecipitation in the Ddc2-13myc strain over that of an isogenic untagged strain) for ChrIII in YMV2 wild type or srs2Δ strains grown in the presence of galactose or glucose. Samples were taken at the indicated time points and processed for ChIP-chip. Chromosomal positions are indicated in kilobases (kb). I note that the gap in signal at the 75 kb position corresponds to the presence of a LTR transposon to which probes were not available. The enrichment ratios shown were averaged from multiple experiments (0 h, n = 2; 3 h and 21 h, n = 3). (B) Rad53 immunoblotting of protein samples taken from the experiment shown in panel (A). Gal: galactose. (C) YMV2 DDC2-13myc wild type or srs2Δ cells grown in the presence of galactose (Gal) to induce HO expression were collected at the indicated time points and subjected to ChIP-qPCR. The x-axis represents the position of the amplicons relative to the leu2-cs locus. Enrichment at the TSC11 locus was used as control and fold-enrichment was determined using the ΔΔCt method. Data are presented as mean ± SEM (n = 3).
Figure 4.3.1
chromosome repair by performing similar ChIP-qPCR experiments with YMV2 and YMV2 srs2Δ strains engineered to express Ddc1-13myc. In both strains, Ddc1-13myc was enriched around the HO-induced DSB 3h following HO induction, but by 21 h, Ddc1-13myc enrichment is no longer detectable by qPCR in either wild type or srs2Δ strains (Figure 4.3.2).

4.4 ssDNA may persist in the srs2Δ mutant

The persistent enrichment of Ddc2-13myc in srs2Δ cells following repair was puzzling since repair of the HO-induced DSB by SSA or BIR should result in the deletion of the chromosome segment between leu2-cs and u2 (Figure 1.5.1,C) and remove all sources of ssDNA. Since bulk chromosome repair of the HO-induced DSB is normal in the srs2Δ mutant (Figure 4.2.2B), I hypothesized that ssDNA, either on the chromosome or as part of extra-chromosomal fragments, persisted in the srs2Δ mutant.

To examine the presence of ssDNA, I engineered a strain that co-expressed Ddc2-YFP and Rfa1-CFP, a subunit of RPA, since RPA can be used marker of ssDNA (Lisby et al., 2004). I monitored the formation and disassembly of foci formed by the two proteins over the course of the checkpoint and recovery (Figure 4.4.1AB). In wild type cells, Ddc2-YFP and Rfa1-CFP formed foci that were dismantled upon the onset of DNA repair and checkpoint recovery. Moreover, in all cases examined, Ddc2-YFP foci colocalized with Rfa1-CFP foci. Intriguingly, the reverse did not hold true as, for example, 10.2% of cells with an Rfa1-CFP focus, 1 h post-HO induction, did not have a Ddc2-YFP focus. In srs2Δ cells, I observed the same colocalization between Ddc2-YFP
Figure 4.3.2. Chromatin association of Ddc1-13myc following DSB induction and repair. YMV2 DDC1-13myc wild type or srs2Δ cells grown in the presence of galactose (Gal) to induce HO expression were collected at the indicated time points and subjected to ChIP-qPCR. The x-axis represents the position of the amplicons relative to the leu2-cs locus. Enrichment at the TSC11 locus was used as control and fold-enrichment was determined using the ∆∆Ct method. Data are presented as mean ± SEM (n = 3).
Figure 4.4.1. RPA and Ddc2-YFP foci dynamics following the induction and repair of a DSB. (A) Wild type or srs2Δ YMV2 RFA1-CFP DDC2-YFP cells were grown in the presence of galactose (Gal) to induce HO expression and were collected at the indicated time points. Ddc2-YFP and Rfa1-CFP foci were quantified by microscopy. The experiment was repeated at least 3 times. Approximately 100 cells were scored per time point. (B) Representative micrographs of the experiment in (A). DIC = difference interference contrast. Scale bar = 1 µm.
Figure 4.4.1
and Rfa1-CFP foci, which persisted beyond the completion of chromosome repair. Since RPA binds to ssDNA, this observation suggested that ssDNA persists and participates in the maintenance of Ddc2-Mec1 and checkpoint signaling in the srs2Δ mutant.

To confirm the persistence of RPA on chromatin, I performed ChIP-qPCR analysis with an antibody that recognizes endogenous Rfa1 (Figure 4.4.2). Prior to galactose induction, Rfa1 was not enriched at any of the 5 loci examined on ChrIII. 3 h following galactose induction, Rfa1 was enriched around the DSB. In agreement with the Rfa1-CFP foci observations, I detected Rfa1 enrichment around the location of the DSB in the srs2Δ mutant but not in the wild type at 21 h after galactose induction. The Rfa1 enrichment at 21 h in srs2Δ cells had spread beyond the DNA between leu2-cs and u2 in a manner similar to the spread of Ddc2-13myc. Although these studies are an indirect measure of ssDNA, they suggest that ssDNA (and RPA filaments) does indeed persist in srs2Δ cells.

Next, to test whether ssDNA persisted in srs2Δ cells more directly, I quantified the relative levels of five loci located around leu2-cs and u2 by qPCR following break induction (Figure 4.4.3A). In particular, I followed two loci, FRM2 and KCC4, which are located between the duplicated leu2-cs and u2 sequences and which should be deleted following DNA repair. The other three loci flank the leu2-cs/u2 interval and should remain unchanged. The amount of DNA at each locus was normalized to that of TSC11, a gene located on ChrV.

As expected from DSB repair via SSA or BIR, I observed that in the wild type strain, the FRM2 and KCC4 loci were depleted over time, compared to the RRP7, LEU2
Figure 4.4.2. Chromatin enrichment of Rfa1 at a DSB. YMV2 wild type or srs2Δ cells grown in the presence of galactose (Gal) to induce HO expression were collected at the indicated time points and subjected to ChIP-qPCR using a yeast RPA antibody. The x-axis represents the position of the amplicons relative to the \textit{leu2-cs} locus. Enrichment at the \textit{TSC11} locus was used as control and fold-enrichment was determined using the \Delta\DeltaCt method. Data are presented as mean ± SEM (n = 3).
Figure 4.4.3. qPCR analysis of ChrIII following a DSB. (A) Relative location of qPCR amplicons across ChrIII (not to scale). DNA content was determined relative to the control locus TSC11 on ChrV. (B) qPCR-based quantification of the indicated 5 loci from genomic DNA prepared from wild type, srs2Δ, rad1Δ, rad51Δ srs2Δ, and rad51Δ cells grown in the absence (0 h) or presence of galactose (Gal) to induce HO expression at the indicated time points. Data are presented as mean ± SEM (n = 3) (C) Histogram of the qPCR-based quantification of the FRM2 and KCC4 loci in YMV2 srs2Δ cells 21 h post-galactose induction. Data are presented as mean ± SEM (n = 3) and were taken from the experiments shown in (B).
Figure 4.4.3
and GPB2 loci which lie outside the predicted deleted region between leu2-cs and u2 (Figure 4.4.3B). In stark contrast, when the same analysis was repeated in the srs2Δ strain, I failed to observe a similar level of depletion of the FRM2 and KCC4 loci. However, I did observe an approximate 2-fold depletion for the FRM2 and KCC4 loci (2.4-fold for FRM2 and 2.6-fold for KCC4), which is consistent with both loci being converted into ssDNA (and therefore losing 50% of the signal) (Figure 4.4.3C). These results suggested that the DNA located between the duplicated leu2 sequences persisted as ssDNA. In parallel, I also quantified the relative abundance of the same five loci in a rad1Δ strain (Figure 4.4.3B). In this strain, the ssDNA flaps produced by end-resection cannot be cleaved. I observed that FRM2 and KCC4 loci were not highly depleted in rad1Δ cells, phenocopying the result observed in the srs2Δ mutant.

Srs2 is a helicase/translocase that disrupts Rad51 nucleofilaments (Krejci et al., 2003; Veaute et al., 2003). Deletion of RAD51 suppresses the checkpoint recovery defect of the srs2Δ mutant, indicating that Rad51 might block checkpoint recovery (Vaze et al., 2002). In light of our observation that DNA between leu2-cs and u2 persisted in srs2Δ cells, I hypothesized that Rad51 protects ssDNA from elimination. To test this possibility, I examined whether the rad51Δ mutation restored the loss of the FRM2 and KCC4 loci in srs2Δ cells following bulk chromosome repair. In rad51Δ srs2Δ cells, I observed a 6- and 7.5-fold depletion of the FRM2 and KCC4 loci over the TSC11 locus, respectively, in stark contrast to the situation seen in srs2Δ cells (Figure 4.4.3B). While the extent of depletion was less than that observed in wild type cells (20- and 22-fold depletion, respectively), it was comparable to that observed in rad51Δ cells (7- and 10-fold depletion, respectively). In rad51Δ cells, the repair of the DSB occurs via Rad51-
independent SSA which is slower than Rad51-dependent SSA in wild type cells. By Southern blot analysis of chromosome repair, I observed that repair is delayed by approximately 3 h in rad51Δ mutants (Figure 4.4.4). The delay in repair most likely accounts for the lower amount of depletion observed in the rad51Δ and rad51Δ srs2Δ cells. Together, these results support a model where the failure to remove Rad51 following DSB repair protects ssDNA and results in continuous association of RPA-Ddc2-Mec1 with chromatin.

The interpretation that ssDNA persists in srs2Δ cells is somewhat complicated by the fact that srs2Δ cells do not divide following DSB induction while cells that undergo recovery do. This situation might artificially inflate the apparent depletion seen in wild type cells. However, I observed persistence of the FRM2 and KCC4 loci in srs2Δ cells at the time point closest to the onset of checkpoint recovery (12 h time point). Furthermore, I carried out time-lapse imaging of Ddc2-GFP in wild type and srs2Δ strains and observed that at the single-cell level, Ddc2-GFP foci (and by inference RPA and ssDNA) persisted for much longer in srs2Δ cells than in wild type cells (Figure 4.4.5AB). The mean duration of a Ddc2-GFP focus following DSB induction in a wild type cell was 251 ± 26 min. Foci in srs2Δ cells lasted much longer with a mean duration of 869 ± 88 min. The mean duration of Ddc2-GFP foci was likely underestimated in the srs2Δ condition since 29% of the foci were still present at the end of the time-lapse experiment (which lasted 1180 min). Spontaneous Ddc2-GFP foci were observed in a wild type strain that had already received and repaired a DSB and the mean duration of these foci was 125 ± 28 min, significantly less than wild type cells that received a DSB. Therefore, it is unlikely that the resumption of cell cycling in wild type cells explains the differences
Figure 4.4.4. Monitoring DSB repair by southern blot analysis. DNA was derived from wild type, srs2Δ, srs2Δ rad51Δ, and rad51Δ cells at the indicated time points following galactose (Gal) induction of HO. A radiolabelled probe directed to LEU2 to measure HO cutting and DSB repair was hybridized to resolved DNA immobilized on nitrocellulose. Strains are isogenic to YMV80. As a loading control, photographs of the agarose gels stained with ethidium bromide are provided beside each blot.
**Figure 4.4.5.** Real-time imaging of Ddc1-GFP foci in YMV2 and YMV2 \(srs2\Delta\) cells. (A) Live-cell imaging of Ddc2-GFP foci in YMV2 wild type and \(srs2\Delta\) cells. Mean elapsed time (minutes) of the duration of a single Ddc2-GFP focus. As a control, spontaneous Ddc2-GFP foci were tracked in a strain isogenic to YMV2 that had already repaired an HO DSB. Data presented are as mean ± 95% C.I. (B) Representative micrographs of live cells (wild type and \(srs2\Delta\)) expressing Ddc2-GFP following an induced DSB. The black scale bars in the bright field panel represent a distance of 1 µm.
Figure 4.4.5
seen by microscopy, ChIP-chip and qPCR.

Finally, I employed slot blotting coupled to Southern hybridization of native (non-denatured) genomic DNA [29] to directly test whether ssDNA persisted in the \textit{srs2}Δ strain following bulk DSB repair. I first validated the system using native and heat-denatured DNA using a probe directed against the \textit{KCC4} locus, which lies in the interstitial region between \textit{leu2-cs} and \textit{u2} and \textit{PGS1}, a locus that lies outside \textit{leu2-cs} and \textit{u2} on \textit{ChrIII} (Figure 4.4.6, A). Under native conditions, no signal was apparent with either probe when 10 µg of DNA was loaded, indicating that the native loading conditions preserved the double-stranded nature of DNA. I next isolated DNA from wild type, \textit{srs2}Δ and \textit{rad1}Δ YMV2 strains at various time points following HO induction and loaded slot blots with native and denatured DNA. By comparing the hybridization signal of the bands in the \textit{KCC4}- and \textit{PGS1}-probed blots, I found that in contrast to wild type cells, the \textit{srs2}Δ samples retained an observable \textit{KCC4} hybridization signal in the native samples, similar to that seen with the samples derived \textit{rad1}Δ, a strain that cannot complete DSB repair by SSA (Figure 4.4.6, B). The ssDNA signal in the \textit{rad1}Δ and \textit{srs2}Δ strains persisted up to 15 h post-HO induction and returned to background levels 24 h post-induction (Figure 4.4.6, BC). These results suggest that while ssDNA persists in the \textit{srs2}Δ strain, long after bulk DSB repair is completed, ssDNA levels decreased over time to bring the overall ssDNA levels below the detection limit of the Southern blot assay.
Figure 4.4.6. Slot blot analysis of ssDNA. (A) *KCC4* and *PGS1* dsDNA probe hybridization to equal amounts of DNA that were either native or denatured. (B) Slot blot analysis of native DNA probed with *KCC4* to observe ssDNA and denatured DNA probed with PGS1 as a loading control. (C) Quantification of *KCC4* signal over *PGS1* signal ($N_{KCC4}/D_{PGS1}$); n = 3.
Figure 4.4.6
4.5 Rad51 chromosomal association persists in the srs2Δ mutant

If the srs2Δ mutant cannot recover from checkpoint arrest because of the inability to remove Rad51 from DNA, then sustained Rad51 enrichment on chromatin should be detectable by ChIP. I tested this prediction by carrying out ChIP-chip of Rad51 in wild type and srs2Δ YMV2 strains (Figure 4.5.1A) and validated the results with ChIP-qPCR (Figure 4.5.1B). Prior to DSB induction, I observed that neither wild type nor srs2Δ cells showed significant Rad51 enrichment on chromatin. In contrast, 3 h after DSB induction, I detected a robust enrichment of Rad51, forming a ~150 kb domain surrounding the break site. Wild type cells had removed the great majority of Rad51 from chromatin by 21 h, 15 h after the appearance of repair products (Figure 4.2.2). However, in srs2Δ cells, I observed a striking persistence of Rad51 in a region that surrounded the HO cut site. I also noted that the domain of Rad51 enrichment in srs2Δ cells far exceeded the region deleted by SSA, in a manner similar to, but much more pronounced than, the enrichment of Ddc2 and Rfa1 (Figure 4.3.1 and Figure 4.4.2). Thus, Rad51 chromatin enrichment is sustained even following bulk chromosome repair in the srs2Δ mutant.

I surmised that the severe recovery defect of srs2Δ cells is the result of persistent Rad51 following bulk chromosome repair. I found that Rad51 has a profound effect on the persistence of ssDNA in srs2Δ cells (Figure 4.4.3). In the YMV2 strain, the HO-induced DSB can be repaired by SSA or BIR (Vaze et al., 2002; Jain et al., 2009). SSA can be accomplished by RAD51-dependent and -independent mechanisms, whereas BIR is strictly RAD51-dependent (Malkova et al., 1996). Alternatively, BIR can repair the DSB but this process is entirely Rad51-dependent (Jain et al., 2009). The choice between SSA and BIR appears to be regulated in part by the distance between the homologous
**Figure 4.5.1.** Persistent chromatin association of Rad51 in *srs2Δ* strains. (A) Genome browser captures of smoothed log2 enrichment ratios for Rad51 (calculated as Rad51 signal over that of control IgG) for *ChrIII* in the YMV2 wild type or *srs2Δ* strains grown in the absence (0 h) or presence of galactose. Samples were taken at the indicated time points and processed for ChIP-chip. Chromosomal positions are indicated in kilobases (kb). Plots are the average of 2 independent experiments. (B) Viability of the indicated YMV2 strains on glucose (no HO expression) or galactose (HO expression). (B) YMV2 wild type or *srs2Δ* cells grown in the presence of galactose (Gal) to induce HO expression were collected at the indicated time points. DNA immunoprecipitated from chromatin with an anti-Rad51 antibody was subjected to ChIP-qPCR. The x-axis represents the position of the amplicons relative to the *leu2-cs* locus. Enrichment at the *TSC11* locus was used as control and fold-enrichment was determined using the ∆∆Ct method. Data are presented as mean ± SEM (n = 3).
Figure 4.5.1
Figure 4.5.2. Survival of YMV2 pol32Δ and srs2Δ cells on galactose media. The indicated YMV2 strains were spotted in serial dilution on glucose (no HO expression) or galactose (HO expression) media.
sequences on the chromosome and BIR is favoured in YMV2 (Jain et al., 2009). Therefore, it was conceivable that the recovery defect of the \textit{srs2}\Delta mutation was simply due to the toxic engagement of the BIR pathway instead of our proposed persistence of ssDNA. To test this possibility, I abrogated BIR by deleting \textit{POL32}, which encodes a non-essential subunit of polymerase \(\delta\) (Lydeard et al., 2007). When \textit{srs2}\Delta and \textit{srs2}\Delta \textit{pol32}\Delta cells were plated on to media containing galactose to induce a DSB, both were equally defective in colony formation, thus indicating a failure of both strains to recover from the checkpoint arrest (Figure 4.5.2). The maintenance of the checkpoint in the \textit{srs2}\Delta mutation is therefore not caused via the engagement of BIR and I propose that Rad51-dependent persistence of ssDNA caused by the \textit{srs2}\Delta mutation is responsible for the inability of those cells to resume cell cycling.

4.6 Discussion

\textit{ssDNA triggers checkpoint activation and its elimination allows checkpoint recovery}

How cells coordinate DNA repair with the termination of DNA damage signaling remains an open question. In particular, it is still unknown whether there is an active signal emanating from repaired chromatin region to activate the phosphatases associated with checkpoint recovery or whether checkpoint recovery is simply the consequence of the loss of checkpoint-inducing DNA structures. Here, using cells lacking the Srs2 helicase, I provide evidence that checkpoint recovery is linked to the loss of ssDNA, the checkpoint signal and it is possible to genetically uncouple the degradation of ssDNA from DNA repair by deleting \textit{srs2}\Delta.

ssDNA bound by RPA is recognized as a potent activator of the DNA damage
checkpoint (Melo et al., 2001; Zou and Elledge, 2003). While it is not surprising that the persistence of ssDNA prolongs checkpoint arrest, I have provided evidence here that ssDNA must be actively removed in a Srs2-dependent manner independently of bulk chromosome repair. In the absence of Srs2, I observed the persistence of DNA damage-induced foci containing Ddc2, Rfa1, and Dpb11 (Section 4.2). A direct interaction with ssDNA is the likely explanation for the persistence of Ddc2 and RPA which is supported by the observation that Ddc1-YFP and Rfa1-CFP foci continue to colocalize even after bulk chromosome repair is completed (Figure 4.4.1).

Rad51 protects ssDNA from degradation during homologous recombination repair

The inability of srs2Δ cells to evict Rad51 from chromatin following repair can be attributed to the persistence of ssDNA and consequently, the maintenance of checkpoint proteins (Figure 4.5.1). I found that in srs2Δ cells, the ssDNA flaps generated by SSA or BIR are protected from degradation by Rad51, in at least a subset of cells (Figure 4.4.3). However, the source of ssDNA is likely not limited to the flaps themselves as we observed Ddc2 enrichment in regions outside the intervening leu2 and u2 sequences. Together, these results indicate that recovery from the DNA damage checkpoint requires the loss of Ddc2-Mec1 from chromatin, following the elimination of ssDNA either via DNA synthesis or the degradation of recombination intermediates such as flaps.

The previous observation by Vaze et al (2002) that deletion of RAD51 can rescue the srs2Δ recovery defect is entirely consistent with our model that Rad51 protects ssDNA from degradation. YMV2 cells are capable of repairing the HO-induced DSB by Rad51-independent SSA; the process of annealing is performed by RPA and this requires
resection to reveal ssDNA regions of homology. RPA-mediated annealing occurs more slowly than Rad51-dependent repair (Supplemental Fig. 1A) (Ivanov et al., 1996). I confirmed this by abrogating BIR in pol32Δ cells, which preferentially use Rad51-dependent SSA (Figure 4.5.2). Furthermore, it is possible that other forms of DNA repair that rely on Rad51 will also result in recovery defects. Indeed, Srs2 is also important for the resumption of the cell cycle following ectopic gene conversion (Vaze et al., 2002), a Rad51-dependent process associated with the generation of extensive ssDNA. Moreover, srs2Δ cells are sensitive to UV radiation and this sensitivity is rescued by rad51Δ as well (Schiestl et al., 1990; Aboussekhra et al., 1992), raising the intriguing possibility that the UV sensitivity of srs2Δ cells might in part be due to a checkpoint recovery defect. Furthermore, Vaze et al (2002) reported that srs2Δ cells are defective for checkpoint recovery following DSB repair by gene conversion. Since srs2Δ cells are also partially defective in repair by GC, it was not an ideal system to examine DNA damage foci disassembly in the same system. The YMV2/YMV80 SSA systems remain the ideal system to study recovery in srs2Δ mutants, as srs2Δ is not required to complete DSB repair in these strains.

Since the output of my ChIP studies represents the average signal of the cell population, this experimental system cannot answer the question of how much ssDNA persists in individual srs2Δ cells. This will be an important question to resolve in future studies in order to determine whether there is threshold of ssDNA under which checkpoint recovery occurs. Nevertheless, our studies suggest that ssDNA can either be chromosomal and flanking the repair site, or can exist as an extra-chromosomal fragment, as in the case of the SSA reporter strain used in this study.
The 9-1-1 checkpoint clamp is dispensable for checkpoint maintenance

In contrast to Ddc2, Ddc1 foci are removed independently of recovery and their disassembly correlated with bulk chromosome repair instead of checkpoint activity (Figure 4.2.3 and Figure 4.3.2). The behaviour of Ddc1 during recovery is strikingly different from that observed in cells that adapt to the checkpoint (Melo et al., 2001). Indeed, in response to a persistent, unrepaired HO break, the ends are continuously resected, providing ssDNA/dsDNA junctions for 9-1-1 loading irrespective of checkpoint activity. I speculate that this ongoing end resection explains the increase in Ddc1 focal intensity observed in cells that adapt to the DNA damage checkpoint (Melo et al., 2001). By contrast, in the YMV2 strains used in this study, end-resection is most likely terminated in a manner that is coupled to the cleavage of the SSA flaps and/or the filling-in of the ssDNA produced by resection past the duplicated region. Interestingly, very little is known about the manner by which end-resection is terminated and checkpoint recovery following SSA might be a useful system to study this process. The apparent lack of the 9-1-1 clamp despite ongoing checkpoint activity in srs2Δ cells suggests that the checkpoint clamp is only involved in initiating the checkpoint but not in maintaining it. Alternatively, our results could equally suggest that despite the initial accumulation of hundreds of 9-1-1 complexes following DSB formation, only a few molecules, under our detection limit, are necessary to maintain checkpoint signaling.

A possible role for Dpb11 in checkpoint maintenance

Even though Ddc1 foci were disassembled in checkpoint recovery mutants, Dpb11-GFP foci remained in the srs2Δ strain (Figure 4.2.5). The initial recruitment of
Dpb11 is dependent on Ddc1 (Navadgi-Patil and Burgers, 2009a, b). While I cannot rule out that a small number of Ddc1 molecules could remain associated with chromatin to sustain Dpb11, our results point to a yet-uncharacterized, 9-1-1-independent means by which Dpb11 association with chromatin and the Ddc2-Mec1 complex is stabilized. Our observations in the srs2Δ mutant suggest that checkpoint initiation and maintenance may be genetically separable. From our data, I conclude that ssDNA and Rad51 play an important role in determining how long the checkpoint is sustained.

I conclude that Srs2 promotes checkpoint recovery by enabling the disassembly of DNA damage foci from chromatin. While a direct role for Srs2 in the eviction of checkpoint proteins from chromatin remains to be determined, I have shown that through the retention of Rad51, checkpoint factors including Ddc2-Mec1, RPA, and Dpb11 remain chromatin-bound. Interestingly, FBH1 was shown to have anti-recombinogenic activities similar to Srs2, suggesting that they are functional homologs (Fugger et al., 2009). Therefore, the FBH1 protein is a potential candidate as a modulator of checkpoint termination in human cells. Since checkpoint failure and aberrant homologous recombination are both associated with tumorigenesis, such a protein could be an important genome caretaker.

4.6. Material and Methods

**Yeast strains and manipulations.** All yeast strains used in this study were isogenic to YMV2 unless otherwise noted (Vaze et al., 2002) (Table 4.6.1). Standard yeast media and manipulations were used. *DDC1-GFP* and *DDC2-GFP* constructs were obtained from the GFP-tagged ORF collection (Huh et al., 2003). C-terminal tags for Ddc1 were
amplified with oligos DDC1-f2 and DDC1-r1 containing a 4-alanine linker. For DDC1-
RFP, a cassette was amplified from pTY24 and for DDC1-13MYC, a cassette was
amplified from pFA6a-13MYC-KANMX. YFP tagging of DDC2 generated from a PCR-
amplified cassette from pDH6 with oligos DDC2-YFP-F and DDC2-YFP-R. 13MYC
tagging of DDC2 containing a 4-alanine linker was PCR-amplified from pFA6a-13MYC-
KANMX using with DDC2-AAAA-F and DDC2-AAAA-R. CFP tagging of RFA1 was
generated from a PCR-amplified cassette from pDH3 with RFA1-f2 and RFA1-r1.
pTY24, pDH3, and pDH6 plasmids were obtained from the Yeast Resource Centre
(Wach et al., 1997). pFA6a-13MYC-KANMX was a gift from J. Pringle (Longtine et al.,
1998). Oligos are listed in Table 4.6.2.

**Microscopy and Foci Assays.** Cells were fixed in 4% (w/v) paraformaldehyde/0.1 M
sucrose for 15 min in the dark at room temperature, then stored and image in a 1.2M
sorbitol, 0.1M sodium phosphate (pH 7.5) buffer. Cells were mounted on Fisherbrand
microscope slides and coverslips (12-550A; 12-542B). For GFP, CFP, and YFP
fluorophores, I captured images at room temperature with a Nikon E600 FN microscope
([http://www.nikonusa.com](http://www.nikonusa.com)) equipped with an ORCA ER2 camera
([http://www.hamamatsu.com](http://www.hamamatsu.com)), Chroma filters (YFP: JP4 C/YFP, dichroic 86002v2, batch
C37370, excitation = 500 ± 20 nm, emission 535 ± 30 nm; GFP: 41025 PSTN GFP,
dichroic Q495LP, batch C37368, excitation = 470 ± 40 nm, emission = 515 ± 30 nm;
[http://www.chroma.com/](http://www.chroma.com/)) and a 100X/1.4 oil emersion Nikon Plan Apo objective.
Approximately 100 cells from each time point were scored for foci. Images were
processed and deconvolved with Velocity v5.3.0. Ddc1-RFP cells were imaged on a
Leica DMIRE2 microscope equipped with a 60X objective. Images were acquired and
processed with Velocity v5.3.0.

**ChIP and ChIP-chip.** ChIP-chip experiments were performed as previously described (Szilard et al., 2010) with the following modifications. Enrichment was determined by co-hybridizing Cy5-labelled experimental DNA and Cy3-labelled control DNA onto a 4 x 44K Agilent *S. cerevisiae* array (Agilent Technologies, http://www.home.agilent.com/). For Ddc2-13myc ChIPs, immunoprecipitations were performed with a 9E10 anti-myc antibody (Santa Cruz Biotechnology, sc-40). Antibodies were coupled to pan-mouse IgG Dynabeads (Invitrogen). Control DNA was immunoprecipitated from an isogenic strains lacking an epitope tag. For Rad51 ChIP-chips, immunoprecipitations were performed with a Rad51 antibody (Santa Cruz Biotechnology, y-180). Anti-Rad51 antibodies were coupled to Protein G Dynabeads (Invitrogen). Control DNA was isolated from a mock IP without antibody. Analysis was completed as previously described (Kanellis et al., 2007). Arrays were scanned with a GenePix 4000B microarray scanner and analyzed with GenePix Pro 6.0 software. Datasets were processed with the Location Analysis Net Application whose access was kindly provided by Dr. François Robert at the Institut de recherches cliniques de Montréal. Results were mapped using the University of California Santa Cruz genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). ChIP experiments for Rfa1 and Ddc1-13myc proteins were performed identically except the purified DNA was immediately subjected to qPCR analysis instead of fluorescence labelling and array hybridization. Rfa1 was immunoprecipitated with a Rfa1 antibody (generous gift of J. Ingles) and Ddc1-13myc was immunoprecipitated by the 9E10 anti-myc antibody (as above). A consolidated BED file with the primary data is available upon request.
**Slot blotting and ssDNA Southern blot assay.** Native and denatured genomic DNA were slot blotted on to a nitrocellulose membrane as previously described (Sugawara and Haber, 2006) with the following modifications. 10 µg of DNA in 200 µL of 10X SCC was loaded per well. dsDNA probes were radiolabelled and hybridized to membrane-bound DNA. A dsDNA probe at the KCC4 locus was used to determine the amount of ssDNA in native genome DNA samples (NKCC4). As a loading control, denatured DNA was probed with a probe directed to PGS1 (DPGS1), an unaffected locus in the recovery assay. The relative amount of ssDNA remaining at KCC4 was determined by a ratio of NKCC4/DPGS1. Band intensities were measured with ImageQuant 5.0 software provided by Molecular Dynamics.

**Quantitative real-time PCR (qPCR).** qPCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on the Applied Biosystems 7500 Fast Real-Time PCR System. In all cases, fold-enrichment at a query locus was determined by comparison to a control locus, TSC11, between total DNA and IP DNA with the ΔΔCt method.
Table 4.6.1. Strains used in Chapter IV.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>YMV2</td>
<td><em>ho hml::ADE1 MATa::hisG hmr::ADE1 his4::URA3-leu2(Xhol-to Asp718) - pBR322-his4 leu2::HOcs ade3::GAL:HO ade1 lys5 ura3-52 trp1 (trp1::hisG)</em></td>
<td>Vaze et al., 2002</td>
</tr>
<tr>
<td>(DD355)</td>
<td><strong>DD972</strong> As DD355, except his4::TRP3-leu2(Xhol-to Asp718)</td>
<td>Keogh et al., 2005</td>
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<tr>
<td>YMV80</td>
<td><em>ho hml::ADE1 mata::hisG hmr::ADE1 his4::NAT-leu2(Asp718-SalI) leu2::HOcs ade3::GAL:HO ade1 lys5 ura3-52 trp1</em></td>
<td>Vaze et al., 2002</td>
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<td>(DD2781)</td>
<td><strong>DD1413, DD1504</strong> As DD355 except <em>DDC2-GFP-NATMX3</em></td>
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<td></td>
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<td><strong>DD2202, DD2203</strong> As DD972 except <em>DDC2-13myc-KANMX6</em></td>
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<td><strong>DD2204, DD2205</strong> As DD2202 except <em>srs2::NATMX3</em></td>
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<td></td>
<td><strong>DD1470, DD1471</strong> As DD355 except <em>srs2::KANMX6</em></td>
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<td><strong>DD2215</strong> <em>ho hml::ADE1 MATa::hisG hmr::ADE1 his4::URA3-leu2(Xhol-to Asp718)-leu2 ade3::GAL:HO ade1 lys5 ura3-52 trp1 (trp1::hisG)</em></td>
<td>This study</td>
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<td></td>
<td><strong>DD3048</strong> as DD972 except <em>DPB11-GFP-NATMX3</em></td>
<td>This study</td>
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<tr>
<td></td>
<td><strong>DD3050</strong> as DD except <em>srs2::KANMX6</em></td>
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Table 4.6.2. Primers used in Chapter IV.

<table>
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<tr>
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<tr>
<td>DDC1-f2</td>
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<tr>
<td>DDC1-r1</td>
<td>CTACTTCATCTAATATTTACACGCTTTTATACTGATTTTGCAATATGGTTGATTAGCGCTTCGTTAAAC</td>
</tr>
<tr>
<td>DDC2-YFP-F</td>
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</tr>
<tr>
<td>DDC2-YFP-R</td>
<td>GGTGAGCGGAGGATTCGAGCTCG</td>
</tr>
<tr>
<td>DDC2-AAAA-F2</td>
<td>TAAATTATATATAGTTAATATGATCAAGATTACAGGTTCTATAAAGCGTTA</td>
</tr>
<tr>
<td>DDC2-AAAA-R1</td>
<td>CCGATCCCCCGGGTTAATTAA</td>
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<td>RFA1-f2</td>
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<tr>
<td>RFA1-r1</td>
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</tr>
<tr>
<td>KCC4-A</td>
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<tr>
<td>KCC4-B</td>
<td>GACAGGGAAGCAGCGATT</td>
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<tr>
<td>GPB2-C</td>
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<td>GPB2-D</td>
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<td>LEU2-B</td>
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<td>RRP7-C</td>
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<td>RRP7-D</td>
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<td>FRM2-D</td>
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<td>TSC11-B</td>
<td>GTTGCCCGCTTCAGTTGTTG</td>
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Table 4.6.2
Chapter V

Thesis Summary and Future Directions

5.1 Summary

Much of my thesis work has focused on two checkpoint complexes that are essential for initiation of DNA damage signaling: Mec1-Ddc2 and the 9-1-1 checkpoint clamp. First, I presented initial characterization of a residue, Rad17-K197, which represents a potential novel receptor site for Rad18-directed ubiquitylation. A rad17-K197R mutant is severely defective in assembling Ddc1-GFP foci and is mildly impaired in robustly phosphorylating Rad53 in response to DSBs (Figure 2.3.3), representing a previously unknown mechanism of regulation of DNA damage signaling. Next, I tested the hypothesis that DNA damage focus disassembly is an important step in checkpoint recovery. The identification of the recovery mutant pph3Δ provided a useful system to examine the hypothesis. Observations of Ddc1-GFP and Ddc2-GFP foci revealed that Ddc2-GFP focus disassembly, but not Ddc1-GFP, correlated with checkpoint recovery (Figure 3.3.1 and Figure 3.3.3). Moreover, experiments with h2a-S129A mutants indicated that γH2A is a positive regulator of DNA damage signaling (Figure 3.2.5 and Figure 3.3.1), in addition to its role as a regulator of genome stability (Figure 3.6.1 and Figure 3.6.2). Finally, I was able to uncover the mechanism by which the helicase Srs2 promotes checkpoint recovery, a long-standing enigma. Cells lacking Srs2 are unable to disassemble Rad51 nucleofilaments (Figure 4.5.1) and subsequently, are unable to eliminate ssDNA which acts as a nucleating factor for continual Mec1-dependent DNA
damage signaling (Figure 4.4.6). This mechanism extends our understanding of the relationship between DNA repair and DNA damage signaling.

5.2 Future Directions

Understanding 9-1-1 localization on damaged chromatin

9-1-1 is loaded on to DSBs at the ssDNA/dsDNA junction by the alternate clamp loading complex Rad24-Rfc2-5 (Majka and Burgers, 2003). When a single DSB is experimentally induced, there are 2 ssDNA/dsDNA junctions that are generated by resection (one on either side of the break). Yet, Ddc1-GFP foci increase in intensity for as long as the DSB is present, indicating continual 9-1-1 loading and accumulation on to DNA. The checkpoint clamp, like PCNA, can slide along ssDNA and dsDNA. It is currently unknown how 9-1-1 is maintained in the vicinity of the DNA damage. ChIP analysis of Ddc1 chromatin enrichment indicated that 9-1-1 is present at both the ssDNA/dsDNA junction and on ssDNA portions of the resected chromosome (Figure 2.3.6); however, the 2 amplicons reported in this chapter provide only a limited view of 9-1-1 dynamics on chromatin as resection progresses. An approach to examine 9-1-1 enrichment on chromatin more thoroughly is ChIP followed by whole-genome analysis of chromatin enrichment (ChIP-chip or ChIP-seq, for example). Such an approach, with frequent time points following DSB induction, will yield valuable insight into the behaviour of 9-1-1 as resection progresses and more generally, the factors that restrict and control DNA damage focus size.
Exploring the role of Rad18 in the DSB response

A role for Rad6-Rad18 in the DNA damage response was originally identified in a screen for mutants that disrupted gene up-regulation in response to MMS (Fu et al., 2008). The effect of rad6Δ and rad18Δ on gene transcription was independent of POL30, the only known substrate of Rad6-Rad18 (Hoege et al., 2002), suggesting that Rad6-Rad18 functions in a different pathway other than the canonical translesion synthesis pathway. The 9-1-1 clamp was a logical substrate to examine based on structural similarity and involvement in the DNA damage response, however, DNA damage dependent 9-1-1 ubiquitylation remains controversial (Davies et al., 2010). DNA damage generated by MMS is likely to be heterogenous. Some DNA damage can be handled by the post-replication repair pathway, a pathway where Rad18 plays a prominent role (Hoege et al., 2002). Furthermore, MMS-induced damage can lead replication stress and potentially fork collapse into DSBs (Lundin et al., 2005). In this respect, a screen with MMS was not optimal to reveal regulators of 9-1-1 in a DSB response. Therefore, a screen for genes that regulate gene transcription following a DSB or multiple DSBs may produce more insightful information.

A yeast-2-hybrid experiment revealed a physical interaction between Rad18 and Rad17 (Fu et al., 2008). Characterizing the nature of the interaction could generate important insight into the mechanism by which Rad18 acts on Rad17. Specifically, mutations in the Rad18 RING and SAP domains along with rad17-K197R should be examined for the fidelity of the Rad18-Rad17 interaction. Results from physical mapping of the Rad18-Rad17 interaction could reveal important clues of the nature of the relationship between the two proteins. Specifically, recent evidence has implicated Rad18
in NHEJ repair of DSBs along with translesion polymerases (Hirano et al., 2009). Therefore, this direction could provide important insight into how Rad18 participates in NHEJ.

*Regulation of Pph3*

Pph3 must be regulated to prevent premature dephosphorylation of its checkpoint substrates. One possibility is a checkpoint-dependent suppression mechanism. There is a SQEL motif beginning on residue 29 which is a possible target of Mec1 and Tel1. Examination of Rad53 and H2A phosphorylation over the course of the checkpoint in a pph3-S29A mutant might reveal important clues about the regulation of the phosphatase. Other candidates for Pph3 regulation may include Psy2 and Psy4, the other components of the phosphatase complex that targets γH2A.

*Reversal of post-translational modification to enable checkpoint recovery*

The DNA damage checkpoint signal transduction cascade involves the phosphorylation of many proteins. In addition to the formation of γH2A, several other DNA-damage induced histone modifications have been identified including the phosphorylation of H2A on serine 122 (Harvey et al., 2005), H2B on serine 14 (Fernandez-Capetillo et al., 2004a), and H4 on serine 1 (Cheung et al., 2005; Altaf et al., 2007). Also, H2A directs NuA4-dependent H2A acetylation (Downs et al., 2004). The Rad9 tudor domain directly binds di-methylated H3 on lysine 79, and although this chromatin association appears to be constitutive, mutations in the tudor domain that disrupt binding have similar cell cycle checkpoint defects as h2a-S129A cells (Wysocki et al., 2005; Hammet et al., 2007). The current understanding of these chromatin
modifications is that H3 diMe-K79 allows for constitutive Rad9 chromatin association while γH2A directs Rad9 relocalization to sites of DNA damage (Hammet et al., 2007). How these histone marks are reversed and how they participate in checkpoint maintenance are important questions to address in the future.

I provided evidence that Pph3 can participate in the dephosphorylation of γH2A and Rad53 (Section 3.2 and Section 3.4). In addition to Pph3, the Ptc2 and Ptc2 PP2A-like phosphatases have also been implicated in the dephosphorylation of Rad53 to promote recovery and adaptation (Leroy et al., 2003). Other proteins such as Rad9 are also phosphorylated. How Rad9 is inhibited in conjunction with Rad53 inactivation is a question that has not been addressed. One possibility is that Rad9 might be dephosphorylated for recovery. Indeed, a Rad9 phospho-dependent mobility shift is sustained in pph3Δ cells (Keogh et al., 2006), although direct biochemical evidence is unavailable to prove that Rad9 is a substrate of Pph3. Continuous Mec1 signaling resulting from elevated levels of γH2A might also sustain phosphorylated Rad9. The fission yeast homolog of Rad9, Crb2, is regulated in part by CDK phosphorylation as well (Nakamura et al., 2005). Exploration of Rad9 CDK sites may improve our understanding of how Rad9 is regulated by phosphorylation. Alternatively, Rad9 could be degraded by proteolysis. For example, the mediator protein Claspin is degraded prior to the onset of recovery after genotoxic stress in mammalian cells (Mamely et al., 2006). As the key mediator of Rad53 activation, Rad9 inhibition may be an important step in checkpoint recovery.

The DNA damage response imparts many different post-translational modifications including phosphorylation and ubiquitylation. It remains to be determined
if prolonged maintenance of these modifications have significant ramifications on the duration of the checkpoint. Chk1 is another conserved effector kinase of the checkpoint (Sanchez et al., 1999). In fission yeast, Dis2 removes phosphorylation on Chk1 imparted by the Rad3 kinase (homolog of S. cerevisiae Mec1) to promote recovery (den Elzen et al., 2004; den Elzen and O'Connell, 2004). Glc7, the Dis2 homolog in budding yeast, is also required for recovery from the DNA damage checkpoint, but its role in regulating Chk1 has not been explored (Bazzi et al., 2010). Instead, the report of Bazzi et al (2010) shows that Glc7 counteracts γH2A. While protein ubiquitylation appears to play a less critical role in the yeast DNA damage checkpoint, ubiquitylation clearly is important for the establishment of the checkpoint in mammals (Kolas et al., 2007; Stewart et al., 2009). Furthermore, the deubiquitylating enzyme OTUB1 was identified in our lab as a protein required for reversal of ubiquitin modifications established by the checkpoint (Nakada et al., 2010). The reversal of post-translation modifications imparted by the DNA damage checkpoint remains an active area of investigation.

*Synthetic genetic screens and proteomic approaches to understand γH2A function*

Two independent screens for genetic interactions with *PPH3* failed to identify synthetically lethal interactions (Table 3.6.1) (Keogh et al., 2006). The redundancy of Pph3 with two other PP2A phosphatases, Ptc2 and Ptc2, might explain the high tolerance of *pph3Δ* cells to disruptions in cooperative genetic pathways. Notably, many of the mild interactions were greatly enhanced in the presence of DNA damaging agents suggesting that many other interactions may exist but only in the condition of excessive DNA damage (Keogh et al., 2006). Therefore, a SGA screen designed to identify genetic
interactions under the condition of DNA damage might reveal more useful insight into the function of Pph3 in the DNA damage response. In addition, to rule out PPH3 genetic interactions that are non-specific to the DNA damage checkpoint, a genetic screen to identify interactions with h2a-S129A might reveal a clearer picture of the role of γH2A in damage signaling and DNA repair.

Chromatin remodelling at a DSB has emerged as an important regulator of repair and recovery. For example, the histone chaperone protein Asf1 re-establishes chromatin by depositing H3 histones following DSB repair (Chen et al., 2008). It is presumed that following repair of a DSB in pph3Δ cells, γH2A is recycled back into chromatin. In light of the observation that Ddc2-Mec1 is retained in pph3Δ cells, how the chromatin environment surrounding repaired damage participates in on-going checkpoint signaling is an important question to address.

To further understand the role of γH2A in checkpoint maintenance, a clear physical interaction network with γH2A would be valuable. Identifying proteins that interact with γH2A in a phospho-dependent manner may reveal proteins that are important for maintaining the checkpoint and promoting DNA repair. Specifically, Rad9 is an attractive candidate for such a study since it interacts with γH2A (Hammet et al., 2007). Recently, a study investigating Rad9 found that the phosphorylation-mediated oligomerization of Rad9 participates in the maintenance of Rad53 activity (Usui et al., 2009). While we understand the mechanisms that activate the checkpoint such as resection and γH2A recognition, the components that participate in the maintenance of the checkpoint are only starting to become clear.
In budding yeast, Mec1 is activated by two distinct mechanisms (Navadgi-Patil and Burgers, 2009b). First, the C-terminal tail of Ddc1 can activate Mec1 directly. The second mechanism, which appears to be the evolutionarily conserved mechanism of Mec1 activation, is activation of Mec1 by Dpb11. Dpb11 is recruited to DSBs through an interaction with the C-terminal tail of Ddc1. The colocalization of Mec1-Ddc2 and the 9-1-1 complex at DSBs is essential for both Mec1 activating mechanisms. In Chapter IV, I discovered that the \textit{srs2Δ} mutant is capable of uncoupling DNA repair from DNA damage signaling. While Ddc1 focus disassembly correlates with DNA repair, Ddc2 focus disassembly correlates with termination of checkpoint signaling. In both wild type and \textit{srs2Δ} cells, Ddc1 foci disassembled at the same time, regardless of ongoing checkpoint signaling. Dpb11 also formed foci at DSBs and the protein appeared to replace Ddc1 and failed to disassemble in \textit{srs2Δ} cells. Therefore, Dpb11-mediated activation of Mec1 is a possible explanation for continued checkpoint signaling in the \textit{srs2Δ} mutant. It is unclear what is sustaining Dpb11 foci after the 9-1-1 clamp has been removed. During initiation of DNA replication, Dpb11 is recruited to the replication origin by an interaction with phosphorylated Sld2 and Sld3 (Muramatsu et al., 2010); however, in response to DNA damage, Dpb11 foci can also form in an Sld2- and Sld3-independent manner (Germann et al., 2011). It might be possible to identify \textit{dpb11} alleles that cannot maintain Mec1 activity and suppress the \textit{srs2Δ} recovery defect. Additionally, it will be important to address the checkpoint signaling proficiency of these alleles, since defects in checkpoint signaling are also known to rescue the \textit{srs2Δ} recovery defect (Vaze et al., 2002). There is a possibility that a small number of 9-1-1 molecules remain at a
DSB below the detectable threshold of ChIP and fluorescence microscopy which sustain Dpb11. If this is the case, it can be tested with the temperature-sensitive \textit{dpb11}\textsuperscript{-1} allele which abrogates the Ddc1-Dpb11 physical interaction at the restrictive temperature (Ogiwara et al., 2006). \textit{dpb11} suppressors of \textit{srs2}\textsuperscript{Δ} could also be informative in characterizing the conserved mode of ATR activation by TOPBP1 in humans.

\textit{Identifying human helicases involved in checkpoint recovery}

The human Fbh1 protein was recently identified and characterized as a helicase with both pro- and anti-recombinase activities, similar to yeast Srs2 (Fugger et al., 2009; Marini and Krejci, 2010). Therefore, Fbh1 is a tempting candidate as a helicase involved in checkpoint recovery in human cells. A challenging aspect of this question is the preference for NHEJ repair of DSB in mammalian cells. In such studies, it may be critical to observe recovery in systems that repair DNA damage by HR.

siRNA screens have proven to be effective approaches to indentifying novel components of the DNA damage response. Currently, a screen for novel regulators of Rad51 is underway in the lab. A future read-out of the screen that may be of interest in understanding recovery in mammalian cells is siRNAs that deplete gene products important for Rad51 focus disassembly. A depleted gene product that leads to persistent Rad51 foci after DNA damage will be a potential candidate as a regulator of checkpoint recovery.
Detecting Extra-chromosomal DNA fragments as a cancer cell biomarker

Extra-chromosomal fragments are not an unusual phenomenon and have been shown to arise from other genome maintenance pathways. dsDNA and ssDNA structures called t-circles and c-circles have been detected in some cancer cells and likely arise from alternate lengthening of telomeres (ALT), a telomerase-independent telomere maintenance pathway that utilizes BIR machinery (Royle et al., 2008) and promotes cell immortalization (Pickett et al., 2009). The number of c-circles seems to correlate with ALT activity and it is even hypothesized that some of these DNA fragments participate in telomere lengthening and consequently, cell immortalization. Indeed, c-circles may eventually be used as a marker for certain cancers (Henson et al., 2009). How these structures are eliminated remains to be clarified, but a Srs2-like mechanism is an attractive model and the mechanism of removal could provide insight into pathways that maintain cell immortality.

5.3 Future Perspectives

The availability of sequence information for entire genomes transformed biological research. The ability to examine the entire genome in a single experiment has been a valuable tool for addressing many biological questions. My own experiments observing DNA damage protein localization on chromatin (ChIP-chip) benefitted from the availability of whole-genome microarrays. Global analyses of whole genomes are proving to be powerful approaches to address biological questions such as how DNA damage and the DNA damage response affect genome-wide changes in chromatin structure and organization. However, a major limitation of these approaches is that they
generate data that are functions of population averages. A major challenge in the field is to begin to look at DNA checkpoint signaling and repair proteins at the single-cell and single-molecule level. Such studies will be instrumental in improving our understanding of focus size restriction, focus architecture, and chromatin occupancy of DNA signaling and repair proteins to a level much more detailed than what current microscopy and genome analysis methodologies presently allow.

Alternatively to the global approaches of genomic studies, future research efforts must also focus on functional analysis of molecular changes induced by DNA damage. The genetic components of the checkpoint in yeast have been indentified and largely characterized; as a signal transduction cascade, the checkpoint pathway is well established. Research in recent years has generated much insight into the modular nature of checkpoint protein activities in different phases of the cell cycle. Overall, however, we have very limited understanding of how post-translational modifications on DNA damage response proteins modulate their activities and coordinate DNA repair, cell cycle arrest, transcription, and apoptosis. The roles of only a handful of Mec1/ATR and Tel1/ATM substrates are well understood. Additionally, ubiquitylation, SUMOylation, acetylation, and methylation are all post-translational modifications that have been associated with the DNA damage response. The DNA damage response is a critical caretaker of genome stability and suppression of carcinogenesis in humans. Characterizing the relationship between DNA damage signaling and the global DNA damage response will be important in generating new knowledge to help us understand the genetic and molecular basis of genome maintenance.


checkpoint Saccharomyces cerevisiae Rad9 protein contains a tandem tudor domain that recognizes DNA. Nucleic Acids Res 35, 5898-5912.


**Appendix 1.** List of oligos used in this work for quantitative PCR analysis and homolog recombination-mediated tagging of loci.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence 5' to 3'</th>
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<tr>
<td>AHD4-G</td>
<td>TTCATTTATTTGCTGCTGTTT</td>
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<tr>
<td>AHD4-H</td>
<td>GGTGGATTTTTTGGCAGGT</td>
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<tr>
<td>CSE1-A</td>
<td>ATTTGAGGCAGTGGAGACG</td>
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<tr>
<td>CSE1-B</td>
<td>GGCAGGTTTGTGGAAGCA</td>
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<td>DDC1-f2</td>
<td>GTCTAACAACACAGTGAAGAAGCCAAAGGGGTATATTTCAGCTGCCGACGGCATCCCGGGTTAATTAA</td>
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<tr>
<td>DDC1-r1</td>
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<tr>
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<tr>
<td>DDC2-AAAA-F2</td>
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<td>GPB2-D</td>
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<td>LEU2-A</td>
<td>AAGAACCACCACACCACCTAAA</td>
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<tr>
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Appendix 2. Asf1 is required for Ddc2-GFP assembly. YMV2 and YMV2 asf1Δ cells expressing DDC2-GFP were grown in media containing 2% galactose and cells were collected, fixed with 4% PFA, and imaged for Ddc2-GFP foci. The percentage of large-budded cells with a Ddc2-GFP focus is plotted against time in galactose (h).
Appendix 2
Appendix 3. A C-terminal HA tag of *RFA1* rescues the *srs2Δ* recovery defect. Serial dilutions of YMV2 wild type and *srs2Δ* cells containing the indicated alleles of *RFA1* and *DDC2* were spotted in serial dilutions on media containing 2% glucose or 2% galactose.
Appendix 3
Appendix 4. Inhibiting CDK activity prevents 9-1-1 accumulation, but not disassembly from a DSB. JKM179 wild type (*CDC28*) and ATP-analog-sensitive *cdc28-as* cells were grown in galactose to induce a DSB. After 1 hour (A) or 6 hours (B), the cultures were split. One half was treated with 5 µM of the ATP analog NM-PP1 and the other half was treated with a carrier control of DMSO. (C) and (D) are western blots with an anti-Rad53 antibody on protein extracts derived from cells in the experiments of (A) and (B), respectively, at the indicated time points.