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The Mec1/ATR-induced checkpoint regulates the Rdh54 DNA translocase in response to chromosome breaks in yeast

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PART 1

CHAPTER - I

INTRODUCTION

The information for a cell's survival, maintenance and function is stored in its DNA, thus it is essential to keep that information intact. However, our DNA is under continuous threat; we are exposed to UV-light, consume genotoxins present in the food, or inhale these via cigarette smoke and fumes, and produce oxygen free radicals during our normal cellular metabolism. Also, our DNA is subject to spontaneous hydrolysis and deamination [*Hoeijmakers JH, 2001*]. All these processes damage our DNA (and proteins) and in this way, each cell of our body suffers an estimated 10,000-1,000,000 DNA lesion every day [*Holmquist GP, 1998; Kunkel TA, 1999*]. DNA damages can perturb the cellular steady-state quasi-equilibrium and activate or amplify certain biochemical pathways that regulate cell growth and division and pathways that help to coordinate DNA replication with damage removal. The four types of pathways elicited by DNA damage known, or presumed, to ameliorate harmful damage effects are DNA repair, DNA damage checkpoints, transcriptional response, and apoptosis. Defects in any of these pathways may cause genomic instability. That most of us do not succumb to lethal DNA damage underlines the success and importance of our cells' DNA repair mechanisms. Deficiencies in our DNA repair mechanisms can give rise to cancer. Cancer can originate from unrepaired (or unsuccessfully repaired) DNA lesions, which give rise to mutations. Mutations in the DNA cause altered functions/behavior of the cells, giving rise to tumor induction and outgrowth [*Cahill DP, et. al., 1999; DePinho RA, 2000; Loeb LA, 1991*].

Basic research not only elucidates why some chemotherapeutic agents are more successful to certain types of cancer than others, but also contributes to the design of new anticancer drugs. In addition, basic science led to the discovery that dysfunction of some DNA repair proteins is linked to specific types of cancer and to syndromes leading to cancer. When a cell is confronted with an altered DNA metabolism due to DNA damage, it can decide to commit suicide (when there is too much damage to repair) or to induce cell cycle arrest in order to create time to repair the lesions. Repair is not only essential to avoid mutations, but also needed to avoid that the lesions interfere with transcription and replication processes. Finally to understand the amount of damage caused and the way it could be repaired, a biochemical approach to study the DNA and proteins at the molecular level is necessary.

1. 1. The Cell Cycle

Most eukaryotic cells proceed through an ordered series of events in which the cell duplicate its contents and then divide into two cells. This cycle of division and duplication is called cell cycle. In order to maintain the fidelity of the developing organism this process of cell division in multicellular organism must be highly ordered and tightly regulated. The cell cycle is finely regulated by numerous regulatory proteins which regulate the cell progression through different stages referred as G_1, S, G_2, and M phases (**Figure 1**). Cells spend majority of their time in interphase which consists of Gap 1 (G1), Synthesis (S), and Gap 2 (G2). In G1 phase, the cells are growing and replicate cytoplasmic organelles. It is also preparing for replication of its DNA by synthesizing the enzymes necessary to make copies of its DNA. During S phase DNA is synthesized and two exact copies of the chromosomes are produced. In G2 phase cells are preparing for division, this involves supercoiling and winding of DNA to package it into chromosomes, and the synthesis of enzymes and structural components required for cell division. Mitosis occurs during the M phase during this cell divides and partitions copies of its chromosomes and about half of its organelles and cytoplasm into each of two daughter cells. Mitosis is divided into four stages: prophase, metaphase, anaphase and telophase. After mitosis is complete the cytoplasmic contents including the chromosomes are divided and distributed into the two daughter cells that result from the process of cell division or cytokinesis. The unicellular eukaryote *Saccharomyces cerevisiae* is used as an ideal genetic model system to study how the highly conserved eukaryotic cellular processes are carried out.

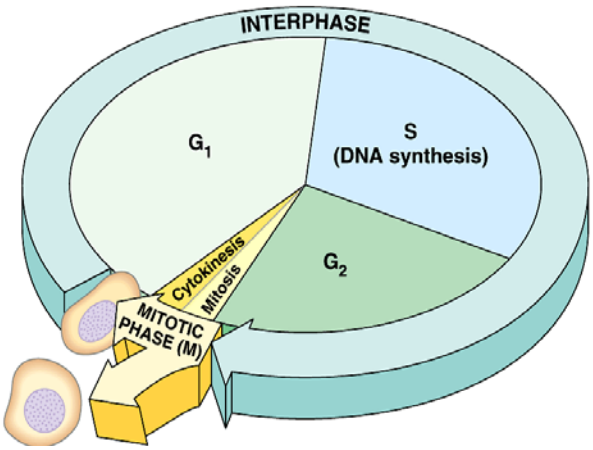

Figure 1. Eukaryotic cell cycle phases

1. 2. Types of DNA damage

DNA can be damaged in a variety of ways. First, energy released by free oxygen radical, generated either by normal metabolic processes or by exposure to an external source of ionizing radiation, can break the phosphodiester bonds in the backbone of the DNA helix. When two of these breaks are close to each other, but on opposite DNA strands, a double-strand break (DSB) is present in the DNA and the cell faces a particularly challenging situation for repair. Second, alkylating chemical moieties can modify purine bases and the size of the chemical adduct determines what repair process is used [*Ford JM, 2004*]. Bifunctional alkylating chemicals can cause intra-strand or inter-strand crosslinks that require additional molecular interventions for them to be reversed. Third, inhibitors of DNA topoisomerases can lead to enhanced single or DSBs depending on which topoisomerase is inhibited and on the phase of the cell cycle [*Froelich-Ammon SJ and Osheroff N, 1995*].

Exogenous and endogenous factors such as irradiation and stalled replication fork leads to double strand DNA break (dsDNA break) in eukaryotic chromosome. These lesions are dangerous to chromosomes that can lead to genome rearrangements, genetic instability, and cancer if not accurately repaired. Cells therefore go to great lengths to repair DSBs, mounting a highly complex multistep response that includes modifications to large chromatin domains (repair foci) through, e.g., ubiquitination, phosphorylation, and binding of numerous repair factors, scaffolding mediators, and posttranslational modifiers (*Harper JW and Elledge SJ, 2007*). In order to repair the damaged DNA eukaryotic cells activate surveillance mechanism called DNA damage checkpoint, which arrest the cells cycle progression and facilitate DNA repair. In the following chapters I described the DNA damage checkpoint mechanism, how it is activated through various signaling molecules at the various stages of the cell cycle phases also in the consecutive chapters I described about the mechanism by which those DNA damages are repaired through specific repair machineries.

1. 3. DNA Damage and DNA Replication Checkpoints

The checkpoint is a signal transduction cascade, that controls the interplay among several DNA transactions and cell cycle progression after DNA damage. The cell cycle can be halted by checkpoint mechanism at different points of cell division, in response to perturbations that risk compromising faithful DNA replication, chromosome segregation and survival. Two checkpoints are sensitive to DNA damage, one that acts before mitosis and a second that acts before DNA

replication. This is relevant to cancer because checkpoint mutants show genetic instability, which is characteristic of many cancers. Studies of checkpoints in normal and cancer cells suggested a mechanistic relationship to the central cell cycle control Cdc28 and its regulators (*Weinert T and Lydall D, 1993)*. Mutations in these genes and those with a role in DNA metabolism may affect the function of checkpoints. The cell cycle is transiently arrest at different stages depending on the phase at which DNA alterations occur (G1, S and G2). Three responses have been characterized in budding yeast, which are known as the G1/S, intra-S and G2/M DNA damage checkpoints. Failure to respond properly to DNA alterations can lead to increased genomic instability, which is one of the most prominent hallmarks of cancer cells (*Hartwell LH and Kastan MB, 1994*). DNA damage checkpoint network is composed of DNA damage sensors, signal transducers and various effector pathways, even though the players might be different, the general mechanism underlying the DNA damage checkpoint response is the same in the different phases of the cell cycle.

	Budding yeast	**Fission yeast**	**Human**
PIKK	Mec1	Rad9	ATR
PIKK	Tel1	Tel1	ATM
Adaptor	Rad9	Crb2	53BP1, MDC1, BRCA1?
Rfc1 homolog	Rad24	Rad17	Rad17
9-1-1 clamp	Rad17	Rad9	Rad9
	Mec3	Hus1	Hus1
	Ddc1	Rad1	Rad1
MRX complex	Mre11	Mre11	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
BRCT domain adaptor?	Dpb11	Rad4/Cut5	TopBP1
Signaling kinase	Rad53	Cds1	Chk2
Signaling kinase	Chk1	Chk1	Chk1
Polo kinase	Cdc5	Plo1	Plk1
Securin	Pds1	Cut2	Securin
Separase	Esp1	Cut1	Separase
APC targeting subunit	Cdc20	Slp1	$_{P}55^{CDC}$/CDC20

Table.1. DNA damage checkpoint proteins [*Harrison JC and Haber JE, 2006*].

1. 4. Sensing the Problem

The checkpoint activation is triggered by the recruitment of PIKKs to DNA damage is considered the most important upstream event in cell cycle arrest. PIKKs bind DNA with the obligatory assistance of one or more partner proteins; in the (**Table 1**) various DNA damage checkpoint proteins which are conserved from yeast to human are listed. The ATM/Tel1 and ATR/Mec1 belong to a structurally unique family of protein serine-threonine kinases, whose catalytic domains share a clear evolutionary relationship with those of mammalian and yeast phosphoinositide 3-kinases (*Hunter 1995; Zakian VA, 1995*). Apical kinase Mec1 plays a major role in the checkpoint pathway by activating and interacting with the downstream checkpoint cascade proteins in response to various genotoxic stresses. These factors play critical roles in early signal transmission through cell cycle checkpoints. Interaction between Mec1/ATR and single stranded DNA coated by Replication Protein A, and between Tel1/ATM and the Mre11-Rad50-Xrs2 complex bound to double strand breaks. Mec1/ATR is active when associated with the Ddc2/ATRIP protein [*Paciott V, et. al., 2000; Rouse J and Jackson SP, 2000; Wakayama T, et. al., 2001*] and ssDNA binding by Mec1-Ddc2 or ATR-ATRIP complexes requires the ssDNA binding complex RPA [*Zou L and Elledge SJ, 2003*]. Alternatively, other checkpoint PIKK Tel1/ATM can be recruited to blunt or minimally processed DSB ends through its interaction with MRX/MRN complex, which triggers its activation [*Falck J, et. al., 2005; Nakada D, et. al., 2003*]. In the case of DSBs generated by the HO endonuclease in *S. cerevisiae* the signal that is required for the activation of the DNA damage checkpoint is not the presence of ssDNA *per se* but rather a continuous resecting activity [*Ira G, et. al., 2004*]. DSBs thus appear to generate two different signaling structures before and after the resection of their ends, detected by two different sensors.

The 9-1-1 complex is strongly required for the checkpoint activation and cell cycle arrest. This heterotrimeric complex is made up of Rad17, Mec3 and Ddc1, all of which show limited sequence homology to the PCNA clamp, therefore referred as a checkpoint clamp [*Venclovas C and Thelen MP, 2000*]. Ddc1 focus formation in the presence of DNA damage does not require Mec1, Ddc2, Rad53, Rad9, or Tel1, suggesting that the 9-1-1 clamp functions as a third independent damage sensor [*Kondo T, et. al., 2001; Lisby M, et. al., 2004; Melo JA, et. al., 2001*]. The 9-1-1 complex is loaded onto DNA by a checkpoint clamp loader RFC which is Rad24, biochemical analysis of Rad24/RFC and the 9-1-1 complex has shown that Rad24/RFC interacts with the 9-1-1 complex and recruits it to DNA [*Bermudez VP, et. al., 2003, Ellison V and Stillman B, 2003; Majka J and Burgers PM, 2003*]. Despite the role for RPA in 9-1-1 DNA loading, extensive

resection is not required even a minimal resection will create the ssDNA/dsDNA junction at which 9-1-1 clamp is likely to be loaded [*Nakada D, et. al., 2004*]. Phosphorylation of Rad9 and Rad53 is reduced in *9-1-1*Δ and *rad24*Δ mutant's shows very strong checkpoint defects [*Emili A, 1998*]. Crippled 9-1-1 clamp have weak phosphorylation of Rad9 by Mec1 and form Rad9-Rad53 complex, in these cells Mec1 cannot phosphorylate Rad53. In contrast, Tel1 can phosphorylate Rad9 and Rad53 in these cells. This result suggests a role for the 9-1-1 clamp in both Rad9 phosphorylation and the subsequent Rad53 phosphorylation, and it further suggests that Tel1 might be significantly less reliant on the 9-1-1 clamp than Mec1 during Rad53 phosphorylation [*Giannattasio M, et. al., 2002*]. Mechanism and the proteins which are involved in activating checkpoint are illustrated in (**Figure 2**).

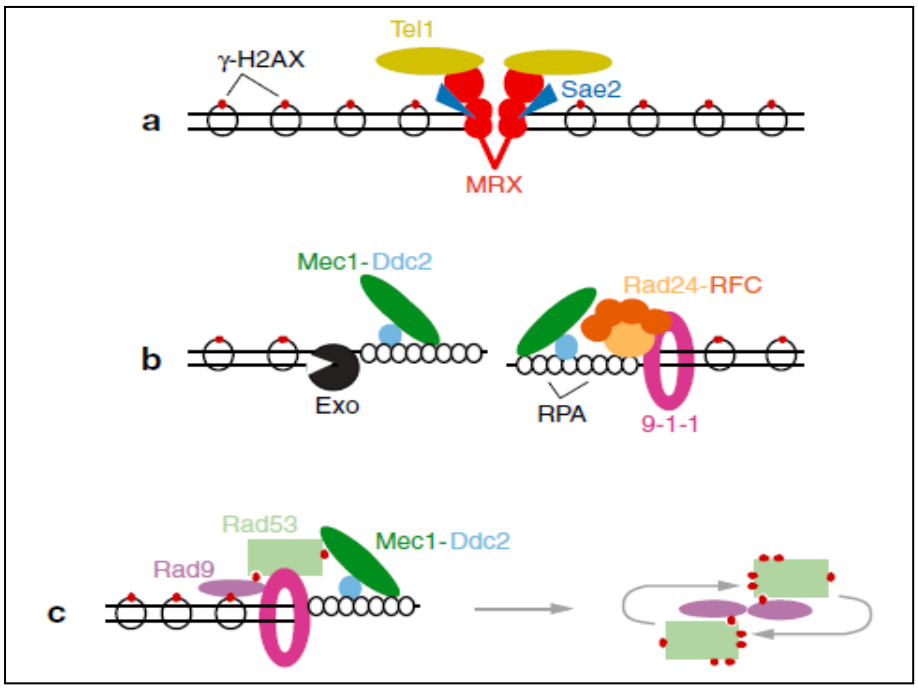

Figure 2. Mechanism of checkpoint activation ***[Harrison JC and Haber JE, 2006].***

1. 5. Role of adaptor and signaling kinases in checkpoint response

After DNA damage is detected by upstream sensors, a kinase cascade amplifies and relays the signal to checkpoint targets. In budding yeast the primary transducer is the Rad53 kinase whose activation requires Mec1 and the Rad9 adaptor protein. Phosphorylated Rad9 may act as an adapter protein that delivers Rad53 to Mec1 allowing Mec1 to activate Rad53, both Mec1 and

Rad9 are targeted to sites of DNA damage, and Mec1 kinase can directly phosphorylate and activate Rad53 *in vitro* [*Ma JL, et. al., 2006*]. During checkpoint activation, Rad53 phosphothreonine binding FHA domains interacts with PIKK-phosphorylated Rad9 leading to catalytic activation of Rad53 and extensive autophosphorylation of Rad53 [*Durocher D, et. al., 2000; Sun Z, et. al., 1998*]. The two FHA domains of Rad53 are only partially redundant for its activation. Loss of either FHA domain or the double FHA1, 2 mutants are as strongly checkpoint defective as the Rad53-kd allele [*Pike BL, et. al, 2003; Schwartz MF, et. al., 2003*]. Hyperphosphorylated forms of Rad9 induced by DNA damage interact with the Rad53 FHA domains, preferentially with FHA2. FHA domains of Rad53 likely to interact with a cluster of 7SQ/TQ motifs in Rad9 central region, mutation of the first 6 of these is sufficient to prevent Rad9 phosphorylation [*Schwartz MF, et. al., 2002*]. Rad53 contains an N-terminal cluster of TQ sites and a C-terminal cluster of SQ sites both these motifs contribute to Rad53 phosphorylation [*Lee SJ, et. al., 2003*]. *In vivo,* many predicted CDK target sites on both Rad9 and Rad53 are also phosphorylated, even in the absence of DNA damage [*Smolka MB, et. al., 2005; Sweeney FD, et. al., 2005*]. The direct phosphorylation of Rad9 or Rad53 by CDK1 contributes to normal checkpoint activation. Thus, checkpoint proteins are recruited to the double strand break sites only after the damaged sites are processed nucleolytically by several nucleases upon different damage condition.

1. 6. Processing the Double Strand Break sites

In *Escherichia coli*, RecBCD, a complex of helicases and a nuclease, is responsible for the formation of 3′ ssDNA tails at DSBs [*Spies M, et. al., 2005*]. In eukaryotic cells, however, the major 5′ end resection activity remains unknown. Several proteins that are required for a normal rate of DSB end resection have been identified in budding yeast and mammals including the MRX/MRN complex (Mre11- Rad50-Xrs2 in yeast and Mre11-Rad50-Nbs1 in human) [*Jazayeri A, et. al., 2006*], Sae2/CtIP [*Clerici M, et. al., 2005; Sartori AA, et. al., 2007*], Exo1 [*Mimitou EP and Symington LS, 2009*] and the chromatin remodeling Ino80 or RSC complex [*Shimada K, et. al., 2008; van Attikum H, et. al., 2004*]. Both MRX and Sae2 belong to one epistasis group with respect to DSB resection [*Clerici M, et. al., 2005*]. Although deletion of any component of the MRX or Sae2 complex decreases the resection rate at DSBs. Mre11 has multiple nuclease motifs but expression of *mre11*-H125N, which completely eliminates nuclease activity *in vitro*, was shown to retain a nearly normal resection rate in the presence of a large number of DSBs [*Llorente B and Symington LS, 2004*], suggesting that the MRX complex may facilitate the access to DSB ends for other nucleases

[*Lee SE, et. al., 2002*]. Paradoxically, MRX's *in vitro* exonuclease has 3′ to 5′ activity rather than the 5′ to 3′ activity seen during DSB resection. Mre11 also exhibits endonuclease activity, which could still be compatible with a helicase/endonuclease mode of resection [*Paull TT and Gellert M, 1998*]. Mre11 nuclease activity is directly responsible for processing Spo11-induced DSBs only in meiotic cells, most likely by removing covalently bound Spo11 from DSB ends [*Moreau S, et al., 1999*; *Neale MJ, et. al., 2005*]. Sae2 also exhibits nuclease activity [*Lengsfeld BM, et. al., 2007*]. However, the role of this nuclease in DSB end resection is not yet defined.

During mitosis in undamaged wild-type cells, the sister chromatids are separated after proteolytic cleavage of cohesin by the pulling forces of the centromere attached at mitotic spindle [*Uhlmann F, 2004*]. If the DNA damage checkpoint is activated, however, cohesins remain intact and the sister chromatids connected. Thus, cohesins can withstand the pulling forces of the mitotic spindle. Lobachev and colleagues reported that this pulling causes a separation of double-strand break ends in cells lacking Rad52 or a functional Rad50-Mre11-Xrs2 complex, but not in wild-type cells [*Lobachev KS, et. al., 2004*]. The ability of the Rad50-Mre11-Xrs2 complex to tether DNA ends lies in a zinc-hook at the tip of the coiled coil structure of Rad50, as a point mutation in the zinc-binding site disrupts tethering of ends similar to a complete deletion of any component of the Rad50-Mre11-Xrs2 complex [*Lobachev KS, et. al., 2004*]. Interestingly, mutation of Mre11 nuclease activity slightly improves the tethering in otherwise wild-type cells, suggesting that intact ends are tethered better than resected ends [*Lobachev KS, et. al., 2004*]. For Rad52, which forms heptameric rings that preferentially bind single-stranded DNA, tethering may occur by the interaction between rings bound to each DNA end [*Stasiak AZ, et. al., 2000; Ranatunga W, et. al., 2001*]. Alternatively, the role of Rad52 in tethering may be indirect, as it is a key component necessary for recruiting many of the later recombination proteins to repair centers [*Lisby M, et. al., 2004*]. Loss of Exo1, a 5′ to 3′ exonuclease, moderately reduces the rate of resection, but the more dramatic defect is observed only when both EXO1 and either the MRX complex or SAE2 are simultaneously deleted [*Clerici M, et. al., 2005; Llorente B and Symington LS, 2004*]. Importantly, gene conversion is still accomplished in *exo1*Δ *mre11*Δ cells, suggesting that additional enzymes are able to generate ssDNA at DNA breaks. Recently, Ira *et. al.,* colleagues identified two new factors involved in 5′ strand resection: Bloom and Werner syndromes helicase ortholog called Sgs1 and the Dna2 nuclease/ helicase. In the absence of Sgs1 or Dna2, resection is slow and depends on yet another nuclease: Exo1 [*Zhu Z, et. al., 2008*].

1. 7. CDK role in double strand break processing

The nucleolytic processing of 5′-to-3′ DNA ends require several factors like Mre11, Sae2, Dna2 and Exo1, this resection process also mediated through cell cycle dependent mechanism by CDK [*Mimitou EP and Symington LS, 2009*]. In *S. cerevisiae* effective resection of double strand break and homologous recombination require sustained CDK1/CyclinB kinase activity [*Aylon Y, et. al., 2004; Caspari T, et. al., 2002; Ira G, et. al., 2004*]. When yeast cells are arrested in G1 phase CDK has little or no resection by HO induced double strand break and in G2 arrested cells when over expressing the CDK1/Clb inhibitor, Sic1, affects the resection process [*Aylon Y, et. al., 2004; Ira G, et. al., 2004*]. Loss of adaptor protein Rad9 binding to H3-K79me leads to increased resection activity and partially bypasses the requirement for CDK activation of DSB processing [*Lazzaro F, et. al., 2008*] interestingly Rad9 has been shown to be phosphorylated by CDK1 [*Grenon M, et. al., 2007; Ubersax JA, et. al., 2003*] and this may modulate chromatin accessibility by nucleases. Cell cycle controlled DSB resection by the CDK phosphorylation in an evolutionarily conserved motif Sae2 protein. The *sae2-S267A* mutant is strong hypersensitivity to camptothecin, which is nearly as sensitive as the *sae2*Δ strain, defective in sporulation, reduced hairpin induced recombination, severely impaired DNA- end processing and faulty assembly and disassembly of HR factors. Furthermore, a Sae2 mutation that mimics constitutive Ser267 phosphorylation complements these phenotypes and overcomes the necessity of CDK activity for DSB resection [*Huertas P, et. al., 2008*]. Recently we published that the over expression of CDC5 which recognize substrates that have been previously phosphorylated by CDK delays the kinetics of DSB ends resection and reduce Rad53 phosphorylation [*Donnianni RA, et. al., 2010*].

1. 8. Triggering checkpoint activation

After the damages sites are processed nucleolytically to produce 3′ overhang ssDNA strand, much evidence suggests that the molecular species recognized by the Mec1-Ddc2 complex is ssDNA [*Costanzo V, et. al., 2003; Zou L and Elledje, 2003*]. Single strand DNA is formed during nucleotide and base excision repair and at stalled replication forks [*Carr AM, 2002; Sogo JM, et. al., 2002*]. In yeast there is a strong connection between the exposure of ssDNA at DSBs, at unprotected telomeres and activation of the DNA damage checkpoint [*Lee SE, et. al., 1998; Garvik B, et. al., 1995*]. When the 3′ single strand DNA exposed after the resection by MRX, Exo1 and an unknown nuclease, immediately ssDNA is coated by the replication protein A (RPA) [*Zou L and Elledge SJ, 2003*], which is formed during DNA replication and DNA repair [*Fanning E, et. al.,*

2006]. Although ATR is activated in response to many different types of DNA damage, including DSBs, base adducts, crosslinks and replication stress, a single DNA structure might be responsible for the activation checkpoint response. The Formation of 3′ ssDNA tails also determines the repair pathway switch from the nonhomologous end joining (NHEJ) to the HR pathway because NHEJ preferentially utilizes the unresected ends for ligation [*Ira G, et. al., 2004*].

1. 9. Histone modification in response to DNA damage

Eukaryotic genomes are packaged into chromatin, which creates a natural barrier against access to DNA during transcription, replication, repair and recombination. Extensive studies have revealed that the basic unit of chromatin, the nucleosome, is reconfigured dynamically during transcription by histone modification, histone variant incorporation, and ATP-dependent chromatin remodeling. The chromatin which harbors repeated nucleosome units each comprising an octamer of four histone proteins H2A, H2B, H3, and H4, and 147bp of DNA [*Luger K, et. al., 1997; Richmond TJ and Davey CA, 2003*]. These mechanisms have been implicated in chromatin alterations in response to DNA double-strand breaks and DNA repair [*Thiriet C and Hayes JJ, 2005; van Attikum H and Gasser SM, 2005*]. Upon different histone modifications, phosphorylation of all four histones play a primary role in DNA damage response by facilitating access of repair proteins to DNA breaks. One immediate target of the ATM/ATR kinase following DNA damage is the histone H2A-variant H2AX, in mammals is phosphorylated on serine 139 in somatic cells [*Redon C, et. al., 2002]* and in Budding yeast H2A is phosphorylated on serine 129 [*Downs JA, et. al., 2000]*. Following Spo11-induced DSB formation [*Mahadevaiah SK, et. al., 2001; Hunter N, et. al., 2001*], phosphorylated H2AX (γ-H2AX) appears within minutes of damage over large adjacent chromatin regions extending tens of kilobase in yeast and up to 2 Mb in mammalian cells [*Rogakou EP, et. al., 1999]*. Although γ-H2AX is central to the DDR, mutation of the H2AX phosphorylation site confers only a moderate sensitivity to DNA damage in comparison to alterations of DNA damage checkpoint proteins [*Celeste A, et. al., 2003; Altaf M, et. al., 2007*]. This suggests that γ-H2AX contributes to the process of DNA DSB repair; however, it is not essential since it is not required for the initial recognition of DNA breaks [*Celeste A, et. al., 2003*]. In addition to damage-induced H2AX phosphorylation, histone H4 phosphorylation at serine 1 by casein kinase 2 (CK2) occurs in response to DSBs, and serine 1 phosphorylation is reported to inhibit histone H4 acetylation by NuA4 acetyltransferase [*Cheung WL, et. al., 2005; Utley RT, et. al., 2005*]. These events may regulate restoration of chromatin structure following repair [*Utley RT, et. al., 2005]*.

The ATR- and ATM-mediated checkpoints maintain the integrity of the genome by arresting the cell cycle. These signaling pathways provide time for DNA repair prior to the onset of mitosis and thus prevent the catastrophic chromosomal breakage that can occur when damaged chromatids are segregated.

1. 10. Cell cycle arrest

The cell cycle block is effect by direct regulation of the cell cycle machinery following activation of the checkpoint signaling kinases. They can occur at all phase of the cell cycle, depending on the type of DNA lesions and the phase during which lesions appear. Cdc28 is part of an extensive genetic network of pathways involved in the maintenance of genome stability, and it cooperates with Homologous Recombination to prevent catastrophic mitotic progression after DNA replication arrest. During an unperturbed cell cycle, the Separase Esp1 promotes sister chromatid separation once its inhibitor, the securin Pds1, has been ubiquitinated by the Anaphase Promoting Complex (APC) assisted by the specificity factor Cdc20 and degraded [*Ciosk R, et. al., 1998*]. In the presence of DNA damage, Pds1 is hyperphosphorylated in a Mec1, Rad9 and Chk1 dependent manner, which blocks its ubiquitination and subsequent degradation, also essential for prevention of anaphase during spindle assembly checkpoint, where as Rad53 inhibits the interaction between Pds1 and Cdc20 [*Agarwal R, et. al., 2003; Sanchez Y, et. al., 1999*], which also block mitotic exit [*Sanchez Y, et. al., 1999*]. Rad53 phosphorylates Swi6, which inhibits the transcription of the *CLN1* and *CLN2* genes and the formation of the cyclin-dependent kinase (CDK)-cyclin complexes, this Cdc28- Cln1/2 required for the G1/S transition [*Sidorova JM and Breeden LL, 1997*]. This stabilizes the CDK inhibitor Sic1, which contributes to maintaining the G1 arrest by sequestering Cdc28-cyclin B complexes [*Wysocki R, et. al., 2006*]. Rad53 is required to maintain CDK activity during the checkpoint arrest and inhibits likely through Cdc5 [Chen *L, et. al., 1998; Sanchez Y, et. al., 1999*]. Cdc5 inhibits Bub2/Bfa1 complex which in turn inhibits the mitotic exit network (MEN) [*Geymonat M, et. al., 2003; Hu F and Elledge SJ, 2002; Hu F, et. al., 2001*]. Rad53 dependent inhibition of Cdc5 could therefore inhibit progression through mitosis and help maintain checkpoint arrest.

The spindle assembly checkpoint appears to contribute to cell cycle arrest following DNA damage in some situation. The Mad2 protein triggers pre anaphase arrest by inhibiting Cdc20 and stabilize Pds1 after microtubule damage. *mad2*Δ can also reduce cell viability and attenuate the DNA damage checkpoint in cells experiencing nucleotide depletion, DNA damaging agents, an

unrepaired DSB, or deprotected telomeres [*Aylon Y and Kupiec M, 2003; Barber PM and Rine J, 2002; Maringele L and Lydall D, 2002*].Whether these Mad2-dependent arrests reflect authentic activation of the spindle assembly checkpoint is not known. Alternatively, deletion of Mad2 may free up more Cdc20/APC to promote Pds1 ubiquitination and mitosis.

1. 11. How DNA Damage checkpoint inactivates?

A stress response consists in a series of targets modified by the components of the stress response pathway, themselves activated by a signal. Each component of the signal transduction cascade can be inactivated or its activation can be prevented by inhibitors. These inhibitors can be specific to the pathway or possess a large number of substrates like proteasome, and some phosphatases; they can act constitutively or be regulated by the stress response pathway itself or by other determinants depending on the phase of the cell cycle. DNA damage checkpoint inactivation can occur either after DNA damage has been repaired, as recovery or return to homeostasis or in the presence of persistent DNA damage as a mechanism of adaptation to DNA lesions. Inactivation of the DNA damage checkpoint response requires inhibitors inactivating the components or the targets of the checkpoint or preventing their activation.

1. 12. Checkpoint adaptation

If the double strand break is not repairable, yeast cells will eventually override the G2/M checkpoint and continue cell cycle despite the persistence of a break, in a process called ***"Adaptation"*** [*Sandell LL and Zakian VA, 1993*]. This process may provide opportunities for the cell to repair the damaged chromosome in a subsequent cell cycle, enhancing its chances for survival [*Galgoczy DJ and Toczyski DP, 2001; Lee SE, et. al., 2000]*. Several proteins are required for adaptation, and their mutation prevents Rad53 inactivation and cell-cycle re-entry. Many of these proteins function in chromatin regulation and recombination, such as Yku70 and Yku80, the Swi2/Snf2/Rad54 homolog Rdh54/Tid1, Rad51, the Srs2 helicase, and Sae2. Others have checkpoint or mitotic roles, such as the PP2Cfamily phosphatases Ptc2 and Ptc3, the CKII subunits Ckb1 and Ckb2, and the Polo kinase Cdc5 (*Clerici M, el. al., 2006; Lee SE, et. al., 1998; 2001; 2003; Leroy C, et. al., 2003; Pellicioli A, et. al., 2001; Toczyski DP, et. al., 1997; Vaze MB, et. al., 2002*). Ptc2 interacts with Rad53 forkhead-associated domains (FHA1 domain) of Rad53 and presumably inactivates Rad53 by dephosphorylation [*Leroy C, et. al., 2003*]. CKII phosphorylate Ptc2 to interact with Rad53 and promote cells to adapt. Ddc2-GFP foci are marinated during the entire checkpoint arrest in cells suffering from unrepairable DSB. At the time of adaptation these foci

show reduced intensity and disappear in many cases. In contrast, Ddc2-GFP foci do not dissociate but maintain intensity or brighten during and beyond adaptation [*Melo JA, et. al., 2001*]. For example the *sae2*Δ cells which frequently fail to adapt, maintain Rad53 phosphorylation in the presence of a single DSB either in the absence of Mec1 and Tel1. Following UV irradiation overexpression of Sae2 can override the checkpoint arrest in the presence or absence of Tel1 [*Clerici M, et. al., 2006*].

The adaptation defective *yku70*Δ cells are apparently the result of significantly increased resection at an unrepairable DSB and are comparable to that seen in cells resecting two DSBs at a normal rate [*Lee SE, et. al., 1998]*. Reducing this resection by deletion of Mre11 suppresses the *yku70*Δ adaptation defect, suggesting that the rate or extent of resection contributes to maintenance of the checkpoint signal and therefore to adaptation [*Lee SE, et. al., 1998]*. The important role of the DNA damage checkpoint is to prevent the segregation of broken or damaged chromosomes. Adaptation promotes the mis-segregation of centric chromosome fragments in as many as 95% of divisions, and the mis-segregation of even centric chromosome fragments is seen in 42% of divisions [*Kaye JA, et. al., 2004]*. This clearly leads to increased genomic instability as has been demonstrated for both chromosome loss and translocations [*Galgoczy DJ and Toczyski DP, 2001]*. Despite these phenotypes, adaptation is required for full viability of yeast cells in response to persistent DNA damage, suggesting that very slow or delayed repair of DNA damage, even after adaptation, aids cell viability [*Galgoczy DJ and Toczyski DP, 2001*].

1. 13. Checkpoint Recovery

When the DSB is repaired, cells turn off the checkpoint and resume cell cycle progression and continue their physiological program called ***"Recovery"***. Interestingly, some adaptation defective mutants, including *yku70*Δ, *rdh54*Δ, and *cdc5-ad*, are not defective in recovery. Other adaptation defective genes instead exhibit only a slow recovery such as *ckb1*Δ, *ckb2*Δ and *rad51*Δ [*Vaze MB, et. al., 2002*]**.** However, *srs2*Δ, *ptc2*Δ, *ptc3*Δ and *sae2*Δ cells have strong recovery defective, [*Clerici M, et. al., 2006; Leroy C, et. al., 2003; Vaze MB, et. al., 2002*]**.** Srs2 helicase shows the ability to remove Rad51 from ssDNA *in vitro*, and deletion of Rad51 substantially alleviates *srs2*Δ's recovery defect [*Vaze MB, et. al., 2002; Veaute X, et. al., 2003*]. One possibility is that Rad51 remains on DNA in *srs2*Δ mutant cells, even after successful repair, and promotes maintenance of the DNA damage checkpoint signal. In addition to Ptc2 and Ptc3 phosphatases, it was also shown that Pph3, a member of the highly conserved PP2A-*like* family, which acts in complex with Psy2

and YBl046w, is required to inactivate DSB-induced checkpoint by dephosphorylating the histone variant γH2A during recovery [*Keogh MC, et. al., 2006*]. Cells lacking any of these subunits have excess γ-H2AX even in the absence of DNA damage and show persistent γ-H2AX foci in irradiated cells. Additionally, the DNA damage checkpoint is significantly prolonged despite normal DSB repair [*Keogh MC, et. al., 2006*]. Studies in human cells have identified that the prolonged checkpoint with excess γ-H2AX is apparently due to defects in DNA repair [*Chowdhury D, et. al., 2005*].

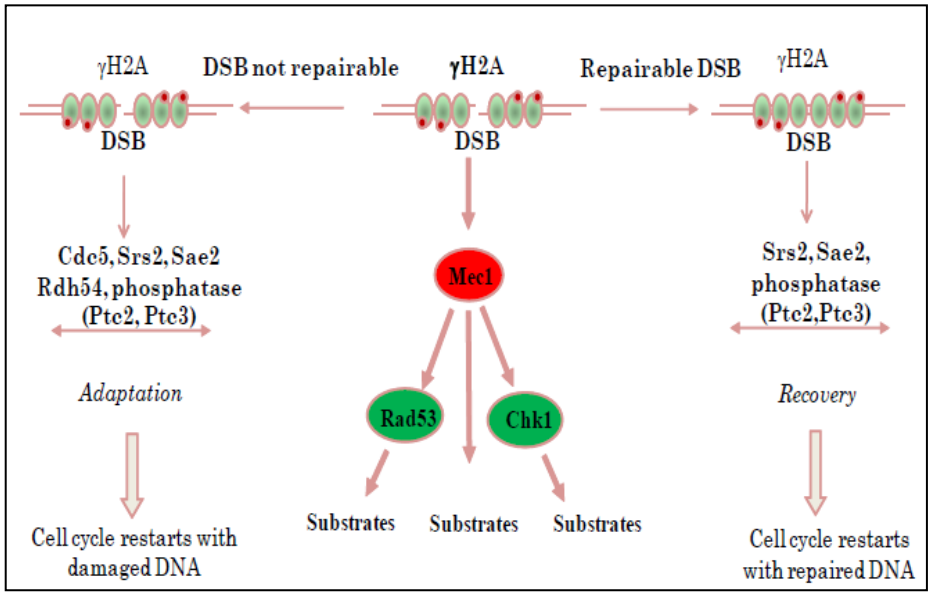

Figure 3. Schematic illustration of checkpoint activation and inactivation pathways in *Saccharomyces cerevisiae.*

2. Double Strand Break repair pathways (DSBs Repair)

All eukaryotic cells must regulate a balance between potentially competing DSB repair mechanisms. Genome stability is of primary importance for the survival and proper functioning of all organisms. In this, DNA double-strand breaks (DSBs) are critical lesions that can result in cell death or a wide variety of genetic alterations including large- or small-scale deletions, loss of heterozygosity, translocations, and chromosome loss. These phenomenons's arise spontaneously during growth, or can be created by external insults. In response to even a single DSB, organisms must trigger a series of events to promote repair of the DNA damage in order to survive and restore chromosomal integrity. The DSB repair pathways appear to compete for DSBs, but the balance between them differs widely among species, between different cell types of a single species, and during different cell cycle phases of a single cell type. The DSB repair pathways are generally classified as either homologous recombination (HR) or non-homologous end joining (NHEJ) in yeast and in the higher eukaryotes. The regulatory factors that regulate DSB repair by NHEJ and HR includes regulated expression and phosphorylation of repair proteins, chromatin modulation of repair factors accessibility and availability of homologous repair templates. The yeast *Saccharomyces cerevisiae* is an ideal model organism for studying these repair processes. Indeed, much of what we know today on the mechanisms of repair in eukaryotes comes from studies carried out in budding yeast. Many of the proteins involved in the various repair pathways have been isolated and the details of their mode of action are currently being unraveled at the molecular level.

2. 1. Non Homologous End Joining (NHEJ)

The NHEJ is a mechanism able to join DNA ends with no or minimal homology. NHEJ repairs DSBs at all stages of the cell cycle, bringing about the ligation of two DNA DSBs without the need for sequence homology, and so is error-prone, and its activity increases as cells progress from G1 to G2/M. In yeast, NHEJ is a minor pathway of DSBs repair, with a strong dominance of HR. Gene-knockout studies, using the budding yeast *S. cerevisiae*, have revealed that most components of NHEJ have been conserved between yeast and mammalian cells [*Critchlow SE and Jackson SP, 1998; Lewis LK and Resnick MA, 2000]*. DSBs with complementary overhangs 5' phosphate and 3' hydroxyl groups, such as those produced by nucleases can be precisely repaired by NHEJ. When ends cannot be precisely rejoined, NHEJ typically involves alignment of one or few complementary bases called "microhomology" to direct repair. The HO endonuclease has been used to create DSBs *in vivo* in chromosomes that lack homologous donor sequences. Here,

about 35% of cells survive HO cleavage [*Moore JK and JE Haber, 1996*]. These events are independent of *RAD52* epistatic group, but requires the Mre11/Rad50/Xrs2 (MRX) complex, the Rad1-Rad10 3'-flap endonuclease, Nej1 and Sae2 [*Lee K and Lee SE, 2007; Ma JL, et. al., 2003*]. Disruption of the *RAD50, MRE11* or *XRS2* genes impairs NHEJ in the same degree as disruption of *YKU70, YKU80 or DNL4*. However, recent studies, using the fission yeast *S. pombe* and vertebrates, suggest that end joining may be independent of this nuclease complex and that these enzymes are not conserved in all eukaryotes [*Harfst E, et. al., 2000; Manolis KG, et. al., 2001*]. NHEJ proceeds in stepwise manner beginning with limited end processing by MRX complex then ends binding by Ku comprising yKu70 and yKu80 subunits (**Figure 4**), and recruitment of the DNA – dependent protein kinase catalytic subunit (DNA-PKcs), forming the trimeric DNA-PK is activated, and it phosphorylate itself and other targets including RPA. In cells lacking ATM, DNA-PK can also phosphorylate histone H2AX. Finally Nej1 (XLF) interacts with DNA ligase IV (Lig4) with its binding partner Lif1 (XRCC4), required to ligate the end [*Schar P, et. al., 1997; Teo SH and Jackson SP, 1997*].

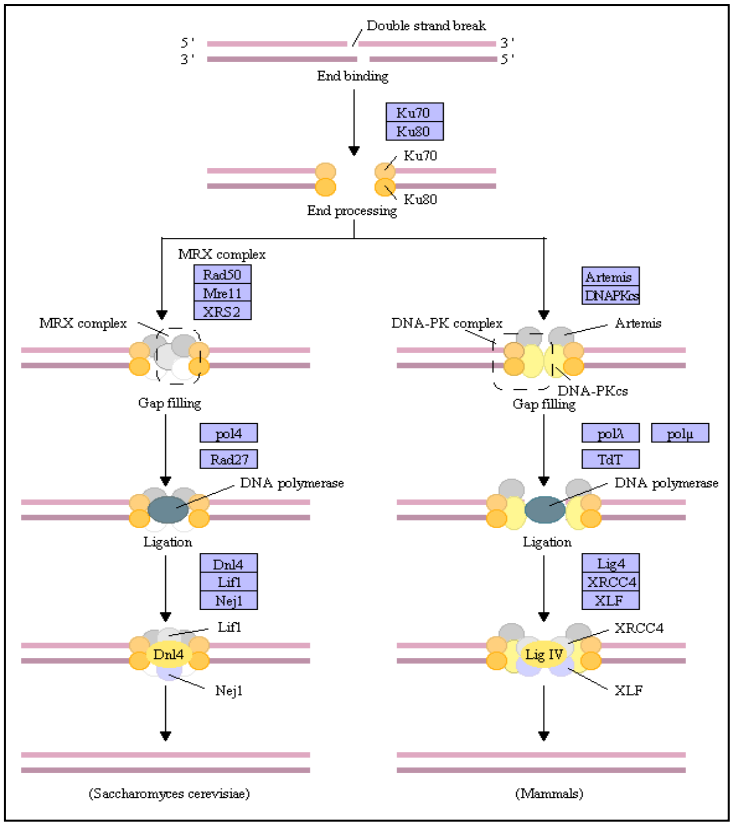

Figure 4. Non Homologous End Joining in Yeast and Human.

2. 2. Meiotic Recombination

Diploid eukaryotes produce haploid gametes through meiosis. During meiosis, a single round of DNA replication is followed by two successive rounds of nuclear division, meiosis I and meiosis II. Homologous chromosomes segregate at meiosis I, and sister chromatids separate at meiosis II. During prophase of meiosis I, homologs recognize each other and undergo high levels of genetic recombination. Reciprocal recombination is crucial for the formation of chiasmata, which are physical connections between homologs that ensure their correct segregation at the first meiotic division. The molecular mechanism of meiotic recombination has been well studied in budding yeast. The process starts with double strand breaks (DSBs) formed by the Spo11 protein, a type II topoisomerase homolog [*Keeney S, 2001*]. The DSB ends are degraded from their 5′ ends, giving rise to single strand DNA (ssDNA) [*Sun H, et. al., 1991; Bishop DK, et. al., 1992*]. This ssDNA is thought to be used for homology searching by strand exchange enzymes (recombinases). Eventually, the ssDNA invades homologous sequences in a non sister chromatid, giving rise to a single-end invasion intermediate and/or a double-Holliday junction [*Schwacha A and Kleckner N, 1994; Hunter N and Kleckner N, 2001*]. It has been proposed that crossovers are formed mainly by resolution of double-Holliday junctions [*Allers T and Lichten M, 2001]*. Budding yeast has two major recombinases Rad51 and Dmc1, both of which are homologs of the bacterial RecA protein [*Bishop DK, et. al., 1992; Shinohara A, et. al., 1992*]. Rad51 is involved in both mitotic and meiotic recombination, whereas Dmc1 is a meiosis-specific protein involved only in meiotic recombination. Rad51 forms a nucleoprotein filament [*Ogawa T, et. al., 1993*] and catalyzes pairing and exchange of strand between homologous DNA molecules [*Sung P, 1994*]. The Dmc1 protein promotes renaturation of complementary ssDNA and assimilation of homologous ssDNA into duplex DNA [*Hong EL, et. al., 2001*]. In the absence of these proteins, meiotic recombination is severely reduced or abolished, and the meiotic cell cycle is delayed because of the persistence of recombination intermediates. Rad51 and Dmc1 appear to perform overlapping as well as distinct functions in meiotic recombination [*Dresser M, et. al., 1997; Shinohara A, et. al., 1997*].

The localization of Rad51 on meiotic chromosomes is independent of Dmc1, whereas that of Dmc1 is largely dependent on Rad51 [*Bishop DK, 1994]*. Meiotic recombination is concurrent with the development of synaptonemal complex [*Roeder GS, 1997; Zickler D and Kleckner N, 1999*]. In preparation for SC formation, two sister chromatids of a single chromosome develop a common proteinaceous core, called an axial element. Within the context of SC, axial elements are referred to as lateral elements. Two lateral elements representing homologs are connected to each

other along their entire length by the central region of the SC. In budding yeast genetic studies have shown that the meiotic recombination machinery has the capacity to detect homology between sequences at ectopic locations almost as efficiently as allelic locations [*Jinks-Robertson S and Petes TD, 1985; Lichten M, et. al., 1987; Haber JE, et. al., 1991*]. Recombination mediated homology searching occurs throughout the entire genome. Fluorescence is situ hybridization (FISH) has demonstrated that some homolog pairing occurs even in the absence of recombination [*Loidl J, et. al., 1994; Weiner BM and Kleckner N, 1994; Nag DK, et. al., 1995*]. The reason that recombination proteins are often associated with meiosis [*Zickler D and Kleckner N, 1999*] is because meiosis needs pairing of the chromosomes before separation into daughter cells. Meiotic recombination requires these recombination repair proteins and some meiosis specific proteins (RAD54 homologue RDH54/TID1). Recombination is a 500-1000 fold increased as compared to mitotic cells [*Roeder GS, 1990*]. Recombination is initiated by breaks caused by meiosis specific endonucleases, rather than by breaks caused by DNA damaging agents. A second essential difference is that mitotic recombination (preferentially) uses the sister chromatid (the exact copy of the broken DNA molecule) while during meiotic recombination the homologous chromosome is used. Mutations resulting from this process are given to the offspring and therefore recombination is considered to be responsible for creating genetic diversity during evolution.

2. 3. Presynaptic Filament required for Homologous Recombination Process

Homologous recombination considered a more accurate mechanism of DSB repair because broken ends use homologous sequences elsewhere in the genome (sister chromatids, homologous chromosomes, or repeated regions on the same or different chromosomes) to prime repair synthesis. If the repair template is perfectly homologous, repair can be 100% accurate. The homologous recombination processes are mediated by genes of the *RAD52* epistasis group, which were first defined in the budding yeast *Saccharomyces cerevisiae*. HR maintains somatic genomic stability by promoting accurate repair of DSBs induced by ionizing radiation and other agents, repair of incomplete telomeres that arise when the enzyme telomerase is nonfunctional, repair of DNA interstrand crosslinks, and the repair of damaged replication forks. Mutations in genes encoding the enzymatic steps of HR result in extreme sensitivity to DNA-damaging agents such as ionizing radiation in model organisms such as *Saccharomyces cerevisiae* [*Game JC, et. al., 1974*]. Homologous recombination initiates with extensive 5′ to 3′ end processing at broken ends, in yeast is regulated by MRX, Exo1 and at least one other nuclease [*Krogh BO and Symington LS, 2004]*. The resulting 3′ ssDNA tails are bound by RPA, which is replaced with Rad51 in a reaction

mediated by Rad52 and two Rad51 paralogs, Rad55 and Rad57. The resulting Rad51 nucleoprotein filament searches for and invades a homologous sequence, a process facilitated by Rad54. The Srs2 helicase is thought to dissociate Rad51 from ssDNA, allowing normal base pairing of the invading and complementary donor strands and subsequent strand extension by DNA polymerase. The extended strand can dissociate and anneal with the processed end of the non-invading strand on the opposite side of the DSB in a process called synthesis-dependent strand annealing [SDSA] (**Figure 5**), or both ends may invade producing a double Holliday junction [dHJ] that is resolved to yield crossover [CO] or non-crossover recombinants [NCO] (**Figure 5**). Once intermediates are resolved, the remaining ssDNA gaps and nicks are repaired by DNA polymerase and DNA ligase. Most HR proteins are conserved through evolution (**Table 2**), although mammals harbor a more elaborate set. There are five paralogs in mammals (Rad51B/C/D and XRCC2/3) but just two in yeast (Rad55/57). During meiotic HR, the Rad51 homolog Dmc1 participates in strand exchange with Rad51, an association conserved from yeast to human (**Table 2**).

The Rad52 epistasis group recombinant mutants are defective in processes that involve the repair of naturally occurring DSBs such as those breaks made during mating-type switching and during meiosis [*Paques F and Haber JE. 1999*]. The first HR model for repair of a DSB was based on observations of transformation in yeast using linear plasmids that carried sequences homologous to yeast chromosomal DNA, which is called double strand break repair (DSBR) [*Orr-Weaver TL, et. al., 1983*]. HR is also required in DNA replication because many replication mutants and mutants in factors required for checkpoint activation when replication is stalled are dependent on HR genes for viability (*Lambert S, et. al., 2007)*. Cells up regulate HR during S and G2 phases of the cell cycle when sister chromatids are available. In fact, sister chromatids are the preferred template for HR repair in yeast and mammalian cells [*Dronkert MLG, et. al., 2000; Kadyk LC and Hartwell LH, 1992*]. This preference probably reflects a proximity effect mediated by the close association of sister chromatids from the time they form in S phase until they segregate in anaphase. Sister chromatid cohesion is mediated by cohesins, and recent evidence indicates that cohesins migrate to DSB repair sites independently of the normal replication cycle [*Watrin E and Peters JM, 2006*]. This finding suggests that replication checkpoints prevent HR at stalled or damaged forks by stabilizing the replication complex at the fork, thus avoiding the occurrence of HR-promoting or HR-like intermediates, also suggests that defective replication can result in HR-provoking intermediates, gaps at or behind the replication fork. Because HR at stalled replication forks can lead to genomic rearrangements, it might be expected that it would be tightly

controlled. In the case of collapsed replication forks, HR is used for one-ended strand invasion events using the sister chromatid to reconstruct the fork. This process may be promoted by sister chromatid cohesion complexes.

Human	*S. cerevisiae*	Biochemical Function	Additional features
Proteins that functions with Rad51			
MRN complex: Mre11-Rad50-Nbs1	MRX complex: Mre11-Rad50-Xrs2	DNA binding Nuclease activities	Involved in DNA damage checkpoint associated with DSB end resection
BRCA2	(None)	ssDNA binding Recombination mediator	Interacts with RPA, Rad51, Dmc1, PALB2, DSS1 member of the Fanconi anemia group
Rad52	Rad52	ssDNA binding and annealing Recombination mediator	Interacts with Rad51 and RPA
No human equivalent has been identified	Rad59	ssDNA binding and annealing	Interacts with Rad52 Homology to Rad52
Rad54 Rad54b	Rad54 Rdh54	ATP-dependent dsDNA translocase induced superhelical stress in dsDNA stimulates the D-loop reaction	Member of the Swe2/Snf2 protein family Chromatin remodeler Interacts with Rad51 The yeast protein remove Rad51 from dsDNA
Rad51B-Rad51C Rad51D-XRCC2 Rad51C-XRCC3	Rad55-Rad57	ssDNA binding recombination mediator activity (Rad55-Rad57 & Rad51B-Rad51C)	Rad51B-Rad51C and Rad51D-XRCC2 form a tertrameric complex Rad51C associates with a Holliday-junction resolvase activity
Hop2-Mnd1	Hop2-Mnd1	Stimulates the D-loop reaction stabilizes the presynaptic filament Promotes duplex capture	Interacts with rad51 and Dmc1
Proteins that function with Dmc1			
Hop2-mnd1	Hop2-Mnd1	Stimulates the D-loop reaction Stabilizes the presynaptic filament Promotes duplex capture	Interacts with Dmc1 and Rad51
No human equivalent has been identified	Mei5-Sae3	Predicted recombination mediator activity	Interacts with Dmc1 Likely functional equivalent of *S .pombe* Sfr1-Swi5
Rad54B	Rdh54	Stimulates the d-loop reaction	Interacts with Dmc1 and Rad51

Table 2. Homologous Recombination Factors [*San Filippo J, et. al., 2008*]

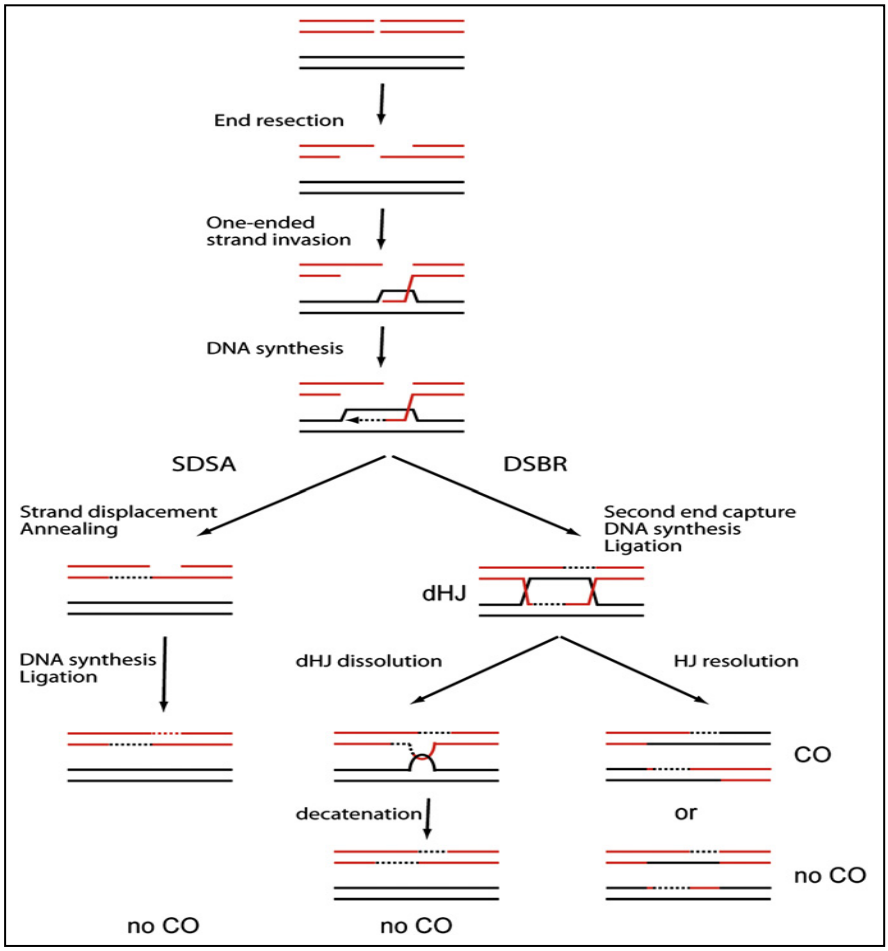

Figure 5. Model of Double Strand Break Repair by Homologous Recombination.

Recombination is initiated by nucleolytic processing of the DSB to generate 30 single-stranded tails that are rapidly covered with RPA. Rad51 recruitment displaces RPA leading to the formation of the presynaptic filament, which searches for an intact homologous template and then catalyzes invasion of the ssDNA into the donor molecule to form a D loop. The invading strand serves as a primer for DNA synthesis to extend the heteroduplex. Different pathways may further process this intermediate. The elongated invading strand can be displaced from the D loop and then anneals to the second end of the DSB (SDSA pathway). A new step of DNA synthesis and ligation leads to repair without production of CO. Alternatively, the capture of the second end results in the formation of a double Holiday Junction (dHJ). These intermediates can be subsequently resolved either by the dissolution pathway, which creates noncrossover products, or by endonucleolytic cleavage of the two Holliday junctions to generate CO or noncrossover products. [*Dupaigne P, et. al., 2008*].

2. 4. Crossovers

Crossovers are associated with a fraction of HR events and can have a stabilizing or destabilizing effect on the genome. In meiosis, crossovers are highly regulated such that at least one crossover occurs between each pair of homologous chromosomes to ensure proper chromosome segregation [*Champion MD and Hawley RS, 2002*]. In mitosis, crossovers pose serious risks of large-scale genome alterations: half of the G2 phase crossovers between homologs result in loss of heterozygosity from the point of the crossover to the telomere, and crossovers between repeated regions on non-homologous chromosomes, the same chromosome, or sister chromatids can result in translocations, inversions, deletions, and gene duplications [*Nickoloff JA, 2002*]. Defects in proteins that suppress mitotic crossovers, such as Sgs1 in yeast and its human homolog BLM, increase genome instability, and BLM defects also predispose to cancer [*Cheok CF, et. al., 2005; Myung K, et. al., 2001*]. Yeast mutants lacking key HR proteins (Mre11, Rad51, Rad52 and Rad54) are viable, but DSBs often go unrepaired and results in cell death in haploids. Diploids usually survive the loss of a broken chromosome, of a full chromosome complement can be retained fusing end invades the homologous chromosome and primer repair synthesis to the ends of the chromosome, a process called break induced replication that results in large scale loss of heterozygosity [*Signon L, et. al., 2001; Krishna S, et. al., 2007*]. In contrast, loss of key HR proteins in higher eukaryotes, including RAD51, BRCA1, and BRCA2, results in cell and/or embryonic lethality; viable mutants in these cases typically carry hypomorphic alleles or lethality is suppressed by p53 mutations. In other cases, as with mutations in higher eukaryotic HR proteins (e.g., RAD52 and RAD54), the HR defects are milder than those of the corresponding yeast mutant [*Dronkert MLG, et. al., 2000; Essers J, et. al., 1997; Bezzubova O, et. al., 1997*], although it has been argued that these differences may reflect changes in the functions of these proteins through evolution [*Sonoda E, et. al., 2006*].

2. 5. Gene Conversion

Gene conversion (GC) is defined as a nonreciprocal transfer of genetic information from one molecule to its homologue. Usually this occurs between two alleles of a gene however, gene conversions can embrace many contiguous genes, including the entire distal part of the chromosome arms. Gene conversions were initially defined in meiosis, where one could observe non-Mendelian segregation of alleles. In meiosis, gene conversion tracts are on average 1 to 2 kb (*Sun Z, et. al., 1996*). In mitosis, some gene conversions cover very short distances [*Nelson HH, et.*

al., 1996] while other extends for hundreds of kilobases. GC is often associated with crossing over. However, some crossovers will not be associated with a detectable gene conversion, either because the interval where crossing over occurs does not contain allelic differences between the homologous sequences or because intermediates that could give rise to a gene conversion can also be restored, with no detectable change in genotype. The proportion of gene conversions that are accompanied by crossing over is much greater in meiosis than in mitosis. In mitosis, only a relatively small fraction of gene conversions are crossover associated, ranging from almost 0% to about 20%. If DSB occur at the 3′ ends of both meiotic and mitotic DSBs are not resected, while the 5′ ends of the DNA can be chewed back for very long distances, often more than 1 kb [*Lee SE, et. al., 1998*]. The 3′ ends are presumed to invade an intact homologous template in a manner similar to the way RecA-catalyzed strand exchange occurs in *E. coli* [*Kowalczykowski SC, et. al., 1994*]. The 3′ ends of the invading strands can then act as primers for the initiation of new DNA synthesis. This process would lead to the formation of two Holliday junctions (HJs), four stranded branched structures whose alternative resolution allows the formation of the crossover products. HJs can be cleaved by a resolvase by cutting either the two non-crossed strands or the two crossed-strands. An equal number of crossovers and non-crossovers would be predicted if HJs were resolved randomly.

2. 6. Break Induced Replication

Break-induced replication (BIR) is a nonreciprocal recombination-dependent replication process that is an effective mechanism to repair broken chromosomes. This plays key role in maintaining genome integrity, including restarting DNA replication at broken replication forks and maintain telomeres in the absence of telomerase [*Kraus E, et. al., 2001*]. In addition to its putative role to restart collapsed replication forks in eukaryotes, BIR is envisioned to elongate telomeres that are lost when telomerase is absent or when telomeres are uncapped [*McEachern MJ and Haber JE, 2006*]. The DSB ends are processed nucleolytically which is similar to the resection that occurs in other DSB HR repair events. The single-strand tail then invades a homologous DNA sequence, often the sister chromatid or homologous chromosome but sometimes a repeated sequence on a different chromosome. The invading end is used to copy information from the invaded donor chromosome by DNA synthesis. The strand invasion step of BIR requires the HR proteins like Rad51, Rad52, Rad54, Rad55 and Rad57 [*Davis AP and Symington LS, 2004*]. When the sister chromatid or homologous chromosome is used, the repair is accurate. When a repeated sequence on a non homologous chromosome is engaged to initiate repair the result is a

nonreciprocal translocation. About 72-bp of homology at one side of an HO-induced DSB is sufficient for BIR, suggesting small homologies can be used [*Bosco G and Haber JE, 1998*]. In another study selecting for gene duplications in haploid yeast, the break point junctions for segmental duplications had no homology or microhomologies, and were independent of *RAD52*, suggesting a non-HR mechanism.

2. 7. Single Strand Annealing

Single strand annealing is a process that is initiated when a double strand break is created between two repeated sequences oriented in the same direction. Single stranded regions are created adjacent to the breaks that extend to the repeated sequences such that the complementary strands can anneal to each other. This annealed intermediate can be processed by digesting away the single stranded tails and filling in the gaps (**Figure 6**). Sometimes a DSB is closely flanked by direct repeats. Indeed, this situation is frequent in higher eukaryotes genomes, which are rich of moderately and highly repeated sequences. This DNA organization provides the opportunity to repair the DSB by a deletion process using the repeated DNA sequences, called single-strand annealing (SSA) [*Fishman-Lobell J, et. al., 1992*]. In the SSA process, the DSB ends are resected and annealed to each other. The process is finished by nucleolytic removal of the protruding single-strand tails, and results in deletion of the sequences between the direct repeats and also one of the repeats. Since strand invasion is not involved, SSA is independent of strand invasion and HJ resolution factors [*Symington LS, 2002*].

SSA probably accounts for most of the spontaneous recombination events that are often call pop-out recombination. SSA has provided a useful assay system to probe aspects of chromosome structure. To explore whether chromosomes lie in separate territories in the nucleus, Haber and Leung created a strain in which two HO-induced DSBs on two different chromosomes could be repaired by competing SSA events: either by two intrachromosomal annealings (creating two deletions) or by two interchromosomal events (creating a pair of reciprocal translocations). Surprisingly, the interchromosomal events were as frequent as the intrachromosomal deletions. This argues that each DSB end could search the entire genome for a partner during SSA mechanism [*Haber JE and WY Leung, 1996*].

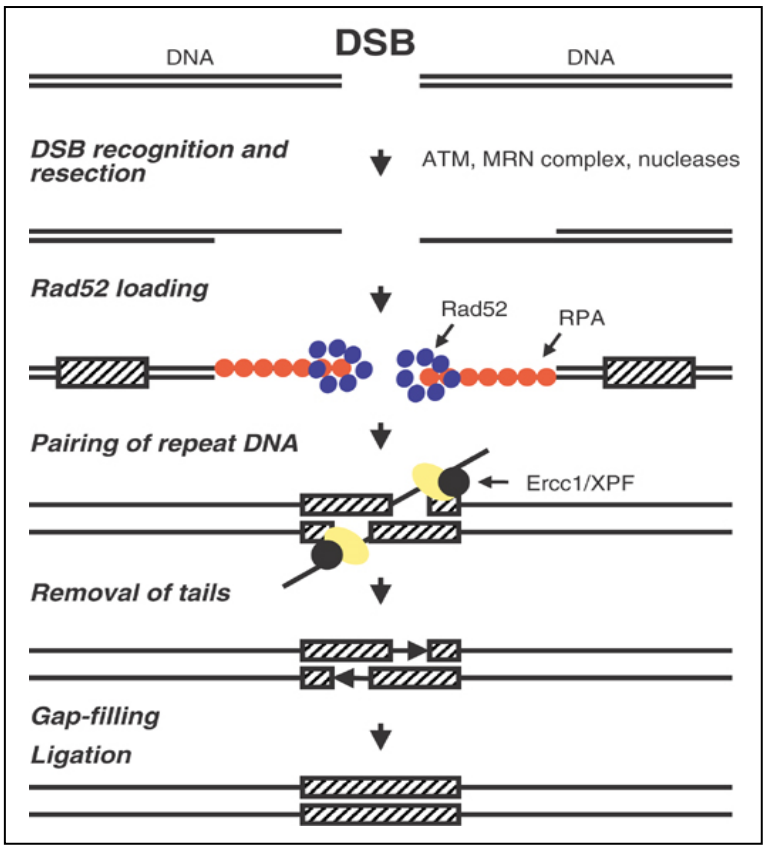

Figure 6. Single Strand Annealing Mechanism.

SSA could occur when, at a DSB, the DNA strand is resected by a nuclease, for example, MRE11 to leave ssDNA overhangs. The length of the overhangs and the extent of homology ranging from microhomologies to several hundred bases or longer most likely determine how SSA is executed. In the case of long homologies between direct repeats on the overhangs, RPA and RAD52 are necessary for facilitating DNA pairing followed by removal of the tails by ERCC1/XPF nuclease and gap filling by DNA polymerase as shown in the figure [*Valerie K and Povirk LF, 2003*].

3. SWI/SNF complex

The destabilization of nucleosome structures, to facilitate the binding of transcription factors to chromatin requires number of genes called SWI and SNF. SWI refers to yeast mating type switching, while SNF is an abbreviation for Sucrose Non-Fermenting. These genes did not appear to encode sequence specific DNA binding proteins but were required to achieve the proper amount of transcription from a limited number of promoters. One of the SWI genes, SWI2 was found to be identical to one of the SNF genes, SNF2 and hence, this gene is referred to as SWI2/SNF2. SWI/SNF complex possesses a DNA stimulated ATPase activity and can destabilize histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner. The SWI2/SNF2 polypeptide contains the characteristic seven conserved protein motif and were labeled sequentially I, Ia, II, III, IV, V and VI, that are present in a large and rapidly growing group of nucleoside triphosphate (NTP)- binding proteins that include DNA and RNA helicases [*Gorbalenya AE and Koonin EV, 1993; Eisen JA, et. al., 1995*]. Motifs I and II are the Walker A and B nucleotide-binding motifs commonly found in ATP-hyrdolyzing enzymes. Proteins containing the helicase motifs are subdivided into several superfamilies on the basis of similarity. The helicase-like enzymes link ATP hydrolysis to a directed change in the relative orientation of these domains [*Singleton MR and Wigley DB, 2002*]. This enzymatic process has been suggested to represent one application of a more general mechanism used in many proteins containing a recA-like domain [*Ye J, et. al., 2004*]. Proteins with a helicase-like region of similar primary sequence to *Saccharomyces cerevisiae* Snf2p comprise the Snf2 family within SF2 (**Figure 7A**). Many of the first identified Snf2 family members were ATPases within chromatin remodeling complexes and it was recognized that the presence of a core polypeptide related to Snf2p is a defining property of ATP-dependent chromatin remodeling [*Becker PB and Horz , 2002*]. It is now apparent that the Snf2 family comprises a large group of ATP-hydrolysing proteins that are ubiquitous in eukaryotes, but also present in eubacteria and archaea. A subset of Snf2 family proteins act as ATP dependent DNA translocases (*Saha A, et. al., 2002; Whitehouse I, et. al., 2003; Jaskelioff M, et. al., 2003; Xue Y, et. al., 2003*]. Some of these proteins have also been found to be capable of generating unconstrained super helical torsion in DNA Neighbour-joining trees from multiple alignments of the set of 1306 sequences revealed a well defined branching structure (**Figure 7B**) and enabled their assignment to 24 distinct subfamilies (**Table 3**). Many Snf2 family proteins are part of larger multi-protein complexes. Accessory motifs within these complexes are also likely to adapt the function of Snf2 motors for different purposes.

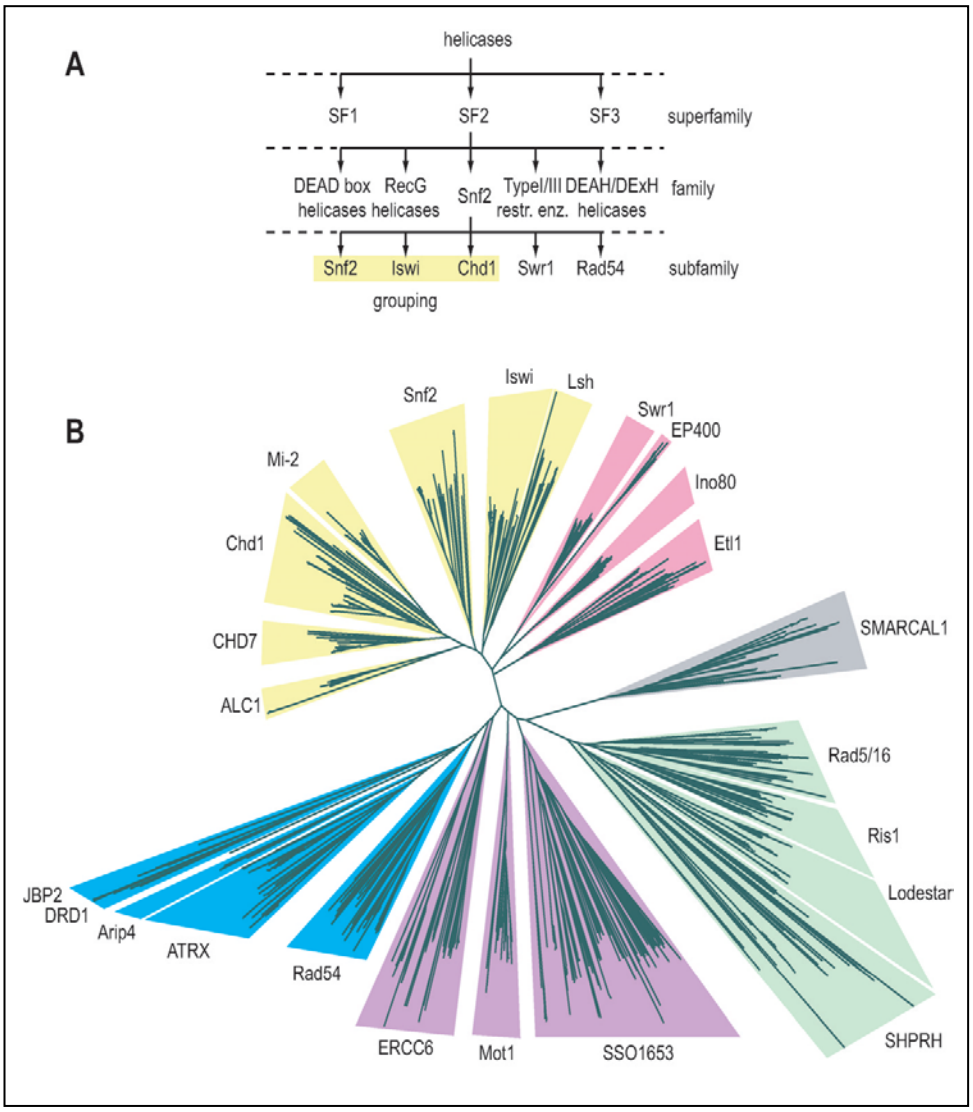

Figure 7. Tree view of Snf2 family.

(A) Schematic diagram illustrating hierarchical classification of superfamily, family and subfamily levels. (B) Unrooted radial neighbour-joining tree from a multiple alignment of helicase-like region sequences excluding insertions at the minor and major insertion regions from motifs I to Ia and conserved blocks C–K for 1306 Snf2 proteins identified in the Uniref database. The clear division into subfamilies is illustrated by wedge backgrounds, colored by grouping of subfamilies. [*Flaus A, et. al., 2006*].

Sub Families	Archetype gene	Assigned from uniref	Other names associated with members
Snf2	*S.cerevisiae* SNF2	117	Snf2p, Sth1p, snf21, SMARCA4, BRG1, BAF190, hSNF2beta, SNF2L4, SMARCA2, hBRM, hSNF2a, SNF2L2, SNF2LA, SYD, splayed, psa-4, brahma
Iswi	*D.melanogaster* Iswi	83	Isw1p, Isw2p, SMARCA1, SNF2L, SNF2L1, SNF2LB, SMARCA5, hSNF2H
Lsh	*M.musculus* Hells	35	YFR038W, SMARCA6, HELLS, LSH, PASG, DDM1, cha101
ALC1	*Homo sapiens* CHD1L	19	SNF2P
Chd1	*M.musculus* Chd1	96	CHD2, CHD-Z, hrp1, hrp3
Mi-2	*H.sapiens* CHD3	88	CHD3, Mi-2a, Mi2alpha, ZFH, PKL, pickle, CHD4, Mi-2b, Mi2beta, let-418, CHD5
CHD7	*H.sapiens* CHD7	53	CHD6, RIGB, KISH2, Kis-L, kismet, CHD8, HELSNF1, DUPLIN
Swr1	*S.cerevisiae* SWR1	44	SRCAP, Snf2-related CBP activator protein, dom, domino, PIE
EP400	*H.sapiens* EP400	27	E1A binding proten p400, TNRC12, hDomino
Ino80	*S.cerevisiae* INO80	34	
Etl1	M.musculus Smarcad1	44	SMARCAD1, hHEL1, Fun30p, snf2SR
Rad54	*S.cerevisiae* RAD54	76	Rad54l, hRAD54, RAD54A, **Rdh54p**, RAD54B, Tid1, okr, okra, mus-25
ATRX	*H.sapiens* ATRX	52	XH2, XNP, Hp1bp2
Arip4	*M.musculus* Srisnf2l	23	ARIP4
DRD1	*Arabidopsis thaliana* DRD1	12	
JBP2	*T.brucei* JBP2	4	
Rad5/16	*S.cerevisiae* RAD5, RAD16	61	rhp16, rad8, SMARCA3, SNF2L3, HIP116, HLTF, ZBU1, RNF80,RUSH-1alpha, P113, MUG13.1
Ris1	*S.cerevisiae* RIS1	35	
Lodestar	*D.melanogaster* Lodestar	40	LDS, TTF2, hLodestar, HuF2,factor 2
SHPRH	*H.sapiens* SHPRH	44	YLR247C
Mot1	*S.cerevisiae* MOT1	45	TAFII170, TAF172, BTAF1, Hel89B
ERCC6	*H.sapiens* ERCC6	71	rad26, rhp26, CSB, csb-1, RAD26L
SSO1653	*S.solfataricus* SSO1653	149	SsoRad54like
SMARCAL1	*H.sapiens* SMARCAL1	54	HARP, DAAD, ZRANB3, Marcal1

Table 3. SNF2 subfamilies [*Flaus A, et. al., 2006*]

Listing subfamily name from prevailing protein name for first characterized member, archetype organism and official gene name, number of subfamily members identified in Uniref, and a non-exhaustive list of alternative names for archetype and other subfamily members.

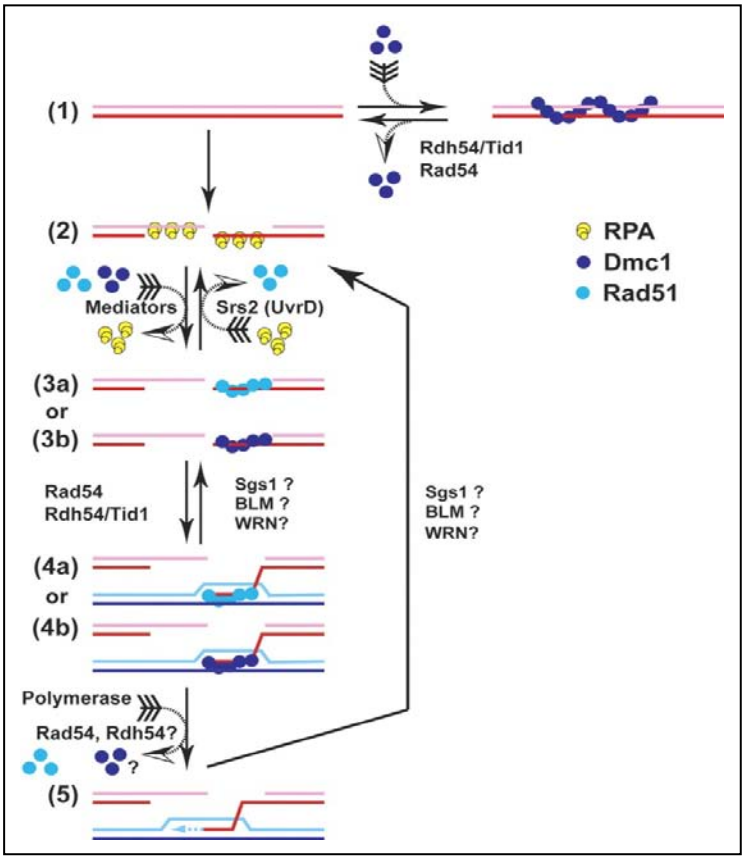

Figure 8. Intermediates during homologous recombination. [*Symington LS and Heyer WD, 2006*].

DNA-based motor proteins, helicases (Srs2, UvrD, Sgs1, BLM, WRN) and dsDNA translocases (Rad54, Rdh54/Tid1), have the ability to dissociate protein–DNA complexes and junction intermediates during homologous recombination (**Figure 8**). Rdh54/Tid1 (and Rad54?) dissociates Dmc1 from dsDNA to feed a pool of free Dmc1 protein. Free Dmc1 and Rad51 protomers are utilized for assembly into ssDNA filaments during DSB-induced recombination. The DNA helicases Srs2 and UvrD catalyze the backward reaction of dissociating the Rad51–ssDNA and RecA–ssDNA filaments, respectively. It has not been tested whether Srs2 can dissociate Dmc1– ssDNA filaments. Rad54 and Rdh54/Tid1 facilitate D-loop formation by Rad51 and can also dissociate the Rad51– dsDNA filament, which is consistent with a function after DNA strand invasion. A similar function is envisioned by [*Holzen TM, et. al., 2006*], for Rdh54/Tid1 dissociating the Dmc1–dsDNA product complex. Sgs1, BLM, and WRN heli- cases are candidate proteins to dissociate nascent D-loops or extended D-loops in a back reaction after initial synapsis.

3. 1. Recombination factor Rdh54/Tid1 (Rad54 homologue in yeast)

The *Saccharomyces cerevisiae* RDH54/Tid1 (YBR073W) was found during yeast genome sequencing project, homologue to Rad54 in yeast and Rad54B in Human, which is located on chromosome II, has a molecular weight of about 110KDa. ORF shares 34% identity with the yeast Rad54 protein (**Figure 9**) [*Van Der Aart QJM, et. al., 1994; Eisen JA, et. al., 1995*]. Rdh54 is a key member of evolutionarily conserved RAD52 epistasis group [*Paques and Haber JE, 2000; Sung P, et. al., 2000*], which is thought to be involved in a late step(s) of recombination [*Shinohara and Ogawa, 1995; Bai Y and Symington LS, 1996*].

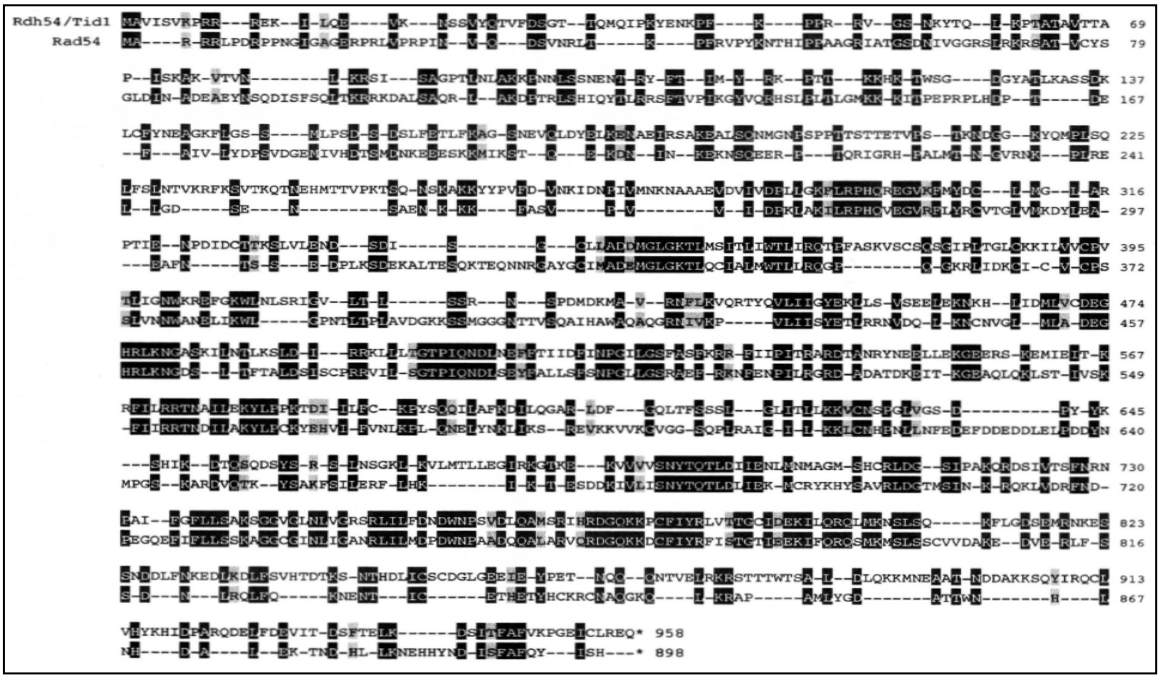

Figure 9. Amino acid comparison of Rad54 and Rdh54/Tid1 protein.
The upper and lower sequences represent the amino acid sequences of the Rdh54/Tid1 and the Rad54 protein, respectively. The gaps were introduced to maximize homology. Identical amino acids are shared, and homologous amino acids are lightly shared. The following groups of amino acids were taken as homologous: D and E; N and Q; S and T; K and R; A, L, M and V; F, H, I, Y and W. [*Shinohara M, et. al., 1997*].

The Rdh54 possess characteristic features of the Swi2/Snf2 family of proteins contain sequence motifs has characteristic of Sf2 helicase but do not have the helicase activity. Instead they couple ATP hydrolysis with the generation of super helical torsion in DNA [*Thoma NH, et. al., 2005*]. Rdh54 found to be essential for a variety of chromosomal transactions [*Eisen JA, et. al., 1995*] but has no role in the normal growth of yeast cells.

3. 2. Biological function of Rdh54 requires ATPase activity

Previous studies shows the Swi2/Snf2 superfamily member Rdh54 protein posses a DNA dependent ATPase activity because the highly conserved Walker type A type motif present which is lysine residue at 318 position of the amino acid sequence is important for the ATPase activity (**Figure 10C**) studies from the *in vitro* purification of Rdh54 protein shows that in the absence of dsDNA there is no ATP hydrolysis occur (**Figure 10D**), and the dsDNA translocase activity of Rdh54 protein is fueled by ATP hydrolysis [*Petukhova G, et. al., 2000*]. The necessary of Rdh54 ATPase activity is different from its biological functions such as modify the DNA topology and enhance D-Loop formation by the Rad51 recombinase, accelerates the rate at which DNA strand are exchanged during HR reaction, and process branched DNA intermediates, including the Holliday structure that are formed during HR as discussed in the previous chapters.

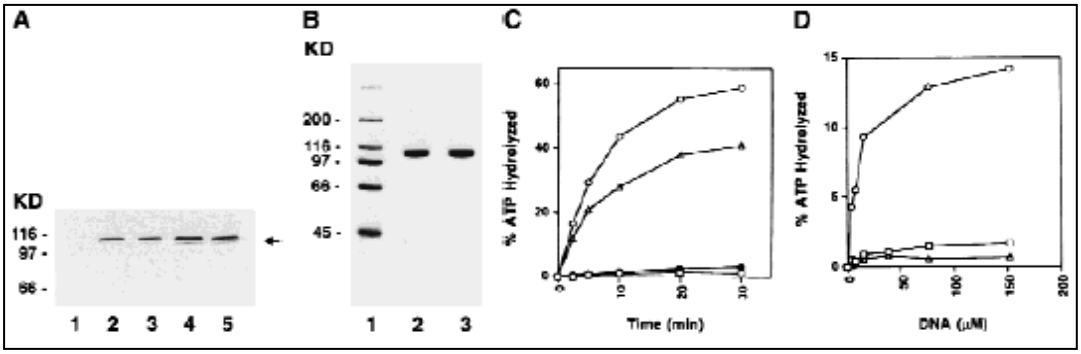

Figure 10. Purified Rdh54 and rdh54K351R possess DNA dependent ATPase activity.
A). Immuno blot analysis of purified 2μ Rdh54 and 2μ *rdh54 K352R* proteins. B) Purified Rdh54 [lane 2] and *rdh54K352R* [lane 3] proteins stained with coomassive blue. C & D shows the percentage of ATP hydrolysis activity Rdh54 in the presence of dsDNA. [*Petukhova G, et. al., 2000*].

3. 3. Role in D-Loop formation

In the initial steps of homologous recombination, exonucleases process broken DNA to generate single-stranded DNA (ssDNA) tails, then Rad51 protein [*Bianco PR, et. al., 1998*] binds these tails to form the Rad51 nucleoprotein filament, which searches for the homologous DNA template and promotes formation of joint molecules also called D-loops. In addition to establishing stable linkage among the recombining DNA molecules, D-loop formation is critical for priming DNA synthesis to replace the genetic information eliminated during end-processing

of DNA double-strand breaks (*Paques F and Haber JE, 1999; Sung P, et. al., 2000*). Main function of D-loop restores the original marker or incorporates information from the transforming fragment into the chromosomal locus. Rad51 inefficiently promotes D-loop formation between single-stranded oligonucleotides and homologous supercoiled DNA [*Mazin AV, et. al., 2000*].

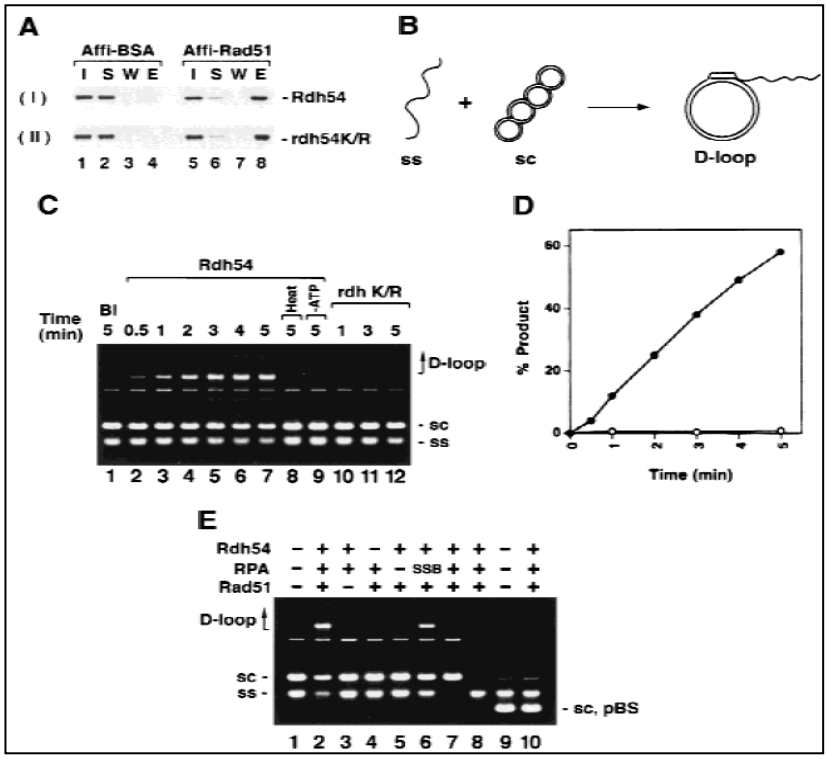

Figure 11. Promotion of D-loop formation.

A). Physical interaction of Rdh54 with Rad51 in Affi-Gel 15 beads bearing bovine serum albumin (Affi-BSA) or Rad51 (Affi-Rad51). B) Schematic diagram of D-loop reaction. C & D) ATP hydrolysis dependent promotion of D - loop formation by Rdh54 (lane 2-7). E) Rad51 dependent (lane 6) formation of D-loop reaction by Rdh54. [*Petukhova G, et. al., 2000].*

The D-loop formed by strand invasion is able to pair with the other side of the DSB, and the 3′ end of the non invading strand is also extended by DNA synthesis, forming a double-Holliday-junction (dHJ) intermediate. Random resolution of the two Holliday junctions is expected to yield equal numbers of crossover and noncrossover products. Two hybrid studies by *Dresser, et. al.,* in 1997 shows Rdh54 interacts with Rad51 and Dmc1 protein. The formation of D-loop requires Rad51 protein, in reaction mix Rad51, Rdh54 and the ssDNA binding protein RPA, an important cofactor for the Rad51recombinase activity [*Sung P, 1994; Sugiyama T, et. al., 1997*]. The unconstrained negative supercoils generated by Rdh54 could render strand invasion by the

Rad51-ssDNA nucleoprotein complex more facile because DNA strand separation in duplex DNA is favored by negative supercoiling. In fact, with the ability to supercoil DNA, even a topologically relaxed DNA template is efficiently used for D-loop formation. Rdh54 modifies DNA topology and generates positive and negative supercoils, the level of D-loop is stimulated by negative supercoiling which are important for the D-loop formation [*Petukhova G, et. al., 2000; Chi P, et. al., 2006*]. Interestingly the N-terminal part which conserved among its orthologues is required for the D-loop formation by interacting with HR factors like Rad51 [*Kwon Y, et. al., 2008*]. Hence Rdh54 is required for Rad51 dependent D-loop formation in recombination process.

3. 4. Chromatin remodeling activity

As described in chapter I section 1.9 the genomic DNA in eukaryotic cells is organized into chromatin, which harbors repeated nucleosome units. The folding of DNA on the surface of histone proteins within the nucleosome inevitably poses an accessibility problem during various DNA transcriptions, including DNA replication, recombination and repair. To overcome this structural hindrance is mediated by ATP hydrolysis dependent chromatin remodeling process, such that remodeling proteins render DNA more accessible by weakening DNA-Histone contacts, sliding nucleosomes along the DNA, or removing H2A-H2B dimmers from the nucleosome [*Becker PB and Horz W, 2002*]. Chromatin remodeling generally refers to a discernable change in histone-DNA interactions in a nucleosome. Chromatin remodeling factors have been observed to catalyze the mobilization and repositioning of nucleosomes, the transfer of a histone octamer from a nucleosome to a separate DNA template and the facilitated access of nuclease to nucleosomal DNA, the creation of dinucleosome-like structures from mononucleosomes, and the generation of super helical torsion in DNA. [*Becker PB and Horz W, 2002; Varga-Weisz P, 2001; Flaus A, et. al., 2001*]. Apart from alteration of histone-DNA contacts, chromatin remodeling factors have been found to function in other chromatin related processes such as facilitating transcription from chromatin templates, catalyze the assembly of periodic nucleosome arrays and participate in homologous strand pairing [*Jaskelioff M, et. al., 2003; Alexiadis V and Kadonaga JT, 2002; Leroy G, et. al., 2000; Tsukiyama T, el. al., 1995].* Because of its relatedness to Swi2/Snf2 chromatin remodeler the Rdh54 has ATP hydrolysis dependent chromatin remodeling activity, *in vitro* studies shows *rdh54K318R* mutant lacking ATPase activity is defective in chromatin remodeling (**Figure 12B and C**). The N-Terminal part which is required for the D-loop formation by interacting with Rad51 also required for the chromatin remodeling activity also ablates the

ability of Rdh54 to repositioning mononucleosome [*Kwon Y, et. al., 2008*], whereas the *Sc*Rad54 protein do not have much impact in chromatin remodeling [*Raschle M, et. al., 2004*].

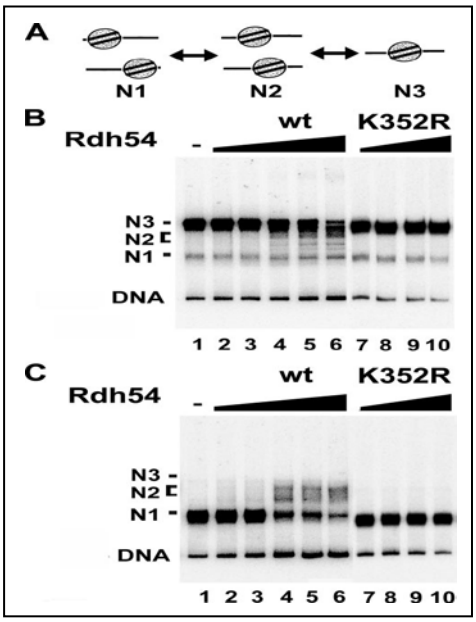

Figure 12. Nucleosome mobility by Rdh54. [*Kwon Y, et. al., 2008*].
Nucleosome sliding assay with substrates that harbor a mononucleosome with Rdh54 in the presence of ATP led to the generation of multiple novel mononucleosome species (N2) and significant amount of free DNA (**Figure 12B and C**). Restriction enzyme accessibility assay by *Kwon, et. al., 2008,* shows that Rdh54 mediated nucleosome repositioning is depending on ATP hydrolysis. Previous study have show that Rad51 stimulates ATPase, DNA supercoiling and DNA strand opening activity of Rdh54 [*Chi P, et. al., 2006*]; interestingly chromatin remodeling activity of Rdh54 protein is independent of Rad51. [*Kwon Y, et. al., 2008*]. Rdh54 is recruited to the DNA in a site specific HO endonuclease induced double strand break by Rad51 and Rad52 dependent manner.

3. 5. Role in Adaptation

Yeast cells when suffer with a single unreparable double strand break arrest the cell cycle progression prior to anaphase to give more time for cells to completely repair the damaged DNA, and the cell cycle arrest is mediated through the cascade of protein kinases. However the arrest is not permanent, cells adapt, escape from G2/M arrest despite the continued presence of the broken chromosome. As mentioned previously the Rdh54 is not an essential gene required for

normal growth of yeast cells, the absence of Rdh54p enhance the mitotic recombination defective in *rad54*Δ cells, but Rdh54 plays much more important role in meiotic recombination by interacting with meiotic specific strand exchange protein Dmc1 as previously discussed in this thesis. The JKM179 derivative of *rdh54*Δ cells when respond to HO endonuclease induced DSB at MAT locus cannot be repaired by homologous recombination as a result of which they block in G2/M cell cycle stage with >90% of rdh54Δ cells at the 2 -cell containing undivided nucleus, this permanent arrest phenotype is depends on a functional DNA damage arrest checkpoint system, as *rad9*Δ *rdh54*Δ double mutant did not show a significant G2/M arrest (**Figure 13**) [*Lee SE, et. al., 1998*].

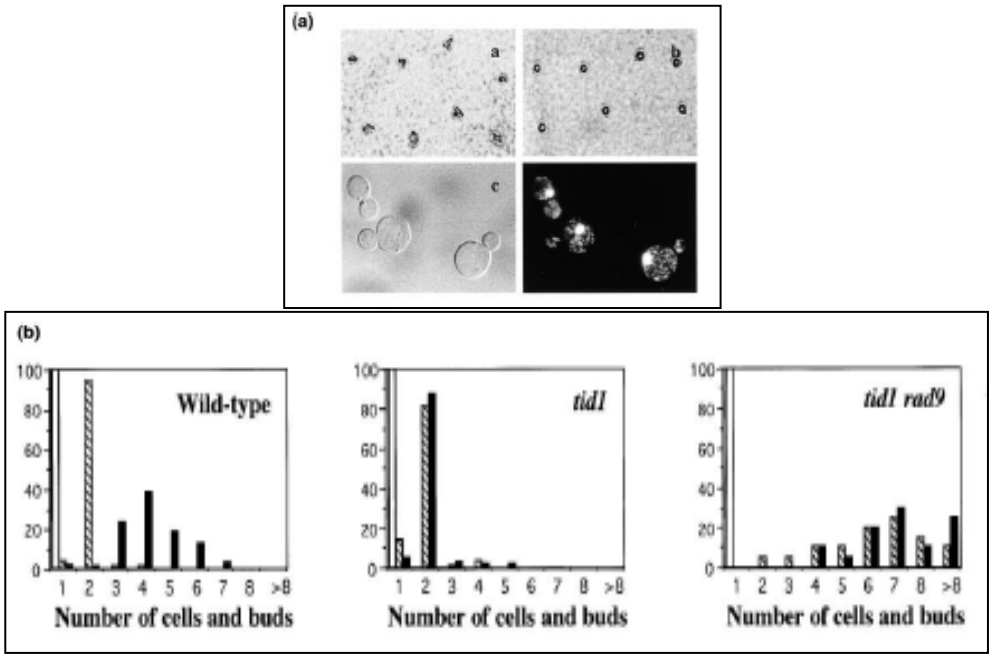

Figure 13. Rdh54 delta cells arrest cell cycle progression in response to single unrepaired DSB.

In response to HO induced unreparable DSB, phosphorylation and kinase activity of Rad53p is increased dramatically after approximately 1-2 hours but after 6-8 hours of HO induction the Rad53p activity peak declined when cells adapt, *rdh54*Δ when respond to such induction they persist high kinase activity and hyperphosphorylation Rad53p for almost 24 hours (**Figure 14**) [*Lee SE, et. al., 2001*]. When *rdh54*Δ cells combined with several recombination mutants such as *rad51*Δ, *rad52*Δ *and rad54*Δ does not alter its adaptation to DSB indicating that the *rdh54*Δ defective in adaptation is distinct from its interaction with its major mitotic recombination

proteins, which suggest that Rdh54p acts in an important cellular process, which is distinct from its several role in recombination [*Lee SE, et. al., 2001*]. Also because Rdh54 localizes to kinetochores even in the absence of recombination proteins, reflects its function during adaptation by communicating between the DNA damage and spindle checkpoints.

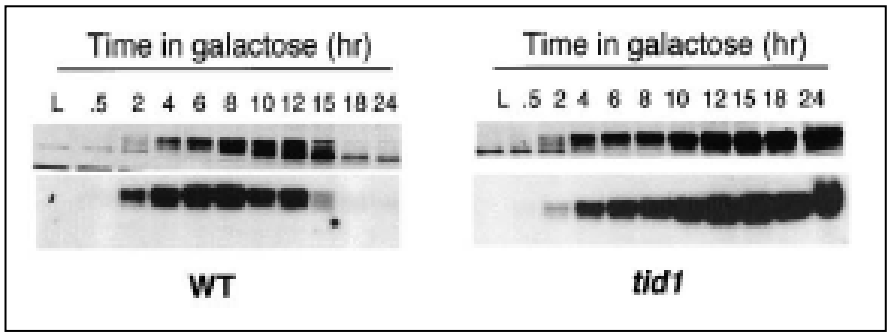

Figure 14. Adaptation defective *rdh54*Δ cells shows hyperphosphorylation and kinase activity of Rad53 checkpoint signaling protein.

3. 6. Role in Recombinational Repair

Recombination is the exchange or transfer of information between DNA molecules, the homologous recombination is a major repair pathway involves the exchange of DNA sequences of perfect or near perfect homology over several hundreds of base pair. The process of homologous recombination plays essential roles in mitotic and meiotic cell cycles of most eukaryotic organisms as discussed previously (section 3.1). The role of Rdh54 in homologous recombination was verified by genetic analysis, which revealed that null mutants are defective in meiotic recombination and crossover interference, and show a deficiency in mitotic recombination, DNA repair and DNA damage checkpoint adaptation, thus placing Rdh54 within the Rad52 epistasis group. The haploid *rdh54*Δ cells do not show sensitivity to MMS, whereas diploid shows slight sensitivity [*Klein HL, 1997*]. The *rad54*Δ *rdh54*Δ haploid strains have similar growth rates and MMS sensitivities to *rad54*Δ haploids, but homozygous *rad54 rdh54* diploids grow slowly and are more sensitive to MMS than *rad54*Δ diploid, but the growth defect of the diploid double mutant can be suppressed by mutation of Rad51, this poor growth phenotype is due to inappropriate recombination [*Klein HL, 1997*]. Although the *srs2*Δ *rdh54*Δ haploids are viable, the homozygous diploids are not; this is again by the fact that the in viability is suppressed by the mutation or checkpoint or homologous recombinational functions [*Klein HL, 2001; 1997*], this suppression lethality by Rad51 and Rad52 suggest that this protein have role in late

recombinational steps. In the absence of DNA damage Rdh54 localizes constitutively to kinetochores in mitotic cells and after damage it is found at repair foci and this recruitment is depends on the recombination proteins Rad51 and Rad52 [*Lisby M, et. al., 2004*]. More over Rdh54 is important for chromosome segregation in meiosis I. In meiotic role, it is thought that Rdh54 cooperates with the meiosis-specific recombinase Dmc1 in the mediation of inter-homologue recombination that links the chromosome homologues which allows proper alignment of the homologues on the spindle apparatus and their faithful disjunction in the first meiotic division [*Shinohara M, et. al., 1997; Symington LS, 2002*].

3. 7. Yeast as Model System

The budding yeast (Baker's yeast) is powerful tool to study the mechanisms of DNA damage checkpoint activation and consequential recombination, because given the possibility to study the molecular events during DSB formation, processing and repair. For example, in a diploid in which the two homologous chromosomes have polymorphic restriction sites flanking a region of interest, it is possible to identify cases in which crossing over has occurred by the appearance of novel restriction fragments generated by reciprocal exchange. This method also permits an analysis of the kinetics of recombination by isolating samples at intervals after the initiation of a recombination event [*Borts RH, et. al., 1986*]. An advantage of the physical assay is that it can give information not only about the products of recombination but also about intermediate steps. One may also examine the extent to which recombination can occur even under conditions where cells are unable to complete recombination. For example, one could ask if recombination can be completed when cells are arrested at different stages of the cell cycle or after the elevation of the cells to the restrictive temperature of a conditional-lethal mutation, determining which steps in recombination are affected by the inactivation of that enzyme and where the mutants become blocked [*Haber JE, 1995*].

3. 8. Synchronous induction of DSB

To analyze the kinetics of checkpoint activation and DSB repair through recombination is dependent upon the ability to initiate recombination synchronously in a large population of cells. In budding yeast cell cycle can be blocked prior to the G2/M (the cell phase in which there is a robust Rad53 activation after DSB) transition by using nocodazole which disrupt and inhibit spindle elongation. In mitotic cells, synchronous initiation of DNA damage checkpoint activation and recombination can be accomplished by the induction of a site-specific endonuclease. The HO endonuclease recognizes a degenerate target of 22bp [*Nickoloff JA, et. al., 1990*] and normally cleaves only one site in the entire yeast genome: the mating-type (*MAT*) locus. Constructs in which the HO gene is fused to a galactose-inducible promoter have made it possible to express HO simply by adding galactose to cells grown on lactate, glycerol or raffinose, these three carbon sources that do not repress the galactose-inducible promoter. About 45 to 90 minutes induction of HO leads to the cleavage of 100% of target sites. HO endonuclease is also turned over rapidly, so that no activity remains 30 minutes after the end of the induction period [*White CI and Haber JE,*

1990]. Once a DSB has been created, intermediate steps in recombination along with the appearance of final products can be identified.

3. 9. MAT switching by HO induced mitotic gene conversion

A good reason to use budding yeast as model genetic system is that this organism physiologically use a particular mechanism based on DSB repair to switch the mating-type from Mat-a to Mat-α and vice-versa. This paradigm of the *MAT* switching is based on mitotic HO endonuclease mediated recombination. During switching (**Figure 15**), the Y**a** or Y**α**-specific sequences at *MAT* that specify the mating type are replaced by sequences copied from two unexpressed donor sequences, *HML***α** and *HMR***a** [*Haber JE, 1998*].The initiating event is of DSB catalyzed by the HO endonuclease at the Y/Z junction of the recipient *MAT* locus. The absence of *Sty*I site in Y**α** sequences and its presence in Y**a** sequences makes it easy to monitor *MAT* switching by the appearance of a novel *Sty*I restriction fragment when *MAT***a** switches to *MAT***α.** It is possible to detect intermediates of recombination. In the time course of *MAT* switching, monitored on alkaline denaturing gels, is possible to observe the transient appearance of one or more higher-molecular-weight DNA restriction fragments. These proved to be the result of extensive 5′-to-3′ degradation of the HO-cut end, so that one or more *Sty*I sites were single stranded and could not be cut by the restriction endonuclease [*White CI and Haber JE, 1990*]. This processing of the DSB ends results in a single strand DNA being the pivotal intermediate in all homologous recombination pathways; this long 3′-ended tail can invade a homologous template. When later steps in recombination are prevented, for example when there is no homologous sequence with which *MAT* can recombine, 5′-to-3′ degradation appears to continue down the chromosome [*Lee SE, et. al., 1998*]. The rate of degradation can be estimated to be 1 to 2 nucleotides per second. The progress of 5′-to-3′ degradation can also be followed on dot blots by using strand-specific DNA probes [*Lee SE, et. al., 1998*]. Although the 5′-ended strand is extensively resected, there is little or no degradation of the 3′-ended strand. In conclusion, this system is useful to study DSB repair and consequential recovery or adaptation if the breakage is not reparable.

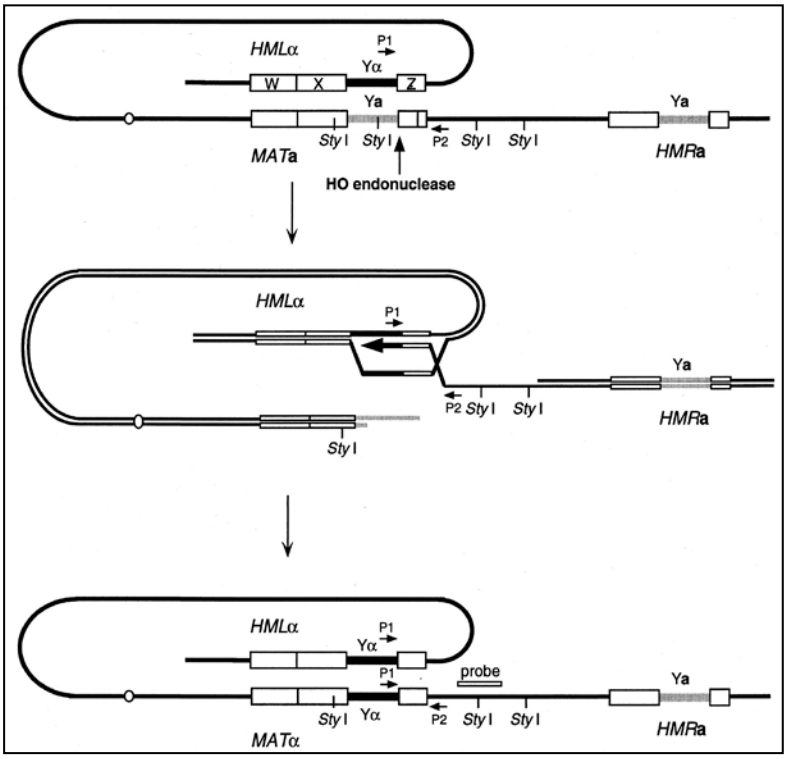

Figure 15. Yeast Mating-type switching.

The *MAT* locus, which determines **a** or a mating type, switches by gene conversion, using one of two silent cassettes, *HMR***a** and *HMLα*, located on the same chromosome. The gene conversion event is initiated by the HO endonuclease, which creates a DSB at the border of the varying region (called Y**a** or Yα, according to the genotype). *MAT*, *HMR*, and *HML* share homology on both sides of the Y regions (W, X, and Z regions). Both strands of DNA are shown in the middle diagram. A PCR assay has been used to detect DNA synthesis during *MAT* switching. Using oligonucleotides P1 and P2, one cannot obtain any PCR products in *MAT***a** cells, but a PCR product appears when the cell switches to *MAT*α, as soon as DNA synthesis initiated from the Z region of *MAT* proceeds to copy Yα from *MAT*α [*Paques F and Haber JE, 1998*].

CHAPTER - II

MATERIALS AND METHODS

4. 1. GROWTH MEDIUM

Escherichia coli:

LD:	Bactotryptone	- 10g
	Yeast extracts	- 5g
	NaCl	- 5g
	H_2O	until to 1000ml
	pH = 7.25	
LD Agar:	LD + 1% Agar	
LD Ampicillin:	LD + 50µg/ml Ampicillin	

Saccharomyces cerevisiae:

YPD:	Yeast extracts	- 10g
	Peptone	- 20g
	H_20	until to 1000ml
	pH = 5.4-5.7	

The required carbon source is added at the moment of use to the final concentration of 2%.

YPD Solid Medium:

Agar at the final concentration of 2% is added to the YPD medium

Synthetic Medium:

400x ml of selective liquid medium:

40ml Yeast Nitrogen Base (10x) DIFCO (6,7g/100ml in water); 16ml di Mix (25x) –histidine, -tryptophan, -uracil, -leucine; Glucose or different carbon source at final concentration of 2%; 2ml of each amino acid or nitrogen base (200x, 5mg/ml); water until 400ml.

400x ml of selective solid medium:

320ml of water added to 8g of agar. When the mixture is dissolved other compounds are added as in the liquid medium

Mix (25x) –Histidine, -Tryptophan, -Uracil, -Leucine 800x ml

L-thr	- 1.250 mg/ml
L-Phe	- 0.625 mg/ml
L-tyr	- 0.625 mg/ml
L-lys	- 0.625 mg/ml
L-Ile	- 0.625 mg/ml
L-Arg	- 0.625 mg/ml
L-Ade	- 0.625 mg/ml
L-Met	- 0.625 mg/ml

VB medium for sporulation:

Sodium Acetate	- 8.2g
Potassium chloride	- 1.9g
Magnesium sulphate	- 0.35g
Sodium chloride	- 1,2g
Agar	- 15g
Water	1000ml.

4. 2. BUFFERS USED:

SDS-PAGE Running Buffer (5X) for 2.5l:

Glycine - 360g

Tris Base - 75g

SDS - 12.5g

Tris-Glycine Buffer 10X (10l):

Tris Base - 302.8g

Glycine - 1440.3g

Distilled water until 10 liter

PBS 10X:

NaCl - 80g

KCl - 2g

KH_2PO_4 - 2g

$Na_2HPO_4 2 H_2O$ - 11.4g

To 1 liter with distilled water

PBST:

PBS - 1X

Tween20 - 0.2%

PBST 5% milk:

PBST - 1X

Non fat dry milk - 5%

SDS-PAGE Running Buffer or SPAG 1X (10 l):

Tris-Glycine Buffer 10X - 1 litre

SDS - 10g

Distilled water until 10litre

Stacking Buffer 4X:

0.5M Tris-HCl pH 6.8

0.4 % SDS

Running Buffer 4X:

1.5 M Tris-HCl pH 8.8

0.4% SDS

TE Buffer:

10mM Tris-HCl pH 7.4

1mM EDTA

TAE Buffer:

0.04M Tris-Acetate

0.01M EDTA

Transfer Buffer 1X (5 l):

0.5l 10X Tris-Glycine Buffer

1l Methanol

Distilled water until 5litre

3x Protein Sample Buffer:

7ml - Stacking buffer 4x (0,5M Tris-HCl pH 6.8; 0.4% SDS)

3ml - Glycerol

1g - SDS

1.2 mg - Bromophenolblue

BFB 6X DNA Loading Buffer:

BFB powder - 0.25%

Glycerol - 30%

1KB DNA Ladder:

DNA Ladder (1µg/µl) - 30µl (New England Biolabs)

Blue 6X DNA - 100µl

Distilled water - 470µl

Zymolyase for yeast ascus dissection:

Glycerol 87% - 5.7ml

Zymolyase - 5mg (US-Biological)

Final volume to 10ml with water

Ponceau S:

Ponceau S - 0.2%

TCA - 3%

Sodium Phosphate 0.1M pH 7 (500ml):

Mix 28.85ml Na_2HPO_4 1M and 21.15ml NaH_2PO_4 1M to 500ml with distilled water.

Ampicillin 500X:

25 mg/ml in distilled water

G418 200X:

80 mg/ml in distilled water

4. 3. RECOMBINANT DNA TECHNIQUES:

PCR (Polymerase Chain Reaction):

PCR is carried out using either plasmid or genomic DNA. In this work are been used three type of DNA polymerase: StS Taq-pol (Genespin), or Pfu Ultra II polymerase (Stratagene). The reaction mix contains

DNA	- 100ng
DNA polymerase buffer 10X	- 10µl
Oligonucleotides	- 20 pmoles each
dNTPs 2mM each	- 5µl
DNA polymerase	- 1 unit
Water	until to 100µl

Reactions condition:

1. Initial denaturation - 2 minutes at 95°C
2. Denaturation - 1 minutes at 95°C
3. Annealing - 1minutes at the T_m of the oligonucleotides
4. Primer extension - 1-2 minutes per kb at 72°C
5. repeat steps - from 2 to 4 for 30-40 times
6. Final extension - 10 minutes at 72°C

T_m is the melting temperature. Conditions can be modified on the basis of oligonucleotides used.

Purification of the PCR products:

PCR products are purified using Promega PCR Purification Kit. Oligonucleotides used to produce PCR deletion cassette (F1 and R1) and to check the genome integration.

DNA Precipitation by Ethanol:

To the DNA containing solution add 1/10 of the volume Sodium Acetate 3M pH5 and 3 volumes of 100% ice-cold ethanol. Incubate the solution -20°C overnight or two hours at -80°C and then centrifuge at 14000 rpm at 4°C for 45 minutes. Wash with 1ml of 70% ice cold ethanol and centrifuge at 14000 rpm at 4°C for 20 minutes. Discard the supernatant and dry the ethanol at room temperature. Finally resuspend the DNA with the fair volume of nuclease-free water.

DNA Digestion with Restriction Enzymes and DNA Purification:

DNA was digested with specific endonucleases in the conditions suggested by New England Biolabs. Samples were added 1/6 of the volume of BFB solution (6X: 0.25% BFB in 30% glycerol), before loading on agarose gel (0.6%-2%). DNA was visualized by Ethidium bromide at the final concentration of 5µg/ml. To identify the size of the DNA fragments, Molecular Weight Marker was loaded along the side from New England Biolabs. DNA was purified from agarose gel using the Gel Extraction Kit (Promega).

Ligation:

A suitable amount of digested DNA and cleaved vector is ligated using Quick ligase kit (New England Biolabs).

Preparation of competent cells and transformation of *E. coli:*

Inoculate the E. coli DH5α™ in 10ml of LD medium and grow overnight at 37°C without agitation. The day after inoculate the culture in 1l of LD and grow at 37°C with agitation until the O.D. $_{(\square=600\ nm)}$ reach 0.4-0.5. At this point keep the cells on ice for 10minutes then centrifuge at 6000 rpm for 15 minutes at 4°C and wash two times with 1l and 500ml respectively of ice-cold water. Competent cells aliquoted in fractions of 50µl and conserve at -80°C. To transform this cells add 0.5-1µg of DNA and transfer the suspension in cuvettes. Perform electroporation at 1700 V, and then grow the cells LD medium for 1hour at 37°C and subsequently plate on selective medium.

Plasmid DNA preparation from *E. coli:*

Wizard Plus SV Miniprep "Promega":

Grow the cells overnight in 3ml of LD + Ampicillin liquid medium and perform extraction using standard protocol provided. DNA concentration obtained 100ng/µl.

Midiprep "Qiagen":

Grow cells overnight in 100ml of LD + Ampicillin liquid medium, prepare extraction using the standard protocol provided, DNA concentration obtain at 1000ng/µl.

Yeast cells Transformation (Gets & Woods-Methods in Enzymology):

Pellet the 10ml of logarithmically growing cell culture for each transformation and wash with 25ml of sterile water, washed again with 0.1mM Lithium Acetate and resuspend in water. Pellet the cells again and resuspend in 360µl of TM 1X solution, after DNA addiction, incubate the cells for 40 minutes at 42°C. Then pellet the cells and wash with water and plate on selective medium. Eventually to express the antibiotics resistance, before plating grow the cells for 2 hours with complete media.

TM 1X	µl X1	µl X5
PEG 4000	240	1200
LiAC 1M	36	180
ssDNA 100mg/ml	10	50
(denaturated)H2O	74	370
Total	360	1800

4. 4. FACS analysis (FLUORESCENCE ACTIVATED CELL SORTER):

1ml of cells are fixed with 70% ice cold ethanol, after fixation pellet the cells and resuspend in 0.5ml of 1mg/ml of RNase A (in 50mM Tris-HCl pH 7.5) and incubate at 37°C 2-4 hours. After RNase treatment, pellet the samples again and resuspend in 0.5 ml of 1mg/ml of Proteinase K (in 50mM Tris-HCl pH 7.5) and incubate at 50°C for 45 minutes. After the incubation time pellet the cells and resuspend in 0.5ml FACS buffer. Stain the DNA with Sytox green (Molecular probes). Sonicate the cells and analyze by cytofluorimeter.

4. 5. CELL SYNCHRONIZATION METHODS:

α – factor:

α -factor pheromone is produced by the MAT**α** cells and is able to cause the cell cycle arrest of MATa cells in G1. This arrest is reversible, α-factor can be bought as a synthetic peptide and is solubilize in water at 10mg/ml concentration.

α - factor synchronization and release:

Exponentially growing cells are treated with α-factor 2μg/ml and the cell cycle arrest is monitored by microscopic observation. When the percentage of unbud cells are about 95%, pellet the culture and wash and resuspend in pheromone free media. In complete media at 28°C, a wild type strain is completely arrest after about 2 hours.

Nocodazole:

Nocodazole is a potent microtubules depolymerizing agent, in response to nocodazole treatment, the cells arrest their cell cycle at the metaphase-anaphase transition as a consequence of spindle assembly checkpoint.

Nocodazole synchronization:

10μg/ml of nocodazole is used in liquid culture; the arrest is monitored by microscopic observation. After 2 to 2.30 hours many cells arrest at the metaphase state. Finally the culture is used for the experiment.

4. 6. TRICHLOROACETIC ACID PROTEIN EXTRACT PREPARATIONS (TCA):

Pellet the cells and resuspend in 1ml of 20% TCA and in a second passage in 50μl of 20% TCA. Add Micro-glass-beads to samples and vortex for 5minutes to break the cells. Transfer the extracts into new tubes and centrifuge 10 minutes at 3000 rpm. Then resuspend the protein pellet in100μl of 2X protein sample buffer and the pH is neutralize with 25μl of Tris-base 2M. Boil the samples 5 minutes at 95°C and centrifuge at top speed for 3 minutes to eliminate the insoluble fraction.

4. 7. SDS-PAGE AND WESTERN BLOTTING:

SDS-PAGE is performed with 10% or 7.5% acrylamide / bis-acrylamide gel (77:1) electrophoresis is performed in SDS-PAGE running buffer. Proteins are electro-transferred on nitrocellulose membrane in a Transfer buffer over night at 200mA or 2 hours at 400mA. Nitrocellulose filters is saturate with 5% non-fat dry milk in PBST for 1hour and incubate with primary antibody in 5% non-fat dry milk 2-3 hours at room temperature. Wash the filters three times with PBST and incubate with secondary antibody even in 5% non-fat dry milk. Then wash the filters again three times with PBST, perform the chemiluminescence reaction and the signals can detect on photograph sheet.

4. 8. LAMBDA (λ) PHOSPHATASE TREATMENT:

Buffers:

Lambda (λ) phosphatase

1X phosphatase buffer (10X)

1X MnCl2 (10X)

60µl of Tris-base 2M/1ml

Protocol:

1. Spin down 10ml of cells (10^7cells/ml concentration) and add 50µl of 20% TCA.

2. Add the micro-glass beads to the cells and vortex for 5 minutes to break the cells. Then transfer the extracts to the 1.5ml eppendorf and centrifuge for 10 minutes at 3000 rpm.

3. Discard the supernatant and resuspend the pellet in 100µl of 1X lambda phosphatase buffer, then check the pH with the pH test strips (sigma pH fix 6-7.7) the pH should be about 7-8.

4. Then add 2000 U of lambda phosphatase and incubate at 30°C for 30 minutes, gently shake every 5 minutes for homogeneous reaction. Add 50µl of 3X lamely buffer and boil for 5 minutes at 95°C.

5. Centrifuge the extract at 3000 rpm for 10 minutes to clarify, and then transfer the supernatant to new 1.5ml eppendorf.

6. Run the samples in SDS gel to visualize the protein.

4. 9. YEAST GENOMIC DNA PREPARATION:

50ml of 10^7cells/ml culture were used following the procedure described below

1. Pellet the cells at 3000 rpm for 5 minutes

2. Wash with 1ml of SE solution and transfer in 1.5ml tubes

3. Pellet the cells, resuspend with the tips in 200µl of SE-β-Z and incubate for 20-30' at 37°C (you can check spheroplast formation at the microscope or by mixing 2µl of cells with 2µl SDS 10% and see if filaments are formed at the tip).

4. Centrifuge at 14000 rpm for 30 seconds and resuspend in 400µl of TE 1X

5. Add 90µl of SDS-solution, mix by inverting and incubate at 65°C for 30 minutes in a water bath

6. Add 80µl of K-Ac 5M (potassium acetate 5M), mix by inverting and incubate on ice for 20 minutes or more

7. Centrifuge at 14000 rpm at 4°C for 15 minutes

8. Carefully collect the clear upper phase in a new 1.5ml tube

9. Add ET-OH 100% pre-warmed at 37°C to fill the tube and mix well

10. Centrifuge at 14000 rpm at 4°C for 15 minutes, empty the tube by inverting

11. Wash pellet with 500 µl of cold ET-OH 70% and spin again at 14000 rpm for 5minutes. Carefully remove supernatant with a Gilson

12. Air-dry pellet, resuspend the pellet in 500µl of TE 1x

13. Add 2.5µl of RNA-se A 10 mg/ml and incubate for 30 minutes at 37°C

14. Add 500µl of Isopropanol, mix by inverting

15. Centrifuge at 14000 rpm for 2-3 minutes and empty the tubes by inverting

16. Wash with cold EtOH 70%, dry the pellet and resuspend in 50µl of TE 1X (or TRIS or water) and store at -20°C.

SE: 0.9M sorbitol, 0.1M EDTA pH 7.5

SE-β-Z: for 1ml of SCE add: 2mg of Zymolyase and 8µl of β-mercaptoethanol

SDS solution: 2% SDS, 0.1M Tris pH 9.0 and 0.05 M EDTA pH 8.0

4. 10. PROTEIN IMMUNOPRECIPITATION (IP)

Lysis Buffer:

50mM Tris Hcl 7.5 - 5ml

50mM NaCl - 1ml

1mM DTT - 15.4 µg

60mM β Glycerol Phosphatase- 1.836g

1M Na3VO4 - 200µl

1% NP40 - 1ml

Final volume prepared for 100 ml

10ml of complete lysis buffer was prepared from the 100ml stock buffer by adding EDTA free rochi mini tablet (Sigma).

Immunoprecipitation Protocol:

1. Spin down 400ml of cells ($9X10^6$ to $1X10^7$ cells/ml concentration) using beckman centrifuge at 6000 rpm for 8 minutes at 4^oC

2. Suspend the pellet with 40ml of cold water and transfer to 50ml falcon tube centrifuge at 4000 rpm for 4 minutes at 4^oC

3. Wash the pellet with 10ml of cold lysis buffer then centrifuge for, trash the supernatant.

4. Add 1ml of complete lysis buffer to the pellet and suspend well with pipette then transfer to screw cap eppendorf

5. Centrifuge at 4°C for 2 minutes at high speed, re-suspend the pellet with 400µl of complete lysis buffer complete (mini rochi tablet)

6. Break the cells using Fastprep machine at 6.5 rpm for 15 minutes each of 5 - 6 cycles

7. Check the breakage of the cells under microscope; transfer the crude to 1.5ml eppendorf

8. Clarify the crude by centrifugation at high speed for 30 minutes at 4°C; transfer the supernatant to the new eppendorf

9. Take 60µl from the supernatant and transfer to new 1.5ml eppendorf containing 30µl of 3X lamely which is the whole cell extract (WCE).

10. Wash the α-HA crosslinked resin 3 times with the complete lysis buffer

11. Mix the remaining supernatant with the α-HA crosslinked resin and incubate for 1 hour and 30 minutes at 4°C in rotator

12. After the incubation time centrifuge the mix at 1500 rpm for 30 seconds and take 60µl from the supernatant and add 30µl of 3X lamely boil at 95°C for 5 minutes which is Flow through (FT) and trash the remaining.

13. Then wash the resin 3 – 4 times with complete lysis buffer, then add 1X lamely buffer vortex little and boil at 95 °C for 5 minutes finally load on the SDS gel.

4. 11. QUANTITATIVE CHROMATIN IMMUNOPRECIPITATION (ChIP) PROTOCOL FOR YEAST

ChIP Buffers:

FA-lysis buffer Buffer III

50mM HEPES-KOH, pH 7.5 10mM Tris-HCL, pH 8.0

140mM NaCl 1mM EDTA

1mM EDTA 250 mM LiCl

1% Triton X-100 1% NP-40

0.1% sodium deoxycholate (w/v) 1% sodium deoxycholate (w/v)

FA500-lysis buffer Elution buffer B

50mM HEPES-KOH, pH 7.5 50mM Tris-HCL, pH 7.5

500mM NaCl 1% SDS

1mM EDTA 10mM EDTA

1% Triton X-100

0.1% sodium deoxycholate (w/v)

Cross-linking:

1. 50ml yeast culture were grown at 30°C to a final density of 1X 10^7cells/ml

2. Add 1.65ml formaldehyde (37% aqueous) directly to the culture to a final concentration of 1.2%. Mix by swirling

3. Incubate for 10 minutes at 30°C under continuous gentle agitation in a rotary shaker (The cross-linking time should be determined empirically for each protein and should be as short as possible)

4. Prepare a 50ml conical flask containing 0.47g glycine (330mM final)

5. Quench the formaldehyde by transferring the yeast culture in this tube. Mix well and incubate for 5 minutes at room temperature. Invert the tube from time to time

6. Place the cell culture on ice for at least 5 minutes (cells can be left on ice for several hours)

7. Pellet cells in a clinical centrifuge at 2000 rpm for 5minutes at 4°C, decant supernatant

8. Wash the pellet twice with 25ml ice-cold Tris-buffered saline (TBS). Decant supernatant

9. Resuspend cells in 1 ml FA-lysis buffer, transfer into a 1.5ml Eppendorf tube and pellet cells at 3000 rpm for 2 minutes at 4°C. Remove all supernatant

! At this stage cells can be frozen in liquid nitrogen and stored at –70°C.

Cell Breakage:

1. Resuspend cells in 50ml ice-cold FA-lysis buffer and add PMSF to a final concentration of 1mM.

2. Add an equal volume of acid-washed glass beads. Break the cells by vortex for 40 minutes at 4°C.

3. Pierce the tube bottom with a 0.45-mm needle, place into a new tube and recover the extract by short spin.

4. Resuspend the pellet (which contains the chromatin and cell debris) by flicking the tube a few times. Add SDS to a final concentration of 0.5%.

Chromatin Shearing:

1. Sonicate 6 times with 10 pulses using a Sonifier Cell Disruptor B-30 (settings: duty 90, pulsed mode, output limit 2500). Cool on ice for at least 1minute between each series of 10 pulses. Avoid foaming as this will reduce the efficiency of DNA fragmentation.

2. Pellet cell debris at 14000 rpm for 15 minutes 4°C, and transfer the supernatant to a new 1.5ml eppendorf tube.

3. Sonicate again 3 times with 10 pulses as before.

4. Centrifuge for 5 minutes and transfer the supernatant to a new 1.5ml Eppendorf tube. Set aside 5µl of the extract to a new 1.5ml tube. This will be referred as the Input sample and can be stored at –20°C until use.

! Extracts may be frozen in liquid nitrogen at this point and stored at –70°C.

Immunoprecipitation:

1. Transfer 100µl of extract (corresponding to about 1 to 2x 10^8 yeast cells) to a new 1.5ml eppendorf. Add 800µl FA-lysis buffer, 40µl protein A Sepharose beads (pre-incubated for 30 minutes at room temperature with rocking in 500µl FA-lysis buffer containing 1µl of sonicate and denatured herring sperm DNA and 10µg of BSA and then wash twice with 1ml FA-lysis buffer), and appropriate amount of the relevant or control antibodies (Ab).

2. Incubate over night at 4°C with gentle mixing on a head-over-tail rotator.

3. Pellet the beads by centrifugation in a microfuge at 3000 rpm for 2 minutes. Remove supernatant.

4. Wash the beads as follows: 1x with 1ml FA-lysis buffer

1x with1ml FA500-lysis buffer

1x with1ml Buffer III

2x with 1ml Tris-EDTA pH 8.0

After the last wash remove as much liquid as possible.

5. Elute the precipitate from the protein A-Sepharose beads by adding 100µl of Elution buffer B. Vortex mildly. Incubate 10 minutes at 60°C.

6. Pellet the beads by centrifugation in a microfuge at 3000 rpm for 2 minutes at room temperature. Transfer elute to a new tube.

7. Repeat steps 5 and 6. Pool the elutes

Reverse cross-linking:

1. Add to the Input (from step 4 in Chromatin Shearing) and Elute samples 200µl of Tris-EDTA pH 8.0 and 3µl of proteinase K at 20mg/ml. Incubate for 4-5 hours (for overnight) at 65°C.

2. Extract samples twice with phenol-chloroform and once with chloroform. Add 1.10 volumes 3M NaOAc (pH 5.2) 3.5g of glycogen as carrier and 2 volume of ethanol, precipitate for at least 1hour at –20°C.

3. Spin down the ethanol precipitate at 14000 rpm for 10 minutes at 4°C. Remove the supernatant (be very careful, the pellet does not stick very well to the side of the tube).

4. Wash with ice-cold 70% ethanol.

5. Dissolve the Input DNA in 120µl and the immunoprecipitated DNA in 120µl of water. Store at –70°C.

PCR quantification of ChIP samples:

A standard PCR amplification method has been used to amplify DNA fragment using specific primers and the gel quantification can be determined by using the NIH Image program. The relative fold enrichments of DSB-bound protein were calculated as follow:

[DSB_IP/CON_IP] / [DSB_input/CON_input],

where IP and Input represent the amount of PCR product in the immunoprecipitates and in input samples before immunoprecipitation, respectively

All PCR reactions are performed in duplicate.

Mix for each PCR reaction:

Input or IP DNA template - 10µl

10X buffer with Mgcl2 - 3µl

10mMdNTPs - 0.6µl

Primer P1 25 µM - 1.2µl

Primer P2 25 µM - 1.2µl

Primer P3 25 µM - 1.2µl

Primer P4 25 µM - 1.2µl

Taq polymerase (gene spin) - 0.4µl

Milli Q H2O to final volume of 30µl

PCR reactions are performed in the following condition:

Step1 – 3 minutes at 94°C Step 2 – 45 seconds at 94°C

Step 3 – 60 seconds at 55°C

Step 4 – 60 seconds at 72°C

Step 5 – repeat the cycle from step 2 for 30 times

Step 6 – 5 minutes at 72°C

Step 7 – 20 minutes at 10°C

Primers used for ChIP sample quantification:

Primer P1	5 – TGGACGGAGGACTTAATATCGTCAC- 3
Primer P2	5 – AGGATGCCCTTGTTTTGTTTACTG – 3
Primer P3	5 –CGTTACTTTCCTCATCACCTTCGC - 3
Primer P4	5 –ACAGAGAGAGTGGGCTCATCTTGC – 3

4. 12. BACTARIAL STRAINS:

DH5α™: F Φ80 dlacZ Δ M15 Δ (lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (r_{K^-},m_{K+}) supE44 λ-thi-1 qyrA96 relA1.

This strain is used as host in order to construct the plasmids.

***Rdh54K318R* mutant:**

The Rdh54K352R mutation was cloned into the pRS306 as XhoI - NgoIV fragment (pHK255) kindly provided by Prof. Hannah Klein. The plasmid was linearized with NruI enzyme and transform into DH5α cells.

4. 13. ONE STEP PCR STRATEGIES FOR C-TERMINAL TAGGING AND GENE DELETION:

The yeast strains used in this study were constructed by one step PCR method [*Longtine MS, et. al., 1998*] using selectable antibiotic resistance markers, and individual mutants used in this work were produced by genetic crossing and tetrad analysis (listed in **Table 4**).

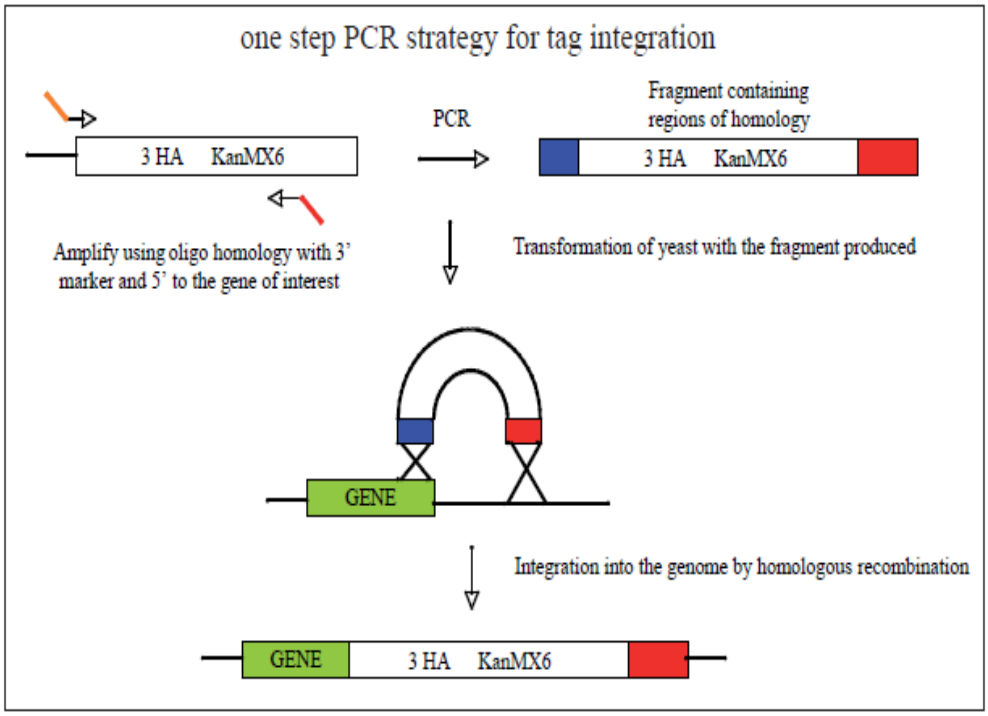

Figure 16. Schematic diagram of one step PCR C-terminal tagging strategy.

Primers used for Rdh54 gene C-Terminal Tagging:

F2 Primer	5′ – TGCGTTTGTAAAGCCCGGCGAGATATGTCTCAGAGAACAA CGGATCCCCGGGTTAATTAA - 3′
R1 Primer	5′– ATAGCTATTTTATTTAGTATATAAGTGTCCATATTTGGCG GAATTCGAGCTCGTTTAAAC - 3′

Primers used for checking the C-Terminal Tagging of Rdh54 gene:

Rdh54 3CF	5′- CGATCCAGCAAGACAAGATG - 3′
Rdh54 4CR	5′- GTGGATATCAACTTGAATCA - 3′
Kan Rev	5′ – GTAACCATGCATCATCAGGAGT – 3′

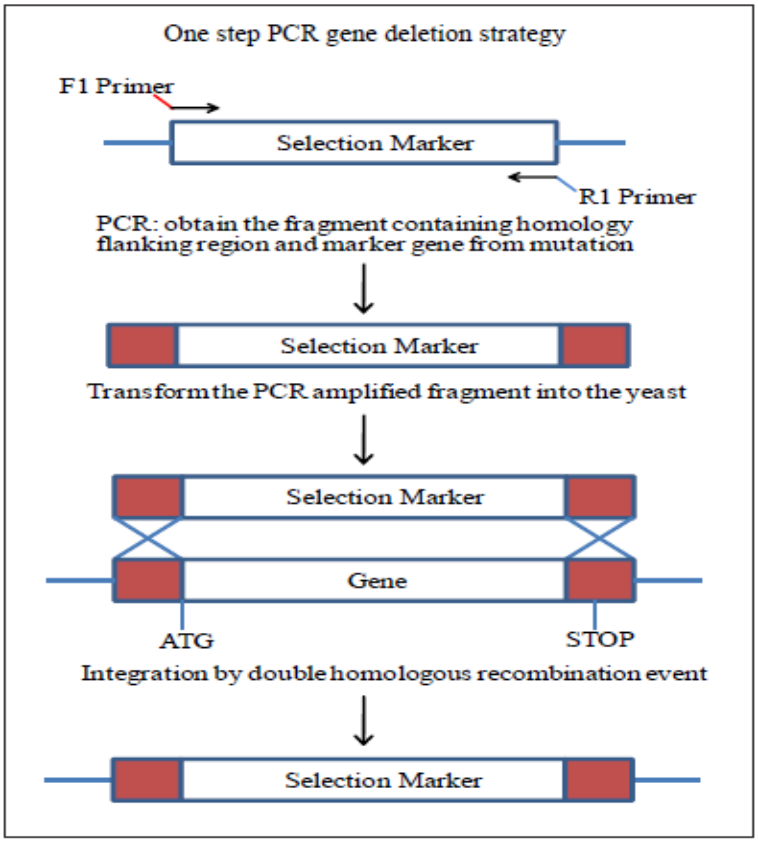

Figure 17. Schematic diagram of one step-PCR gene deletion strategy.

4. 14. YEAST STRAINS

Strains	Genotype	Reference/Source
Y1	*ho Δ MATα hml Δ::ADE1hmRΔ::ADE1ade1-100 leu2-3 leu2-112 lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO*	Jim Haber Lab
Y117	*ho Δ MAT**a** hml Δ::ADE1hmRΔ::ADE1ade1-100 leu2-3 leu2-112 lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO*	Jim Haber Lab
Y143	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::his G, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, delta srs2::LEU2*	Jim Haber Lab
Y316	*hoΔ, Mat-a, hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112, lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO, sml1::KANMX6, rad53::TRP1 <YCplac111, LEU2, CEN, ARS>*	Lab permanent
Y454	*Mata, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::his G, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, RDH54::3HA::kanMX6*	This study
Y462	*MAT-a, ade2-1, trp1-1, leu2-3112, his3-1115, ura3, can1-100 GAL PSI1+ RDH54-3HA::KanMX6*	This study
Y521	*hoΔ, Mat-α, hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112, lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO, sae2::KANMX6, RDH54::3HA-KanMX6*	This study
Y522	*hoΔ, Mat-a, hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112, lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO, sml1::KANMX6, mec1::TRP1, RDH54::3HA-KanMX6*	This study
Y608	*Δho Mat-a, Δhml::ADE1 Δhmr::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO rad51::LEU2*	Lab permanent
Y623	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, RDH54::4HA::kanMX6*	This study

Y625	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, GAL1:: SIC1stable@URA3*	Dr. Federico Lab 15
Y627	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, GAL1::SIC1stable@URA3 RDH54::4HA-KanMX6*	This study
Y629	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, GAL1::SIC1stable@URA3 RDH54::4HA-KanMX6*	This study
Y639	*hoΔ, Mat-α, hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112, lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO, sml1::KANMX6, rad53::TRP1 RDH54::3HA-KanMX6*	Lab permanent
Y640	*hoΔ, Mat-a, hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112, lys5 trp1Δ::hisG ura3-52 ade3::GAL::HO, sml1::KANMX6, rad53::TRP1 RDH54::3HA-KanMX6*	This study
Y736	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, rad51 delta::LEU2*	Lab permanent
Y738	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, RDH54::4HA::kanMX6 rad51delta::LEU2*	This study
Y740	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, RDH54::4HA::kanMX6 rad51delta::LEU2*	This study
Y814	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, delta srs2::LEU2 RDH54-3HA::KanMX6*	This study
Y815	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, delta srs2::LEU2 RDH54-3HA::KanMX6*	This study

Y841	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta:: hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO rdh54-K352R::3HA::kanMX6*	This study
Y873	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta:: hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, rdh54-K352R::3HA::kanMX6 rad51::LEU2*	This study
Y874	*Mat-α, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta:: hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, rdh54-K352R::3HA::kanMX6 mec1::TRP sml1::KanMX6*	This study
Y876	*Mat-a, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta:: hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO, rdh54-K352R::3HA::kanMX6 mec1::TRP sml1::KanMX6*	This study

CHAPTER - III

AIM OF THE WORK

The ScRad54 homologue Rdh54 belongs to the Rad52 epistasis group gene involved in recombinational DNA repair and, as discussed in this thesis introduction part, Rdh54 is required for various biological activities such as adaptation in response to damage, D-loop formation, chromatin remodeling and gene conversion recombination between inter homologs, specifically in meiotic cells. In mammals, RAD54B is likely the homolog of Rdh54. However, mutagenic studies on mammalian Rad54B in chicken and mice do not show hypersensitivity to MMS as their yeast counterpart Rdh54. In mitotic cell cycle Rad54B does not possess sister chromatid mediated repair in response to ionizing radiation [*Miyagawa K, et. al., 2002*]. Yeast Rdh54 interacts with Rad51 and meiotic recombination factors Dmc1, but there are contradictory results about the interaction of Rad54B in human with the hRad51 and hDmc1. In one study, the hRad54B does not interact with hRad51 and hDmc1 even though it possesses similarities in its N-terminal with the ScRdh54 [*Tanaka K, et. al., 2002*]; in another study, hRad54B does interact with hRad51 and this interaction is highly specific [*Wesoly J, et. al., 2006*]. Both hRad54B and ScRdh54 has ATP hydrolysis dependent DNA translocase activity, which is required for negative supercoiling activity that cause transient opening of the DNA double helix [*Wesley J, et. al., 2006; Chi P, et. al., 2006; Petukhova G, et. al., 2000*]. In colon cancer cell lines inactivation of hRad54B severely reduces the frequency of targeted integration [*Tanaka K, et. al., 2002; Miyagawa K, et. al., 2002],* suggesting that hRad54B also play role in homologous recombination.

During my Doctoral Thesis, I investigated how ScRdh54 protein is regulated in response to DNA damage, focusing on DSB-induced checkpoint process.

RESULTS

5. 1. Analysis of Rdh54 protein:

To visualize Rdh54 protein by commercial antibodies, I inserted 3HA epitope cassette at the STOP codon of *RDH54* gene by one-STEP PCR-based method (see Materials and Methods) [*Longtine MS, et. al., 1998*] into JKM background strain, in which one single irreparable DSB can be induced by site-specific HO endonuclease [*Lee SE, et. al., 2001*].

First I analyzed the Rdh54 protein stability and modification in synchronized cells during an unperturbed cell cycle. Exponentially growing cells of the *RDH54*-3HA strain was synchronized in G1 cell cycle phase by adding α-factor into the culture. The G1 block was visualized by microscope and when 90% of cells have been unbudded, the culture have been released from α-factor by centrifugation and resuspended in fresh pheromone-free YPD medium. Samples were collected at the indicated time points and were processed for protein extract by TCA and FACS analysis. A part of the cell culture has been treated with galactose to induce over-expression of HO, which causes the formation of single irreparable DSB in this yeast background. In **Figure 1A**, cell cycle progression was analyzed by FACS and we can see that G1-blocked cells restart synchronically the cell cycle progression by replicating their DNA content starting from 30 minutes after released from G1 block. Protein extracts were run in SDS-PAGE and transferred in nitrocellulose membrane and the Western blotting was performed as previously described [*Pellicioli A, et. al., 1999*]. In **Figure 1B**, by using commercial antibodies anti HA and monoclonal antibodies anti Rad53, I compared the Rdh54 protein levels to those of Rad53, which is a very stable protein in all the cell cycle phases. The results indicate that protein levels of both Rdh54 and Rad53 proteins do not fluctuate significantly during unperturbed cell cycle, moreover Rdh54 does not show any visible modification. Interestingly, in the protein sample prepared from cells experiencing DSB formation after 6 hours of HO induction, Rdh54 shows a band shift. In these cells, DNA damage checkpoint is activated, as indicated by Rad53 phosphorylation.

In many cases, proteins are regulated by phosphorylation in response to DNA damage and it is well known that DNA damage checkpoint is based upon phosphorylation signaling (see

Introduction). Therefore, I decided to test the possibility that Rdh54 band shift was due to protein phosphorylation. To this purpose, I treated the protein sample prepared from cells experiencing DSB with λ phosphatase before loading into the SDS page gel. Surprisingly, the electrophoretic mobility shift of Rdh54 in response to DNA damage was abolished quantitatively by λ phosphatase treatment (F**igure 1C**), indicating that the mobility shift of Rdh54 protein was due to phosphorylation event. These results indicate that Rdh54 protein is phosphorylated in the presence of DSB lesions, while it is not during unperturbed cell cycle.

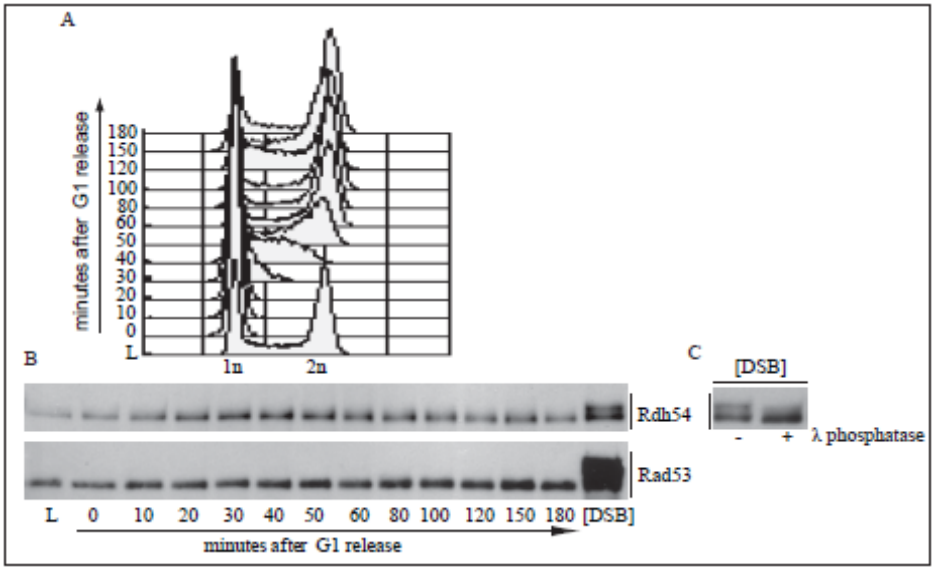

Figure 1. Rdh54 is phosphorylated in response to DNA damage.

Exponentially growing cells of the Y454 (wild type) indicated Mata hml/r strain was grown in YPD medium at 25°C; α-Factor (10μg/ml) was added to the culture to arrest the cells in G1. After the arrest in G1, cells were released into fresh YPD medium. A part of the culture was grown in YP + raffinose medium and galactose was added for 6 hours to induce the expression of site specific HO endonuclease and the formation of single irreparable DSB. At indicated time samples were taken for FACS analysis (**Figure 1A**) and Western blotting analysis (**Figure 1B**). Protein extracts prepared from cells grown in galactose were treated with/without λ phosphatase before loading on to the SDS page gel (see Material and Methods) (**Figure 1C**). Rdh54 Protein was analyzed by western blot using 12CA5 monoclonal antibodies and Rad53 protein was analyzed by western blot using Mab.EL7 monoclonal antibodies (**Figure 1B and C**).

5. 2. Analysis of Rdh54 phosphorylation in G1 and G2 phases:

Previous experiment showed that Rhd54 protein is phosphorylated in exponentially growing cells experiencing irreparable DSB formation. It is known that DSB activates a robust checkpoint response in the G2 cell cycle phase. I wanted to establish whether the Rdh54 phosphorylation could also occur in cells arrested either in G1 and G2 phases of the cell cycle by treatment with different DNA damaging agents. Thus I performed the following experiment in which exponentially growing cells were divided equally into two flasks: in one flask α-factor was added to block cells in G1; in the second flask the microtubule inhibitor nocodazole was added to block the cells in G2 phase of the cell cycle. Cells were monitored under the microscope and when the cell cycle block has been established, the cell cultures were equally divided into three separate flasks. The first flask contained the same medium and I used this cells culture as untreated control (mock), while the second and third flask contained the DNA damaging agents zeocin (Z) or 4-Nitroquinoline (Q), respectively. Z causes formations of many DSBs, while Q causes UV-like DNA damage. The cell cultures were incubated for 30 minutes at 28°C and samples were then processed for FACS and Western blot analysis.

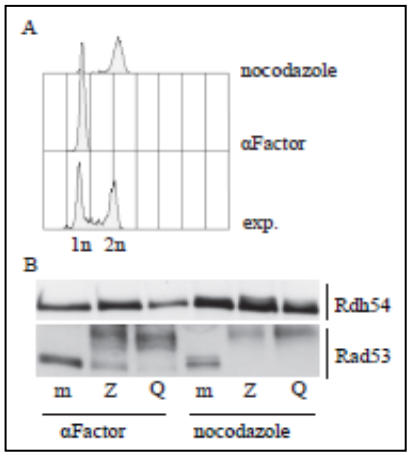

Figure 2. Rdh54 is phosphorylated in G2 phase in response to DNA damage.
Exponentially growing cells of the indicated Y454 (wild type) Mata hml/r strain has been blocked in G1 with α-Factor (10μg/ml) and in G2 with nocodazole (20μg/ml). The cell cycle block has been visualized by microscope. The cultures have been untreated (m), treated with Zeocin (Z, 200μg/ml 30 minutes) and 4-Nitroquinoline (Q, 200μg/ml 30 minutes). Samples were taken for FACS analysis (**2A**); protein extracts were analyzed by western blot using specific antibodies (**2B**).

FACS analysis in **Figure 2A** shows cell cycle blocks in G1 or G2. Protein extracts were separated in SDS PAGE, and analyzed by Western blotting. In **Figure 2B**, I observed that Rad53 kinase is fully phosphorylated in cells treated with zeocin or 4-Nitroquinoline, both in G1 and G2 phases. Interestingly, in G2 arrested cells I observed Rdh54 phosphorylation in both the damage conditions (Z and Q) when compared with untreated samples (m). On the contrary, Rdh54 protein was not phosphorylated in G1 phase when cells were treated with zeocin or 4-Nitroquinoline, even if the checkpoint kinase Rad53 protein was fully activated in response to such damages. These results indicate that Rdh54 is phosphorylated in response to DNA damage in a cell cycle specific way. Interestingly, these results are similar to what previously found for Srs2, a DNA helicase involved in recombination and checkpoint response [*Liberi G, et. al., 2000*], and may suggest that recombination factors, such as Rdh54, Srs2 and others are not recruited to DNA lesions in G1.

5. 3. Analysis of Rdh54 phosphorylation in response to replication stress:

Hydroxyurea (HU), an inhibitor of ribonucleotide reductase, causes nucleotide depletion and blocks DNA replication process. It has been shown that yeast cells respond to HU treatment by activating a signal transduction pathway that leads to Rad53 phosphorylation [*Sanchez Y, et al., 1996*]. We analyzed whether the cells released from G1 block in the presence of hydroxyurea phosphorylates Rdh54 as a result of replication stress. Exponentially growing cells in YPD medium at 28°C were arrested in G1 phase by adding α-Factor into the culture medium; cell cycle block was monitored under microscope. After cells block has been established, cells were released into fresh YPD medium containing 0.2M HU to induce replication block. FACS analysis in **Figure 3A** confirmed that in response to hydroxyurea cells progressively arrested in S-Phase. Interestingly, Western blotting analysis in **Figure 3B** revealed that Rdh54 protein does not show any significant phosphorylation in cell treated with HU, although Rad53 protein is fully hyperphosphorylated and active. These results indicate that Rdh54 protein is not phosphorylated in response to replication block induced by hydroxyurea treatment, suggesting that Rdh54 protein is likely not implicated in response to DNA replication intermediates generated by replication fork stalling.

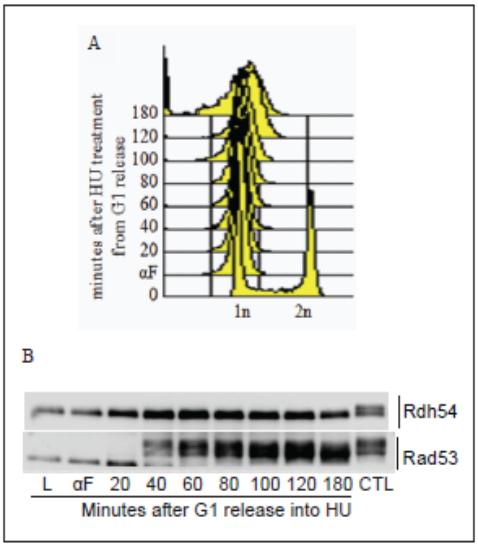

Figure 3. Analysis of Rdh54 protein in cells treated with hydroxyurea.
Indicated Y454 (wild type) Mata hml/r strain was grown exponentially and blocked in G1 with α-Factor (10µg/ml) for 1 hour and 30 minutes, and then cell cycle block was visualized under microscope. Cells were centrifuged and released into fresh YPD medium containing 0.2M hydroxyurea (HU). At indicated time points samples were collected and analyzed by FACS (**3A**) and Western blotting (**3B**). Untreated (L), α-Factor arrests (αF). As a positive control of Rdh54 phosphorylation, (CTL), cells were treated with zeocin for 30 minutes (200µg/ml).

5. 4. Rdh54 phosphorylation requires Mec1-signaling:

Previous experiments indicate that Rdh54 is phosphorylated in response to DNA damage. To test if kinases involved in DNA damage checkpoint signaling are required for Rdh54 phosphorylation, I used different experimental approaches.

In one first experiment, cells of the strain Y454, carrying *RDH54*-3HA gene, were grown exponentially in YP + raffinose medium and galactose was added to induce DSB. After 6 hours of galactose induction, the culture was devised into two parts. In one half, the PIKK inhibitor caffeine was added to the culture medium and incubated for 30 minutes. In this condition, PIKKs are inhibited and checkpoint signaling is abrogated [*Sarkaria JN, et. al., 1999; Zhou BB, et. al., 2000*]. Samples were collected at indicated time and Western blot analysis was performed. In **Figure 4A**, Western blot results indicate that caffeine treatment abrogated DSB-induced Rad53

phosphorylation. Surprisingly, also Rdh54 phosphorylation is completely abrogated by caffeine, suggesting that the PIKKs are required to induce Rdh54 phosphorylation in response to DNA damage.

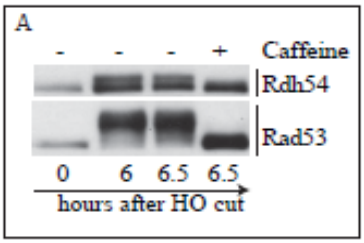

Figure 4. Mec1 and Rad53 are required for Rdh54 phosphorylation.
(A) Culture of Y454 (wild type) JKM strain was grown in YP + raffinose medium, galactose was added for 6 hours to induce DSB. The culture was divided in 2 parts and 5mM caffeine was added to one half for another 30 minutes at 28°C. Samples were collected at indicated time and western blot was performed using specific antibodies to test Rdh54 and Rad53 proteins. (B) Exponentially (0) growing culture of the strain Y454 (wild type), Y522 (*mec1*Δ) and Y640 (*rad53*Δ) were grown in YP + raffinose. Galactose was added to induce irreparable DSB and samples were taken at the indicated time points. Western blotting was performed using specific antibodies to test Rdh54 and Rad53 proteins. (C) Cells grown in YP + raffinose were arrested in G2 by nocodazole. In one half of the culture galactose was added for 6 hours to induce irreparable DSB. Cells were collected and Rdh54-3HA protein was immunoprecipitated using 12CA5 monoclonal antibodies (see materials and methods). Samples were analyzed by Western blotting using commercial antibodies raised against phospho-(S/T)-Q motifs (Cell Signaling Technology). HA-tagged protein was detected with the 12CA5 monoclonal antibody.

In order to test genetically the involvement of the main checkpoint kinases Mec1 and Rad53 in promoting Rdh54 phosphorylation, I generated *mec1*Δ and *rad53*Δ mutations in Y454 strain, expressing Rdh54-3HA protein. Cells of the corresponding strains were grown in YP + raffinose medium then galactose was added to induce the site specific HO nuclease. In these conditions, an irreparable DSB is formed. Samples were collected and Western blot was performed to analyze the phosphorylation of Rdh54 and Rad53 respectively. In **Figure 4B**, Western blot results shows that in wild type, Rdh54 protein is phosphorylated in response to single HO-induced irreparable DSB; the Rdh54 phosphorylation is maintained for several hours

(until end of the experiment), mirroring the kinetic of Rad53 phosphorylation. It is also shown that in *mec1Δ* and *rad53Δ* cells, Rdh54 protein phosphorylation is affected, with a stronger inhibition in *mec1*Δ cells, which also prevented the full activation of Rad53. The results shown in **Figure 4B** indicate that the two checkpoint protein kinases Mec1 and Rad53 are both necessary for a proper DNA damage induced phosphorylation of Rdh54 protein. Although both Mec1 and Rad53 kinases can phosphorylate directly Rdh54, previous genetic evidences (**in Figure 4B**) do not prove this model. Moreover, Mec1 is very upstream in the checkpoint signaling pathway, and it is required to activate Rad53. Therefore, it is also possible that only Rad53 phosphorylates directly Rdh54, while Mec1 is required to activate Rad53.

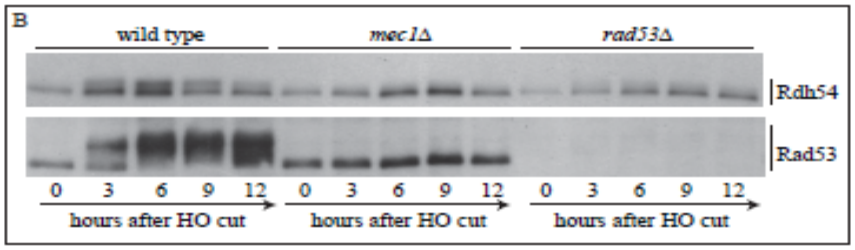

Figure 4B.

General consensus phosphorylation sites for Mec1 and Rad53 have been proposed [Kim ST, et. al., 1999]: i) the canonical serine/threonine (S/T)-Q motifs favor the phosphorylation of specific proteins by ATM/ATR kinases; ii) (S/T) -Ψ (where Ψ denotes hydrophobic amino acid) is a general Rad53 phosphorylation motif. Interestingly, Rdh54 protein sequence shows many S/T-Q and S/T-Ψ motifs (see **Figure 9**) supporting the hypothesis that it could be a Mec1 and/or Rad53 substrate. To test more directly if Mec1 phosphorylates Rdh54, I took advantage of commercial specific monoclonal antibodies raised against phosphor - (S/T) - Q motifs. Wild type cells, carrying *RDH54*-3HA gene, were grown in YP + raffinose medium. Nocodazole was added in the culture to block the cell cycle in G2 phase. Then the culture was devised in two half and galactose was added in one half to induce irreparable DSB. After 6 hours of induction, samples were collected and immunoprecipitation of Rdh54 protein was prepared using α-HA crosslinked resin (Refer materials and methods of this thesis). The immunoprecipitated proteins were separated by SDS PAGE gel electrophoresis, and Western blotting was performed with specific monoclonal antibodies anti phosphor - (S/T)-Q motifs and anti-HA. As shown in **Figure 4C,** Rdh54 protein is detected by the specific anti phosphor - (S/T) - Q motifs only in sample

prepared from the cells with irreparable DSB. These results, together with previous genetic evidences (**Figure 4B**), strongly support the hypothesis that Mec1 directly phosphorylates Rdh54 in the presence of DSBs.

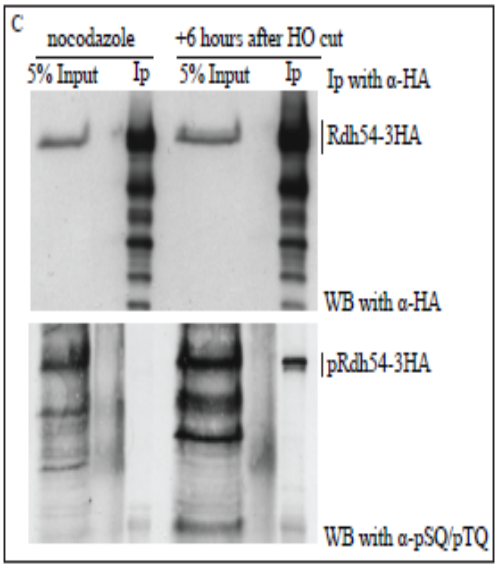

Figure 4C.

Therefore, Rdh54 is a novel DNA damage checkpoint target that is phosphorylated both by Mec1 and by a Rad53-dependent mechanism. Interestingly, Rdh54 is also required to silence checkpoint signaling during adaptation, suggesting a feedback regulation of the protein (see **Discussion**).

5. 5. Analysis of the ATPase defective Rdh54 protein variant:

As discussed in the introduction chapter, Rdh54 protein displays DNA-dependent ATPase activity which is essential for its biological function. Mutation in the highly conserved Walker type A motif on the lysine residue 318 to arginine displays only 1%-2% of the ATPase activity of wild type protein [*Petukhova G, et. al., 2000*]. Mutation in this residue also abolishes Rdh54 functions in checkpoint adaptation [*Lee SE, et. al., 2001*] and causes greater increase in MMS sensitivity [*Kwon YH, et. al., 2008; Chi P, et. al., 2006*].

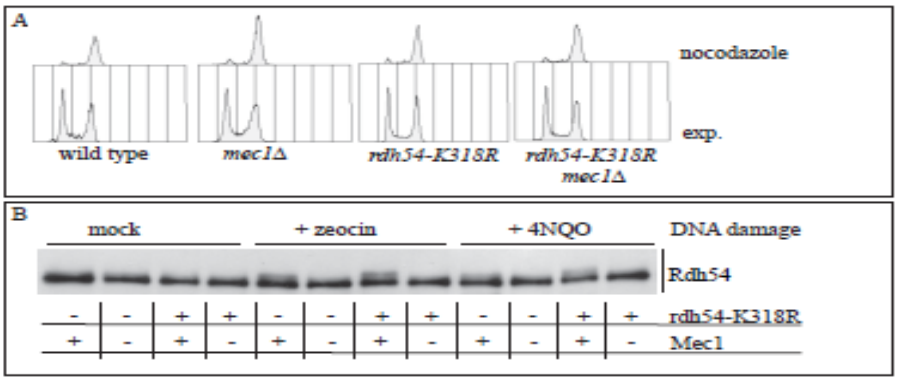

Figure 5. Analysis of Rdh54-K318R protein variant in G2-blocked cells.
Exponentially growing cells of the indicated Y454 (wild type), Y522 (*mec1*Δ), Y841 (*rdh54K318R*) and Y876 (*rdh54K318R mec1*Δ), Mata hml/r strains have been blocked in G2 with nocodazole (20μg/ml). The cell cycle block has been visualized under microscope. The cultures have been treated with Zeocin (200μg/ml 30 minutes) and 4-Nitroquinoline (4NQO, 200μg/ml 30 minutes) (**Figure 5A**) Samples were taken for FACS analysis; (**Figure 5B**) protein extracts were analysed by western blot using monoclonal αHA. Rdh54 Protein was analysed by western blot using 12CA5 monoclonal antibody.

I tested if the Rdh54-K318R protein variant can be phosphorylated in the presence of DNA damage. In the experiment in **Figure 5A**, cells of the indicated strains, carrying *RDH54*-3HA or *rdh54*-K318R-3HA genes, with or without *MEC1* gene, were grown in YPD medium and arrested in G2 phase by adding nocodazole. Then culture was divided into 2 parts: one was untreated (mock), and the second half was treated with zeocin or 4NQO for 30 minutes. Samples were collected after the treatment for FACS and protein analysis. Western blotting analysis in **Figure 5B** shows that both the wild type and the Rdh54-K318R protein variant are phosphorylated by a

Mec1 dependent manner in response to DNA damage (zeocin and 4NQO). These results indicate that the ATPase activity and the ability to translocate along DNA are dispensable to induce the checkpoint-dependent phosphorylation event of Rdh54.

Then I tested Rdh54-K318R phosphorylation in the presence of irreparable HO-induced DSB. Cells of wild type and *rdh54-K318R* strains were grown in YP + raffinose medium and galactose was added in the cell culture medium to induce irreparable DSB. Samples were collected at indicated time and protein extracts were analyzed by Western blot.

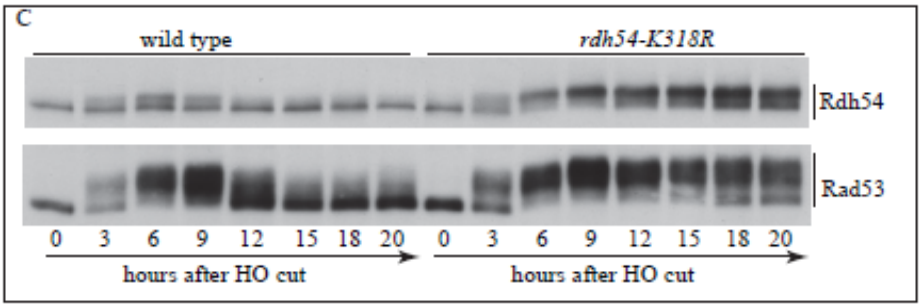

Figure 5C. Analysis of Rdh54-K318R protein variant phosphorylation in the presence of irreparable DSB.

Exponentially growing cells of the indicated Y454 (wild type) and Y841 (*rdh54K318R*) Mata hml/r strains were grown in YP + raffinose. Galactose was then added to induce the single double strand break at HO cleavage site and samples have been taken at the indicated time points and western blot was performed using specific antibodies.

As shown in **Figure 4B**, the wild type protein is phosphorylated in response to irreparable DSB and its phosphorylation is maintained for many hours; then, at about 12 hours, the checkpoint adaptation takes over and the Mec1/ATR checkpoint is switched off, as judged by the phosphorylation state of Rad53. Concomitantly with the inactivation of the checkpoint signaling, the phosphorylation of Rdh54 is no longer accumulated, strongly supporting the previous evidences that it is due to checkpoint signaling. Interestingly, the phosphorylation of the Rdh54-K318 protein variant is induced and accumulated till the end of the experiment. The prolonged phosphorylation state or Rdh54-K318R protein is correlated to the defect in checkpoint adaptation and in switch off the Mec1/ATR signaling in *rdh54*-K318R mutant cells [*Lee SE, et. al., 2004*]. In other words, the prolonged Mec1 activity and checkpoint signaling in *rdh54*-K318R mutant cells leads to a prolonged phosphorylation of Rdh54-K318R protein variant. Moreover, a careful

analysis of the blot in **Figure 5C** and **Figure 5B** reveals that the phosphorylation shift of the Rdh54-K318 protein variant is sensitive higher than the one visible for wild type protein. There are several explanations for this result. One interpretation is that the Rdh54-K318R protein is stuck into some DNA intermediates because it is defective in moving along the DNA and, for that reason, the protein remain exposed for a longer time to the upstream kinase(-s), which in turn may phosphorylate the protein in several residues. A second hypothesis is that the ATPase defective protein variant is phosphorylated by one additional kinase.

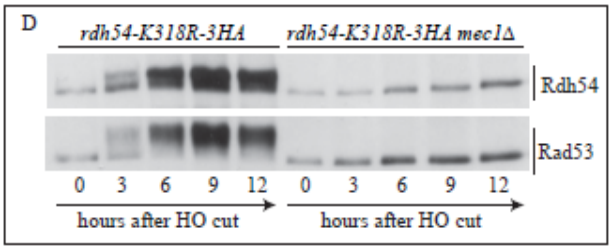

Figure 5D. DSB-induced Hyper-phosphorylation of Rdh54-K318R protein variant requires Mec1.

The indicated Y841 and Y876 Mata hml/r strains were grown in YP + raffinose. Galactose was then added to induce the single double strand break at HO cleavage site. Samples were taken at the indicated time points and protein extracts were analysed by western blot using specific antibodies.

To further address this possibility, I tested Rdh54-K318R phosphorylation in *mec1*Δ mutant. The cells of the indicated strains were grown in YP + raffinose medium and galactose was added to induce the irreparable DSB formation (**Figure 5D**). Samples were taken at the indicated time points and phosphorylation of Rdh54 and Rad53 proteins have been analyzed by Western blotting. Rad53 is not phosphorylated and activated in *mec1*Δ cells, as expected. In these experimental conditions, also the phosphorylation of rdh54-K318R is affected, indicating that it still requires the Mec1-signaling. However, our results do not rule out the possibility that additional kinase (-s) other than Mec1 and Rad53 may phosphorylates Rdh54 and that the phosphorylation event is much more evident for the ATPase defective rdh54-K318R protein variant.

5. 6. CDK1 role in Rdh54 phosphorylation:

CDK1 participates in several processes and it phosphorylates several substrates in cells responding to DSBs (see **Introduction**). Inhibition of CDK1 activity by the overexpression of the physiological inhibitor Sic1, leads to a notable reduction of 5' to 3'DSB ends processing [*Aylon Y, et. al., 2004; Ira G, et. al., 2004*]. Since Rdh54 protein is recruited to the double strand break site, I asked whether the inhibition of CDK activity affect the phosphorylation of Rdh54 protein in response to irreparable DSB. In nocodazole arrested wild type and *Gal::Sic1* cells galactose was added to induce the expression of *HO* and *SIC1* genes. Samples were collected at indicated time points and protein extracts were analyzed by Western blotting.

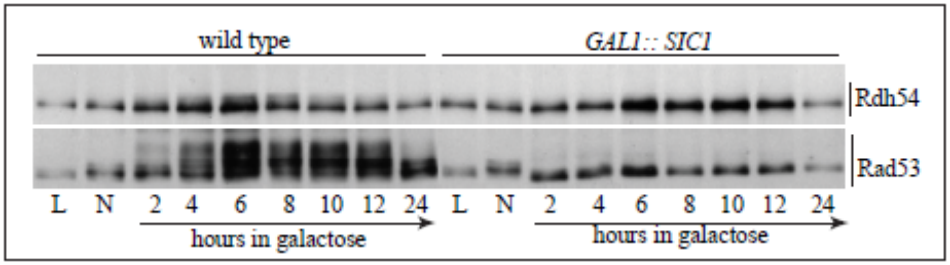

Figure 6. Analysis of DSB-induced Rdh54 phosphorylation in *SIC1* over-expressing cells. The indicated Y454 (wild type) and Y625 (*GAL1::SIC1*) Mata hml/r strains were grown in YP + raffinose. Nocodazole was added (20μg/ml) to the cultures to arrest the cells in G2 cell cycle phase. Then galactose was added to induce the over-expression of HO nuclease and of *SIC1*. Samples were collected at indicated time and protein extracts were analyzed by Western blotting. 12CA5 antibodies were used to visualize Rdh54-3HA protein; EL7 monoclonal antibodies were used to test Rad53 protein.

The results in **Figure 6** show that Rdh54 phosphorylation correlates with the kinetic of Rad53 activation by Mec1/ATR signaling, confirming the previous evidences (see **Figure 4**); on the contrary, inhibition of CDK1 by the over-production of Sic1 strongly prevents the Mec1/ATR signaling and Rdh54 phosphorylation. Although CDK1 may phosphorylate directly Rdh54, one plausible hypothesis is that the inhibition of CDK1 activity affects the DSB resection and generation of ssDNA, which is necessary to recruit checkpoint and recombination factors to the DNA lesion. To further address this hypothesis, others experiments are required (see **Discussion**).

5. 7. Analysis of Rdh54 phosphorylation in mutants altered in recombination:

The DNA DSBs are nucleolytically resected to generate a 3' single stranded tail that serves as a template for the assembly of a presynaptic Rad51 filament. The Rad51 filament invades an undamaged double-stranded DNA (dsDNA) where homologous sequences can pair in a synaptic complex. Once homology is identified, the dsDNA is used as a template to extend the invading strand by DNA synthesis. Rad51-ssDNA nucleoprotein filament formation is a critical step in mediating DSB induced homologous recombination. Rdh54 through its N-terminal domain interacts with Rad51 recombinase to mediate strand exchange and catalyze the removal of Rad51 from DNA *in vitro* [*Chi P, et. al., 2006*]. The *rad51*Δ mutant cause delayed in adaptation, as only about 30% of cells have adapted after 24hours; further, the *rad51*Δ *rdh54*Δ double mutant does not show any difference from *rdh54*Δ single mutant in checkpoint adaptation [*Lee SE, et. al., 2001*]. These data suggest that the amount of Rad51-nucleoprotein filament is a critical parameter to establish checkpoint adaptation. The deletion of DNA helicase *SRS2* is sensitive to genotoxic agents and failed to adapt from DNA damage checkpoint mediated cell cycle arrest, slow growth, chromosomal loss and hyper recombination [*Klein HL, 2000; Vaze, MB, et. al., 2002*]. Interestingly, Srs2 uses the free energy from ATP hydrolysis to dislodge Rad51 from the presynaptic filament and deletion of RAD51 gene suppresses *srs2*Δ phenotypes [*Krejci L, et. al., 2003*]. Thus we asked whether the deletion of DNA helicase *SRS2* and presynaptic filament binding protein *RAD51* affect the phosphorylation of Rdh54 protein in response to HO induced irreparable DSB.

Cells of the Y454 (wild type) and Y814 (*srs2*Δ), both carrying *RDH54*-3HA gene, were grown in YP + raffinose and galactose was added to induce the DSB formation. Samples were taken at the indicated time points and Rdh54 and Rad53 proteins were analyzed by Western blotting. In **Figure 7A** we observed that in *srs2*Δ cells both Rdh54 and Rad53 proteins phosphorylation are maintained until after 20 hours since DSB formation, indicating that defect in switching off checkpoint signaling through adaptation leads to the maintenance of Rdh54 phosphorylation.

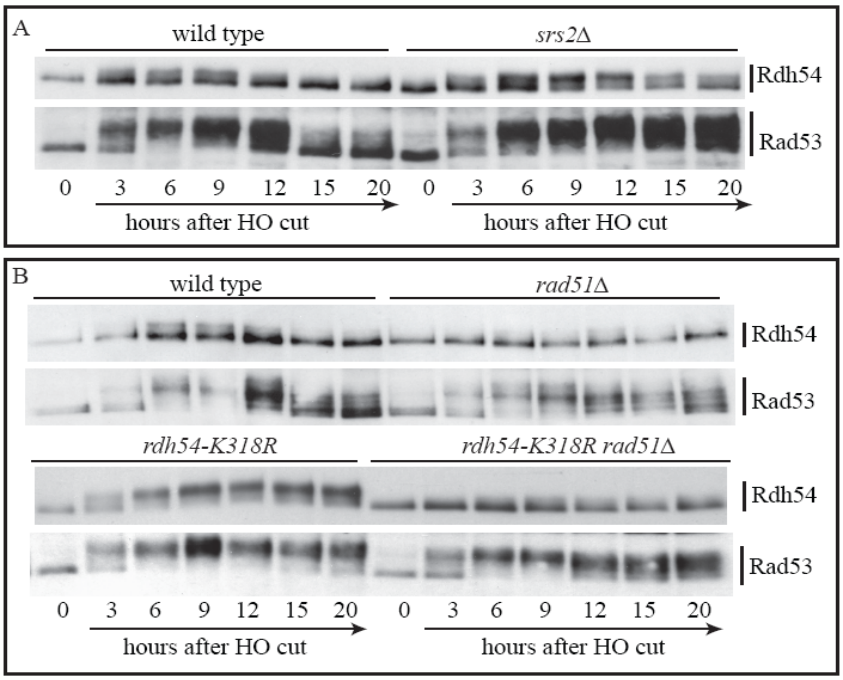

Figure 7. Rad51-nucleoprotein filament is required for the DSB-induced Rdh54 phosphorylation.

The indicated strains Y454 (wild type), Y814 (*srs2*Δ), Y740 (*rad51*Δ), Y 841 (*rdh54K318R*) and Y873 (*rdh54K318R rad51*Δ) were grown logarithmically in YEP + raffinose medium, galactose was added to induce single irreparable DSB. Samples were collected at indicated time and protein extracts were analyzed by Western blotting. 12CA5 antibodies were used to visualize Rdh54-3HA protein; EL7 monoclonal antibodies were used to test Rad53 protein.

Then we performed the same experiment in *rad51*Δ cells, both in wild type and in *rdh54*-K318R mutant cells (**Figure 7B**). In wild type cells we observed that Rdh54 phosphorylation started to decrease at 12 hours after galactose induction, as in the previous experiment (**Figure 7A**); surprisingly, in *rad51*Δ cells we do not see the same intensity of Rdh54 phosphorylation we observed in wild type cells, although Rad53 gets phosphorylation till the end of the experiment because of the adaptation defect in *rad51*Δ cells. The defect in Rdh54 phosphorylation is particularly evident in the double mutant *rdh54*-K318R *rad51*Δ cells, in which checkpoint adaptation is strongly compromised by the two mutations.

Taken together the results in **Figure 7A** and **7B** indicate that Rad51 protein is necessary to induce the Mec1/ATR-dependent Rdh54 phosphorylation in the presence of DSBs. In *srs2*Δ cells, since checkpoint signaling cannot be switched off and Rad51 cannot be dislodged from DNA, Rdh54 phosphorylation is accumulated. Interestingly, Rad51 is required to recruit Rdh54 to the DSBs [*Kwon Y, et. al., 2008*] and one possibility is that Rdh54 is phosphorylated after it is recruited on to the lesions (see also the **Discussion**).

5. 8. Analysis of Rdh54 recruitment on to DSB:

Several checkpoint and repair proteins are recruited to the DSB sites to facilitate the proper repair mechanism. Previous work demonstrated that Rdh54 is recruited to irreparable DSBs through a Rad51 and Rad52 dependent mechanism (*Kwon Y, et. al., 2008*). We also found that Rad51 is required to promote Mec1-dependent Rdh54 phosphorylation (**Figure 7**). Thus, we were interested to test if the recruitment of Rdh54 to the irreparable DSB site requires Mec1 and the checkpoint signaling. In a preliminary experiment, I compare by ChIP analysis, the recruitment of Rdh54 and the ATPase defective Rdh54-K318R protein variant, which is unable to move along the DNA. Cells of the indicated strains were grown in YP + raffinose and galactose was added to induce the DSB formation. Samples were taken at the indicated time points and processed by ChIP procedures (see Material and Methods). In **Figure 8B**, we observed that both Rdh54 and Rdh54-54K318R protein variant have been recruited on to DSB, indicating that ATP hydrolysis by Rdh54 is not required for such recruitment. Interestingly, we also noted that Rdh54-K318R protein variant has a 2 folds increase in its binding to DSB site than the wild type protein. To explain this result, we are tantalizing to suggest that the Rdh54-K318R protein variant is recruited on to DSB sites and, because of its defect in moving along DNA, it accumulates at the DSB sites. On the contrary, the binding to DSB of the wild type Rdh54 protein is dynamic and transient (see also **Discussion**).

Then, I tested the recruitment to DSB of both Rdh54 and Rdh54-K318R protein variant in *mec1*Δ cells. Cells of the indicated strains were grown and treated as in previous experiment in **Figure 8B**. Surprisingly, in *mec1*Δ cells the binding to DSB of both the wild type and the mutant Rdh54 proteins is affected. Thus the chromatin immunoprecipitation data indicate that the recruitment of Rdh54 protein to the DSB site, which is promoted by Rad51 recombinase, also requires Mec1 activity.

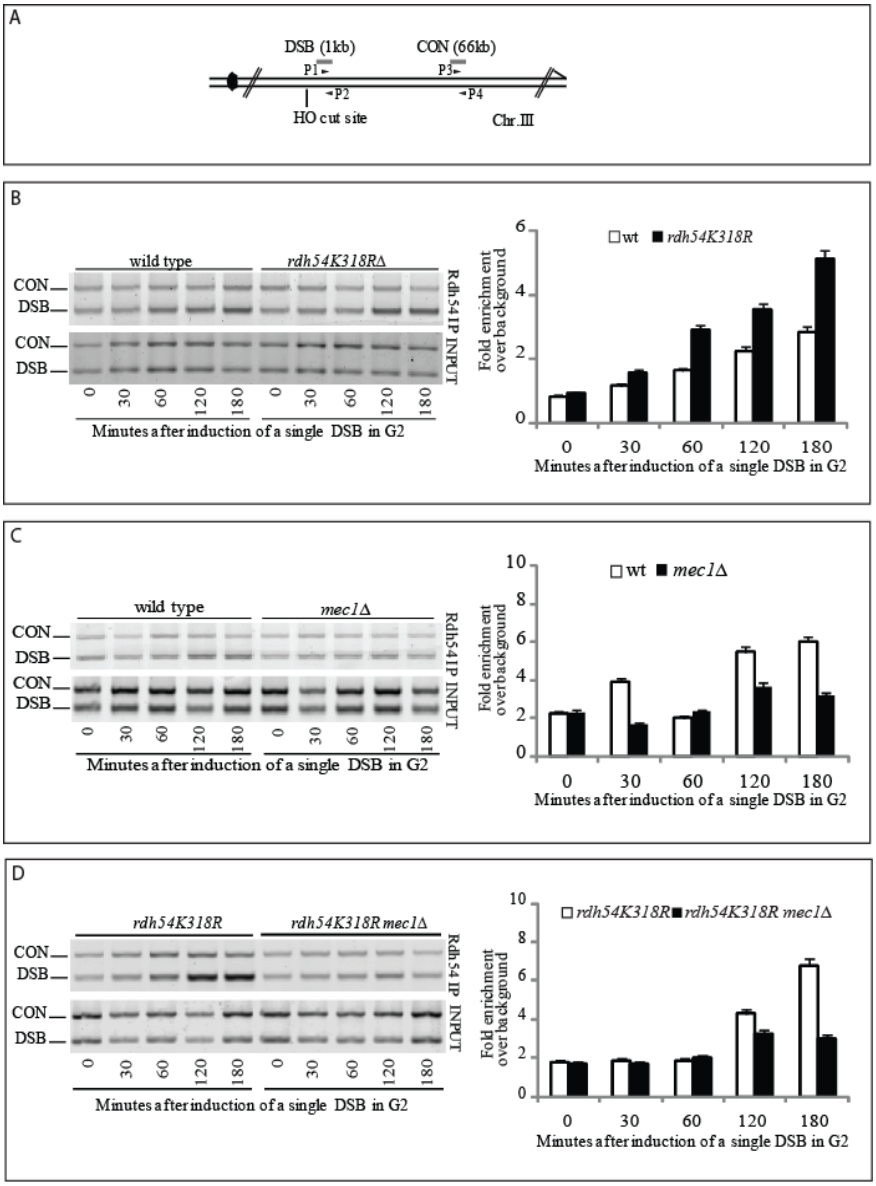

Figure 8. Recruitment of Rdh54 to DSB site.

Schematic representation of the HO cleavage site with the position of the primers used to amplify regions 1 kb (DSB) and 66 kb (CON) from the HO cut site (A). PCR analysis at the CON site is used as a control of background signal. (B-D) YP + raffinose nocodazole arrested cell cultures of wild type JKM and isogenic *mec1*Δ cells expressing the *RDH54*-3HA allele, and cell culture of isogenic *mec1*Δ cells expressing *rdh54K318R-3HA* mutant allele were transferred to nocodazole-containing YP + raffinose + galactose (time 0). Cells were collected at indicated time and subjected to chromatin immunoprecipitation. Representative ChIP time course analysis of protein-DSB association is shown for each protein tested with the relative quantitative PCRs before (Inputs) and after protein immunoprecipitation (IP).

CHAPTER - IV

DISCUSSION

The maintenance of genomic integrity is crucial for the survival of all the living organisms; the compromised integrity of human genome contributes to genetic disorders, aging and cancers. DNA damage checkpoints control the interplay among cell cycle progression and several DNA transactions, including DNA replication, DNA repair, senescence and apoptosis. In the last two decades unicellular budding yeast *Saccharomyces cerevisiae* has been extensively used as a model system to study this complex processes, as its genetic system is highly conserved among the higher eukaryotes. Most cellular processes are regulated by reversible protein phosphorylation, which is controlled by protein kinases and phosphatases. Extensive studies have revealed that kinase–substrate interaction, their subcellular localization, and substrate specificity of protein kinases are all involved in determining their substrates in the cells. In *Saccharomyces cerevisiae* cells with a single double-strand DNA break (DSB) activate ATR/Mec1, which belongs to the structurally unique family of protein serine/threonine kinase whose catalytic domain shares a clear evolutionary relationship with those of mammalian and yeast phosphoinositide 3-kinases (*Hunter 1995; Zakian VA, 1995*). In DNA damage response (DDR), Mec1 plays central role by activating and interacting with the downstream checkpoint cascade proteins to send early signal transmission through cell cycle checkpoints. Mec1 is targeted to single stranded DNA covered by the replication protein A (RPA) through its DNA binding subunit Ddc2, which is a regulatory partner of ATR. The activation of Mec1 kinase activity requires the proper loading of the 9-1-1 complex (composed of Rad17, Mec3 and Ddc1) by its clamp loader Rad24-RFC onto partial duplex DNA [*Majka J, et. al., 2006*]; both these sensors independently recognize the DNA damage and their colocalization greatly enhance the activation of Mec1 [*Melo JA, et. al., 2001*]. Then DNA damage signal is relayed through the damage specific mediator Rad9 to the effector kinase Rad53, which finally activate the full checkpoint response [*Harper JW and Elledge SJ, 2007*].

The checkpoint inactivation is a process by which the cell cycle restarts after the successful completion of repairing the damaged DNA. In response to single site specific HO induced DSB, budding yeast can activate two different cellular processes: i) by "*Adaptation*" the cell can restart the cell cycle progression even though the damage is not repairable; ii) by "*Recovery*" the cell repairs the damaged site and restart the normal cell cycle. Both these physiological processes are

regulated by several checkpoint proteins and phosphatases, which have been identified and characterized in budding yeast.

In this Doctoral Thesis, I reported that the budding yeast Rdh54/Tid1 DNA translocase, which is involved in recombination and checkpoint adaptation, is phosphorylated in response to DSB formation by the ATR/Mec1 dependent signaling pathway. It has been shown that in mitotic cells Rdh54 proteins localized constitutively in the kinetochores, but in response to DNA damage they move to the Rad52 foci [*Lisby M, et. al., 2004*]. I found that Rdh54 is phosphorylated in the presence of DNA damage (**Figure 1B and 1C**), and such phosphorylation occurs only in G2 cells (**Figure 2B**). Similarly, it has been shown that Srs2, which is a DNA helicase/translocase required for proper recombination and checkpoint adaptation [*Vaze MB, et. al., 2002*), is regulated in response to DNA damage through phosphorylation only in S/G2 phase, and it is not phosphorylated in G1 phase even if Mec1-signaling is very active [*Liberi G, el. al., 2000*]. Further, treatment with caffeine, which is a specific PIKKs inhibitor, or the analysis of *mec1* and *rad53* mutant cells, indicated that Rdh54 phosphorylation requires an active Mec1-dependent signaling (**Figure 4A and 4B**).

ATM/ATR-like kinases preferentially phosphorylate their substrates on serine or threonine residues that precede glutamine residues, the so called (S/T)-Q motif. I collected evidences that Rdh54 protein, immunoprecipitated from cells with irreparable DSBs, is phosphorylated at (S/T)-Q motifs (**Figure 4C**). Indeed, inspection of Rdh54 protein sequence reveals the presence of 11 consensus (S/T)-Q motifs (**Figure 9**), and some of those sites can be potentially phosphorylated by Mec1 or Tel1 kinases. Our genetic results in *mec1*Δ cells indicated that Mec1 plays the major role (**Figure 5B**).

Recently it has been shown by *in vitro* assay that Rdh54 and its ortholog Rad54 are phosphorylated by meiotic specific kinase Mek1 (*Niu H, et. al., 2009*). Mek1 plays important regulatory role in promoting inter-homologue recombination in meiotic cells, and it is regulated by Mec1 in the pachytene checkpoint [*Niu H, et. al., 2005; Bailis JM and Roeder GS, 2000*]. Mek1 protein has a kinase domain and a FHA interacting domain similar to Rad53 kinase and it is considered a functional ortholog of Rad53 in meiotic cells [*Niu H, et. al., 2005].* It was shown by mass spectrometry that Rad54 and Rdh54 are phosphorylated by Mek1 in the N-terminal region of the protein (T58, T132 and T231 in the case of Rad54; S85 and T89 for Rdh54) [*Niu H, et. al., 2009*]. In the case of Rdh54, the numbering of the amino acid residues is misleading because of

previous mistakes in mapping the first ATG of the gene. Therefore, S85 and T89 should be correctly numbered as S51 and T55, respectively. In meiotic cells, it was also shown that Mek1 phosphorylates Rad54 at T132 *in vivo;* indeed, this amino acid residue is highly conserved in the Rad54 orthologs of fungi and nematode, and it is also conserved in human and in fruit flies. In diploid *dmc1*Δ cells the overexpresssion of multicopy *Rad54-T132A* mutant produce more than 10 times as many asci as Rad54, majority of spores are viable suggesting that this mutant promotes interhomologue recombination in the absence of *dmc1*Δ [*Niu H, et. al., 2009*]. Whereas the Rdh54-S51A and T55A nonphosphorylatable mutants complement the sporulation and spore viability defects of *rdh54*Δ, but they did not improve the sporulation of *dmc1*Δ [*Niu H, et. al., 2009*], suggesting that the phosphorylation of these sites is not important for the *in vivo* role of Rdh54 in meiotic cells. Anyway, the *in vivo* phosphorylation of Rdh54 was never shown directly, raising the possibility that the Mek1-dependent phosphorylation was induced only by the *in vitro* kinase assay [*Niu H, et. al., 2009*]. In the case of Rad54, the Mek1-dependent phosphorylation at the N-terminal region of the protein interferes the interaction with Rad51, preventing recombination between sister chromosomes [*Niu H, et. al., 2009*].

At the moment, we do not know if the *in vivo* Mec1-dependent Rdh54 phosphorylation we described in this work does also occur in meiotic cells; however it will be interesting to test by sites-specific mutagenesis of the phosphorylation residues if it is an important event in regulating the interaction between Rad51 and Rdh54, influencing Rdh54 functions both in mitotic and meiotic cells. In mitotic cells, Rdh54 is also required for checkpoint adaptation and we are working to understand whether Mec1 and/or Rad53 dependent phosphorylation is involved in this process. It is also possible that such phosphorylation event may interfere with ATPase activity of Rdh54. In that case, we expect that phosphosite mutants may affect both recombination and checkpoint adaptation. Indeed, it was already shown that the ATPase defective mutation *rdh54*-K318R prevents adaptation to irreparable DSB (**Figure 5C)** and [*Lee SE, et. al., 2001*], and causes sensitivity to MMS *[Chi P, et. al., 2006]*, although the protein is phosphorylated in the presence of DNA damage. Interestingly, I found that Rdh54-K318R protein variant is phosphorylated in the presence of DNA damage and it became hyper-phosphorylated likely because it remained stuck in some intermediate which is exposed to Mec1-signaling for prolonged time. In fact the Rdh54-K318R protein variant does not retain the capacity to move along DNA, and we found that its loading to DSB lesion increases (**Figure 8D**).

I also found that the inhibition of CDK1 in G2 cells by overexpressing *SIC1* affects the DSB-dependent Rdh54 phosphorylation (**Figure 6**); raising the possibility that Rdh54 is regulated through phosphorylation by multiple kinases, including Mec1, Rad53 and CDK1. Interestingly, it was shown that Srs2, which is required to dislodge Rad51 from DNA (*Krejci L, et. al., 2003; Antony E, et. al., 2009*], is regulated by Mec1, Rad53 and CDK1 phosphorylation events [*Liberi G, et. al., 2000*]. Inspection of Rdh54 protein sequence revealed the presence of many (S/T)-P motifs, the typical consensus for CDK1. Anyway, CDK1 inhibition may affect indirectly the Rdh54 phosphorylation by preventing DSB resection and Mec1 activation [*Ira G, et. al., 2004*]. Therefore, to address if CDK1 phosphorylates directly Rdh54 protein, biochemical evidences are required.

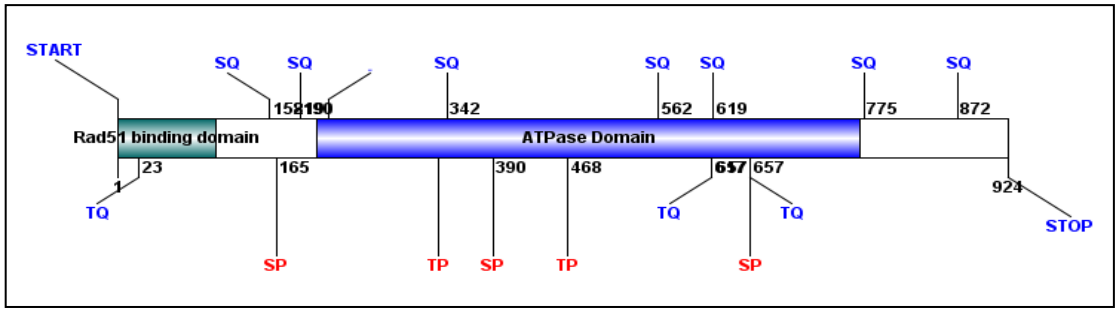

Figure 9. (S/T)-Q are typical consensus for ATM and ATR; (S/T)-P are CDK1 phosphorylation motif.

In conclusion, a working model (**Figure 10**) can be proposed to summarize the data I described in this Thesis on Rdh54 protein.

Assembly of Rad51 on to resected DSB is needed to search for the homologous sequence during recombination-based repair. *In vitro* studies have shown that Rdh54 through its N-terminal domain interacts with Rad51 recombinase to catalyze the removal of Rad51 from DNA. Moreover, it was shown that Rad51 is an important factor to recruit Rdh54 on to DSB, likely because it serves a sort of platform through which Rdh54 interacts. Once Rdh54 is recruited on to DSB, it may contribute to regulate Rad51-based recombination and, moving along DNA, it may also contribute to remodel chromatin around the DSB lesions, likely affecting checkpoint response and adaptation. We can hypothesis that Rdh54 is phosphorylated by Mec1 and Rad53 when it is recruited on to DSB. Indeed, Rdh54 phosphorylation is affected in *rad51*Δ cells, likely because it is not stably recruited on to the DNA lesion. Moreover, the Mec1-dependent Rdh54 phosphorylation may have a role in maintaining Rdh54 binding to DSB, as we found that its

loading is affected in *mec1*Δ cells (**Figure 8C**). In meiotic cells, the meiosis specific Mek1 signaling on *Rad54T132* residue down regulates the interaction between Rad51-Rad54, preventing recombination between sister chromosomes [*Niu H, et. al., 2009*]. From our results in mitotic cells responding to DNA damage, we can hypothesize that checkpoint pathway may affects the balancing of recombination rate and the recombination partner choice, likely down-regulating interhomologous recombination. Interestingly, mutations in checkpoint factors cause an increase in recombination between non sister chromatids in mitotic cells [*Klein HL, 2001*], supporting the notion that checkpoint signaling may influence recombination pattern choice.

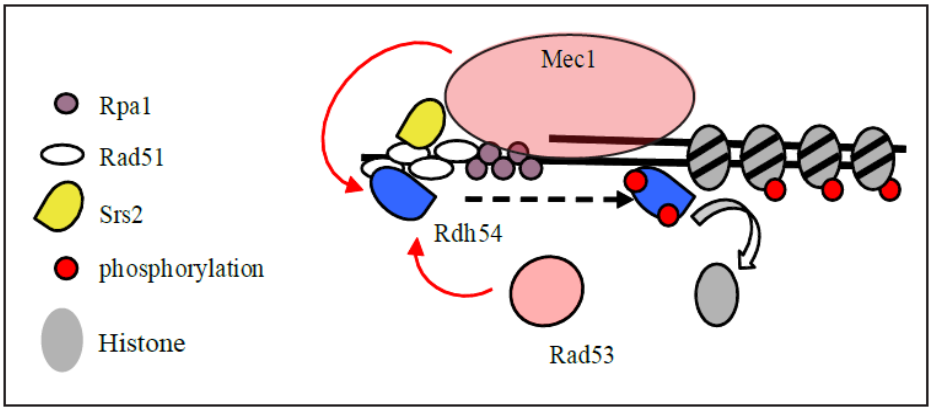

Figure 10. Scheme of Rdh54 functions and regulations at the DSB lesion.

In conclusion, taken together the results presented in this Thesis, along with the previous genetic observations, provide novel insights on the regulation of Rdh54 translocase, also suggesting a plausible mechanism by which checkpoint signaling affect recombination in cell responding to DNA damage.

CHAPTER - V

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PART II

Publications Contributed:

I have attached two published articles in which I have contributed as co author.

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Saccharomyces CDK1 Phosphorylates Rad53 Kinase in Metaphase, Influencing Cellular Morphogenesis*[S]

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Rad53 is an essential protein kinase governing DNA damage and replication stress checkpoints in budding yeast. It also appears to be involved in cellular morphogenesis processes. Mass spectrometry analyses revealed that Rad53 is phosphorylated at multiple SQ/TQ and at SP/TP residues, which are typical consensus sites for phosphatidylinositol 3-kinase-related kinases and CDKs, respectively. Here we show that Clb-CDK1 phosphorylates Rad53 at Ser^{774} in metaphase. This phosphorylation event does not influence the DNA damage and replication checkpoint roles of Rad53, and it is independent of the spindle assembly checkpoint network. Moreover, the Ser-to-Asp mutation, mimicking a constitutive phosphorylation state at site 774, causes sensitivity to calcofluor, supporting a functional linkage between Rad53 and cellular morphogenesis.

Protein kinases are fundamental regulators of cellular metabolism and cell cycle progression. Therefore, understanding how they are regulated is a very important challenge for the comprehension of several cellular pathways.

Saccharomyces cerevisiae Rad53 is a serine/threonine/tyrosine kinase, and it is well established that Rad53 family members, including the Chk2 protein kinase in human cells, play a central role in the signal transduction pathway activated in response to DNA lesions and help prevent genome rearrangements and cancer (1, 2). Rad53 is phosphorylated by the upstream phosphatidylinositol 3-kinase-related kinases Mec1 and Tel1 (ATR and ATM in human cells); this triggers autophosphorylation events, leading to full activation of the kinase (3). In addition to the kinase domain, Rad53 presents two FHA domains through which the protein interacts with substrates and regulators (4). Moreover, Rad53 also contains a bipartite NLS domain in the C-terminal region (5, 6), required for efficient translocation of Rad53 into the nucleus, where it exerts its checkpoint functions as a guardian of the genome.

Rad53 has been reported to be also phosphorylated in a Mec1-independent manner in response to spindle damage, but the kinase responsible for this modification has not been described (7). However, it is unlikely that Rad53 kinase activity plays a role in response to spindle damage because Rad53, prepared from cells treated with nocodazole (an agent that cause spindle depolimerization and triggers the spindle assembly checkpoint), does not exhibit autophosphorylation activity, as determined by *in situ* kinase assay (8); moreover, *rad53*Δ cells are not sensitive to spindle-damaging agents.

Finally, Rad53 appears to be involved in cellular morphogenesis (9, 10). In fact, *rad53*Δ cells have an abnormal shape and are sensitive to morphogenesis-stressing agents (9). Genetic evidence suggests that the roles of Rad53 in morphogenesis are not dependent on Mec1-dependent phosphorylation, and it is unknown whether the role of Rad53 in morphogenesis is regulated during the cell cycle.

Mass spectrometry analysis revealed that Rad53 is phosphorylated at many serine and threonine sites, and, interestingly, it is phosphorylated at proline-directed Ser^{175}, Ser^{375}, and Ser^{774} sites in exponentially growing cells, without any DNA and/or spindle damage (5, 11). These findings suggested that the CDK1 cell cycle kinase may directly phosphorylate Rad53 and influence its activity, as supported by recent observations implicating CDK1 in DNA damage checkpoint activation (12, 13).

Here, we provide genetic and biochemical evidence that CDK1 phosphorylates Rad53 in metaphase, without any damage to the spindle or genomic DNA, and that most of the modification that had been previously described as induced by spindle damage (7) is instead due to the accumulation of cells in metaphase induced by the spindle-damaging agent. The Ser^{774} residue is the main target for CDK1-dependent Rad53 modification in metaphase. Interestingly, the Ser-to-Asp mutation of residue 774, mimicking a constitutively phosphorylation state, causes sensitivity to calcofluor (a morphogenetic stress agent), further suggesting a role for Rad53 in controlling cellular morphogenesis. Our findings raise the possibility that the role of Rad53 in morphogenesis (9) may be modulated during the cell cycle by CDK1. Furthermore, none of the CDK1-dependent phosphorylation events occurring on Rad53 at metaphase appear to have any role in promoting Rad53 activation in response to DNA damage or replication stress.

EXPERIMENTAL PROCEDURES

Yeast Strains and Cultures—All strains are isogenic derivatives of W303. The genotypes of the yeast strains utilized in this

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Fig. S1.

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study are listed in supplemental Table S1. To obtain strains Y849 and Y850, plasmid pVF6 (generously provided by F. Vanoli and M. Foiani), carrying the *cdc28*-as1 mutant allele, was cut with ClaI and integrated in the *CDC28* locus.

All the experiments were done in YPD medium containing 10 g yeast extract, 20 g peptone, glucose 2% final concentration, H_2O to 1 liter; the pH was adjusted to 5.4 with HCl. *E. coli* strain (DH5α) was used for the production of mutants and cloning; *E. coli* cultures were grown in LD medium containing 10 g bactotryptone, 5 g yeast extract, 5 g NaCl, H_2O to 1 liter, and the pH was adjusted to 7.25.

Construction of Rad53 Mutants—The plasmid pCH10, carrying *RAD53*-9myc under the control of its own promoter (14), was cut with Not1 and ligated to obtained the pCla6 plasmid, carrying *RAD53* without the 9myc epitope cassette. pCla6 was used in PCR-based site-directed mutagenesis to generate the mutations *rad53*-S175A/S175D, *rad53*-S375A/S375D, and *rad53*-S774A/S774D. Two complementary oligonucleotides (listed in supplemental Table S3) containing the selected mutation were used to amplify the plasmid pCla6. The PCR product was subsequently digested with DpnI to eliminate the wild type template, and the obtained DNA was used to transform *E. coli* DH5α cells. The resulting plasmids (listed in supplemental Table S2) were verified by DNA sequencing. This kind of mutagenesis has been described previously (15). To construct yeast strains, standard genetic procedures for transformation and tetrad analysis were followed.

Western Blot Analysis—The TCA protein extraction and the Western blot procedures have been described previously (15). The Rad53 protein was analyzed using the specific monoclonal antibodies (Mab.EL7) that we have recently produced (15). In some experiments (Fig. 3), the SDS-PAGE analysis was performed in a larger apparatus and at 4 °C, conditions that improved the separation of the Rad53 phosphorylated isoforms.

In Vitro Dephosphorylation Assay—Crude extracts were prepared as described (14) and resuspended in λ phosphatase buffer with or without 4000 units of λ phosphatase (Biolabs). Samples were incubated for 30 min at 30 °C and resuspended in Laemmli buffer.

Cell Synchrony and Flow Cytometry—Cells were presynchronized in G_1 with α-factor (2 μg/ml) and then released in fresh medium. Cells were arrested in G_1 and G_2/M with α-factor (10 μg/ml) or nocodazole (15 μg/ml), respectively. DNA content was analyzed by FACSCalibur (Bekton-Dickinson) and CellQuest software (Bekton-Dikinson).

RESULTS

CDK1 Promotes Rad53 Phosphorylation in Nocodazole-treated Cells—Several amino acid residues of the Rad53 protein have been found to be phosphoryated *in vivo*. Most of these modifications occur in cells treated with DNA damaging agents and likely drive full activation of the kinase toward specific substrates (5, 11). However, mass spectrometry analysis has revealed that some Rad53 residues are phosphorylated even in cells growing in unperturbed conditions, raising the possibility that Rad53 protein is regulated throughout a normal cell cycle (5, 11).

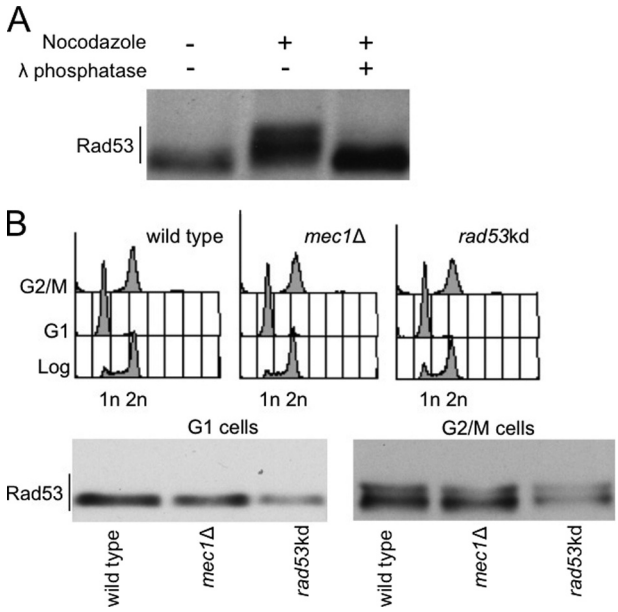

FIGURE 1. **Analysis of Rad53 phosphorylation in nocodazole-blocked cells.** *A*, Western blot analysis of protein extracts prepared from wild type cells 3 h after treatment with/without nocodazole (15 μg/ml). Samples were treated with/without λ phosphatase before gel electrophoresis. Rad53 protein was tested with Mab.EL7 monoclonal antibodies. *B*, exponentially growing cells of wild type, *mec1*Δ, *rad53*-kd strains were treated for 3 h with α-factor (10 μg/ml) or nocodazole (15 μg/ml) to block cell cycle in G_1 or G_2/M, respectively. Samples were taken and processed to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies.

Interestingly, we observed a slower migrating Rad53 isoform in nocodazole-treated cells in the absence of any DNA damage (Fig. 1*A*). This nocodazole-induced Rad53 isoform is due to phosphorylation events because it is reverted by *in vitro* treatment with λ phosphatase (Fig. 1*A*). Our results, together with previous findings (7, 8, 12), indicate that Rad53 is phosphorylated in cells responding to spindle defects caused by the nocodazole treatment.

Because the genetic requirements for the nocodazole-induced Rad53 modification has not been extensively studied, we then analyzed whether such Rad53 modification was due to *in trans* phosphorylation events, mediated by specific kinases, or whether it had to be ascribed to autophosphorylation. Taking advantage of the biochemical features of the Rad53-K227A (Rad53-kd) kinase-defective protein variant (6), which receives *in trans* phosphorylation from the upstream kinase Mec1 but is defective in the autophosphorylation reaction (14), we analyzed Rad53 modification in *mec1*Δ or *rad53*-kd cells arrested with α-factor or nocodazole. Exponentially growing cells (Fig. 1*B*) have been blocked with α-factor or nocodazole in G_1 or M phase, respectively; cell cycle arrest was confirmed by FACS analysis (Fig. 1*B*). By Western blotting, Rad53 does not show any gel mobility shift in G_1-blocked cells. On the other hand, the protein is clearly phosphorylated in nocodazole-treated cells, and this modification is not mediated by Mec1, in agreement with previous indications (7), or by autophosphorylation events. In fact, the retarded form of Rad53 observed in metaphase-arrested cells is still evident in *mec1*Δ and *rad53*-kd cells.

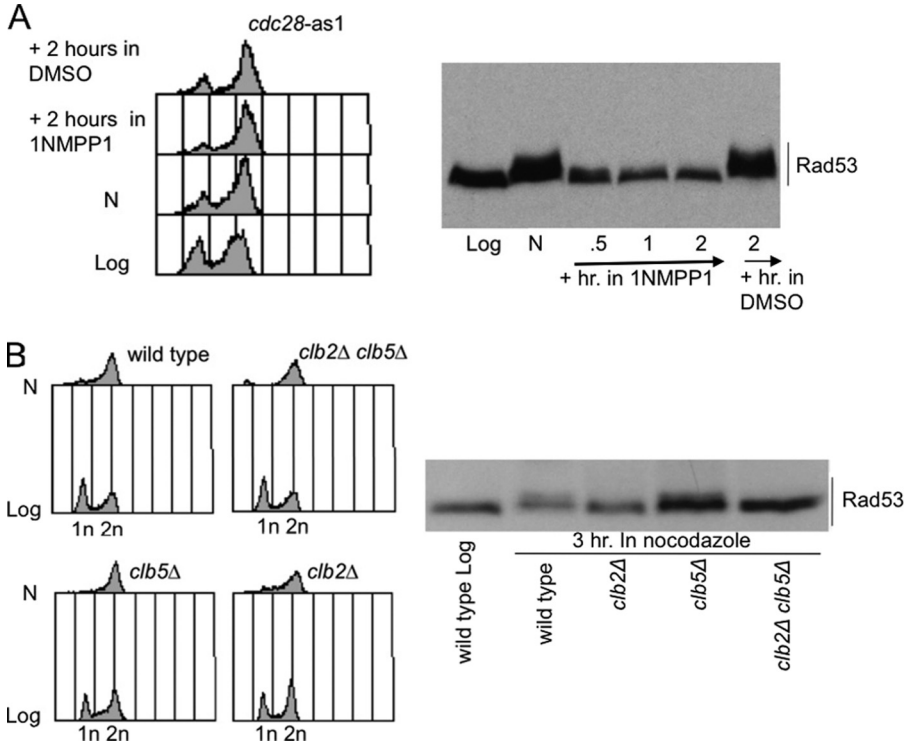

FIGURE 2. **CDK1 is required for nocodazole-induced Rad53 phosphorylation.** *A*, exponentially growing *cdc28*-as1 cells were treated for 3 h (*lane N*) with nocodazole (15 μg/ml). 1NMPP1 (dissolved in dimethyl sulfoxide (*DMSO*)) was then added to one-half of the culture. Samples were taken at the indicated time points to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies. *B*, exponentially growing cell cultures of wild type and isogenic *clb2*Δ, *clb5*Δ, and *clb2*Δ*clb5*Δ strains were treated for 3 h (*N*) with nocodazole (15 μg/ml). Samples were taken to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies.

It is known that nocodazole treatment causes spindle damage and cell cycle block in metaphase due to activation of the spindle assembly checkpoint (SAC)[3] (16). SAC prevents activation of the anaphase-promoting complex, whose activity is required to inhibit Clb-CDK1; indeed, nocodazole-treated cells are characterized by high activity of the Clb-CDK1 complex (17). We thus decided to explore the possibility that Clb-CDK1 may be responsible for Rad53 phosphorylation in metaphase.

In *S. cerevisiae*, the catalytic subunit of the Clb-CDK1 complex is expressed by the essential gene *CDC28* (18). We took advantage of the *cdc28*-as1 mutant, sensitive to the ATP analogue 1NMPP1 (19), to inhibit CDK1 activity in nocodazole-treated cells. Presently, this mutation, used in conjunction with the 1NMPP1 molecule, is the most tunable way to specifically switch off CDK1 activity. Indeed, it has been demonstrated that 1NMPP1 does not inhibit wild type CDK1 or other kinases at the experimental concentrations used, while specifically inhibiting CDK1 activity in the *cdc28*-as1 mutant background, that has been engineered to become a specific target of 1NMPP1 (19). *cdc28*-as1 exponentially growing cells were treated with nocodazole for 3 h to induce cell cycle arrest and phosphorylation of Rad53 protein. We then divided the cell culture in two parts and added 1NMPP1 to one-half to inhibit Cdc28 kinase. Western blot analysis (Fig. 2*A*) revealed that inhibition of CDK1 rapidly leads to the disappearance of phosphorylated Rad53 isoforms, indicating that CDK1 activity is required to maintain Rad53 phosphorylation in nocodazole-treated cells.

CDK1 activity is cell cycle-regulated and its specificity toward substrates is determined by the association with various cyclin subunits (18). Cyclins Cln1–3 are specific for G_1 phase, Clb5 and 6 are mainly required for DNA replication, and Clb1–4 are more important in G_2/M and exit from mitosis. To determine which cyclin is involved in cells responding to nocodazole, we studied Rad53 phosphorylation in *clb5*Δ, *clb2*Δ, and *clb2*Δ*clb5*Δ double mutants. Cells were treated with nocodazole for 3 h, and Rad53 phosphorylation was analyzed by Western blotting. We found that Rad53 phosphorylation is prevented in *clb2*Δ and *clb2*Δ*clb5*Δ cells, but it is maintained in *clb5*Δ cells (Fig. 2*B*). Taken together, the results described in Fig. 2 indicate that CDK1 kinase, mainly associated with cyclin Clb2, is required for Rad53 phosphorylation in cells treated with nocodazole.

CDK1 Kinase Promotes Rad53 Phosphorylation in Metaphase, also in the Absence of Spindle Defects—The results described so far may indicate a direct effect of CDK1 on Rad53, or they might be explained by assuming that nocodazole treatment activates the SAC, and one or more kinases of this pathway may phosphorylate Rad53 in a CDK1-dependent manner. Indeed, mutations in spindle assembly checkpoint factors, such as *mad2*Δ and *bub2*Δ, prevent nocodazole-induced Rad53 phosphorylation (7). However, abrogation of the spindle assembly checkpoint through specific mutations also eliminates the cell cycle block at metaphase caused by nocodazole treatment, preventing the possibility to maintain high levels of Clb-CDK1 activity. Moreover, previous work (7) failed to identify a kinase among the spindle assembly checkpoint factors that is responsible for the nocodazole-induced Rad53 phosphorylation. Thus, we investigated the possibility that CDK1 may directly induce Rad53 phosphorylation at the metaphase-anaphase transition independently on SAC activation. To visualize CDK1-dependent Rad53 phosphorylation through a normal cell cycle, wild type cells were synchronized in G_1 by α-factor treatment and released into fresh medium without the pheromone. Cell cycle progression after the release from the G_1 block was followed by FACS analysis (Fig. 3*A*). Rad53 protein level and its modifications were analyzed (Fig. 3*B*) using the

[3] The abbreviations used are: SAC, spindle assembly checkpoint; FACS, fluorescence-activated cell sorter; kd, kinase defective.

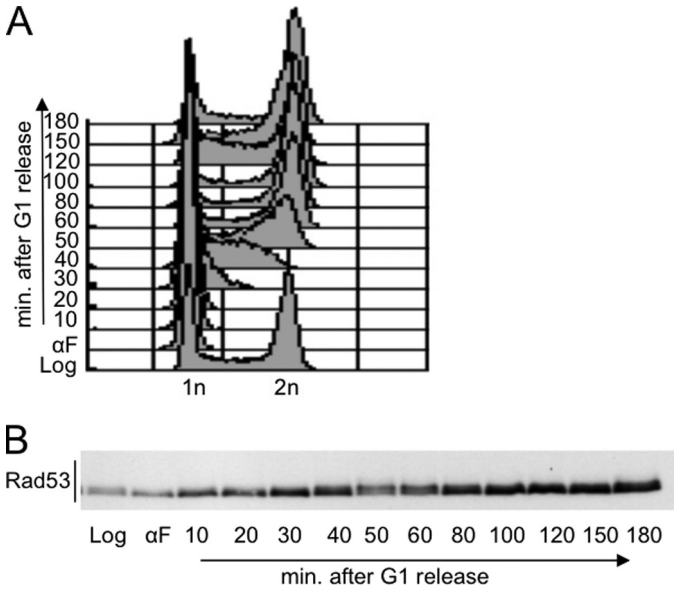

FIGURE 3. **Analysis of Rad53 phosphorylation throughout the cell cycle.** Exponentially growing wild type cells were presynchronized in G_1 by α-factor (αF) treatment and released from the G_1 block in fresh YPD medium. Samples were taken at the indicated time points to test FACS profiles (*A*) and Rad53 phosphorylation by Western blot (*B*) with Mab.EL7 monoclonal antibodies.

highly specific Mab.EL7 monoclonal antibody that we recently described (15).

Fig. 3*B* shows that the monoclonal antibody Mab.EL7 detects a modification of Rad53 in samples taken at different time points during an unperturbed cell cycle. The slower migrating isoform of Rad53 is accumulated at time points corresponding to the G_2/M transition, and it is not visualized in G_1 and at the early stage of S phase, as supported by the FACS profiles (Fig. 3*A*). The possibility that this cell cycle-dependent Rad53 modification may be associated to the kinase activity of Rad53 itself is unlikely. In fact, by using a highly sensitive *in situ* assay, we and others (8, 14) failed to gain any evidence indicating that Rad53 kinase activity may fluctuate during an unperturbed cell cycle.

The cell cycle-dependent modification of Rad53 at G_2/M under unperturbed conditions, suggests that the Rad53 phosphorylated form detected after nocodazole treatment is simply related to a G_2/M cell cycle block, rather than to SAC activation. Therefore, we assumed that the accumulation of the CDK1-dependent phosphorylated form of Rad53 could be better visualized in a cell population uniformly arrested in mitosis.

To block cell cycle in metaphase without triggering the SAC, we took advantage of a genetic system in which we can deplete Cdc20 by switching off the expression of the corresponding gene. Indeed, Cdc20 is an unstable protein required to activate anaphase-promoting complex and drive the exit from mitosis (20). In this strain, expression of *CDC20* under the control of the inducible *GAL1* promoter is repressed by addition of glucose into the culture medium. Because Cdc20 is one of the final targets of the spindle assembly checkpoint cascade, this genetic system is widely used to block cell cycle progression in metaphase, mimicking the effect of the spindle assembly checkpoint activation without generating any damage to the spindle (20).

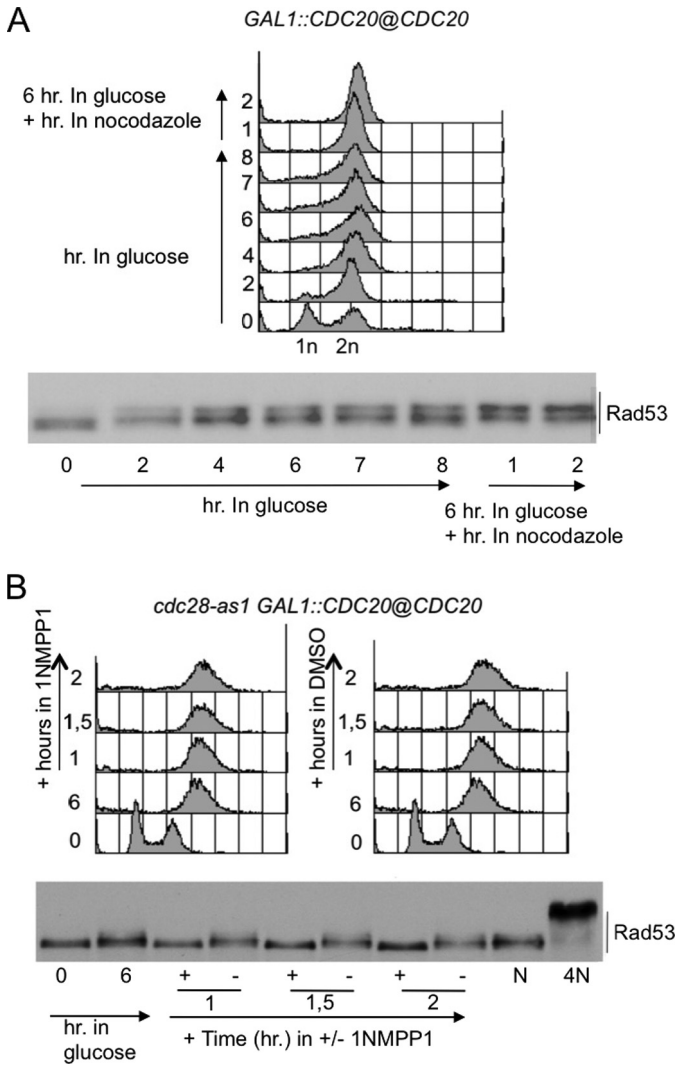

FIGURE 4. ***CDC20* shut-off leads to CDK1-dependent Rad53 phosphorylation in metaphase.** *A*, yeast extract peptone and galactose cells of the *GAL1*::*CDC20*, carrying endogenous *CDC20* gene under *GAL1* promoter, were treated with 2% glucose to shut off the *GAL1* promoter. 6 h after glucose addition, a small aliquot of the culture was treated with nocodazole (15 μg/ml). Samples were taken at the indicated time points to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies. *B*, yeast extract peptone and galactose cells of the *cdc28*-as1 *GAL1*::*CDC20*, were treated with 2% glucose to shut off the *GAL1* promoter. 6 h after glucose addition, 1NMPP1 (dissolved in dimethyl sulfoxide (*DMSO*)) was added to one-half of the culture. Aliquots of the culture were treated with nocodazole (15 μg/ml for 3 h; *lane N*) or 4NQO (2 μg/ml for 30 min., *lane 4N*). Samples were taken at the indicated time points to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies.

Addition of glucose to exponentially growing *GAL1*::*CDC20* cells represses the *CDC20* expression, leading to a progressive accumulation of cells in metaphase due to the inability to activate anaphase-promoting complex as a consequence of Cdc20 depletion. At different times after glucose addition, samples were taken to monitor FACS profiles and Rad53 protein levels and modifications. Fig. 4*A* shows that Rad53 phosphorylation correlates with the accumulation of cells in mitosis. Moreover, treatment with nocodazole 6 h after Cdc20 depletion only led to a mild but noticeable increase in the fraction of cells blocked in M phase and in the intensity of the Rad53-phosphorylated band. These results suggest that the SAC is not involved in

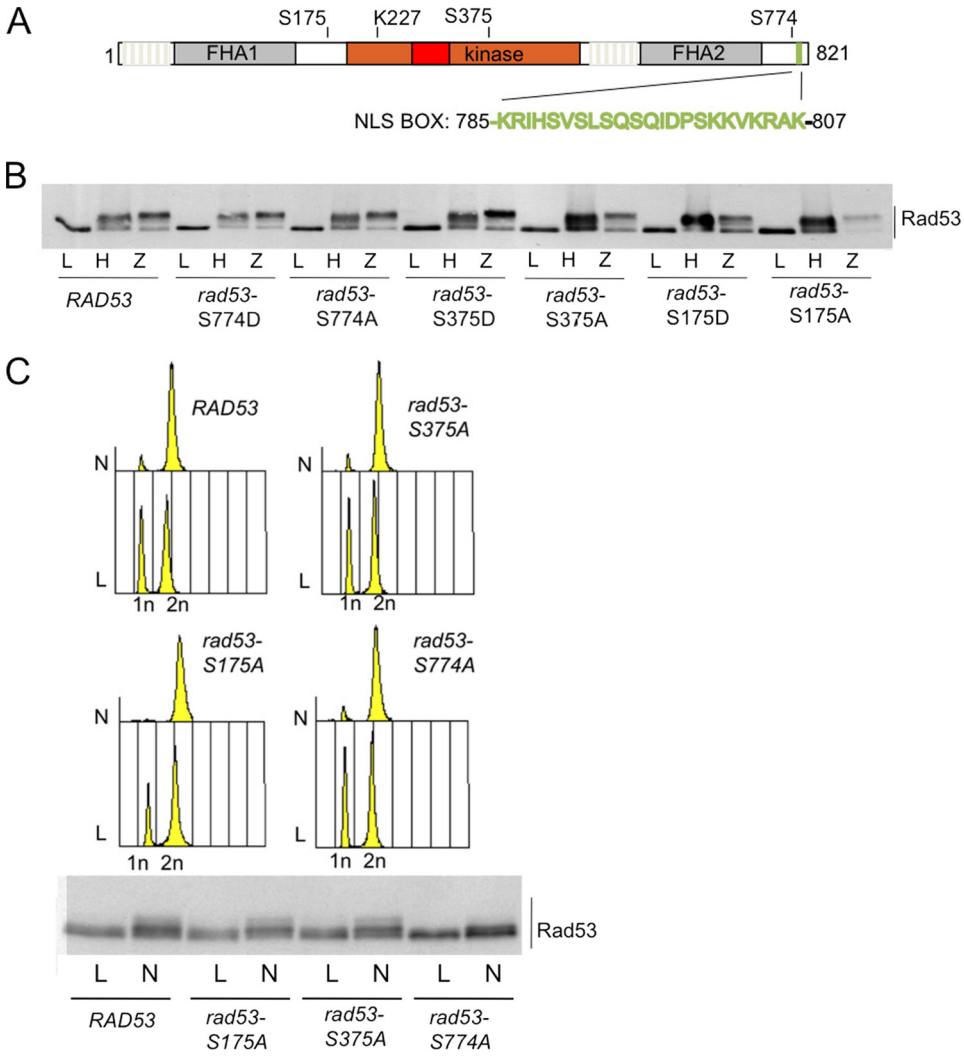

FIGURE 5. **CDK1 phosphosites mutagenesis of *RAD53*.** *A*, schematic representation of Rad53 protein showing the phosphorylatable proline-directed serine sites (Ser[175], Ser[375], and Ser[774]). Relevant Rad53 domains are indicated as follows: *gray boxes* indicate the fork head domains (FHA1 and FHA2); the *red box* inside the kinase domain (*brown*) represents the activation segment; the *green box* at the carboxyl end of the protein represents a bipartite nuclear localization signal. *B*, analysis of the phosphorylation state of Rad53 protein in various *rad53* alleles, as indicated. Exponentially growing cell cultures of *rad53*Δ *sml1*Δ strains, carrying the indicated *rad53* allele under its own promoter on a centromeric plasmid, were treated for 3 h with hydroxyurea (200 mM, *lane H*) or for 30 min with zeocine (50 μg/ml, *lane Z*). Samples were taken to test Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies. Please note that *lane Z* for *rad53*-S175D was accidentally underloaded. *C*, exponentially growing cell cultures of *rad53*Δ *sml1*Δ strains, carrying the indicated *rad53* allele under its own promoter on a centromeric plasmid, were treated for 3 h (*N*) with nocodazole (15 μg/ml). Samples were taken to test FACS profiles and Rad53 phosphorylation by Western blot with Mab.EL7 monoclonal antibodies.

phosphorylating Rad53 in mitosis, whereas it sustains the idea that CDK1, whose activity is high in *CDC20*-depleted cells, is directly involved in phosphorylating Rad53.

To test whether the Rad53 phosphorylation detected in cells blocked by *CDC20* depletion was due to CDK1 activity, we repeated the previous experiment in a *GAL1*::*CDC20* strain carrying the *cdc28*-as1 mutation (Fig. 4*B*). 6 h after the addition of glucose, the culture was split into two, and the 1NMPP1 inhibitor was added to half of the culture. Fig. 4*B* clearly shows that inactivation of CDK1 prevents Rad53 phosphorylation in cells arrested in mitosis. The findings described above indicate that high activity of the Clb-CDK1 kinase complex in mitosis promotes Rad53 phosphorylation, similarly to what is found in nocodazole-treated cells, also suggesting that Rad53 phosphorylation induced by nocodazole treatment is due, at least partially, to CDK1 activity and ruling out the possibility that it requires activation of the spindle assembly checkpoint (7).

Mutagenesis of Proline-directed Sites in Rad53—CDK1 consensus phosphorylation sites are serine or threonine residues followed by a proline (SP/TP). Mass spectrometry analysis revealed that Rad53 is phosphorylated at three proline-directed sites: Ser[175], Ser[375], and Ser[774] (5, 11), supporting our data suggesting that CDK1 directly phosphorylates Rad53 in metaphase. In agreement with this hypothesis, Xenopus p34, the frog homolog of Cdc28, phosphorylates Rad53 *in vitro* (5).

We explored whether one or more of the three proline-directed sites indicated above are responsible for Rad53 phosphorylation in mitotic cells. We mutagenized the three sites to alanine or aspartate to mimic the nonphosphorylatable or the constitutively phosphorylated state, respectively, generating the *rad53*-S175A, *rad53*-S375A, *rad53*-S774A, *rad53*-S175D, *rad53*-S375D, and *rad53*-S774D alleles.

First, we analyzed the level and the activity of the mutant Rad53 proteins in cells growing in normal condition or experiencing DNA damage (zeocine treatment) or replication stress (hydroxyurea treatment). Fig. 5*B* shows that none of the alleles display any significant change in Rad53 protein level in exponentially growing conditions, and the mutations do not affect Rad53 phosphorylation in response to DNA damage or replication stress. We then tested the effect of these site-specific mutations on the phosphorylation of Rad53 in cells arrested in mitosis by nocodazole treatment. We found that although the Rad53-S175A and Rad53-S375A mutant forms are phosphorylated as the wild type protein in nocodazole-treated cells, the Rad53-S774A form is not (Fig. 5*C*). In nocodazole-blocked cells, Rad53-S774A protein variant lost the hyperphosphorylation shift in the Western blotting analysis, although we cannot rule out completely the possibility that the Rad53-S774A variant may still retain a minor modification that proved to be difficult to be visualized under our gel electrophoresis conditions.

This finding suggests that in mitotic cells, Rad53 is mainly phosphorylated by CDK1 on serine 774 and that S774 phosphor-

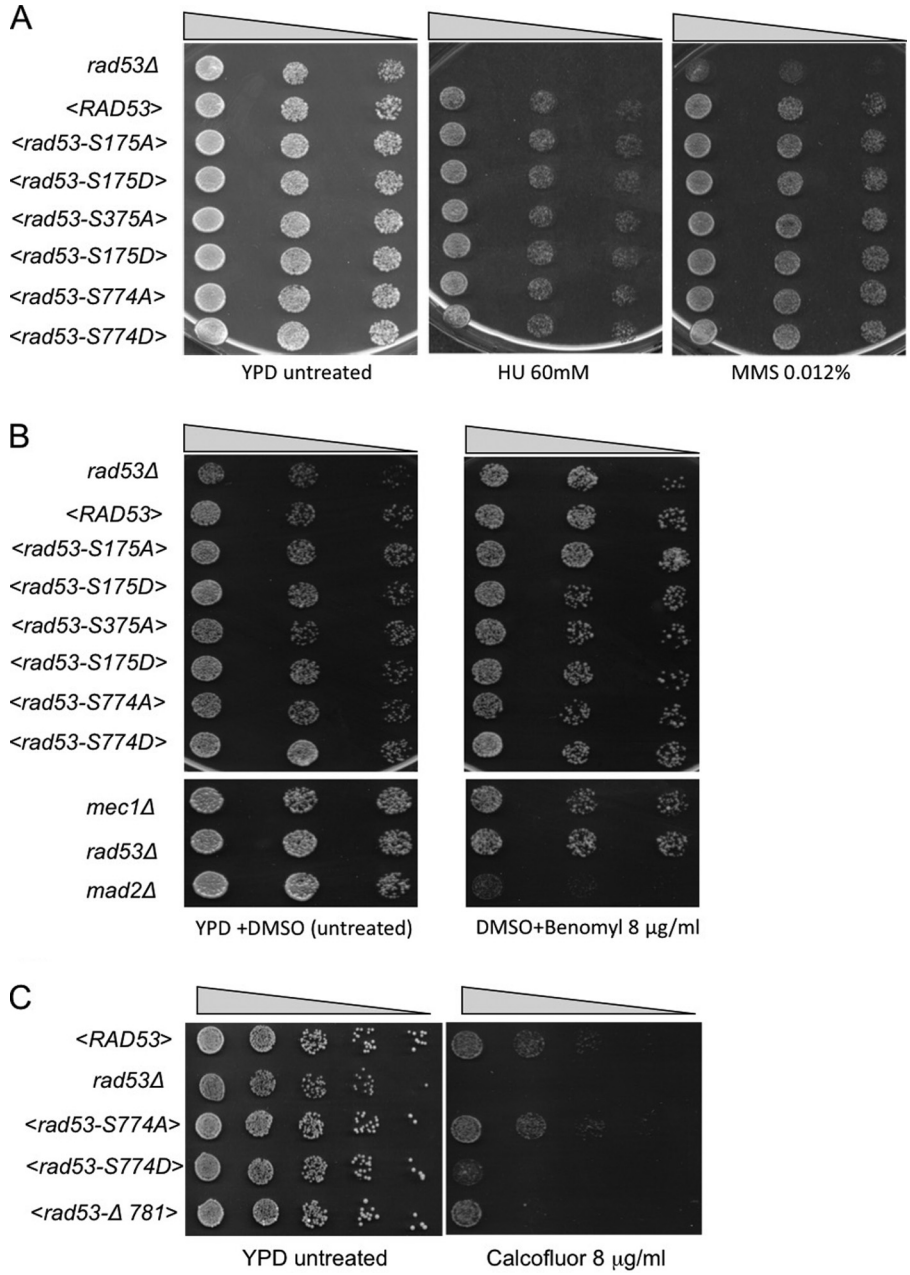

FIGURE 6. **Viability of CDK1-phosphosites *rad53* mutants in different stress conditions.** Drop test analysis of serial 5-fold dilutions of exponentially growing cell cultures of *rad53*Δ *sml1*Δ strains, carrying the indicated *rad53* allele under its own promoter on a centromeric plasmid. Isogenic *mec1*Δ, *mad2*Δ, and *rad53*-Δ781 strains were also tested as indicated. *A*, *B*, and *C*, cells were plated on YPD and YPD plus hydroxyurea (*HU*), methyl methanesulfonate (*MMS*), benomyl, or calcofluor white at the indicated concentrations. Plates were incubated 3 days at 28 °C (*A* and *C*) or 16 °C (*B*).

ylation is responsible for the major Rad53 electrophoretic mobility shift observed in nocodazole-treated cells. To explore the functional role of the CDK1-dependent phosphorylation of Rad53, we analyzed the sensitivity of the *rad53* mutant strains to different toxic agents.

Serial dilutions of cells of the various *rad53* strains were plated in the presence of different concentrations of hydroxyurea treatment or methyl metanesulfonate (a DNA-alkylating agent). Wild type or *rad53*Δ strains were used as controls in the plates. The plates were analyzed after 3 days of incubation at 28 °C (Fig. 6*A*). We found that none of the *rad53* alleles tested were sensitive to hydroxyurea treatment or methyl metanesulfonate. This result is in agreement with our previous finding (Fig. 5*B*), indicating that the various Rad53 mutations do not prevent Rad53 activation following DNA damage and replication stress.

By a similar approach, we then tested the sensitivity to benomyl, a spindle-damaging agent. In this case, as controls we used *mec1*Δ and *mad2*Δ strains (Fig. 6*B*). As shown previously (21), *mad2*Δ cells are sensitive to spindle damage, whereas strains mutated in the *MEC1* and *RAD53* kinases do not cause any sensitivity. We found also that the CDK1-phosphosite mutants of Rad53 are not sensitive to benomyl, ruling out the possibility that CDK1-dependent phosphorylation of Rad53 might have a functional role in the spindle assembly checkpoint.

Recently, Rad53 was implicated in morphogenesis (9). The relationship between the function of Rad53 in the DNA damage checkpoint and its role in cellular morphogenesis is not yet understood. Moreover, the observation that the Mec1 kinase and other DNA damage checkpoint factors are not involved in cellular morphogenesis (9, 10), leads to the hypothesis that Rad53 function in this pathway is separated from its role in response to DNA damage. Because serine 774 is the main CDK1-dependent modification of Rad53 in metaphase, we tested the sensitivity of the *rad53*-S774A and *rad53*-S774D mutant strains to calcofluor white. This agent interferes with cell wall synthesis and cellular morphogenesis (9). Serial dilutions of cells were plated in the presence of various concentrations of calcofluor white, and the plates were incubated at 28 °C for 3 days. *rad53*Δ mutants are very sensitive to morphogenetic stress, as reported previously (9). Surprisingly, we found that phospho-mimicking *rad53*-S774D mutant cells are sensitive to calcofluor white, albeit less than *rad53*Δ cells, whereas *rad53*-S774A cells are not (Fig. 6*C*). We also found that *rad53*-Δ781, expressing a truncated variant of Rad53 protein that lacks the NLS motif (5, 6), is also sensitive to calcofluor white.

The results described in Fig. 6 suggest that CDK1-dependent phosphorylation of Rad53 does not play any role in the response to DNA or spindle damage. Moreover, the sensitivity of *rad53*-

S774D mutant to calcofluor rises the possibility that CDK1-dependent phosphorylation of Rad53 may influence Rad53 function during morphogenesis and/or in the response to morphogenesis stress.

DISCUSSION

Biochemical and genetic analysis have shown that Mec1 and/or Tel1, the upstream kinases in the checkpoint cascade, phosphorylate Rad53 at multiple SQ/TQ residues, through the action of a mediator protein (Rad9 or Mrc1), which likely promote the optimal interaction between Rad53 and the phosphatidylinositol 3-kinase-related kinases (1, 3, 22). The in *trans* phosphorylation of Rad53 is followed by its autophosphorylation, leading to full activation of the kinase in the presence of DNA damage or replication stress.

Whether Rad53 is activated in unperturbed exponentially growing cells was an argument of discussion: on one hand, mutations abrogating the catalytic activity of Rad53 cause cell lethality (23), suggesting that its kinase activity is essential in unperturbed conditions; on the other hand, the methods currently used to measure Rad53 kinase activity do not reveal any active Rad53 form in unperturbed exponentially growing cells (14, 15). One possible interpretation is that the procedures to test Rad53 kinase activity are not sensitive enough to detect a very low level of activity. Hence, most of the details leading to phosphorylation and activation of Rad53 have been analyzed in response to DNA damage and replication stress, and the regulation of Rad53 in untreated exponentially growing cells is much less studied.

Recently, accurate mass spectrometry analysis revealed that Rad53 is phosphorylated at SP/TP residues in exponentially growing cells (5, 11), suggesting that during an unperturbed cell cycle, Rad53 may be the target of a kinase different from Mec1 and Tel1 phosphatidylinositol 3-kinase-related kinases. Here, we provide genetic and biochemical evidence that CDK1 phosphorylates Rad53 at the G_2/M transition in an unperturbed cell cycle, and this modification is clearly visible in metaphase-arrested cells (Figs. 2 and 3). These findings support the hypothesis that CDK1 is at least one of the kinases modifying Rad53 in exponentially growing conditions.

Previous observations (7) led to the hypothesis that an undescribed kinase, likely activated by the SAC pathway, phosphorylates Rad53 in response to spindle damage. Our findings indicate that the Rad53 phosphorylation detected in mitotic cells does not require DNA damage or spindle checkpoint pathways but, instead, it is a metaphase-anaphase event carried out by CDK1, which targets the serine 774 residue of Rad53. We also found that inactivation of CDK1 abrogates Rad53 phosphorylation observed after spindle damage (Fig. 2). Although alternative hypothesis can be suggested, we favor the idea that CDK1 phosphorylates Rad53 at metaphase and that the Rad53 phosphorylation observed in cells with damaged spindles is possibly due to the fact that the SAC pathway blocks the cell cycle at the metaphase-anaphase transition with high Clb-CDK1 activity. Moreover, we failed to observe any sensitivity to benomyl in various *rad53* mutant cells (Fig. 6), and there is no evidence that Rad53 activity is required in response to spindle damage.

What is the functional significance of the cell cycle-dependent phosphorylation of Rad53 mediated by CDK1? Interestingly, *rad53*-S774D mutant cells, in which the main CDK1 phosphorylation site mimics a constitutively phosphorylated state, are sensitive to calcofluor white (Fig. 6), suggesting morphogenetic dysfunctions. Because the Ser^{774} residue is the main target for the CDK1-dependent Rad53 phosphorylation at metaphase, it is tempting to speculate that CDK1 may regulate Rad53 to orchestrate cellular morphogenesis during the cell cycle. Surprisingly, the nonphosphorylatable *rad53*-S774A mutant allele does not show any calcofluor sensitivity. Opposing phenotypes of mutations altering the phosphorylation of a specific residue have been observed in the regulation of kinases (24) and of other relevant proteins (for an example, see Ref. 25). Such an effect has been extensively characterized biochemically for certain protein kinases (24), whereas for most proteins, the biochemical details remain obscure. Concerning the effect of opposite mutations at the Ser^{774} residue of Rad53, an attractive hypothesis may be related to the location of the phosphorylatable residue, which is positioned near the NLS motif (amino acids 781–801; see Fig. 4*A*). We speculate that such a residue may influence the subcellular localization of Rad53 or its interaction with specific cellular structures. Interestingly, the Rad53-Δ781 mutant protein, which lacks the NLS (5, 6), is not phosphorylated in metaphase and in the presence of DNA damage (supplemental Fig. S1). Moreover, *rad53*-Δ781 cells are sensitive to calcofluor white, DNA damage, and replication stress (Fig. 6*C* and see Ref. 10). It is possible that mimicking the constitutive phosphorylation of Ser^{774}, the *rad53*-S774D mutation may deregulate the dynamics of Rad53 localization or intracellular interactions. However, because Rad53-S774D is properly activated in response to genotoxic stress (Fig. 5), and mutant cells are not sensitive to DNA damage (Fig. 6), we have to infer that in this mutant, a sufficient amount of Rad53 is localized in the nucleus. Direct microscopic examination of wild type and mutant Rad53 tagged with green fluorescent protein or other epitopes suitable for immunofluorescence analysis, failed to detect any evident alteration in the partition of Rad53 between the cytoplasm and the nucleus.[4] Rad53 is an abundant protein that appears diffusely stained both in the cytoplasm and in the nucleus (10). Partial relocalization or accumulation of Rad53 to specific cellular compartments or structures is thus difficult to test by microscopic analysis.

Recently, it was shown that Rad53 interacts with septins (10) and regulates the phosphorylation and timely degradation of Swe1 (9), which is the main kinase involved in the morphogenesis checkpoint controlling bud emergence and growth (26). It will be a challenge for further studies to investigate whether the *rad53*-S774A/S774D mutations will influence Swe1 level and function in the cell, perhaps causing abnormalities in the cell wall and/or bud formation.

The interaction of Rad53 with other proteins localized at specific cellular structures may be addressed by genetic screenings to identify synthetic interactions between the *rad53*-S774D/S774A mutant alleles and mutations in other yeast

[4] L. Diani and A. Pellicioli, unpublished observations.

genes. Alternatively, the Rad53 interactors so far identified by tandem affinity purification-tagging methods (10), can be challenged for their biochemical and genetic interactions with the Rad53-S774D/S774A protein variants. In conclusion, our findings indicate that CDK1 phosphorylates Rad53 in metaphase, and such modification may influence its role in modulating morphogenetic events, also supporting the notion of a molecular link between cell growth and genome integrity checkpoints (9, 10).

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Elevated Levels of the Polo Kinase Cdc5 Override the Mec1/ATR Checkpoint in Budding Yeast by Acting at Different Steps of the Signaling Pathway

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Abstract

Checkpoints are surveillance mechanisms that constitute a barrier to oncogenesis by preserving genome integrity. Loss of checkpoint function is an early event in tumorigenesis. Polo kinases (Plks) are fundamental regulators of cell cycle progression in all eukaryotes and are frequently overexpressed in tumors. Through their polo box domain, Plks target multiple substrates previously phosphorylated by CDKs and MAPKs. In response to DNA damage, Plks are temporally inhibited in order to maintain the checkpoint-dependent cell cycle block while their activity is required to silence the checkpoint response and resume cell cycle progression. Here, we report that, in budding yeast, overproduction of the Cdc5 polo kinase overrides the checkpoint signaling induced by double strand DNA breaks (DSBs), preventing the phosphorylation of several Mec1/ATR targets, including Ddc2/ATRIP, the checkpoint mediator Rad9, and the transducer kinase Rad53/CHK2. We also show that high levels of Cdc5 slow down DSB processing in a Rad9-dependent manner, but do not prevent the binding of checkpoint factors to a single DSB. Finally, we provide evidence that Sae2, the functional ortholog of human CtIP, which regulates DSB processing and inhibits checkpoint signaling, is regulated by Cdc5. We propose that Cdc5 interferes with the checkpoint response to DSBs acting at multiple levels in the signal transduction pathway and at an early step required to resect DSB ends.

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Introduction

Saccharomyces cerevisiae cells suffering a double stranded DNA break (DSB) activate a robust Mec1-dependent checkpoint response when DSB ends are processed to expose single-stranded DNA (ssDNA), and progression through the cell cycle is arrested prior to anaphase. Several well conserved factors are recruited at the DSB lesion, and contribute to the activation of a signaling pathway based on sequential phosphorylation events driven by the upstream kinases Tel1/ATM and Mec1/ATR which, in turn, activate the transducer kinases Rad53/Chk2 and Chk1 [1,2]. The checkpoint response is influenced at several levels by kinases such as CDK1, CKII and Polo-like Cdc5, all involved in promoting key events throughout an unperturbed cell cycle, supporting the notion that the cellular response to DNA damage is tightly linked to cell cycle events [3]. The intensity of the DSB-induced checkpoint response correlates to the amount of the ssDNA that is accumulated at DSB lesions [4]. 5′-to-3′ nucleolytic processing of DNA ends is dependent upon several factors, including CDK1 and the nucleases Mre11, Sae2, Dna2 and Exo1 [5]. Moreover, the checkpoint is a reversible signaling pathway which is turned off when DNA lesions are repaired, thus permitting the resumption of cell cycle progression [6]. Different types of phosphatases (Pph3, Ptc2 and Ptc3) dephosphorylate and inactivate Rad53 and other checkpoint kinase targets [7]. Further, mutations in several DNA repair genes, including *SAE2*, *KU70/80*, *RAD51*, *RDH54*, *SRS2*, affect the inactivation of the DSB-induced checkpoint response [7,8]. These observations suggest that the attenuation, as well the activation, of the checkpoint pathway are related to the metabolism of DSB ends, in a way that is not yet completely understood. It is also known that the checkpoint response can be attenuated when an irreparable DNA lesion is formed in the cell, leading to adaptation to DNA damage. Checkpoint inactivation during recovery and adaptation to DNA damage is a phenomenon described also in higher eukaryotes [6]. The functional role of adaptation is not completely understood; however, it was suggested that it may be partly responsible for chromosomal rearrangements, genome instability and tumorigenesis [6,9]. Interestingly, the well conserved family of Polo-like kinases (Plks) has been involved in checkpoint adaptation and/or recovery both in budding yeast and vertebrates [10]. Cdc5 is the only polo kinase expressed in yeast, whereas higher eukaryotes usually express three or four Plks [11]. However, only Plk1, which is the most extensively studied, is a true mitotic kinase homolog to the *Drosophila* Polo kinase [11]. In yeast, *CDC5* is an essential gene and the point mutation *cdc5*-ad (a Leucine-to-Tryptophan substitution

Author Summary

Double strand DNA breaks (DSBs) are dangerous chromosomal lesions that can lead to genome rearrangements, genetic instability, and cancer if not accurately repaired. Eukaryotes activate a surveillance mechanism, called DNA damage checkpoint, to arrest cell cycle progression and facilitate DNA repair. Several factors are physically recruited to DSBs, and specific kinases phosphorylate multiple targets leading to checkpoint activation. Budding yeast is a good model system to study checkpoint, and most of the factors involved in the DSBs response were originally characterized in this organism. Using the yeast *Saccharomyces cerevisiae*, we explored the functional role of polo kinase Cdc5 in regulating the DSB–induced checkpoint. Polo kinases have been previously involved in checkpoint inactivation in all the eukaryotes, and they are frequently overexpressed in cancer cells. We found that elevated levels of Cdc5 affect the cellular response to a DSB at different steps, altering DNA processing and overriding the signal triggered by checkpoint kinases. Our findings suggest that Cdc5 likely regulates multiple factors in response to a DSB and provide a rationale for a proteome-wide screening to identify targets of polo kinases in yeast and human cells. Such information may have a practical application to design specific molecular tools for cancer therapy. Two related papers published in *PLoS Biology*—by Vidanes et al., doi:10.1371/journal.pbio.1000286, and van Vugt et al., doi:10.1371/journal.pbio.1000287—similarly investigate the phenomenon of checkpoint adaptation/overriding.

at residue 251, within the kinase domain) causes the inability to adapt to one irreparable DSB lesion and to turn off Rad53 kinase [12,13]. However, *cdc5*-ad cells can recover from checkpoint when the DSB is repaired, suggesting that adaptation and recovery are two genetically separate processes [14]. A corresponding *cdc5*-ad mutation in Plks has not yet been isolated in mammals; however, it was found that Plk1 depletion severely blocks checkpoint recovery and adaptation [10,15,16], and rapidly causes cell death in cancer cells [17,18]. Based on the fact that the DNA damage checkpoint pathway is well conserved in all the eukaryotes, it is reasonable to expect that the functional role of Cdc5 in budding yeast and of Plk1 during adaptation (and perhaps in recovery) may be conserved. Polo-like kinases contain in the C-terminal region of the protein a polo box which mediates the interaction of Plks with substrates previously phosphorylated by CDK or MAPK kinases [19]. Indeed, Cdc5 targets multiple substrates during an unperturbed cell cycle [20] and could functionally interact with several checkpoint proteins as well. In vertebrates, polo kinases regulate the DNA damage checkpoint acting on multiple factors. They phosphorylate Claspin [21–24], a Chk1 kinase regulator, and the Fanconi-Anemia protein FANCM [25], promoting their degradation and checkpoint inactivation. Further, Plk1, Plk3 and Plk4 interact with and phosphorylate Chk2, the ortholog of Rad53 in human cells, likely influencing its activity [26–28]. Interestingly, yeast Cdc5 is phosphorylated and inhibited in a Mec1- and Rad53-dependent manner [29], and several studies indicate that in mammals Plk1 activity is inhibited by ATM/ATR-signaling in response to DNA damage [30–33]. Further, the DNA damage checkpoint regulates Plk1 protein stability in response to DNA damage in mitosis [34]. It was also shown that Aurora kinase A phosphorylates and re-activates Plk1 to promote recovery from DNA damage [35]. Altogether, these informations suggest that the DNA damage checkpoint inhibits Plk1, thus contributing to block cell cycle progression in response to DNA damage; however, the re-activation of Plk1 is a crucial event of a feedback regulatory loop in the inactivation of the DNA damage checkpoint during recovery and adaptation.

Therefore, the activity of Plks must be finely regulated during the DNA damage checkpoint response, and it is worth mentioning that the expression of a constitutively active Plk1 protein variant overrides the G2/M arrest induced by DNA damage [30]. Indeed, Plks are frequently overexpressed in tumor cells with uncontrolled proliferation and genome instability [36–39], and high level of Plk1 is predictive of a bad prognosis in several cancers [40–44].

To further characterize the functional link between Plks and the DNA damage checkpoint and, possibly, to understand why Plks are frequently overexpressed in cancer cells, we used budding yeast as a model system to study DNA damage related events in the presence of high levels of Cdc5.

Here, we show that overproduction of Cdc5 impairs the Mec1-signaling pathway in response to an inducible DSB lesion, altering phosphorylation of Ddc2, Rad9, Rad53 and other Mec1 targets. We also found that elevated levels of Cdc5 slow down DSB ends processing, although it does not prevent the formation of ssDNA, which triggers the recruitment of checkpoint factors. Consistently, we observed that overexpression of Cdc5 does not alter the loading of the apical Mec1 kinase checkpoint complex and recruitment of the checkpoint mediator Rad9, but surprisingly it physically interact with the checkpoint inhibitor Sae2, inducing its hyper-phosphorylation and an increased and persistent binding onto a DSB lesion.

We propose that high levels of polo kinase Cdc5 override Mec1-induced checkpoint response to DSB lesions, likely by regulating multiple factors, previously phosphorylated by CDK1, involved in both DSB processing and checkpoint signaling. Our work may represent a simple model to further understand why polo kinases are frequently overexpressed in cancer cells.

Results/Discussion

Elevated levels of Cdc5 override Mec1 signaling

DNA damage checkpoints represent a barrier to oncogenesis; in fact, loss of these surveillance mechanism is a characteristic of early tumor development [45]. Several evidences indicate that Plks are targets of the DNA damage checkpoint in all the eukaryotes [29–34], suggesting a functional model in which the DNA damage checkpoint inhibits Plks to maintain a cell cycle block at the metaphase to anaphase transition. Indeed, numerous cancer cells have been reported to display overexpression of Plks, and this may contribute to their transformed phenotype [36–39].

In budding yeast, overproduction of the polo kinase Cdc5 in *cdc13-1* mutant cells with uncapped telomeres has been reported to override the checkpoint-dependent cell cycle block in the G2 phase of the cell cycle [46,47]. We found that overproduction of Cdc5 impairs the replication checkpoint, which delays S phase in the presence of the alkylating agent MMS (methylmetane sulfonate, Figure 1A). Indeed, Figure 1A shows that MMS treated wild type cells accumulate in S phase for a very long period (1C<DNA<2C), while Cdc5 overproducing cells rapidly go through the replication phase and reach a G2/M DNA content (2C). Moreover, the DNA damage-induced phosphorylation of Rad53 is essentially abolished in Cdc5 overproducing cells treated with zeocin, an agent causing DSBs (Figure 1B).

We have to assume that, although the DNA damage checkpoint inhibits Cdc5 [29,46], contribuiting to block cell cycle in the presence of DNA damage, when *CDC5* is placed under the control

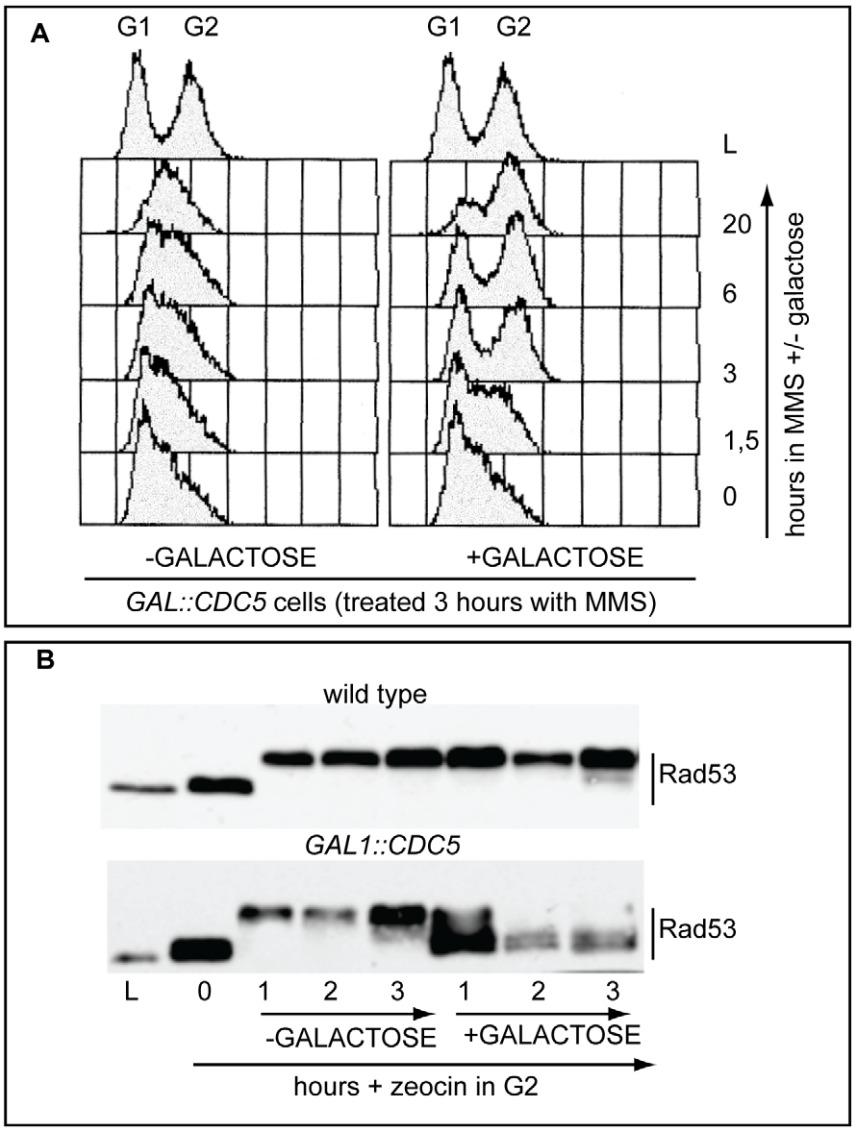

Figure 1. Overproduction of Cdc5 overrides the DNA replication and DNA damage checkpoints. (A) Exponentially (L) growing culture of the strain Y114 (*GAL1::CDC5*) was grown in YEP+3%raffinose and treated for 3 hours with 0.02% MMS (time 0). Then the culture is split in two and 2% galactose was added to one half, while the other half was maintained in raffinose. Samples were taken at the indicated time and analysed by FACS. (B) Cultures of the strains Y79 (wild type), Y114 (*GAL1::CDC5*), exponentially (L) growing in YEP+3%raffinose were blocked in G2/M by nocodazole treatment (0). Zeocin (50 µg/ml) was then added to cause DSBs formation and after 30 minutes of treatment, 2% galactose was added. Samples were taken at the indicated time and Rad53 protein was analyzed by western blotting with Mab.EL7 antibody.
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of the *GAL1* promoter, the DNA damage-induced inhibition on overproduced Cdc5 is not complete. This is likely due to the elevated Cdc5 levels, which are higher than the endogenous amount (see also Figure S1), leading to the override of the checkpoint response. Indeed, it was previously shown that the overproduction of Cdc5, which is a finely regulated protein [29], causes severe phenotypes during an unperturbed cell cycle [48–51].

In order to expand the analysis on the crosstalk between polo kinases and checkpoint pathways, and possibly to understand why overexpression of Plks is often found in tumor cells characterized by uncontrolled proliferation and genome instability, we analysed the effects of elevated Cdc5 levels on the DSB-induced checkpoint cascade in *S. cerevisiae*. We took advantage of a standard yeast genetic system (JKM background) in which one irreparable DSB can be induced at the *MAT* locus by expressing the site-specific HO nuclease [8]. We overexpressed wild-type *CDC5* and the two *cdc5*-ad and *cdc5*-kd mutant alleles (adaptation-defective and kinase-dead alleles, respectively [51]) from the galactose-inducible promoter and examined Rad53 phosphorylation and *in situ* auto-phosphorylation activity, which are routinely used as markers of DNA damage checkpoint activation [52]. To prevent variations due to cell cycle differences, we first arrested cells with nocodazole in mitosis, a cell cycle stage in which the DSB-depended checkpoint can be fully activated [12], and subsequently added galactose to induce Cdc5 overproduction and HO-break formation, while maintaining the cell cycle block. Figure 2A shows the FACS profiles of the cell cultures. We observed that overproduction of Cdc5 impairs the accumulation of hyper-phosphorylated Rad53 forms and prevents Rad53 auto-phosphorylation activity in

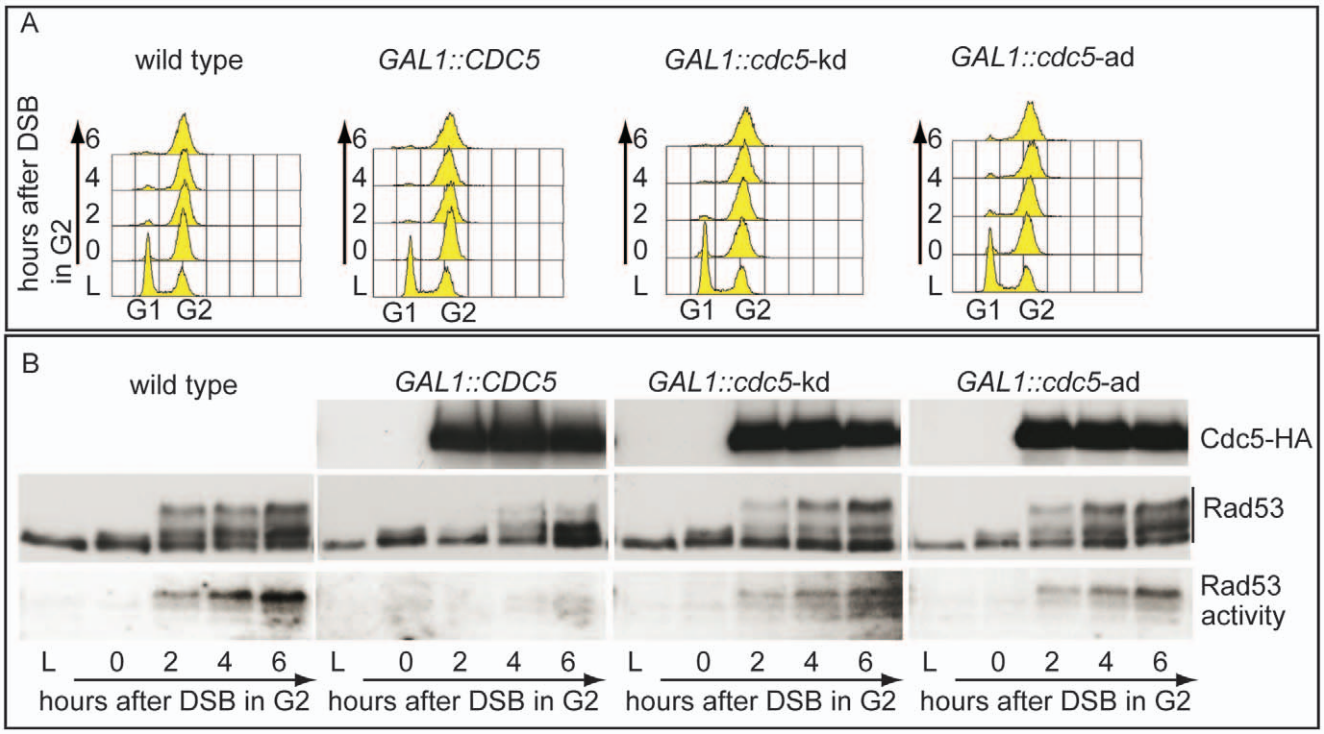

Figure 2. Overproduction of Cdc5 affects DSB–induced Rad53 phosphorylation and activity. (A,B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5*, *GAL1::cdc5*-kd (kinase dead, K110A mutation) and *GAL1::cdc5*-ad (adaptation defective, L251W mutation) strains were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A) Samples were taken at the indicated time points and analyzed by FACS. (B) Overproduced Cdc5 proteins have an additional HA epitope and their accumulation in galactose was analyzed by western blots using 12CA5 antibody. Rad53 was analyzed by western blots with Mab.EL7 antibodies. Rad53 in situ auto-phosphorylation activity was analyzed by ISA assay.
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response to DSB formation (Figure 2B). Interestingly, overproduction of the protein variants Cdc5-kd or Cdc5-ad did not significantly interfere with Rad53 phosphorylation and activation, suggesting that the kinase activity of Cdc5 and its capacity to interact with specific target(s) are required to override the DSB-induced Rad53 activation.

In vertebrates, polo kinases regulate the DNA damage checkpoint response by affecting the signal transduction pathway at different levels; interestingly, Chk2, the homologue of Rad53 in human cells, interacts with and is phosphorylated by the polo kinases Plk1, Plk3 and Plk4 [26–28].

Therefore, we tested whether the overproduction of Cdc5 might override Rad53 activation by targeting directly the Rad53 protein and/or by acting on other upstream checkpoint factors.

We failed to co-immunoprecipitate Rad53 and Cdc5, when expressed at endogenous levels or by using the polo box of Cdc5 in a standard GST pull down assay; however, we retrieved Rad53 with overproduced Cdc5 (Figure S2). Considering such physical interaction, we analyzed how overproduction of Cdc5 might affect the events leading to full activation of Rad53, which involves a two steps-based mechanism: an *in trans* phosphorylation event mediated by PIKKs, followed by auto-phosphorylation [53]. In theory, Cdc5 might affect any of these events required to activate Rad53. We analysed the effect of Cdc5 overexpression on the PIKKs-dependent phosphorylation of Rad53 by taking advantage of the catalytically inactive *rad53*-K227A mutant. Such protein can be phosphorylated *in trans* by the upstream kinases, but does not undergo auto-phosphorylation in the presence of DNA damage [52], allowing us to separate and discriminate the two steps.

In nocodazole blocked cells, induction of a single irreparable HO cut induced Mec1-dependent phosphorylation of the Rad53-K227A protein variant (Figure 3A). As expected, the corresponding phosphorylated bands of Rad53-K227A protein were not visualized by western blot using the monoclonal antibody (Mab.F9) which is specific for the auto-phosphorylated and active Rad53 isoform [54]. Moreover, the same phospho-specific antibody did not significantly detect Rad53 in wild type cells responding to DSB when Cdc5 is overproduced, confirming the results of the *in situ* kinase assay (Figure 2B). A residual shifted band of Rad53, visualized in *CDC5* overexpressing cells through the highly sensitive Mab.EL7 antibody (both in Figure 2B and Figure 3A, and in other figures below), could reflect low levels of Rad53 activation not detected by the antibody against the active form; this is consistent with the residual Rad53 activity in the *in situ* analysis in Figure 2B. In any case, it is unlikely that this remaining Rad53 activity is sufficient to maintain a full checkpoint response, since overproduction of Cdc5 functionally overrides the cell cycle block in the presence of DNA damage.

Significantly, Cdc5 overproduction abolished DSB-induced *in trans* phosphorylation of the Rad53-K227A variant (Figure 3A). This result rules out the hypothesis that Cdc5 may override the DSB-induced checkpoint acting only on the auto-phosphorylation step of Rad53 activation, and suggests that *CDC5* overexpression likely impairs the Mec1-dependent *in trans* phosphorylation and activation of Rad53.

The residual Rad53 phosphorylation and activity in the presence of high levels of Cdc5 might suggest that the upstream Mec1 kinase, which is mainly responsible of the Rad53 activation in the presence of a single DSB in wild type cells [55], is strongly

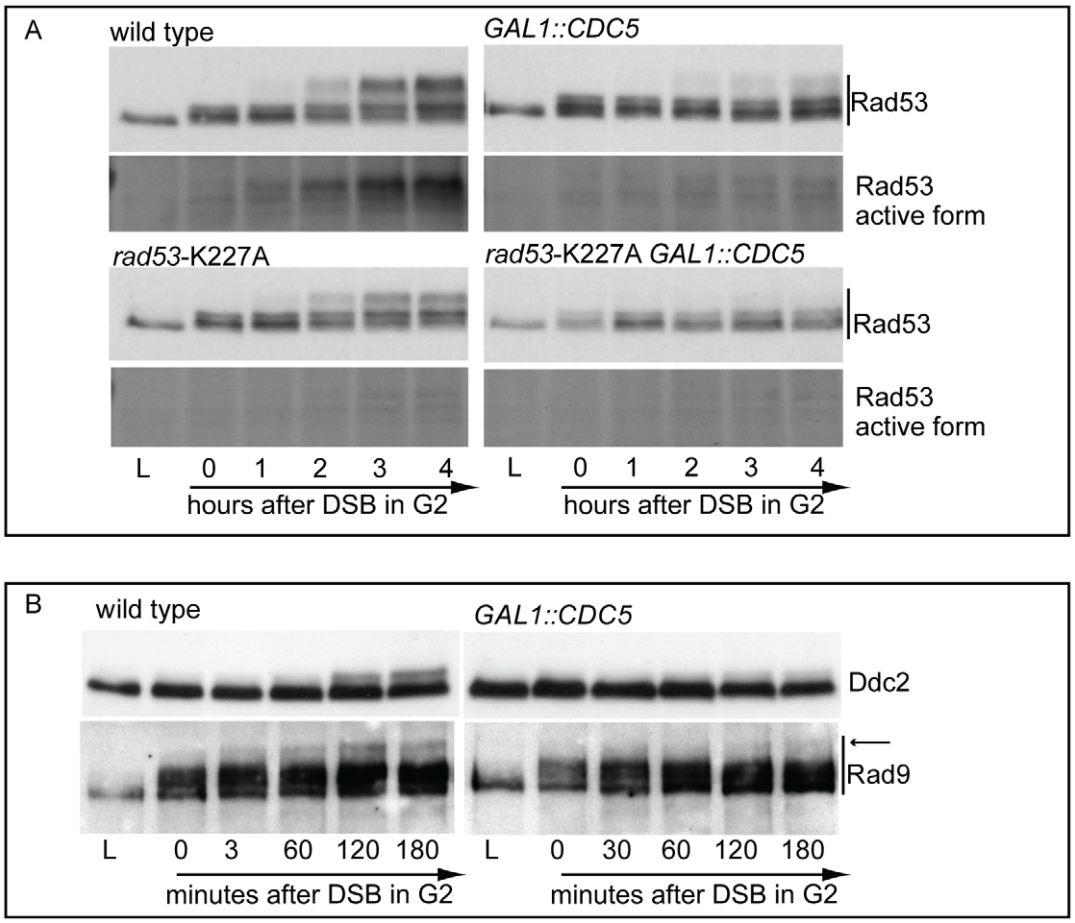

Figure 3. Overproduction of Cdc5 overrides Mec1 checkpoint signaling. (A) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM and isogenic *rad53*-kd (kinase dead, K227A mutation) derivative strains, with or without *GAL1::CDC5*, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Samples were taken at the indicated time points and Rad53 was analyzed by western blots using monoclonal antibodies Mab.EL7 or Mab.F9, which recognized, respectively, all the forms of Rad53 or only the auto-phosphorylated and active forms. (B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5* derivative strains, expressing *DDC2*-HA, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Ddc2 protein was analyzed by western blots using 12CA5 antibody; Rad9 protein was analyzed by polyclonal antibodies. An arrow denotes the hyper-phosphorylation band of Rad9 accumulated specifically in response to DNA damage.
doi:10.1371/journal.pgen.1000763.g003

but not fully inhibited. Alternatively, Mec1 may still be functional as a kinase, but impaired in fully trans-activating Rad53. To test more directly the activity of the upstream kinase Mec1, we analysed the phosphorylation state of its interacting subunit Ddc2, the ortholog of human ATRIP, and that of the checkpoint mediator Rad9, which are known to be directly phosphorylated by Mec1 [1]. Cells were arrested with nocodazole and *CDC5* overexpression and induction of a single unrepairable DSB were induced by galactose addition (Figure 3B). Western blot analysis indicate that phosphorylated isoforms of Ddc2 and hyper-phosphorylated Rad9 (indicated by the arrow in Figure 3) accumulated after the formation of the HO cut in wild type cells, as expected; however, overexpression of Cdc5 reduced the DSB-induced hyper-phosphorylated form of both Ddc2 and Rad9, suggesting that the activity of Mec1 kinase is strongly impaired in the presence of high level of Cdc5. A careful analysis of the blot shown in Figure 3B or in analogous experiments indicates that reduced levels of phosphorylated Rad9 isoforms are present in *CDC5* overexpressing cells, suggesting that Mec1 could still retain a flebile activity toward Rad9 and Rad53, as discussed above. In addition, it is known that Rad9 is a target of multiple kinases [56] and we cannot rule out the possibility that the residual phosphorylation of Rad9 observed in cells with elevated levels of Cdc5 may be due to other kinase(-s), including Cdc5 itself.

Taken together the results shown in Figure 1, Figure 2, and Figure 3 indicate that Cdc5 activity overrides the DSB-induced checkpoint by influencing an early step of the Mec1 signaling pathway, likely reducing the functionality of Mec1 activity. However, it is possible that Cdc5 may target multiple substrates, including the Mec1 interactor Ddc2, the checkpoint mediator Rad9, whose role in promoting Mec1-to-Rad53 signaling is well established, and Rad53 itself, thus counteracting the checkpoint signaling pathway at several levels.

High levels of Cdc5 affect DSB resection

Robust Mec1 and Rad53 activation is not triggered by the DSB itself, but requires multiple interconnected events following the formation of the lesion, including the generation of nucleolytic-dependent 5′-to-3′processing of the DNA ends and recruitment of various DNA repair and checkpoint factors onto the long stretches of the generated ssDNA [4].

Therefore, we investigated whether Cdc5 may control Mec1 signaling by affecting DSB processing. We measured the kinetic of

ssDNA formation after a single unrepairable DSB in cells overexpressing *CDC5*. Cells were arrested in mitosis, to prevent cell cycle-dependent effects on resection [57], and samples were collected at various time points after HO nuclease induction (Figure 4). The kinetic of production of ssDNA regions in genomic DNA was tested by the loss of restriction sites distal to the HO-cut site which leads to the accumulation of undigested ssDNA fragments detectable with a strand-specific probe after alkaline electrophoresis (see the scheme of the unprocessed and processed DNA locus in Figure 4A). *CDC5* overexpressing cells reproducibly

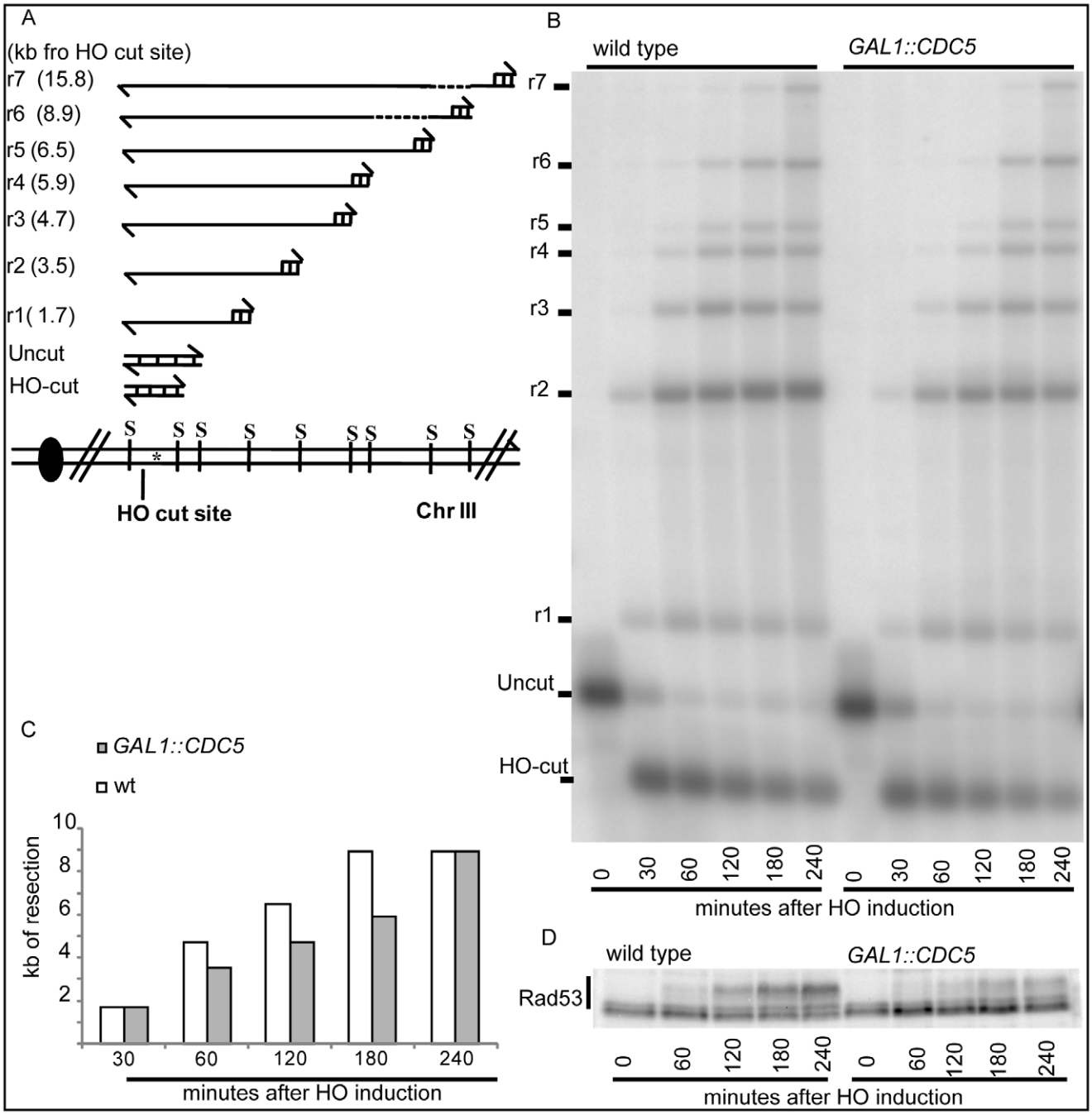

Figure 4. Overproduction of Cdc5 affects DSB processing. (A–D) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM MATα and isogenic *GAL1::CDC5* strain were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A) Schematic representation of the system used to detect DSB resection. Gel blots of *SspI*-digested genomic DNA separated on alkaline agarose gel were hybridized with a single-strand RNA probe specific for the un-resected strand at the MAT locus, which shows HO-cut and uncut fragments of 0.9 and 1.1 kb, respectively. 5′-to-3′ resection progressively eliminates *SspI* sites located 1.7, 3.5, 4.7, 5.9, 6.5, 8.9, and 15.8 kb centromere-distal from the HO-cut site, producing larger *SspI* fragments (r1–r7) detected by the probe. (B) Analysis of ssDNA formation as described in (A). (C) The time of the first appearance over the background of each undigested band in the blot shown in (B) was graphically represented for both the wild type and *GAL1::CDC5* strains. (D) Western blot analysis of protein extracts with anti-Rad53 Mab.EL7 antibody.
doi:10.1371/journal.pgen.1000763.g004

exhibited a slower DSB resection, measured by the kinetic of appearance of DNA fragments, which correlated with a reduced phosphorylation of Rad53 (Figure 4B–4D). However, we found that, although the kinetic of DSB ends resection was delayed, high levels of Cdc5 do not prevent the generation of a long ssDNA track (25 kb) which is required to repair the DSB in a specific yeast genetic background [14] by the single-strand annealing process (Figure S3).

We previously identified a role for the checkpoint mediator Rad9 in inhibiting the kinetic of DSB ends resection, likely by generating a non-permissive chromatin configuration around the DSB and/or interfering with the action of nucleases [58]. Therefore, we analyzed the Rad9 contribution in delaying DSB processing in *CDC5* overexpressing cells. Wild-type or *rad9*Δ cells, with or without *GAL1::CDC5*, were arrested in mitosis by nocodazole treatment and the same experiment described in Figure 4B was performed. We found that the kinetic of appearance of ssDNA fragments was accelerated in *rad9*Δ strains, despite the high levels of Cdc5 kinase (Figure 5A and 5B). Moreover, the faster DSB resection in *CDC5* overexpressing *rad9*Δ cells also correlated with a modest increase in Ddc2 phosphorylation (Figure 5C); however, the phosphorylated state of Ddc2 did not reach the same level found in wild-type and *rad9*Δ cells, suggesting that overproduction of Cdc5 impaired Mec1-dependent signaling also in a *rad9*Δ background. These results suggest that elevated levels of Cdc5 may slow down DSB processing through the action of the Rad9-dependent barrier on resection [58], likely targeting Rad9 itself or other factors involved in this mechanism. Interestingly, many of the proteins involved in DSB ends processing (i.e. Rad9, Dna2, Xrs2 and Sae2) are phosphorylated

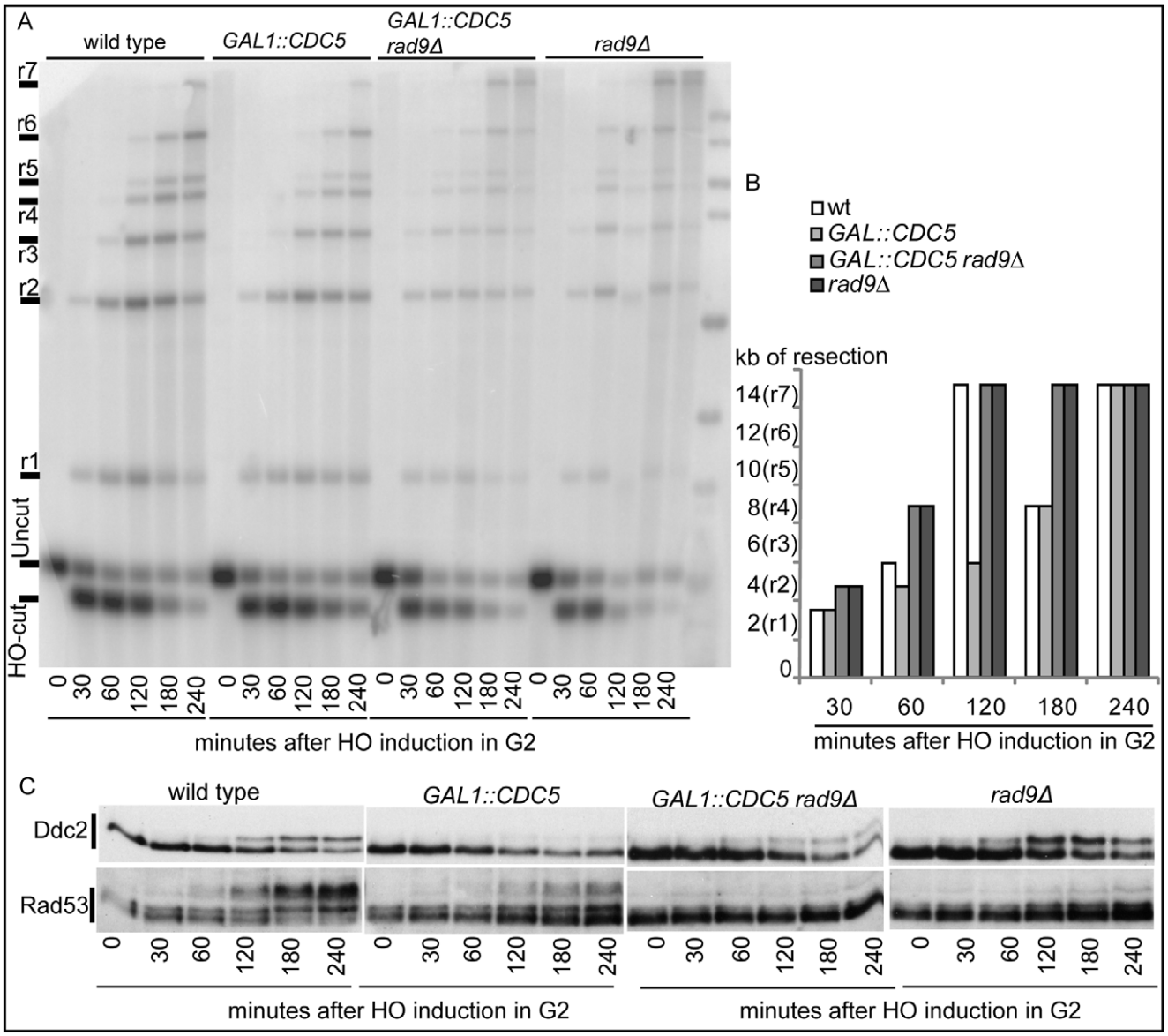

Figure 5. Deletion of *RAD9* gene accelerates DSB resection despites high Cdc5's levels. YEP+raffinose nocodazole-arrested cell cultures of wild type JKM MATa and isogenic *rad9*Δ strains, with or without *GAL1::CDC5*, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A,B) Analysis of ssDNA formation as described in Figure 4. (C) Ddc2 protein was analyzed by western blots using 12CA5 antibody; Rad53 protein was analysed by monoclonal antibody Mab.EL7.
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by CDK1 [59,60] and inspection of their protein sequence reveals that they may be potential targets of Cdc5.

Hence, Cdc5 may influence the DSB response acting on multiple factors, affecting DSB processing and Mec1-signaling; moreover, the possibility that Cdc5 might specifically regulate Rad53 by influencing its interaction with the checkpoint mediator Rad9 cannot be excluded.

Recruitment of checkpoint factors in *CDC5*-overexpressing cells

Since high levels of Cdc5 did not prevent the generation of long ssDNA regions but inhibit Mec1-signaling, we tested, by chromatin immunoprecipitation (ChIP), whether overexpression of *CDC5* affected the recruitment of checkpoint factors onto the HO-induced DSB lesion in nocodazole-arrested cells. Sheared chromatin from formaldehyde crosslinked cells taken at different time-points after galactose addition was immunoprecipitated to recover checkpoint proteins (i.e. Ddc2, Ddc1, Dpb11, Rad9) carrying the MYC or HA epitope tags at their carboxyl-terminal end. Quantitative multiplex PCR was then used to monitor co-immunoprecipitation of DNA fragments located either 66 kb centromere-proximal to the *MAT* locus (CON) or 1 kb away from the HO-cut site (DSB) (Figure 6A).

Ddc2 and Ddc1 association at the DSB was not significantly affected in *CDC5* overexpressing cells blocked by nocodazole treatment (Figure 6B and 6C). The Mec1 interacting factor Ddc2 and Ddc1, one of three subunits of the stable PCNA-like 9-1-1 checkpoint complex, are recruited early onto a DSB lesion [61–63]. We, therefore, assume that Cdc5 overproduction does not prevent the recruitment of upstream checkpoint protein complexes onto damaged DNA. This observation also confirms that elevated levels of Cdc5, while delaying resection, do not prevent the generation of ssDNA (see Figure 4, Figure 5, and Figure S3) which is required for the recruitment of checkpoint factors [4].

Similarly, we found that overproduction of Cdc5 did not prevent the localization near the DSB of Dpb11 (Figure 6D), the yeast ortholog of TopBP1, which, together with the 9-1-1 complex, stimulates the Mec1 kinase activity [64].

Moreover, when we tested by ChIP analysis the binding of the checkpoint mediator Rad9, we found that also its localization onto the DSB was not altered in *CDC5* overexpressing cells (Figure 6E).

Taken together, the ChIP analyses of checkpoint factors at a DSB site indicate that high levels of Cdc5 kinase do not significantly interfere with the binding of checkpoint proteins to a processed DSB.

We then tested the DSB binding of Sae2, which is a protein regulated by CDK1 [60] and PIKKs [65] after DNA damage and is involved in DSB processing [5] and checkpoint inactivation [66,67]. Surprisingly, while in wild-type cells Sae2 loading was not significantly enriched at the HO-cut site (Figure 7A), likely because of its dynamic and transient binding to DSBs [67], Sae2 localization near the break greatly increased in *CDC5* overexpressing cells (Figure 7A). To test whether Cdc5 may specifically target Sae2 influencing its binding onto DSBs, we analysed the level and modification of Sae2 by western blotting following DSB formation. In nocodazole-blocked cells, induction of the HO cut caused PIKKs-dependent phosphorylation of Sae2 at the same time-points at which Rad53 phosphorylation was observed (Figure 7B). Interestingly, although high levels of Cdc5 impair Rad53 phosphorylation, they seem to cause hyperphosphorylation of Sae2. Infact, in *CDC5* overexpressing cells we observed the appearance of a ladder of slower migrating forms of Sae2 (Figure 7B), which are abolished by *in vitro* treatment with λ phosphatase (Figure 7C), indicating that they are due to phosphorylation events of Sae2. We then found that overproduction of Cdc5 induces Sae2 hyper-phosphorylation in untreated cells and in nocodazole-blocked cells without the HO-cut formation (Figures 7D), supporting the idea that Sae2 might be a direct target of Cdc5. Indeed, as mentioned above, Sae2 protein sequence reveals several sites that could be bound and/or phosphorylated by Cdc5 (Figure 8A). The C-terminus of Cdc5, like other Polo-like kinases, contains a phospho-serine/phospho-threonine binding domain called the Polo-box Domain (PBD) [19]. The PBD is known to bind Plk substrates after they have been "primed" by a preliminary phosphorylation by another protein kinase [19]. Interestingly, the putative PBD binding motif of Sae2 has been previously shown to be phosphorylated by CDK1 [60], making it a perfect candidate for mediating the interaction between Sae2 and Cdc5. Indeed, by a 2-hybrid assay we found that the PBD of Cdc5 interacts with Sae2 (Figure 8B), and a recombinant GST-PBD fusion protein, purified from *E. coli*, precipitated Sae2-3HA from yeast extracts (Figure 8C).

Taken together, the results shown in Figure 7 and Figure 8 indicate that Cdc5, through its PBD, interacts with Sae2, causing its hyper-phosphorylation and accumulation at the DSB (see also a model in Figure 8D). It is interesting to point out that CtIP, the functional ortholog of Sae2 in human cells, was found to be associated to chromatin following DNA damage and its chromatin binding is promoted by phosphorylation and ubiquitination [68]. Indeed, recent evidences indicate that CtIP and Ctp1 (the CtIP counterpart in *S. pombe* [69]), are recruited to DSB sites through their interaction with Nbs1 [70–72], a subunit of Mre11 complex, and BRCA1 [73,74]. Moreover, CtIP is phosphorylated and regulated by CDK1 [74,75]. In yeast, Sae2 is involved both in promoting an early step of DSB ends resection [5] and in inactivating checkpoint signaling during recovery and adaptation [66,67], although the exact role of Sae2 in these processes is not yet fully understood. Interestingly, the overproduction of Sae2 also causes the overriding of the Mec1-signaling [66], while deletion of *SAE2* gene prevents switching off of the checkpoint [65,66].

One possible working model (Figure 8D), which needs to be verified, predicts that the increased and persistent binding of Sae2 to a DSB, induced by overproduction of Cdc5, may affect both DSB resection and Mec1-signaling. It is tempting to speculate that even physiological levels of Cdc5 may regulate Sae2 during recovery and adaptation, contributing to switch off the checkpoint signal. It is also possible that Sae2 is regulated by Cdc5 only when this kinase is expressed at elevated levels, leading to the checkpoint overriding. Indeed, such situation is frequently observed in cancer cells, when Plks are overexpressed [36–39], suggesting that what we found in yeast may represent a model for a pathological condition in human cells. Future works, requiring the analysis of *sae2* mutations in the sites regulated by Cdc5, may help to discriminate between the two possibilities.

In conclusion, in the present study we further explored the role of the polo kinase Cdc5 in attenuating the DNA damage checkpoint in budding yeast. We found that overproduction of Cdc5 affects different parameters of the cellular response to an inducible DSB: i) it overrides Mec1 signaling and prevents the phosphorylation of various Mec1 targets (Rad53, Rad9, Ddc2); ii) it causes a slower resection of DSB ends in a *RAD9*-dependent manner; iii) it binds Sae2 protein, causing its hyper-phosphorylation and leading to its increased and persistent binding onto DSB.

The emerging scenario suggests that Cdc5 may target multiple factors involved in various aspects of the cellular response to DSB lesions and DNA damage checkpoint signaling. Indeed, Cdc5 is a fundamental regulator of cell cycle progression and targets many

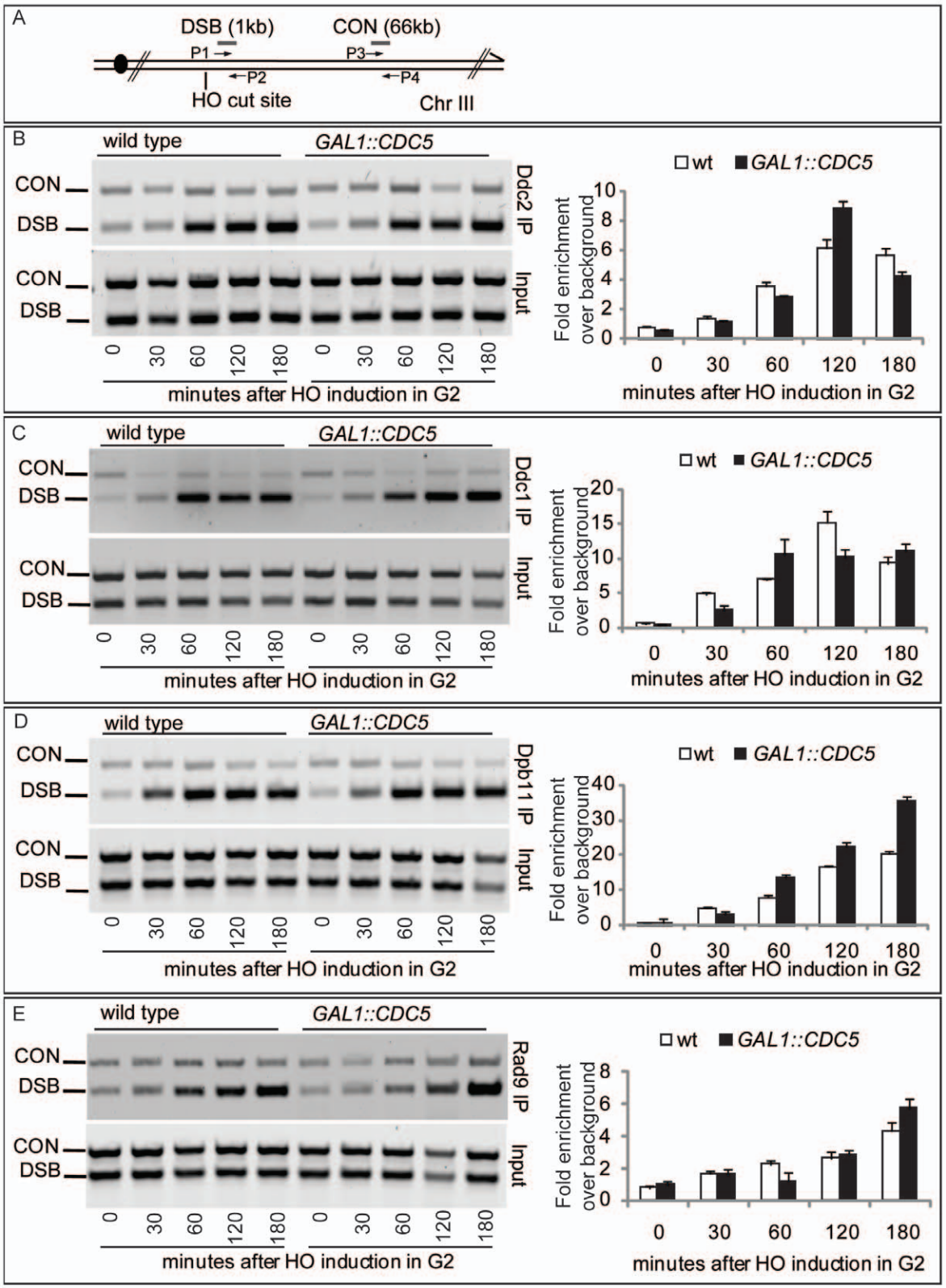

Figure 6. Recruitment to DSB of checkpoint factors in *CDC5*-overexpressing cells. (A) Schematic representation of the HO cleavage site with the positions of the primers used to amplify regions 1 kb (DSB) and 66 kb (CON) from the HO cut site. PCR analysis at the CON site is used as a control of background signal. (B–E) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM and isogenic *GAL1::CDC5-MYC or GAL1-CDC5-HA* strains, expressing *DDC2*-HA, *DDC1*-MYC, *DPB11*-MYC, and *RAD9*-MYC alleles, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Cells were collected at the indicated times and then subjected to chromatin immunoprecipitation. Representative ChIP time-course analysis of protein-DSB association is shown for each protein tested before (Inputs) and after protein immunoprecipitation (IP).
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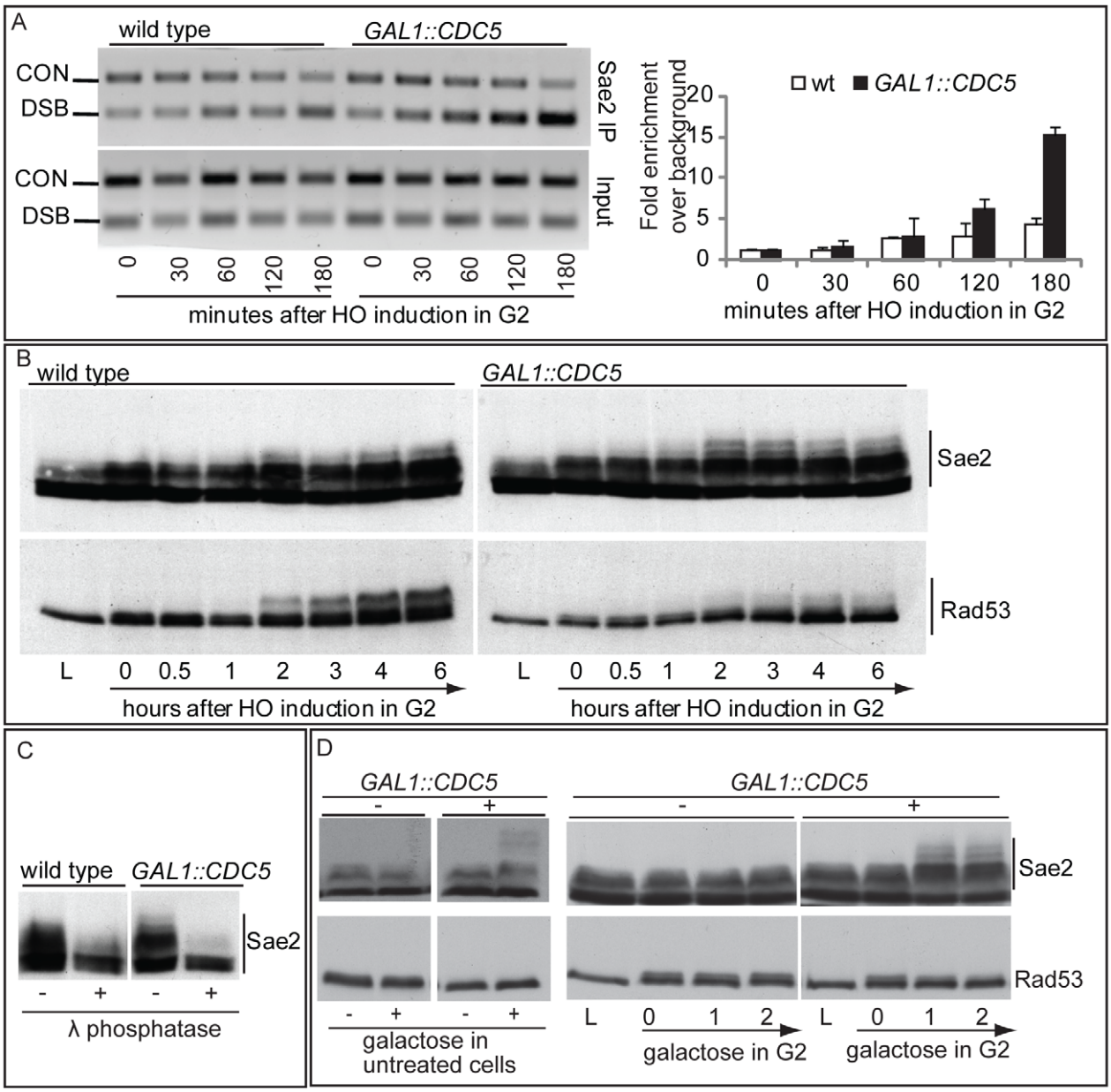

Figure 7. Analysis of Sae2 protein in *CDC5* overexpressing cells. (A,B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5-MYC* strain, expressing *SAE2*-3HA allele, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Cells were collected at the indicated times and then subjected to chromatin immunoprecipitation (ChIP) as described in Figure 6. Representative ChIP time-course analysis of protein-DSB association is shown before (Inputs) and after protein immunoprecipitation (IP). (B) Western blot analysis of protein extracts. (C) Western blot analysis of protein extracts prepared 3 hrs after HO induction and treated with or without λ phosphatase before gel electrophoresis. (D) YEP-raffinose growing cells of wild type and of wild-type JKM MATa-inc and isogenic *GAL1::CDC5-MYC* strains, expressing *SAE2*-3HA allele, were split in two. One half was treated with nocodazole to block cells in G2. Galactose was then added to the cultures to induce overproduction of Cdc5. Cells were collected at the indicated times after galactose addition. (B–D) Sae2-HA protein was analyzed by western blots using 12CA5 antibody; Rad53 protein was analysed by monoclonal antibody Mab.EL7.
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proteins throughout a normal cell cycle [20]. Most of the Cdc5 substrates are proteins previously phosphorylated by CDK1, which is the principal regulator of the DSB-induced response, regulating DSB processing, recombination and checkpoint signaling [57]. Here we found that high levels of Cdc5 separately affected Mec1 signaling and DSB processing, leading us to speculate that Cdc5 may regulate multiple targets in response to DNA damage, including factors phosphorylated by CDK1. In support of such hypothesis, Plks phosphorylate, in vertebrates, several proteins involved in various aspects of the DNA damage response, such as FANCM [25], Claspin [21–24], Chk2 [26–28], MCM5 [76], MCM7 [77] and others. Moreover, our findings on the functional role of Cdc5 in responding to a DSB in yeast rise the possibility that Plks may also regulate CtIP.

Recently, a proteome-wide screening led to the identification of novel Cdc5 targets in a normal cell cycle [20]; we believe that a similar approach is promising to identify Cdc5 targets regulated in response to DSBs. Good experimental evidence indicates that the

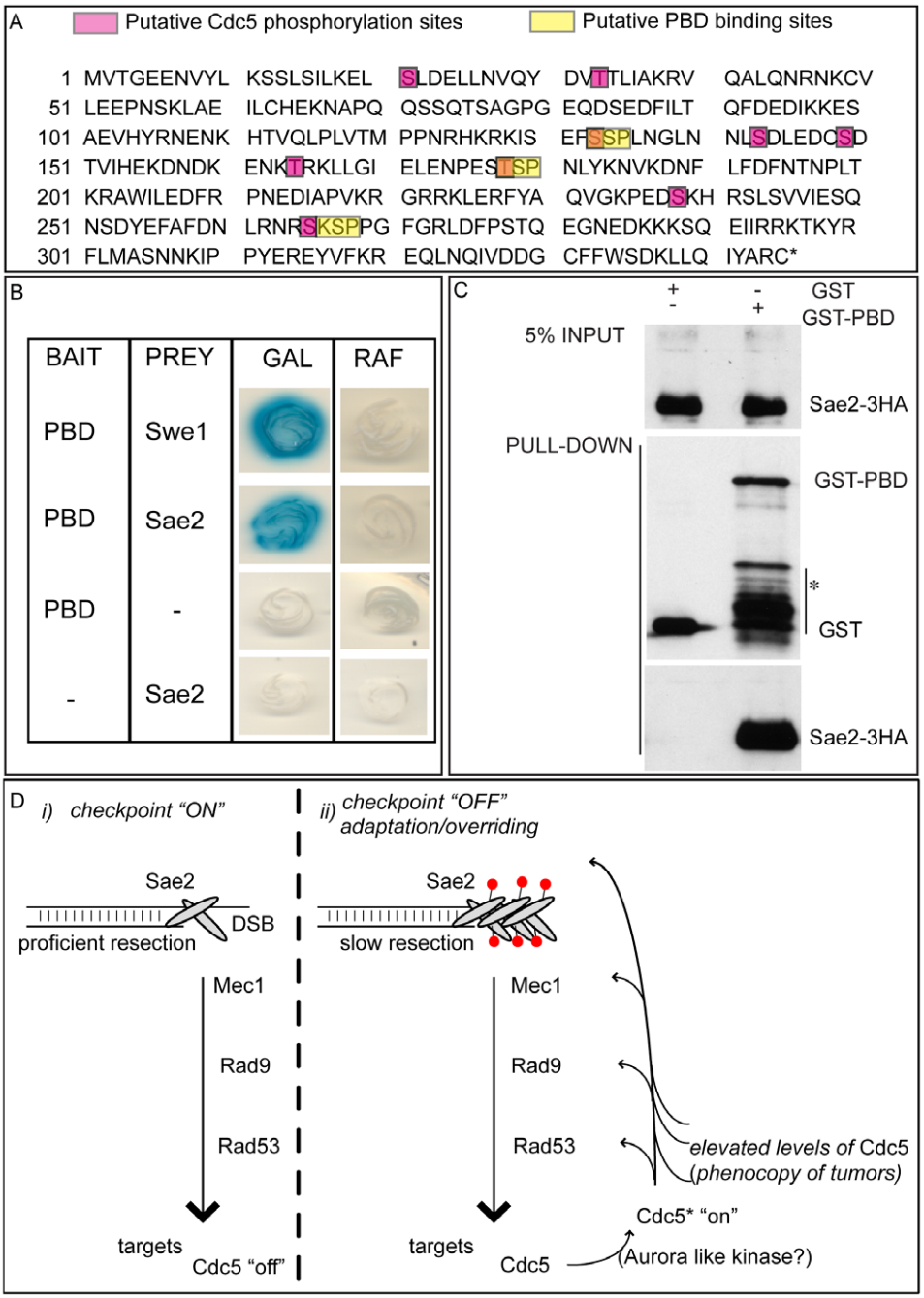

Figure 8. Sae2 protein interacts with PBD of Cdc5. (A) Sae2 protein sequence. The putative Cdc5 phosphorylation sites and PBD binding sites are indicated. (B) Plasmid pEG202-PBD$_{340–705}$, carrying the polo box domain of Cdc5 (PBD, aa 340 to 705), and pJG4-5-SAE2, carrying the full length *SAE2* gene under the *GAL1* promoter, were co-transformed with pSH18-34, a β-galactosidase reporter plasmid in the wild type yeast strain EGY48. To assess two-hybrid interaction, these strains were patched on to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X Gal) plates containing either raffinose (RAF, prey repressed) or galactose (GAL, prey expressed). Accordingly to [50], the strain Y692 (PBD versus Swe1$_{173–400}$ protein fragment) was used as positive control. (C) Cells of the strain Y202, expressing *SAE2*-3HA gene, were blocked in G2/M by nocodazole treatment. Whole cell protein extract was prepared and incubated with glutathione-Sepharose beads carrying GST or GST-PBD$_{357–705}$. Input and pull-down samples were analyzed by western blotting with monoclonal antibody 12CA5 (αHA) or polyclonal antisera raised against GST (αGST). Asterisk denotes bands of GST-PBD degradation or expression of truncated proteins. (D) Schematic model to summarize the results presented in this work. (i) Sae2 transiently binds DSB, regulating ends resection and influencing Mec1-signaling. The checkpoint signal is amplified downstream, regulating several targets, including Cdc5. (ii) After a prolonged checkpoint response, adaptation to damage takes over and Cdc5 is re-activated, likely by an activating kinase (in human cells, it is aurora A [35]); Cdc5 then inhibits checkpoint signaling in a feedback regulatory loop, by likely targeting several factors, including Sae2 whose loading on the irreparable DSB increases, slowing down resection and contributing to counteract the checkpoint signaling (red circles denote phosphorylation). Alternatively, or in addition, Cdc5 function on several targets, including Sae2, is enhanced in the presence of elevated levels of Cdc5, a situation frequently found for Plks in tumor cells.
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functional role of Cdc5 in the DNA damage response is evolutionary conserved and the outputs of such a screening may provide important information for new cancer therapy strategies, targeting Plks and their substrates with specific tools.

Materials and Methods

Yeast strains and plasmids

Strains are listed in Table S1. All the strains were constructed during this study, and all were derivatives of JKM (*MATα*, *hmldelta::ADE1*, *hmrdelta::ADE1 ade1-100*, *trp1delta::hisG*, *leu2-3*, *leu2-112*, *lys5*, *ura3-52*, *ade3::GAL::HO*), with the exception of strain Y38, which was generated from strain Y5 (YMV80, *matΔ::hisG1*, *hmlΔ::ADE*, *hmrΔ::ADE1*, *lys5*, *ura3-52*, *leu2::HOcs*, *ade3::GAL::HO*, *his-URA3-5′Δleu2-is4*). To construct strains, standard genetic procedures for transformation and tetrad analysis were followed. Y38 and Y210 were obtained by integration of ApaI-digested plasmid pJC57 (p*GAL1*::*CDC5*-3HA) at the URA3 locus. Y215 was derived by integration of ApaI-digested pJC59 (p*GAL1*::*CDC5*-3myc) at *URA3* locus. Y220 was obtained by integration of ApaI-digested plasmid pJC62 (p*GAL1*::*cdc5*-K110A-3HA) at *URA3* locus. Y222 was obtained by integration of ApaI-digested plasmid pJC69 (p*GAL1*::cdc5-L251W-3HA) at *URA3* locus. Deletions and tag fusions were generated by the one-step PCR system [78]. The yeast two-hybrid assay was performed using the B42/lexA system with strain EGY48 (*Mata his3 ura3 trp1 6lexAOP-LEU2*; *lexAOP-lacZ* reporter on plasmid pSH18-34) as the host strain [79]. Bait plasmid pEG202-$PBD_{340–705}$ for the two-hybrid assay, expressing lexA fusion with polo box domain of Cdc5, was obtained by amplifying the corresponding coding sequence of *CDC5* gene (aa 340 to 705) from genomic DNA and ligating the resulting fragment into pEG202 (kind gift from R. Brent). Prey plasmids pJG4-5-$Swe1_{173–400}$ and pJG4-5-SAE2, expressing B42 activating domain fusions, were obtained by amplifying the corresponding coding sequence of *SWE1* (aa 173 to 400) and *SAE2* (full length) from genomic DNA and ligating the resulting fragments into pJG4-5.

Western blot analysis

The TCA protein extraction and the western blot procedures have been previously described [29]. Rad53, Rad9, Sae2-HA, Ddc2-HA, Ddc1-myc, Dpb11-myc, Cdc5-HA, Cdc5-myc were analysed using specific monoclonal or polyclonal antibodies: anti-Rad53 Mab.EL7 and Mab.F9 monoclonal [54], anti-HA 12CA5 monoclonal, anti-myc 9E10 monoclonal, anti-Rad9 polyclonal (a kind gift from N Lowndes's lab).

In situ auto-phosphorylation assay

It was performed as previously described [52].

Immunoprecipitation analysis

Yeast whole cell extracts were prepared by FastPrep (MP Biomedicals) in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mm Tris (pH 7.5), 1 mM dithiothreitol (DTT), 60 mM β-glycerophosphate, 1 mM $NaVO_3$, cocktail proteases inhibitors (Roche)). HA-tagged proteins were immunoprecipitated using anti HA monoclonal antibody (12CA5) conjugated to protein G Agarose.

GST pulldown assay

GST and GST-PBD were induced in BL21 *E. coli* cells as previously described [80] and conjugated to glutathione-Sepharose 4B beads (GSH beads, Amersham). Yeast whole cell extracts, prepared as indicated above, were incubated with GST or GST-PBD GSH beads and rotated for 1 hour at 4°C. Samples were washed three times with NP-40 buffer, boiled in SDS-based sample buffer, and analyzed by Western blotting analysis.

In vitro dephosphorylation assay

Crude extracts were prepared as described [52], and resuspended in λ phosphatase buffer with or without 4000 U of λ phosphatase (Biolabs). Samples were incubated 30 min at 30°C and resuspended in Laemmli buffer.

Measurements of DNA resection and SSA at DSBs

Cells grown in YEP-raffinose 3% medium at 28°C to a concentration of 5×10^6 cells/ml were arrested with nocodazole (20μg/ml). A DSB was produced by adding 2% galactose and inducing the production of the HO endonuclease. The maintenance of the arrest was confirmed by FACS analysis and monitoring of nuclear division. Genomic DNA was isolated at intervals, and the loss of the 5′ ends of the HO-cleaved MAT locus was determined by Southern blotting [14,81,82]. To visualize the kinetics of resection, the graphs shown in Figure 4C and Figure 5B display, for each strain and for each ssDNA fragment (r1–r7), the time of the first appearance in the blot. In particular, since the appearance of a ssDNA fragment signal in the gel was due to the loss of the internal *Ssp*I sites, we represented the length of the minimal resection for each time point in the graph (see scheme in Figure 4A). All the experiments have repeated al least 3 times. In the corresponding figures, one representative example is shown with its graphic representation.

Chromatin immunoprecipitation analysis (ChIP)

ChIP analysis was performed as described previously [83,84]. Multiplex PCRs were carried out by using primer pairs complementary to DNA sequences located 1 kb from the HO-cut site at MAT (DSB) and to DNA sequences located 66 kb from MAT (CON). Gel quantitation was determined by using the NIH Image program. The relative fold enrichments of DSB-bound protein were calculated as follow: [DSB_IP/CON_IP]/[DSB_input/CON_input], where IP and Input represent the amount of PCR product in the immunoprecipitates and in input samples before immunoprecipitation, respectively.

Supporting Information

Figure S1 Cellular levels of endogenous and overproduced Cdc5 protein. (A) Exponentially (L) growing culture of the strain Y79 (wild type) and Y114 (*GAL1::CDC5*) were grown in YEP+3%raffinose. The cell cultures were treated with nocodazole to block and maintained the cells in G2/M. Galactose was then added to induce the overproduction of Cdc5 and sample have been taken at the indicated times. (A) The cell cycle block in G2/M was analyzed by FACS. (B) Cdc5 protein was analysed by western blotting with polyclonal antibody, which recognized both the endogenous Cdc5 and the overproduced Cdc5-myc tagged protein.
Found at: doi:10.1371/journal.pgen.1000763.s001 (0.96 MB TIF)

Figure S2 Overproduced Cdc5 co-immunoprecipitates with Rad53. (A) Cultures of the strains Y79 (wild type), Y114 (*GAL1::CDC5*-MYC), exponentially (L) growing in YEP+3%raffinose were blocked in G2/M by nocodazole treatment (N) and zeocin (50 μg/ml) was then added to cause DSBs formation. After 30 min. of treatment with zeocin, 2% galactose was added and samples were taken after 1 hour. Overproduced Cdc5 protein has been immunoprecipitated with anti MYC antibody. Cdc5-MYC

and Rad53 proteins were analysed by western blotting with monoclonal antibodies 9E10 (αMYC) and Ma.EL7 (αRad53).
Found at: doi:10.1371/journal.pgen.1000763.s002 (0.32 MB TIF)

Figure S3 Overproduction of Cdc5 does not prevent DSB repair by Single Strand Annealing (SSA). (A) Schematic representation of the YMV80 system used to detect DSB repair by SSA. Vertical bars show the relevant *KpnI* sites. After the HO cleavage, DNA is resected. When the left and right *leu2* sequences have been converted to ssDNA, repair by SSA can take place and can be monitored by the appearance of a SSA product in a Southern blot. (B,C) YEP+raffinose nocodazole-arrested cell cultures of wild type YMV80 and isogenic *GAL1::CDC5* strain were transferred to nocodazole-containing YEP+raffinose+galactose (time zero). (B) *KpnI*-digested genomic DNA, prepared from cells collected at the indicated times, was analysed by Southern blotting with a *LEU2* probe. Two fragments, 8 and 6 kb long (*his4::leu2*, *leu2::HOcs*) are evident in the absence of HO cut, whereas the HO-induced DSB causes the disappearance of the 6-kb species and the formation of a 2.5-kb fragment (HO-cut fragment). Repair by SSA converts such fragment to a repair product of 3.5-kb (SSA-product). (C) Western blot analysis of protein extracts with anti-Rad53 antibodies (Mab.EL7).

Found at: doi:10.1371/journal.pgen.1000763.s003 (0.81 MB TIF)

Table S1 Yeast strains used in this study.
Found at: doi:10.1371/journal.pgen.1000763.s004 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: RAD MF FL AP. Performed the experiments: RAD MF FL BTN AP. Analyzed the data: RAD MF FL MC BTN AP. Contributed reagents/materials/analysis tools: MC. Wrote the paper: PP MMF AP.

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