ORCHESTRATION OF THE DNA DAMAGE CHECKPOINT RESPONSE THROUGH THE REGULATION OF THE PROTEIN KINASE RAD53

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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In order to maintain genome stability, DNA damage needs to be detected and repaired in a timely fashion. To cope with damaged DNA, cells have evolved mechanisms termed "checkpoints", where, upon damage, cells initiate a signal transduction cascade that results in the slowing or halting of the cell cycle, allowing efficient DNA repair. Defects in the DNA damage checkpoint result in an overall increase in genomic instability and are thought to fuel cancer progression. To facilitate our understanding of how DNA damage leads to cancer progression, it is crucial to fully comprehend how these signal transduction mechanisms function. In this work, we have characterized in great detail the mechanisms of regulation of Rad53 (a central regulator of the DNA damage response in *Saccharomyces cerevisiae*) at the genetic, biochemical and structural level. Firstly, we describe a complex biochemical two-step mode of activation of Rad53 by protein-protein interaction and multi-step phosphorylation. We also shed light onto the mechanisms by which Rad53 is turned off to allow the cell cycle to resume, a process termed DNA damage recovery and adaptation. We found that during adaptation, the polo-like kinase Cdc5 is required to attenuate Rad53 catalytic activity. Finally, the study of Rad53 at the molecular and atomic level revealed that in addition to being regulated through a complex network of protein-protein interactions, Rad53 autophosphorylation is orchestrated by a mechanism of dimerization, activation segment phosphorylation via A-loop exchange, as well as through an autoinhibition mechanism regulated by a specific α-helical region at the C-terminal extremity of its kinase domain. Such work is important in understanding the function of different proteins in DNA damage signaling. This knowledge will enhance our understanding of the progression of DNA damage related diseases such as cancer, and could eventually help in the long term the development of novel therapeutics as treatments against these conditions.
ACKNOWLEDGEMENTS

It is with tremendous humility that I present this work. During my graduate career, I realized early on that the greatest accomplishments are only possible with the help of highly motivated, intelligent, generous and resourceful people. I wish to thank the following people who have made this work possible: First and most importantly, my supervisor and great friend, Dr. Daniel Durocher who provided me with unprecedented support, giving me not only all the resources possible to perform my work, but also the moral support allowing me to take risks and to benefit from the rewards. I would also like to thank my committee members, Dr. Frank Sicheri and Dr. Grant W. Brown who guided me throughout these exciting times. Moreover, this work would have not been possible without the help, advice and support of my fellow lab members, particularly Sarah Jane Galicia, Dr. Pamela Kanellis and Dr. Rachel Szilard. Within all these individuals, I did not only find labmates but also mentors and friends. I also extend my appreciation to my collaborators, especially Dr. Nadine Kolas, Dr. Anne-Claude Gingras, Dr. Cynthia Ho, Genevieve Vidanes and Dr. David Toczyński whose work was instrumental in many of my discoveries.

Lastly, I would like to thank my parents, Pierre Sweeney and Johanne Demers Sweeney for their unconditional love and support. You have seen me grown intellectually and emotionally during this process and sacrificed a lot to provide me with all the tools necessary to succeed. Without you, this work would have never been possible and I strongly believe that we have earned this degree together.
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The following material consists of published and unpublished data for which I contributed significantly. This material is not presented as an integral part of this thesis but can be found at the end of this document in appendices.

PUBLISHED MATERIAL:

The characterization of RNF8 FHA domain:


The study of Rad53 activation:

LIST OF ABBREVIATIONS

4-NQO - 4-Nitroquinoline Oxide
APC/C - Anaphase Promoting Complex / Cyclosome
ADP - Adenosine diphosphate
AMP - Adenosine monophosphate
AMP-PNP - 5'-adenylyl-beta,gamma-imidodiphosphate
ATP - Adenosine Triphosphate
ARS - Autonomous Replicating Sequence
as - Analog Sensitive
ATM - Ataxia Telangiectasia Mutated
ATR - Ataxia Telangiectasia Related
ATRIP - Ataxia Telangiectasia Related Interacting Protein
BER - Base Excision Repair
BIR - Break-induced replication
BRCT - Brca1 C-Terminal
BSA - Bovine serum albumin
CDC - Cell Division Cycle
cDNA - Complementary DNA
CDK - Cyclin dependent kinase
CMK - chloromethylketone-containing pyrrolopyrimidines
dd - Double distilled
DDK - Dfb4-Dependent Kinase
DDR - DNA Damage Response
DNA - Deoxyribonucleic Acid
DSB - Double-Stranded Break
dsDNA - Double-Stranded DNA
ssDNA - Single-Stranded DNA
DTT - Dithiothreitol
EDTA - Ethylenediamine tetraacetic acid
EtBr - Ethidium bromide
FEAR - Cdc14 Early Anaphase Release
FHA - Forkhead Associated
GAP - GTPase Activating Protein
GCR – Gross Chromosomnal Rearrangement
GEF - GTP Exchange Factor
GFP - Green Fluorescence Protein
GST - Glutathione-S-Transferase
HR - Homologous recombination
HU - Hydroxyurea
IB - Immunoblot
IP - Immunoprecipitation
IR - Ionizing Radiation
ISA - In situ assay
kb - kilobases
kDa - kilo Dalton
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
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<td>MEN</td>
<td>Mitotic Exit Network</td>
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<td>MMR</td>
<td>Mismatch Repair</td>
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<td>MMS</td>
<td>Methylmethane sulfonate</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>MRX</td>
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<td>PRR</td>
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<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
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<td>RFB</td>
<td>Replication Fork Barrier</td>
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<td>RNA</td>
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<td>RNAi</td>
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<td>Trichloroacetic Acid</td>
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<td>ts</td>
<td>Temperature Sensitive</td>
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<td>UV</td>
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<td>Western Blot</td>
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"I'm not young enough to know everything"
-OSCAR WILDE
CHAPTER 1

INTRODUCTION

DNA DAMAGE, GENOMIC INSTABILITY AND CANCER
1.0 DNA DAMAGE AND CARCINOGENESIS

1.0.0 DNA DAMAGE IN THE LIFESPAN OF A CELL

One of the fundamental processes of life is the ability of cells to faithfully transmit their genetic information. However, this task is complicated by the fact that cells are constantly subjected to DNA damaging events, whether from endogenous or exogenous origin (Friedberg et al., 1995). In addition, the DNA molecule by its very nature is chemically reactive which can potentially cause adverse events during the normal metabolic processes of DNA replication and chromosome segregation (Branzei and Foiani, 2005). For instance, spontaneous alterations of the DNA such as tautomeric shifts often take place, where the nitrogen base of a nucleic acid spontaneously isomerizes, resulting in a mutation (von Borstel, 1994). Base loss can also create a mutation if the abasic site is repaired with the incorrect nucleotide. This type of mutagenesis is particularly prevalent after cytosine deamination to uracil (Boiteux and Guillet, 2004) (Norbury and Hickson, 2001). Finally, small and highly reactive molecules called reactive oxygen species (ROS) also cause DNA damage. ROS are created during normal metabolism and can be found in different forms, such as free radicals and oxygen ions (Bertram and Hass, 2008). The highly reactive nature of ROS originates from its unpaired electron, which tends to attack DNA, creating single- and double strand-DNA breaks (SSBs and DSBs). ROS can also be formed as a by-product of ionizing radiations (IR). In addition to creating ROS, IR directly ionizes the DNA molecule, creating SSBs and DSBs (DiMauro and Schon, 2003; Finkel, 2000; Wallace, 2005). A myriad of other exogenous factors have the potential to damage DNA: UV radiation (creating DNA thymidine dimers for example), alkylating agents and DNA cross-linking agents
(Norbury and Hickson, 2001). These aforementioned DNA damaging agents can interfere with the normal DNA replication process (reviewed in (Norbury and Hickson, 2001)) which can result in replication fork collapse and attendant DSBs.

Despite many mechanisms that ensure faithful DNA replication, mutations and DNA lesions can occur as a result of DNA replication. For example, nucleotide misincorporation, leading to DNA base pair mismatch or DNA replication fork collapse are major sources of DNA damage (Branzei and Foiani, 2005). Moreover, during the process of DNA lesion bypass, the replication machinery can be error-prone and eventually lead to mutations (Broomfield et al., 2001).

Overall, individual cells are exposed to thousands of DNA damaging events per day (Billen, 1990). Evolutionarily speaking, DNA damage and the ensuing mutations are essential as these genotypic changes can lead to genetic and phenotypic variation, which may create a competitive advantage (Maresca and Schwartz, 2006). However, mutations in certain key genes can lead to cellular lethality or, in multicellular organisms, to malignant transformation.

1.0.1 HOW GENOMIC INSTABILITY LEADS TO CANCER PROGRESSION

In order to become cancerous, a cell needs to acquire the unique capabilities that will provide it with a significant growth advantage relative to its neighboring cells. A cancerous cell has to be self-sufficient in growth signal, insensitive to anti-growth signals and have a limitless replicative potential, which will allow it to divide indefinitely (Hanahan and Weinberg, 2000). Importantly, cells also have to evade apoptosis, or programmed cell death, a phenomenon acting as a fail-safe mechanism to preserve
cellular homeostasis (Hanahan and Weinberg, 2000). In addition, or as an alternative, cancer cells must also evade senescence, a cellular process by which cells lose the ability to replicate (Campisi and d'Adda di Fagagna, 2007). Finally, cancer cells must eventually acquire the potential of creating a self-sustaining environment by the induction of angiogenesis and be able to spread and invade other tissues in an event called metastasis (Hanahan and Weinberg, 2000). As a means to acquire so many proliferative advantages in such a short period of time, cells must acquire a series of mutations in “key genes” that create a growth advantage (often known as “driver” mutations). For instance, these key genes could be involved in cell cycle control, apoptosis, growth inhibition, etc. As acquisitions of these mutations are stochastic events, they are often accompanied by mutations that do not contribute to the cancer phenotype. These mutations are often referred to as “passenger” mutations. The number of key mutations required to achieve a fully malignant phenotype is small and does not reconcile well with the mutation frequency in a normal cell to explain the cancer rate observed in the population (Hahn, 2004; Zhao et al., 2004). As a solution to this conundrum, the mutator hypothesis was developed, which is based on the idea that the loss-of-function of genes that act as genomic gatekeepers is necessary to promote tumorigenesis (Loeb et al., 2003).

Although the mutator gene hypothesis has largely been validated in a number of settings (Beckman and Loeb, 2005; Rheinwald et al., 2002; Venkatesan et al., 2006; Westermarck and Hahn, 2008), an alternative model of the relationship between the DNA damage response and tumorigenesis has emerged. Indeed, this alternative model is based on the following question: where do the genomic rearrangements associated with cancer originate from? By examining the genotype, the DNA damage response and the genomic
instability of precancerous lesions, as well as comparing the same metrics against cancerous cells originating from these same lesions, it was found that “endogenous” DNA damage was a characteristic that differentiates precancerous cancer lesion from normal cells. This damage could be recapitulated by the mis-expression of a strong oncogene (Bartkova et al., 2005; Bartkova et al., 2006; Campisi, 2005; Di Micco et al., 2006; Gorgoulis et al., 2005). This DNA damage activates the DNA damage checkpoint, leading to cell cycle arrest and senescence. Therefore, DNA damage in this context is seen as a tool to keep cancer cells in check. Evidently, cells evading the checkpoint response would resume proliferation, with the added advantage that the DNA damage experienced might fuel genome rearrangements, particularly at fragile sites (Bartkova et al., 2006; Di Micco et al., 2006) although not necessarily limited to those sites. Fragile sites are defined as regions of the genome that are prone to chromatid breaks, especially in response to replication stress (Bartkova et al., 2006; Di Micco et al., 2006). Put together, this model of cancer progression is depicted in Figure 1.1.
Figure 1.1 Schematic representation of the carcinogenesis model proposed by Gorgoulis et al and Bartkova et al.

An important corollary of this model indicates that DNA damage checkpoint response is not only important to ensure that DNA damage is properly detected and timely repaired, but also plays a crucial role in acting as a barrier to cancer progression. Many additional questions regarding the checkpoint as a cancer barrier are left unanswered, and a deeper molecular understanding of the DNA damage response is critical in order to make use of this model and be able to address cancer with potent and specific therapies.
1.1 *Saccharomyces cerevisiae* as a model to study DNA damage response

1.1.0 The use of *S. cerevisiae* as a genetic tool

The budding yeast *S. cerevisiae* is a unicellular eukaryotic organism that has been a model of choice for genetic and cell biological studies over the years. More recently *S. cerevisiae* has also been used as a critical test-bed for genomic and proteomic approaches. Since budding yeast can be grown either as a haploid or diploid, it has allowed the rather facile identification of recessive mutations in haploid that would otherwise silent in diploid organisms, unless subjected to crosses. In particular, the identification of temperature sensitive (ts) mutations in essential genes has been instrumental to unravel the basic machinery of the cell division cycle.

More recently, efforts have been made to develop a comprehensive genetic interaction map in budding using robotic-assisted, large-scale genetic screens. The generation of genetic networks provides “bird’s eye view” of the way different genes and pathways interact with each other. A particularly useful technique for studying genetic interaction networks is the synthetic genetic array (SGA) developed at the University of Toronto (Ho et al., 2002a; Parsons et al., 2004; Tong et al., 2001). In brief, this technique allows one to carry out genetic crosses in a high-throughput manner. Although this technique has been particularly useful to uncover synthetic-lethal interactions among gene deletions, it has now been used for different applications, such as synthetic dosage lethality (controlled expression of essential genes) and chemical genetic screens (Baetz et al., 2006; Parsons et al., 2004).
1.1.1 HISTORICAL CONSIDERATIONS

1.1.1.0 THE DISCOVERY OF THE CELL CYCLE GENES

In the early 1970s, the seminal work by Hartwell and colleagues revealed the genetic basis of cell cycle regulation in *S. cerevisiae*: Genetic screens and time lapse photomicroscopy was utilized to identify mutants that were incapable of cycling through cellular division (Culotti and Hartwell, 1971; Hartwell et al., 1974; Hartwell et al., 1970; Hartwell et al., 1973). By using morphological and nuclear positioning, mutants that impact specific stages of the cells were identified. For instance, cells in G1 (Growth Phase 1) were characterized by being unbudded. A small budded cell, however, is characteristic of a cell performing DNA replication in S phase, whereas a large budded cell is a cell in G2 (Growth Phase 2) or M (Mitosis) phase of its cell cycle. This allowed the group to characterize these mutants according to the specific stages at which they arrested and led to the identification of key regulators of the cell cycle, such as *CDC28*, which codes for the main cyclin-dependent kinase Cdk1, the replication licensing genes (*CDC6-ATPase* and *CDC7-Kinase*), the DNA ligase gene *CDC9*, as well as a component of the anaphase-promoting complex (APC) coded by *CDC20*, amongst many others. To this day, cell cycle regulatory genes are still being uncovered, underlining the complexity of how cells regulated their division (Jorgensen et al., 2002; Kanemaki et al., 2003; Stevenson et al., 2001; Zettel et al., 2003). Strikingly, the basic mechanisms that underlie cell division are conserved in most eukaryotes, as revealed by studies in *Schizosaccharomyces pombe* (Nasmyth and Nurse, 1981; Nurse, 1975; Nurse et al.,
1976), and *Xenopus Laevis* (Masui and Markert, 1971; Miake-Lye et al., 1983; Newport and Kirschner, 1984).

1.1.1.1 The discovery of the DNA damage checkpoint genes

The discovery of genetic determinants that control cell division was a major milestone in biology. However, the intrinsically fragile nature of DNA hinted at other mechanisms, working in parallel to regular cellular division, which would ensure that the damaged DNA is properly repaired before cell division. These mechanisms would prevent mutations caused by DNA damage from being passed from mother to daughter cells (Weinert and Hartwell, 1988).

In the mid 1970s, Tobey et al. proposed that a “surveillance mechanism” could be involved to ensure that the cellular DNA is faithfully replicated and that gross genetic mutations are not passed on from generation to generation (Tobey, 1975). In 1980, Painter and Young also reported that cells derived from an ataxia-telangiectesia patient were unable to stop DNA synthesis in response to IR (Painter and Young, 1980). However, it is not until the late 1980s that the group Weinert and Hartwell identified the first gene involved in pausing or arresting the cell cycle following DNA damage, *RAD9* (Weinert and Hartwell, 1988). Weinert and Hartwell reasoned that if cells were unable to arrest cell division after DNA damage, they would divide with broken DNA and would not be able to form colonies. They therefore examined a collection of radiation-sensitive mutants to isolate mutants would continue cycling normally after IR (usually 2 to 4 cycles) before dying as small “microcolonies”. Of all the genes they originally tested,
mutation in the RAD9 gene displayed this remarkable phenotype (Weinert and Hartwell, 1988).

Genetic analysis of RAD9 with DNA repair genes revealed that DNA repair was not dependent on RAD9. Moreover, artificial arrest of yeast cultures at the G2/M transition by incubation with a microtubule-depolymerizing drug suppressed to a great extent the radiation hypersensitivity of rad9 cells. This non-epistatic relationship between RAD9 and the genes involved in DNA repair pathway indicates that the RAD9 is part of a surveillance mechanism independent of other self-sustaining cellular mechanisms (Weinert and Hartwell, 1993).

The groundbreaking discovery of RAD9 provided the stepping stone for the discovery of many other DNA damage checkpoint genes within the following years. Most genes were identified under the same experimental design: genetic screens to isolate mutants that failed to arrest under different DNA damaging conditions, again highlighting the visionary experimental approach of Hartwell and colleagues. During the early to mid 1990s, many other checkpoint genes were identified such as the mitotic entry checkpoint genes (MEC1, MEC2 - later renamed RAD53 - and MEC3), S-phase arrest defective mutants (SAD1 - also RAD53 -) and other checkpoint-associated radiation sensitive genes (RAD17, RAD24, amongst many others) (Allen et al., 1994; Weinert and Hartwell, 1993; Weinert et al., 1994). The function and description of these genes will be examined in more detail later in this dissertation.
1.1.2 *S. cerevisiae* DNA DAMAGE CHECKPOINT GENES AND THEIR MAMMALIAN HOMOLOGS

Shortly after the discovery of *RAD9* and the other genes participating in the DNA damage checkpoint in budding and fission yeasts, it was rapidly appreciated that these organisms share striking homologies within the genes involved in this process. Moreover, many mutations in *S. cerevisiae* genes have been identified in human as being key in the development of radiosensitive, immunological and neurodegenerative diseases (noted in Table 1.1) (Taylor et al., 2004). Using yeast *S. cerevisiae* as a tool to characterize the genetic and biochemical functions of these genes, and their corresponding proteins, is key in order to effectively move forward in the investigation of DNA damage related human diseases. In Table 1.1, I describe the main participants in the checkpoint response in budding and fission yeast, as well as their human counterparts.
<table>
<thead>
<tr>
<th>(S.\text{cerevisiae})</th>
<th>(S.\text{pombe})</th>
<th>(H.\text{sapiens})</th>
<th>Catalytic/modular function</th>
<th>Classification</th>
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<tr>
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<td>DNA DAMAGE SENSORS</td>
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<td>Nuclease, complex with Rad50 and Xrs2</td>
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<td>H2AX</td>
<td>Histone, DNA binding</td>
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<td>53BP1/BRCA1</td>
<td>Molecular adaptor</td>
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<tr>
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</tbody>
</table>

Table 1.1 A non-exhaustive list of genes involved in the DNA damage response

* Germline mutations found in human
1.2 MECHANISMS OF DNA DAMAGE REPAIR AND CHECKPOINT.

1.2.0 DNA REPAIR

DNA repair is not conditional upon checkpoint activation. In fact, some types of DNA damage such as UV and oxidative damage can go undetected by the checkpoint response, and be repaired without the assistance of the checkpoint (Callegari and Kelly, 2007; Leroy et al., 2001; Siede et al., 1994). However, this is not the case for all types of DNA damage. Indeed, of all types of DNA damage, the double stranded DNA break (DSB) is arguably the most lethal. A single DSB can result in robust activation of checkpoint signaling, and if not repaired properly, can quickly lead to chromosome loss, gross rearrangements and mitotic catastrophe (Lobrich and Jeggo, 2007). Below I provide a brief survey of selected repair mechanisms utilized by *S. cerevisiae* that are relevant to the work done in my thesis.

1.2.0.0 RECOMBINATION REPAIR (HR AND SSA)

DSBs can be repaired in three different ways in *S. cerevisiae*: homologous recombination (HR), single strand DNA annealing (SSA) and non-homologous end joining (NHEJ).

The most prominent form of DSB repair used by yeast is HR. Depending on the availability of homology sequences in parent chromosomes, the yeast also utilizes a specialized process of homologous recombination called SSA (van den Bosch et al., 2002).

HR depends on the *RAD52* epistasis group (*RAD50, RAD51, RAD52, RAD54, RAD57, RAD59, MRE11 and XRS2*) (Norbury and Hickson, 2001). Upon the formation of a DSB, the MRX complex (Mre11, Rad53, Xrs2) will resect the dsDNA into ssDNA
which will then promote the binding of ssDNA-binding protein RPA. Rad52 will then promote the exchange of RPA molecules for Rad51, which then initiate the stand-exchange process (van den Bosch et al., 2002). Rad51 promotes strand annealing and exchange between homologous chromosomes. Following strand exchange, Rad51-coated DNA molecules contribute to the initiation of DNA synthesis by a process called strand invasion, where the Rad51-coated DNA molecule will use its homologous DNA strand as a template for repair (Krogh and Symington, 2004; Norbury and Hickson, 2001; Paques and Haber, 1999). After the synthesis is completed, the resulting crossover is resolved and the DNA molecules are ligated, resulting in most cases in an error-free DNA molecule. Notably, other mechanisms using Sgs1-Top3-Rmi1 can be utilized to resolve HR intermediate as well as stalled DNA replication forks (Chang et al., 2005; Wu et al., 2006; Zhang et al., 2006). HR is arguably the most reliable repair system for faithfully repairing long stretches of DNA (Krogh and Symington, 2004).

Similarly to HR, Rad52 is required for SSA and binds the broken DNA ends (Norbury and Hickson, 2001). However, during SSA, the break is resected to ssDNA (by the MRX complex) until sequences of sufficient homology are revealed (Ira et al., 2004; Llorente and Symington, 2004; Zierhut and Diffley, 2008). Following the identification of homologous sequences, Rad52 promotes strand annealing; the remaining, non-annealed ssDNA is removed by endonucleolytic trimming with the help of the Rad1/Rad10 complex (Krogh and Symington, 2004; Norbury and Hickson, 2001; van den Bosch et al., 2002). Evidently, this process would result in deletions of varying lengths (Norbury and Hickson, 2001; Paques and Haber, 1999).
1.2.0.1 NON-HOMOLOGOUS END JOINING (NHEJ)

In the event that a sister chromatid or neighboring homology is not available to serve as a DNA repair template for recombination repair (as it is the case in G1 haploid cells), the cell repairs DSB using non-homologous end joining (NHEJ). NHEJ is characterized by the repair of a DSB by the joining of the DNA ends and the religation of these ends, with or without the assistance of microhomology sequences (Burma et al., 2006). Most of the naturally occurring DSB are not precise breaks (DNA ends with exposed 5’-phosphaste and 3’-hydroxyl group); the majority of breaks observed result in imprecise, incompatible or partially resected DNA ends. During the NHEJ process, these ends are trimmed and religated, which has a high probability of introducing small deletions and mutations in the repaired DNA strands (Daley et al., 2005; Dudasova et al., 2004).

Although homologous recombination is highly favored in ascomycetes, NHEJ is more commonly used in metazoans and has been extensively studied, especially in the mammalian immunoglobulin maturation process known as V(D)J recombination. V(D)J recombination relies on the induction of DSBs coupled with the error-prone characteristics of NHEJ repair in order to generate a library of different immunoglobulin variants. These variants are later selected based on their immunogenicity to a specific antigen (Soulas-Sprauel et al., 2007).

In *S. cerevisiae*, the current NHEJ working model is described as follows: Following a DSB, the yeast proteins Yku70/Yku80 are recruited to the break (Norbury and Hickson, 2001). This is thought to protect the DNA ends from further degradation and/or resection and to help to recruit the MRX complex (Mre11/Rad50/Xrs2) which is believed to bridge the two DNA ends with the use of the elongated, condensin-like
structure of the Rad50 protein (D'Amours and Jackson, 2002). If the DNA break is precise with exposed 5’-phosphatase and 3’-hydroxyl group, the DNA ligase complex Lig4/Lif1/Nej1 will be recruited to the break site and stimulate religation (Norbury and Hickson, 2001). In most cases, however, the break is imprecise. Microhomology alignment will then be used to attach the two DNA ends, and the Rad27 nuclease may participate in the removal of excess nucleotides. DNA PolIV will then stimulate DNA synthesis to complete the repair process. In most of the case, the checkpoint will then be relieved and the cell will resume cycling (Daley et al., 2005; Dudasova et al., 2004).

1.2.0.2 BASE EXCISION REPAIR AND NUCLEOTIDE EXCISION REPAIR

Oxidative damage, hydrolytic DNA base damage and UV-type damage such as thymidine dimers create “small” DNA lesions are repaired by processes such as base excision repair (BER) and nucleotide excision repair (NER). BER is mostly responsible for removing damaged bases resulting from oxidative and hydrolytic-type damage (Boiteux and Guillet, 2004; Friedberg, 2001, 2005; Norbury and Hickson, 2001). It does so by resolving small patches (1 or 3-10 nucleotides) of DNA by excising the damaged base(s) using a DNA glycolsylase (e.g. Ung1/Mag1) and apurinic/apyrimidic endonucleases (Apn1/Apn2) (Boiteux and Guillet, 2004). Once the base(s) are removed, the gap is filled using DNA PolIV. In the event that the gap is larger than simply one DNA base, RFC/PCNA will contribute and load the DNA polymerase Pol3 to help DNA synthesis. Once the synthesis is completed, the endonuclease Rad27 will trim the repair site of all excess nucleotides and Cdc9 will ligate the phosphate DNA backbone (Boiteux and Guillet, 2004; Krogh and Symington, 2004; Norbury and Hickson, 2001).
NER, on the other hand, is genetically distinct from BER as mutations in NER genes such as \textit{RAD1}, \textit{RAD2}, \textit{RAD4} or \textit{RAD10} are synergistic with \textit{apn1}Δ, a gene of the BER pathway. NER is thought to remove larger, bulkier types of lesions such as UV-like damage and other chemical damages creating distortion of double stranded DNA molecules. Although, distinct from BER, NER’s mechanism of action appears similar to BER. The damage is recognized by a large group of proteins that include Rad4, Rad7, Rad14 and Rad16. The lesion is removed with the help of the endonucleases Rad1/Rad10 as well as Rad2. Finally, the lesion is filled and ligated with the help of the same enzymes utilized by BER; Pol3 and Cdc9, respectively (Boiteux and Guillet, 2004; Norbury and Hickson, 2001).

1.2.1 Turning on the DNA damage checkpoint

The DNA damage checkpoint acts as a surveillance system present at every cell cycle transition from G1 to S phase, intra-S phase and at the G2/M boundary (Melo and Toczyski, 2002). These checkpoints allow the cell to slow down or halt its cell cycle to perform timely repair of the DNA (Harrison and Haber, 2006). It is important to note that the DNA damage checkpoint is not only used to halt the cell cycle but also plays an active role in the control of the transcription of DNA damage genes, chromatin structure and telomere length maintenance (Nyberg et al., 2002).
1.2.1.0 DNA DAMAGE CHECKPOINTS, AN OVERVIEW

The DNA damage checkpoint response is largely composed of four major protein groups: sensor, mediator/adaptor, regulator and effector proteins. The current working model is that the presence of DSBs is recognized by the DNA damage sensor Mre11/Xrs2/Rad50 (also known as MRX). Nucleolytic activity initiated by MRX and carried out in cooperation with Sgs1, Exo1 and Dna2 resects DNA ends into ssDNA tracts (ssDNA) using Mre11 3’-5’ exonuclease activity (D'Amours and Jackson, 2001, 2002; Grenon et al., 2001; Hopfner et al., 2002; Hopfner et al., 2001; Paull and Gellert, 1998; Usui et al., 1998; Zhu et al., 2008). Although Mre11 is often characterized as a 3’-5’ exonuclease as identified by its \textit{in vitro} activity, mutations in MRE11 leads to decreased 5’-3’ activity \textit{in vivo} (Haber, 1998). In any case, this ssDNA is thought to be the original activator of the DNA damage checkpoint. Moreover, it has been shown that the strength of the signal is proportional to the length of exposed ssDNA, which furthermore strengthens the hypothesis that ssDNA activates the checkpoint response (Lee et al., 1998; Vaze et al., 2002; Zierhut and Diffley, 2008). ssDNA is coated with replication protein A trimer molecules (RP-A), which has been proposed to act as a landing pad for the DNA damage sensor Ser/Thr kinase Mec1, along with its binding partner Ddc2 (Bartrand et al., 2004; Pellicioli et al., 2001; Zou and Elledge, 2003).

Mec1 is part of the P(1)3-Kinase-like family of kinases (PIKK) due to its sequence similarity to other lipid binding kinases, such as Phosphatidyl-3’-OH Kinase (PI3K), Target of Rapamycin (TOR) as well as ATM and ATR in mammalian cells. Interestingly, Mec1 has never been shown to have lipid binding activity and is localized
exclusively in the nucleus, which is different than its lipid-binding family members, localizing predominantly to the inner cellular membrane (Datta et al., 1999).

In parallel to Mec1 recruitment to the site of the DNA breaks, the Rad24-containing RFC complex and the Rad17/Ddc1/Mec3 complex (also known as the 9-1-1 complex) (Majka and Burgers, 2003, 2005; Majka et al., 2006) also act as an additional sensor of the checkpoint response. The Rad24/Rad17/Ddc1/Mec3 complex facilitates the activation of the Mec1/Ddc2 complex, and also acts as Mec1 substrate (Green et al., 2000; Melo et al., 2001; Paciotti et al., 1998). Moreover, recent evidence highlighted that artificial recruitment of the 9-1-1 trimer using LacI-based localization system is sufficient to activate the DNA damage response, despite the absence of damage. This suggests that the role of resection and RP-A is to concentrate the amount of 9-1-1 complexes to the break (Bonilla et al., 2008; Redon et al., 2003).

Once recruited to the site of DNA damage, Mec1 will phosphorylate a selected number of targets, preferring [Ser/Thr]-Gln sequences. This phosphorylation sequence is not exclusive, and largely depends on the components of the signaling complexes involved. It has been shown that molecular adaptors can increase the promiscuity of Mec1 (Lim et al., 2000; Pellicioli and Foiani, 2005; Sweeney et al., 2005). This unusual property of Mec1 is discussed further in Chapter 2.

Catalytic activation of Mec1 is conditional on its interaction with Ddc2 and helps to strengthen the checkpoint signal. For instance, Mec1 will phosphorylate histones H2A at the extreme C-terminus on Ser129 (H2A phospho-Ser129 is also referred to as γH2A) (Downs et al., 2000). This phosphorylation event helps to amplify and maintain the checkpoint signal, likely by orchestrating the recruitment of Rad9 onto chromatin
Mec1 also induce the activation of downstream kinases by hyperphosphorylating the BRCT-domain containing protein Rad9 (Emili, 1998; Vialard et al., 1998). Rad9 shares homology to Crb2/Rhp9 in fission yeast and to 53BP1 in mammalian cells and is often classified as a “checkpoint mediator”. Rad9 is recruited to the chromatin surrounding DNA lesions via the combined action of its Tudor domain, which binds in budding yeast to Dot1-methylated histone H3 K79; and its BRCT domains, which bind to exposed DSB and γH2Ax (Hammet et al., 2007; Lazzaro et al., 2008; Naiki et al., 2004). Hyperphosphorylated Rad9 then recruits Rad53 via its two FHA domains flanking its Ser/Thr kinase domain (Durocher et al., 1999; Durocher et al., 2000; Jia-Lin Ma and Stern, 2008; Lee et al., 2004; Lee et al., 2003; Schwartz et al., 2002; Schwartz et al., 2003; Sun et al., 1998). Upon the interaction of Rad9 and Rad53, Rad9 acts as a molecular adaptor by allowing Rad53 to become a potent Mec1 substrate (Sweeney et al., 2005). Mec1 phosphorylation activates Rad53, which then autophosphorylates, a process also coordinated by Rad9. According to a model based on a study by Gilbert et al. (Gilbert et al., 2001), Rad9 activates Rad53 by increasing the local Rad53 concentration which results in an increase in Rad53 concentration that is thought to trigger trans-autophosphorylation and catalytic activation. This autophosphorylation might inhibit the Rad9-Rad53 interaction thereby liberating hyperphosphorylated Rad9 for another round of Rad53 activation (schematics described in Figure 1.2) (Gilbert et al., 2001; Lee et al., 2003; Ma et al., 2006; Schwartz et al., 2002; Sweeney et al., 2005). Activated Rad53 then phosphorylates a number of substrates that affect many aspects of cell cycle progression, DNA repair and transcriptional regulation.
The DNA damage checkpoint signaling modulates the transcriptional regulation of a cluster of genes that is collectively called the DNA damage regulon (Huang et al., 1998). The DNA damage regulon is largely under the control of a Rad53 paralog, Dun1. Dun1 is recruited to Rad53 in a DNA damage and FHA-dependent manner (Bashkirov et al., 2003; Chen et al., 2007). Dun1 activation is achieved by Rad53 phosphorylation, and activated Dun1 then phosphorylates the transcriptional repressor protein Crt1. Crt1 is normally found bound to 13-mer DNA sequences, called X-Boxes. Hypophosphorylated Crt1 forms a complex with the Ssn6/Tup1 transcriptional co-repressor. Crt1 therefore represses the DNA damage regulon in the absence of DNA damage. Upon phosphorylation of Crt1 by Dun1, Crt1 is released from the chromatin and the transcriptional repression of the DNA damage regulon is relieved (Huang et al., 1998; Woolstencroft et al., 2006). The relief of Crt1-mediated repression upregulates the transcription of genes such as those coding for ribonucleotide reductase (RNR) subunits. RNR catalyzes the rate-limiting step in the synthesis of deoxynucleotides, the conversion of NTPs to dNTPs (Huang and Elledge, 1997). Other genes such as Crt1 itself, as well as a number of genes from the recombination repair pathway (RAD51, RAD54) and the nucleotide excision repair pathway (RAD2, RAD16, RAD18), are also transcribed (Huang et al., 1998).
Following Mec1/Ddc2 activation by DNA lesions (1), Mec1 phosphorylates the adaptor molecule Rad9 on multiple S/T-Q motifs (2). Phospho-Rad9, in turn, recruits Rad53 in an FHA-dependent manner to DNA lesions (3). We propose that Rad53 recruitment leads to direct phosphorylation of Rad53 by Mec1 (4). Mec1 phosphorylation of Rad53 leads to Rad53 activation and subsequent autophosphorylation (5). Hyperphosphorylated Rad53 is then released from Rad9 (6) freeing Rad9 to perform another round of Rad53 activation (7).

1.2.1.1 THE G2/M CHECKPOINT

The G2/M checkpoint is an important safeguard system, as it is the last checkpoint before cells attempt to divide. The robustness of the G2/M checkpoint is demonstrated by its complexity and its redundancy. At least two distinct mechanisms act to prevent mitotic progression in budding yeast: firstly by inhibiting anaphase entry and secondly by ensuring the nucleolar sequestration of the protein phosphatase Cdc14, an essential activator of the mitotic exit pathway (Sanchez et al., 1999; Schollaert et al., 2004).

Firstly, Chk1, a Ser-Thr kinase and DNA damage checkpoint effector, will initiate cell cycle arrest by phosphorylation and stabilization of Pds1, an inhibitor of Esp1 (Sanchez et al., 1999; Wang et al., 2001). Under uninhibited circumstances, Esp1 is a
separase protein that promotes sister chromatid separation and spindle elongation through the cdc fourteen early release (FEAR) network. By stabilizing Pds1 and activating the GTPase activating protein Bfa1, the Rad53 and Chk1 kinase ensure that the Cdc14 phosphatase will remain sequestered in the nucleus, the sister chromatids are held in place and Cdc28 substrates remain activated (by remaining phosphorylated and not being targeted to APC-mediated proteosome-mediated degradation) (reviewed in (D'Amours and Amon, 2004) and schematics of the MEN and FEAR pathways in Figure 1.3).

The G2/M checkpoint is further strengthened by the regulation of Bfa1 by the Rad53 and Dun1 kinases. Bfa1 forms a dimeric GTPase activating protein (GAP) with Bub2. The Bfa1/Bub2 GAP promotes the hydrolysis of Tem1-bound GTP to Tem1-GDP. Since Tem1 is a key regulator of mitotic exit, inhibition of Tem1 catalytic activity by the checkpoint-dependent inhibition of its GAP will result in the prolonged sequestration of Cdc14 in the nucleolus (Hu et al., 2001b; Wang et al., 2000). Cdc14 is the key mitotic phosphatase involved in the dephosphorylation of Cdc28 (Cdk1) substrates (Jaspersen et al., 1999; Noton and Diffley, 2000). The combined action of Rad53 and Chk1 therefore enforces the G2/M checkpoint at multiple points.
1.2.2.2 THE S PHASE CHECKPOINT

Contrary to the G1/S and G2/M checkpoint, the intra-S phase checkpoint is triggered by replication stress events normally caused by the prolonged pausing and/or the collapse of the DNA replication fork (Branzei and Foiani, 2005, 2006; Lopes et al., 2001). This checkpoint event eventually leads to the stabilization of stalled replication forks, to the transcription of DNA metabolism genes and to the inhibition of firing of late origins of replication (Duncker and Brown, 2003).

Fork pausing events naturally occur when the DNA replication machinery encounters certain types of DNA regions, which are more complex or difficult to duplicate. For instance, specialized protein-mediated Replication Fork Barrier (RFB),
tRNA genes, Replication Slow Zones (RSZ), as well as inverted repeats are more challenging to replicate resulting in the slowing down or pausing of the replication forks (Labib and Hodgson, 2007). These DNA regions are also associated with so-called fragile sites, which as we discussed earlier, are sites of DNA break, recombination and genomic instability. The checkpoint machinery monitors the excess of RPA-bound ssDNA that can occur if the replisome and the DNA processing machinery (such as the unwinding helicase) unpair and leave tracts of ssDNA exposed. Moreover, stalled forks can be processed by the nuclease Exo1, resulting in more ssDNA (Segurado and Diffley, 2008). This exposed ssDNA is rapidly coated with RPA molecules and the DNA damage checkpoint is activated.

In some cases, instead of pausing, replication forks collapse, resulting in a DSB. Replication forks that encounter a DSB or ssDNA nicks or gaps invariably collapse. Treatment of cells in S phase with chemicals such as alkylating, cross-linking or intercalating agents promote replication fork collapse. Often, converging replication forks from neighboring unidirectional or bidirectional replication zone can resolve replication fork collapse (Branzei and Foiani, 2005). As is the case with paused forks, collapsed DNA replication forks can be substrates for the Exo1 exonuclease. The resulting ssDNA is coated with RPA which activates the DNA damage checkpoint (Morin et al., 2008).

The S-phase DNA damage checkpoint is organized in a similar manner as the G1/S and G2/M checkpoint in terms of the key players involved. Mec1 (along with its homologue Tel1) is the primary sensor kinase, and is activated upon its recruitment to the site of paused or collapsed fork (Branzei and Foiani, 2005). Activated Mec1 then
phosphorylates an S-phase specific mediator protein Mrc1, which in turn promotes the 
recruitment, activation and autophosphorylation of the regulator kinase Rad53. Mrc1 has 
been proposed to be a Rad9-equivalent specific to S-Phase, although \textit{mrc1}\textsubscript{Δ} cells still 
exhibit slight S-phase specific Rad53 activation, presumably through residual Rad9 
activity (Alcasabas et al., 2001; Lee et al., 2004; Osborn and Elledge, 2003). Mrc1 is a 
component of the moving replication fork and also plays an important role in controlling 
replication fork stability independently of its role in Rad53 activation (Hodgson et al., 
2007; Katou et al., 2003; Xu et al., 2007). Consistent with the idea that Mrc1 and Rad9 
are the two main mediators of Rad53 activation, the \textit{mrc1}\textsubscript{Δ} \textit{rad9}\textsubscript{Δ} mutation is lethal 
unless dNTP production is boosted by the overexpression of the RNR or by the deletion 
of the gene \textit{SML1}.

The activation of the DNA damage checkpoint in S-phase results in a marked 
slow-down of DNA replication progression, which is mainly the consequence of the 
inhibition of late-origin firing (Duncker and Brown, 2003; Duncker et al., 2002; 
Santocanale and Diffley, 1998). The firing of late origins is controlled by the interaction 
of the kinase Cdc7 to its regulatory subunit Dfb4 via the N-Terminus of Cdc7. The two 
proteins share an interaction domain with Rad53 following DNA damage (Duncker and 
Brown, 2003; Duncker et al., 2002). The current working hypothesis of how Rad53 
regulates the inhibition of late origin firing suggests Rad53, once activated, 
phosphorylates and interacts with Dfb4, disrupting its interaction with Cdc7. Cdc7 is 
inactivated following the loss of interaction with Dfb4 and is no longer able to fire late 
origins. It is not known, however, whether the disruption of the Dfb4/Cdc7 complex is 
sufficient for the inhibition of the firing or whether Dfb4 once phosphorylated plays an
active role in repressing Cdc7 activity (Duncker and Brown, 2003; Duncker et al., 2002). Nevertheless, the result is a halt of the cell cycle coupled with the induction of gene transcription, which will allow timely repair of the DNA damage.

In addition to its role in gene transcription of the DNA damage regulon, Dun1, once activated by Rad53, acts on the small inhibitor of the ribonuclease reductase complex Sml1. Sml1, which normally represses deoxynucleotide metabolism by inhibiting RNR activity, is phosphorylated by Dun1 and targeted to the proteosome system for degradation (Chen et al., 2007; Uchiki et al., 2004; Zhao and Rothstein, 2002). The degradation of Sml1 provides a fast acting mechanism by which dNTPs can be generated in response to DNA damage which is also reinforced by the transcriptional response from the inhibition of the Crt1 DNA damage regulon repressor (Huang et al., 1998; Woolstencroft et al., 2006).

1.2.1.3 THE G1/S CHECKPOINT

The G1/S checkpoint is less predominant in yeast. The regulation of the G1/S transition by DNA damage occurs primarily via the regulation of the Swi6 via Rad53 phosphorylation (Sidorova and Breeden, 1997, 2003). Swi6 is a component of both the MBF and SBF transcription factors, which are key multimeric regulators of the G1/S transition. Rad53-dependent phosphorylation of Swi6 in particular, disrupts its interaction with Swi4, its partner in SBF, which is the main transcription factor responsible for the induction of the transcription of the CLN1 and CLN2 genes during the G1/S transition of the cell cycle (Schneider et al., 1998). The lack of Cln1 and Cln2 expression in response to DNA damage delays entry into S-phase (Sidorova and Breeden, 1997, 2003).
Emerging evidence also highlighted the importance of histone methylation at the G1/S transition of the checkpoint. Dot1-dependent K79-methylation of histone H3 was shown to be required for checkpoint at the G1/S and intra-S phase (Wysocki et al., 2005). This methylation event is thought to act as a docking site recognized directly by the Tudor domain of Rad9 (Grenon et al., 2007; Wysocki et al., 2005). Interestingly, this mechanism is dispensable for the G2/M checkpoint, indicating that checkpoint mechanisms are controlled differently depending of the stage of the cell cycle. It is unclear at this point why the G1/S checkpoint requires Dot1-dependent recruitment of Rad9 onto chromatin whereas the G2/M checkpoint does not.

1.2.2 TURNING OFF THE DNA DAMAGE CHECKPOINT RESPONSE

The re-entry into the cell cycle after the DNA damage checkpoint is a critical but, compared to the initiation of checkpoint signaling, much less understood process. The DNA damage checkpoint can be turned off in coordination with the termination of DNA repair, a process termed checkpoint recovery. In addition to checkpoint recovery, cells can also turn off the DNA damage checkpoint when exposed to unrepairable DNA lesion, a phenomenon termed checkpoint adaption (Harrison and Haber, 2006).

1.2.2.0 DNA DAMAGE CHECKPOINT RECOVERY

The DNA damage checkpoint is mostly regulated by reversible protein phosphorylation. It is therefore not surprising that of the action of protein phosphatases plays a critical role in checkpoint recovery. The first class of phosphatases that have been shown to regulate checkpoint recovery is the PP2C-type phosphatases Ptc2 and Ptc3. Deletion of both
phosphatases leads to a strong checkpoint recovery defect, the maintenance of a checkpoint arrest despite the completion of DNA repair. In addition to a defect in the normal termination of the checkpoint after DNA repair, the Ptc2/3 phosphatases are also required for the adaptation to DNA damage (Guillemain et al., 2007; Heideker et al., 2007; Leroy et al., 2003; Travesa et al., 2008). Conversely, overexpression of the PTC2 gene dampens the activity of Rad53 (Leroy et al., 2003) suggesting that the Ptc2/3 phosphatases either directly dampen Rad53 activity or they act to dephosphorylate one or more key Rad53 target.

At the molecular level, Ptc2 and Ptc3 physically interact with Rad53 via a phosphorylation-dependent interaction with the FHA1 domain of Rad53. This interaction is mediated by CKII-dependent phosphorylation of Ptc2. Remarkably, mutation of the CKB2 gene coding for a CKII catalytic subunit results in defective in checkpoint adaptation (Toczyski et al., 1997).

In addition to PP2C-type phosphatases, the budding yeast PP4 homolog also plays an important role in the recovery from the checkpoint arrest. In budding yeast, PP4 is a trimeric complex consisting of the Pph3 catalytic subunit and the Psy2 and Psy4 regulatory subunits. Deletion of PPH3 results in a severe delay in the recovery from the DNA damage checkpoint but does not impact checkpoint adaptation (Keogh et al., 2006).

At the molecular level, the PP4 complex likely acts at two distinct steps. Firstly, Pph3 is the main γ-H2A phosphatase in yeast. Mutation of the Ser129 residue of H2A into a non-phosphorylatable residue suppresses largely (but not completely) the checkpoint recovery defect of pph3Δ cells, indicating that H2A dephosphorylation is a key step in checkpoint recovery. Secondly, mainly from studies examining the replication
checkpoint, it was recently suggested that Pph3 might also regulate the activity of Rad53 directly (Heideker et al., 2007; Keogh et al., 2006; O'Neill et al., 2007; Travesa et al., 2008).

Finally, helicases and nucleases have also been shown to promote recovery from the DNA damage checkpoint. The DNA helicase Srs2 has been reported to downregulate the DNA damage checkpoint through an epistatic relationship with RP-A and Rad51 as well as other proteins involved in sensing the DNA damage response from the repaired DNA break. Evidence includes the observation that Rad53 remains hyperphosphorylated in $srs2A$ cells and that the recovery defects in these cells can be suppressed by deleting $RAD52$ and $RAD51$ (Liberi et al., 2000; Vaze et al., 2002).

1.2.2.1 DNA DAMAGE CHECKPOINT ADAPTATION

Conceptually related to the DNA damage checkpoint recovery, the DNA damage checkpoint adaptation is a phenomenon by which the checkpoint is turned off and cell cycle resumes despite the fact that a DNA break has not been properly repaired. In the case of unicellular organisms such as $S. cerevisiae$, one could easily conceptualize such a process as a “survival at any cost” method utilized by the cell. However, this same rationale is difficult to reconcile in the case of multicellular organisms although there is evidence that checkpoint adaptation does exist in metazoans. Indeed, the adaptation process could be detrimental to multicellular organisms, as mitosis in cells that harbor unrepaired DSBs would likely result in genomic aberrations.

DNA damage adaption, although not termed as such at the time, was first observed by Sandell and Zakian (Sandell and Zakian, 1993). Toczyski and Hartwell then
performed a genetic screen in order to identify mutants that resume cell division despite the presence of irreparable damage where they identified the genes \textit{CDC5} and \textit{CKII} in cells that can arrest following DNA damage but resume cell division despite a persistent DNA damage signal.

Large epistatic groups are now known to play a role in adaptation such as repair pathways (YKU70, YKU80, RAD51), chromatin remodelers \textit{SWI2/SNF2}, as well as other ATPases such as \textit{TID1}. Some of the same genes are also involved in recovery such as \textit{PTC2/PTC3}, \textit{CKB1/CKB2} (forming CKII), \textit{SRS2} and \textit{SAE2} (Branzei and Foiani, 2006). The distinct mechanisms by which these proteins perform their adaptation function are not clear. However, an elegant GFP-localization study by Toczyski et al. demonstrated that the timing of adaptation is dependent on the removal of the Mec1/Ddc2 complex from the break and not of the 9-1-1 clamp. In these experiments, Ddc1 and Ddc2 were GFP-tagged and localization studies demonstrated that Ddc2-GFP resides at the site of the break during the entire checkpoint, but Ddc1-GFP stays at the break site well beyond when adaptation has taken place (Melo et al., 2001).

In addition to the action of phosphatases (Downey and Durocher, 2006; Keogh et al., 2006; O'Neill et al., 2007), Yku70/Yku80 are also involved in adaptation by protecting DNA ends from exonucleolytic degradation and therefore by limiting the formation of ssDNA, a potent checkpoint signal. Indeed resection rates are increased in the adaptation-defective \textit{ykuΔ} cells and this adaptation defect can be suppressed by deleting an exonuclease involved in DSB resection, Mre11 (Barlow et al., 2008; D'Amours and Jackson, 2002). Vaze and colleagues also identified Srs2, by virtue of
being a helicase known to remove Rad51 from ssDNA, to participate in the adaptation process by stripping key checkpoint proteins from the site of damage (Vaze et al., 2002).

Another interesting player in the process is the Polo kinase Cdc5. The Haber group isolated the first allele of CDC5 defective in adaptation, cdc5-ad (Pellicioli et al., 2001; Toczyski et al., 1997). Despite providing genetics evidence that CDC5 plays a role in adaptation, nothing is known about the biochemical mechanisms by which this takes place. A limited amount of evidence has recently been demonstrated in Xenopus laevis suggesting that the Polo-like kinase Plx could play such a role. Plx phosphorylates Claspin (Rad9/Mrc1 in S. cerevisiae) and this phosphorylation disrupts the Claspin:Chk1 interaction, which leads to Chk1 inactivation and relief from the checkpoint (Mamely et al., 2006; Yoo et al., 2004). Moreover, the human homolog of Cdc5, Plk1, is phosphorylated in a DNA damage dependent manner and interacts with Chk2 (Tsvetkov and Stern, 2005; Tsvetkov et al., 2003; Tsvetkov et al., 2005). This observation strongly suggests an involvement in the Polo-like kinases in the regulation of the DNA damage response.

Whether the same mechanism is utilized to regulate adaptation in yeast is unknown but strong evidence presented in this dissertation suggests that Cdc5 directly downregulates Rad53 activity by interaction and phosphorylation. The work presented in Chapter 3 provides additional biochemical evidence supporting this possibility.
1.2.3 The Rad53 Protein Kinase

RD53 is an essential gene that codes for a protein kinase paramount to the DNA damage checkpoint response. The gene was identified in a number of functional and genetic screens: SPK1 from a screen to identify tyrosine kinases in budding yeast, MEC2 from another screen designed to identify mitotic exit checkpoint genes as well as SAD1 later on in a screen geared towards isolating S-phase arrest defective genes (Allen et al., 1994; Stern et al., 1991; Sun et al., 1996; Weinert et al., 1994; Zheng et al., 1993). Consensus then reverted to name the gene RD53, the name of the original mutant strain identified in 1978 as sensitive to X-rays and bleomycin (Moore, 1978).

The Rad53 protein is an essential player in the DNA damage response. It has a central Ser-Thr kinase domain that also possesses some levels of tyrosine kinase activity (Stern et al., 1991). Flanking the kinase domain are two (N-terminal and C-terminal) FHA domains (denoted FHA1 and FHA2, respectively), which are required for DNA damage signaling but dispensable for the essential function of Rad53 (Sweeney and Durocher, unpublished). Interestingly, this observation suggests a separation of function between the DNA damage signaling role of Rad53 and its essential function.

The essential function of Rad53 relates to a role in DNA replication. The lethality of the RD53 deletion can be suppressed simply by increasing the levels of deoxynucleotides in the cell (Huang and Elledge, 1997; Zhao et al., 1998a). This can be achieved either by overexpression of the gene coding for the large catalytic subunit of the ribonucleotide reductase gene (RNRI) or by a loss-of-function mutation in SML1, a gene encoding an an inhibitor of Rnr1 (Huang and Elledge, 1997; Zhao et al., 1998a). Formally, the essential function of Rad53 is therefore to promote the synthesis of enough
dNTPs to complete DNA replication. Interestingly, Rad53 also plays a key role in promoting DNA replication fork integrity (Lopes et al., 2001; Osborn and Elledge, 2003; Pellicioli et al., 1999b). Increasing the endogenous level of nucleotides, by deleting the \textit{SML1} for instance, might increase the processivity of the DNA polymerase and decrease the frequency of pausing, circumventing the need for Rad53. Some elegant two-dimensional gel electrophoresis studies coupled with electron microscopy performed by Lopes and colleagues clearly demonstrated a role of Rad53 in replication fork stability, although I note that all these studies were done in the presence of DNA damaging agents (Lopes et al., 2001; Tercero and Diffley, 2001). The relevant Rad53 targets at the DNA replication fork remain, however, elusive.

In addition to its role in regulating the progression of DNA replication, Rad53 was found by Gunjan et al. to regulate histone levels in \textit{S. cerevisiae} (Gunjan and Verreault, 2003). An imbalance in histone levels can be particularly harmful and histone gene transcription and translation is tightly coordinated (Gunjan and Verreault, 2003). Remarkably, yeast cells carrying a kinase defective \textit{RAD53} allele are sensitive to histone overexpression. This sensitivity is not simply a DNA damage response since the sensitivity is specific to \textit{rad53} mutants. Moreover, Rad53 interacts with histone H3 in \textit{vivo} and \textit{in vitro} suggesting a model where excess histones are detected via a direct binding to Rad53 (Gunjan and Verreault, 2003).

Remarkably, increasing dNTP levels not only suppresses \textit{rad53}Δ lethality but also the lethality of \textit{MEC1} deletion (Zhao et al., 1998a). Interestingly, \textit{MEC1} and \textit{RAD53} are the only two essential DNA damage checkpoint genes (if genes such as \textit{DBP11} or \textit{POL2} are excluded due to their role in DNA replication). Moreover, \textit{RAD53} overexpression
rescues mec1Δ lethality, but the opposite is not true (Zhao et al., 1998a). This result suggests that Rad53 acts downstream of Mec1 with regards to its essential function. Moreover, the fact that the sml1-1 allele suppresses the DNA damage sensitivity of downstream effectors of RAD53, but does not suppress the DNA damage sensitivity of rad53 and mec1 loss-of-function mutants themselves (Chen et al., 2007; Uchiki et al., 2004; Zhao et al., 1998a; Zhao and Rothstein, 2002).

Rad53 possess orthologs in all eukaryotes. In particular, the Rad53 ortholog in Schizosaccharomyces pombe is Cds1 whereas in mammals it is Chk2 (Matsuoka et al., 1998). Chk2 is phosphorylated in a DNA damage dependent manner by ATM/ATR (Tel1 and Mec1 in S. cerevisiae) and this phosphorylation requires, in some instances, Brca1 (Foray et al., 2003; Matsuoka et al., 1998; Matsuoka et al., 2000). Chk2 also plays a critical role in apoptosis by phosphorylating p53 (Hirao et al., 2000; Tominaga et al., 1999). The gene coding for Chk2, CHEK2, is a potent tumor suppressor and several CHEK2 germline mutations have been identified in patients with a variant Li-Fraumeni syndrome, which results in a heightened propensity to develop sarcoma, breast, brain and colorectal cancers (Meijers-Heijboer et al., 2002).
1.3 MODULAR PROTEIN INTERACTION DOMAINS

The precise and ordered assembly of the different components of the DNA damage checkpoint is tightly regulated via post-translational modifications (Melo and Toczyński, 2002). A large majority of these modifications are phosphorylation events, which then coordinate protein:protein interaction through modular domains (Pawson, 2007). Two of these domains are present in a number of DNA damage signaling proteins and are of key importance in this signalization process: the FHA domain and the BRCT domain.

1.3.0 THE FHA DOMAIN

The forkhead-associated (FHA) domain was discovered by Hofmann and Bucher, who identified a conserved 55-75 amino acids sequence in over 200 proteins of different function such as transcription factors of the forkhead family, RNA-binding proteins, kinesins, metabolic enzymes as well as kinases and phosphatases (Hofmann and Bucher, 1995). This domain was present across all phylogenies indicating the importance of this evolutionarily conserved motif.

The first evidence that the FHA domain mediates protein-protein interaction came from studies in John Walker’s laboratory on the interaction between the plant KAPP protein, a protein phosphatase, and a receptor protein kinase. These studies established a region on KAPP that bound specifically to the phosphorylated receptor kinase (Stone et al., 1994). Although unrecognized at the time, the receptor-interacting region encompassed an FHA domain.

It is only a few years later that the first evidence showing a phosphoregulated interaction between Rad9 and the C-terminal FHA domain of Rad53 suggested that the
FHA domain acted as a phosphopeptide recognition motif (Sun et al., 1998). Durocher and colleagues then confirmed this hypothesis with biochemical experiments that took advantage of degenerate phosphopeptide libraries to characterize the phosphopeptide recognition properties of the N-terminal FHA1 domain of Rad53 (Byeon et al., 2001; Durocher et al., 1999; Durocher et al., 2000). The Rad53 FHA1 domain binds exclusively to phospho-Thr containing peptides with high affinity to peptides that contain an Asp residue located at the +3 position relative to the phosphothreonine (Durocher et al., 1999). Importantly, Durocher and colleagues also showed that a variety of FHA domains were able to bind to phosphothreonine-containing peptides, suggesting that phosphopeptide-binding is a general property of the FHA domain. These studies were then complemented by X-ray crystallography and nuclear magnetic resonance experiments where the structure of the FHA domain of both Rad53 (FHA1 and FHA2) and the Rad53 human homologue hChk2 were solved (Durocher et al., 2000; Li et al., 2002). The elucidation of the FHA domain structure revealed that the domain formed an 11 β-sheets sandwich (Figure 1.4). Moreover, the phosphorylated peptide binds to the FHA via the β3/β4, β4/β5 and β6/β7 loops and the phospho-Thr residue is stabilized by a highly conserved Arg residue at the end of the β3 strand (Arg70 and Arg605 in Rad53 FHA1 and FHA2, respectively). The ability of FHA domains to selectively bind to phospho-Thr and not to phospho-Ser is striking and can be explained, at least in part, by the fact that the γ-methyl group of the Thr seems to make a side chain interaction with a conserved Asn residue in the β6/β7 loop of the FHA domain (Durocher et al., 2000).

Despite having no clear primary sequence homology, the FHA domain is structurally very similar to MH2 domains, a phospho-Ser binding domain found in
SMAD proteins, which are essential for TGFβ signaling (Durocher et al., 2000). This observation suggests that the two domains might share a common ancestral phosphoregulated binding domain (Durocher and Jackson, 2002).

Finally, the integrity of the FHA domain in some FHA-containing proteins is vital for preventing genomic instability as mutations in the sequence coding for the FHA domain of CHK2 and NBS1 have been mapped in human patients suffering from Li-Fraumeni Syndrome (Li et al., 2002) and Nijmegen Breakage Syndrome (Kobayashi et al., 2002) respectively. Both diseases are characterized by genomic instability and cancer predisposition, as well immunological and neurological defects in the case of hNBS1 patients.

Figure 1.4 The FHA domain (Reproduced with permission from (Durocher et al., 2000))
1.3.1 The BRCT Domain

The BRCT domain is a ~100 amino acids domain which was first identified in the \textit{BRCA1} gene (Wu et al., 1996) and which is present in proteins found in bacteria to mammals. A large number of BRCT-containing proteins are involved in the DNA damage response and some budding yeast proteins containing BRCT domains include Esc4, Dpb11 and Rad9. BRCT domains are most exclusively found in tandem or multiples and have very versatile functions. BRCT domains can dimerize \textit{in cis} (with another BRCT domain of the same protein) or \textit{in trans} (with the BRCT domain of another protein) (Manke et al., 2003; Soulier and Lowndes, 1999; Yu et al., 2003). Importantly, tandem BRCT domains were shown to act as phospho-Ser binding modules with a preference for pSer-X-X-Phe sequences (Manke et al., 2003; Yu et al., 2003).

In \textit{S. cerevisiae}, the Rad9 protein is thought to be an ortholog of mammalian 53BP1. As it is the case with its mammalian ortholog, the integrity of Rad9 BRCT domain is essential for checkpoint signaling (Soulier and Lowndes, 1999). It has been suggested that the Rad9 BRCT domain is responsible for protein oligomerization and, although the domain has been shown to bind phospho-Ser \textit{in vitro}, there is no evidence so far to indicate that Rad9 oligomerization upon DNA damage is regulated via Ser phosphorylation (Soulier and Lowndes, 1999).
1.4 Objectives

The main objective of my doctoral work presented herein was to shed light on the mechanisms that govern the activation and termination of checkpoint signaling. Using *S. cerevisiae* as a model system, I focused my attention on the protein kinase Rad53.

Firstly, we will examine the role of the mediator protein Rad9 in the Rad53 activation cascade. Understanding the role of checkpoint mediators is important, as these proteins play a key role in the signaling pathways that they participate in. I determined that Rad9 acts as an adaptor that bridges the protein kinase Mec1 to Rad53.

We will then explore the role of Cdc5 in the termination of the DNA damage checkpoint. Briefly, I found evidence that Cdc5 acts directly on Rad53 by interacting with and also by phosphorylating Rad53. I hypothesize that Rad53 phosphorylation by Cdc5 is responsible for the down regulation of Rad53 activity during checkpoint adaptation.

Finally, I will present my contribution to a collaborative study that led to solving the atomic structure of Rad53. I will present a model of Rad53 activation based on these studies.
CHAPTER 2

RESULTS

REGULATION OF RAD53 THROUGH MODULAR PROTEIN: PROTEIN INTERACTIONS AND
MULTI-STEP PHOSPHORYLATION

Adapted from: Sweeney, Yang, Chi, Shabanowitz, Hunt and Durocher
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2.0 INTRODUCTION

The DNA damage checkpoint is a signal transduction system that detects and signals physical alterations in DNA as well as monitoring the integrity of replication forks (Rouse and Jackson, 2002). When this highly conserved genome surveillance mechanism is disrupted, cells become sensitive to DNA damaging agents, DNA replication inhibitors and accumulate genetic lesions at high rates (Kolodner et al., 2002a, b). In human cells, disruption of the components of the DNA damage checkpoint, such as ATM, Chk2 and p53, is associated with an aberrant response to DNA damage resulting in genome instability and heightened predisposition to cancer (Motoyama and Naka, 2004).

In the budding yeast \textit{S. cerevisiae}, the DNA damage checkpoint is in large part under the control of the PI(3) kinase-like kinase (PIKK) Mec1, the ortholog of the ATR Ser/Thr protein kinase (Abraham, 2001a; Durocher and Jackson, 2001). In complex with its interacting protein Ddc2, Mec1 has been proposed to detect processed DNA lesions via an interaction with RPA-coated single-stranded DNA (Zou and Elledge, 2003). Upon Mec1 activation, the tandem BRCT domain-containing protein Rad9 becomes highly phosphorylated on putative Mec1 consensus sites characterized by Ser/Thr-Gln (S/T-Q) motifs(Schwartz et al., 2002). Rad9 phosphorylation triggers the binding of Rad53, the Chk2 ortholog, to Rad9 in a manner that depends on both the N-terminal (FHA1) and C-terminal (FHA2) FHA domains of Rad53 (Durocher et al., 1999; Schwartz et al., 2002; Schwartz et al., 2003; Sun et al., 1998; Vialard et al., 1998). Rad9 is essential for DNA damage signaling in the G1 and G2/M phases of the cell cycle, whereas in S-phase, its function in the replication checkpoint is dispensable and appears to be substituted by
Mrc1, a replication fork-associated protein (Alcasabas et al., 2001; Osborn and Elledge, 2003).

In vertebrates, there is no single Rad9 ortholog readily identifiable, but rather a family of tandem BRCT domain-containing proteins that act in the checkpoint response. These Rad9 homologs are Brca1, 53BP1 and MDC1 and are commonly referred to as checkpoint mediators (Zhou and Elledge, 2000). Like Rad9, human checkpoint mediators localize directly to sites of DNA damage, are often substrates of PIKKs and are required for the downstream propagation of the DNA damage signal (Canman, 2003; Foray et al., 2003; Wang et al., 2002). The shared properties between budding yeast Rad9 and human checkpoint mediators suggest that a common underlying role in the checkpoint response may govern checkpoint mediator action.

Two main models attempt to explain the requirement for checkpoint mediators in Chk2-like kinase activation. The first model, based on a study by Gilbert et al. (Gilbert et al., 2001), proposes that Rad9 activates Rad53 in a manner akin to a solid-state catalyst by increasing the local Rad53 concentration. The resulting increase in Rad53 concentration is thought to be sufficient to trigger trans-autophosphorylation and catalytic activation. This model (called herein the ‘solid-state catalyst’ model) was inferred from hydrodynamic data of the Rad9 complex and on the observation that recombinant Rad53 produced from *Escherichia coli* is extensively phosphorylated and active. However, the solid-state catalyst model of Rad53 activation does not satisfyingly account for the observation that Rad53 is phosphorylated in response to DNA damage independently of its kinase activity ((Lee et al., 2003; Pellicioli et al., 1999b; Sun et al., 1996) and below) or that covalent linking of Rad53 to Ddc2 partially alleviates the requirement of Rad9 for
Rad53 activation (Lee et al., 2004). A second model has been proposed since the realization that Rad9 (or other checkpoint mediators) are critical for the PIKK-dependent phosphorylation of Chk2-like kinases in response to DNA damage. This second model suggests that the main role of Rad9 is to act as a protein that recruits Rad53 to Mec1. In this model Rad9 acts as a *bona fide* signaling adaptor which can be defined as a protein lacking known catalytic domains which physically links at least two other proteins to facilitate a signaling event (Pawson, 2007; Pawson and Nash, 2003; Pawson and Scott, 1997). However, direct biochemical evidence to support the adaptor model has remained elusive.

In this study, we investigated how Rad53 phosphorylation influenced its catalytic activation. Using mass spectrometry coupled to metal-affinity enrichment of phosphopeptides, we mapped unambiguously phosphorylation sites on Rad53 purified from cells subjected to DNA damage. Results from the mutational analysis of these sites are consistent with a model whereby PIKKs play a direct and essential role in converting a catalytically latent Rad53 enzyme to an active state by phosphorylating multiple sites on Rad53. Furthermore, we have tested the adaptor model in biochemical reconstitution assays using purified Mec1, Rad53 and Rad9 and our results indicate that Rad9 acts as a signaling adaptor necessary for efficient Rad53 phosphorylation by Mec1. Since MDC1, Brca1 and 53BP1 are analogous to Rad9 in many respects and are required for multiple PIKK phosphorylation events, our work supports a model whereby BRCT domain-containing checkpoint mediators act as PIKK phosphorylation adaptors to ensure the proper spatio-temporal regulation of the DNA damage response.
2.1 Results

2.1.0 FHA Domains and DNA Damage Dependent Phosphorylation of Rad53

In this study, we examined the contribution of the Rad53 FHA domains to the regulation of Rad53 kinase activation in response to DNA damage. We mutated a critical Arg residue in each of the Rad53 FHA domains to Ala (R70A in FHA1 and R605A in FHA2; Figure 2.1A) in order to impair the phosphopeptide-binding activity of the mutated FHA domain (Durocher et al., 1999; Durocher et al., 2000). These mutations were generated alone or in combination to yield the rad53<sup>FHA1</sup>, rad53<sup>FHA2</sup> or rad53<sup>FHA1,FHA2</sup> alleles. We also generated a kinase-dead allele of RAD53, rad53<sup>D339A</sup> (Fay et al., 1997). Each plasmid-borne allele (including the wild-type allele) was introduced in a rad53<sup>Δ</sup>sml1<sup>-1</sup> strain (SML1 mutation is required to maintain the viability of the RAD53 deletion; (Zhao et al., 1998a)). DNA damage-induced Rad53 activity was elicited by addition of the UV-mimetic drug 4-nitroquinoline oxide (4-NQO) and Rad53 activity in the resulting strains was monitored by in situ kinase assays (ISA; (Pellicioli et al., 1999b), or by examining protein gel mobility shifts due to phosphorylation). As shown in Figure 2.1B, we observe that Rad53 catalytic activation absolutely requires at least one functional FHA domain, an observation also corroborated by others (Pike et al., 2003; Schwartz et al., 2002). This result indicates that FHA-dependent binding of Rad53 to upstream activators is required for its conversion from a latent state to an active one.

We also noticed that in stark contrast to the lack of a DNA damage-induced mobility shift displayed by Rad53 in the rad53<sup>FHA1,FHA2</sup> strain (Figure 1B, lane 10, lower panel), Rad53<sup>D339A</sup> displays a noticeable mobility shift after DNA damage, albeit not at the level seen with the wild-type allele (Figure 2.1B, lane 4, lower panel). This mobility
shift, also observed in (Sun et al., 1996), is abrogated by treatment with λ protein phosphatase (data not shown) indicating that the mobility shift is due to a phosphorylation event independent of Rad53 catalytic activity but which requires the phospho-Thr binding activity of the FHA domains. Since both Rad53 FHA domains are required for optimal binding to Rad9 ((Schwartz et al., 2002) and data not shown), we next examined whether the Rad53 kinase-independent phosphorylation event requires Rad9 residues necessary for its interaction with Rad53. Thus, the kinase-dead rad53^{D339A} allele was introduced in a RAD9 strain, a strain lacking RAD9 (rad9^{Δ}), or in a rad9^{9TA} strain which harbors mutations in nine TQ motifs required for the phosphorylation-dependent Rad53-Rad9 interaction (Schwartz et al., 2003). As shown in Figure 2.1E, the DNA damage-induced phosphorylation of the Rad53^{D339A} protein in G1 (a cell cycle phase where Rad53 activation is totally dependent on RAD9) is totally abolished in the rad9^{9TA} strain indicating that this Rad53 kinase-independent phosphorylation event is entirely dependent on a functional Rad9-Rad53 interaction. Furthermore, this phosphorylation step is dependent on Mec1 and generates phospho-[S/T]-Q epitopes, suggesting that Mec1 may be the protein kinase responsible for this phosphorylation event (Figure 2.1CD).
Figure 2.1 Rad53 is modified in two distinct phosphorylation events.

(A) Domain architecture of Rad53 showing the two FHA domains (FHA1 and FHA2) flanking the central catalytic protein kinase domain. Also indicated are the sites of mutations abolishing phosphopeptide-binding activity in the FHA1 (R70A) or FHA2 (R605A) domains. The site of the D339A mutation in the Rad53 kinase domain, which abolishes kinase activity, is also indicated. (B) Analysis of Rad53 activity and phosphorylation after DNA damage. Strains harboring the indicated RAD53 alleles were (+) or were not (-) treated with the UV-mimetic drug 4-NQO. Whole-cell extracts (WCE) were prepared by glass bead lysis in TCA and proteins were separated by SDS-PAGE. Rad53 activity was measured by in situ kinase assays (ISA, top panel) and Rad53 phosphorylation was estimated by examining gel mobility shifts following immunoblot analysis (bottom panel). (C) Rad53 activation is entirely dependent on Mec1. Cells treated with 4-NQO from MEC1 or mec1Δ strains were analyzed for Rad53 activation by ISA (top panel) and electrophoretic mobility shift (bottom panel). (D) Rad53 is phosphorylated on [S/T]-Q residues in a Mec1-dependent manner. Strains harboring the indicated genotypes were treated with 4-NQO to induce DNA damage. Rad53 from native extracts was then immunoprecipitated and the immunoprecipitate was subjected to immunoblot analysis using a phospho[S/T]Q epitope specific antibody. (E) Rad53 kinase independent phosphorylation depends on Rad9. rad53ΔD339A cells containing either the RAD9, rad9Δ or rad99TA alleles (the rad99TA allele contains T→A mutations on nine TQ motifs) were arrested in G1 by exposure to α-factor and then treated with 4-NQO. Phosphorylation of Rad53 was examined by immunoblot as described above. Rad53D339A phosphorylation is dependent on the PIKK phosphorylation sites on Rad9. (F) Model of Rad53 activation following DNA damage. Rad53 is first phosphorylated by an upstream kinase in a MEC1-dependent manner on [S/T]-Q residues. This phosphorylation is followed by Rad53 activation and autophosphorylation.
2.1.1 PHOSPHORYLATION OF RAD53 IN RESPONSE TO 4-NITROQUINOLINE OXIDE (4-NQO)

Since phosphorylation of Rad53\textsuperscript{D339A} following DNA damage is dependent on the same genetic determinants required for Rad53 activation, we hypothesize that the trans-phosphorylation event observed participates in Rad53 catalytic activation. We therefore sought to identify the phosphorylation sites involved in this event in order to characterize their function (termed “Class I” phosphorylation sites). As outlined in Figure 2.2A, our strategy was to map phosphorylation sites on Rad53\textsuperscript{D339A} in order to identify sites that are independent of Rad53 kinase activity. In parallel, we also mapped sites on wild-type Rad53 after DNA damage in order to deduce which sites are likely to represent autophosphorylation events (termed “Class II” phosphorylation sites). Therefore, to map Rad53 phosphorylation sites \textit{in vivo}, we introduced FLAG epitope-tagged \textit{RAD53} or \textit{rad53}\textsuperscript{D339A} alleles under the control of the inducible \textit{GAL1/10} promoter into a \textit{rad53}Δ \textit{sml1-1} strain to facilitate purification. The C-terminal Flag epitope does not alter Rad53 function as the \textit{RAD53-FLAG} plasmid restores tolerance to DNA damage when introduced in a \textit{rad53}Δ strain. Rad53-Flag and Rad53\textsuperscript{D339A}-Flag were both immunopurified from 4-NQO-treated, asynchronously dividing cells to identify phosphorylation sites that are dependent or independent of Rad53 catalytic activity according to the scheme described in Figure 2.2A.

After immunopurification (Figure 2.2B), Rad53-Flag or Rad53\textsuperscript{D339A}-Flag was digested with trypsin or a combination of trypsin-Glu-C or trypsin-chymotrypsin proteases. The resulting peptides were split in two aliquots. One aliquot was analyzed by tandem mass spectrometry to estimate protein coverage. Our analysis indicates that we obtained peptide coverage of 74.7% of the total protein sequence (data not shown). The
other aliquot was enriched for phosphopeptides by passing the peptide mixture over a Fe (III)-IMAC column and phosphorylation sites were identified by tandem mass spectrometry (Ficarro et al., 2002a). Using this method, we unambiguously detected 42 phosphorylated peptides (Tables 2.1) representing 14 sites on the Rad53\textsuperscript{D339A} sample (Figure 2.2D). In addition to identifying phosphopeptides corresponding to most of the sites found on the Rad53\textsuperscript{D339A} sample (boxed residues, Figure 2.2D), we identified 13 additional sites on the wild-type protein which we termed Class II phosphorylation sites (Figure 2.2D).
Figure 2.2 Phosphorylation site mapping of 4-NQO dependent Rad53 sites

(A) Strategy for the identification Rad53 phosphorylation sites by mass spectrometry. Rad53 phosphoregulation occurs in two distinct steps. (A) The Rad53^{D339A} kinase-dead variant was used to identify sites that are phosphorylated independently of Rad53 catalytic activity. Conversely, sites that are present only on wild-type Rad53 are likely to represent autophosphorylation sites. (B) Immunopurification of Rad53 proteins. Equal fraction of crude cell lysates, proteins bound to anti-Flag beads and proteins eluted from the beads were stained with colloidal Coomassie. The eluate fraction corresponds to 5% of the total quantity of protein used for phosphorylation site mapping by mass spectrometry. (C) Typical MS/MS spectrum obtained from phospho-Rad53 showing a fragmentation pattern identifying doubly phosphorylated Ser350 and phosphorylated Thr354 residues in the activation segment of Rad53. Please refer to Tables S1-S3 for a complete list of the peptide sequences and the position of the phosphorylation sites. (D) In vivo phosphorylation sites identified by mass spectrometry from the Rad53^{D339A} sample (sites above the drawn Rad53 molecule) and wild-type Rad53 (sites below). Boxed residues represent sites that we also found in the wild-type Rad53 sample.
Analysis of the phosphorylation sites mapped on Rad53\textsuperscript{D339A} reveals that 5 of the 14 sites mapped conform to the [S/T]-Q consensus for PIKK phosphorylation (Table 2.1). However, we also noted four additional sites that contain a hydrophobic residue immediately following the phosphoacceptor residues (S/T-Ψ; where Ψ is either Ala, Val, Ile, Leu, Phe or Trp residues), a motif that is found phosphorylated by PIKKs \textit{in vitro} and \textit{in vivo} (Kim et al., 1999). Of the remaining 5 sites, one appears to be a proline-directed site (S375) perhaps indicative of Cdk-dependent phosphorylation. As for the 13 sites found solely in the Rad53 wild-type sample, we predict that most correspond to autophosphorylation sites. Satisfyingly, two of the 13 sites (S350 and T354) map to the predicted activation segment of Rad53 (Table 2.1, Figures 2.2DE and discussed in Section 2.1.6), a functionally relevant motif in kinases which is often phosphorylated. In addition, many of the phosphorylation sites are found as clusters (e.g. S745, S746, S748, S750). Whether Rad53 acts as a processive kinase, prefers previously phosphorylated substrates or whether the observed clusters of phosphorylation sites represent particularly accessible regions of the protein is unknown due to the paucity of \textit{bona fide} Rad53 substrates. Lastly, from the analysis of these 13 sites, we could not detect any obvious Rad53 consensus phosphorylation site or any site that conforms to the Rad53 phosphorylation consensus derived from the analysis of Swi6 phosphorylation (Sidorova and Breeden, 2003). Most of the discussions in this Chapter revolve around the role of Class I phosphorylation sites in Rad53 activation. However, we strongly believe that Class II phosphorylation is of critical importance for Rad53 activity, most likely in regulating the off-rate of Rad53 from Rad9. In this regard, we will share some unpublished data of the role of Rad53 Class II phosphorylation as well as a discussion on
some of the possible mechanistic role(s) played by these phosphorylation events (Section 2.1.6 and 2.2.4, respectively).

2.1.2 PHOSPHORYLATION OF RAD53 IN RESPONSE TO HYDROXUREA (HU)

As discussed in great details in Chapter 1, the function of Rad53 is essential for checkpoint function during replication stress caused by prolonged replication forks pausing, collapse or intrinsic DNA damage. The mechanisms by which Rad53 is activated following these events are similar to the ones utilized by the cell during the G1/S and G2/M checkpoint but differ in some details. For instance, Rad9 is thought to be dispensable for the intra-S phase the checkpoint, replaced by its paralog Mrc1. Moreover, in S-Phase, Rad53 performed a checkpoint-independent essential function most probably related to the maintenance of replication fork integrity (Lopes et al., 2001). The identification of Rad53 S-phase specific phosphorylation sites could help to shed light onto the mechanism of Rad53 activation differenting from S-phase and allow us to identify other kinases responsible for Rad53 activation in S-phase.

The positive experience from the strong phospho-mapping protocol that we established by mapping Rad53 4-NQO-dependent phosphorylation sites allow us to utilize the same protocol to pursue, although in less detail, the phosphomapping of Rad53 in response to replication stress induced by the nucleotide-depleting drug Hydroxurea.

In brief, the same strains as detailed in Section 2.1.1 were used and treated with 200mM HU for 3 hours after which, the cells were lysed, Rad53 was purified and subjected to tandem mass spectrometry (as described in Section 2.1.1). We uncovered unambiguously 19 sites, 6 sites which were found in the Rad53D339A (Class I sites)
sample as well as 13 sites which were found only in the Rad53\textsuperscript{WT} sample (Class II sites), indicative of sites dependent of Rad53 kinase activity (Figure 2.3 and Table 2.2). Interestingly, all but two of the Class I sites identified (S485, S485, S793 and S795) were Mec1 consensus phosphorylation sites (\([S/T]-Q\)) as the other two sites (S789 and S791) fit the consensus sequence \([S/T]-Ψ\) (where \(Ψ\) is an hydrophobic residue) identified in Section 2.1.1. The Class II sites were significantly more abundant, indicating perhaps that upstream phosphorylation of Rad53 is not as frequent in S-phase, that phosphatases are more active on Rad53 in this phase of the cell cycle or simply that the purification and phosphomapping method was not as efficient for the Rad53\textsuperscript{D339A} sample. The latter argument is supported by the observation that 5 of the 13 sites were identified as Class I 4-NQO dependent sites suggesting that the phosphomapping was more efficient for these latter samples. However, we identified a novel Ser-Pro site, indicative of a Cdk-dependent phosphorylation site (S774) as well as a new PIKK consensus phosphorylation site (Thr-Gln) in S473.

Overall, only 3 sites out of the 16 identified in the HU sample were not observed in the 4-NQO-dependent sample (T191, S473 and S774, see Figure 2.3). Of the conserved sites, most were Mec1 consensus phosphorylation sites (10 of 16) and some were Class II phosphorylation sites such as the S568, S746, S748 and S750. The striking similarity between the HU and 4-NQO dependent phosphorylation sites allows us to confirm the validity and the strong reliability of the phosphomapping protocol that we have develop and feel confident that the sites identified within these two experiments are actual \textit{in vivo} phosphorylation sites.
2.1.3 Multisite transphosphorylation is required for Rad53 function

With a strong reliable phosphomapping protocol, we felt confident that the sites identified in vivo were involved in Rad53 activity. In order to test the importance of these sites in the activation of Rad53, we next turned our attention to the sites mapped on kinase-dead Rad53\(^{D339A}\) following DNA damage by 4-NQO, as they may represent sites that directly participate in Rad53 transactivation. To determine whether these phosphorylation sites play a critical role in catalytic activation of Rad53, we mutated each of the 14 phosphorylation sites individually or in pairs. Rad53 catalytic activity was then evaluated by ISA, mobility shift on SDS-PAGE and by examining the ability of the phosphorylation site mutants to complement the DNA damage sensitivity of the rad53\(^{Δ}\) sml1-1 strain. None of the single or double site mutants displayed sensitivity to DNA damage or loss of catalytic activity, as measured by ISA (Figure 2.4AB and data not shown). However, mutation of residues S485/489 (Rad53\(^{A1}\)) and S560/563 (Rad53\(^{A5}\)) lead to a reproducible reduction in Rad53 mobility shift after DNA damage, suggesting that these sites are indeed phosphorylated in vivo (Figure 2.4B, lower panel).
Next, we considered the possibility that multiple sites contribute to Rad53 kinase activation. Therefore, we combined phosphorylation site mutations and analyzed their phenotypes, as described above. To our surprise, even though we could nearly abolish the mobility shift of Rad53 following DNA damage when we mutated a total of six sites (Rad53A6 mutant), its activity as measured by ISA (Figure 2.4B, upper panel) and DNA damage sensitivity (Figure 2.4C), remained similar to wild-type levels. However, when we mutated additional residues resulting in mutations of 8 or 9 of the 14 sites (e.g. the Rad53A8 or Rad53A9 mutants) we reached a critical point where Rad53 cannot be converted into an active protein kinase after exposure to DNA damaging agents, as demonstrated by the lack of activity in the ISA (Figure 2.4B, upper panel) and DNA damage sensitivity (Figure 2.4C). These results suggest that Rad53 activation is dependent on the contribution of multiple phosphorylated residues.

The Rad53A8 and Rad53A9 mutants are expressed at wild-type or near wild-type levels in yeast cells and interact with Rad9 (data not shown). These observations rule out the possibility that the defect in Rad53 activity is simply due to loss of protein expression or loss of interaction with Rad9. Importantly, expression of Rad53A8 in bacteria results in an active protein kinase as demonstrated by the substantial autophosphorylation observed on Rad53A8 (Figure 2.4D). This latter result indicates that the rad53A8 allele is likely to be inactive in yeast due to the disruption of regulatory residues rather than protein misfolding. Based on the above results, we conclude that MEC1-dependent Rad53 trans-phosphorylation is required to convert the latent Rad53 enzyme into an active protein kinase.
Figure 2.4 Putative Mec1 phosphorylation sites are required for Rad53 activity.
(A) Series A mutations. (B) Strains harboring the indicated RAD53 alleles were (+) or were not (-) treated with 3 μM 4-NQO. WCE were prepared and proteins separated by SDS-PAGE. Rad53 activity was monitored by in situ kinase assays (ISA, top panel), and Rad53 phosphorylation was estimated by gel mobility shift after immunoblotting (bottom panel). Combinations of 8 or 9 mutations lead to a defect in Rad53 activation (mutants Rad53A8 and Rad53A9). However, note that some combinations of mutants affect Rad53 mobility shift after DNA damage without affecting its kinase activity. (C) Strains containing the alleles described above were diluted serially ten-fold and spotted on agar plates containing 4-NQO at the indicated concentrations. (D) Bacterially produced Rad53 A8 has protein kinase activity. His-tagged Rad53 and Rad53 A8 were expressed and purified in E. coli as described in Materials and Methods. Note that Rad53 A8 displays an extensive mobility shift comparable to wild-type Rad53 indicating that it has protein kinase activity.

2.1.4 RAD9 STIMULATES MEC1 PHOSPHORYLATION OF RAD53 IN VITRO

The observation that putative Mec1 phosphorylation sites are required for Rad53 activation in vivo is at odds with the solid-state catalyst model of Rad53 activation in which the role of Mec1 is mainly to phosphorylate Rad9 (Gilbert et al., 2001; van den Bosch and Lowndes, 2004). However, considering our new data on the phosphoregulation of Rad53, we reasoned that instead of acting as a solid-state catalyst,
Rad9 may be acting to recruit Rad53 to sites of DNA damage to allow it to be efficiently phosphorylated by Mec1.

To directly test the adaptor model, we first examined whether Mec1 can phosphorylate Rad53 directly in vitro. To do so, we optimized a purification procedure to produce full-length, recombinant, Rad9 from yeast and performed large-scale immunoprecipitations of FLAG-tagged Mec1 and kinase-dead Mec1 proteins (Mec1-kd, Figure 2.5AB). With these purified proteins, we examined the capacity of Mec1 to phosphorylate Rad53\textsuperscript{D339A} directly in vitro. We incubated purified Mec1 with increasing amounts of bacterially produced Rad53\textsuperscript{D339A} or the model PIKK substrate, PHAS-I (Banin et al., 1998). The protein kinase reactions were carried out in the presence of radiolabeled ATP, and phosphorylation was detected and quantified by phosphorimaging. At equimolar concentrations, Rad53 is consistently phosphorylated less than PHAS-I (Figure 2.5CD) even though there is only one PIKK site, S111, on PHAS-I (Abraham, 2001b) compared to 14 potential phosphoacceptor sites on Rad53 (i.e. all sites mapped on Rad53\textsuperscript{D339A}). The observation that Rad53 behaves as a poor Mec1 substrate relative to the non-physiological PIKK substrate, PHAS-I, is clearly at odds with the quantitative phosphorylation of Rad53 observed in vivo (Figure 2.1). As a control, we ensured that the phosphorylation of both Rad53 and PHAS-I is dependent on Mec1 kinase activity by carrying out kinase assays with the Mec1-kd protein instead of wild-type Mec1. As expected, Mec1-kd is unable to support efficient phosphorylation of either PHAS-I or Rad53 (Figure 2.5E, lanes 5 and 8).
Figure 2.5 Direct phosphorylation of Rad53 by Mec1

(A)(B) Purified recombinant proteins used in the in vitro phosphorylation assays of this study. Full length GST-TEV-Rad9 (A) was purified from yeast cells using heparin-sulfate (F1), GST affinity followed by TEV cleavage (F3). Full length FLAG-His$_{10}$-Mec1 or FLAG-His$_{10}$-Mec1-kd (B) were purified from yeast cells using Ni$^{2+}$-NTA-agarose affinity columns followed by FLAG immunopurification. The residue mutated in the kinase-dead mec1-kd allele is D2224A (Paciotti et al., 2001).

(C) Rad53 is a poor Mec1 substrate relative to PHAS-I. In vitro Mec1 kinase assays using equimolar concentrations of Rad53$^{D_{339}A}$ or PHAS-I as substrates were performed in the presence of [γ-$^{32}$P]ATP. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and radioactivity incorporation was measured by phosphorimaging (top panel). The presence of Rad53 and PHAS-I was monitored by immunoblotting using anti-HIS tag and anti-PHAS-I antibodies, respectively (bottom panels).

(D) Phosphorimager quantification of the in vitro kinase assays performed in (B). Note that at equimolar concentrations, Rad53 is markedly less efficiently phosphorylated than PHAS-I.

(E) Kinase reactions as described in (B) using 2 μM of the indicated substrates were carried out using either wild-type Mec1 or Mec1-kd. The presence of substrate was monitored by immunoblotting using an anti-HIS tag antibody which recognizes either substrate (bottom panels).
In vivo, Rad53 is efficiently phosphorylated in a RAD9-dependent manner and in a Rad53 FHA domain-dependent manner (Figure 2.1). Therefore, the observation that Rad53 is a relatively poor Mec1 substrate prompted us to test whether Rad9 can stimulate phosphorylation of Rad53<sup>D339A</sup> by Mec1. Kinase assays were performed with fixed amounts of Rad53<sup>D339A</sup> and Mec1, and increasing amounts of Rad9. Under these conditions, Rad9 is clearly able to stimulate Rad53 phosphorylation in a dose-dependent manner (Figure 2.6A). Intriguingly, we observe that Rad9 does not appear to incorporate as much <sup>32</sup>P as Rad53 in these assays. This observation can be reconciled with our finding that Rad9 purified from yeast cells is substantially phosphorylated on multiple sites, including at some [S/T]-Q residues (data not shown), a condition that may preclude additional Mec1 phosphorylation. Nevertheless, the stimulation of Rad53 phosphorylation is not due to contaminating kinase activity in the Rad9 preparation since: (1) omission of Mec1 does not lead to appreciable Rad53 phosphorylation (Figure 2.6A); (2) Rad53 phosphorylation is not observed when Mec1-kd is used instead of wild-type Mec1 (Figure 2.6B); and (3) Rad53 phosphorylation by Mec1 is inhibited by the PIKK inhibitor wortmannin (Figure 2.6D). We therefore conclude that Rad9 facilitates Rad53 phosphorylation by Mec1 in vitro.
Figure 2.6 Rad9 acts as an adaptor to facilitate Rad53 phosphorylation by Mec1 in vitro.

(A) Rad9 stimulates phosphorylation of Rad53 by Mec1 in vitro. In vitro kinase assays were carried out using constant amount of Mec1 and Rad53D339A in the absence or increasing amounts of purified Rad9. The presence of Rad9 was monitored by silver staining and the levels of Rad53 were monitored by anti-HIS tag immunoblotting (bottom panels).

(B) Rad9 does not increase Mec1 intrinsic kinase activity. Protein kinase reactions were carried out as above with the exception that phosphorylation of PHAS-I was also measured in the presence and absence of Rad9. Addition of Rad9 does not significantly increase Mec1 activity towards PHAS-I.

(C) Quantification of the PHAS-I/Rad53 kinase assays described in (B). Please note that the data for Rad53 phosphorylation +/- Rad9 was quantitated from the kinase reaction shown in panel D.
(lanes 7 versus 5). (D) In vitro Mec1 kinase assays using equimolar concentration of Rad53D339A or Rad53D339A, FHA1,2 as substrates were performed in the presence of [γ-32P]ATP in the presence or absence of Rad9. In one reaction (lane 9), the PIKK inhibitor wortmannin was also included. Proteins were separated on SDS-PAGE, transferred onto a PVDF membrane and radioactivity incorporation was measured by phosphorimaging. The presence of Rad53 was monitored by immunoblotting using anti-HIS tag antibody (bottom panel). Please note that the apparent ‘shift’ displayed by the Rad53D339A, FHA1,2 protein is due to a longer hexahistidine tag. (E) In vitro Mec1 kinase assay was performed in the presence of Rad53D339A and equimolar concentration of purified Rad9, dephosphorylated or mock-dephosphorylated. The proteins were incubated in the presence of [γ-32P]ATP, separated by SDS-PAGE and transferred to PVDF membrane. The presence of radioactivity incorporation was monitored by phosphorimaging (top panel) and the presence of Rad53D339A was monitored by immunoblotting using an anti-HIS antibody (bottom panel).

Two possible models can explain the observed effect of Rad9 on Rad53 phosphorylation by Mec1: Firstly, Rad9 may directly stimulate Mec1 catalytic activity. If this is the case, one would expect that phosphorylation of other substrates such as PHAS-I may also be stimulated by Rad9. Secondly, Rad9 may act as an adaptor (or scaffold) to bring Rad53 in close proximity to Mec1. To test the first model, we examined the effect of Rad9 on the phosphorylation of PHAS-I by Mec1. As shown in Figures 2.6BC, addition of Rad9 does not lead to appreciable stimulation of PHAS-I phosphorylation indicating that Rad9 does not act by increasing Mec1 catalytic activity.

Next, we examined whether Rad9 stimulation of Rad53 phosphorylation in vitro depends on the same determinants for Rad53 activation in vivo, namely a requirement for functional Rad53 FHA domains and Rad9 phosphorylation. First, we employed the kinase assay described above but in some reactions, substituted Rad53D339A with a mutant version of this protein incorporating the R70A and R650A mutations which disable the Rad53-Rad9 interaction in vivo (Rad53D339A, FHA1,2). As shown in Figure 2.6D (lane 8), the Rad9 stimulation of Rad53 phosphorylation clearly requires the Rad53-Rad9 phospho-dependent interaction as mutation of the Rad53 FHA domains completely abrogates the effect of addition of Rad9. Secondly, we examined whether dephosphorylation of Rad9 prior to kinase assays affects its ability to stimulate
phosphorylation of Rad53 by Mec1. As shown in Figure 2.6E, phosphorylation of Rad9 is essential for its ability to stimulate Rad53 phosphorylation by Mec1. We therefore conclude that Rad9 stimulates Rad53 phosphorylation by Mec1 in vitro via a FHA domain-dependent interaction between Rad53 and phospho-Rad9, thus recapitulating the requirement for the Rad9-Rad53 interaction essential for Rad53 activation in vivo.

2.1.5 In vitro Rad53 phosphorylation sites overlap with those observed in vivo

To verify that the reconstitution of Rad9-dependent Rad53 phosphorylation by Mec1 recapitulates Rad53 phosphorylation after DNA damage we examined whether the sites phosphorylated by Mec1 in vitro overlap with the sites mapped on Rad53 purified from yeast cells. First, we tested whether the Rad53 D339A, A8 mutant, which is not appreciably phosphorylated in vivo (Figure 2.7), can act as an efficient substrate in Mec1 kinase reactions. As shown in Figure 2.7A, substitution of Rad53 D339A by the Rad53 D339A, A8 mutant in Mec1 kinase assays (in the presence of Rad9) results in a marked decrease in incorporation of radioactivity onto Rad53, suggesting that some of the sites mapped in vivo are essential for Rad53 phosphorylation by Mec1 in vitro.

In parallel to these experiments, Rad53 D339A was phosphorylated by Mec1 in the presence of Rad9 on a preparative scale (Figure 2.7B) and Rad53 phosphorylation sites were then mapped by mass spectrometry, as described above. Satisfyingly, we unambiguously identified 13 sites, 7 of which were also identified in vivo (Figure 2.7C and Table 2.3). Of these seven sites, three correspond to S/T-Q sites, three to S/T-Ψ sites and two to S-S motifs (Table 2.1). Interestingly, some of the sites mapped in our in vitro kinase reactions were previously mapped on wild-type Rad53 and not on Rad53 D339A (see
Table 2.1 and Figure 2.2D), perhaps indicating that some sites were missed in our analysis of the Rad53$^{D339A}$ sample. Therefore, we conclude that we were, at least partially, able to reconstitute the phosphorylation of Rad53 observed *in vivo* following DNA damage. Overall, the reconstitution assays described herein support the adaptor model for Rad9 function, indicate that Mec1 directly phosphorylates Rad53 *in vitro* and *in vivo*, and suggest that Mec1 phosphorylation of Rad53 is essential for its subsequent catalytic activation.

**Figure 2.7** *In vitro* Rad53 phosphorylation sites overlap with those observed *in vivo.*

(A) The Rad53$^{D339A,A8}$ mutant is not efficiently phosphorylated by Mec1. *In vitro* Mec1 kinase assays using equimolar concentration Rad53$^{D339A}$ or Rad53$^{D339A,A8}$ as substrates were performed in the presence of [γ-$^{32}$P]ATP. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and analyzed by phosphorimaging (upper panel) or immunoblotting using an anti-His tag antibody (lower panel). The difference in size between Rad53$^{D339A}$ or Rad53$^{D339A,A8}$ is due to an additional 31 amino acids residue between the C-terminal hexahistidine sequence and the stop codon (see Materials and Methods). (B) Recombinant Rad53$^{D339A}$ was phosphorylated by Mec1 in the presence of Rad9 on a preparative scale. After termination of the kinase reaction, proteins were separated by SDS-PAGE and visualized by colloidal Coomassie-staining (left panel). In a separate reaction, radiolabeled [γ-$^{32}$P]ATP was added to monitor phosphorylation by phosphorimaging (right panel). (C) Phosphorylated residues identified on Rad53$^{D339A}$ phosphorylated by Mec1. Sites that were also identified *in vivo* are boxed. The sequence of these sites can be found in Table 2.3.
2.1.6 **CLASS II PHOSPHORYLATION IS REQUIRED FOR RAD53 ACTIVITY**

Rad53 autophosphorylation is a crucial component of its activation and has been reported by numerous groups as being essential for the proper activation of the checkpoint (Pellicioli and Foiani, 2005). As Rad53 is regulated by a two-step phosphorylation mechanism, we sought to decipher the importance of each phosphorylation steps (Rad53 kinase dependent or independent, or Class I and Class II, respectively). In order to achieve this, we tested if mutations of critical Class II residues on Rad53 would render the kinase unable to autophosphorylate and to perform its DNA damage signaling function. Cells carrying the corresponding mutant alleles would then be sensitive to DNA damage.

To test the function of Class II sites, we undertook to mutate Class II phospho-acceptor residues to alanine by site-directed mutagenesis. We tested the resulting rad53 alleles for DNA damage sensitivity and Rad53 activation. The first set of mutants examined were mutations of phosphorylation sites in the activation segment of Rad53 (S350A and T354A). As shown in Figure 2.8, mutation of these residues led to rad53 alleles that were severely defective in catalytic activation and resistance to genotoxins, indicating that Rad53 activity is controlled by the phosphorylation status of its activation segment. Furthermore, since these residues are targets of Class II phosphorylation, they likely represent autophosphorylation events. Thus, the activation mechanism of Rad53 may not require phosphorylation of the activation segment by upstream kinase(s) and may be regulated by autophosphorylation. The phosphorylation of the activation segment of Rad53 will be discussed in greater details in Chapter 4.
Figure 2.8 Rad53 in phosphorylated on its activation segment

(A) An alignment of the activation segment of Rad53 the activation segment of different kinases revealed that Rad53 is phosphorylated on two conserved residues of its activation segment. (B) Phosphorylation of Rad53 on its activation segment is required for full catalytic activation as demonstrated by in situ kinase assay. Yeast cells carrying activation segment phospho-site mutant are defective in Rad53 activation (top panel) and show absence of slower mobility forms of protein as demonstrated by western analysis (bottom panel) (C) 10 fold serial dilution of yeast cells carrying the activation segment phospho-sites mutations display DNA damage sensitivity on agar media containing genotoxins (4-NQO and HU).
Each remaining Class II site was mutated singly or in combination. In summary, we identified a combination of five Class II phospho-acceptor site mutations that led to a defect in Rad53 activation, tolerance to DNA damage and replication block \textit{in vivo} (Figure 2.9). The apparent requirement of these sites for Rad53 activation may seem surprising considering that, according to the results observed in Section 2.1.3, Class I phosphorylation appears to be the predominant activation mechanism of Rad53. However, it is possible to reconcile this apparent paradox by taking a closer look at our proposed model of Rad53 activation (see Chapter 1). This model suggests that autophosphorylation of Rad53 occurs after its initial interaction with Rad9 and is required to negatively regulate the Rad9-Rad53 interaction. Gilbert et al. (Gilbert et al., 2001) developed an assay to measure the release of Rad53 from Rad9. In this “release” assay, Rad9 is immunoprecipitated (along with Rad53 that co-purifies) from cells treated with a DNA damaging agent. The resulting Rad9 beads are then extensively washed and finally incubated in a Rad53 kinase buffer along with ATP. Rad9-bound Rad53 autophosphorylates with the ensuing release of Rad53 from Rad9, which is monitored by western blotting. Thus, a Rad53 mutant that is unable to be released from Rad9, once activated, will be "locked" on Rad9 and unable to propagate the DNA damage checkpoint signal to downstream effectors, hence, leading to a defective checkpoint response. Indeed, using this release assay, preliminary experiments demonstrated that the mutant Rad53$^{45A/S568A}$ does not release from Rad9 following DNA damage despite being phosphorylated on Class I residues (data not shown).
Figure 2.9 Class 2 phospho-sites are required for Rad53 activation

(A) Diagram showing the Class 2 phospho-sites required for Rad53 activation. (B) Phosphorylation of Rad53 on five (5) Class 2 phospho-sites is required for Rad53 full catalytic activation as demonstrated by in situ kinase assay. Yeast cells carrying alleles of Rad53 Class 2 phospho-site mutants treated with damage are defective in Rad53 activation (top panel) and show absence of slower mobility forms of protein as demonstrated by western analysis (bottom panel). (C) 100 folds serial dilution of yeast cells carrying Rad53 Class 2 phospho-sites mutants display DNA damage sensitivity on agar media containing genotoxins (4-NQO and HU).
2.2 DISCUSSION

2.2.0 PHOSPHORYLATION OF RAD53 IN RESPONSE TO DIFFERENT GENOTOXIC INSULTS

In recent years, numerous studies investigated the post-translational modifications of Rad53 (Lee et al., 2008; Ma et al., 2006; Smolka et al., 2005; Sweeney et al., 2005; Usui and Petrini, 2007). Major studies were done in our lab where we investigated the in vivo phosphorylation of Rad53 in response to 4-NQO and hydroxurea. Moreover, another study by Smolka et al. obtained quantitative data on Rad53 phosphorylation in response to methylmethane sulfonate (MMS) ((Smolka et al., 2005) and Table 2.4). Interestingly, the two studies performed in our laboratory revealed very strong overlap between the 4-NQO and HU-dependent phosphorylation sites with only 3 sites out of 19 not overlapping. Two of these non-overlapping sites (T473 and S774) are Mec1 and CDK consensus sites, respectively. Interestingly, Rad9 is dispensable for Rad53 activation in response to HU and its role is thought to be performed by Mrc1 (discussed in section 2.2.3.1). It is tempting to hypothesize that Mrc1 can endow Mec1 with a different affinity for Rad53, resulting in phosphorylation site profile that is slightly different than the Rad9-dependent sites. The identification of novel CDK consensus phosphorylation sites (also identified in the study by Smolka et al.) suggests that Rad53 might be phosphorylated by CDKs during S-Phase. In particular, phosphorylation on S774 and S375 are interesting candidates for a possible regulation of Rad53 activity during the cell cycle and perhaps even its essential role during DNA replication.

The sites identified by Smolka et al vary somewhat from the sites identified in our laboratory (6 sites out of 15 are not overlapping). Technically, Smolka et al utilized a glass beads lysis system relying on a N-terminal TAP tagging method of purification.
Using this method, they were successful in mapping N-Terminal phosphorylation sites (S24, S49). Our approach relied on mechanical grinding of cells frozen in liquid nitrogen coupled with a C-terminal Flag purification system. Some *in vitro* studies have suggested that Rad53 might be phosphorylated on its N-Terminal and that these phospho-residues would be important for Rad53 activation (Lee et al., 2008; Lee et al., 2003; Sidorova and Breeden, 2003; Smolka et al., 2005). However, we did not identify any N-terminal phosphorylation sites in our studies. Our study identified a greater number of *in vivo* sites than the other comprehensive study of Rad53 phosphorylation sites from Smolka and colleagues (27 sites from our study vs. 15 sites from (Smolka et al., 2005)). These differences rely most likely on the type of construct used, sample preparation, different MS techniques including differences in data analysis.

2.2.1 PIKK-DEPENDENT ACTIVATION OF RAD53 ORTHOLOGS

In both fission yeast and in human cells, the Rad53 orthologs (Cds1 and Chk2, respectively) are also subject to direct phosphorylation by PIKKs. However, Cds1 and Chk2 are phosphorylated by PIKKs on important N-terminal TQ motifs (T11 in *S. pombe* and T68 in human cells) in response to DNA damage (Matsuoka et al., 2000; Tanaka et al., 2001). In human cells, T68 phosphorylation of Chk2 by ATM is thought to promote the formation of Chk2 multimers, which are then competent to activate via trans-autophosphorylation (Oliver et al., 2006; Xu et al., 2002). The formation of these multimers is dependent on the Chk2 FHA domain. However, in the case of Rad53, the analogous phospho-residues appear rather to be involved in the interaction with Asf1 (chromatin remodeling protein) and Dun1 (Emili et al., 2001; Hu et al., 2001a; Lee et al.,
2008; Lee et al., 2003), and not directly in Rad53 activation. Furthermore, despite detecting the phosphorylation of the analogous T12 and T15 residues in bacterially produced hyperphosphorylated Rad53, we have failed – despite multiple attempts – to detect any phosphorylation on these residues from Rad53 purified from yeast cells (data not shown). The bacterially produced Rad53 protein is hyperphosphorylated to a great extent and many of these autophosphorylation sites are artifactual as illustrated by the identification by mass spectrometry of at least 70 distinct phosphorylation sites on recombinant Rad53 (Sweeney FD, Durocher D and Hunt DF, unpublished). Therefore, bacterially produced Rad53 appears to be a poor model to investigate the phosphoregulation of Rad53. In any case, our data suggests that phosphorylation of this cluster might not a universally conserved mechanism for the regulation of Chk2 orthologs.

2.2.2 BRCT DOMAIN PROTEINS AS MOLECULAR ADAPTORS TO THE DNA DAMAGE SIGNALING CASCADE

2.2.2.0 RAD9 AS A MOLECULAR ADAPTOR

Rad9 was the first checkpoint protein identified in yeast by the pioneering work of Weinert and Hartwell (Weinert and Hartwell, 1988). As the founding member of the group of checkpoint mediators, the elucidation of the function of Rad9 should bring new light on the role of checkpoint mediators in DNA damage signaling. In this study, we found that direct phosphorylation of Rad53 by Mec1 is required for its catalytic activation. Furthermore, based on biochemical reconstitution experiments, our results suggest that the role of Rad9 in Rad53 activation is to promote phosphorylation of Rad53 by its upstream activator,
Mec1. In that sense, the function of Rad9 in Rad53 activation is more akin to classical signaling adaptors. To our knowledge, our data presents the first direct biochemical evidence supporting the adaptor model of checkpoint mediator function. However, our data does not exclude a mixed model whereby Rad9 would play two distinct roles during Rad53 activation: first as a Mec1 adaptor and then as a facilitator of Rad53 autophosphorylation, perhaps of the activation segment, by increasing the local concentration of Rad53 on the Rad9 surface. As we will discuss in Chapter 5, local concentration of Rad53 molecules is crucial to promote its dimerization, which we hypothesize to be responsible for catalytic activation.

Mec1 therefore plays two distinct roles in the Rad53 activation cycle (Figure 2.10A): Firstly, following detection of DNA damage, Mec1 phosphorylates multiple Rad9 residues at [S/T]-Q sites (Schwartz et al., 2002). Rad9 phosphorylation presumably occurs at regions of DNA damage (Lisby et al., 2004; Naiki et al., 2004) and multisite-phosphorylated Rad9 recruits Rad53 to DNA lesions in an FHA-dependent manner (Durocher et al., 1999; Schwartz et al., 2003; Sun et al., 1998; Vialard et al., 1998). Mec1 then acts a second time to phosphorylate Rad9-bound Rad53 on multiple sites. This multisite phosphorylation of Rad53 presumably contributes to the relief of catalytic autoinhibition allowing Rad53 to become active. The relief of catalytic autoinhibition by protein phosphorylation is often used as a catalytic switching mechanism by protein kinases (Huse and Kuriyan, 2002). Following Mec1 phosphorylation, Rad53 autophosphorylates itself on many residues including S350 and T354 in the activation segment. We propose that activation segment autophosphorylation completes the Rad53 activation process, as in most kinases (Nolen et al., 2004). Active Rad53 is finally released
from Rad9 (Gilbert et al., 2001), liberating Rad9 for another round of Rad53 activation or for inactivation by the action of protein phosphatases (Figure 2.10A). The role of autophosphorylation outside the activation segment remains to be ascertained but it is tempting to speculate that at least some of these sites promote the release of Rad53 from Rad9 or promote the phosphorylation-dependent Rad53-Dun1 interaction.
2.10 Model of DNA damage checkpoint activation

(A) Model of Rad53 activation. Following Mec1/Ddc2 activation by DNA lesions (1), Mec1 phosphorylates the adaptor molecule Rad9 on multiple S/T-Q motifs (2). Phospho-Rad9, in turn, recruits Rad53 in an FHA-dependent manner to DNA lesions (3). We propose that Rad53 recruitment leads to direct phosphorylation of Rad53 by Mec1 (4). Mec1 phosphorylation of Rad53 leads to Rad53 activation and subsequent autophosphorylation (5). Hyperphosphorylated Rad53 is then released from Rad9 (6) freeing Rad9 to perform another round of Rad53 activation (7).

(B) We propose that checkpoint mediators act as PIKK adaptors during the DNA damage response, linking PIKKs to their substrates. The diversity of mediators in metazoans suggests that the spatio-temporal control of the checkpoint response is regulated by a network of protein-protein interactions controlled by checkpoint mediators.

2.2.2.1 Mrc1, a potential molecular adaptor for S-phase specific Rad53 activation

Interestingly, functional RAD9 is dispensable in the S-phase checkpoint in response to DNA replication stress. In addition, rad9 loss-of-function mutations display only a slight sensitivity to the DNA replication inhibitor hydroxyurea (HU) (Alcasabas et al., 2001; Osborn and Elledge, 2003). In 2001, a genetic screen revealed MRC1 as a gene required
for the activation of Rad53 and the tolerance of cells to DNA replication stress and S-phase specific DNA damage. (Alcasabas et al., 2001; Osborn and Elledge, 2003). Mrc1 is structurally similar to Rad9 in that it is a large (125 kDa) chromatin-binding protein with no catalytic domains. MRC1 has been linked to the DNA replication stress response pathway by evidence suggesting that it is phosphorylated in a MEC1-dependent manner following exposure to HU. Moreover, phosphorylation of Mrc1 is required for the proper DNA damage-dependent activation of Rad53 in S-phase. In addition, it has been suggested that Rad53 can, in turn, phosphorylate Mrc1, suggesting an elegant feedback loop mechanism, a phenomenon not observed with the G1 and G2 DNA damage adaptor Rad9 (Osborn and Elledge, 2003). Finally, Mrc1 along with its interacting partners Tof1 and Csm3, have been shown to be an integral component of the DNA replication fork machinery, as they are loaded shortly after DNA replication and travel along with the replication fork (Katou et al., 2003).

Upon exposure of cells to HU, DNA replication forks stall due to a depletion of dNTPs. In wild-type cells, the structural integrity of the stalled fork is maintained in part by Rad53 (Lopes et al., 2001). rad53Δ cells cannot sustain replication fork pausing and the DNA replication forks collapse. Interestingly, mrc1Δ cells do not display overt fork collapse but are rather defective in replication fork recovery from HU (Alcasabas et al., 2001; Osborn and Elledge, 2003). Consequently, mrc1Δ mutants are unable to reinitiate DNA replication after the removal of the drug. Genetic evidence implicating MRC1 in the replication stress, S-phase specific DNA damage and normal DNA replication processes are compelling and suggest a complex mechanism of regulation by which Rad53 and Mrc1, along with Mec1, act in concert to police the proper behavior of the DNA
replication machinery under normal and stress-induced conditions. Unfortunately, due to a lack of biochemical data, it is impossible for now to propose a clear mechanism on how Mrc1 and Rad53 regulate this DNA damage response in S-phase. Nevertheless, with the structural and genetic similarities between MRC1 and RAD9, it is tempting to speculate that Mrc1 could act as the “Rad9 of S-phase”. It would be interesting to test such a hypothesis using a similar approach as described in this Chapter. A combination of HU-dependent phosphomapping of Mrc1 couple with some *in vitro* reconstitution experiment using recombinant Mec1, Mrc1 and Rad53 could prove determinant in testing whether Mrc1 perform a similar function to Rad9 in S-phase of the cell cycle.

2.2.2.2 CHECKPOINT MEDIATORS AS PIKK ADAPTORS

In ‘classical’ signal transduction pathways such as those operating at the cell surface, signaling adaptors are usually polypeptides that lack known catalytic domains but possess at least two protein-protein interaction domains, usually of a modular nature (Pawson and Nash, 2003). In a general sense, signaling adaptors function to couple upstream signaling with downstream events by bridging at least two distinct proteins. The modular nature of signaling adaptors enables the use of a relatively small subset of protein domains and target motifs to construct elaborate signaling networks. In such networks, adaptors also act as regulatory nodes where signaling can be regulated via signal-dependent protein-protein interactions.

Based on the above considerations and on the following observations, we propose that Rad9 acts as a *bona fide* signaling adaptor during the DNA damage response: (1) Rad9 physically co-localizes with Mec1 at sites of DNA damage suggesting that both proteins
may physically interact (Lisby et al., 2004); (2) the Rad9-Rad53 interaction is essential for Rad53 activation \textit{in vivo} (Schwartz et al., 2003; Sun et al., 1998); and (3) Rad9 greatly stimulates Rad53 phosphorylation by Mec1 \textit{in vitro} (this Chapter). We propose that Rad9 functions to physically bridge Rad53 to Mec1 and that Rad9 may be able to physically interact with Mec1, at least transiently. It is tempting to speculate that this latter interaction may be mediated via the tandem BRCT domains of Rad9 as the Rad9 BRCT domains are required for checkpoint signaling \textit{in vivo} (Soulier and Lowndes, 1999). Interestingly, tandem BRCT domains can act as phosphopeptide recognition modules (Manke et al., 2003; Yu et al., 2003) but whether the Rad9 BRCT domains act as \textit{bona fide} phosphopeptide recognition modules to bind to Mec1 or any other protein remains to be determined.

Finally, given the similarities between Rad9 and human checkpoint mediators containing tandem BRCT domains, we propose that Brca1, MDC1 and 53BP1 also act as PIKK adaptors during the DNA damage response and that this activity represents a common function of checkpoint mediators. Interestingly, each known mammalian checkpoint mediator harbors a unique protein-interaction module such as the FHA domain (Mdc1), RING-finger (Brca1), or the Tudor domain (53BP1) indicating that they promote either a unique set of protein-protein interactions or engage an overlapping set of proteins in response to different upstream signals. The requirement for multiple checkpoint mediators in mammals may reflect the increased complexity of the mammalian genome or may indicate that novel layers of regulation were added during metazoan evolution. By virtue of these properties, we therefore propose that checkpoint
mediators are PIKK adaptors that orchestrate the DNA damage response in space and time.

2.2.3 REGULATION OF RAD53 OFF-RATE VIA CATALYTIC AUTOPHOSPHORYLATION

As mentioned previously, a combination of 5 Rad53 Class II phospho-acceptor site mutations resulted in a defect in Rad53 release from Rad9 (Figure 2.9C). Interestingly, modeling of the position of these residues on the Rad53 FHA2 NMR structure (Byeon et al., 2001) predicts that they would be situated in close proximity to the site of the FHA-phosphopeptide interaction. I hypothesize that phosphorylation of these Class II sites may regulate the release of Rad53 from Rad9 either by electrostatic repulsion of the FHA2 domain from the phospho-Thr epitope(s) of Rad9 or via an intramolecular engagement of the phosphorylated Class II sites in the phosphate-binding residues of the FHA2 domain. However, some key experiments need to be performed before reaching that conclusion. First and foremost, it is crucial to test whether the defect in Rad53 release of the Class II mutant is not due to a general defect in kinase activation. Gilbert et al. demonstrated that a kinase-defective Rad53 is not released from Rad9 (Gilbert et al., 2001). In order to address whether the Class II sites are specifically required for Rad9 release, one needs to examine the Rad53 activation status of Rad9-bound Rad53 by examining the phosphorylation status of the Rad53 activation segment on the Rad9 bound Rad53 molecules. A true “release-deficient” Rad53 mutant is predicted to be highly phosphorylated on the activation segment when compared to Rad9-bound wild-type Rad53. Moreover, one would expect that mutation of the Class II Serine residues to acidic residues would render Rad53 unable to interact with the phosphorylated threonine
residue(s) on Rad9, a situation that could be tested by co-immunoprecipitation experiments. Another strategy could also be to test if the Rad53-Rad9 interaction can be competed out by the presence of phosphorylated peptides corresponding to the phosphorylated sequences of the crucial five Class II phospho-sites.

However, other mechanisms can be involved in this process and be explained by different hypothesis. For instance, these phosphorylated Class II sites could form docking site for a "release factor" or alternatively, these phosphorylation sites could targeted by other kinase(s) activated in a DNA damage and Rad53-dependent manner.
### 2.3 Tables

#### Table 2.1a. 4-NQO dependent Rad53 phosphorylation sites mapped by mass spectrometry

<table>
<thead>
<tr>
<th>Sites found on Rad53&lt;sup&gt;D339A&lt;/sup&gt;</th>
<th>Sites found only in the Rad53 sample</th>
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</thead>
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<tr>
<td>S165 RIRSNLK</td>
<td>T170 LKNTSKI</td>
</tr>
<tr>
<td>S198 KDFSIID&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S171 KNTSKIA</td>
</tr>
<tr>
<td>S375 TSVSPDE</td>
<td>S184 STASSMV</td>
</tr>
<tr>
<td>S485 ISLSQSL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S185 TASSMVA</td>
</tr>
<tr>
<td>S489 QSLSQQK&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S350 GNGSFMK&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<td>T543 IRYTQP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T354 FMKTF&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>S373 KDTSVSP</td>
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<td>S411 FSGSTQD</td>
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<td>T563 SNNTENV</td>
<td>S568 NVKSSKK</td>
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<td>S748 SSSSMA</td>
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<td>S795 LSQSQID&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>Potential PIKK consensus phosphorylation sites (Ser/Thr-Gln or Ser/Thr-Y where Y denotes hydrophobic residue).

<sup>b</sup>Activation segment phosphorylation sites.
Table 2.1b. Phosphopeptides identified from Rad53 and Rad53<sup>D339A</sup> following DNA damage. (*) denotes the phosphorylated residue.

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<th>Sequence</th>
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<td>IHS<em>VS</em>LS*QSQIDPSK</td>
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Table 2.2a Rad53 HU-dependent phosphorylation sites mapped by mass spectrometry

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<td>S791 HSVLSQS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T543 IRYTQP&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S793 VSLQSQS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S547 QPKSIEA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S795 LSQSQID&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S560 LLHSNNT</td>
</tr>
<tr>
<td></td>
<td>T563 SNNTENV</td>
</tr>
<tr>
<td></td>
<td>S568 NVKSSKK&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>S569 VKSSKKK</td>
</tr>
<tr>
<td></td>
<td>S746 SASSSSM</td>
</tr>
<tr>
<td></td>
<td>S748 SSSSMSA</td>
</tr>
<tr>
<td></td>
<td>S750 SSMSAKK</td>
</tr>
<tr>
<td></td>
<td>S774 LVESPIN</td>
</tr>
</tbody>
</table>

*Potential PIKK consensus phosphorylation sites (Ser/Thr-Gln or Ser/Thr-Y where Y denotes hydrophobic residue).

Table 2.2b Phosphopeptides identified from Rad53 and Rad53<sup>D339A</sup> following replication stress. (*) denotes the phosphorylated residue.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Residue#</th>
<th>*site position</th>
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</thead>
<tbody>
<tr>
<td>1 M.VANKT*GIFK.D</td>
<td>186-196</td>
<td>T190</td>
</tr>
<tr>
<td>2 K.TGIFKDFS*IIDE.V</td>
<td>190-203</td>
<td>S198</td>
</tr>
<tr>
<td>3 R.GKDTSVS*PDEYEER.N</td>
<td>368-383</td>
<td>S375</td>
</tr>
<tr>
<td>4 M.SPLGS*QSYGD.F.S</td>
<td>468-480</td>
<td>S473</td>
</tr>
<tr>
<td>5 Y.GDFSQISLS<em>QSLS</em>QQK.L</td>
<td>476-493</td>
<td>S485, S489</td>
</tr>
<tr>
<td>6 K.IPAHAPIRYT*QPK</td>
<td>533-547</td>
<td>T543</td>
</tr>
<tr>
<td>7 R.YTQPKS*IEAETR.E</td>
<td>541-554</td>
<td>S547</td>
</tr>
<tr>
<td>8 K.LLHS*NNTENVK.S</td>
<td>556-568</td>
<td>S560</td>
</tr>
<tr>
<td>9 K.LLHSNNT*ENVK.S</td>
<td>556-568</td>
<td>T563</td>
</tr>
<tr>
<td>10 K.LLHS<em>NNT</em>ENVS*KSK.K</td>
<td>556-571</td>
<td>S560, T563, S568</td>
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<tr>
<td>11 K.LLHS<em>NNTENVKSS</em>K.K</td>
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<td>S560, S569</td>
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<td>12 K.LLHSNNTENVKSS*K.K</td>
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<td>S569</td>
</tr>
<tr>
<td>13 R.ANQPSASS<em>SS</em>MS*AK.K</td>
<td>738-573</td>
<td>S746, S748, S750</td>
</tr>
<tr>
<td>14 L.VES*PINANTGNILK</td>
<td>771-785</td>
<td>S774</td>
</tr>
<tr>
<td>15 N.DVES*PINANTGNILK</td>
<td>769-785</td>
<td>S774</td>
</tr>
<tr>
<td>16 R.HIS<em>VLSL</em>QSQIDPSK.K</td>
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<td>S789, S793</td>
</tr>
<tr>
<td>17 R.IHS<em>VLSL</em>QSQIDPSKK.V</td>
<td>786-803</td>
<td>S789, S793</td>
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<tr>
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<td>S789, S795</td>
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<td>19 R.IHS*VLSQSQIDPSK.K</td>
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<td>S789, S791</td>
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<tr>
<td>20 R.IHS*VLSQSQIDPSK.K</td>
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<tr>
<td>21 R.IHS<em>VLSL</em>QSQIDPSKK.V</td>
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<td>S793</td>
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<tr>
<td>22 R.IHSVLSQSQQIQIDPSK.K</td>
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</tr>
<tr>
<td>23 R.IHSVLSLSQSQIDPSK.K</td>
<td>786-802</td>
<td>S793</td>
</tr>
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</table>
### Table 2.3 Sites found on Rad53 phosphorylated by Mec1 in vitro

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
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<tr>
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<td>ANKTGIF$^b$</td>
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<tr>
<td>S568</td>
<td>NVKSSKK$^{a,b}$</td>
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<td>RFLTLKP</td>
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<td>KKLQOMM</td>
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<td>SASSSSM$^{a,b}$</td>
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<tr>
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<tr>
<td>S811</td>
<td>DQTSKGP</td>
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<tr>
<td>S821</td>
<td>LQFS$^*$</td>
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</table>

$^a$Sites found in the Rad53 samples purified *in vivo* (4-NQO)

$^b$Sites found in the Rad53 samples purified *in vivo* (HU)
**Table 2.4** Comparative Analysis of Rad53 *in vivo* phosphorylation sites from different genotoxic insults

<table>
<thead>
<tr>
<th>Rad53 phosphorylation Sites</th>
<th>4-NQO (Sweeney et al.)</th>
<th>Hydroxyurea (Sweeney et al.)</th>
<th>MMS (Smolka et al.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EKFSSEQQ</td>
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<td>RIRSNLQK</td>
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<td>T170</td>
<td>LKNTSKI</td>
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<td>S171</td>
<td>KNTSKIA</td>
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<tr>
<td>T354</td>
<td>FMKTFCG&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>S795</td>
<td>LSOSSQID&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Potential PIKK consensus phosphorylation sites (Ser/Thr-Gln or Ser/Thr-Y where Y denotes hydrophobic residue).
2.4 EXPERIMENTAL PROCEDURES

DNA DAMAGE SENSITIVITY ASSAYS

4-nitroquinole oxide (4-NQO) was purchased from Sigma-Aldrich Chemicals. Drop tests were performed using ten-fold serial dilutions of an overnight culture. A 8 µl aliquot of the diluted culture was spotted on corresponding dropout media plates with or without genotoxins (see figure legends for details) and were grown for 3-4 days at 30°C.

RAD53 IN SITU KINASE ASSAY (ISA) AND IN VITRO KINASE ASSAYS

The Rad53 in situ kinase assays were performed as described (Pellicioli et al., 1999a). In vitro kinase assays were performed as follows. Purified recombinant kinase, adaptor and substrate were incubated in Mec1 kinase buffer (25 mM Hepes pH 7.5, 400 mM NaCl, 20mM MnCl₂, 1mM DTT, 5 mg/ml ethidium bromide, 40 mM ATP and 0.5 ml [γ-³²P]ATP (Perkin-Elmer)) for 90 min unless indicated otherwise. Similar results are obtained if ethidium bromide is omitted from the reactions. The reactions were stopped by adding SDS sample buffer and by boiling the sample for 5 min. Half of the reactions were then loaded on 8% SDS-PAGE gel and transferred to PVDF membrane (Millipore). The membrane was then exposed overnight on a phosphor screen (GE bioscience) and revealed by phosphorimaging (GE Bioscience). All quantifications were done with ImageQuant

STRAINS AND PLASMIDS

All strains are derivatives of W303a. The strain MATα rad53::HIS3 sml1-1 (DD002) was obtained as a gift from Xiaolan Zhao and Rodney Rothstein (Zhao et al., 1998b). The strain rad9::HIS3 rad53::HIS3 sml1-1 was generated by standard genetic techniques
from DD002 and the loss of the Rad9 protein was confirmed by Western blotting. The strain *MATa mec1::TRP1 sml1::HIS3* (DD005) was a kind gift of Rodney Rothstein. All the other strains were generated by introducing the plasmids mentioned above according to standard yeast genetic techniques.

The plasmid YCplac33-*RAD53* is a kind gift from Nori Sugimoto (Sugimoto et al., 1997), the YCplac33 plasmid was obtained from Allison Bardin (MIT) and the His-tagged Rad53 expression plasmid is a gift of Noel Lowndes. All Rad53 mutants used in this study were generated by site directed mutagenesis (Stratagene) unless indicated otherwise. The pET15b-*rad53* plasmid was generated by introducing the *RAD53* ORF from YCplac33-*rad53* in the *Nco*I site of the pET15b plasmid. The pET15b-*rad53* was then used as a template for mutagenesis to generate the pET15b-*rad53,D339A* plasmid. pGAL1/10-*RAD53-FLAG* originates from a previous study (Ho et al., 2002b). The pGAL1/10-*FLAG-MEC1* plasmid was generated by cloning two sets of complementary oligos coding for the 3xFLAG-10xHIS-Linker-TEV sequence into a *Bam*HI digested pFA6-*TRP1* plasmid (Longtine et al., 1997). The *MEC1* genomic locus was then tagged by integration of the new pFA6-3xFLAG-10xHIS-Linker-TEV and cloned into pRS426-*MEC1* (a gift from Nori Sugimoto) by gap repair. pEG-*RAD9* was constructed by PCR cloning using Pfu polymerase (Stratagene). Full-length *RAD9* was amplified from pRS426-*RAD9* (gift from David Lydall) and cloned *Bam*HI into pEG-KG (gift from Frank Sicheri). The plasmid encoding the *rad9* allele was generated by site directed mutagenesis on the pRS426-*RAD9* template. All plasmids and mutants derived from these plasmids were sequenced.
IMMUNOBLOTTING, SMALL-SCALE IMMUNOPURIFICATION AND PHOSPHATASE TREATMENT

Immunoblotting of Rad53 was performed as described (Kanellis et al., 2003). The antibodies against GST, hexahistidine tag and PHAS-we were obtained from Santa Cruz Biotechnologies. The antibody against the Flag epitope was obtained from Sigma. The antibody against the PIKK consensus sites phospho-Ser/Thr-Gln was obtained from Cell Signaling Technologies. All SDS-PAGE were performed using an acrylamide to bis-acrylamide ratio of 37.5:1 unless indicated otherwise (Bio-Rad).

Small-scale immunopurification of Rad53 was preformed by immunoprecipitating 1 mg of native crude extract obtained from glass bead lysis for 3 hrs at 4°C in lysis buffer (50mM Tris pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM Microcystin-LR and 1x protease inhibitor cocktail (Roche). 5 ml of Rad53 polyclonal antibody DAB001 was used per sample. 25 ml of Protein A-agarose (Pierce) was then added and the samples were incubated for an extra 45 min. Immunoprecipitates were washed 3 times with lysis buffer supplemented with a total of 500 mM NaCl and proteins were finally eluted by addition of SDS-PAGE loading buffer.

Cultures of BL21 E. coli cells transformed with plasmids encoding recombinant His6-Rad53 were grown to OD600 0.6. Protein expression was induced by adding 1 mM IPTG for 3 hrs at 30°C. Cells were lysed by sonication in lysis buffer (50 mM Hepes pH 7.4, 250 mM NaCl, 5 mM imidazole). The clarified lysate was incubated with Ni2+-NTA agarose resin (Qiagen) for 1 hr at 4°C and the beads were washed 3 times with lysis buffer supplemented with 20 mM imidazole. The protein was then eluted from the beads with two subsequent washes of one bead volume of lysis buffer supplemented with 300 mM imidazole.
Protein phosphatase treatments were performed as follows. The eluted proteins were diluted in 1X l protein phosphatase buffer containing 2 mM MnCl₂ and incubated with 400 U of l protein phosphatase for 30 min at 30°C. The reactions were stopped by adding 1X SDS-PAGE loading buffer.

**RAD53-FLAG PURIFICATION**

Cultures (6 L) of the appropriate strains were grown to OD₆₀₀= 1 in rich media containing 2% raffinose. Protein expression was induced for 3 hrs following addition of 2% galactose. Cells were then treated with 3 mM 4-NQO for 1 hr. Cells were then harvested by centrifugation and lysed by nitrogen grinding in lysis buffer (0.5% Triton X100, 25 mM HEPES pH7.5, 150 mM NaCl, 30 mM b-glycerophosphate, 20 mM NaF, 100 mM Sodium orthovanadate, 1 mM DTT, 1 mm Microcystin LR and 1x protease inhibitor cocktail (Boehringer Mannheim). 1.2 ml of Anti-Flag M2-Agarose resin (Sigma) was added to 40 ml of lysate and immunoprecipitation was performed for 1 hr at 4°C. The resin was sedimented by centrifugation and washed with alternating buffers of High Stringency Detergent (0.5% Triton X100, 25mM Hepes ph7.5, 150mM NaCl, 1mM DTT and 0.5% Deoxycholic Acid) and High Salt (0.5% Triton X100, 25mM Heps ph7.5, 1000 mM NaCl) for three washes per buffer. The resin was finally resuspended in 5 ml of Elution Buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT and 220 mg/ml M2-FLAG peptide) and the elution was performed for 10 min at room temperature. The elution procedure was repeated a second time and both supernatants were combined and dialyze 3 times in 20mM HEPES pH 7.5, 250mM NaCl ,1mM DTT and concentrated to a final volume of 500 ml.
PHOSPHOPEPTIDE ANALYSIS USING OFF-LINE Fe (III)-IMAC FOLLOWED BY THE nRP-HPLC-µESI-MS/MS

Protein aliquots (300 μl) were added to 150 μl of ammonium bicarbonate (100 mM, pH 8.5), reduced with 300 mM dithiothreitol (DTT, 7 μl) at 37°C for 1 h, carboxyamidomethylated with 1 M iodoacetamide (13 μl) in the dark at room temperature for 1 h, and digested with modified trypsin (1.5 μg) (Promega) at 37°C for 12 h. One third of the trypsin digest (~150 μl) was further digested with endoproteinase Glu-c (0.5 μg) for 8 h at 37 °C and pH 8. Chymotrypsin (0.5 μg) was used to further digest another one third of trypsin digested samples (150 μl) for 8 h at 37 °C and pH 8. Each of the three digest samples was concentrated under vacuum on a Speed Vac and acidified with glacial acetic acid to pH ~3.

The Iron (III)- Immobilized Metal Affinity Chromatography (IMAC) was performed according to (Ficarro et al., 2002b). Briefly, a 360 × 100-µm fused silica column (Polymicro Technologies, Phoenix, AZ) was packed with 8 cm POROS 20 MC (Perspective Biosystems, Farmingham, MA). EDTA (100 mM, pH 10) was used to rinse the column for about 10 minutes to strip the free metal off the packing material. The column was then rinsed with nanopure water for 5 minutes to remove excess EDTA. Next, the column was activated with 100 mM FeCl₃ (Aldrich, Milwaukee, WI), rinsed with 0.1% acetic acid to remove excess metal and equilibrate the column. Thirty percent of each digest for the same type of sample (Rad53 WT or KD) was combined (~40 μl), and loaded on this conditioned Fe (III)-IMAC column to enrich for phosphopeptides. The IMAC column was washed with 10 μl of wash buffer (100 mM NaCl, 1% HOAc, 25%
MeCN) to remove nonspecific binding peptides and then re-equilibrated with 20 μl of 0.1% HOAc. Phosphopeptides were eluted from Fe (III)-IMAC column to a nano-HPLC pre-column [360 × 100 μm, 5 cm 5-20 μm C18 beads (YMC ODS-AQ,Waters) with 15 μl of 500 mM ascorbic acid. Sample was washed with 0.1% HOAc and the pre-column was then connected to the analytical column [360 × 50 μm, 7 cm 5 μm C18 beads (YMC ODS-AQ, Waters)]. Samples were then analyzed by a combination of a nano-HPLC/micro-electrospray ionization on a LCQ Deca mass spectrometer (ThermoFinnigan, San Jose, CA) as described previously (Ficarro et al., 2002b).

LC/MS/MS PARAMETERS ON THE LCQ QUADRUPOLE ION TRAP MASS SPECTROMETER

Samples were analyzed by nanoflow HPLC-microelectrospray ionization on a ThermoFinnigan LCQ Deca mass spectrometer (San Jose, CA) operating in the data-dependant mode. An HPLC gradient consisting of 0-60%B in 80 minutes, 60-100%B in 10 minutes (solvent A = 0.1M acetic acid in nanopure water, solvent B = 70% acetonitrile in 0.1 M acetic acid) was used to elute peptides from the reverse-phase column to the mass spectrometer through the emitter tip on the analytical column.

DATABASE SEARCH

All MS/MS spectra recorded on tryptic phosphopeptides derived from Rad53 samples were searched against Rad53 WT or KD database by using SEQUEST algorithm (Eng et al., 1994). Search parameters included a differential modification of +80 Da (presence or absence of phosphate group) on serine, threonine, and tyrosine and a static modification of +57(carboxyamidomethylation) on cysteine. MS/MS spectra for phosphopeptides with
Xcorr > 1.2 were manually confirmed. Spectra with low cross correlation scores but high neutral loss counts have been also manually confirmed.

**RAD9 PURIFICATION**

pEG(KG)-RAD9 was transformed in DD002 (MAT a rad53::HIS3 sml1-1). 6 L of cells were grown in SD-LEU 2% raffinose to log phase (OD\textsubscript{600}=1) and GST-Rad9 expression was induced by the addition of 2% galactose. Cells were then allowed to grow for another 4 hrs and were then harvested by centrifugation, washed with one packed cell volume of water and 2X Rad9 lysis buffer without KOAc (100 mM HEPES-KOH pH 7.4, 1 mM EDTA, 20% glycerol, 8 mM b-mercaptoethanol, 10 mM magnesium acetate, 2X complete protease inhibitor mix (Boehringer Mannheim). The compact cell pellet was then extruded through a syringe into liquid nitrogen and the frozen cell pellet was mechanically ground with a mortar and a pestle until a fine powder was obtained. The powder was resuspended in 1 volume (of the starting packed cell pellet) of 2x Rad9 lysis buffer supplemented with 20mM KOAc and clarified by centrifugation. The concentrated lysate was diluted 1:5 with 1X Rad9 lysis buffer (10mM KOAc) and incubated with 5 ml of Affi-Gel Heparin (Biorad) for 1 hr at 4°C. The column was then washed with 10 bead volume of 1X Rad9 lysis buffer (with 100 mM KOAc). GST-Rad9 was then eluted from the column with 3 bead volumes of 1X Rad9 lysis buffer (with 500 mM KOAc). 2 ml of glutathione sepharose 4B was then added to the eluate and equilibrated at 4°C for 30 min. The column was then washed extensively (~10 bead volume) with H1 buffer (30 mM Hepes pH 7.5, 1 mM DTT, 0.25 mM EDTA, 0.01%NP-40, 0.1% Inositol) supplemented with 1 M NaCl. The same beads were then washed with H1 buffer containing 250 mM NaCl, 2 mM DTT and 4 mM
EDTA and incubated with TEV protease overnight at 4 degrees (1 bead volume). The flow-through was then collected and beads were washed with one bead volume of H1 supplemented with 250 mM NaCl. The resulting eluate was then concentrated (Vivaspin, 2ml concentrators, MWCO 30,000 Da). The presence and relative purity of GST-Rad9 and Rad9 were monitored by SDS-PAGE and colloidal coomassie staining (Pierce, GelCode Blue staining). The purified protein was then aliquoted in small amount and stored at -80°C.

**MEC1 IMMUNOPURIFICATION**

 Yep-FLAG-MEC1 was transformed into DD029 (Mat a rad53::HIS3 rad9::HIS3 sml1-1) and cells were grown and lysed as described for GST-Rad9 in 2x Mec1 lysis buffer (20% Glycerol, 100 mM Hepes pH 7.4, 200 mM KCl, 0.2 mM EDTA, 0.4% Tween, 8 mM b-mercaptoethanol, 5 mg/ml ethidium bromide) supplemented with 10mM Imidazole at pH 8.0. The lysate was clarified by centrifugation and diluted 5-fold in 1X Mec1 lysis buffer containing 5 mM Imidazole. 2 ml of Ni-NTA agarose (Qiagen) was washed with 1X Mec1 lysis buffer (with 5 mM imidazole), added to the diluted lysate and incubated for 1 hr at 4°C. The column was washed with 15 bead volumes of 1X Mec1 lysis buffer supplemented with 20 mM Imidazole pH8.0. Flag-Mec1 was then eluted with 5 bead volumes of 1X Mec1 lysis buffer containing 500 mM Imidazole pH 8.0 and the resulting eluate was incubated with 0.5ml Anti-Flag agarose resin (Sigma) for 1 hr at 4°C. The beads were then washed with 10 bead volumes of 1X Mec1 lysis buffer supplemented with 250 mM NaCl, resuspended in 2 bead volumes of the same buffer and were frozen in liquid nitrogen in small aliquots.
CHAPTER 3

RESULTS

REGULATION OF THE DNA DAMAGE CHECKPOINT RECOVERY AND ADAPTATION BY CDC5-DEPENDENT RAD53 DOWNREGULATION

Adapted from
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3.1 INTRODUCTION

The DNA damage checkpoint is critical for the timely repair of damaged DNA, which can occur at different periods of the cell cycle. Although the mechanisms by which cells activate DNA damage checkpoint signaling allowed us to obtain a precise, although not exhaustive, comprehension of how they are able to slow down or halt their cell cycle in response to DNA damage (Melo and Toczyski, 2002) the mechanisms that lead to the resumption of the cell cycle (referred here as checkpoint recovery) remains less understood. Moreover, in some other instances, cells can resume division despite the presence of irreparable damage, a process called DNA damage adaptation. The genetic and biochemical processes by which recovery and adaptation occur have been described in detail in Section 1.2.2. Briefly, a cohort of phosphatases, kinases and other ATPase are involved in shutting down the key DNA damage checkpoint kinases proteins, resulting in the termination of the checkpoint.

One family of kinases important for the adaptation process is the Polo-like kinases (Plks). Plks are serine/threonine protein kinases that are involved in different aspects of mitosis such as mitotic entry, mitotic exit and cytokinesis (Lee et al., 2005; Malumbres and Barbacid, 2007; van de Weerdt and Medema, 2006). The catalytic domain of Plks is usually located in the N-terminus of the protein whereas the C-terminus contains usually tandem Polo Box Domains (PBD), which are conserved modular interaction domains that possess the ability to recognize phospho-Ser/Thr epitopes (Lowery et al., 2005). Structural and biochemical studies of Xenopus Plk1 shed light onto the role of Plks PBD (Lowery et al., 2005; Lowery et al., 2004). In addition to its role in regulating subcellular regulation through phospho-Ser/Thr dependent protein-protein interactions, the PBD also
determines an auto inhibitory regulation of the kinase domain. In particular, the PBD recognizes phosphopeptides from the consensus motif Ser-[pThr/pSer]-Pro (Elia et al., 2003). The mammalian Plk family consists of four different kinases (Plk1, Plk2, Plk3 and Plk4/Sak) whereas the *Xenopus laevis* shares three homologs (Plx1, Plx2 and Plx3). The remaining organisms possess only a single Plk: Polo in *Drosophila melanogaster*, Plo1 in *Schizosaccharomyces pombe* and Cdc5 in *S. cerevisiae* (Lee et al., 2005; van de Weerdt and Medema, 2006).

Although the precise mechanisms by which the Polo-like kinases direct mitotic progression are well characterized, how Cdc5 performs its adaptation function in *S. cerevisiae* remains elusive and relies on evidence from one allele isolated by Toczyski and Hartwell which is defective in adaptation: *cdc5-ad* (Pellicioli et al., 2001). However, as mentioned in Section 1.2.2, Plk1 in human and Xenopus Plx1 were shown to play an active role in recovery/adaptation by acting on Chk2 and Claspin, respectively (Mamely et al., 2006; Tsvetkov and Stern, 2005; Tsvetkov et al., 2003; Tsvetkov et al., 2005).

In this Chapter, we provide the first biochemical evidence explaining the role of the Polo-like kinase Cdc5 in the regulation of adaptation in *S. cerevisiae*. This evidence converges towards a working model in which Cdc5 would bind to and directly downregulate Rad53 activity via phosphorylation. Moreover, we have identified some candidate phosphorylation sites *in vitro* and, in accordance with the results obtained by atomic resolution of the Rad53 kinase domain presented in Chapter 4, we can hypothesize that this phosphorylation event by Cdc5 might regulate the dimer to monomer transition of Rad53, therefore influencing its activity.
3.1 RESULTS

3.1.0 OVEREXPRESSION OF CDC5 LEADS TO RAD53 INACTIVATION

In order to investigate further the role of Cdc5 in the regulation of DNA damage signaling and adaptation, the group of David Toczyski tested the effect of Cdc5 overexpression on Rad53 activity in vivo. In order to do so, yeast cells carrying the temperature sensitive (ts) mutated allele cdc13-1 were transformed with plasmids containing the CDC5 gene under the control of the GAL1/10 promoter. Cdc13 is a telomere capping protein essential to protect DNA ends and to prevent these DNA ends to be recognized as DNA damage (Gardner et al., 1999; Weinert et al., 1994). Upon shifting the cdc13-1 ts strain at restrictive temperature (32 °C), telomeres become uncapped and detected by the DNA damage checkpoint system. On the other hand, the GAL1/10 promoter, as described in Chapter 2, is strongly induced in the presence of galactose and allow the timely overexpression of the genes it promotes, in this case CDC5. This cdc13-1 pGAL-HA-CDC5 strain (the CDC5 gene is tagged with the HA epitope to facilitate immunoblotting analysis) was grown to log phase at permissive temperature and galactose was added to the media in order to overexpress Cdc5. As a control, a strain carrying the same CDC5 gene without the GAL1/10 promoter was utilized. Following the overexpression of Cdc5, DNA damage was induced by shifting cells at restrictive temperature and the activity of Rad53 was monitored by in situ kinase assay and immunoblotting using a polyclonal antibody specific to Rad53 (Pellicioli et al., 1999b; Sweeney et al., 2005). Cdc5 overexpression was monitored by immunoblotting using an HA antibody and Cdc28 was also immunoblotted and acted as a loading control. As seen in Figure 3.1A, Cdc5 overexpression clearly reduced Rad53 kinase activity and
phosphorylation state indicating that Cdc5 acts on the Rad53 pathway to inhibit or down regulate its activity.

In order to test whether the phenotype observed with \( \textit{CDC5} \) overexpression is relevant to adaptation, it is important to test whether this phenomenon is observed with all types of DSBs. Moreover, one should also examine the importance of Cdc5 catalytic activity in Rad53 down regulation. In order to address these two issues, we generated yeast strains carrying the wild-type \( \textit{CDC5} \) gene as well as the catalytically inactive \( \textit{cdc5} \) allele (\( \textit{cdc5-K110A} \) or simply \( \textit{cdc5-kd} \)). These strains were grown to log phase separately and DNA damage was induced using the drug zeocin. Zeocin is a drug from the bleomycin family, which intercalates DNA and promotes double stranded DNA breaks (DSBs) (Sleigh and Grigg, 1977). After the induction of DNA damage, \( \textit{CDC5} \) and \( \textit{cdc5-kd} \) were overexpressed with the addition of galactose to the media (or glucose as a control) and samples were taken for analysis pre-induction as well as 3 hrs post-induction. As seen in Figure 3.1B, Rad53 phosphorylation and activity was downregulated in the presence of overexpressed Cdc5\textsuperscript{wt} but not Cdc5\textsuperscript{kd}. These results suggest that the downregulation of Rad53 by Cdc5 requires Cdc5 catalytic activity and that Cdc5 can downregulate Rad53 in response to different types of DSBs (telomeric and drug-induced).
Overexpression of Cdc5 downregulates the DSB-dependent Rad53 activity

(A) Yeast strains carrying the cdc13-1 pGAL CDC5 or pGAL cdc5-kd alleles were shifted at restrictive temperature following the galactose-induced overexpression of Cdc5. The activation status of Rad53 was monitored by in situ kinase assay (ISA, top panel) and by electrophoretic mobility shift using a Rad53 specific antibody (2nd panel from the top). The overexpression of Cdc5 was monitored using an anti-HA antibody (3rd panel from the top) and Cdc28 western was utilized as a loading control (bottom panel). (B) A similar experiment using CDC13 CDC5 or CDC13 cdc5-kd strains was performed where DNA damage was induced by the DSB-causing drug Zeocin. Rad53 activity was monitored by ISA and western blot after pre and post induction (3hrs) and 1hr after drug treatment (two first panel from the top, respectively). PGK3 was monitored by immunoblotting as a loading control (bottom panel).

The Cdc5-dependent down regulation of Rad53 could occur at different steps in the Rad53 activation process (Figure 3.2). For instance, Cdc5 could act on Mec1 or other Mec1-dependent mechanisms. On the other hand, Cdc5 could also regulate the binding of Rad53 to Rad9, similarly to how Plx1 phosphorylation of Claspin prevents Chk1 binding in Xenopus (Yoo et al., 2004). Another hypothesis could be that Cdc5 acts
directly on Rad53 either by (a) preventing its activation or (b) promoting its inhibition once activated (Figure 3.2).

**Figure 3.2 Cdc5 as a potential direct down regulator of DNA damage signaling?**
A non-exhaustive description of the mechanisms by which Cdc5 could potentially down regulated the DNA damage signaling cascade. Cdc5 could act as a downregulator of Mec1 (1), act directly on Rad9 (2) or modify Rad53 directly (3).

### 3.1.1 CDC5 PHOSPHORYLATES RAD53 IN VITRO

To identify the mechanism by which Cdc5 inhibits, we first examined whether the Mec1-dependent phosphorylation of Rad53 on [Ser/Thr]-Gln sites was affected by *CDC5* overexpression. Yeast strains carrying an overexpression vector for *CDC5* or *cdc5-kd* were grown to log phase, treated with damage and Cdc5 protein expression was induced for 1 hr and 3 hrs. After these time points, cells were harvested and Rad53 was immunoprecipitated from native cells extracts using a polyclonal Rad53 antibody. The immunoprecipitates were then assayed by immunoblot using an antibody specific to
phosphorylated [Ser/Thr]-Gln epitopes (p[S/T]Q). Interestingly, Rad9 p[S/T]Q phosphorylation can also be monitored as Rad9 coimmunoprecipitates with Rad53 during the process (Figure 3.3). We found that overexpression of CDC5 or cdc5-kd does not significantly alter the p[S/T]Q status of either Rad9 or Rad53. This result indicates that Cdc5 most likely acts downstream of Rad9, possibly at the level of Rad53 itself.

A direct action of Cdc5 on Rad53 might involve a direct interaction between Cdc5 and Rad53 and/or phosphorylation of Rad53. We therefore first tested whether Cdc5 can phosphorylate Rad53 in vitro in immune complex assays. We immunopurified HA-tagged Cdc5wt, Cdc5kd or Cdc5ad from yeast under stringent conditions. cdc5ad is also known as cdc5L251W (Pellicioli et al., 2001). The catalytically inactive Rad53D339A protein was utilized as the substrate in order to avoid autophosphorylation. The immune complex kinase reactions were performed in the presence on [γ-32P]-ATP. In addition to kinase-
dead Rad53, we also tested whether a potential phosphorylation was dependent on Rad53 FHA domains. We therefore also employed as substrates, Rad53$^{D339A}$ proteins where the FHA domain was mutated individually or in combination (R70A, R605A or R70A/R605A, respectively). As shown in figure 3.4A, Cdc5 can phosphorylate Rad53 in vitro. Moreover, this phosphorylation is dependent primarily on the Rad53 FHA1 domain as the Rad53 R70A mutant displays a drastic decrease in phosphorylation compared to the wild type and R605A substrates. Interestingly, this phosphorylation event is not dependent on DNA damage as Cdc5 immunoprecipitated from extract mock-treated is still able to phosphorylated Rad53. Surprisingly, immunoprecipitated Cdc5$^{ad}$ protein still phosphorylates Rad53 in vitro (Figure 3.4B). However, as the “ad” mutation protein has been proposed to reduce but not eliminate the affinity of Cdc5 towards certain substrates, it might be difficult to detect the impact of this mutation on Rad53 phosphorylation under the conditions we employed. Nevertheless, these results indicate that Cdc5 might directly regulate Rad53 activity in vivo by phosphorylation.
Figure 3.4 Cdc5 phosphorylates Rad53 in a FHA1 dependent manner in vitro

(A) In vitro kinase assays were performed using equimolar amounts of Rad53_{D339A}, Rad53_{R70A}, D339A Rad53_{R605A}, D339A or Rad53_{R70A}, R605A, D339A and Cdc5 immunoprecipitated from extracts mock treated (lanes 1-4) or treated with Zeocin (lane 5-8). The presence of Rad53 and Cdc5 was monitored by anti-Rad53 and anti-HA immunoblotting respectively (bottom panels) whereas presence of Rad53 labelled with $\gamma^{32P}$-ATP was monitored by phosphorimaging (top panel).

(B) In vitro kinase assays under the same conditions as in (A) were carried out using constant amount of Rad53_{D339A} or Rad53_{R70A,D339A} and increasing amounts of immunoprecipitated Cdc5_{WT} or Cdc5_{KD} as well as Cdc5_{KD} as a negative control. The presence of Rad53 and Cdc5 was monitored by anti-Rad53 and anti-HA immunoblotting respectively (bottom panels) whereas presence of Rad53 labelled with $\gamma^{32P}$-ATP was monitored by phosphorimaging (top panel).
3.1.2 **RAD53 INTERACTS WITH CDC5 IN AN FHA-DEPENDENT MANNER**

The FHA-dependent phosphorylation of Rad53 by Cdc5 suggested that the two proteins might interact in an FHA-dependent manner. We therefore performed GST pull-down assays using various portions of Rad53 coupled to glutathione GST. These portions of Rad53 encompassed the FHA1 or FHA2 domains and we also used the R70A and R605A mutants (Figure 3.5A). These Arg-to-Ala mutants abolish phospho-binding ability of FHA domains without affecting the fold (Durocher et al., 1999; Durocher et al., 2000; Pellicioli et al., 2001). The four recombinant proteins were then incubated with whole cell extracts obtained from zeocin-treated cells expressing HA-Cdc5\textsuperscript{wt} or HA-Cdc5\textsuperscript{kd}. After incubation, the beads were extensively washed and the precipitated proteins analyzed by immunoblotting. As displayed in Figure 3.5B, Cdc5 can be retrieved by the Rad53 FHA1 domain in an Arg70-dependent manner, suggesting that the interaction is phospho-dependent. This interaction, however, does not seem to be dependent on the kinase activity of Cdc5 as it is preserved in pull-downs from cell extracts containing Cdc5\textsuperscript{kd} and Cdc5\textsuperscript{ad} (Figure 3.5C).
Figure 3.5 Rad53 interacts with Cdc5 in a FHA1 dependent manner

(A) Schematics of the GST constructs used in the pull down experiments described below (B) Pull down experiment was performed using recombinant portion of Rad53 as indicated and incubated with native extracts also as indicated. The presence of GST-Rad53 and Cdc5 was monitored by Ponceau-S and anti-HA immunoblotting respectively (bottom and top panel, respectively). (C) Pull down experiment was performed identically as in (A) using extracts containing Cdc5 WT, Cdc5 KD or Cdc5 AD. The presence of GST-Rad53 and Cdc5 was monitored by Ponceau-S and anti-HA immunoblotting respectively (bottom and top panel, respectively).

Preliminary experiments have suggested that this Cdc5-Rad53 interaction is slightly strengthened when cells are exposed to DNA damage compared to mock treated
cells (data not shown). These results therefore suggest that the interaction between Cdc5 and Rad53 is regulated in a Cdc5-kinase independent manner where Cdc5 would be phosphorylated in a DNA damage- or cell cycle-dependent manner prior to its FHA-dependent interaction with Rad53. Importantly, this interaction was reproduced in co-immunoprecipitation experiments (Genevieve Vidanes and David Toczyski, personal communication). An interesting kinase candidate for Cdc5 phosphorylation is Cdc28. Cdc28 is required for DSB signaling (Ira et al., 2004) and has been reported to phosphorylate Cdc5 at numerous Ser and Thr residues (Ira et al., 2004; Mortensen et al., 2005). It is tempting to speculate that this phosphorylation would create a Rad53 FHA1 binding sites. This hypothesis still is currently being tested in the Durocher and Toczyski laboratories.

3.1.3 IDENTIFICATION OF Cdc5 POTENTIAL RAD53 BINDING SITES

We next sought to identify candidate phosphorylation sites that mediate the Cdc5-Rad53 interaction by identifying peptides derived from Cdc5 that could be bound by the Rad53 FHA domains. In brief, a peptide library encompassing all possible threonine residues either phosphorylated or not was synthesized on cellulose support. The resulting membrane was then incubated with GST-FHA1 or GST-FHA1 R70A (see Figure 3.5A). The FHA-bound molecules to the peptide spots were then detected by immunoblotting against the GST moiety of the recombinant proteins. As observed in Figure 3.6, four phospho-threonines (T186, T463, T484 and T664) displayed a phospho-dependent affinity for Rad53 FHA1.
Figure 3.6. Far western identifies 4 Threonines with high phospho-specific affinity for Rad53 FHA1

(A) A membrane was spotted with different peptides (phosphorylated and non-phosphorylated side-by-side) corresponding to Cdc5 as described in (B). The membrane was then incubated with recombinant GST-FHA1 or GST FHA1 R70A and bound recombinant proteins were revealed by anti-GST immunoblotting with the strongest spot binding in a FHA-dependent manner boxed in red. (B) List of the peptides used in (A) with the corresponding columns (number A-CC) and rows (1-4). Potential interacting peptides are shaded in yellow and the strongest interacting peptides are boxed in red.

3.1.4 MAPPING OF CDC5-DEPENDENT RAD53 PHOSPHORYLATION SITES

Conversely, we sought to map the sites on Rad53 that are phosphorylated by Cdc5. The position of these sites could give an indication as to their precise role in downregulating Rad53 catalytic activity.

We carried out a large-scale immune complex kinase reaction essentially as described above with the exception that we used unlabelled ATP. The reaction was then subjected to SDS-PAGE and the proteins were stained by colloidal coomassie. The band
corresponding to Rad53$^{D339A}$ was then excised from the gel, digested by trypsin and subjected to tandem mass spectrometry in collaboration with the laboratory of Dr. Anne-Claude Gingras. The analysis of the spectra revealed the presence of two unambiguous phosphorylated peptides (GKDTS*VSPDEYEER and DTS*VSPDEYEER) where Ser373 is phosphorylated. Interestingly, this site was also identified as a 4-NQO and MMS dependent Rad53 phosphorylation site (Smolka et al., 2005; Sweeney et al., 2005) and is located within the dimerization portion of the kinase domain of Rad53 (discussed in Chapter 5). These results could potentially explain how Cdc5 might regulate Rad53 activity. Additional in vivo mutational analyses are being performed to confirm the physiological importance of these phosphorylation sites on the response of Rad53 to DNA damage.
3.2 DISCUSSION

The results presented in this section provide an emerging biochemical basis of the role of Cdc5 in regulating adaptation, via the control of Rad53 activity. I hypothesize that Rad53 is turned off during the adaptation process following a direct interaction and phosphorylation by Cdc5. This interaction is phospho-dependent, not dependent on Cdc5 catalytic activity and only slightly influenced by DNA damage.

3.2.0 PHOSPHORYLATION OF Cdc5 CREATES Rad53 FHA1 BINDING SITE(S)

We demonstrated that the interaction between Cdc5 and Rad53 occurs in a FHA-dependent manner. Moreover, peptide-binding studies demonstrated that in vitro, the favored Rad53 FHA1 binding sites on Cdc5 are T186, T463, T484 and T664. Unfortunately, these sites do not conform to any clear consensus phosphorylation site. Therefore, the putative kinase phosphorylating these sites remains unknown. As mentioned previously, Cdc28 is required for DSB signaling (Ira et al., 2004) and has been shown to phosphorylate Cdc5 on 11 Ser/Thr sites (Mortensen et al., 2005), none of which however correspond to the sites identified in the spot blot analysis (Mortensen et al., 2005). These potential phosphorylation sites are not predicted to be strong Cdc28 sites, perhaps suggesting that they might only become phosphorylated after a long cell cycle arrest. In the context of adaptation, Cdc5-dependent phosphorylation of Rad53 would not be directly regulated by DNA damage but rather by extended cell cycle arrest due to irreparable DNA damage. It will be interesting to test this hypothesis.

Genetics as well as biochemical approaches can be used in order to identify the candidate kinase(s) responsible for the phosphorylation of Cdc5 during the adaptation
process. It would be interested to perform an overexpression genetic screen using a kinase overexpression library utilized by Sopko et al to identify which kinases, once overexpressed, can suppress the cdc5-ad phenotype (Sopko et al., 2007). Once candidate kinases are identified, they can be tested individually for their ability to phosphorylate Cdc5 in vitro. Moreover, phospho-specific antibodies to Cdc5 pT186, pT463, pT484 and pT664 can be generated to verify if this phosphorylation event occurs in vivo. All of these hypotheses are presently being examined in our laboratory.

3.2.1 Downregulation of Rad53 by Cdc5 Phosphorylation

This study identifies Rad53 as a strong candidate for being a bona fide Cdc5 substrate. Numerous studies have looked at the various Cdc5 substrates. One study by the group of Kevan Shokat at the University of California in San Francisco is of particular interest (Snead et al., 2007). In this study, an analogue sensitive mutation of Cdc5 was genetically manipulated by a mutation in a conserved residue of its kinase domain to render it sensitive to a chloromethylketone-containing pyrrolopyrimidines (CMK). This mutation, cdc5-L158G or cdc5-as1 did not influence the normal catalytic of the kinase in the presence of ATP but CMK act as an irreversible competitive inhibitor of ATP by creating a covalent bond using a reactive Cystein residue (C96) in Cdc5. Therefore, the use of such analogue-sensitive kinase variants allows one to shut down the modified kinase at any time without affecting the activity of other cellular kinases. This technique has been pioneered by the Shokat laboratory and used for many kinases such as Cdc28, Fus3, and Cdk2 as well as Rad53 in our laboratory (Bishop et al., 2000). By using cdc5-as1, Shokat and colleagues identified numerous potential Cdc5 substrates, some of which
are part of the DNA damage checkpoint including Rad53 (Rad9, Xrs2, Chk1, Dun1, Rad24 and Rad53).

Our mapping experiments identify Ser373 as a site phosphorylated by Cdc5 \textit{in vitro}. Interestingly, this site has been identified as phosphorylated \textit{in vivo} in response to 4-NQO and MMS but not in response to HU (Smolka et al., 2005; Sweeney et al., 2005). Although one should be careful in interpreting mass spectrometry results, it is tempting to speculate that this Rad53 phosphorylation event is dependent on DNA damage only in G2/M and dispensable in S-phase, which would correlate nicely with the role of Cdc5 in adaptation.

Finally, we will learn in Chapter 5 that Ser373 maps into the dimer interface of Rad53. Moreover, dimerization of Rad53, like that of other Chk2 relatives, is crucial for its activity. Taken together, these results suggest a model whereby phosphorylation of Rad53 by Cdc5 inhibits the former kinase by modulating its ability to dimerize.

\section*{3.2.2 Future direction and unanswered questions}

Most of the conclusions and hypotheses formulated in this chapter are based on \textit{in vitro} results and it will therefore be imperative to confirm the relevance of the Cdc5 interaction and phosphorylation of Rad53 \textit{in vivo}. In order to do so, the mutation of phosphoacceptor sites on Rad53 and Cdc5 need to be carried out.

Monitoring and understanding checkpoint adaptation is important in order to understand cancer progression. Plk1 for instance, is expressed in a number of cancer types and research is underway in order to find Plk1 and other Plk-specific inhibitors to treat different types of cancer (Malumbres and Barbacid, 2007). In addition to its role in
normal cell cycle progression, Plks could also be involved in shutting off the DNA damage response in human cells. However, if Plks possess the ability to shut off Chk2 for instance, the overexpression of these kinases might leads to a premature termination of the checkpoint, resulting in premature cell cycle progression in the presence of deleterious mutations.
3.3 EXPERIMENTAL PROCEDURES

YEAST STRAINS AND PLASMIDS

The CDC5 yeast strain utilized in this Chapter were kindly provided by the laboratory of David Toczyski and are described in (Charles et al., 1998). The GST recombinant Rad53 plasmids were constructed by PCR-mediated cloning and are described in (Durocher et al., 1999).

cdc13-1 AND ZEOCIN DNA DAMAGE ASSAYS

cdc13-1 strain containing the indicated CDC5 alleles were grown in raffinose rich media to log phase (OD$_{600}$= 0.200) at 23 degrees and then shifted at restrictive temperature (32 degrees) Simultaneously, 2% Galactose was added to the media in order to induce the overexpression of CDC5. Sample were collected every hours for 7 hrs and subjected to glass beads TCA-mediated lysis as described in Section 2.4 Zeocin assays were performed by growing cells in raffinose rich media at 30°C until they reach log phase (OD$_{600}$= 0.2). Cells were then synchronized by adding 10 μg/ml of nocodazole (Sigma) for 3 hrs. After the synchronization was confirmed by the formation of large budded cells observed under light microscopy, the media was spiked with an additional 5 μl/ml of nocodazole and 2% galactose was added to the media in order to induce the overexpression of the indicated CDC5 allele. 30 min after the addition of galactose, 0.4 mg/ml of Zeocin (Invitrogen) was added to the media and samples were taken at the indicated times. Cells were pelleted by centrifugation and subjected to native glass beads lysis as indicated in Section 2.4.
IMMUNOBLOT

All western blots performed in the Chapter utilized the procedure recommended by the manufacturer with the exception of Rad53 immunoblot, which is described in Section 2.4. The HA, Pgk1 and Cdc28 antibodies were purchased from Santa Cruz Biotechnologies. The p[S/T]Q was purchased from Cell Signaling Technologies.

IMMUNOPRECIPITATION, \textit{IN SITU AND IN VITRO} RAD53 KINASE ASSAYS

Rad53 immunoprecipitations are described in Chapter 2, Section 2.4. HA-CDC5 immunoprecipitation were performed by growing cultures (250 ml) of the appropriate strains to OD$_{600}$= 1 in rich media containing 2% raffinose. Protein expression was induced for 3 hrs following addition of 2% galactose. Cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH 8.0, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma)). Immunoprecipitation were performed using HA-coupled dynabeads (Invitrogen) for 1 hours and the pellets was washed 3 times in RIPA buffer and 2 times in kinase buffer (25 mM Hepes pH 7.5, 250 mM NaCl, 20mM MgCl$_2$, 20mM MnCl$_2$, 1mM DTT ). \textit{In vitro} kinase assays were performed as follows. Purified recombinant Rad53$^{D339A}$ was incubated in immunoprecipitated Cdc5 in kinase buffer (25 mM Hepes pH 7.5, 250 mM NaCl, 20mM MgCl$_2$, 20mM MnCl$_2$, 1mM DTT, 40 mM ATP and 0.5 ml [g-$^{32}$P]ATP (Perkin-Elmer)) for 30 min unless indicated otherwise. The reactions were stopped by adding SDS sample buffer and by boiling the sample for 5 min. Half of the reactions were then loaded on 8% SDS-PAGE gel and transferred to PVDF membrane (Millipore). The membrane was then exposed overnight on a phosphor screen (GE bioscience) and
revealed by phosphorimaging (GE Bioscience). All quantifications were done with ImageQuant 5.0

**GST Pull-Down Assays**

Recombinant GST-Rad53 was purified by growing cultures of BL21 *E. coli* cells transformed with plasmids encoding recombinant GST-Rad53 to OD$_{600}$ 0.6. Protein expression was induced by adding 1 mM IPTG for 3 hrs at 30°C. Cells were lysed by sonication in lysis buffer (50 mM Hepes pH 7.4, 500 mM NaCl, 1x AEBSF). The clarified lysate was incubated with GST-Sepharose (GE Healthcare) for 1 hr at 4°C and the beads were washed 3 times with lysis buffer, aliquoted and frozen at -80°C.

The pull down assays were performed by incubating recombinant GST-Rad53 with 1 mg of native cell extracts overexpressing HA-Cdc5 for 3 hrs at 4°C. Cells were then washed 3 times with lysis buffer and boiled in SDS sample buffer for 5 min. Half of the reactions were then loaded on 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore). The membrane was then subjected to immunoblot using the indicated antibodies.

**Spot Blot Synthesis Protocol**

A peptide library was created on a spot blot membrane to determine the binding sites of the Cdc5 protein. The peptide fragment sequences were of the 4 amino acids flanking the C and N terminal regions surrounding every threonine. The phosphorylated and non-phosphorylated versions were synthesized side-by-side using the Automated Multiple Peptide Synthesizer (Multipep) by Intavis AG. The peptides were synthesized using
standard Fmoc chemistry. Deprotection was completed by incubating the membrane with 82.5% Trifluoroacetic Acid (Sigma), 5% Thioanisole (Sigma), 5% Phenol (Fluka), 2.5% 1,2-Ethanedithiol (Sigma), ddH2O for 4hrs in the fumehood. After deprotection, The membranes were washed 4x with N,N-dimethylformamide (EM Science), 4x with Methylene Chloride (EM Science), and 4x with 95% Ethanol (Fisher) and dried overnight in the fumehood. The blots were stored dry at –20°C. 0.5 mmol of each amino acid is used by the spot blotter, these should be stored at least 4°C until use or –20 °C for long-term storage both conditions under dessicant.

**Overlay Western Blot**

The spot blot membrane was rinse 3x with TBS-T (0.1%) for 5min each and blocked with 5% milk/TBS-T (0.1%) for 1 hr at room temperature. The membrane was then incubated with 10 uM GST-FHA overnight at 4°C in incubation buffer (5% milk, 50 mM Tris-Cl pH7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT). The membrane was then washed 4x TBS-T (0.1%) for 5 min each at room temperature and incubated in 1:2000 (v/v) GST polyclonal antibody (Santa Cruz Biotechnologies) in 5% milk TBS-T (0.1%) for 1hr at room temperature. The membrane are then washed 4x TBS-T (0.1%) for 5 min and incubated with secondary HRP (rabbit) 1:10000 (v/v) in 5% milk/TBS-T (0.1%) for 30 min at room temperature and proceed with standard chemiluminescence techniques.
CHAPTER 4

RESULTS

ATOMIC RESOLUTION OF RAD53 REVEALS THREE DIFFERENT MODES OF REGULATION

In collaboration with:

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4.0 INTRODUCTION

The serine/threonine protein kinase Rad53 and its mammalian ortholog Chk2 are key regulators of DNA damage checkpoint signaling (Allen et al., 1994; Hirao et al., 2000; Matsuoka et al., 1998; Sun et al., 1996). Loss-of-function mutations of RAD53 result in a DNA damage sensitivity phenotype and a defective checkpoint response in yeast (Allen et al., 1994; Fay et al., 1997; Pellicioli and Foiani, 2005; Sun et al., 1996; Sweeney et al., 2005; Weinert et al., 1994). In mammalian cells, loss-of-function mutations in the tumor suppressor CHK2 also lead to a defective checkpoint response as well as a deficiency in DNA damage induced apoptosis (Bartek et al., 2001; Hirao et al., 2000; Matsuoka et al., 1998; Matsuoka et al., 2000; Tominaga et al., 1999).

Studies of the phospho-regulation of Rad53 and Chk2 have provided us with a wealth of information in regards to potential phospho-regulatory sites involved in the activation of these checkpoint kinases (Matsuoka et al., 2000; Melchionna et al., 2000; Smolka et al., 2005; Sweeney et al., 2005). For instance, as seen in Chapter 2, some DNA-damage dependent phospho-acceptor residues within the Ser/Thr-rich kinase domain extension of Rad53 have been shown to be functionally important as their mutation to Ala contributes in part to the loss of Rad53 activation in vivo. This observation suggests a regulatory role for this region of Rad53 in its catalytic activation in vivo. Such regions flanking protein kinase domains commonly serve to regulate kinase function, either negatively or positively. For instance, kinase inhibition can be achieved by directly regulating catalysis by mimicking the substrate interactions, as in the case of twitchin kinase, or by distorting the ATP-binding pocket as in calcium/calmodulin-dependent protein kinase I (CamKI) (Goldberg et al., 1996). In other cases, the kinase
extension plays a positive regulatory role and functions in substrate recognition (e.g.: MAPK family), or promotes a stable productive conformation of the activation segment, as seen for the kinase extensions of CK2 and yeast Sky1p kinases (Litchfield, 2003; Nolen et al., 2001). Whether the kinase extension of Rad53 serves a regulatory function will be examined in this Chapter.

To investigate the structural basis of Rad53 regulation, I collaborated with Cynthia Ho in the Sicheri lab to analyze x-ray crystallographic studies of the kinase and adjoining C-terminal Ser/Thr-rich sequence of Rad53. Dr. Ho’s contribution was invaluable to this work as she solved the atomic structure of the kinase as I focused my work on the biochemical characterization and validation of this same crystallographic structure.

The Rad53 crystal structure reveals that the Ser/Thr-rich sequence at the end of the kinase domain forms a novel helical extension that closely associates with the protein kinase domain. *In vitro* and *in vivo* functional analyses show that this association between the helical extension and the kinase domain is important to inhibit kinase function. The Rad53 structure also reveals the functionally significant position of an invariant Thr-Gln motif at the head of helix αG in the C-terminal lobe of the kinase. Phospho-mimic mutation of the Thr-Gln motif to Glu-Gln influences the kinetics of Rad53 activation *in vitro* and *in vivo*. Moreover, we demonstrate that Rad53 forms a dimer *in vitro*. The aforementioned conserved TQ motif, as well as a conserved hydrophilic patch in the C-lobe of the kinase region seems to play an important role in maintaining this dimerization, which is essential for the preservation of Rad53 catalytic activity; critical mutations disputing dimer formation lead to DNA damage sensitivity and defective
Rad53 activation \textit{in vivo}. Further studies demonstrate that this essential dimerization process is most likely required to favor trans-autophosphorylation of Rad53 activation segment by T-Loop exchange.
4.1 RESULTS

4.1.0 RAD53 KINASE EXTENTION PLAYS AN INHIBITORY ROLE IN VITRO

Analysis of the Rad53 amino sequence revealed that the sequences residing immediately at the C-terminus of the Rad53 kinase domain most likely formed one or two alpha-helices (data not shown). Since helical extensions play a critical role in the regulation of CamKI and cAPK (Goldberg et al., 1996; Narayana et al., 1997) we sought to determine the crystal structure of the Rad53 kinase domain along with C-terminal sequences. The crystallographic studies were done by Cynthia Ho, a post-doctoral fellow in the laboratory of Frank Sicheri. I generated many of the reagents used by Cynthia and carried out most of the in vivo characterization of the structure. I will point out our respective contribution in the figure legends.

We hypothesized that Rad53 C-terminal kinase extension could play a role similar to analogous helical extensions in other kinases, in inhibiting Rad53 activity. Moreover, Rad53 Ser485 and Ser489 are located in the predicted helical region and these two residues have been shown to be phosphorylated in vitro and in vivo and required for the activation of Rad53 (Smolka et al., 2005; Sweeney et al., 2005). An attractive hypothesis in regards to Rad53 activation is that these sites, amongst others, need to be phosphorylated by Mec1 in the first step of Rad53 activation to release Rad53 from auto-inhibition.

To test the hypothesis that the sequence C-terminal to the kinase domain plays a role in Rad53 auto-inhibition, I generated various hexahistidine-tagged fragments of Rad53 by in vitro translation (Figure 4.1A). These in vitro translated Rad53 protein fragments contained the kinase domain as well as the C-terminal extension domain
(Rad53$^{170-512}$) or fragments deprived form the kinase extension domain (Rad53$^{170-470}$). Equimolar quantities of protein fragment were utilized in an in vitro kinase assay where I assayed the ability of Rad53 to phosphorylate the myelin binding protein (MBP). As a control, a Rad53 fragment coding for a kinase inactive version of Rad53 (Rad53$^{170-512}$, D339A) was utilized. As seen in Figure 4.1B, truncation of the C-terminal portion of Rad53 leads to a marked increase of MBP phosphorylation. This result suggested that the C-terminal extension of Rad53 kinase domain might play an auto-inhibitory role. In order to examine this phenomenon in more details, we pursued to solve the structure of Rad53 by X-Ray crystallography.
Figure 4.1 The C-Terminal extension of Rad53 Kinase domain inhibits its activity in vitro

(A) Schematics of the strategy used to create fragments of Rad53 (with or without C-terminal kinase extension) by PCR and *E. coli* mediated in vitro translation. (B) *E. coli* extracts containing in vitro translated Rad53 (bottom panel) were incubated with MBP, an exogenous Rad53 substrate and an in vitro Rad53 kinase assays was in the presence of \( \gamma^{32}P \) ATP. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and radioactivity incorporation was measured by phosphorimaging (top panel). The presence of Rad53 monitored by immunoblotting using anti-HIS antibody (bottom panel).

4.1.1 OVERALL DESCRIPTION OF RAD53 ATOMIC STRUCTURE: 3 IMPORTANT FEATURES

The kinase-inactive Rad53\(^{170-512,\,D339A}\) protein was expressed in *E. coli* and purified to homogeneity for crystallization trials using sparse matrix screening. Reproducible Rad53 crystals were observed in solutions containing PEG. Following optimization of the crystallization process, 2.6 Å and 2.9 Å datasets were collected for Apo and AMP-PNP complex crystals, respectively. The two structures were solved by molecular replacement using the Death-Associated Protein Kinase (PBD# 1IG1) as the search model. The AMP-PNP bound Rad53 structure could not be refined beyond \(R_{\text{factor}}/R_{\text{free}}\) statistics of 29.9%/33.8% although clear interpretable electron density is seen for the adenine ring and ribose backbone of AMP-PNP. Moreover, the terminal phosphates of AMP-PNP are disordered which is typical in the case of the kinase inactivating mutation to the magnesium-binding site, here D339A. The Apo-Rad53\(^{170-512,\,D339A}\) structure was refined to \(R_{\text{factor}}/R_{\text{free}}\) values of 24.3%/28.9% and displayed good geometry and Ramachandran statistics by keeping with the resolution of the data. Data collection and refinement statistics for Apo- Rad53\(^{170-512,\,D339A}\) are listed in Table 4.1. Cynthia Ho performed these studies.
4.1.1.0 General Description

Rad53\textsuperscript{170-512, D339A} displays the typical bilobal architecture of other protein kinase domains which consist of a smaller N-terminal lobe (N-lobe), comprised mainly of $\beta$-sheets, followed by a larger C-terminal lobe (C-lobe) that is mostly $\alpha$-helical (Figure 4.2A). Overall, the Rad53\textsuperscript{D339A} structure is well ordered with the exception of the N-terminal 23 residues, the C-terminal 9 residues, residues 347 to 357 of the activation segment and residues 369 to 383 in the C-lobe connecting helices $\alpha$EF and $\alpha$F (Figure 4.2A, broken lines mark regions of disorder).
Figure 4.2 Structure of Rad53

(A) Ribbon diagram of the structure of Rad53 demonstrating the N-lobe (light green), C-lobe (dark green) of the kinase along with the C-Terminal helical extension $\alpha$HE1 and $\alpha$HE2 (purple). Also noted, the disordered region of the activation segment (red, dotted line) and the $\alpha$G (green, dotted line). (B) Superposition of Rad53 structure along with protein kinase of the same family highlights structural similarities. (C) Electron density diagram highlighting the two helical extensions at the back of the kinase domain (purple) as well as two critical aspartic acid residues neutralizing the dipole moment of the helices (orange). A modeled docking peptide is also represented in yellow.
Interestingly, the activation segment of Rad53^{170-512, D339A} is disordered and could not be modeled. This observation suggests that phosphorylation of the activation segment could be required in order to obtain a productive activation segment conformation. This observation supports our data of Chapter 2 indicating that the phosphorylation of Ser350 and Thr354 of Rad53 activation segment is required for the catalytic activation of the kinase. In activated kinases, an invariant Arg side chain in the so-called RD pocket projects outward to make direct contact with the phosphorylated residue in the activation segment (Huse and Kuriyan, 2002). In Rad53^{170-512, D339A}, this Arg side chain (Arg318) is also disordered and modeled as an Ala. However, the overall arrangement of other key catalytic residues in Rad53^{170-512, D339A} is consistent with the active conformation. For example, the kinase domain characteristic Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) "anchor" points of the activation segment, residues Asp339-Phe340-Gly341 and Ala363-Pro364-Glu365 in Rad53, are well ordered and adopt canonical conformations. The orientation of the invariant Lys and Glu that positions the terminal phosphates of ATP, Lys227 and Glu244 in Rad53, as well as the putative catalytic base, Asp317 in Rad53, are also in the active conformation. These observations reveal that non-phosphorylated Rad53 is inactivated by the disordering of the activation segment and suggests that activation of Rad53 will not involve extensive structural rearrangements of the catalytic residues in the active site.

4.1.1.1 A UNIQUE HELICAL EXTENSION

As predicted, the Ser/Thr-rich kinase extension of Rad53 forms a unique structure consisting of two successive amphipathic α-helices, αHE1 (residues 479 to 490) and
αHE2 (residues 493 to 501), not observed in any other kinase domain structures to date (Figure 4.2, purple ribbons). The two helices are structurally distinct from the core kinase domain and are not involved in crystal packing interactions. The C-terminal helices associate with the lower back of the C-lobe relatively far away (25Å) from the active site. Moreover, this conformation is conserved in the two distinct Rad53 molecules of the asymmetric unit.

The αHE1 and αHE2 helices are positioned in a shallow groove formed by the terminal ends of helix αD, αE, and αH in the C-lobe (Figure 4.2 and Figure 4.4AB). Additional stability is provided by two conserved acidic residues in the β6 to β7 loop, Asp330 and Asp331 in Rad53 or two Glu in mammals, which extend over the N-terminus of αHE1 (Figure 4.4AB, orange). These two residues may act to cap the alpha helix, helping in the stability of the latter by partially neutralizing the helix dipole moment. The second helical element, αHE2, appears stabilized by side chain interaction of Glu503, the last ordered residue in Rad53, with two conserved glutamate residues, Glu437 and Glu438, in the C-lobe.

Interestingly, two in vivo phosphorylation sites (Ser485 and Ser489) lie along the same lateral face of αHE1 with their side chains oriented away from the kinase Figure 4.4AB, in red). As observed in Chapter 2, these Ser residues are phosphorylated following DNA damage independently of Rad53 kinase activity and are potential sites of phosphoregulation by the upstream kinase Mec1.
4.1.1.2 Location of an Invariant TQ Motif

Rad53 and Chk2 kinases share a strikingly conserved Thr-Gln (TQ) motif at the solvent exposed head of helix αG in the C-lobe of the kinase (Figure 4.2 red ribbon and Figure 4.5A, in red). In fungal orthologs, the Ser residue preceding the TQ motif, substituted for Lys in mammals, is also highly conserved and extends this motif to residues Ser411-Thr412-Gln413 (STQ) in fungal Rad53. The side chains of Ser411 and Thr412 are oriented down and away from the kinase while the side chain of Gln413 is directed up toward the disordered activation segment (Figure 4.5A). Phosphopeptide mapping studies of human Chk2 and *S. cerevisiae* Rad53 have detected phosphorylation of this motif both *in vitro* and *in vivo*, respectively (Ahn et al., 2000; Bartek et al., 2001; Matsuoka et al., 2000; Melchionna et al., 2000). In Rad53, the STQ motif is phosphorylated on Ser411 (Smolka et al., 2005; Sweeney et al., 2005) and Thr412 (Don Hunt, personal communication) in a manner that depends on Rad53 kinase activity. In other kinases such as the RNA-dependent protein kinase PKR, the same region of helix αG has been shown to be a docking site for the recognition of specific substrates. Moreover, this region can also form part of an extended substrate-binding groove, as observed in the cAMP-dependent kinase family (Dar et al., 2005; Dar and Sicheri, 2002; Dey et al., 2005). Together, these observations suggest that the Rad53 STQ motif and the Chk2 TQ motif could be play an important role in the catalytic activation of the kinase or in the support of the dimerization interface.
4.1.1.3 Dimerization

Both Rad53 molecules of the asymmetric unit form identical antiparallel dimers related by a crystallographic 2-fold axis (Figure 4.5A). The dimer is oriented such that the residues on the front face of the N-lobe in each molecule contact the C-lobe of the other molecule at two distinct regions. The primary interface of the Rad53 dimer is comprised of basic residues, Lys231-Arg232-Lys233, located at the N-terminal end of the \( \beta_3 \) to \( \alpha_C \) loop in the N-lobe that contact three Asp residues, Asp280, Asp283 and Asp323, located in helix \( \alpha_D \) and the loop preceding \( \beta_3 \) of the C-lobe, respectively (Figure 4.5A). The Lys231-Arg232-Lys233 sequence is well conserved with Rad53 and its ortholog Chk2 with Lys233 varying to an Arg in Chk2. This sequence forms a localized basic patch on the front face of the kinase N-lobe above the active site (Figure 4.5A). The three Asp residues form a localized acidic patch immediately below the ATP-binding pocket (Figure 4.5A). The secondary interface of the Rad53 dimer involves side chain to backbone interaction of non-conserved residues at the C-terminus of \( \alpha_C \) in the N-lobe with the highly conserved N-terminus of helix \( \alpha_G \) in the C-lobe that encodes the STQ motif (Figure 4.5A). The side chain of Asp239 in \( \alpha_C \) of Rad53, substituted for Pro or Leu in mammals, packs against the backbone of the invariant Thr at the head of helix \( \alpha_G \). The electrostatic surface potential of this interaction interface is neutral (Figure 4.5A).

The observation that both Rad53 molecules in the asymmetric unit form identical dimers suggests a non-random interaction that may represent a biologically relevant conformation.
4.1.2 A PHOSPHO-SPECIFIC ANTIBODY TO RAD53 ACTIVATION SEGMENT

As discussed in Chapter 2, the current *in vivo* model of Rad53 activation involves a two-step phosphorylation mechanism where Rad53 is recruited to phosphorylate Rad9. We and others have previously identified numerous Rad53 kinase-dependent autophosphorylation sites, two of which reside in the activation segment of Rad53 (Ser350/Thr354) and their phosphorylation is essential for Rad53 activation (Fiorani et al., 2008; Sweeney et al., 2005; Usui and Petrini, 2007). In light of these facts, I generated a rabbit phospho-specific antibody (Rad53 T354-P) that specifically recognizes the phosphorylated activation segment of Rad53 (pSer350 and pThr354). This antibody displayed specificity for activated Rad53 *in vivo* and *in vitro*. As displayed in Figure 4.3, a band corresponding to Rad53 is clearly visible in cells *RAD53* cells treated with 200 mM HU. The specificity of the immunoblot is also significantly increased when the primary phospho-specific antibody is incubated with approximately a 1:1 molar ratio of non-phosphorylated peptide corresponding to the same antigen utilized to generate the antibody in rabbit, albeit non-phosphorylated. This antibody provided us with an invaluable tool to monitor the kinetics of Rad53 activation *in vivo* and *in vitro*. 
Figure 4.3 A Rad53 phosphospecific antibody detecting activation segment phosphorylation
Log phase yeast cells were treated or mock treated with 200mM Hydroxurea for 60min and TCA cell lysates were separated by SDS-PAGE, transferred onto a PVDF membrane and blotted using a phosphospecific antibody raised to detect the phosphorylated activation segment of Rad53.

4.1.3 C-TERMINAL α-HELICAL KINASE EXTENSION INHIBITS RAD53

The in vitro studies from Section 4.1.0 suggested that the two α-helices at the extension of the kinase domain play an inhibitory role at the level of Rad53 catalytic regulation. However, the position of these two helices revealed by X-Ray crystallography (on the lower back of the kinase C-lobe and situated relatively far from the kinase active site) do not suggest an obvious role for this motif in either directly regulating catalysis or substrate recognition of Rad53. Nevertheless, we tested the functional significance of the helical extension by making use of a recombinant purified Rad53 from construct encoding amino acids Thr170 to Gln512 (Rad53170-512) as well as one truncated and one elongated construct Thr170 to Pro470 (Rad53170-470) and Thr170 to Lys572 (Rad53170-572), respectively. The truncated fragments were soluble and expressed to a similar level.
as the parental construct, indicating that the deletion of the helical extension did not adversely affect Rad53 stability.

To determine whether the results from the in vitro translated kinase assay observed in Section 4.1.0 could be reproduced using the proteins used for crystallization experiments, the catalytic activity of the parental and modified Rad53 proteins were tested by in vitro kinase assay. The phosphorylation status of Thr354, a key phosphoregulatory site in the activation segment required for Rad53 catalytic activation in vivo, was monitored using a phosphospecific antibody (Rad53\textsuperscript{T354-P} and Section 4.1.2). Although the overall phosphorylation of the recombinant parental and truncated Rad53 kinase molecules was monitored, the interpretation of such phosphorylation must be made with caution, as critical phosphorylation sites are located in the $\alpha$-helical extensions, which are truncated in some cases. In order to circumvent this problem, recombinant, full length and catalytically inactive Rad53 (Rad53\textsuperscript{D339A}) was used as substrate to test the ability of Rad53\textsuperscript{170-512}, Rad53\textsuperscript{170-470} and Rad53\textsuperscript{170-572} to trans-autophosphorylate on its activation segment. In comparison to Rad53\textsuperscript{170-512}, the truncated Rad53\textsuperscript{170-470} protein, which lacks the helical extension, had significantly more activation segment phosphorylation and the elongated Rad53\textsuperscript{170-572} protein showed even greater inhibition in activation segment phosphorylation (Figure 4.4B, lanes 1-2, 9-10, 13-14).
Figure 4.4 The C-terminal extension of Rad53 Kinase domain inhibits its activity in vivo
(A) Ribbon and electron density diagram representing the residues predicted to play a role in maintaining the stability of the α-helical extension of the kinase domain of Rad53. (B) Mutational analysis of the residues highlighted in (A) where equimolar quantities of recombinant protein carrying the indicated mutation(s) were incubated with full-length Rad53 \( ^{D339A} \) (substrate) and the activity of Rad53 (autophosphorylation on the bottom panel and trans phosphorylation of the top panel) was monitored by immunoblotting using a phosphospecific Rad53 antibody Rad53 \( ^{T354P} \). (C) Strains harbouring the indicated \( RAD53 \) alleles were treated with 75mM HU. WCE were prepared at the indicated time points and proteins separated by SDS-PAGE. Rad53 activity was monitored by gel mobility shift after immunoblotting using a Rad53 antibody. Panel (B) was generated by Cynthia Ho, panel (C) was generated by myself.
As previously noted, phosphorylation of Rad53 within the Ser/Thr-rich C-terminal sequence contributes in part to its catalytic activation in vivo. Two potential phosphoregulatory sites identified by in vivo phosphopeptide mapping (Ser485 and Ser489) are located in the Ser/Thr-rich C-terminal extension (Figure 4.2A and Figure 4.4AB, in red). To test the functional significance of Ser485 and Ser489, we generated the phospho-mimic mutant Rad53^{(170-512)-S485E/S489E} and the double Ala mutant Rad53^{(170-512)-S485A/S489A} was used as control. The wild type and mutant proteins were also purified from bacteria and tested for in vitro kinase activity using Rad53^{T354-P} to monitor trans-autophosphorylation of Rad53^{D339A} activation segment. As demonstrated in Figure 4.4B (lane 3-6), Rad53^{(170-512)-S485E/S489E} had significantly more activation segment trans-autophosphorylation. By contrast, Rad53^{(170-512)-S485A/S489A} had similar kinetic activity to Rad53^{170-512}.

This significant increase in activation segment trans-autophosphorylation provides some insight as to how Rad53 autophosphorylates during its activation cycle. Moreover, the importance of the kinase helical extension’s integrity as well as the phenotype of Rad53^{(170-512)-S485E/S489E} suggests that the disruption of the kinase extension alleviates an inhibitory effect on Rad53 function. These in vitro results are consistent with previous studies demonstrating that multisite phosphorylation is important for Rad53 activation in vivo and supports a model wherein the disassociation of the C-terminal helices from the kinase, perhaps in part through phosphorylation of Ser485 and Ser489, promotes catalytic activation of Rad53.

Additional evidence to support this hypothesis comes from similar in vitro kinase reactions using Rad53^{(170-512)-D330A/D331A} as a candidate kinase. According to the crystal
structure and as mentioned in Section 4.1.1, Rad53 Asp330 and Asp331 are situated in the C-lobe of the kinase domain and act as N-terminal capping structure of the first α-helix of Rad53 kinase domain extension motif. Often, these negatively charged capping residues neutralize the dipole moment of these helices resulting in an overall stabilization of the helices. Dipole moments are formed by the additive effect of the individual dipoles from the carbonyl groups of the peptide bond pointing along an α-helix axis.

One can destabilize the helix by adding a neutralizing mutation to the capping residues, in this case by mutating the Asp330/Asp331 to Ala330/Ala331. When Rad53^{170-512-}\text{D330A/D331A} is tested using \textit{in vitro} kinase assays, the resulting trans-autophosphorylation of Rad53^{D339A} is clearly increased compared to its parent Rad53^{170-512}. This suggests that the destabilization of the helix or helices by the neutralizing mutation of the capping Asp residues relieve the inhibition of Rad53 provided by these same helices.

It is paramount to demonstrate the physiological relevance of these mutations \textit{in vivo}. In order to do so, I tested the mutant alleles rad53^{D330A/D331A} and rad53^{S485E/S489E}. These mutations were introduced in a rad53Δ sml1-1 strain. Rad53 activation was elicited by addition of HU and the kinetics of Rad53 activity in the corresponding strains was monitored by examining protein gel mobility shifts due to phosphorylation at different time points after the addition of HU to the media. As demonstrated in Figure 4.4C, the Rad53^{D330A/D331A} activation is a more rapid, as shown by the marked increase in electrophoretic mobility shift compared to the \textit{RAD53} strain. This indicates that this structural element of the kinase domain, perhaps through the electrostatic stabilization of the helical extension conformation, is important for the inhibition of Rad53 catalytic activity \textit{in vivo}. Next, we tested the kinetics of activation of the Rad53^{S485E/S489E} mutant.
Interestingly, Rad53<sup>S485E/S489E</sup> displayed a marked increase in catalytic activity in vitro but this phenotype was moderate in the in vivo setting (Figure 4.4C). This observation suggests that other structural elements in addition to Ser485/Ser489 are required to activate Rad53 in vivo. This observation correlates with our previous findings where at least 8 phosphorylation events, including these latter two Ser residues, are required for the activation of Rad53 in vivo.

4.1.4 Dimerization

Interestingly, in this study, Rad53 was found as a dimer. Although clear dimerization of Chk2, was demonstrated (Ahn and Prives, 2002; Ahn et al., 2000; Oliver et al., 2007; Oliver et al., 2006; Schwarz et al., 2003), the dimerization of Rad53 has not been as readily demonstrated, although the group of David Stern has presented evidence supporting the possibility that Rad53 dimerization occurs in vivo (Jia-Lin Ma and Stern, 2008; Ma et al., 2006).

Both Rad53 molecules of the asymmetric unit form identical antiparallel dimers related by a crystallographic 2-fold axis. The dimer is oriented such that the residues on the front face of the N-lobe in each molecule contact the C-lobe of the other molecule at two distinct regions. The primary interface of the Rad53 dimer is comprised of basic residues, Lys231-Arg232-Lys233, located at the N-terminal end of the β3 to αC loop in the N-lobe that contact three Asp residues, Asp280, Asp283 and Asp323, located in helix αD and the loop preceding β3 of the C-lobe, respectively (Figure 4.5A). The Lys231-Arg232-Lys233 sequence is well conserved among Rad53 and Chk2 proteins with Lys233 varying to an Arg in Chk2. This surface is also well conserved among Rad53
and Chk2 orthologs where Asp283 is invariant but two Glu in Chk2 replaces Asp280 and Asp323.

The secondary interface of the Rad53 dimer involves side chain to backbone interactions of non-conserved residues at the C-terminus of αC in the N-lobe with the highly conserved N-terminus of helix αG in the C-lobe that encodes the STQ motif (Figure 4.5A). The side chain of Asp239 in αC of Rad53, substituted for Pro or Leu in mammals, packs against the backbone of the invariant Thr at the head of helix αG. The electrostatic surface potential of this interaction interface is neutral (Figure 4.5B).

The observation that both Rad53 molecules in the asymmetric unit form identical dimers suggests a non-random interaction that may represent a biologically relevant conformation. Similar N-lobe to C-lobe dimers have been observed previously in crystal structures of phosphorylase kinase for instance (Lowe et al., 1997; Venien-Bryan et al., 2002). Despite our best efforts to identify a phosphoregulatory mechanism at the level of the STQ motif, the alleles corresponding to the phosphoacceptor site mutants Rad53S411A and Rad53T412A, singly or in combination, failed to display a defect in activation in vitro and only showed slight DNA damage sensitivity in vivo when combined with the basic patch mutant Rad53D280A/D323A (Figure 4.5D). A closer look at the Rad53 dimer reveals that the STQ motif might be important for dimerization by making contact with the Asp239 of the opposite Rad53 molecule. In order to test whether the dimerization of Rad53 regulates kinase activity, we generated Alanine mutants of the acidic and basic patches as well as the STQ motif (identified in Figure 4.5A). These mutants were first introduced in the crystallization constructs and test for in vitro kinase as described in
Section 4.1.3 corresponding alanine or reverse-charge mutations individually or in combination.

In order to test whether the disruption of dimerization interface revealed by the crystal structure of Rad53 impacts Rad53 catalytic activity in vitro, we generated a series of recombinant Rad53^{170-512} mutants within the dimerization interface. The D280, D283, D323, K233, R232, K231, S411 and T412 residues were mutated to Ala individually or in combination (Figure 4.5A). As a negative control, we employed Rad53^{170-512, T354A}. We then tested the resulting mutants for in vitro kinase activity using Rad53^{T354-P} to monitor trans-autophosphorylation of Rad53^{D339A} activation segment as well as autophosphorylation of the recombinant protein Rad53^{170-512}. As demonstrated in Figure 4.5B, mutation of a number of dimerization interface residues compromised trans-autophosphorylation (lane 4-12, lane 21-26). Interestingly, the charge-reversal mutant of the dimerization interface residues (lane 11-12) as well as the Ala mutation of the conserved STQ motif (lane 24-26) resulted in an additional defect in autophosphorylation. Despite the fact that a clear DNA damage sensitivity phenotype was observed in charge-reversal mutants, we were unable to observe such a phenotype in strains carrying a RAD53 allele mutated at the STQ conserved motif. Nevertheless, these results indicate that the residues in the dimerization interface of Rad53 are required for the protein catalytic activity in vitro.
Figure 4.5 Residues involved in Rad53 dimerization are required for Rad53 catalytic activity.

(A) Ribbon and cartoon diagram representing the residues predicted to play a role in maintaining the stability of the Rad53 dimer. (B) Mutational analysis of the residues highlighted in (A) where equimolar quantities of recombinant protein carrying the indicated mutation(s) were incubated with full-length Rad53^{D339A} (substrate) and the activity of Rad53 (auto-phosphorylation on the bottom panel and trans phosphorylation of the top panel) was monitored by immunoblotting utilizing a phosphospecific Rad53 antibody.

Table: Summary of mutational analysis results.

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SD-URA 0 mM HU

SD-URA 20 mM HU

DD02 smd1-t rad53A (rad53)

DD02 smd1-t rad53A (rad53)

SD-URA 0 mM HU

SD-URA 20 mM HU

Rad53^{D339A} (substrate)
antibody Rad53$^{T354P}$ (C) Strains harbouring the indicated RAD53 alleles were treated with 100mM HU. TCA extracts were prepared at the indicated time points and proteins separated by SDS-PAGE. Rad53 activity was monitored by gel mobility shift after immunoblotting using a Rad53 antibody. (D) Strains containing the alleles described above were diluted serially five-fold and spotted on agar plates containing HU at the indicated concentration. Panels A and B were generated by Cynthia Ho whereas I generated panels C and D.

Next, we generated the same mutants in strain in full-length, yeast expression plasmids under the control of Rad53 endogenous promoter (yCPlac33-RAD53). Again, these mutations were generated according to the residues identified at the dimerization interface and were mutated alone or in combination to yield the rad53$^{D323A}$, rad53$^{D323A}$, rad53$^{D280A/D283A}$, rad53$^{D280A/D283A/D323A}$ and rad53$^{K231A/R232D/K233E}$ strains. We also utilized a kinase-dead allele of RAD53, rad53$^{D339A}$, an activation segment phosphorylation mutant rad53$^{T354A}$ and a hyperactive mutant rad53$^{D330A/D331A}$ as controls. Each plasmid-borne allele was introduced in a rad53Δsml1-1 strain and DNA damage-induced Rad53 activity was elicited by addition of 100 mM Hydroxurea. Rad53 activity was assayed by electrophoretic mobility shift on Rad53 immunoblot as well as by assaying sensitivity to 20 mM HU following serial dilution of cultures on drop-out media containing the drug. As shown Figure 4.5CD, Rad53 phosphorylation and cellular survival is significantly compromise in the strains containing the acidic and basic patch mutants (top panel lane 11-16 and bottom panel lane 1-4). Interestingly, although the STQ motif alanine and reverse charge mutants (rad53$^{S411A/T412A}$, rad53$^{S411E/T412E}$, respectively) did not show a change in mobility (data not shown) and cell survival (bottom panel, lane 8-9), the combination of the moderate allele rad53$^{D323A}$ with the STQ mutant rad53$^{S411A/T412A}$ display a clear additive effect in survival under DNA damaging conditions (Figure 4.5C, bottom panel lane 10). These results suggest that all the structural components identified
in the Rad53 structure are likely involved in maintaining the structural integrity of the Rad53 dimer.

4.1.5 T-LOOP EXCHANGE

An intriguing possibility raised by the Rad53 dimer structure is the potential for inter-molecular exchange of the activation segment and helix $\alpha$EF across the face-to-face dimer to the active site of the opposite Rad53 molecule. An intermolecular helix EF strand-exchange phenomenon has been observed in the crystal structures of two CAMK family members, Checkpoint kinase 2 (Chk2, the mammalian orthologue of Rad53) (Oliver et al., 2007; Oliver et al., 2006) and Death-associated protein kinase 3 (DAPK3) (Bialik and Kimchi, 2006), as well as two STE family members, STE20-like kinase (SLK) and Lymphocyte-originated kinase (LOK) (Oliver et al., 2007). It has been proposed that exchange might be critical for auto-activation by trans-phosphorylation of the activation segment (Oliver et al., 2007). Whether exchange of helix $\alpha$EF is relevant for kinase domain function \textit{in vitro} and \textit{in vivo} has yet to be determined.

The association of helix $\alpha$EF with its binding pocket on the kinase C-lobe (i.e. the $\alpha$EF binding pocket) is stabilized by a kinase domain invariant salt bridge. In Rad53, this interaction is mediated by Glu365 in helix $\alpha$EF and Arg454 in a linker region connecting helices $\alpha$I and $\alpha$J (Figure 4.6A). We predicted that mutation of either charged side chain to opposite polarity would perturb kinase function, in part by destabilizing the association of helix $\alpha$EF with the kinase domain (Figure 4.6B, left and central panel, respectively). This would be the case if kinase function was dependent on the precise association of helix $\alpha$EF with the kinase domain in either an intra or inter-molecular manner. A loss of
kinase function would manifest itself as a loss of the ability to auto-phosphorylate on Thr354 in trans. If the binding mode in trans was critical for kinase function, we predict that the mixing of the two charge reversal mutants might restore catalytic function of the paired proteins by reconstituting the stabilizing salt interaction in opposite polarity (Figure 4.6B, right panel).
Figure 4.6 Dimerization promotes T-Loop exchange, trans-autophosphorylation and activation of Rad53

(A) Ribbon diagram representing the residues involved in electrostatic interaction promoting T-Loop exchange of the Rad53 dimer. (B) Cartoon diagram representing the residues important for T-Loop exchange and their respective mutation for residues of the opposite charge utilized to perform the mutagenesis and complementation analysis in C and D. (C) In vitro complementation assay using mutants of the residues highlighted in (A) where equimolar quantities of recombinant protein carrying the indicated mutation(s) were incubated with full-length Rad53 D339A (substrate) and the activity of Rad53 (auto-
phosphorylation on the bottom panel and trans phosphorylation of the top panel) was monitored by immunoblotting utilizing a phosphospecific Rad53 antibody Rad53<sup>Thr354P</sup>. In vivo complementation studies where strains containing the alleles described above (YcP = YePlac33(URA3) backbone, pGAL = pGAL1/10(LEU2) backbone) were diluted serially 5-fold and spotted on agar plates containing 15mM HU (Figure 4.6D was performed by Frederic Sweeney and Cynthia Ho).

To test this possibility, the charge reversal mutations were introduced into the Rad53<sup>170-512</sup> constructs (in ampicillin and kanamycin-based vectors) to generate Rad53<sup>170-512 (E354K)</sup> and Rad53<sup>170-512 (R454E)</sup> for single or co-expression. As shown in Figure 4.6C, both single site mutants were severely compromised in their ability to phosphorylate Thr354 as assessed by anti-Rad53<sup>Thr354-P</sup> immunoblotting of bacterial lysates (Figure 4.6B, lanes 1-4, 5-6 and 10-11). However, when Rad53<sup>170-512 (E354K)</sup> and Rad53<sup>170-512 (R454E)</sup> were co-expressed in bacteria, the ability of both proteins to accept phosphate on Thr354 was restored (Figure 4.6B, lanes 7-10). The level of Thr354 phosphorylation is not optimal but this might be expected since invariant residues mediate the salt bridge. Nevertheless, the functional rescue by the complementary charge-reversal mutations provides a very strong indication that strand-exchange of the activation segment and helix αEF can occur in the Rad53 dimer.

We tested whether the charge-reversal mutations would have an effect on full-length wild type Rad53 function in vivo and whether co-expression of the paired mutant proteins in yeast would functionally complement to rescue yeast growth following DNA damage. To test this possibility, the two charge reversal mutations were introduced into <i>RAD53</i> constructs to generate <i>rad53<sup>E354K</sup></i> and <i>rad53<sup>R354E</sup></i> for single expression alone or co-expression together. The wild type full-length <i>RAD53</i> was used as a positive control in the experiment for intact Rad53 function. The catalytically inactive <i>rad53<sup>D339A</sup></i> was used as a loss-of-function negative control and as a point of comparison for any functional
complementation. As shown in Figure 4.6D, both single site mutants were severely compromised for rescue of yeast cell growth following DNA damage compared to wild type RAD53, although the rad53^{E354K} mutant displayed some growth compared to the rad53^{R454E} mutant and the rad53^{D339A} negative control, which had no detectable growth on HU plates (Figure 4.6D, left and central panel). However, when the rad53^{E354K} and rad53^{R454K} mutants were co-expressed, an increase in survival on media containing HU was clearly observed although the level of the rescue is far below that of wild type Rad53. From these observations, we can conclude that strand-exchange of the activation segment and helix $\alpha EF$ can occur in full-length Rad53 and that the precise orientation of the exchanged elements mediated by the invariant salt bridge is important for Rad53 function in vivo.
4.2 DISCUSSION

4.2.0 THREE DIFFERENT MODES OF REGULATION

The atomic resolution of Rad53 coupled with biochemical and genetic mutational analyses revealed that Rad53 is regulated by three different modes of action. First, Rad53 is auto-inhibited by two α-helices situated at the C-terminal extension of the protein kinase domain. Intriguingly, as mentioned earlier in this Chapter, these helices sit on the back of the kinase domain, do not interact with the activation segment, ATP binding or substrate binding region of the kinase and are not involved in the dimer formation. Although clear biochemical evidence demonstrates the importance of these helices in the inhibition of the kinase, it remains mysterious how these helices do so.

Secondly, the kinase forms a dimer. This dimerization which occurs via a conserved acidic and basic patches and a highly conserved motif (STQ) is required for Rad53 catalytic activity in vitro and in vivo. Although mutation of these sites yields a clear decrease in Rad53 activity, the dimerization of Rad53 in vivo still remains to be clearly demonstrated. This dimerization promotes the third activation mechanism of Rad53 by activation loop exchange where the T-loop of the first molecule extends and makes contact with the kinase domain of the second molecules leading to the activation segment trans-autophosphorylation. Rad53 joins its corresponding orthologs Chk2 and Cds1 (mammalian and Schizosaccharomyces pombe respectively) in sharing an evolutionary conserved activation mechanism whereby dimerization of the kinase is required for activation. However, contrary to Chk2 and Cds1, and in accordance with the Rad9 solid-state catalyst model of Rad53 activation (Gilbert et al., 2001; Pellicioli and Foiani, 2005; Sweeney et al., 2005), the dimerization of Rad53 does not seem to be
orchestrated by the phosphorylation of Rad53 FHA binding sites on the kinase but rather by the multi-site phosphorylation of numerous sub-optimal sites on Rad9.

4.2.2 PROPOSED MODEL OF RAD53 ACTIVATION

Although the data described above point to a complex regulation of Rad53 activation, we would like to propose the following model: Firstly, the activation of Rad53 is initiated following its interaction with phosphorylated Rad9. This interaction favors the phosphorylation of Rad53 by Mec1 on multiple residues. Of the potential Mec1 phosphorylation sites mapped, Ser485 and Ser 489, reside in the α-helices identified in this Chapter as being important for Rad53 auto-inhibition. I propose that phosphorylation of these residues, would relieve Rad53 auto-inhibition of Rad53 dimer. The mechanisms by which the relief on inhibition occurs are unknown. The “primed” Rad53 would then be phosphorylated on its activation loop via T-loop exchange with its corresponding dimerization partner. The hyperphosphorylation subsequent to Rad53 activation would promote its release from Rad9, perhaps by electrostatic repulsion of the Rad53 FHA:phospho-Rad9 interaction (as discussed in Chapter 2). Finally, the Cdc5 phosphorylation site identified in Chapter 3 maps at the dimer interface of Rad53. Cdc5 would then, in a context of DNA damage adaptation, phosphorylate Rad53 and prevent this dimer formation, hence breaking the Rad53 activation cycle by preventing T-loop exchange.

Interestingly, Gilbert et al. suggested that the interaction of Rad53 and Rad9 promotes Rad53 aggregation and autotransphosphorylation of the kinase, which is one of the proposed mechanisms of activation of Rad53 (the catalyst model described in Chapter
2) (Gilbert et al., 2001). However, it has been clearly shown that Rad9 can be bypassed in the Rad53 cascade if the protein (Rad53) is genetically modified and to be artificially recruited to upstream components of the DNA damage response (Lee et al., 2004). This observation suggests that the recruitment of the DNA damage sensor Mec1 is required for Rad53 activation, in which case would relieve the inhibitory status of Rad53 by phosphorylating the appropriate residues (e.g. Ser485 and Ser489). Rad53 activation segment phosphorylation does not require Rad9 in this case; the activation would rather happen via auto-transphosphorylation due to the Rad53 dimer. It would be interesting to test whether the dimerization mutants identified in this Chapter would impede Rad53 activity in this context (Lee et al., 2004).
4.3. Table

Table 4.1 Apo-Rad53 Data Collection, Structure Determination and Refinement Statistics

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<tr>
<td>Most favoured/disallowed regions (%)</td>
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</tr>
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</table>

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$^a$ Numbers given in parentheses refer to data for the highest resolution shell (2.65-2.60Å).

$^b$ R$_{sym}$ = \(100 \times \frac{\sum \left| I - \langle I \rangle \right|}{\sum \langle I \rangle}\), where \(I\) is the observed intensity and \(\langle I \rangle\) is the average intensity from multiple observations of symmetry-related reflections.
4.4. EXPERIMENTAL PROCEDURES

CLONING, EXPRESSION AND PURIFICATION

The kinase domain and C-terminal kinase extension sequence of *S. cerevisiae* Rad53, residues 170 to 512, was cloned by PCR using primers containing additional flanking restriction enzyme sites (NcoI and EcoRI) to facilitate ligation into pPROEx-Hta-TEV (a bacteria expression vector with a TEV protease-cleavable N-terminal 6xHis-tag). The QuickChange kit (Stratagene) was used to generate kinase inactive Rad53 by point mutation of Asp339 to Ala (Rad53<sup>D339A</sup>). The His-Rad53<sup>D339A</sup> construct was transformed in *E. Coli* BL-21 cells and grown in LB media (supplemented with 100 μg/ml ampicillin) with overnight induction at 15°C (A<sub>600</sub> = 0.6 and 0.2 mM IPTG final concentration). Cells were collected by centrifugation, resuspended in buffer A (20 mM HEPES pH 7.5, 200 mM NaCl, 5 mM imidazole supplemented with 1 mg/ml lysozyme and 5 mM β-Mer) and lysed by sonication on ice. Following centrifugation and filtering to remove cell debris, the crude lysate was applied to a HiTrap chelating HP Ni<sup>2+</sup> column (GE Healthcare). The column was washed using 5 column volumes of 5 mM and 30 mM imidazole buffer (20 mM HEPES pH 7.5 and 200 mM NaCl) and the His-Rad53<sup>D339A</sup> proteins eluted in the final step by 5 column volumes of 350 mM imidazole buffer. Removal of the His-tag from 6xHIS-Rad53<sup>D339A</sup> was achieved by addition of His-tagged TEV protease (with a non-cleavable His-tag), 1 mM DTT and 0.5 mM EDTA directly to the final 350 mM imidazole wash fraction (overnight, 4°C). Following dialysis to remove DTT and imidazole, the TEV-cleaved fraction was applied to a Ni<sup>2+</sup> column and successive 5 mM, 30 mM and 350 mM imidazole washes were performed as described above. The free Rad53<sup>D339A</sup> protein flowed-through were eluted following 5 mM
imidazole wash. The fractions were combined and concentrated by ultrafiltration and further purified by gel filtration chromatography (20 mM HEPES pH 7.5, 100 mM NaCl and 2 mM DTT). Aliquots were flash frozen in liquid nitrogen and stored at -80 °C until required.

IN VITRO TRANSLATION

In vitro translation experiments (Figure 4.1) were performed using the EasyXpress Protein Synthesis System (Qiagen) according to manufacturer’s instruction.

CRYSTALLIZATION, DATA COLLECTION, STRUCTURE DETERMINATION AND MODELING

Rad53D339A crystals (a = 76.1, b = 79.1, c = 227.3, space group = C222_1) were produced in hanging drops containing 0.5 µl of protein (30 µg/µg) mixed with an equal volume of well buffer (30% v/v PEG400, 50 mM sodium citrate, 100 mM Tris pH 8.0). Crystals were stabilized by glutaraldehyde crossing-linking treatment for 10 min (Lusty et al., 1999) prior to flash freezing in liquid nitrogen directly from crystallization condition. Diffraction data was collected at the National Synchrotron Light Source (Brookhaven National Laboratory, Long Island, NY) beamline x29 using an ADSC Q315 detector. Data processing and reduction was carried out with the HKL and XDS program suites. The Rad53D339A structure was solved by molecular replacement with CNS using Death-associated protein kinase (PDB#: 1IG1) as a search model. The Rad53D339A structure was initially modified and refined with iterative cycles of manual building and simulated annealing using O and CNS (R_{factor}/R_{free} = 27.8/33.1%). The last stages of refinement and model building were performed using coot and REFMAC (R_{factor}/R_{free} = 24.3/28.9%) with
residues of the final structure in either the most favored (88.4%) or additional allowed regions (11.6%) of the Ramachandran map. Relevant data collection and refinement statistics are listed in Table 1.

CLONING AND MUTAGENESIS

The QuickChange kit (Stratagene) was used to generate all site-directed *S. cerevisiae* Rad53 mutants and *M. musculus* Chk2 mutants used *in vitro* and the *H. sapiens* Chk2 mutants used *in vivo*.

GENERATION OF PHOSPHOSPECIFIC RAD53^{T354-P} ANTIBODY

A polyclonal rabbit antibody specific to phosphorylated residues Ser350 and Thr354 of Rad53 was generated by injecting three animals with a cationic BSA-coupled phosphopeptide corresponding to Rad53 phosphorylated activation segment (G-N-G-pS-F-M-K-pT-F-C-G). In parallel, one rabbit was injected with the non-phosphorylated peptide to be used later on in the protocol as a control to assess the phospho-specificity of the antibody. The BSA coupling was performed using the Imject Maleimide-Activated Bovine Serum Albumin Kit (Pierce Biotechnology, # 77116), as per manufacturer’s instructions. The phospho-specificity of the various sera was tested by standard ELISA techniques using the serum from the non-phosphorylated rabbit as a benchmark control. The phospho-specific anti-sera were then purified against an agarose column containing KLH-coupled non-phosphorylated peptides and the eluate was finally bound, purified and eluted from an agarose column containing KLH-coupled phosphorylated peptides. The coupling of the KLH and peptides was performed using the Imject Maleimide-Activated
Bovine Serum Albumin Kit (Pierce Biotechnology, # 77116) and the agarose column and purification methods were done using the SulfoLink Immobilization (Pierce Biotechnology, # Kit 44995).

DNA DAMAGE SENSITIVITY ASSAYS

Hydroxurea (HU) was purchased from Sigma-Aldrich Chemicals. Drop tests were performed using five-fold serial dilutions of an overnight culture. A 8 µl aliquot of the diluted culture was spotted on corresponding dropout media plates with or without genotoxins (see figure legends for details) and were grown for 3-4 days at 30°C.

STRAINS AND PLASMIDS

All strains are derivatives of W303a and are described in (Sweeney et al, 2005). All the other strains were generated by introducing the plasmids mentioned above according to standard yeast genetic techniques.

TIME COURSE OF RAD53 ACTIVATION

Yeast strains were grown to log phase (OD 2.0) and treated with 75mM Hydroxurea. Samples were taken at the indicated times and the cells were lysed by standard TCA precipitation methods (as described in Sweeney et al, 2005). Standard Rad53 immunoblotting was then performed as described below.
IMMUNOBLOTTING AND IN VITRO KINASE ASSAY

Immunoblotting of Rad53 was performed as described in (Sweeney et al, 2005). Phospho-specific Rad53 immunoblotting was performed using the method described below. Each Rad53 mutant was diluted in kinase reaction buffer (50 mM HEPES pH 7.5, 250 mM NaCl, 2 mM DTT, 20 mM each of MgCl₂ and MnCl₂) and incubated with purified His-tag full-length kinase inactive Rad53 in the presence of 1 mM cold ATP at room temperature (see figure legends for specific protein concentrations and reaction incubation times). The reactions were mixed with 6x SDS loading buffer, boiled, electrophoresed and transferred to PVDF membrane. T354 trans-autophosphorylation, of both the His-tag full-length kinase inactive Rad53 substrate and the kinase active Rad53 mutants, was detected by immunoblotting with a phospho-specific α-Rad53 T354-P primary antibody (1:1000), followed by incubation with HRP-conjugated goat α-rabbit secondary antibody (1:10 000) and visualized using chemiluminescence (Super Signal West Pico, Pierce).
CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

RAD9, RAD53 AND THE MOLECULAR MECHANISMS OF DNA DAMAGE SIGNALING
5.0 SUMMARY

The frequency at which cells are exposed to DNA damaging events requires not only a faithful DNA repair mechanism but also a surveillance mechanism by which the damage can be properly detected and the cell cycling can be regulated to ensure that adequate repair is performed. This surveillance mechanism, the DNA damage checkpoint, is reliable, efficient and tightly regulated.

The efficiency of the system does not come solely from its redundancy but also from a controlled mechanism by which insignificant damage is repaired and significant damaging events trigger cell cycle arrest. As we explored Section 1.2, the robustness of the DNA damage checkpoint allows for a subcritical level of damage to be repaired without triggering a checkpoint event. On the other hand, a single DSB, which is lethal to the cell, triggers a checkpoint. The mechanisms by which this “dosage” of DNA damage is interpreted by the cell remain unknown. In this dissertation, we provided evidence that the molecular adaptor Rad9 serves as a “buffer” that sets the threshold for Rad53 activation. This activation is counteracted by the action of phosphatases and also by the Cdc5 kinase after a long checkpoint arrest.

In this section, I will speculate on how the cell sets the threshold for activation of the DNA damage checkpoint. Moreover, I will reconcile some of the data presented on Chapter 3 regarding Cdc5 regulation of Rad53 with the atomic resolution of Rad53 in order to isolate the mechanism by which Cdc5 down regulates Rad53 during adaptation.
5.1 DISCUSSIONS

5.1.0 RAD9 AS AN ULTRA-SENSITIVE SWITCH TO THE DNA DAMAGE CHECKPOINT

Molecular adaptors are proteins with modular interaction domains that coordinate signal transduction events. Many such molecules have been identified in different systems and were found to be critical for either increasing the affinity (lowering Km) of a particular kinase for its substrate, increasing the affinity of the kinase for ATP or for proper spatio-temporal organization of the required signaling molecules (Pawson, 2007). For example, adaptor molecules can simply serve to bridge two proteins together, as it is often the case during receptor tyrosine kinase signaling (Pawson, 2007). During insulin signaling, for instance, the insulin receptor trans-autophosphorylates on Tyr residues, which recruits the IRS1 protein via its PTB domain. IRS1 also interacts with p110, the regulatory subunit of PI3K, which is activated upon its recruitment to the cell membrane. IRS1 in this case acts as an adaptor bridging the receptor of its regulatory kinase (Meijers-Heijboer et al., 2002).

More recently, strong evidence is showing that adaptors are not just used to couple two proteins together but also play a complex integrative role in directing signals and greatly influence the rate of catalysis of the associated reactions (Dueber et al., 2007; Sallee et al., 2007).

The idea that enzymes in signal transduction pathways are not necessarily regulated in an allosteric or gradient fashion was coined by the group of Goldbeter and Koshland when they made the observation that some enzymes does not exhibit a typical Michaelis-Menten behavior as their rates of catalysis are less or more steep than the classical curve (Goldbeter and Koshland, 1984). They termed “ultrasensitivity” and
“subsensitivity” enzymatic reactions that tend to convert graded inputs into all-or-none responses. The theoretical and experimental framework on ultrasensitive behavior in cellular systems has been more recently supported by pioneering work from the laboratory of James Ferrell (Huang and Ferrell, 1996; Tsai et al., 2008). Ferrell’s discovery that the MAPK pathway is regulated in an all-or-none fashion in *Xenopus laevis* oocyte in response to progesterone has shed light onto the way many cellular processes are regulated, with main focus on cell cycle regulation (Huang and Ferrell, 1996).

Furthermore, work by Nash et al. and Tang et al., provided a biochemistry and structural explanation on how such all-or-none responses occur (Nash et al., 2001; Tang et al., 2007). In these studies, the kinetics of degradation of the protein Sic1, an inhibitor the Clb-Cdc28 kinases that is responsible for S-Phase entry, was investigated. Sic1 is phosphorylated on multiple low affinity SCF*^cde4* binding sites. Upon phosphorylation of Sic1 on numerous residues, SCF*^cde4* binds to Sic1 and targets it for degradation. Even though these residues are of low affinity for binding to Cdc4, the probability of binding of Sic1 to Cdc4 increases exponentially accordingly to the number of low affinity phosphorylated sites on Sic1 (Nash et al., 2001; Tang et al., 2007). This behavior converts the graded response translated by Sic1 phosphorylation into a biological all-or-none response displayed by Sic1 degradation and cell cycle progression (Klein et al., 2003).

As it is the case for the cell cycle, the regulation of the DNA damage checkpoint must also be achieved in an all-or-none fashion. It would be logical to hypothesize that similar all-or-none mechanisms occur during the checkpoint response (Figure 5.1).
Figure 5.1 An ultrasensitivity switch-like mechanism for the DNA damage checkpoint response? (A) Although DNA damage is a gradient-like phenomenon, the DNA damage checkpoint is all or none response. (B) The theoretical curve of Mec1 activation displays a typical Michaelian response where Mec1 activity is proportional to the amount of ssDNA (left panel) as well as the theoretical curve of Rad53 activation (right panel), which shows cooperativity where (C) Rad9 multisite phosphorylation is predicted to convert the Mec1 Michealian response into a switch-like response.

Interestingly, a study by Schwartz et al. showed that the Rad53-Rad9 interaction seems to be controlled in such a similar fashion (Schwartz et al., 2002). In this study, potential Mec1 phosphorylation sites (Thr-Gln residues) on Rad9 were mutated to Ala residues. It was found that Rad53 was only able to bind Rad9 when multiple low affinity FHA binding sites were present on Rad9. The in vivo activation of Rad53 is a tightly regulated
process, which is initiated once the kinase binds to its molecular adaptors Rad9. Hence, a series of evidence points towards the fact that DNA damage checkpoint is regulated using an ultrasensitivity switch controlled by Rad9. Additional mutagenesis, biochemical and \textit{in vitro} work would be required before confirming this hypothesis. In particular, it will be critical to characterize \textit{in vitro} the binding affinity of the Rad53 FHA domains to different phosphorylated forms of Rad9.

5.1.1 TOWARDS SOLVING THE DNA DAMAGE PROTEOME

The DNA damage response is a complex, but well organized and tightly controlled network of protein-protein interactions that propagates signals mostly through a series of phosphorylation events. It is crucial to keep in mind that these signals do not progress in a linear fashion but rather influence each other through crosstalk between different pathways and via complex positive and negative feedback regulatory mechanisms. In order to understand more clearly how this pathway operates, it is essential to understand how protein phosphorylation affects the components of the checkpoint response. The phosphomapping of Rad53 presented in this dissertation is a relatively small but powerful example of the discoveries that cascade from what appears to be a straightforward phosphomapping analysis. New protein purification and mass spectrometry techniques such as SILAC allow us not only to map phosphorylation sites on a giving protein, but also to get a quantitative and temporal representation of these phosphorylation events (Ong et al., 2002). By pursuing the mapping of the DNA damage phosphoproteome, possibly in combination with the mapping of other post-translational modifications and compiling the data acquired in a comprehensive format, important information can be
obtained from such phospho-maps. Novel kinase, regulatory, feedback and crosstalk pathways as well as novel roles for existing key player can be identified. In combination with the current genetic and biochemical knowledge of DNA damage signaling, we could be able to get a more precise idea on where these phospho-proteins reside in the signaling cascade and how these phosphorylation events influence the checkpoint response.

5.1.2 Cdc5 phosphorylation regulated Rad53 by influencing dimer formation

In trying to understand the regulation of Rad53 and its implication into checkpoint activation and recovery/adaptation, we identified that Cdc5, the yeast polo-like kinase, interacts and phosphorylated directly Rad53 on Ser373. At this point, it is unclear if this phosphorylation event is relevant in vivo as most of the data presented in Chapter 3 rest on in vitro evidence. In Chapter 4, however, I collaborated with the laboratory of Frank Sicheri to characterize the crystal structure on Rad53 and identified, along with others, that the dimerization of the kinase is essential for Rad53 regulation (Gilbert et al., 2001; Jia-Lin Ma and Stern, 2008). However it is unclear whether this dimerization promotes the activation or the inhibition of the kinase. It is also unclear at which point in the signaling cascade Rad53 dimerize although most evidence points towards a mechanism where Rad53 promotes A-Loop trans-autophosphorylation.

The Ser373 residue is located within the dimer interface of the kinase domain in between the two dimerization motifs (basic/acidic and STQ motifs) we identified in our structural studies. It is tempting to speculate how Cdc5 promotes the downregulation of Rad53 via Ser373 phosphorylation. Indeed, phospho-Ser373 might prevent Rad53 from dimerizing, thus precluding its trans-autophosphorylation. Since active Rad53 is negatively
regulated by phosphatases, the inability of Rad53 to dimerize would rapidly reduce the pool of active Rad53 in the cell.

This working hypothesis is currently being tested in our laboratory. Interestingly, phospho-Ser 373 has been detected by both our laboratory in response to 4-NQO and the group of Smolka et al. in response to MMS (Table 2.4), but not in response to HU. Phospho-specific antibodies are being generated to confirm presence of phosphorylated Ser373 in response to these types of DNA damage. Moreover, the Ala mutant is being investigated for adaptation defects as well as Rad53 activation phenotypes and genetics analysis such as observing the phosphorylation status of Ser373 in different stage of the cell cycle as well as in response to overexpression of CDC5 or a dominant negative of Cdc5 such as \textit{cdc5-kd} should help us elucidate in detail the mechanism by which Cdc5 modifies Rad53 in order to promote adaptation.
5.1.3 UNDERSTANDING MOLECULAR MECHANISMS OF CANCER PROGRESSION

5.1.3.0 UNDERSTANDING THE CHECKPOINT AT THE MOLECULAR LEVEL.

Historically, the investigation of the DNA damage checkpoint focused on understanding the key genetic pathways implicated. This allowed us to identify key regulatory genes and proteins critical for checkpoint function. However, the molecular mechanisms by which these protein function have only recently started to be understood. As biochemical tools evolved, researchers can investigate these molecular mechanisms more readily. For instance, mass spectrometry and automation technologies have greatly improved in the last decade and allowed us to unravel with great efficacy in vivo protein: protein interaction networks as well as site-specific, quantitative post-translational modifications. One of the ultimate goals in understanding the molecular mechanisms of protein signaling in this post-genomic era is to be able to script disease pathways with great precision and allow diseases to be identified by molecular diagnostics. In addition, because most diseases are heterogeneous by nature, being able to do so on an individual per individual basis would revolutionize conventional medicine towards a more personalized medicine approach.

5.1.3.2 BIOMARKERS, RATIONAL DRUG DESIGN AND PERSONNALIZED MEDICINE

The development of genomic and proteomic tools promoted the discovery a wealth of proteins, genetic mutations or other genotypic/phenotypic variations indicative of specific diseases, called biomarkers. Biomarkers drastically changed the way disease classification and diagnostics are performed. Cancer, for instance, is now characterized largely by its genotypic signature (e.g.: HER2+ breast cancer) rather than simply by its
pathology (breast vs. liver cancer). Biomarker-based diagnostics now allow diseases to be detected at an earlier stage, also allowing for the selection of more optimal therapies as well as reduce adverse drug reaction. Moreover, it is because of the increasing understanding of the molecular mechanisms of these diseases that drugs based on rational design can be developed. For example, the molecular understanding of receptor tyrosine kinase signaling led to the development of Herceptin, a monoclonal antibody therapy targeting specifically cancer overexpressing the Her2 receptor. Another example of rational drug design is the development of Imatinib (also know as Gleevec) for the treatment of CML or Chronic Myeloid Leukemia. Imatinib is a small molecule inhibitor designed to inhibit specifically the Abl kinase once it has been fused to the BCR gene, which a very common genotype in CML patients. Both drugs are a major success and molecularly diagnosed patients who received the treatments demonstrated impressive remission rates. Many other diagnostic and personalized treatment methods will become available in the near future based on the molecular understanding of diseases. The work presented in this thesis is a modest exemple on how we could try to understand molecular signatures of diseases such as Cancer.


