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Investigation into the regulation of DNA repair by the S.pombe cell cycle kinase Cdc2-cyclinB

Ewert-Krzemieniewska, Katarzyna

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Investigation into the regulation of DNA repair by the *S. pombe* cell cycle kinase Cdc2-cyclin B

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Katarzyna Ewert-Krzemieniewska

Bangor University, UK

School of Biological Sciences

The North West Cancer Research Fund Institute

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ABSTRACT

Investigation into the regulation of DNA repair by the *S. pombe* cell cycle kinase Cdc2-cyclin B

This PhD project investigated the role of the main *Schizosaccharomyces pombe* cell cycle regulator Cdc2 (CDK1 in *Homo sapiens*, Cdc28 in *Saccharomyces cerevisiae*) in the cell cycle-dependent regulation of homologous recombination. Increasing evidence in the literature suggests that cells suppress homologous recombination in S phase, whereas this process is promoted in G2 to repair ssDNA gaps or DNA breaks that arose during DNA replication. Suppression of recombination in S phase appears to be important because ssDNA gaps are a very good substrate for DNA recombination and the latter process could interfere with DNA replication. Although recombination proteins travel with replication forks and perform repair functions during S phase, cells do not engage full homologous recombination until they enter G2. The underlying mechanisms of this regulation are still enigmatic, but it is known that DNA helicases play a crucial role.

The work presented in this thesis proposes two novel functions for *S. pombe* Cdc2 in the cell cycle-dependent coordination of DNA recombination: (i) during unperturbed cell cycle and (ii) in response to camptothecin (CPT)-induced DNA breaks.

In unperturbed cells, *in vivo* elevated Cdc2 activity causes problems during DNA replication that lead to an increase in spontaneous gene conversion between sister

chromatids and enhanced loss of a non-essential minichromosome. Data presented here suggest that Cdc2 regulates the anti-recombinogenic activity of the Srs2 DNA helicase to prevent such spontaneous gene conversion events in S phase. Both proteins associate with PCNA in distinct protein complexes, which may allow them to regulate DNA repair in S phase. Elevated Cdc2 activity leads to constitutive phosphorylation of the checkpoint kinase Chk1, indicating that the inability to regulate Srs2 DNA helicase causes DNA replication lesions, which engage the G2-M checkpoint.

Cells with elevated Cdc2 activity are specifically sensitive to the Topoisomerase I (Top1) poison CPT. The camptothecin sensitivity of *cdc2.1w* mutant cells increases in the absence of Tyrosyl-DNA-phosphodiesterase (Tdp1), which cleaves immobilised Top1 releasing it from the 3'-end of DNA in S phase. As during the unperturbed cell cycle, Cdc2 appears to regulate Srs2 DNA helicase under these circumstances. Srs2 may unwind the blocked 3'-strand in the absence of Tdp1 to allow a nuclease to access the damaged site. Both Mus81 and Rad16 are potential candidates since both nucleases act in the Cdc2-dependent pathway in response to CPT.

Although all "wee" mutants with elevated Cdc2 activity are defective in this CPT repair pathway, both Wee1 and Mik1 kinase may perform independent repair functions. Wee1 kinase is required for homologous recombination upstream of Rqh1 DNA helicase and for the repair of UV induced DNA damage when nitrogen-starved cells exit the cell cycle in G1. Mik1 kinase, which regulates Cdc2 activity in S phase, appears to act in a novel repair pathway depending on the Ku80-Ku70 heterodimer but not on DNA ligase IV.

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1 Introduction: The role of Cdc2 in the cell cycle-dependent regulation of DNA double-strand break repair

One in three people suffer from cancer once in their lifetime and there are more than 200 different types of cancer (WHO 2006). Cancer is characterized by unregulated cell growth and the invasion and spread of cells from the site of origin. It is a genetic disease, which requires accumulation of several mutations occurring in genes critical for the control of cell growth, differentiation and cell death. Hence, prevention of genetic instability is crucial to evade carcinogenesis. The main driving force of tumourigenesis is advanced age. More than 65% of all malignancies are diagnosed in patients older than 60 years (Cancer Research UK 2007). Since stem cells persist throughout our lifetime, it is reasonable to assume that DNA mutations accumulate over time in stem cells or their fast dividing progenitors, impairing genome maintenance pathways (Feinberg et al. 2006). Such dividing cells may accumulate mutations allowing damaged cells to survive evolving slowly into a tumour. The latter hypothesis is consistent with the observation that many tumours are "monoclonal" (Hoeijmakers 2001; Pecorino 2005).

One of the earliest signs of cancer development is the accumulation of DNA double-strand breaks (DSBs) as a result of DNA replication stress (Bartkova et al. 2005). DNA replication stress can be defined as any state in a cell that leads to either replication fork arrest or dissociation of replication proteins from the DNA (Halazonetis et al. 2008). Such aberrant DNA replication can be induced by activation

of oncogenes or loss of tumour suppressors, or other changes that affect the integrity of the genome. For example, the oncogenic protein c-Myc can induce unscheduled replication in a novel manner independent of its activity as transcription factor (Dominguez-Sola et al. 2007). Since DNA replication problems are common, cells have evolved multiple pathways that allow forks to bypass DNA lesions, that stabilise stalled forks or that repair damaged forks. If however the replication machinery cannot replicate past a DNA lesion, replication can be re-initiated down-stream of the lesion leaving a single-stranded gap opposite the damaged DNA (Sogo et al. 2002). Research in model systems has shown that recombinogenic repair of such gaps is postponed until cells exit S phase. This delayed recombination response seems to be necessary, because homologous recombination, which is one of the major repair pathways of DNA gaps, is actively suppressed while chromosomes are being replicated in S phase (Hyrien 1999; Rothstein et al. 2000). However, the prolonged persistence of single-stranded gaps until cells enter G2 increases the risk of secondary breaks or nucleolytic activities which may convert a gap into a double-stranded break (Figure 1-1). This may explain why DNA replication stress leads to a sharp increase in DSBs in pre-cancerous tissues. The mechanisms behind this cell cycle-dependent oscillation of DNA recombination are not well understood and are therefore the subject of this PhD project.

Cell cycle progression is regulated by cyclin-dependent kinases (CDKs) and many oncogenes can interfere directly or indirectly with CDK activity resulting in aberrant entry into DNA replication (Hartwell and Kastan 1994; Pecorino 2005). Based on recent evidence suggesting that aberrant function or abundance of cyclins impair DSB repair, cancer development may be seen as a vicious cycle such that changes to CDKs not only force cells into an unscheduled S phase resulting in chromosomal breakage

but also compromise error-free repair of these breaks in late S phase and G2 (Caspari et al. 2002; Aylon et al. 2004; Ira et al. 2004) (Figure 1-2). Taken together, the source of genetic mutations that drive tumour evolution appears to be unscheduled DNA replication combined with an inability to coordinate DNA repair.

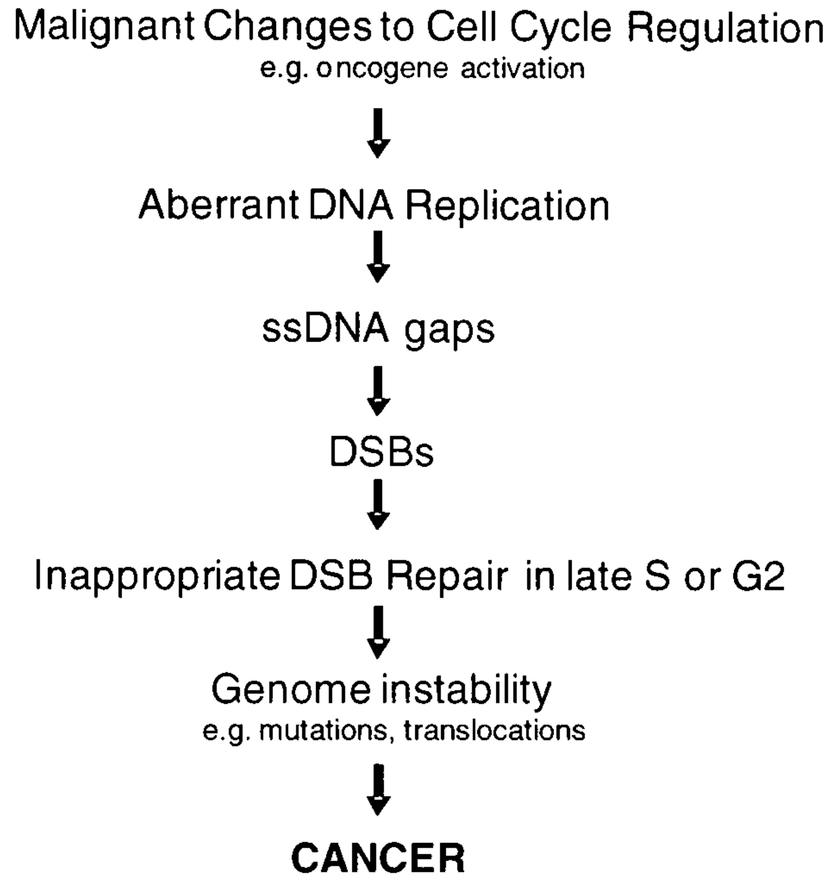


Figure 1-1. A model for cancer development.

Genetic instability, a driving force of tumour development, may result from aberrant repair of chromosomal breaks that arise due to unscheduled DNA replication.

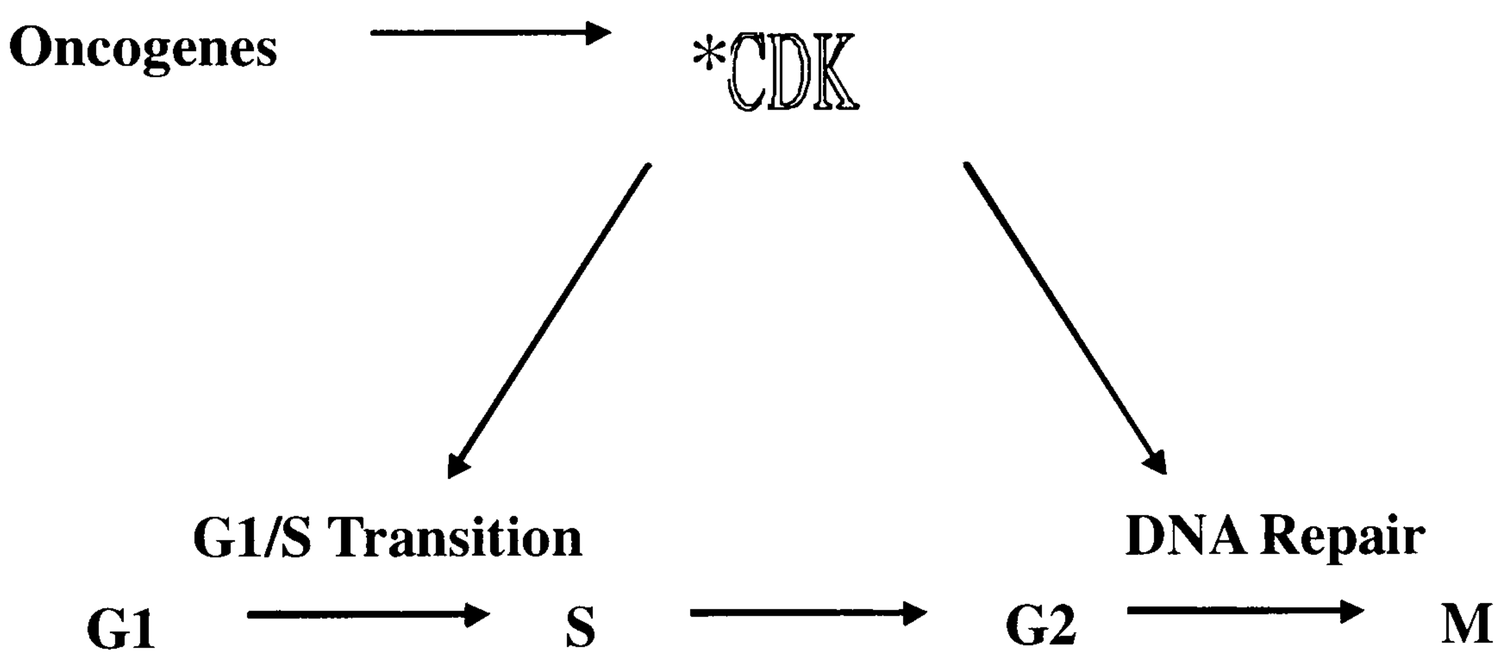


Figure 1-2. Oncogene-induced changes of cell cycle regulators

Oncogene-driven changes of cell cycle regulators such as cyclin-dependent kinases (CDKs) may promote chromosome breakage in an aberrant S phase and may compromise error-free repair of these breaks in G2.

1.1 The DNA double-strand break repair pathways

Accumulation of broken chromosomes in response to replication stress is one of the earliest signs of cancer development (Bartkova et al. 2005). Since DNA double-strand breaks (DSBs) are detected as a very early malignant marker in many different tumours such as breast, bladder or lung, DNA replication stress caused by the activation of oncogenic proteins or by loss of tumour suppressors may be a major driving force of tumourigenesis. Mutations that affect the balance between DNA replication lesions and the appropriate DNA repair pathways are expected to promote cancerous cell growth. Indeed, hereditary mutations in DNA damage checkpoint proteins as well as in proteins that are required for DSB repair predispose patients to an increased tumour risk (Bartek et al. 2007). The accumulation of DSBs in cancerous tissue could be explained by an increase in single-stranded DNA (ssDNA) gaps, which occur when leading and lagging strand synthesis become un-coupled and the replication fork is being re-assembled downstream of the DNA lesion. While this model is widely accepted, such ssDNA gaps have so far only been observed in yeast when DNA replication was challenged with UV light (Lopes et al. 2006).

DSBs are repaired by two main DNA repair pathways, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR), and their interplay as well as their regulation is important to protect cells from the accumulation of DSBs.

1.1.1 Non-Homologous End Joining (NHEJ)

NHEJ can occur throughout the cell cycle and is the main DSBs repair pathway in mammalian cells catalysing the ligation of broken chromosomes independently of their DNA sequence (Riballo et al. 2004). While higher eukaryotic cells possess more NHEJ factors, research in lower eukaryotic cells has established that only three protein complexes are essential to re-join broken chromosomes: the Ku70-Ku80 heterodimer, the trimeric Mre11-Rad50-Nbs1 complex and DNA Ligase IV complex (Dudasova et al. 2004; Daley et al. 2005).

The initial step is the independent and rapid binding of the Ku hetero-dimer and the MRN complex to the chromosomal ends immediately after DSB formation (Wu et al. 2008). The MRN complex composed of Mre11, Rad50 and Nbs1 in *Homo sapiens* (*Hs*) or Xrs2 in *Schizosaccharomyces pombe* (*Sp*) and *Saccharomyces cerevisiae* (*Sc*) is required for limited end processing, which then allows binding of the Ku70-Ku80 complex that encircles the end of the chromosome. The MRN complex, unlike Ku, plays also a role in HR where more extensive 5' to 3' end processing is required (Krogh and Symington 2004). The Ku heterodimer consists of the Ku70 and Ku80 subunits, which form a channel-like structure accommodating the DNA molecule (Aylon and Kupiec 2004; Ira et al. 2004). Having bound, the Ku heterodimer recruits DNA-PKcs kinase in higher eukaryotic cells. So far, no DNA-PKcs related kinase has been identified in yeast. DNA-PKcs is a large protein kinase, which shares sequence homology with ATM and ATR, two other kinases crucial for both DSB repair and checkpoint activation (Walker et al. 2001). DNA-PKcs regulates several NHEJ proteins at the DNA lesion. DNA-PKcs activates mammalian Artemis, an endonuclease required to process chromosomal ends that cannot be directly rejoined.

Since trimming of the ends can result in the deletion of a few nucleotides, NHEJ is potentially an error-prone repair pathway. To promote ligation of the ends, DNA-PKcs activates the scaffold protein XRCC4, the interaction partner of DNA Ligase IV (Lombard et al. 2005) (Figure 1-3). Interestingly, termination of NHEJ by dissociation of Ku and Ligase IV from irreparable DSBs seems to be dependent on the presence of the intact MRN complex as well as ATP binding by Rad50, which suggests a possible role of the MRN complex in the switch between the different DSB repair pathways (Wu et al. 2008). The latter function would be in keeping with the structure of the MRN complex, in which between two and four MRN complexes associate to connect and stabilise broken chromosomes (Hopfner et al. 2000a; Hopfner et al. 2000b; Hopfner et al. 2001; Hopfner et al. 2002).

In addition to general DSB repair, NHEJ plays a crucial role in antibody diversity (V(D)J recombination). V(D)J recombination is the process by which the Variable, Diversity and Joining segments of both the immunoglobulin and T cell receptor genes are rearranged when haematopoietic precursor cells develop into B and T cells. NHEJ is essential to repair the genetically programmed DSBs at the interface between the different segments. Since NHEJ is error-prone, genetic variation at the joints increase the diversity of the variable region of antibodies (Lobrich and Jeggo 2005).

Figure 1-3. The Non-Homologous End Joining pathway.

In mammalian cells, NHEJ joins ends at a DSB without any template using the end-binding Ku70-Ku80 complex, DNA-PKcs kinase, the endonuclease Artemis and the XRCC4-Ligase IV complex. Figure taken from Lombard et al. (Lombard et al. 2005).

1.1.2 Homologous Recombination (HR)

Homologous Recombination is the second major DSB repair pathway in eukaryotic cells. Since HR utilises the genetic information stored in the sister chromatid, it is an error-free pathway and restricted to late S and G2.

The initial step in HR is processing of both chromosomal ends to generate 3'-ssDNA tails. Binding of the MRN complex composed of the proteins Mre11, Rad50 and Nbs1 proceeds 3'-ssDNA formation and is required to recruit ATM kinase to the DNA

lesion (Connelly and Leach 2002; Symington 2002). Although Mre11 possesses nuclease activity, there is clear evidence that additional nucleases and DNA helicases have to bind to the break site (Aylon and Kupiec 2004; Lombard et al. 2005). For example, the nuclease activity of Mre11 possesses the wrong polarity and the protein appears to process secondary DNA structures at the break site rather than being required for ssDNA formation (Kelly and Brown 2000). One good candidate for these unknown nucleases is Exo1, since Exo1 deletion in mouse impairs upstream induction of DNA damage responses by reducing ssDNA formation and the recruitment of both Replication Protein A (RPA) and ATR at DNA breaks (Schaetzlein et al. 2007). Two newly published studies have now provided firm evidence that Exo1 resects DSB ends that have been initially cleaved by the MRN complex (Mimitou and Symington 2008; Zhu et al. 2008). Very recent studies in *S. cerevisiae* propose a model such that DSB ends are processed to 3'-ssDNA tails in two stages. First, the MRX complex in cooperation with Sae2 endonuclease ($Ctp1^{Sp}$, $CtIP^{Hs}$) is required for only limited DSB end resection to generate ends that can be efficiently processed by other nucleases and/or DNA helicases. Subsequently, the 3' end is further extended by either Exo1 nuclease or Sgs1 helicase in association with Dna2, which has both helicase and nuclease activities (Figure 1-4) (Barlow et al. 2008; Gravel et al. 2008; Mimitou and Symington 2008; Terasawa et al. 2008; Zhu et al. 2008). It is currently unclear why different pathways are required for the generation of ssDNA tails at broken chromosomes. The pathway choice may depend on the cell cycle stage, the type of DNA end or the chromatin context of the break.

Figure 1-4. A model for DSB end processing in yeast.

In the first step, the MRX complex together with Ctp1 catalyzes a limited amount of DSB end resection.

The 3'-tailed DNA ends are then processed by either the coordinated helicase and nuclease activities of Sgs1 and Dna2, or possibly Exo1, or by the 5' to 3' exonucleolytic activity of Exo1 (Raynard et al. 2008).

During its formation, ssDNA is coated by Replication Protein A (Alani et al. 1992; Krogh and Symington 2004). RPA is a hetero-trimeric protein complex with high-affinity to ssDNA that helps to 'melt' secondary structures in ssDNA and ensures its stability. Subsequently, the recombination protein Rad51 is loaded by Rad52 onto the ssDNA tail, a process that requires several associated factors. In *Homo sapiens* the functional homologue of Rad52 is the breast cancer protein BRCA2. BRCA2 interacts with monomeric Rad51 and catalyses Rad51 polymerization along the ssDNA to form the Rad51-ssDNA nucleoprotein filament. The factors that regulate BRCA2 recruitment and the putative 'switch' from the signalling function of RPA-coated ssDNA to its repair function include CDK kinase and yet unknown factors (Esashi et

al. 2005; Scully and Xie 2005). The binding of the BRCA2 C-terminal site to Rad51 is negatively regulated by cyclin-dependent kinases (CDKs) *via* phosphorylation of S3291 within the C-terminal domain of BRCA2, suggesting that BRCA2 function in HR is regulated in a cell cycle-dependent manner (Esashi et al. 2005). Interestingly, CDK-dependent phosphorylation of BRCA2 peaks when cells enter mitosis, coinciding with a sharp increase in CDK1-cyclin B activity, indicating that BRCA2 may be inactivated when cells exit G2 to prevent unscheduled recombination in mitosis or to reset the system for the subsequent cell cycle. BRCA2 is a tumour suppressor protein and mutations in its gene predispose to breast and ovarian cancer as well as Fanconi Anaemia (FA), a cancer predisposition syndrome (Kennedy and D'Andrea 2005; Patel 2007). A related role in the G2-specific activation of HR has recently been reported for the second important breast cancer factor, BRCA1, in chicken DT40 cells (Yun and Hiom 2009).

In yeast and mammalian cells, the Rad51 protein associates with a number of HR factors, including Rad52, Rad54 and Rad51 paralogues (XRCC2, XRCC3, Rad51B, Rad51C, Rad51D), which regulate strand invasion and stimulate Rad52 assembly of the Rad51 filament (Aylon and Kupiec 2004). In association with this auxiliary factors, the Rad51 nucleoprotein filament invades the sister chromatid to search for homology, generating a structure known as the D-loop intermediate (Figure 1-5). The 3'-end of the invading Rad51-ssDNA strand provides the primer for DNA polymerases which extend the invading strand thereby copying the lost genetic information from the sister chromatid. The subsequent steps depend on whether the invading DNA strand is being returned to its initial position within the broken chromatid (Figure 1-6, 4a) or whether its 3'-end captures the opposite end of the broken chromatid (Figure 1-6, 4b).

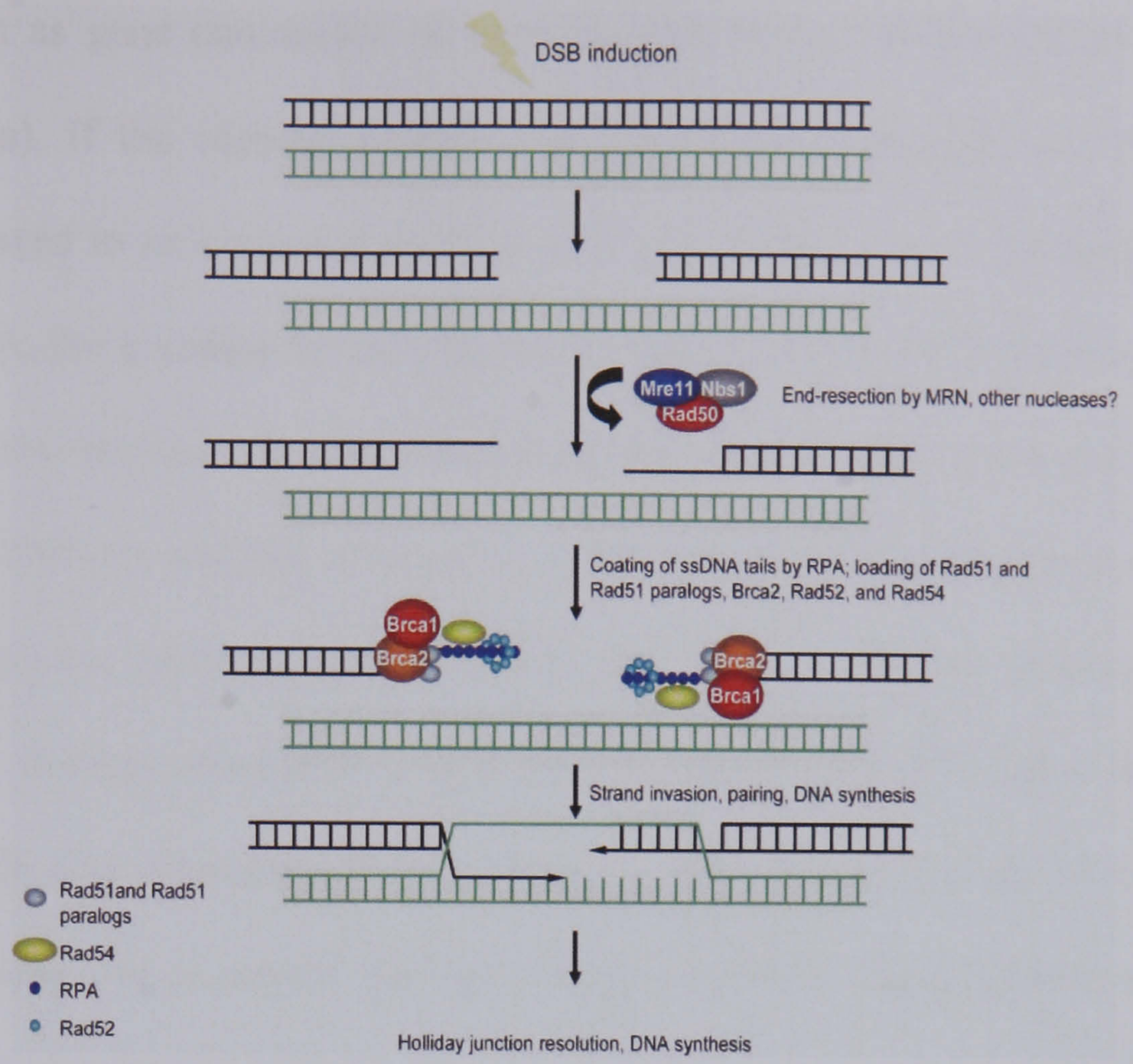


Figure 1-5. The Homologous Recombination pathway.

The initial step of HR is resection of the broken end resulting in 3'-ssDNA. The Rad50-Mre11-Nbs1 complex is required for the initial resection step, which provides access for either Exo1 or the Sgs1 DNA helicase in association with Dna2. The result of this collaborative step is an extended ssDNA tail. This tail is covered by ssDNA-binging protein RPA, which is required for DNA damage signalling and protection of the processed end. Next, the Rad51-ssDNA filament is formed, displacing RPA from the resected DNA, a process facilitated by the BRCA2 ($Rad52^{Sc}$) protein. The nucleoprotein filament probably includes Rad51-related proteins XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D. Rad54 is an ATP dependent DNA translocase of the Swi2/Snf2 family, which acts at the invasion step to stabilise Rad51 on ssDNA and later to promote branch migration of dHJ. The Rad51 and BRCA2 proteins associate with BRCA1, which may perform scaffolding function, potentially coordinating the formation of functional repair complexes at DSBs (Dong et al. 2003). The Rad51 nucleoprotein filament invades the sister chromatid to search for homology providing the 3'-ends to DNA polymerases as primers so that they are able to restore the missing genetic information. The processes of exchange and extension can generate double Holliday junctions (dHJs) when the invading strand captures the opposite end of the broken chromatid. Figure taken from Lombard et al., 2005 (Lombard et al. 2005).

In the first case, the lost information is copied from the intact sister chromatid in a process known as gene conversion or synthesis-dependent-strand-annealing (SDSA) (Figure 1-6, 6a). If the copying process continues further along the chromatid, the process is referred to as break-induced repair (BIR) (Figure 1-6, 6b) (Malkova et al. 1996). In SDSA, the invading strand is disconnected after some DNA synthesis and re-annealed with the broken chromatid, always leading to non-crossover outcomes. This pathway can involve a number of invasion, synthesis and disengagement events. The re-annealing process seems to involve Rad52 and BRCA2 proteins (Petalcorin et al. 2006) and the D-loop dissolution engages BLM ($Sgs1^{Sc}$, $Rqh1^{Sp}$) DNA helicase in association with Topoisomerase III (Adams et al. 2003; Bachrati et al. 2006). In both yeasts *S. cerevisiae* and *S. pombe*, the Sgs1-TopIII and Rqh1-TopIII protein complexes perform similar functions, respectively (Ira et al. 2003; Laursen et al. 2003). SDSA may be an important repair pathway in S phase, since it would re-establish a DNA replication fork. If the DNA synthesis continues to the end of the chromosome (BIR), telomeric sequences may be extended, providing an alternative pathway for telomere lengthening (Dunham et al. 2000; Lydeard et al. 2007).

In the second case, capture of the opposite end of the broken chromatid generates a double Holliday Junction (dHJ) (Figure 1-6, 5c), which can be either resolved by endonuclease such that there is no crossing over of genetic material (Figure 1-6, 6d) or that genetic material is exchanged between the two chromatids (crossover) (Figure 1-6, 6c) (Szostak et al. 1983; Malkova et al. 1996; Sugiyama et al. 1998; Sugiyama et al. 2006). In mitotic DSB repair, HJs are thought to be resolved primarily *via* disengagement without crossover and not by cleavage with crossover (Lombard et al. 2005). Disengagement without crossovers is catalysed by the mammalian Bloom's Syndrome DNA helicase (BLM^{Hs}, $Rqh1^{Sp}$, $Sgs1^{Sc}$) in association with type I

Topoisomerase TOPOIIIα (Figure 1-6, 6e) (Wu and Hickson 2003; Plank et al. 2006; Wu et al. 2006). The importance of this process is reflected by the severity of the cancer-prone Bloom's Syndrome (German 1993; Bachrati and Hickson 2003; Hickson 2003).

Since recombination in mitotic cells is rarely associated with crossovers to avoid genomic rearrangements and loss of heterozygosity (LOH) (Moynahan and Jasin 1997; Richardson and Jasin 2000; Elliott and Jasin 2002), gene conversion (or SDSA) and non-crossover dissolution of dHJ by BLM-TOPOIIIα seem to be likely models for DSB repair in mammalian cells. This is contrary to the situation in meiotic cells, where DSBs are actively generated by the Rec12 (Spo11) protein to promote crossovers and exchange of genetic information (Keeney et al. 1997).

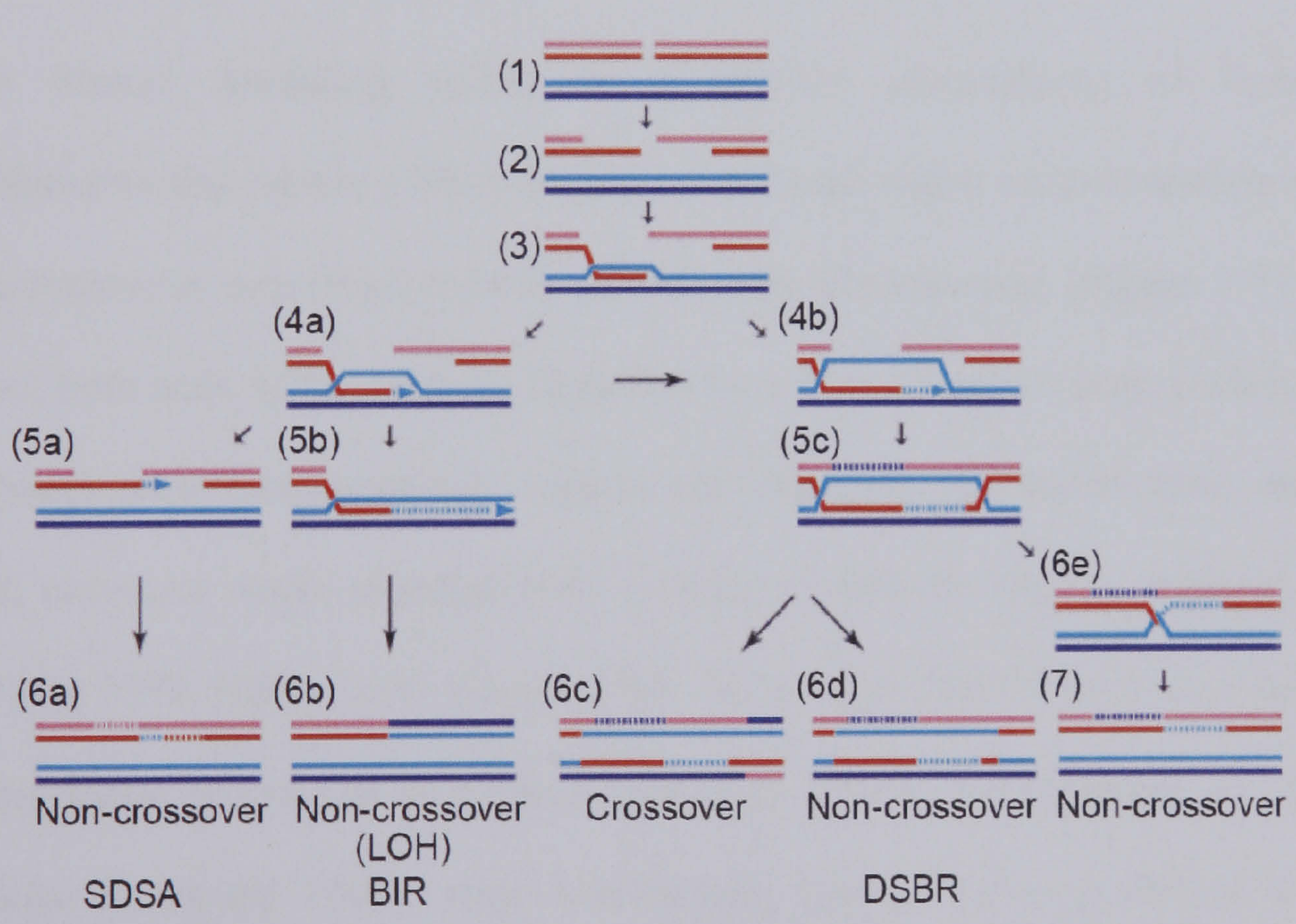


Figure 1-6. Recombination pathways in mitotic DNA DSB repair.

All pathways share the same initial steps: (1) DSB end recognition, (2) 3'-ssDNA generation followed by (3) Rad51-ssDNA strand invasion and homology search. The outcome is a joint molecule (3) which

can be dealt with in at least three different ways, leading to cross- or non-crossover products. In synthesis dependent strand annealing (SDSA - 4a, 5a, 6a), the invading strand is disconnected after DNA synthesis and re-annealed with the broken chromatid leading to gene conversion (non-crossover) products. In break-induced repair (BIR - 4a, 5b, 6b), the invading strand is assembled into a replication fork and the distal arm of the intact chromosome is copied, leading to loss of heterozygosity (LOH). In double-strand break repair (DSBR - 4b, 5c, 6c-e, 7), the invading strand captures the opposite end of the broken chromatid leading to formation of a double Holliday junction (dHJ) (5c). The dHJ can be either resolved by an endonuclease to non- (6d) or crossover (6c) products or by BLM DNA helicase in association with Topoisomerase TOPOIIIα resulting in non-crossover products (6e, 7). Figure adapted from Li and Heyer, 2008 (Li and Heyer 2008).

1.1.3 Single Strand Annealing (SSA) and Microhomology-Mediated End Joining (MMEJ)

Single Strand Annealing (SSA) is a specific sub-pathway of homologous recombination that repairs a DNA double-strand break which occurs between or within two homologous sequences located on the same chromosome (Figure 1-7). In this process, both ends of the DSB are degraded by a 5' to 3' exonuclease (4kb/hour in *S. cerevisiae*) until complementary regions are exposed. The homologous sequences anneal, excessive single-stranded DNA is removed and the break is repaired (Paques and Haber 1999; Haber 2000; Karran 2000; Aylon et al. 2003).The SSA pathway was first suggested by Lin and co-workers (Lin et al. 1985). The efficiency of SSA in *S. cerevisiae* is around 100%, when homologous regions are over 400bp long. The smallest amount of homology, which is recombined at a level of 0.1%, is a stretch of 29bp. Such limited homology allows the ligation of DNA ends in G1 in a Ku-independent pathway known as microhomology-mediated end-joining (MMEJ) (Ma et

al. 2003). SSA repair is efficient even when the homologous regions are separated by as much as 25kb (http://www.bio.brandeis.edu/haberlab/jehsite/ssa.html 2008).

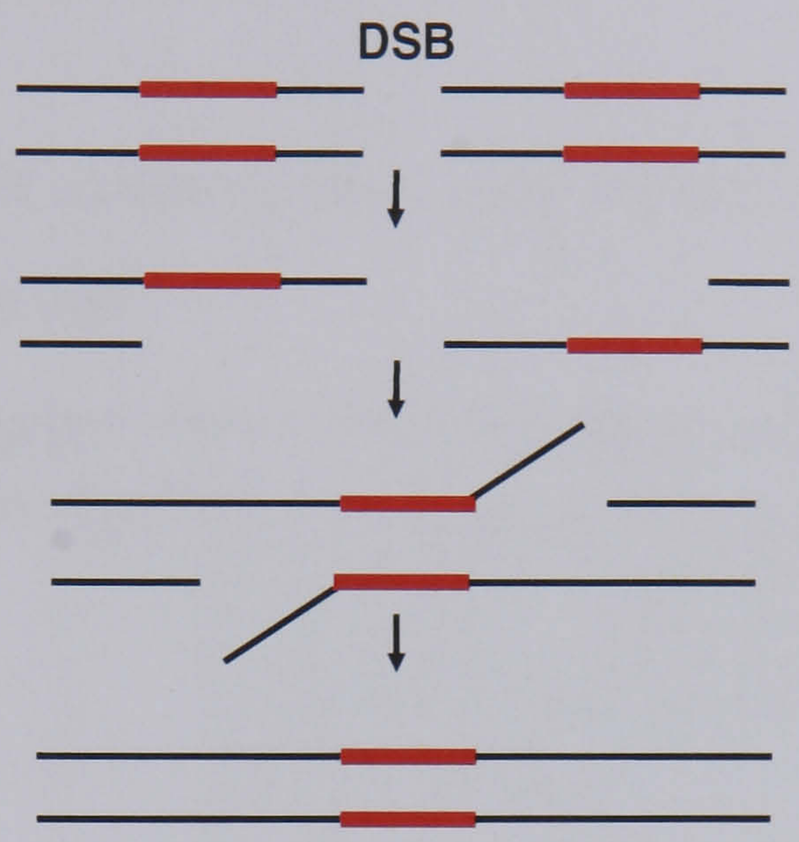


Figure 1-7. Single Strand Annealing (SSA) repair of DNA double strand breaks located between two homologous sequences on the same chromosome.

In this process 5' to 3' exonucleases create single stranded DNA such that the complementary regions can anneal to each other, followed by removal of excessive single stranded DNA.

1.2 The role of Cyclin-dependent kinase Cdc2-cyclin B in the regulation of DNA double strand break repair

Chromosomal breakages can arise at any stage of the cell cycle. While non-homologous end joining appears to act throughout the cycle, homologous recombination requires the sister chromatid and is therefore restricted to late S phase and G2 (Rothkamm et al. 2003; Aylon et al. 2004; Aylon and Kupiec 2005). The molecular mechanisms ensuring this cell cycle-dependent regulation of DNA recombination are not well understood, but emerging evidence suggests that the Cdc2-

cyclin B kinase (Cdk1 ^{Hs}, Cdc28 ^{Sc}), the main regulator of G2-M progression, is required for this control (Table 1-1). Work in yeast has shown that NHEJ and HR activity oscillate throughout the cell cycle with a NHEJ peak in G1 and a HR peak in G2 (Ferreira and Cooper 2004).

Table 1-1. The known Cdc2 phosphorylation events involved in the response to DNA DSB. Reviewed in (Yata and Esashi 2009).

Cdc2 target			Target site			Function of the phosphorylation event		
Hs	*Sp*	*Sc*	*Hs*	*Sp*	*Sc*	*Hs*	*Sp*	*Sc*
CtIP	Ctp1	Sae2	S327	-	S267?	Stimulation of interactions with a tumor supressor BRCA1. CtIP-BRCA1 interact with MRN and stimulate Mre11 nuclease activity promoting DNA end resection. Phosphoryl. in S, G2.	Although there is no analogue site, Ctp1 expression increases in S & G2, which is directly regulated by Cdc2.	Control of DNA-end resection and homologous recombination
			T847	-	S267	Facilitation of DNA end resection. Phosphorylation in G2.	-	Facilitation of DNA end resection. Phosphorylation in G2.
BRCA2		-	S3291		-	Prevention of Rad51 filament formation by blocking interaction of the C-terminal region of BRCA2 with Rad51. Phosphorylation low in S phase, increases in G2/M, decreases after DNA damage.		-
53BP1	Crb2	Rad9		T215	Multiple sites	Phosphorylation is dependent on the cell cycle and increases after DNA damage. The exact role is not yet uncovered.	Stimulation of interactions with Rad3 (ATR) activator Cut5 (TopBP1) leading to accumulation of Crb2 at the damage site. Futher phosphorylation of Crb2 by Rad3 dependent on T215 phosphoryl. is required for subsequent activation of DSB repair.	Activation of downstream checkpoint kinase Rad53. Possibly, down-regulation of Rad9 function in protecting DNA ends from resection.
RPA			S23 & S29 of the N-terminal region of RPA32.			Possibly, promotion of the recruitement of DNA recombination factors such as Rad51, Rad52, BRCA2. Phosphorylation only in the presence of ATR depedent phosphorylation at the S33.		
Chk1	Cds1	Rad53	S286 & S301			Not known. Phosphorylation in M or upon stalled DNA replication (Hu-treatment) and DNA damage checkpoint stimulation (UV-irradiation).		
Recql5 RTEL-1 Fbh1 (?)	Srs2	Srs2		S21?	Multiple sites			Recquired for subssequent phosphorylation by Mec1 (ATR) after DNA damage. Regulation of recombination.

In *S. cerevisiae*, Cdc28-Clb5 activity seems to control resection of the DSB (i.e. 3'ssDNA formation) by regulating the binding kinetics of the MRN (MRX^{Sp}) complex. Although Cdc28 activity promotes ssDNA formation by the MRN complex in G2, no direct molecular link between the kinase and the MRN complex has yet been reported. This link may be provided by CtIP ($Sae2^{Sc}$) which associates with the MRN complex as well as with BRCA1. Phosphorylation of human CtIP at S327 by CDK2 is required for the association with BRCA1 and activation of HR in G2 (Yun and Hiom 2009) as well as for foci formation in the presence of DNA damage (Yu et al. 1998). Recently it has been shown that CtIP contains a second CDK phosphorylation site, T847, near its C-terminus that is conserved between human and yeast. In *S. cerevisiae*, Cdc28 phosphorylates the corresponding site, S267, in G2, promoting the end resection activity of the MRN complex (Sartori et al. 2007; Huertas et al. 2008). Interestingly, the *S. pombe* homologue of CtIP, Ctp1, lacks this conserved CDK phosphorylation site, and it seems that CDK regulation has been replaced by the cell cycle-dependent expression of Ctp1, which increases once cells have entered S phase (Yu and Baer 2000; Limbo et al. 2007). Cdc2-cyclin B activity is however also important in *S. pombe* to activate HR in G2, but the kinase appears to target a yet unknown protein that acts in a parallel pathway with regard to the MRN complex (Caspari et al. 2002). In addition to this early HR function, Cdc28 activity promotes DNA synthesis after the invasion step (Ira et al. 2004). Whether these observations apply to other eukaryotic organisms is not yet clear since S. *cerevisiae* is somehow unique in that cells arrest with high Cdc28 activity in presence of DNA damage (Li and Cai 1997) whereas Cdc2 activity is down-regulated by the DNA damage checkpoint in all other systems.

The decision whether to utilize NHEJ (no or little ssDNA) or to activate HR (long stretches of ssDNA) is clearly linked with the generation of ssDNA tails and the

activity of the MRN complex (Figure 1-8). For example, *S. cerevisiae* cells use NHEJ in G1, when no 3'-ssDNA can be detected, while they employ HR in G2 when ssDNA is formed. Interestingly, the ability to produce ssDNA in G2 does not require replicated chromatids further supporting the hypothesis that cell cycle regulators play an important role (Aylon and Kupiec 2004; Esashi et al. 2005; Scully and Xie 2005). This 'point of commitment' between NHEJ and HR may be controlled by CDK activity *via* the MRX complex. In G1, the MRX complex allows binding of the Ku heterodimer to promote NHEJ, whereas in G2, the MRX complex facilitates binding of nucleases and DNA helicases generating ssDNA (Aylon et al. 2004). However, data in *S. pombe* suggest that the *in vivo* situation may be more complex, because a mutation in cyclin B compromises an early event at a DSB site that does not require the MRN complex (Caspari et al. 2002).

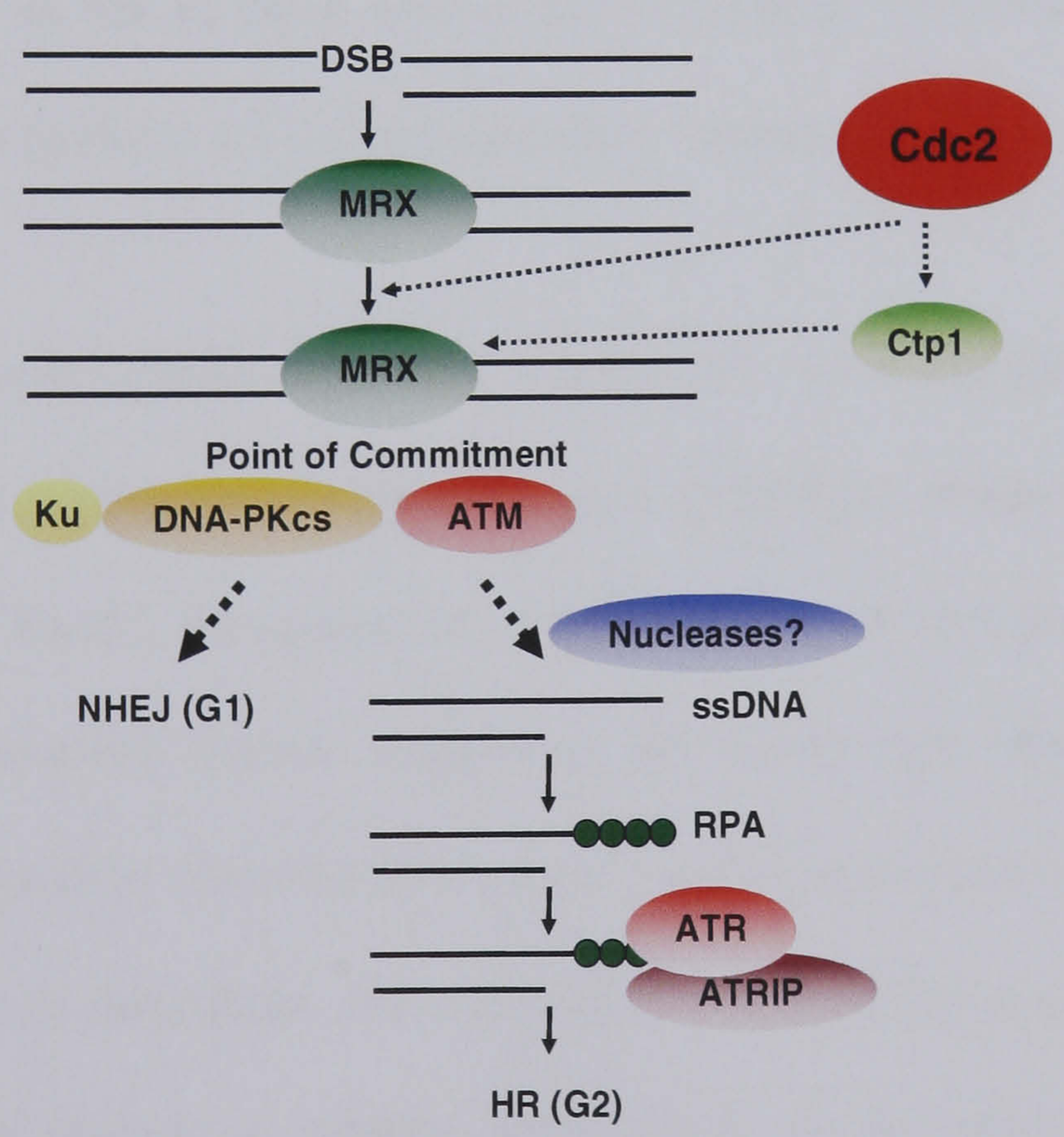


Figure 1-8. Detection of the chromosomal break and decision between NHEJ and HR.

Mre11-Rad50-Nbs1 (MRN) protein complex localises to DSBs recruiting either DNA-PKcs *via* Ku or the checkpoint kinase ATM^{Hs} ($Tel1^{Sp}$). CDK activity modulates the function of the MRN complex *via*

its interaction with CtIP (Ctp1). In *S. pombe* expression of $Ctp1^{Sp}$ ($CtIP^{Hs}$) increases when cells pass through the G1-S transition, allowing the association of Ctp1 with Mre11 in late S and G2. In other organisms, CDK phosphorylation of CtIP within the C-terminal domain promotes its binding to the MRN complex. Binding of CtIP to MRN would switch its function from NHEJ to HR, allowing MRN to recruit nucleases and DNA helicases that would resect the broken end to long 3'-ssDNA tails. The outcome of this regulation would determine whether ssDNA is generated, promoting HR in G2, or whether the Ku heterodimer binds to the break preventing ssDNA formation in G1. The presence of RPA-coated ssDNA would recruit the checkpoint kinase ATR^{Hs} ($Rad3^{Sp}$) and favour association of recombination proteins ($BRCA1^{Hs}$, $BRCA2^{Hs}$, $53BP1^{Hs}$, $Rad9^{Sc}$, $Crb2^{Sp}$).

In addition to this early event, Cdc2-Cyclin B plays a second role in DSB repair by regulating the activity of the Rqh1 (BLM in *H. sapiens*)-TopIII protein complex probably in the context of the resolution of Holliday Junctions (Caspari et al. 2002). Although cyclin B mutant cells possess a reduced Cdc2 activity, it has not yet been shown that the effect on HR in these cells is due to reduced Cdc2-cyclin B activity or whether cyclin B may perform a Cdc2-independent function.

Cdc2-dependent phosphorylation can also inhibit HR. Mammalian Cdc2-Cyclin B phosphorylates S3291 within the C-terminal region of BRCA2 blocking the interaction between BRCA2 and Rad51. Phosphorylation of S3291 is low in S phase but increases when cells progress towards mitosis, suggesting that Cdc2 may either switch off HR when cells enter M phase or reset the HR system for the subsequent cell cycle (Esashi et al. 2005). This idea is consistent with the peak in Cdc2-cyclin B activity at the G2-M transition and may provide a common mechanism preventing recombination at a time cells intend to separate sister chromatids. A similar regulation may exist in *S. pombe*, where Cdc2 phosphorylates two residues within the N-terminal domain of Crb2 ($Rad9^{Sc}$, $53BP1^{Hs}$) S43 and T215, when cells enter mitosis (Esashi and Yanagida

1999). Since phosphorylation of T215 is required for the DNA damage response and for re-establishing cell cycle progression after activation of the DNA damage checkpoint, modification of Crb2 may re-set the system for the next cell cycle round. This notion would be consistent with the observation that T215 phosphorylation is dispensable for the DNA damage response in non-cycling G0 cells (Mochida and Yanagida 2006). Interestingly, both Crb2 modifications are present in cells that express a mutant version of cyclin B, indicating that cyclin B does not act by changing the phosphorylation status of Crb2 (Caspari et al. 2002). Intriguingly, the regulation of Rqh1-TopIII DNA helicase by cyclin B requires Crb2 but not its Cdc2 phosphorylation events (Caspari et al. 2002). Since human BLM helicase unwinds double Holliday junctions in presence of Topoisomerase III (Wu et al. 2005), Cdc2-cyclin B may stimulate resolution of late recombination intermediates in a crossover-free manner.

CtIP, BRCA2 and Crb2 (53BP1) are not the only CDK targets involved in the response to DSBs. The N-terminal domain of human RPA32, one of the three RPA subunits, becomes phosphorylated by both CDK1 and CDK2 at S23 and S29 in response to DNA damage, but only when RPA32 is already phosphorylated at S33 by ATR kinase (Anantha et al. 2007). Since the N-terminal domain neither binds to ssDNA nor to any known protein, the physiological functions of this CDK phosphorylation are not known. Another enigmatic target of CDK1 is human Chk1 kinase, which becomes phosphorylated at two sites, S286 and S301, when cells enter mitosis or when DNA replication stalls in presence of hydroxyurea (Ikegami et al. 2008).

1.3 Suppression of recombination during DNA replication in S phase.

Another important aspect of cell cycle-dependent regulation of DNA recombination is the active repression of HR during S phase in eukaryotic cells. Although HR plays an important role in repairing DNA lesions simultaneously with DNA replication in prokaryotic cells [reviewed in (Michel et al. 2007)], both processes are separated in eukaryotic cells. For example, certain recombination factors such as the endonuclease Mus81 are excluded from chromatin when replication forks stall (Kai et al. 2005) and Rad22 recombination foci sharply increase when cells exit S phase (Meister et al. 2005). This suppression of recombination is mirrored by a broad spectrum of different pathways that deal with DNA replication lesions during replication without activating HR. Interestingly, HR proteins such as Rad51 travel with the replication fork but they are not required for bulk replication (Bailis et al. 2008). The role of recombination factors during DNA replication is somehow confused by the fact that recombination is crucial to re-start DNA replication forks in prokaryotic cells and that individual HR factors can perform DNA repair functions during S phase in eukaryotic cells without initiating the full recombination pathway (Lisby et al. 2003).

In what way could HR be toxic during DNA replication? The answer may be provided by the competition for ssDNA. Normally ssDNA is not exposed at DNA replication forks, but fork damage or inactivation of the DNA damage checkpoint functions can lead to larger sections of ssDNA being accessible for HR factors. Exposure of small stretches of ssDNA is actually important to activate the DNA damage response (Tourriere and Pasero 2007). In prokaryotic cells, HR factors would bind and re-start

DNA replication, but in eukaryotic cells this does not happen. The reason could be the complexity of the eukaryotic genome, which contains more repetitive or micro-homologue sequences than a smaller prokaryotic genome. If HR were to act on ssDNA exposed at broken forks, DNA could become attached to distant regions in the genome, impairing successful replication of chromosomes. This may explain why eukaryotic cells have evolved multiple pathways that allow forks to bypass DNA lesions, to stabilise stalled forks (i.e. prevent their breakage) or to repair replication lesions by template switching at the fork (McGowan 2003; Friedberg 2005). If however, the replication machinery can not replicate past a DNA lesion, a new polymerase complex appears to be loaded downstream of the lesion, leaving ssDNA gaps behind the fork which are not repaired before cells exit S phase and gain access to HR pathways (Carr 2002; Cha and Kleckner 2002; Sogo et al. 2002; Zachos et al. 2005). The prolonged persistence of single-stranded gaps until cells enter G2 may be an economic way to deal with major replication obstacles without compromising replication of the remaining genome. This may be the reason why eukaryotic cells possess an oscillating requirement for DNA replication and DNA recombination (Hyrien 1999; Rothstein et al. 2000; Kolodner et al. 2002).

1.3.1 Topic of the PhD project

Despite its importance for genomic stability, little is known about the molecular mechanisms underlying the oscillation between DNA replication and DNA recombination. Although the role of CDK kinases in the activation of recombination in G2 becomes much better understood, hardly any information is available whether and how they promote recombination avoidance pathways in S phase. Avoidance of

recombination seems to depend on the coordinated interplay between the intra-S checkpoint, some circadian clock proteins, Proliferating Cell Nuclear Antigen (PCNA) modifications and DNA helicases (Figure 1-9). The changing roles of DNA helicases such as Rqh1^{Sp} (BLM^{Hs}, Sgs1^{Sc}) and Srs2^{Sp} (Rtel-1, RecQL5β^{Hs}) are of particular interest because they promote DNA recombination in G2, while they prevent the same process in S phase. This is why this PhD project investigates the potential links between Cdc2 and DNA helicases in the context of DNA recombination.

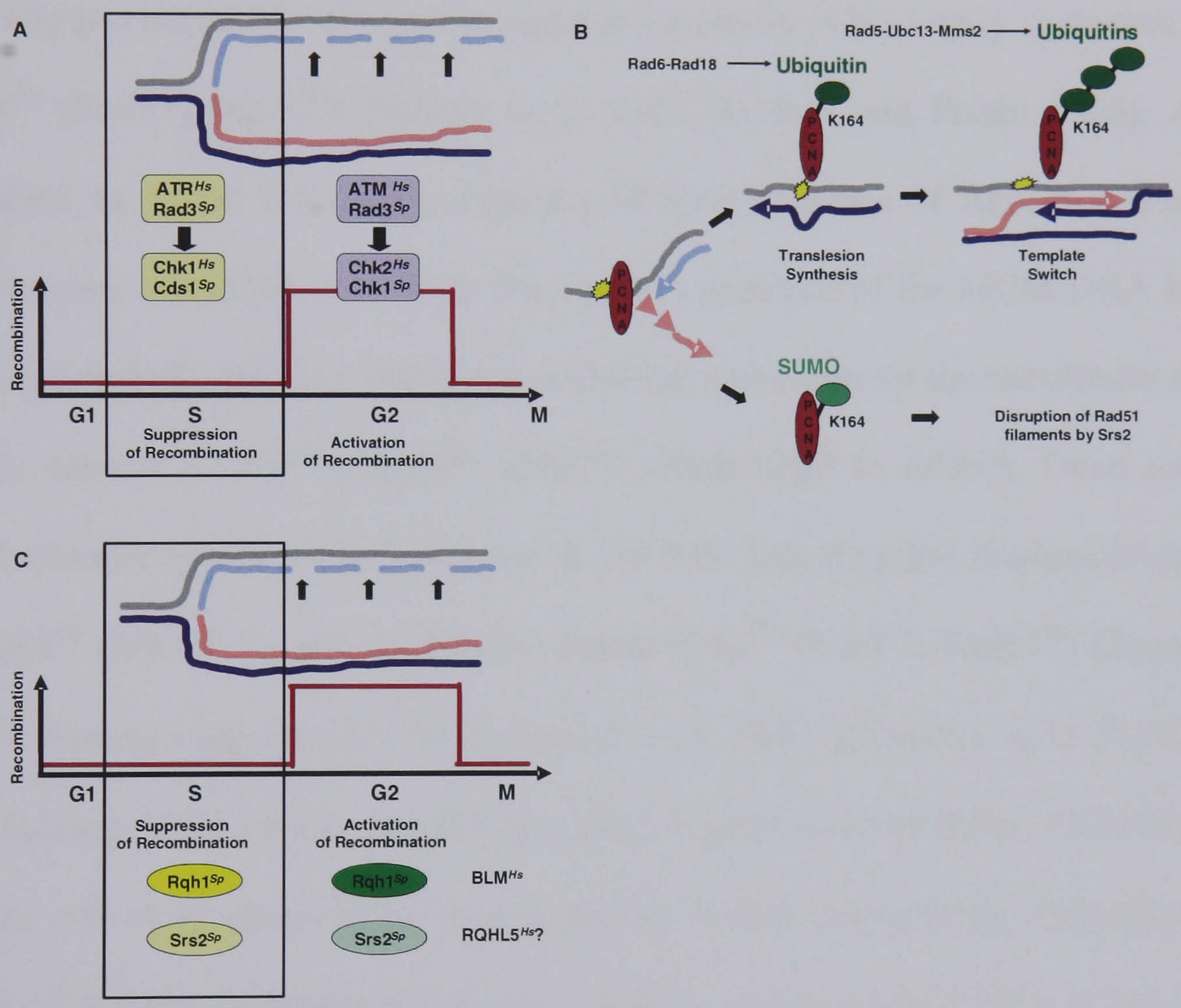


Figure 1-9. The DNA recombination-avoidance mechanisms.

A) The intra-S checkpoint (ATR – Chk1 pathway) suppresses recombination during DNA replication by stabilising stalled forks (see chapter 1.3.2). B) PCNA modification by Ubiquitin and SUMO promote alternative DNA repair mechanisms at stalled replication forks like Translesion Synthesis or Template Switch (see chapter 1.3.4). C) DNA helicases such as Rqh1^{Sp} or Srs2^{Sp}, dependent on the phase of cell cycle, can either suppress or promote HR (see chapter 1.3.5).

1.3.2 The intra-S checkpoint as a recombination-avoidance mechanism

The importance of the intra-S checkpoint in the repression of recombination during DNA replication is demonstrated by the detection of pathological recombination intermediates and recombination foci when checkpoint defective cells progress through S phase in the presence of DNA damage (Lopes et al. 2001).

The key protein of this checkpoint signalling pathway is the highly conserved kinase ATR^{Hs} ($Rad3^{Sp}$, $Mec1^{Sc}$) (Nyberg et al. 2002; Branzei and Foiani 2006). ATR is recruited to stalled forks upon exposure of short stretches of RPA-coated ssDNA. These areas of ssDNA arise from the limited separation of the MCM DNA helicase from the stalled replication machinery and act as a platform for the recruitment of ATR by its subunit $ATRIP^{Hs}$ ($Rad26^{Sp}$, $Ddc2^{Sc}$) which binds to ssDNA. Once activated, ATR phosphorylates several proteins at the fork including the checkpoint-mediator $Claspin^{Hs}$ ($Mrc1^{Sp,\ Sc}$) and the effector kinase $Chk1^{Hs}$ ($Cds1^{Sp}$, $Rad53^{Sc}$) (Brush et al. 1996; Kumagai and Dunphy 2000; Paciotti et al. 2000; Alcasabas et al. 2001; Rouse and Jackson 2002; Osborn and Elledge 2003; Namiki and Zou 2006). Activated Chk1 blocks cell cycle progression, down-regulates further origin firing, stabilises stalled replication forks, facilitates translesion synthesis and suppresses DNA recombination (Figure 1-10) (Zhou and Elledge 2000; Melo and Toczyski 2002; Nyberg et al. 2002; Sancar et al. 2004; Kai et al. 2005).

ATR-dependent stabilisation of stalled forks seems to be crucial in preventing inappropriate and elevated levels of HR during S phase. ATR and Chk1 phosphorylate several target proteins directly related to this function (Lisby et al. 2004; Meister et al.

2005; Paulsen and Cimprich 2007). Both kinases stabilise stalled replication forks by preventing disassociation of replicative polymerases from the replication machinery and by inhibiting further unwinding of DNA by the replicative MCM DNA helicase. The latter regulation is important to suppress the formation of excessive ssDNA at stalled forks which may otherwise recruit HR proteins. In addition, the *S. pombe* Rad3 (ATR^{Hs}) – Cds1 ($Chk1^{Hs}$) pathway prevents fork cleavage by Mus81 endonuclease upon its phosphorylation causing its dissociation from chromatin. Furthermore, this pathway may regulate fork reversal by modulating RecQ (BLM^{Hs}) helicase activity. Fork reversal is a process in which the newly synthesized DNA strands anneal, allowing the controlled dissociation of leading and lagging strand synthesis to bypass DNA lesions (Sogo et al. 2002; Cobb et al. 2003; Cobb et al. 2005; Kai et al. 2005).

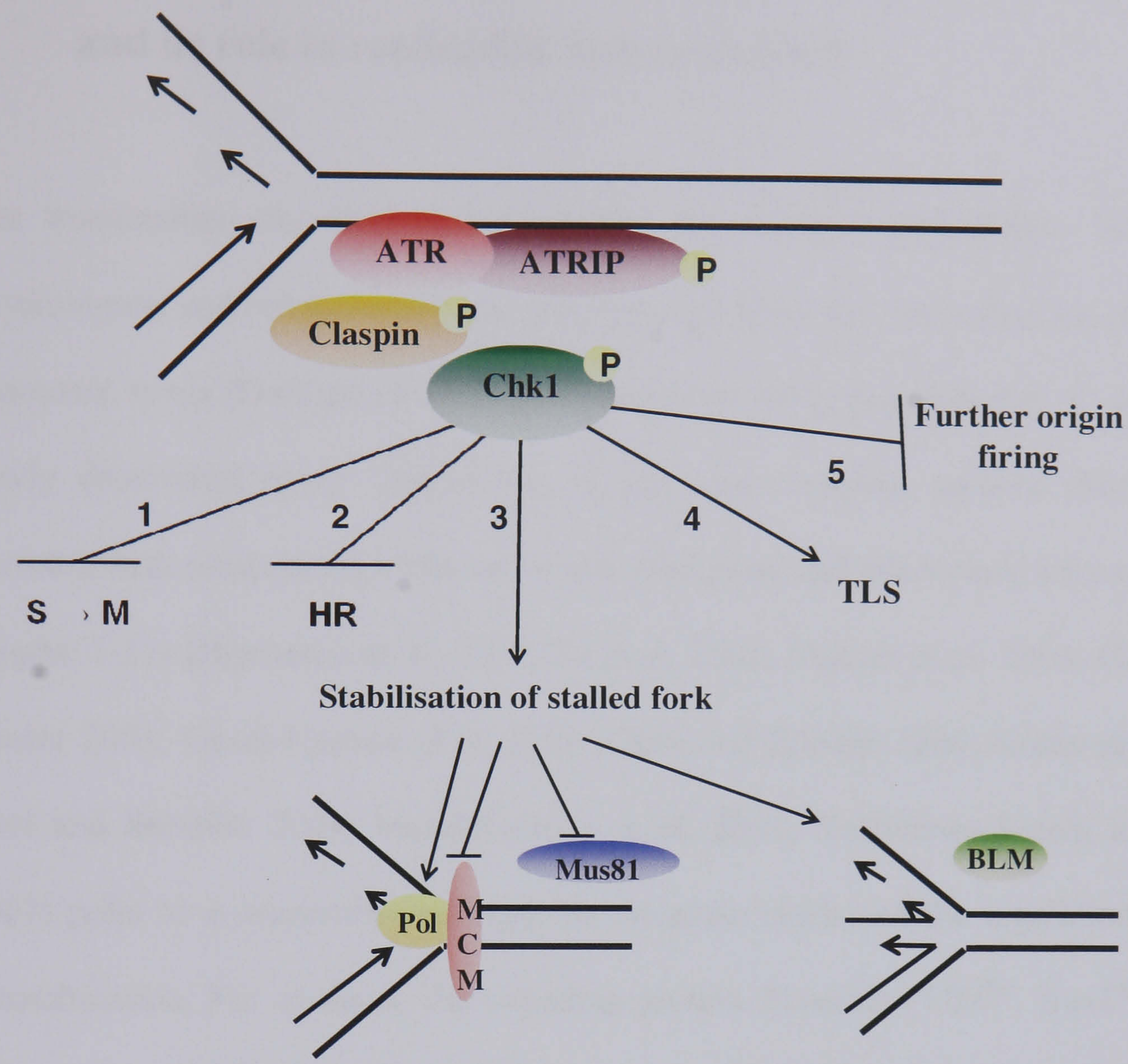


Figure 1-10. Schematic of the intra-S checkpoint functions in response to stalled replication fork.

In response to stalled replication fork the ATR-Chk1 pathway (1) delays onset of mitosis to provide time to complete replication before cell division; (2) blocks HR factors; (3) stabilises stalled forks by preventing disassociation of replicative polymerases from the replication machinery by inhibiting the replicative MCM DNA helicase, by preventing fork cleavage by Mus81 endonuclease or/and by promoting the bypass of DNA lesions by fork reversal through modulating BLM DNA helicase activity; (4) promotes alternatives to HR such as Translesion Synthesis (TSL) or Template Switching TLS, and (5) blocks origin firing.

1.3.3 The circadian clock as a regulator of the intra-S checkpoint and its role in replication fork protection

The mammalian circadian clock regulates the 24-hour periodicities of multiple physiological and behavioural processes including those that determine the response to genotoxic stress (Duffield et al. 2002; Panda et al. 2002; Kondratov et al. 2007). The newly discovered direct interactions of the core circadian proteins Period-1 and Period-2 with components of the cell cycle checkpoint and DNA replication machinery (Figure 1-11) (Bjarnason et al. 2001; Fu et al. 2002; Matsuo et al. 2003; Gauger and Sancar 2005; Unsal-Kacmaz et al. 2005; Chou and Elledge 2006; Gotter et al. 2007; Levi and Schibler 2007; Unsal-Kacmaz et al. 2007; Yoshizawa-Sugata and Masai 2007) point to a connection between the circadian clock and the regulation of DNA recombination. For instance, the circadian protein Timeless (TIM^{Hs}, $Swi1^{Sp}$, $Tof1^{Sc}$) affects the ATR-Chk1 pathway through its interaction with Claspin ($Mrc1^{Sp\ Sc}$), which binds to Chk1 and replicative polymerases (Gotter et al. 2007). In addition, TIM and its non-circadian partner TIPIN form a heterodimer (Gotter 2003) that associate with protein complexes assembled at the replicative fork and their yeast homologues Tof1-Crm1 have been shown to travel with the replication fork (Foss 2001; Chou and Elledge 2006; Gotter et al. 2007; Unsal-Kacmaz et al. 2007; Yoshizawa-Sugata and Masai 2007) suggesting that the circadian clock regulates the oscillation between DNA replication and DNA recombination. Consistent with this idea, down-regulation of TIM-TIPIN by siRNA increases levels of DNA DSBs probably as a result of impaired regulation of DNA repair in S phase (Chou and Elledge 2006). Taken together these findings indicate a role for the TIM-TIPIN heterodimer in the protection of replicative fork. This is supported by TIM yeast homologues $Tof1^{Sc}$ and $Swi1^{Sp}$ also being

implicated in DNA replication and the regulation of DNA damage responses in S phase (Dalgaard and Klar 2000; Foss 2001).

A possible explanation for why this circadian requirement may have evolved is provided by the metabolomic clock in *S. cerevisiae*. Yeast cells that grow under physiological, low glucose conditions oscillate between respiration and fermentation. DNA replication is restricted to the low-oxygen fermentation stage of this cycle to avoid DNA damage by active oxygen radicals (Chen et al. 2007). Consistent with the role of circadian proteins in the regulation of the ATR-Chk1 pathway, inactivation of the *S. cerevisiae* homologue of Cds1, Rad53, abolishes this regulation and cells accumulate point mutations because DNA replication takes place during the high-oxygen stage.

Figure 1-11. Roles of mammalian circadian proteins in the regulation of cell cycle checkpoint and DNA replication.

Upon DNA damage the circadian proteins PER1 and TIM1 activate cell cycle checkpoint effector kinases Chk2 and Chk1, respectively. The interaction between PER1 and TIM1 links the G1-S

checkpoint (ATM-Chk2 pathway) with the G2-M (ATR-Chk1) checkpoint responses. Additionally, TIM and its non-circadian partner TIPIN, under both normal and stress conditions, associate with protein complexes at the replicative fork and thus regulate DNA replication. Figure taken from Kondratov and Antoch, 2007 (Kondratov and Antoch 2007).

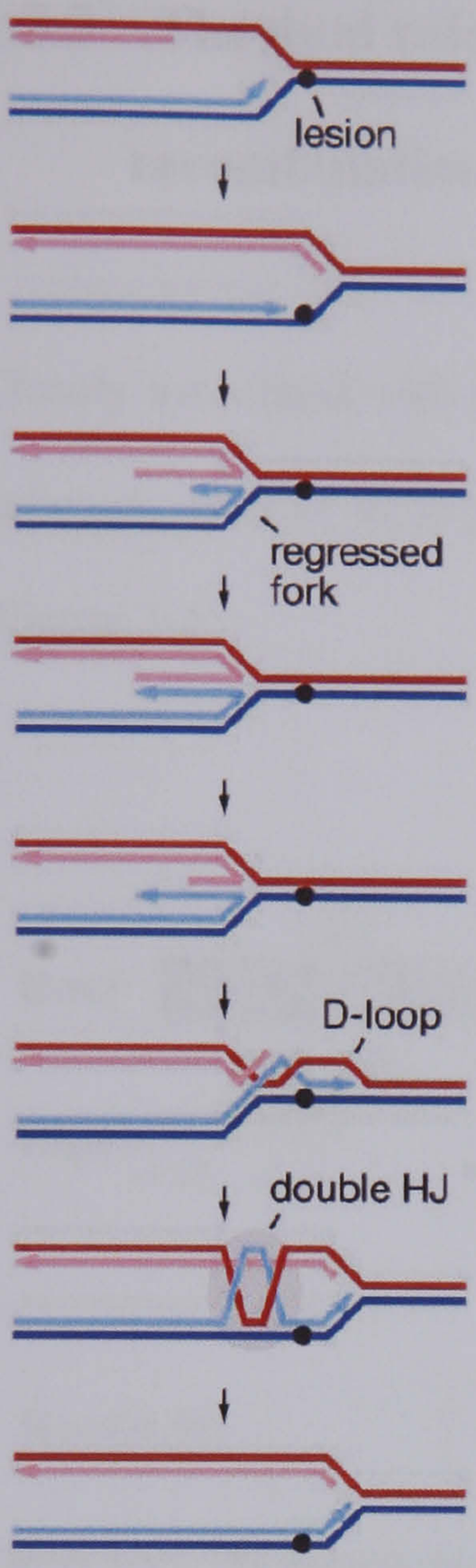
1.3.4 Proliferating Cell Nuclear Antigen (PCNA) modifications as recombination-avoidance mechanisms

The process of recombination avoidance includes also the regulation of PCNA by covalent linkage to small protein modifiers. Data from *S. cerevisiae* show that modification of PCNA at lysine 164 (K164) with either SUMO or Ubiquitin activates distinct recombination avoidance pathways (Figure 1-9, B) (Papouli et al. 2005; Pfander et al. 2005).

Mono-ubiquitination of PCNA at lysine 164 is induced by the Rad6 – Rad18 (Rhp6 – $Rhp18^{Sp}$) pathway in response to replication fork stalling and triggers the potentially error-prone Translesion Synthesis pathway (TLS). Mono-ubiquitinated PCNA displaces the blocked replicative DNA polymerases Polδ and Polε, and allows synthesis across a DNA lesion by the DNA polymerases Polη and Polζ (Hoege et al. 2002; Stelter and Ulrich 2003; Kannouche et al. 2004; Moldovan et al. 2007). This polymerase switch is an important mechanism for cells to proceed with DNA replication in presence of modified DNA bases without having to reassemble the replication machinery down-stream of the blockage. This process is not recombinogenic, because it does not generate ssDNA gaps. If TLS fails to permit replication of a damaged template, the Rad5-Ubc13-Mms2 pathway attaches additional ubiquitin units to PCNA *via* lysine 63 (K63), facilitating an error-free template switch

(TS) mechanism (Ulrich and Jentsch 2000). This enigmatic template switch pathway utilizes the recombination protein Rad52 in *S. cerevisiae* ($Rad22^{Sp}$) (Zhang and Lawrence 2005) and may be functionally related to Rad52-dependent Break-Induce-Replication (BIR) (Malkova et al. 1996). The mechanistic details of TS are not well understood and it is possible that this pathway is closely related to a process known as "regression of stalled DNA replication forks" (Figure 1-12) (Higgins et al. 1976; McGlynn and Lloyd 2000; McGlynn and Lloyd 2002; Blastyak et al. 2007). Stalled forks are predicted to be regressed by DNA helicases such as Rqh1 ($Sgs1^{Sc}$, BLM^{Hs}) and/or FANCM ($Fml1^{Sp}$) such that the two newly synthesized DNA molecules anneal forming a "chicken foot" structure (Ralf et al. 2006; Gari et al. 2008; Sun et al. 2008). The benefit of this pathway is that partial uncoupling of leading and lagging strand would permit the usage of the newly synthesized DNA strand as a template instead of the damaged, original strand. This would result in DNA synthesis past the lesion before normal DNA replication would resume. Since this process involves the switch from the original, damaged template to the newly synthesized strand, it could be classified as TS.

Interestingly, only ubiquitination of K164 is conserved across species but not SUMO-ylation, which has not yet been found in *S. pombe* and human cells (Frampton et al. 2006). In *S. cerevisiae,* SUMO-ylation of PCNA at K164 and K127 occurs during unperturbed S phase and in response to DNA replication stress in an Ubc9-dependent manner to recruit Srs2 DNA helicase to stalled replication forks. Srs2 is an anti-recombinogenic helicase which prevents unwanted Sister-Chromatid Exchange (SCE) by disrupting Rad51 nucleoprotein filaments (Papouli et al. 2005; Pfander et al. 2005; Branzei and Foiani 2006).



Replication fork stalls at lesion in leading strand.

Uncoupling of lagging strand polymerase.

BLM or FANCM-mediated fork regression.

Leading strand polymerase switch from damage template to nascent lagging strand.

Double-stranded DNA end resection by exonuclease.

Recombination-mediated re-initiation of replication.

Dissolution of dHJ by BLM.

Bypassed lesion.

Figure 1-12. Model for regression of stalled DNA replication forks and recombination-mediated re-initiation of replication.

A lesion on the leading strand template during DNA replication may cause replication fork stalling and uncoupling of polymerases. BLM or FANCM-mediated fork regression allows then template switching so that the leading strand can be extended pass the lesion. The reversed fork can be reset and replication restarted after double-stranded DNA end resection by exonuclease and Rad51-mediated strand invasion of the template DNA ahead of the lesion, resulting in D-loop and dHJ formation, which can be dissolved by BLM helicase. Figure adapted from Sun et al, 2008 (Sun et al. 2008).

1.3.5 The dual roles of DNA helicases in the regulation of DNA recombination

Closely associated with the anti-recombinogenic pathways discussed above are DNA helicases, such as the highly conserved RecQ and UvrD/Rep family of DNA helicases (Figure 1-13).

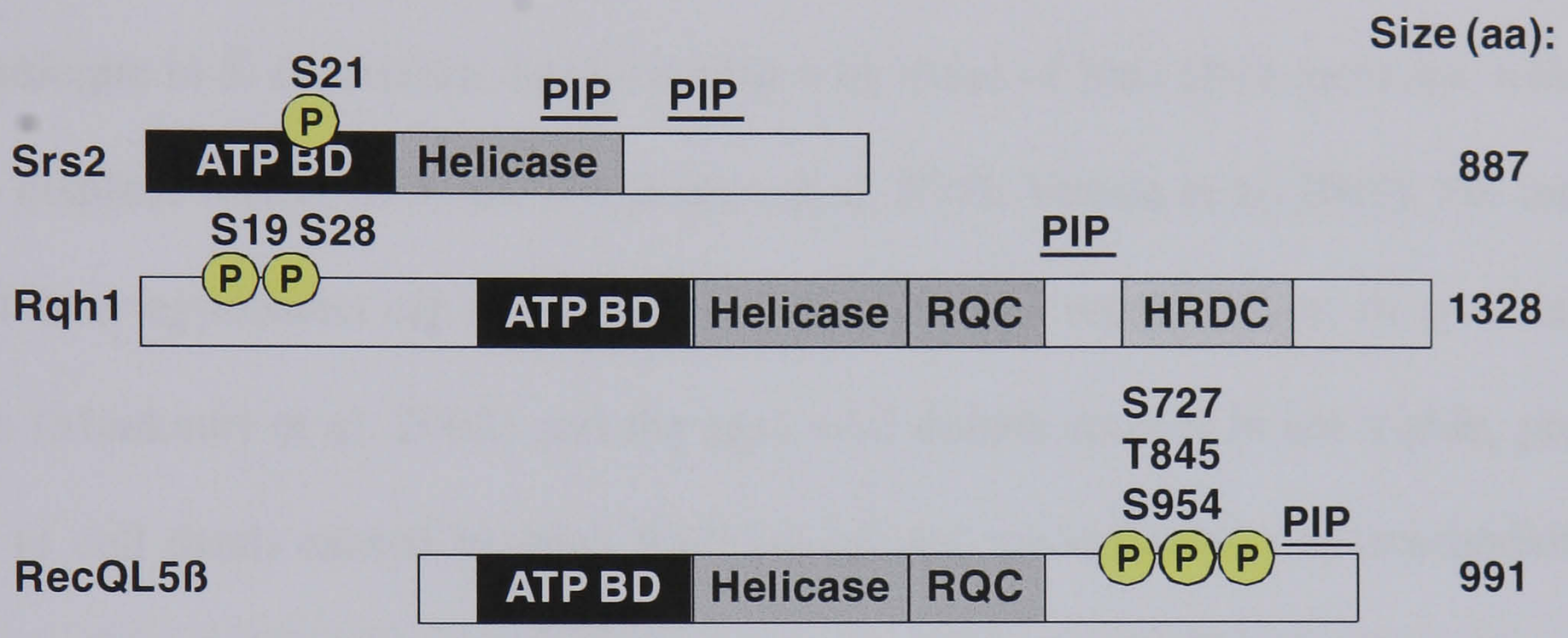


Figure 1-13. The domain structure of Srs2, Rqh1 and Recql5 helicases.

The ATP BD represents ATP binding domain. The RQC domain is unique for RecQ helicases and is important for DNA binding and overall protein stability. The HRDC is another DNA binding domain (Wu 2007). PIP is the conserved PCNA interaction domain. "P" represents potential Cdc2 phosphorylation sites.

The RecQ family plays crucial roles in maintaining genome stability (Sharma et al. 2006; Bugreev et al. 2007; Hanada and Hickson 2007) and mutations in the human RecQ DNA helicase BLM ($Rqh1^{Sp}$, $Sgs1^{Sc}$) cause Bloom's Syndrome (BS), an autosomal recessive disorder, characterized by a strong cancer predisposition due to hyper-recombination between sister chromatids and homologous chromosomes (German 1993; Bachrati and Hickson 2003; Hickson 2003). The BLM helicase suppresses HR at stalled replication forks by disrupting formation of the Rad51-

filament and thus promotes alternative repair mechanisms, which include replication fork regression (Figure 1-14) (Bugreev et al. 2007). Intriguingly, it has been shown that BLM was only able to disrupt Rad51-ssDNA filaments in their inactive form (Bugreev et al. 2007) and that some auxiliary proteins are required to maintain Rad51 in an active ATP-bound form (Shim et al. 2004). This may imply that BLM mediated disassociation of Rad51 filaments depends on other protein interactions and occurs when cells are not fully prepared for later steps of HR, as would be expected as long as cells are in S phase. *In vivo* experiments revealed that the functions of the BLM homologue in *S. cerevisiae*, Sgs1, overlap with those of Srs2 DNA helicase, which can also displace Rad51 from ssDNA (Krejci et al. 2003; Veaute et al. 2003). For instance, Sgs1 over-expression can suppress recombination and repair defects of *srs2* deficient cells (Mankouri et al. 2002) and the *sgs1 srs2* double mutant is not viable, probably due to cell death caused by high Rad51-mediated recombination or unscheduled HR taking place during DNA replication (Lee et al. 1999; Gangloff et al. 2000; Wang et al. 2001). The latter is consistent with the observation that loss of Rad51 suppresses the synthetic lethality of a *sgs1 srs2* double mutant (Branzei and Foiani 2007). The same applies to *S. pombe* and probably to human cells.

As mentioned above, recent evidence indicates that *S. cerevisiae* Srs2 counteracts loading of Rad51 onto ssDNA by Rad52 ($BRCA2^{Hs}$) to remove unwanted recombination filaments. While SUMO-ylated PCNA is required for this anti-recombinogenic function in S phase, a yet unknown mechanism recruits Srs2 to sites of recombination in G2 (Burgess et al. 2009). So far, no sequence homologue of Srs2 has been found in human cells, but emerging evidence suggests that Recql5β, one of the five RecQ DNA helicases, is a functional homologue of Srs2 (Hu et al. 2007). Like Srs2, Recql5 interacts with Rad51 and displaces Rad51 from ssDNA, suppressing HR

(Figure 1-14). Deletion of both BLM and Recql5β increases further recombination between sister chromatids, although it has not yet been shown that simultaneous loss of Rad51 suppresses this hyper-recombination phenotype. Another functional human homologue of yeast Srs2 appears to be Rtel-1 helicase, which has recently been identified in a synthetic lethality screen in *C. elegans* searching for mutant alleles which are lethal when combined with a loss-of-function mutation in BLM (Barber et al. 2008).

In addition to its important role as anti-recombinogenic helicase, Sgs1 was also shown to stabilise DNA replicative polymerases at stalled forks as it travels along with the fork (Frei and Gasser 2000).

There is sufficient evidence to suggest that RecQ DNA helicases switch from anti- to pro-recombination functions once cells progress into G_2 (Bugreev et al. 2007; Laursen et al. 2003; Caspari et al. 2002). Promotion of HR can happen at at least two stages, at the early stage of DNA end resection in association with either Exo1 or DNA2 (Mimitou et al. 2008; Zhu et al. 2008) and at the late stage of dissolution of double Holliday Junctions in association with Topoisomerase III (Figure 1-14) (Wu et al. 2005). The latter process is important to prevent the crossover of genetic material during the mitotic cell cycle.

These antagonistic functions of RecQ DNA helicases raise the important, and yet unanswered, question of their regulation. Since their function switches from anti- to pro-recombination, it is very likely that the cell cycle regulator Cdc2 (CDK1) is required for this switch. Human BLM helicase is *in vitro* phosphorylated at multiple sites, including Ser-714 and Thr-766, during mitosis by Cdc2 (Bayart et al. 2006). The

biological functions of these phosphorylations are not well understood but mitotically phosphorylated BLM associates with Topoisomerase III and becomes quickly de-phosphorylated in response to DNA damage (Dutertre et al. 2002). Since DNA damage inactivates mitotic Cdc2, de-phosphorylation could activate the BLM-TOPOIIIα helicase complex, allowing it to rapidly promote dissolution of double Holliday Junctions.

S. cerevisiae Srs2 DNA helicase is also phosphorylated by Cdc2 ($Cdc28^{Sc}$) at multiple sites and these modifications are important to regulate the dynamics of the Srs2 protein complex (Chiolo et al. 2005). Srs2 associates with both Sgs1 and Mre11 in a large protein complex, which dissociates in response to DNA damage into two smaller sub-complexes containing either Srs2 and Mre11, or Sgs1 and Mre11. This dissociation does not happen in either checkpoint-deficient cells or cells expressing a Srs2 variant that cannot be phosphorylated by Cdc28. Again, the biological role of this regulation is unknown, but it may be linked with end processing of DSBs by Sgs1 and the MRN (Rad50-Mre11-Nbs1) protein complex (Mimitou et al. 2008; Zhu et al. 2008). Cdc28 may exert a second role in the regulation of Srs2, since it has been reported that the checkpoint-dependent phosphorylation of Srs2 prematurely declines upon inhibition of Cdc28 kinase (Liberi et al. 2000). This suggests that high Cdc28 activity has to be maintained for Srs2 to fulfil its DNA response activities. Since this happens when cells arrest in S phase, this second Cdc28-dependent regulation may be distinct from the regulation of the Srs2-Sgs1-Mre11 protein complex. These two regulations may be related to the two different mechanisms of Srs2 activation in S and G2, respectively. In S phase, SUMO-ylated PCNA recruits Srs2 to stalled replication complexes, whereas a yet unknown pathway ensures that Srs2 can remove Rad51 from unwanted recombination sites (Burgess et al., 2009). In this respect it is however important to

remember that *S. cerevisiae* is unique, in that cells arrest with elevated Cdc28 activity in response to DNA damage or DNA replication stress (Amon et al. 1992).

Overall, it appears that DNA helicases have opposing roles in suppressing and promoting recombination, which may possibly be accomplished through their engagement in different, specialised protein complexes that in addition could be regulated by the cell cycle and additional post-translational modifications. The involvement of DNA helicases in the regulation of HR can be additionally supported by the fact that the *S. cerevisiae* Srs2 as well as human BLM and Recql5β helicases all associate with replication machinery *via* their interactions with PCNA (Kanagaraj et al. 2006).

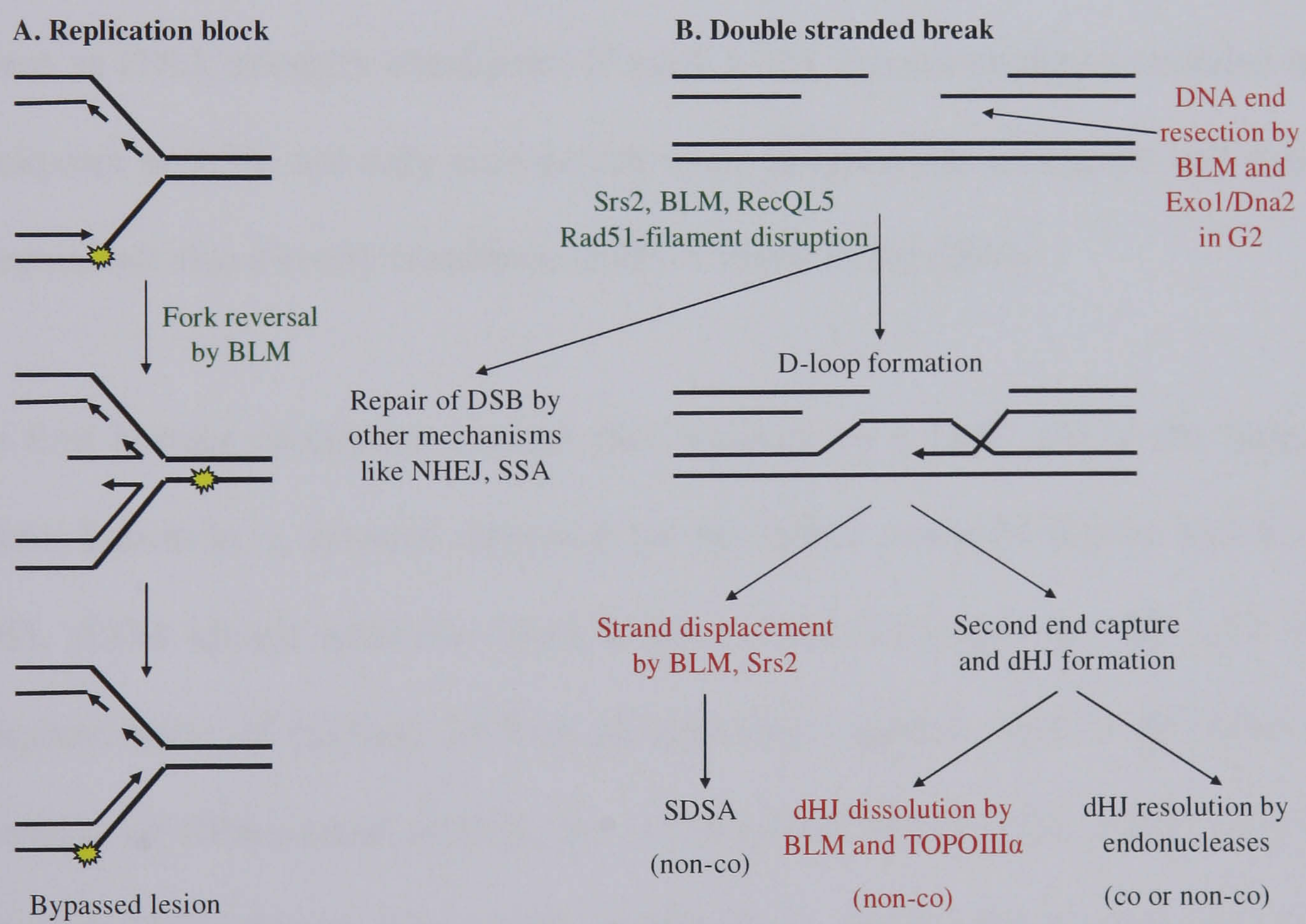


Figure 1-14. The pro- and anti-recombination functions of DNA helicases in DNA double-strand break and stalled replication fork repair.

The pro-recombination functions of DNA helicases are indicated in red and the anti-recombination functions are in green. A) At a stalled replication fork BLM suppresses HR by disrupting Rad51-

filaments and promoting alternative repair mechanisms which may involve fork regression ("chicken foot"). SUMO-ylated PCNA would recruit Srs2 helicase in *S .cerevisiae* to remove Rad51 from ssDNA (not shown). B) At a DNA double-strand break BLM, RecQL5β and Srs2 can prevent HR by disrupting Rad51 filaments or the subsequent D-loop structure, thereby channelling DSBR toward other pathways. In G2, BLM would promote HR at an early stage by facilitating DNA ends resection and at a later stage by resolving dHJs in association with TopIIIα.

1.4 How do DNA damage checkpoint control and recombinational repair interact?

An essential element of the DNA damage response is a transient cell cycle arrest known as DNA integrity checkpoint (Figure 1-15). Recent evidence revealed that the checkpoint proteins not only prevent cell cycle progression, giving the cell extra time to repair, but also directly contribute to DNA repair (Carr 2002).

The first human checkpoint protein that localises to a DSB site is the large ATM protein kinase in a process catalysed by the MRN complex (Lisby and Rothstein 2005). ATM kinase activates Chk2 kinase, which impinges on p53, and initiates phosphorylation of Histone 2AX in chromosome regions close to the break. Upon formation of RPA-coated ssDNA, the ATM-related kinase ATR binds to the break activating CHK1 kinase (Carr 2002; Scully et al. 2005). Independently of ATR and ATM, other checkpoint proteins such as the PCNA-like sliding clamp consisting of Rad9-Hus1-Rad1 (9-1-1) are loaded onto the junction between double-stranded and single-stranded DNA. Loading of the 9-1-1 complex requires the RFC like protein complex containing Rad17 (Lindsey-Boltz et al. 2001; Zou et al. 2002; Bermudez et al.

2003; Wu et al. 2005). Although the role of 9-1-1 complex in DSB repair in mammalian cells is not yet fully understood, some data indicate that Rad9 plays an active role in DSB repair induced by ionizing radiation independently of its C-terminal domain, suggesting that Rad9 may influence HR independently of its DNA signalling function (Parrilla-Castellar et al. 2004). This idea is consistent with the recent observation that human Rad9 interacts with Rad51 specifically in G2 (Pandita et al. 2006). Whether the 9-1-1 complex slides along the chromosome is not yet proven but, like PCNA, the 9-1-1 complex associates with a large number of different DNA repair factors facilitating base excision and mismatch repair (Sancar et al. 2004; Moldovan et al. 2007).

Upon assembly of the checkpoint complex, ATR (Rad3^{Sp}, Mec1^{Sc}) phosphorylates the C-terminus of Rad9, which promotes binding and activation of Chk1 kinase (Furuya et al. 2004), delivering the DNA damage response signal to downstream targets (Scully et al. 2005). Upon its activation, Chk1 negatively regulates the phosphatase Cdc25C by phosphorylating the protein at Ser-216. Cdc25C promotes mitotic entry by removing the inhibitory phosphate at tyrosine 15 within the ATP binding domain of Cdc2-cyclin B kinase (Gould and Nurse 1989). Phosphorylation of Ser-216 at Cdc25C creates a binding site for a 14-3-3 protein resulting in a less active enzyme and its re-localisation to the cytoplasm. In addition to Cdc25C, CHK1 activates the main inhibitor of Cdc2, Wee1 kinase, which phosphorylates Y15 (Lee et al. 2001).

Although the 9-1-1 complex plays a central role in DNA repair and DNA damage signalling, little is known about its regulation. Constitutive phosphorylation of human Rad9 by Protein Kinase Cδ is important for the formation of the 9-1-1 complex (Yoshida et al. 2003). Once formed, the 9-1-1 complex undergoes several constitutive

and DNA damage induced phosphorylation events within its C-terminal tail domain, which are partly interdependent (St Onge et al. 2001; Roos-Mattjus et al. 2003). Amongst these are several Cdc2 phosphorylation events, which occur as in the case of BLM and BRCA2, during mitosis (St Onge et al. 2003). The timing of these Cdc2-dependent modifications suggests a common switch that regulates HR during the cell cycle.

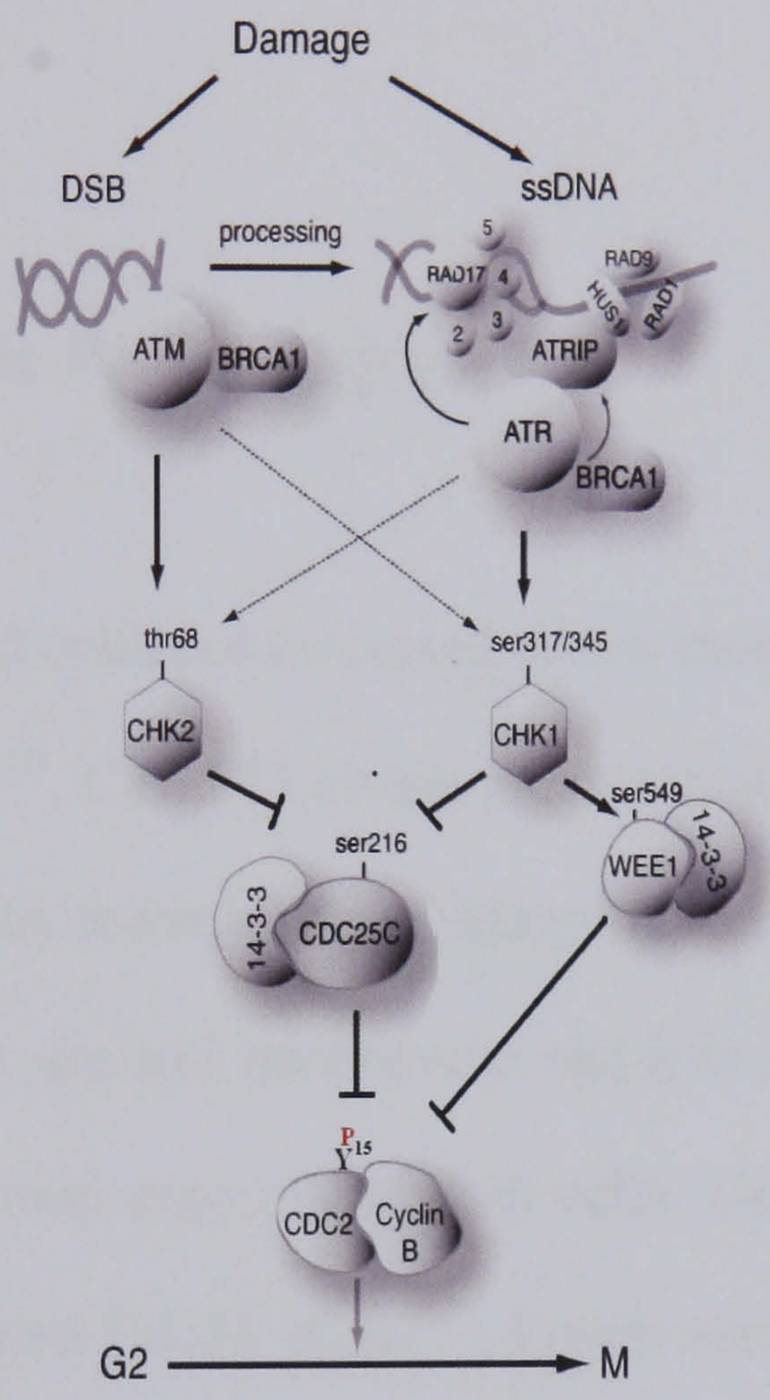


Figure 1-15. The DNA damage G2/M checkpoints summary.

ATM kinase localises to DSB and activates Chk2 kinase, which impinges on p53, and initiates phosphorylation of Histone 2AX in chromosome regions close to the break. Upon formation of RPA coated ssDNA, the ATM-related kinase ATR binds to the break activating the CHK1 kinase. The break site is independently recognized by ATR/ATRIP and by Rad17-RFC. The latter complex loads the PCNA-like sliding clamp Rad9-Hus1-Rad1 onto ssDNA/RPA. Whether the 9-1-1 complex slides along dsDNA or along ssDNA/RPA is not yet proven. ATR ($Rad3^{Sp}$, $Mec1^{Sc}$) is activated by binding to ssDNA and phosphorylates the C-terminus of Rad9. Together, ATR/ATRIP and the 9-1-1 complex synergize to generate maximal activation of CHK1 and deliver the DNA damage response signal to

downstream targets. CHK1 then negatively regulates Cdc25C by phosphorylating it at Ser^{216}, a kinase that promotes mitosis by removing phosphates at Cdc2 inhibitory sites. This creates a binding site for 14-3-3 proteins and in a bound state renders Cdc25C less inactive or/and sequestrated Cdc25 C to the cytoplasm. In addition to activation by ATR, CHK1 may be activated by BRCA1 to induce a G2/M arrest. The 14-3-3 not only regulates Cdc25C, but also Wee1, which is a kinase that inhibits Cdc2 by phosphorylating it at Y^{15}. CHK1 phosphorylates Wee1 and promotes 14-3-3 binding. This significantly enhances the inhibitory kinase activity of Wee1 toward Cdc2 so that cells do not progress into mitosis.

Figure adapted from Nyberg et al. 2002 (Nyberg et al. 2002).

1.5 Objectives of this PhD project

Taken together, the published evidence discussed above strongly indicates that the cell cycle regulator Cdc2 ($CDK1^{Hs}$, $Cdc28^{Sc}$) kinase plays crucial roles in the coordination of DNA replication with DNA recombination. Many important questions, such as the regulation of DNA helicases, are still unanswered and it is unclear how well the data obtained with *S. cerevisiae* map onto mammalian cells. Unlike mammalian cells, *S. cerevisiae* arrests with elevated Cdc28 ($Cdc2^{Sp}$) kinase activity in response to DNA damage to preserve the pre-mitotic structures, which already assemble during S phase in this yeast. This may have allowed for unique functions to evolve that are neither present in *S. pombe* nor in human cells, which both share a normal G2 phase. Some functions such as the phosphorylation of Sae2 ($CtIP^{Hs}$, $Ctp1^{Sp}$) by Cdc28 ($Cdc2^{Sp}$) are conserved, whereas other functions such as SUMO-ylation of PCNA may not happen in mammalian cells. The main aim of this work is to gain further insight into how Cdc2 regulates DNA repair throughout the cell cycle using the model organism *S. pombe*.

2 Materials and methods

2.1 Materials

2.1.1 Media

YEA (Yeast extract with supplements):

- 0.5% w/v yeast extract (Melford, cat no Y1333)
- 3.0% w/v glucose
- 225mg/L of supplement: adenine / histidine /uracil / leucine
- + H_20
- Solid media was made with 2% w/v agar (Lab M limited, cat no Q30320/310)

YEP (Yeast extract with phloxin B - checking ploidy):

- YEA
- 5mg/L phloxin B (Sigma No. P4030), added when the medium had cooled below 60°C from a 5g/L stock solution in sterile distilled water.

EMM (Edinburgh minimal medium):

- 3.0% w/v glucose
- 0.67% w/v yeast nitrogen base (Melford, cat no Y2004)
- 0.5% w/v NH_4Cl
- 225mg/L of supplement: adenine / histidine /uracil / leucine

- pH = 5.5 adjusted with KOH
- + H_2O
- Solid media was made with 2% w/v agar (Fluka, cat no 05040)

ME (Malt extract):

- 3% w/v Bacto-malt extract
- 225mg/L of supplement: adenine / histidine /uracil / leucine
- + H_20
- pH = 5.5 adjusted with NaOH.
- Solid media was made with 2% w/v agar (Lab M limited, cat no Q30320/310)

SPA mating media:

- 1% w/v glucose
- 7.3mM KH_2PO_4
- 1000 x vitamins (w/v)
- 45mg/L of supplement: adenine / histidine /uracil / leucine
- + H_20
- Solid media was made with 2% w/v agar (Lab M limited, cat no Q30320/310)

1000 x vitamins stock:

- 4.20mM pantothenic acid
- 8l.2mM nicotinic acid
- 55.5mM inositol
- 40.8µM biotin

2.1.2 Buffers

TrisHCl (TrizmaHCl):

- 1M Tris Base 121.1g
- + ddH20 up to 1000ml

Buffer H:

- 50mM HEPES, pH = 8.0
- 150mM NaCl
- 0.1% NP40
- 10% glycerol
- 5mM EDTA

Protease inhibitors for soluble protein extract:

- 50mM NaF
- 1mM Na_3VO_4
- 5mM N-ethylmethimid
- 1mM PMSF
- 1 Protease Inhibitor Cocktail Tablet (Roche, cat no 11836 153 001) per 10ml of buffer H

Laemmli buffer 2 x (loading buffer; Laemmli, 1970):

- 4% SDS
- 10% 2-mercaptoehtanol
- 20% glycerol
- 0.004% bromophenol blue

- 0.125M TrisHCl pH 6.8

Running buffer for SDS-PAGE (Tris-glycine buffer):

- 25mM Tris base
- 190mM glycine
- 0.1% SDS
- pH around 8.3

Transfer buffer for Western blotting:

- 25mM Tris base
- 190mM glycine
- 10-20% methanol
- (0.1% SDS)
- pH around 8.3

For proteins larger than 80kD, it was recommended that SDS was included at a final concentration of 0.1% and concentration of methanol was reduced to 10%.

Tris Buffer Saline (TBS) 10 x :

- 24.23g Trizma HCl
- 80.06g NaCl
- Mix in 800ml ultra pure water.
- pH = 7.6, set up with pure HCl.
- Top up to 1L.

Tris Buffer Saline Tween20 (TBST):

- 100ml of TBS 10 x

- 900ml ultra pure water
- 1ml Tween20
- Kept no longer than 1 week in 4°C.

Blocking membrane buffer (milk buffer):

- 3 - 5% powder milk or BSA in TBST buffer.

TBE buffer for agarose gel electrophoresis 10 x:

- 0.89M Tris base
- 0.02M EDTA
- 0.89M Boric acid

2.1.3 SDS - polyacrylamide gel

Resolving gel (total of 10 ml):

Reagent	**Gel percentage**		
	7.5%	**10%**	**12.5%**
10% SDS	100 µl	100 µl	100 µl
1.5M Tris pH = 8.8	2.5 ml	2.5 ml	2.5 ml
30% Acrylamide	2.5 ml	3.34 ml	4.2 ml
Distilled water	4.84 ml	4 ml	3.14 ml
10% APS	50 µl	50 µl	50 µl
10µl Temed	10 µl	10 µl	10 µl

Stacking gel:

Reagent	**Volume**
10% SDS	50 µl
0.5M Tris pH = 6.8	1.25 ml
30% Acrylamide	0.65 ml
Distilled water	3.05 ml
10% APS	50 µl
10µl Temed	10 µl

2.1.4 Agarose gel:

- 1 x TBE buffer
- 1% Agarose
- 0.5μg/ml ethidium bromide

2.1.5 Antibodies

Table 2-1. List of antibody used in this study

Antibody to	Company	Catalog Number	Description	Contcenrtations
Cdc13	abcam	ab10873	Mouse monoclonal	WB 1:1000
Cdc2	abcam	ab5467	Mouse monoclonal	WB 1:2000
c-MYC tag	Covance	MMS-150R	Mouse monoclonal	WB 1:500
c-MYC tag	Santa Cruz Biotechnolc	9E10: sc-40	Mouse monoclonal	WB 1:500
HA tag	Covance	MMS-101R	Mouse monoclonal	WB 1:1000
HA tag	Santa Cruz Biotechnolc	F-7: sc-7392	Mouse monoclonal	WB 1:1000
Mouse IgG	DacoCytomation	P 0161	Horseradish Peroxidase-conjugated Rabbit poly	WB 1:5000
Mouse IgG, Light Cl	Jackson ImmunoResea	115-035-174	Horseradish Peroxidase-conjugated Affinity Pur	WB 1:5000
PCNA	Santa Cruz Biotechnolc	PC10: sc-56	Mouse monoclonal	WB 1:1000
Rabbit IgG	Sigma	A 6154	Horseradish Peroxidase-conjugated Affinity Pur	WB 1:5000
Rabbit IgG	DacoCytomation	P 0399	Horseradish Peroxidase-conjugated Swine poly	WB 1:5000
Ubiquitin	abcam	ab19247	Rabbit polyclonal	WB 1:1000

2.1.6 *S. pombe* strains used in this study

Table 2-2. List of *S. pombe* strains used in this study

Strain	Genotype
cdc13-245	*cdc13-245 ura4.D18 ade6.M210 leu1.32, h-*
cdc13-245	*cdc13-245 ura4.D18 ade6.M210 leu1.32, h+*
cdc13-245 Ku70-d mik1-d	*cdc13-245 Ku70::KanMX6 mik1::ura4+ ura4.D18 ade6.M210 leu1.32, h+*
cdc18 –K46 cdc2.1w	*cdc18 –K46 cdc2.1w ura4+ ade6.M210 leu1.32*
cdc18-K46	*cdc18-K46 ura4+, h-*
cdc1-P13	*cdc1-P13 ura4.D18 ade6.M210 leu1.32 h-*
cdc2.1w	*cdc2.1w ura4.D18 ade6.M210 leu1.32, h+*
cdc2.1w	*cdc2.1w ura4.D18 ade6.M210 leu1.32, h-*
cdc2.1w AUA (A)	*cdc2.1w ura4.D18 ade6.M210 leu1.32 ade6.L496-ura4^{+} -ade6.M375*
cdc2.1w AUA (B)	*cdc2.1w ura4.D18 ade6.M210 leu1.32 ade6.L496-ura4^{+} -ade6.M375*
cdc2.1w AUA (C)	*cdc2.1w ura4.D18 ade6.M210 leu1.32 ade6.L496-ura4^{+} -ade6.M375*
cdc2.1w Ch16	*cdc2.1w Ch16, ura4.D18 ade6.M210/M216 leu1.32 mat-a KAN^{R} his^{+},*
cdc2.1w pREP42-rqh1	*ura4.D18 leu1.32, Leu2 [pREP41], h^{90}*
cdc2.1w pREP42-rqh1-K547I	*ura4.D18 leu1.32, Leu2 [pREP41], h^{90}*
cdc2.33	*cdc2.33 ura4.D18 ade6.M210 leu1.32, h-*
cdc2.3w	*cdc2.3w ura4.D18 ade6.M210 leu1.32, h+*
cdc2.3w AUA (A)	*cdc2.3w ura4.D18 ade6.M210 leu1.32 ade6.L496-ura4^{+} -ade6.M375*
cdc2.3w AUA (B)	*cdc2.3w ura4.D18 ade6.M210 leu1.32 ade6.L496-ura4^{+} -ade6.M375*
cdc2.L7	*cdc2.L7ura4.D18 ade6.M210 leu1.32, h-*
cdc20-M10	*cdc20-M10 ura4.D18 leu1.32, his2-, h^{+}*
cdc22-M45	*cdc22-M45 ura4+ leu1.32, h-*
cdc22-M45 cdc2.1w	*cdc22-M45 cdc2.1w ura4+ ade6.M210 leu1.32, h-*
cdc25.22	*cdcc25.22 ura4.D18 leu1.32 h-*
cdc25.22 cdc2.1w	*cdcc25.22 cdc2.1w ura4.D18 ade6.M210 leu1.32*
cdc27-P11	*cdc27-P11 ura4+ ade6.M210 leu1.32*
cdc27-P11 cdc2.1w	*cdc27-P11 cdc2.1w ura4+ ade6.M210 leu1.32*
cdc6.23	*cdc6.23 ura4.D18 ade6.M210 leu1.32*
cdm1-SP212	*cdm1::ura4+ SP212 ura4.D18, h+*
chk1-d	*chk1::ura4+ ura4.D18 ade6.M210 leu1.32*
chk1-d cdc2.1w	*chk1::ura4+ cdc2.1w ura4.D18 ade6.M210 leu1.32*
chk1-HA	*chk1-HA ura4.D18 ade6.M210 leu1.32, h+*
chk1-HA cdc2.1w (I)	*chk1-HA cdc2.1w ura4.D18 ade6.M210 leu1.32*
chk1-HA cdc2.1w (II)	*chk1-HA cdc2.1w ura4.D18 ade6.M210 leu1.32*
DMCdc2	*cdc2::DMCdc2 ura4+ leu2+ his3+, h-*
DMCdc2	*cdc2::DMCdc2 ura4+ leu2+ his3+, h+*
DMCdc2	*cdc2::DMCdc2 ura4.D18 leu1.32 ade6.216, h+*
DMCdc2	*cdc2::DMCdc2 ura4.D18 leu1.32 ade6.216, h-*
fbh1-d	*fbh1::leu2 leu1.32 16387, h-*
fbh1-d cdc2.1w	*fbh1::leu2 leu1.32 16387 cdc2.1w*
His$_6$-HA-wee1	*His6-HA-wee1 ura4.D18 leu1.32, h^{+}*
Ku70-d	*Ku70::Kan MX6 ura4.D18 ade6.M210 leu1.32, h+*
Ku70-d	*Ku70::Kan MX6 ura4.D18 ade6.M210 leu1.32, h-*
Ku70-d	*Ku70::his3+ his3.D1 ura4.D18 ade6.216 leu1.32*
Ku70-d cdc13-245	*Ku70::his3+ cdc13-245 his3.D1 ura4.D18 ade6.216 leu1.32*
Lig IV-d	*Lig IV-d::Kan MX6 ura4.D18 ade6.M210 leu1.32 his3.D1, h+*
Lig IV-d cdc13-245	*Lig IV-d::Kan MX6 cdc13-245 ura4.D18 ade6.M210 leu1.32 his3.D1, h+*
mik1-d	*mik1::ura4+ ura4.D18 ade6.M210 leu1.32, h-*
mik1-d cdc13-245	*mik1::ura4+ cdc13-245 ura4.D18 ade6.216 leu1.32*
mus81-d	*mus81:: Kan MX6 ura4.D18 leu1.32, h+*
mus81-d cdc2.1w	*mus81:: Kan MX6 cdc2.1w ura4.D18 leu1.32, h+*
PCNA-K164R	*PCNA-K164R ura4+, h+*
PCNA-K164R rqh1-MYC	*PCNA-K164R rqh1-MYC [KAN MX6] ura4+*
rad16-d	*rad16::ura4+ ura4.D18, leu1.32, h+*
rad16-d cdc2.1w	*rad16::ura4+ cdc2.1w ura4.D18 ade.M210 leu1.32, h+*
rad2-d	*rad2::ura4+ ura4.D18 ade.M210, h-*
rad2-d cdc2.1w	*rad2::ura4+ cdc2.1w ura4.D18 ade.M210 leu1.32, h-*
rad32-D65N	*rad32-D65N ura4.D18 leu1.32, h+*
rad32-D65N cdc2.1w	*rad32-D65N ura4.D18 ade.M210 leu1.32*
rad50-d	*rad50::Kan MX6 ura4.D18 leu1.32 ade6.216, h+*
rad50-d	*rad50::Kan MX6 ura4.D18 leu1.32 ade6.216, h-*
rad50-d cdc2.1w	*rad50::Kan MX6 cdc2.1w ura4.D18 leu1.32 ade6.216, h+*
rad50S	*rad50S ura4.D18, h+*
rad50S cdc2.1w	*rad50S cdc2.1w ura4.D18 ade6.M210 leu1.32*

2.1.7 Primer sequences

- Leu2-3 - GCCGAAGCCATTAAGG
- Leu2-4 - TCACCGACTTCGGTGG
- Leu2-5 - GTTAGGTGCTGTGGGTGG (sequencing)
- Ura4-3 - gaagcttagctacaaatccc
- Ura4-4 - ttgctgtcccagcccg
- Srs2-1 (wild type) - tac gga cat ATGGAAACGA AATCATCATA CTTGAAGTTT CTGAATGAAG AACAAAGGAT TAGTGTTCAG AGTCCTCATA AGTATACTCA AATTTTAGCC
- Srs2-2 (S21A) - tac gga cat ATGGAAACGA AATCATCATA CTTGAAGTTT CTGAATGAAG AACAAAGGAT TAGTGTTCAG gcc CCTCATA AGTATACTCA AATTTTAGCC
- Srs2-3 (S21E) - tac gga cat ATGGAAACGA AATCATCATA CTTGAAGTTT CTGAATGAAG AACAAAGGAT TAGTGTTCAG gagCCTCATA AGTATACTCA AATTTTAGCC
- Srs2-4 - tcg gac cat gga tcc cta taa cat tcg tga aac tcg tag

2.2 Methods

2.2.1 Construction of yeast strains

Schizosaccharomyces pombe strains were constructed by standard genetic techniques (Forsburg 2001). Strains with opposite mating types were mixed on SPA or ME plates (http://www-rcf.usc.edu/~forsburg/main.html) and incubated for 2 - 3 days at 25°C. Random spore analysis was performed after treatment of asci with 30% EtOH for 30 min at RT. Surviving spores were plated on YEA (http://www-rcf.usc.edu/~forsburg/media.html) plates and analysed for the desired genotype.

2.2.2 Yeast strain storage and culturing

Yeast strains were long-term stored in YEA medium with 25% glycerol at -80°C. To recover frozen strains and prepare working stocks, small portion of the frozen glycerol stock was streaked onto YEA agar plate and incubated at 30°C until colonies reached ~2mm in diameter (3 - 5 days). Working stocks on agar plates were sealed with Parafilm and stored at 4°C up to two months. To prepare liquid overnight cultures, single colonies from the working stocks were inoculated in ~3ml of YEA medium and incubated overnight at 30°C at 180 rpm. For physiological experiments cultures should be maintained in mid-exponential growth between 2 x 10^6 and 1 x 10^7 cells/ml. To calculate the volume of a pre-culture from which to generate an overnight culture grown at 180 rpm, the following formula was used: $[(Y \times 0.4) / 2^n] \times OD^{-1}$ where Y = volume of required culture; OD = OD_{595} of the pre-culture; n = the expected number of

generations -1, to allow the cells to recover from stationary phase; 0.4 refers to a suitable OD for an exponential culture (Nurse).

2.2.3 Measuring cell number

Cell number per 1 ml was estimated by measuring the OD_{595} of an exponentially growing culture. An OD_{595} = 0.5 was equivalent to 1 x 10^7 cells/ml. To accurately determined cell number, a haemocytometer was used (Nurse Fission Yeast Handbook).

2.2.4 Molecular Biological Techniques

The *E. coli* Top10 strain was transformed with the pPW52 plasmid upon treatment with CaCl as described in (Hanahan 1983). Plasmids were isolated according to the protocol published by (Birnboim and Doly 1979).

2.2.5 Transformation of *S. pombe*

S. pombe transformation was performed as described by Okazaki and co-workers (Okazaki et al. 1990).

2.2.6 Preparation of genomic DNA from *S. pombe*

Isolation of genomic DNA as described in (Grallert et al. 1993).

2.2.7 The HO Induction Protocol

HOSSA strains transformed with the HO plasmid pPW52 were grown from single colonies in EMM minimal medium (http://www-rcf.usc.edu/~forsburg/media.html) containing adenine (250µg/ml), leucine (250µg/ml), and thiamine (final concentration 100µm) at 25°C with shaking over 3 nights until stationary phase was reached (OD 595 nm: ~3-4). Stationary cells were spun down, washed once with sterile water and resuspend in 3 ml of sterile water. Subsequently, 0.3 ml of this stationary culture were added into 2.7 ml of EMM +A+L+U lacking thiamine (= HO endonuclease induction, nmt promoter on) and incubated for 24h at 25°C with shaking. Following induction, approximately 500 cells were plated on YEA + Thiamine medium (Thiamin final concentration: 100 µm) (= HO endonuclease repression, nmt promoter off) and incubated for 2-3 days at 25°C until single colonies appeared. For phenotypic analysis, colonies were replica plated on selective EMM plates or YEA plates containing hygromycin (50-100µg/ml). Plates were incubated at 25°C or at 37°C to test temperasensitive mutants.

2.2.8 Polymerase Chain Reaction

In the first experiment, genomic DNA was isolated from HOSSA isolates, whereas in later experiments Colony PCR was performed.

Genomic DNA PCR:

- 2µl genomic DNA (1:10 dilution), 5 µl primer I (5µM), 5 µl primer II (5µM), 5 µl 10-times buffer Fermentas $(NH_4)_2SO_4$, 5 µl 1mm dNTPs, 6 µl 25 mm $MgCl_2$, 0.5 µl Taq polymerase (Fermentas 1 u/µl), 21.5 µl sterile H_2O.

- Initial denaturation for 1 min at 94°C, 30 cycles of 1 min 94°C denaturation, 1 min 50°C annealing, 2 min 72°C extension, 1 cycle of 10 min 72°C and 20 min 4°C final hold.

Colony PCR:

- Single colonies were grown in liquid media to stationary phase and subsequently 10 µl of each were centrifuged (10,000 x g for few seconds) and incubated for 5 min at 37°C in 10 µl of zymolase 2.5 mg/ ml in buffer containing 1.2 m sorbitol, 0.1 m Na_2HPO_4 at pH=7.4.
- 2µl of previously prepared DNA, 5 µl primer I (5µM), 5 µl primer II (5µM), 5 µl 10 times buffer Promega Mg free, 5 µl 1mM dNTPs , 6 µl 25 mM $MgCl_2$, 0.25 µl Taq polymerase (Promega 5 u/µl), 21.75 µl sterile H_2O.
- Initial denaturation at 94°C for 3 min, 30 cycles of 30 sec 94°C denatutarion, 30 sec 50°C annealing, 2.5 min 72°C extension, 1 cycle of 7 min 72°C and 20 min 4°C final hold.

PCR fragments for sequencing analysis were extracted out of an agarose gel (1%) using HiYield Gel/PCR DNA Fragments Extraction Kit, RBC cat. No. YDF100.

2.2.9 Agarose gel electrophoresis

1% agarose was dissolved in 1 x TBE buffer by heating up in microwave. The solution was cooled down to 55°C and ethidium bromide was added at a final concentration of 0.5µg/ml. The gel tray was prepared by sealing its ends with tape and placing a comb about 1 inch from the top of the tray. The gel was poured into the tray to a depth of 5 mm and allowed to set for 20 minutes at room temperature, followed by removing of the comb and placing the tray in an electrophoretic chamber. The tray with gel was

covered with 1 x TBE buffer. 5µl of a dye marker and 1 – 5µl of DNA sample mixed with 6 x loading buffer were loaded onto the gel and electrophoresis was run at 120 V until the dye marker migrated to the bottom of the gel or to an appropriate distance. The DNA fragments were visualized by ultra-violet trans-illumination.

2.2.10 Drop Tests

Strains from single colonies were freshly grown in 3 ml of YEA medium at 180 rpm until OD_{595} = 0.5. Cells were then counted under the microscope using a haemocytometer and each cells suspension was adjusted to a concentration of 10^7 cells ml^{-1}. 10µl of a 10-fold serial dilution series were dropped onto selective media plates and grown at indicated temperatures for ~ 4 days.

2.2.11 Acute Survival Experiments

Strains were freshly grown in 3 ml of YEA medium at 180 rpm until OD_{595} = 0.5 and after that cells were counted under the microscope using a haemocytometer. For UV sensitivity experiments, 5 x 10^2 cells (100µl of 5 × 10^3 cells/ml) of each strain was plated on a number of YEA media plates, allowed to dry and exposed to different UV intensities. For other DNA damaging agents, 5 x 10^3 cells in 1 ml YEA were incubated in water bath at indicated temperature and 100µl samples were collected at different time points, starting from time-point 0 and plated on YEA plates. After 4 days incubation at indicated temperatures, colonies were calculated on each plate and

survival for each strain (%) estimated. Each experiment was repeated at least three times and average survival for each strain was determined.

2.2.12 Total Cell Extract with Trichloroacetic acid (TCA)

Approximately 10^7 - 10^8 logarithmically growing cells were harvested (OD_{595} = 1 x 10^5 cells) and washed in water followed by 20% (w/v) trichloroacetic acid (TCA). Cells were then re-suspended in 200 µl of 20% TCA and 150µl of glass beads were added. After that cells were broken in Fastprep machine 4 × 20 s at speed 6.0. Samples were kept cold on ice all the time. 400 µl of 5% TCA was added and cells homogenate was transferred to a new tube using syringe or spun down to new tube. The homogenate was centrifuged at 10,000 x g for 5 minutes. Supernatant was discarded and pellet resuspended in ~ 200 µl of loading buffer. If solution was to acidic (yellow colour) then Tris-HCL pH = 8.8 was added to alkaline it (blue colour) (Caspari et al. 2000). Samples were boiled in 95°C for 5 minutes and either divided into aliquots at stored at -80°C or used directly for further analysis

2.2.13 Soluble Protein Extract

Approximately 10^7 - 10^8 logarithmically growing cells were harvested, spun down, washed in buffer H and spun again. Buffers should have been cold and samples kept on ice for the whole procedure. Pellet was re-suspended in 1ml of buffer H plus protease inhibitors (buffer H+) and transferred to a screw cup tube and spun down. Supernatant was discarded. 150µl of glass beads was added to the pellet and overlaid

with cold buffer H plus inhibitors. After that cells were broken in Fastprep machine 4 × 20 s at speed 6.0. Cells lysate was transferred to a new tube; 600 µl of buffer H+ was used to wash glass beads and combined with the cells lysate. Sample was micro-centrifuged for 5 minutes, 10,000 x g, at 4°C. The supernatant was either divided into aliquots and stored at -80°C or used directly for further analysis.

2.2.14 Bradford Assay Procedure for Protein Concentration:

Protein concentrations were determined by Micro Bradford Assay as described at (http://www-class.unl.edu/biochem/protein_assay/bradford_assay.htm 2008).

2.2.15 SDS – polyacrylamide gel electrophoresis (SDS - PAGE) and Western blotting

Before SDS – PAGE, samples were denaturised by boiling in 95°C for 5min with loading buffer. Proteins, depended on size, were separated by electrophoresis on a 7.5%, 10% or 12.5% SDS -polyacrylamide gel in 1 x Tris-Glycine running buffer. Pre-stained protein ladder was used as a molecular weight marker (Sigma Pre-stained Molecular Weight Marker, 26 - 180kDa). Electrophoresis and Western blotting procedures were processed as described on (http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf 2008) using Geneflow PAGE Sub Vertical Electrophoresis System VS10D, VS10DSYS, VS10DCAST and OmniPAGE Molecular Electroblotting Units SB10, VS10BI. Antibody-protein

complexes were visualized using an ECL kit (Amersham Pharmacia Biotech) and membranes were exposed to a Fuji film (SuperRX).

2.2.16 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is an *in-vitro* method for discovering protein complexes. In this technique, protein that is believed to be a member of a larger complex is being bound to the specific antibody and removed from the solution by addition of an insoluble form of the antibody biding protein such as Protein G or Protein A. This allows pulling the entire protein complex out of solution and thus identifying unknown members of the complex. Samples for Co-IP were prepared in PCR tubes as followed: 100 µl of soluble protein extract without and with antibody were incubated on a rotating platform, at 4°C, over night. Optimum concentration of antibody was determined individually for different proteins by using a range of concentrations in pre-experiment (1:100 – 1:10). After incubation, 25 µl of slurry of Protein G Plus-Agarose (Calbiochem, cat no IP08) pre-washed 2 x in milk buffer and 3 x in buffer H was added to each sample. After that samples were incubated on a rotating platform, at 4°C, for 2 - 4h, followed by centrifuging for 10 seconds at 10,000 x g. Supernatants were kept and re-suspended in 2 x loading buffer to be used as a control, whereas beads were washed 4 x in buffer H and re-suspended in 1 x loading buffer. After boiling at 95°C for 5 minutes samples were either frozen in - 80°C or analysed straight away by SDS – PAGE and Western blotting (Harlow et al. 1999; Bonifacino et al. 2001).

2.2.17 Spontaneous intra-chromosomal recombination assay

Mitotic spontaneous recombination frequencies were measured by using strains containing an intra-chromosomal recombination substrate consisting of non-tandem direct repeats of *ade6*$^{-}$ hetero-alleles flanking a functional *ura4*$^{+}$ gene. Frequencies of spontaneous recombination were determined by recovery of Ade^{+} recombinants. Strains were grown on minimal medium minus uracil to select for *ura4*$^{+}$ marker gene and containing adenine and leucine to prevent selection for Ade^{+} recombinants (EMM –U+A+L). Next single colonies from each strain were grown in 2 ml of YEA medium till stationary phase, cells were counted and plated at the density of 5 x 10^{2} on YEA to estimate total cell number and 10^{5} on EMM+U-A+L to select for Ade^{+} recombinants. After 4 days number of recombinants and cells in total were determined. The Ade^{+} recombinants were replica plated onto selective plates lucking uracil to determine proportion of conversion type (*ade6*$^{+}$ *ura4*$^{+}$) to deletion type (*ade6*$^{+}$ *ura4*$^{-}$) recombinants. For each strain 4 independent colonies were used for each assay and each assay was repeated at least three times. Median recombination rates of each strain were determined from three independent assays. The average recombination frequencies and percentage of conversion types were determined from these medians (Osman et al. 2000).

2.2.18 The Minichromosome Assay

Strains containing the Ch^{16}-*rad21::MATa-kanMX6* non-essential minichromosome were transformed with pACYCREP81X-HO plasmid. pACYCREP81X-HO is a multicopy plasmid that contains the *S. cerevisiae* HO-endonuclease under the control

of the rep81X nmt promoter (Osman et al. 1996) To repress the nmt promoter 8mM thiamine was added to EMM (Maundrell 1990). Prior to performing the analysis, strains were maintained on EMM +U +H +T to select for Ch16-MG and the pREP81X-HO plasmid. Cells were then washed twice in phosphate-buffered saline (PBS), followed by culturing for up to 48 h in log phase, in either EMM +A +U +H +T or EMM +A +U +H media to maintain plasmid selection but allow Ch16-MG loss. To carry out the DNA repair assay, cells were plated onto non-selective YEA plates, followed by incubation at 30°C and the total colonies numbers were calculated. Colonies were replica-plated onto *ade*$^{+/-}$ (EMM +U +H +L +T) plates or YEA with G418 (500mg/l) in order to determine the percentage of the population that had become *ade*$^{+/-}$ or G418 sensitive, respectively. Cell viability was verify by spotting serial dilutions of cultures onto plates with EMM +U +H +A and EMM +U +H +A +T medium, incubating at 30°C and determining colony-forming ability.

2.2.19 Size exclusion chromatography on Superdex 200 HR gel filtration column.

Size exclusion chromatography (SEC), also known as gel filtration chromatography, separates molecules according to their size. Smaller molecules are able to enter the pores of the media and thus take longer to elute, whereas larger molecules elute faster since they are excluded from the pores. Superdex 200 HR column (Amersham Pharmacia Biotech) was connected to the ACTA design system, equilibrated with buffer H at +4°C and calibrated as described in the manual. 0.2 – 0.5 ml of soluble protein extract was loaded on the column and eluted at a low rate of 0.25 ml/min. 1 ml fractions were collected of the total of 24 ml. Fractions were stored in -80 °C.

2.2.20 Synchronisation of *S. pombe* cells in G2 phase of the cell cycle on 15 ml lactose gradients

S. pombe cells can be synchronised in G2 by separation in lactose gradients. *S. pombe* cannot metabolise lactose thus minimum disturbance to growth occurs. Using this technique, multiple strains can be synchronized in parallel and the yield is sufficient for multiply time-points for DAPI/Calcofluor staining. To synchronised cells on the 15 ml lactose gradient the 30% and 7% lactose stock solutions made up in media were mixed in a set of tubes as followed:

30% (ml)	7% (ml)
10	0
8.75	1.25
7.5	2.5
6.25	3.75
5	5
3.75	6.25
2.5	7.5
1.25	8.75
0	10

Gradient was started by layering 1.5 ml of 30% lactose in 15 ml Greiner tube, followed by the 8.75 / 1.25 mix and so on until layering 7% lactose on the top. To lay each gradation a cut off blue tip was used and pipette tip was carefully put just in the layer below and emptied slowly. To prepare cells for synchronization, strains were grown in a YEA pre-culture, in 25°C, over night, 180 rpm. After that, OD_{595} was measured and cells were re-suspended in 150 ml of YEA such that they would reach OD_{595} = 0.5 the next day (the doubling time is about 2.5h for wild type) kept growing in the same conditions. Cells were then spun at 3000 x g for 5 minutes in 50 ml Greiner tube, pellet re-suspended in 1 ml of YEA and cells were moved to 1.5 ml tubes and sonicated to prevent them from sticking together. Such prepared cells were put on top of the gradient and spun 1000 x g for 8 minutes. After spinning, at the bottom of the lactose

gradient, a white, fussy layer of approximately 2 ml of cells was formed. 0.4 ml of this layer, which should be the small G2 cells, was picked with a pipette and used immediately for further analysis.

2.2.21 DAPI/Calcofluor white staining

DAPI is a blue-fluorescent dye that binds strongly to DNA and is compatible with both live and fixed cells. Calcofluor white has binding affinity to cellulose and is commonly used to visualise cell wall and septum. 5 µl of sample was placed on a microscope slide and allowed to dry. 15 µl of DAPI/Calcofluor solution in H_2O (DAPI: 1000 x stock solution 1mg/ml in DMSO; Calcofluor: final concentration of 1 µg/ml) was placed on a cover glass and the cover was put over cells on the slide. Samples were then analysed under a fluorescence microscope (Sherman et al. 1986).

2.2.22 DNA damage checkpoint analysis of *S. pombe* cells

200ml of synchronised cells were resuspended in 1.5 ml tube in 500 µl of YEA and another 200ml in 500 µl of YEA with an indicated DNA damaging agent. Tubes were then incubated in 30°C water bath for a certain period of time and 30 µl samples were collected at equal time-points, starting from the time-point 0. After collecting, every sample was spun down at 10^4 rpm for 1 minute and the pellet was fixed in a drop of methanol. 5 µl of each sample was stained with DAPI/Calcofluor solution and analysed using fluorescence microscopy. For each sample 100 cells were counted, for

which a percentage of septated cells (G1/S cells) was calculated. The percentage of G1/S cells was put against time in a graph.

2.2.23 Construction of the Srs2 plasmids

To construct the Srs2 plasmids, the *srs2* gene was amplified from genomic DNA isolated from wild type 804 cells with primers:

1) Srs2-1 + Srs2-4 (no mutation, wild type)

2) Srs2-2 + Srs2-4 (S21A)

3) Srs2-3 + Srs2-4 (S21E)

Reaction conditions:

- 10 µl 5x Phusion buffer
- 5 µl 2mM dNTPS
- 2.5µM 10uM primer 1
- 2.5 µl 10µM primer 2
- 1 µl genomic DNA
- 0.5 µl phusion DNA polymerase
- 29 µl water
- 3 min 98ºC
- 30 cycles of: 10 sec 98ºC, 30 sec 55ºC, 2 min 72ºC
- 10 min 72ºC

The fragments were purified and digested with NdeI (cuts at the ATG) and BamHI (cuts downstream from the stop codon). Both sites were encoded by the primers. Purified, cut fragments were ligated with NdeI-BamHI cut pREP41 or pREP41-Myc

(encodes N-terminal Myc epitope in frame). Correct plasmids were amplified in *E. coli* and used for yeast transformation.

2.2.24 FACS Analysis (Fluorescence Activated Cell Sorter)

3×10^7 cells/ml was centrifuged for 1 min at 3000 x g, followed by washing in 1 ml of 50mM sodium citrate of pH=7.0 and fixed in 1 ml of ice-cold 70% EtOH. Cells were then washed twice in 1 ml of 50mM sodium citrate pH=7.0 and incubated either at 50ºC for 1 h or at 37°C over night with 0.025 mg/ml RNase A. Cells were washed twice and pellet was re-suspended in 1 ml of sodium citrate. Subsequently, propidium iodide (16µg/ml) was added, cells were mixed and incubated for 30 min at RT. Approximately 300 µl of cells were transferred to FACS tubes and analyzed.

3 *S. pombe* mutant cells with *in vivo* elevated Cdc2 activity suffer from problems during DNA replication

Cdc2 activity can be manipulated *in vivo* by the use of dominant active *cdc2* alleles, which harbour point mutations that render the kinase either insensitive to inactivation by the Wee1 kinase (*cdc2.1w [G146D]*) (Booher and Beach 1986) or overcome the requirement for activation by Cdc25 phosphatase (*cdc2.3w*) (Enoch and Nurse 1990). Since *in vivo* elevated Cdc2 activity drives cells prematurely into mitosis, without giving G2 cells sufficient time to grow, the mutant strains are classified as "*wee*" (Nurse 2004). The shortened G2 phase is compensated by an extended G1 phase so that the total cell cycle transition time of *wee* mutants is comparable to wild type cells (Fantes 1979). This change in cell cycle distribution results in an increased G1 cell population, which is normally not detectable in wild type cells. Although the cell cycle changes are comparable in the different *wee* mutants, their sensitivity to DNA damage is distinct. Loss of Wee1 kinase renders cells sensitive to UV light and ionising radiation (Alkhodairy and Carr 1992; Barbet and Carr 1993), while the phenotypically similar *cdc2.1w* strain fails to display either sensitivity. The *cdc2.3w* strain, which does not require activation by Cdc25 phosphatase, possesses a defect in the DNA replication checkpoint (i.e. sensitivity to hydroxyurea), which is detected neither in *cdc2.1w* nor in *wee1* deletion cells (Enoch and Nurse 1990). The *cdc2.1w* strain is unique amongst these *wee* mutants because no DNA damage sensitivity or checkpoint defect has so far been reported for this hyperactive kinase mutant.

This results chapter reports experiments designed to reveal yet unknown DNA repair defects of the *cdc2.1w* mutant strain, which was isolated as the *wee2.1* mutant by

Nurse and Thuriaux (Nurse and Thuriaux 1980). This Cdc2 mutant was chosen because it does not show any of the previously reported DNA repair (i.e. *wee1-d*) or checkpoint impairments (i.e. *cdc2.3w*), which may be only indirectly related to the elevated Cdc2 activity. Hence, any defects in the response to DNA damage or DNA replication stress displayed by the *cdc2.1w* strain are more likely to be caused by high *in vivo* levels of Cdc2 activity possibly revealing yet unknown target proteins of the kinase.

3.1 *In vivo* elevated Cdc2 activity impairs repair of camptothecin-induced DNA damage

3.1.1 *Cdc2.1w* cells are specifically defective in the repair of camptothecin-induced DNA damage

To identify yet unknown DNA damage repair defects in *cdc2.1w* cells, the different *wee* mutants were screened for impaired growth in the presence of different DNA damaging agents or upon irradiation with UV light. The strains listed in Table 3-1 were analysed.

Table 3-1 . *Wee* mutant strains used in this study

Strain	Description	References
cdc2.1w	Elevated Cdc2 activity; insensitive to Wee1 inactivation	(Nurse and Thuriaux 1980; Booher and Beach 1986)
wee1-d	Elevated Cdc2 activity; deletion of the Cdc2 inhibitor Wee1 kinase	(Enoch and Nurse 1990)
wee1-50	Temperature-sensitive loss of function mutant of Wee1 kinase	(Nurse 1975)
mik1-d	Slightly elevated Cdc2 activity; deletion of the S-phase specific Cdc2 inhibitor Mik1 kinase	(Lee et al. 1994)
cdc2.3w	Elevated Cdc2 activity; no requirement for activation by Cdc25 phosphatase	(Enoch and Nurse 1990)
cdc2.L7	Reduced Cdc2 activity	(Kanaoka and Nojima 1994)
cdc2.33	Reduced Cdc2 activity	(Nurse et al. 1976; Nurse and Bissett 1981)

3.1.1.1 Elevated *in vivo* Cdc2 activity renders *S. pombe* cells specifically sensitive to the DNA replication poison Camptothecin (CPT)

Camptothecin is a plant alkaloid of which derivatives, irinotecan and topotecan, are approved for the treatment of some metastatic cancers. CPT kills rapidly dividing cells by trapping the enzyme Topoisomerase I (TopI) on DNA and thus causing chromosome breaks when replication forks collide with the trapped enzymes. TopI normally binds to DNA during the DNA replication process to release structural tension that arises from local unwinding of the double helix. CPT binds to and transiently stabilises the TopI-DNA cleavage complex which forms when TopI binds to the 3'-end of the cleaved DNA single strand (Figure 3-1) (Pommier 2006).

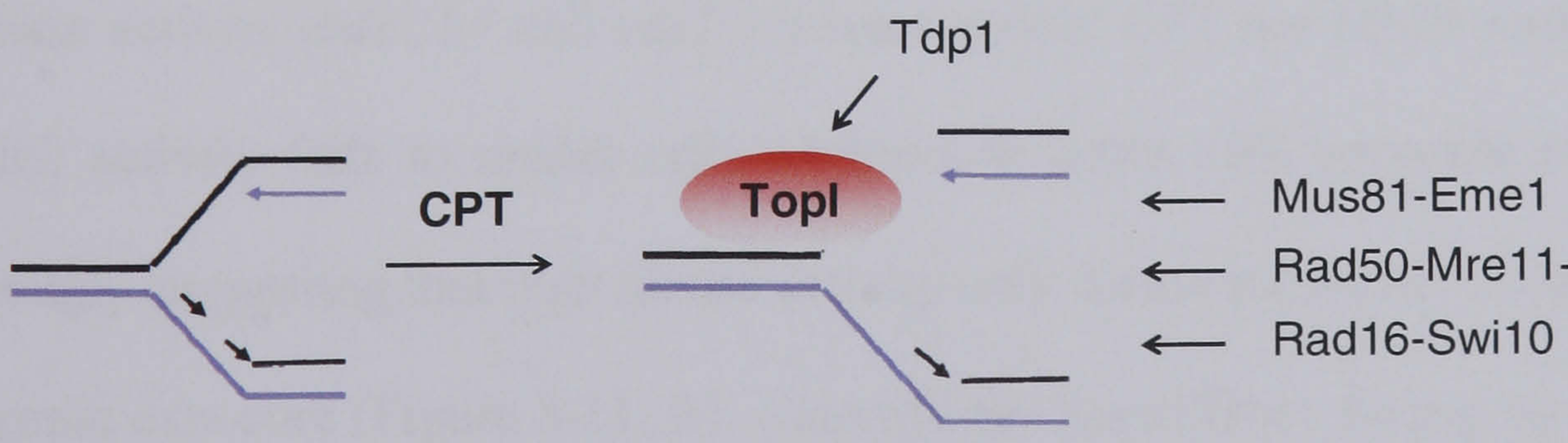


Figure 3-1. Camptothecin traps Topoisomerase I on DNA and the collision of a DNA replication fork with this DNA-CPT-TopI complex causes a broken leading stand.

Topoisomerase I forms a covalent linkage with the 3'-end of the DNA and CPT binds exclusively to this protein-DNA intermediate. When TopI becomes attached to the leading strand, the DNA replication

machinery is thought to synthesise DNA up to the last base flanking the TopI cleavage site (i.e. run-off) generating a replication double-strand break. The trapped TopI can be released by Tyrosyl-DNA-Phosphodiesterase (Tdp1) which cleaves the enzyme next to the catalytic tyrosine residue that binds to the 3' end of DNA. The endonucleases Mus81-Eme1, Rad16-Swi10 and Mre11, in the Rad50-Mre11-Nbs1 protein complex, can all repair CPT-induced DNA damage and act in parallel pathways (Liu et al. 2002; Deng et al. 2005; Hartsuiker et al. 2009).

S. pombe cells expressing different mutated variants of Cdc2 with either elevated or reduced kinase activity were grown at 30°C on rich medium plates containing increasing concentrations of the DNA replication poison camptothecin (CPT) or the DNA alkylating agent methyl-methanesulfonate (MMS) (Figure 3-2). Both agents impair DNA replication, but only MMS activates the intra-S checkpoint whereas the DSBs generated by CPT are not detected by this pathway (Redon et al. 2003). Cells lacking the DNA damage checkpoint protein Rad9 (*rad9-d*) were used as a control for the activity of these drugs. The experiment revealed that all strains with elevated *in vivo* Cdc2 activity are sensitive to medium to high doses of CPT while they are resistant to DNA replication stress caused by DNA alkylation (MMS). The CPT sensitivity correlates well with the degree of Cdc2 activation, which is lower in *mik1* deletion cells (Lee et al. 1994). Cells expressing mutated Cdc2 proteins with reduced kinase activity (*cdc2.L7* and *cdc2.33*) were neither CPT nor MMS sensitive. Elevated Cdc2 activity fails to render cells sensitive to acute CPT exposure (up to 8 hours) strongly suggesting that high kinase activity only compromises the cellular response to chronic exposure (Figure 3-11, B). Amongst the tested DNA damaging agents, CPT is the only drug to which *cdc2.1w* cells are sensitive (Figure 3-2; Figure 3-3). While *cdc2.3w* cells fail to grow when DNA replication is challenged by lack of nucleotides (i.e. hydroxyurea), by DNA methylation (i.e. MMS) and by CPT, *cdc2.1w* cells are only sensitive to the latter drug (Figure 3-3). This is a novel observation and indicates

that *in vivo* elevated Cdc2 activity specifically impairs the repair of chromosomal breaks that occur in S phase in the presence of the TopI poison CPT.

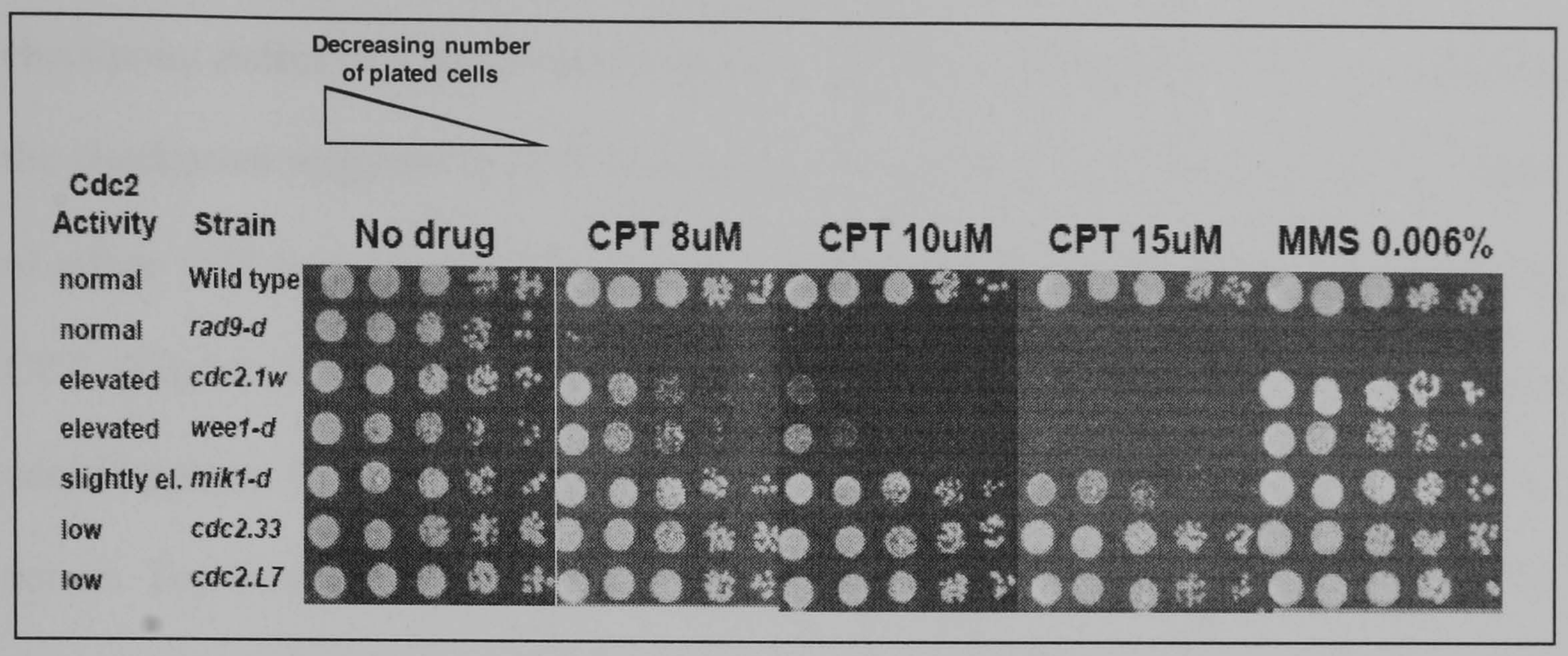


Figure 3-2. Elevated *in vivo* activity of the main cell cycle regulator Cdc2 renders *S. pombe* cells sensitive to CPT.

S. pombe cells with the indicated genotypes were grown at 30°C on rich medium plates containing increasing camptothecin concentrations or the DNA alkylating agent methyl-methanesulfonate (MMS). Cells lacking Rad9 checkpoint protein (*rad9-d*) were used as a control for the activity of drugs. All strains expressing high Cdc2 activity show sensitivity to CPT, whereas cells with reduced activity (*cdc2.L7*; *cdc2.33*) are resistant. Cells with slightly elevated Cdc2 activity (*mik1-d*) show weaker CPT sensitivity than cells with high activity (*cdc2.1w*; *wee1-d*). Strain description: *rad9-d* – no DNA damage response, *cdc2.1w* – activating point mutation in Cdc2, *wee1-d* – deletion of the major Cdc2 inhibitor Wee1 kinase, *mik1-d* - deletion of the minor Cdc2 inhibitor Mik1 kinase, *cdc2.L7* and *cdc2.33* – inactivating point mutations in Cdc2.

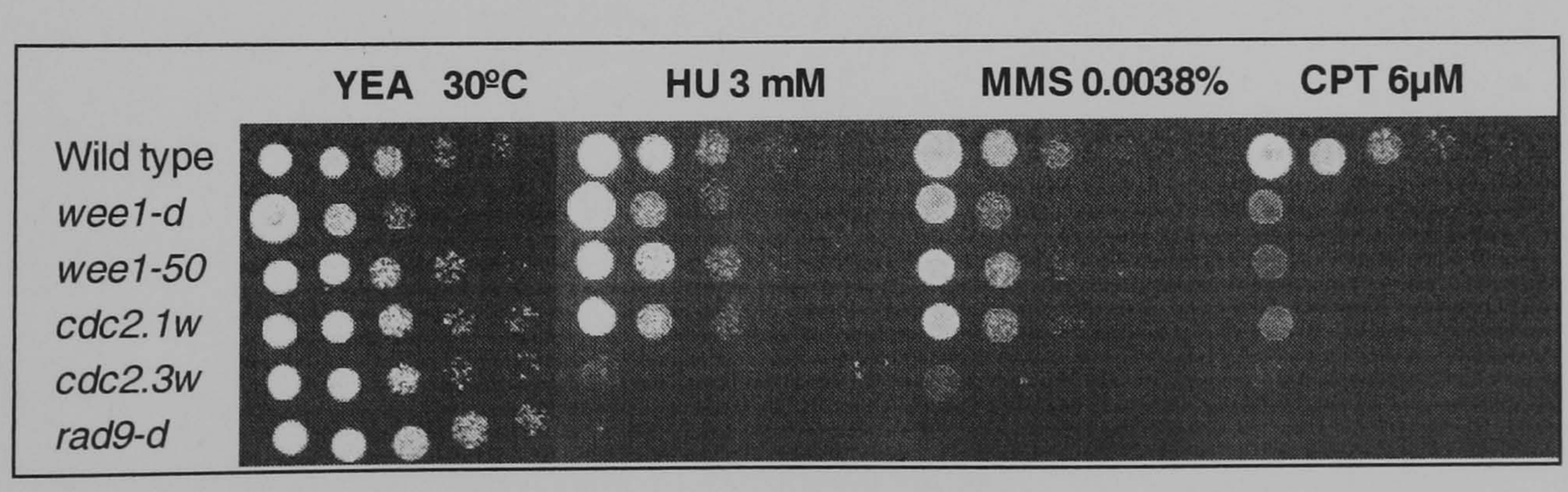


Figure 3-3. *Cdc2.3w* is sensitive to hydroxyurea, MMS and CPT.

Serial dilutions of indicated strains were dropped on EMM medium plates with either 3mM hydroxyurea, 0.0038% MMS or 6µM CPT. Plates were incubated at 30°C for 4 days.

3.1.1.2 Hyperactive *cdc2.1w* does not impair the DNA damage checkpoint in response to CPT

Sensitivity to DNA damaging agents can be caused by a DNA repair defect, a DNA checkpoint defect or a combination of both. To distinguish between these possibilities, the checkpoint response to CPT was analysed by releasing an enriched G2 population of either wild type (strain 804) or *cdc2.1w* cells into rich medium containing 200µM CPT (Figure 3-4). Cells were synchronized in G2 by lactose density gradient centrifugation. Since G2 cells have to progress into the next S phase before CPT can act on Topoisomerase I, wild type cells possess a transient cell cycle arrest in the second cell cycle phase when released into medium containing the drug. Chromosomal breakage caused by CPT in S phase (i.e. first peak of septation in Figure 3-4) leads then to a transient delay of the following G2-M transition consistent with the observation that the G2-M but not the intra-S checkpoint becomes engaged by CPT (Collura et al. 2005). Like wild type cells, cells with elevated Cdc2 activity (*cdc2.1w*) are able to delay cell cycle progression in the presence of CPT showing that their CPT sensitivity is a DNA repair defect and not DNA checkpoint impairment.

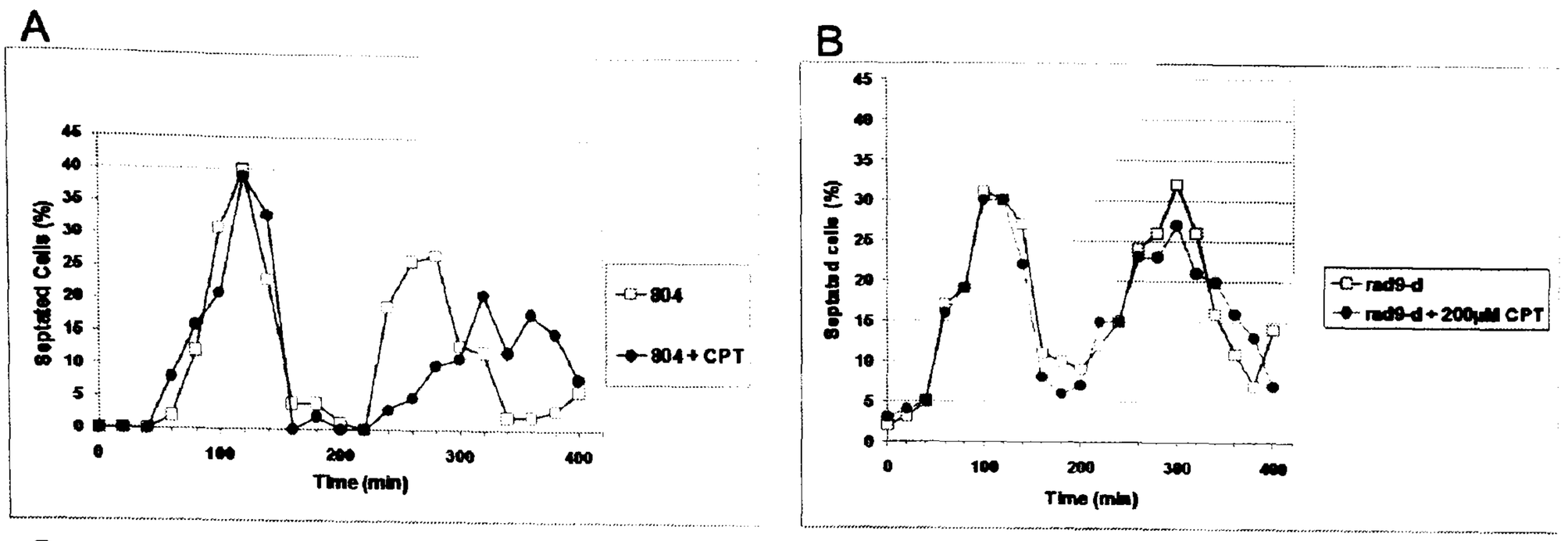


Figure 3-4. DNA damage checkpoint analysis of G2 synchronized *S. pombe* cells upon treatment with camptothecin.

Cells were synchronized in G2 by lactose gradients and checkpoint analysis were carried out upon release of G2 cells into rich medium containing 200µM CPT. *S. pombe* G1 is short and thus the septation index peak corresponds to S phase. In response to CPT, the DNA damage checkpoint delays entry into mitosis in wild type cells (A) whereas the DNA damage checkpoint defective *rad9-d* cells do not arrest cell cycle (B). *Cdc2.1w* cells do still show a DNA damage checkpoint defect in response to CPT (C).

3.1.1.3 The sensitivity to CPT correlates with Cdc2 activity

To obtain further evidence for the notion that elevated Cdc2 activity is responsible for the sensitivity to CPT a genetic trick was used to lower Cdc2 activity in *cdc2.1w* cells. Simultaneous loss of the Cdc2 inhibitor Wee1 kinase and the Cdc2 activator Cdc25 phosphatase suppresses elevated Cdc2 activity and restores normal cell cycle distribution (Russell and Nurse 1986). The combination of temperature sensitive point mutation *cdc25.22* with *cdc2.1w* also results in a reduction of *in vivo* elevated Cdc2 activity since both mutations compensate each other. This can be seen from the

suppression of the temperature sensitive phenotype of *cdc25.22* upon introduction of *cdc2.1w* (Figure 3-5). The double mutant is no longer CPT sensitive at 10μM supporting the argument that elevated Cdc2 activity is responsible for the sensitivity (Figure 3-5).

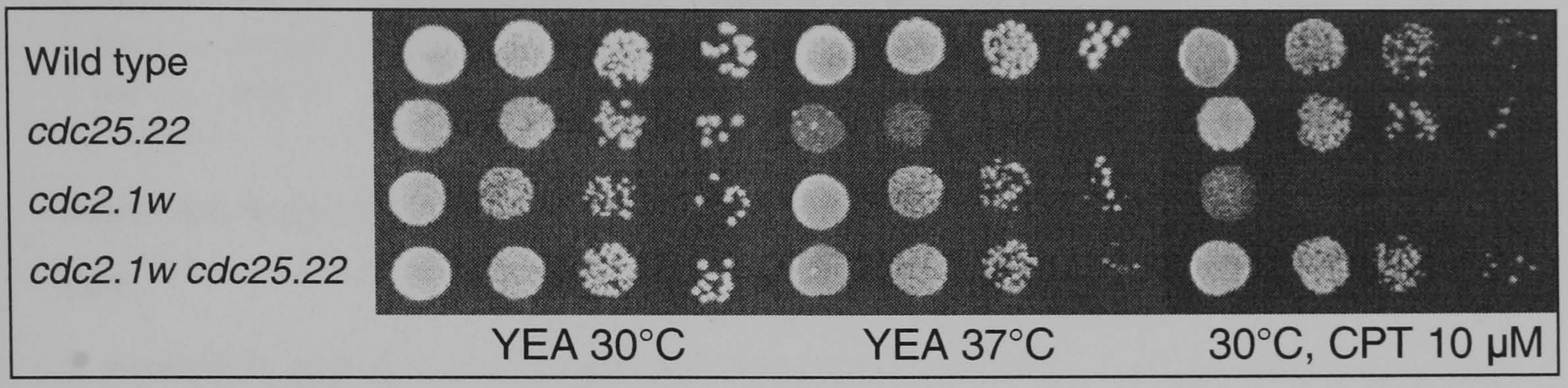


Figure 3-5. Inactivation of the main Cdc2 activator Cdc25 kinase results in a reduction of the elevated *in vivo* Cdc2 activity and cells resistant to CPT.

S. pombe strains with the inactivating temperature-sensitive mutation of the main Cdc2 activator Cdc25 phosphatase (*cdc25.22*) and elevated *in vivo* Cdc2 activity (*cdc2.1w*) were grown at 30°C and 37°C on YEA plates and at 30°C on plates containing the DNA damaging agent CPT. The inactivation of Cdc25 in *cdc2.1w* results in the suppression of the temperature sensitive phenotype of *cdc25.22* and the suppression of the CPT sensitivity of *cdc2.1w*.

3.1.1.4 Replacement of yeast Cdc2 by *D. melanogaster* Cdc2 renders *S. pombe* cells sensitive to CPT

Jimenez and colleagues reported that replacement of *S. pombe* Cdc2 with its *Drosophila melanogaster* paralogue *DMCdc2* results in *wee* cells (Jimenez et al. 1990) strongly indicating that the fly kinase is incompletely down-regulated in yeast. Consistent with the previous observations, *S. pombe* cells expressing *DMCdc2* as sole CDK are sensitive to CPT but resistant to MMS (Figure 3-6).

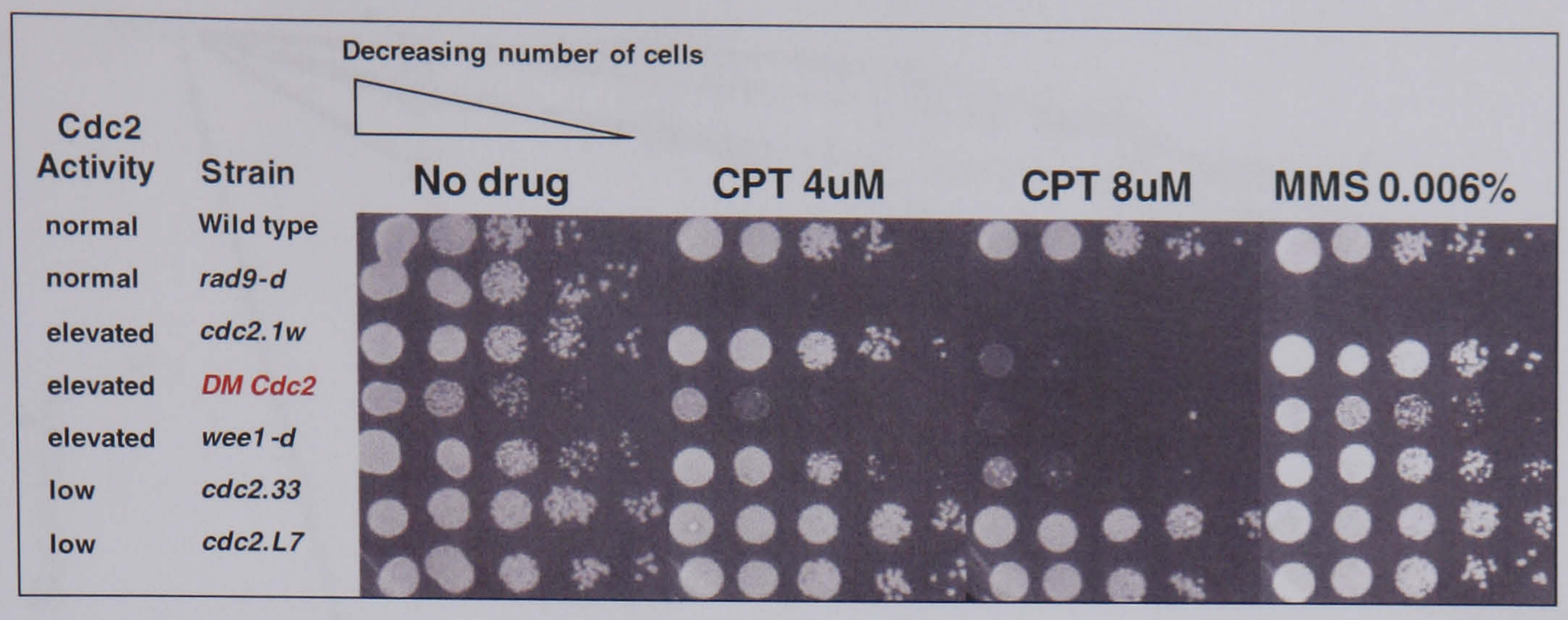


Figure 3-6. Replacement of *S. pombe* Cdc2 with *D. melanogaster* Cdc2 leaves cells sensitive to CPT.

S. pombe cells with the indicated genotypes were grown at 30°C on rich medium plates containing increasing CPT concentrations or MMS. Cells lacking the Rad9 checkpoint protein (*rad9-d*) were used as a control for the activity of the drugs. The replacement of yeast Cdc2 by fly Cdc2 rendered *S. pombe* cells sensitive to CPT.

In contrast to *cdc2.1w* mutant cells, cells expressing *DMCdc2* are significantly more UV sensitive (Figure 3-7). This difference indicates that elevated Cdc2 activity caused by the replacement of glycine 146 by an aspartate residue within the T-loop domain of *S. pombe* Cdc2 affects specifically repair of CPT damage, whereas expression of the homologue kinase causes a wider repair deficiency. The T-loop is phosphorylated by CDK-activating Kinases (CAKs), Mcs6 in *S. pombe*, resulting in activation of Cdc2 (Hermand et al. 2001). This indicates a requirement for CAK enzymes in the regulation of the DNA repair function of Cdc2.

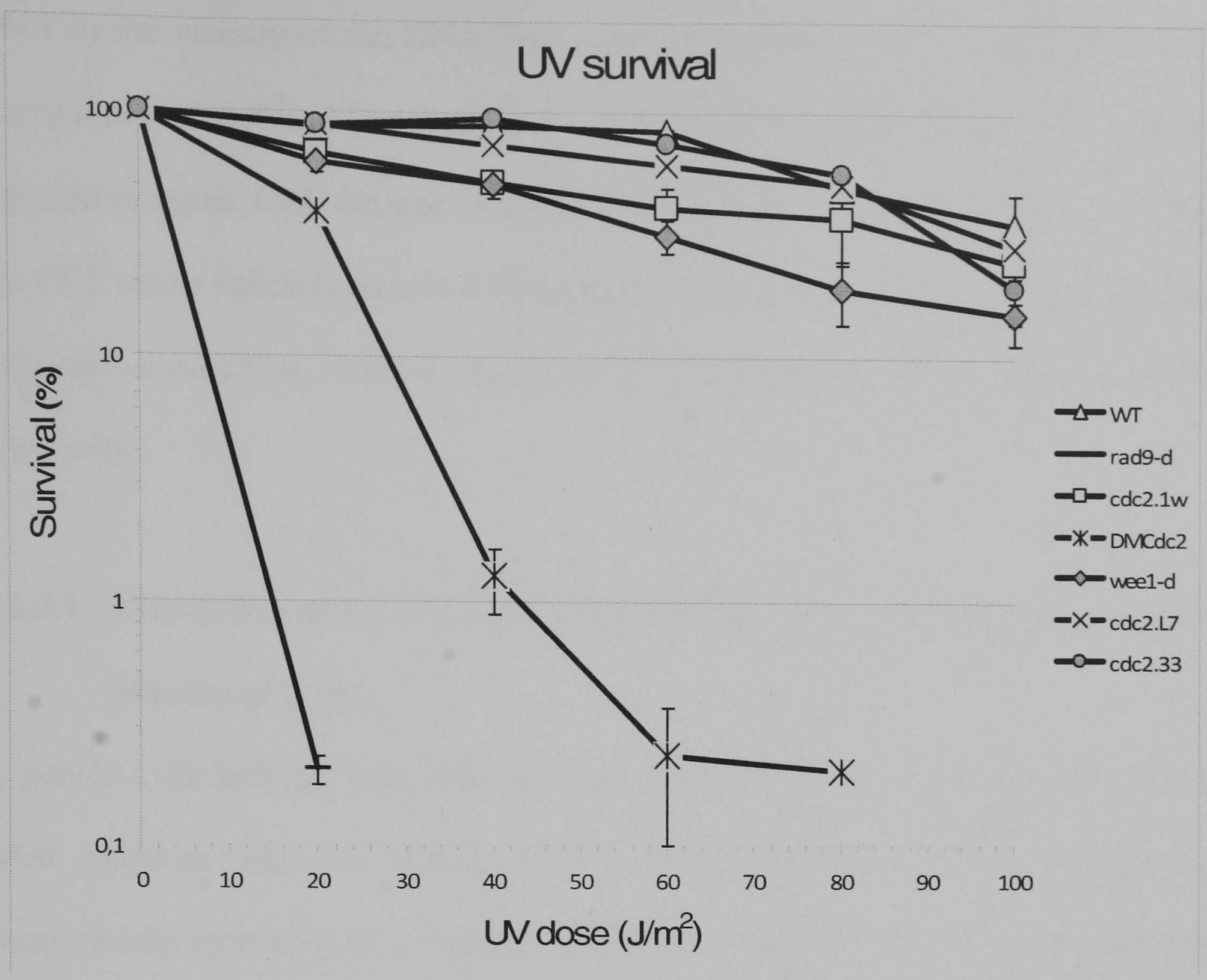


Figure 3-7. Acute UV sensitivity analysis of mutant cells with *in vivo* elevated or decreased Cdc2 activity.

After plating on rich medium plates, the indicated strains were exposed to a range of UV light and incubated for four days in 30°C. Colonies were then counted and the percentage of surviving cells was calculated for each mutant. *Cdc2.1w* cells do not show hyper sensitivity to UV light. The error bars indicate standard errors of the mean.

3.1.2 Cdc2 activity is required for CPT response pathways acting in parallel to Tdp1 and Rqh1 DNA Helicase

Camptothecin-induced DNA damage is somewhat different from other DNA lesions in that an unusual large number of redundant pathways have been reported to contribute to its repair. While Tyrosyl DNA Phosphodiesterase (TDP1) acts directly on the DNA-TopI complex, the endonucleases Mus81-Eme1, Mre11 and Rad16-Swi10 cut the

DNA in the vicinity of the DNA-TopI complex (Liu et al. 2002; Deng et al. 2005; Hartsuiker et al. 2009). Moreover, the DNA helicases Rqh1 (BLM) and Srs2 have been reported to repair CPT damage in a redundant manner (Doe et al. 2002). To identify the CPT repair function impaired by elevated Cdc2 activity, *cdc2.1w* double mutants with *tdp1-d*, *mus81-d*, *rad50-d*, *rad16-d*, *srs2-d* and *rqh1-d* were tested for their CPT sensitivity.

3.1.2.1 Elevated *in vivo* Cdc2 activity increases the CPT sensitivity of cells in the absence of Tdp1.

S. pombe cells lacking Tdp1 (*tdp1-d*) were crossed with cells carrying the *cdc2.1w* point mutation and the double mutant was analysed for their sensitivity to camptothecin by means of a standard drop tests. Elevated Cdc2 activity renders cells without Tdp1 very sensitive to CPT showing that the kinase regulates a DNA repair function acting in parallel to this enzyme (Figure 3-8).

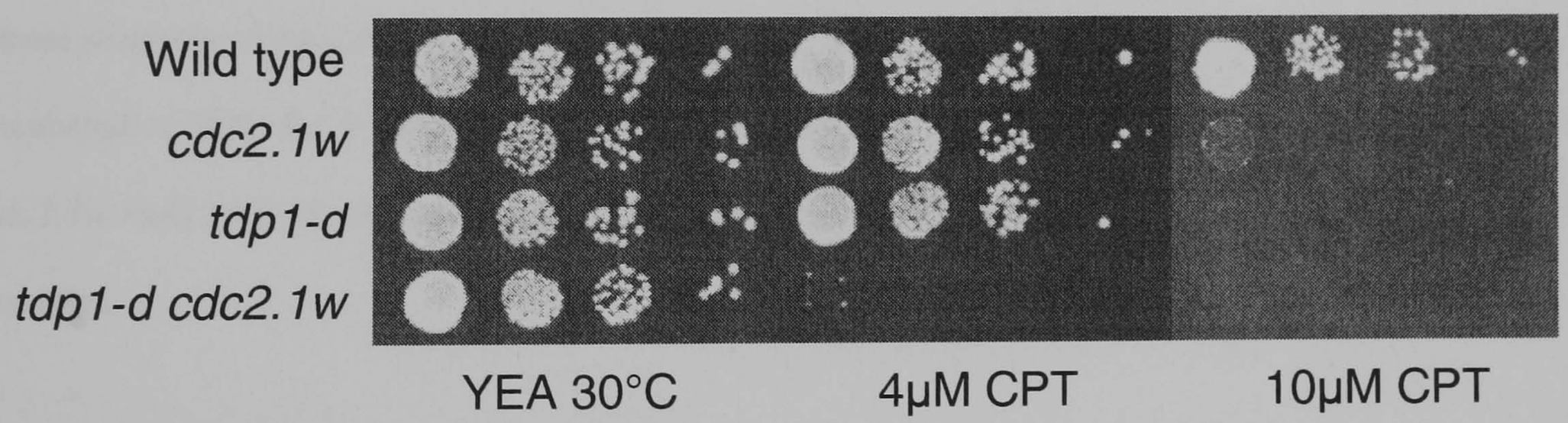


Figure 3-8. Cdc2 activity is not required for the repair activities of Tdp1.

Cdc2.1w point mutation increases the CPT sensitivity of *tdp1-d* mutants. Serial dilutions of indicated strains were spotted onto YEA agar plates containing different concentration of CPT and plates were incubated for 4 days at 30°C. Tdp1 was deleted with the G418 resistance marker (*kanMX6*).

3.1.2.2 *Cdc2.1w* seems to act in a parallel pathway to *rad50S*, a point mutant of Rad50 specifically sensitive to CPT.

To overcome the genetic instability of the *cdc2.1w rad50-d* strain, *cdc2.1w* was combined with either the *rad50S* (*rad50-K81I*) or the *mre11/rad32-D65N* nuclease-dead mutants. Both point mutations impair specifically the role of the Rad50-Mre11-Nbs1 (MRN) protein complex in the removal of trapped Top 1 (Hartsuiker et al. 2009). The combination of the hyperactive *cdc2* allele with either *rad50S* or *rad32-ND* resulted in both cases in increase in CPT sensitivity (Figure 3-9). This is noteworthy because the *S. cerevisiae* MRN complex has been identified as a target of Cdc28 (Cdc2) kinase (Ira et al. 2004).

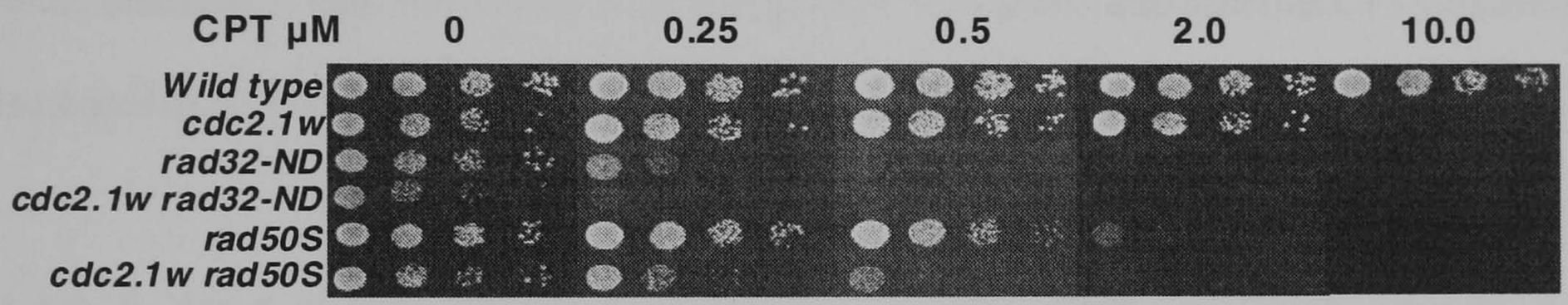


Figure 3-9. Elevated *in vivo* Cdc2 activity increases the CPT sensitivity of *rad50S* or *rad32-ND*. Serial dilutions of indicated strains were spotted onto YEA plates with different CPT concentrations and incubated at 30°C for 4 days. The increased CPT sensitivities of double mutants *cdc2.1w rad50S* and *cdc2.1w rad32-ND* in comparison to single mutant cells suggests that Cdc2 acts in parallel to the MRN complex.

3.1.2.3 Loss of the Endonuclease Rad16 does not increase the CPT sensitivity of *cdc2.1w* cells.

The Rad16 (Swi9)-Swi10 3'-flap endonuclease is important for many DNA repair pathways including Nucleotide Excision Repair and the repair of CPT-induced lesions due to its ability to remove blocked 3'-ssDNA tails from branch intermediates (Liu et al. 2002). To test whether inactivation of this 3'-flap endonuclease would influence the CPT sensitivity of *cdc2.1w* cells, a *rad16-d cdc2.1w* double mutant was constructed.

Loss of Rad16 does not affect the CPT sensitivity of *cdc2.1w* cells indicating that Cdc2 may act in the same pathways as the 3'-flap endonuclease (Figure 3-10). Since deletion of *rad16* on its own fails to render cells sensitive to CPT in the concentration range tested, the latter conclusion is not well supported.

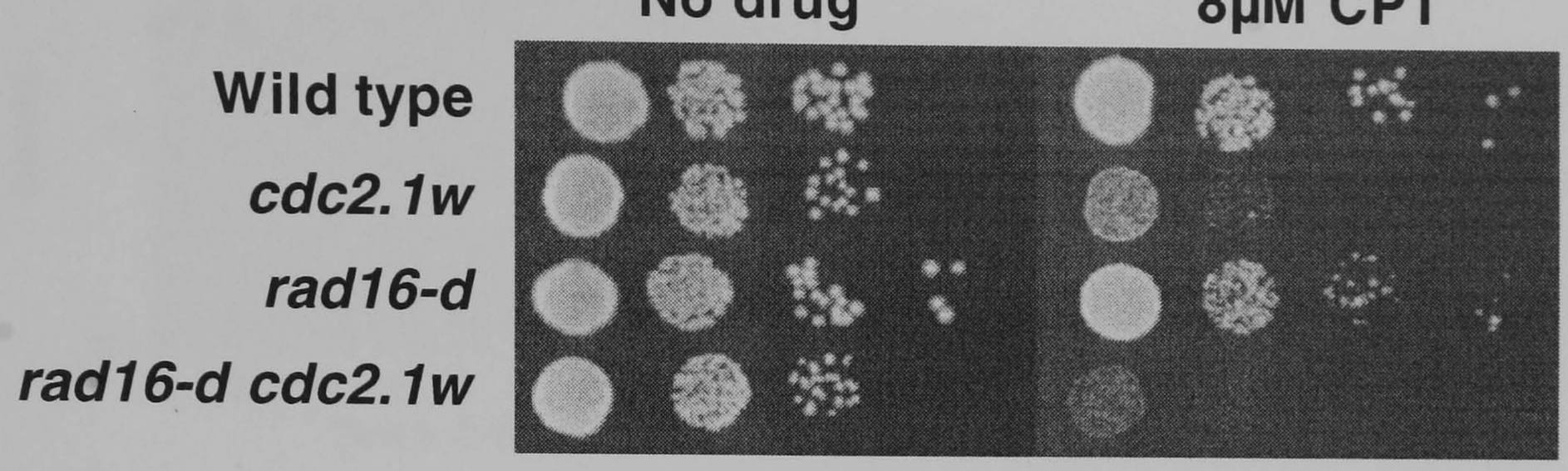


Figure 3-10. Loss of Rad16 does not affect the CPT sensitivity of *cdc2.1w* cells.

Serial dilutions of indicated strains were spotted onto YEA plates with different CPT concentrations and incubated at 30°C for 4 days.

3.1.2.4 High Cdc2 activity does not increase the CPT sensitivity of cells devoid of the structure-specific Endonuclease Mus81-Eme1.

The structure-specific endonuclease Mus81-Eme1 cleaves different branched DNA structures and fission yeast cells devoid of this enzyme are highly CPT sensitive (Doe et al. 2002). To test the true CPT sensitivity of a *mus81-d cdc2.1w* double mutant, existing suppressor mutations (not shown) were crossed out and the survival of the resulting strain was assayed in response to chronic and acute CPT exposure (Figure 3-11). In both assays, the *mus81-d cdc2.1w* double mutant was not more sensitive than the highly sensitive *mus81-d* single mutant strongly indicating that Cdc2 and Mus81-Eme1 act in the same repair pathway.

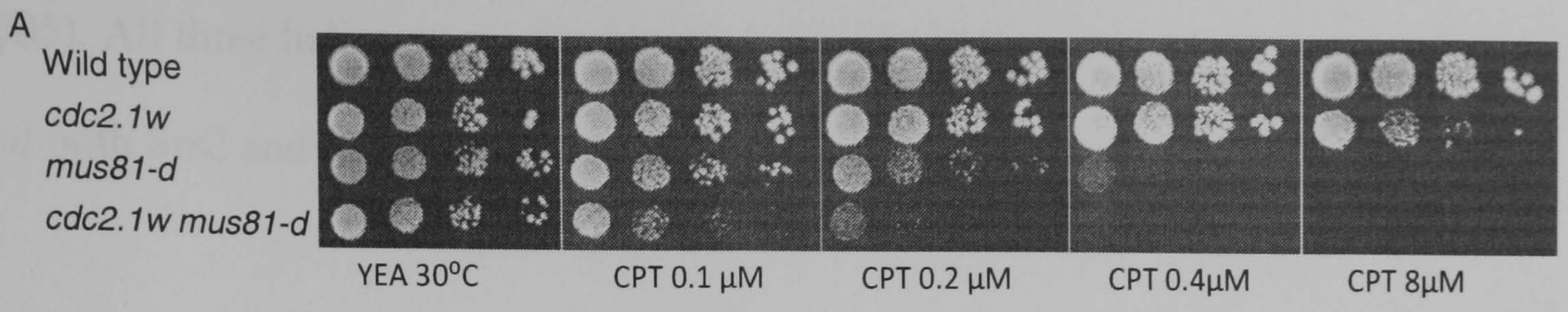


Figure 3-11. Cdc2 and Mus81 act in the same CPT repair pathway.

A) Serial dilutions of indicated strains were spotted onto YEA plates with different CPT concentrations and incubated at 30°C for 4 days. B) Indicated strains were incubated at 30°C in the presence of 40µM CPT and aliquots at the indicated times were removed and plated on rich medium plates. The number of surviving colonies was determined after 4 days at 30°C. The double mutant *mus81-d cdc2.1w* was not more sensitive than either single mutant.

In summary, elevated Cdc2 activity seems to impair not one but several DNA repair pathways dependent on Rad16 and Mus81, which are known to act in parallel to Tdp1. Consistent with this conclusion, *tdp1* deletion cells become very CPT sensitive upon introduction of the *cdc2.1w* allele (Figure 3-8).

3.1.2.5 Elevated Cdc2 activity specifically increases the CPT sensitivity of cells devoid of Rqh1 DNA helicase

In addition to Tdp1, Mus81, Rad16 and Mus81, at least three DNA helicases, Rqh1, Srs2 and Fbh1, contribute to the repair of CPT induced DNA damage (Osman et al.

2005). All three helicases are functionally linked with Rad51-dependent recombination and both Srs2 and Rqh1 act in parallel to Mus81 (Laursen et al. 2003).

To test the requirement of Cdc2 activity, the *cdc2.1w* allele was introduced into strains deleted either for *fbh1*, *srs2* or *rqh1*, and the CPT sensitivities of the corresponding double mutants were assayed. While the *fbh1-d cdc2.1w* and *srs2-d cdc2.1w* double mutants are only slightly more CPT sensitive than *fbh1-d* and *srs2-d* single mutants, the CPT sensitivity of the *rqh1-d cdc2.1w* double mutant is significantly increased (Figure 3-12). This strong increase in CPT sensitivity suggests that Cdc2 regulates one or more repair pathways acting in parallel to Rqh1 DNA helicase. Interestingly, the increase is specific to the *cdc2.1w* point mutation and does not occur upon deletion of Wee1 kinase (Figure 3-12, B). This is a significant observation because it may be the first genetic background distinguishing between *wee1-d* and *cdc2.1w* mutants. Importantly it shows that the genetic interaction with *rqh1-d* is caused by elevated Cdc2 activity and not by the indirect changes to the cell cycle. The *wee1-d rqh1-d* mutant was backcrossed to confirm that this difference is not caused by an additional suppressor mutation.

Elevated Cdc2 activity increases not only the CPT sensitivity of *rqh1-d* cells but it also reduces cell viability, promoting the accumulation of genetic suppressors as in the case of the *cdc2.1w mus81-d* and *cdc2.1w rad50-d* mutants (not shown). Neither of these phenotypes was observed in *cdc2.1w srs2-d* nor in *cdc2.1w fbh1-d* double mutants.

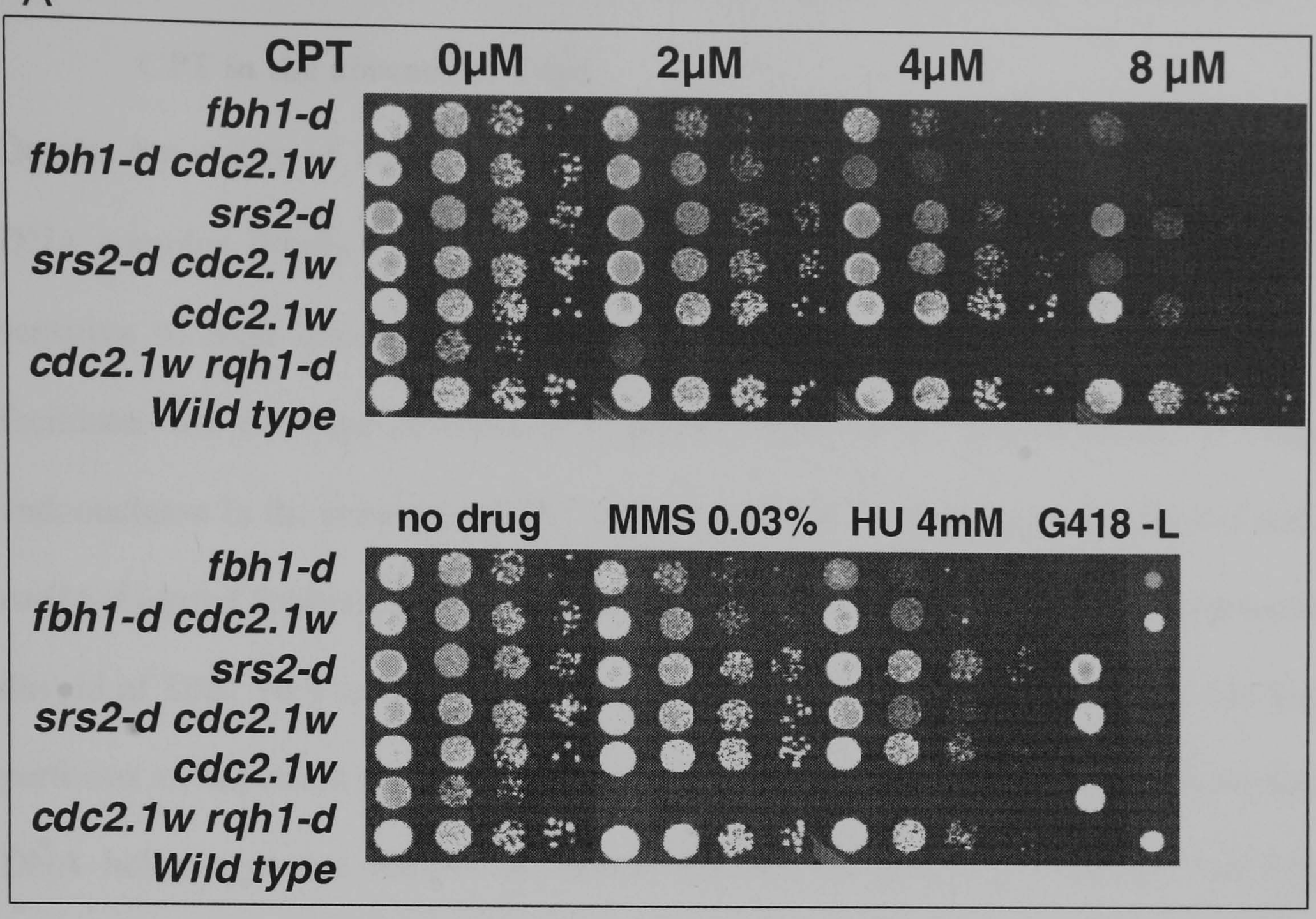


Figure 3-12. Elevated Cdc2 activity specifically increases the CPT sensitivity of cells devoid of Rqh1 DNA helicase

A) Analysis of the CPT sensitivity of *cdc2.1w* double mutants lacking either *rqh1*, *srs2* or *fbh1*. The sensitivity to the anti-cancer drug camptothecin (CPT) caused by elevated Cdc2 activity (*cdc2.1w*) is not significantly increased upon loss of Srs2 or Fbh1, but inactivation of rqh1 renders the double mutant much more sensitive. B) Loss of Wee1 kinase does not result in a similar increase in CPT sensitivity as the cdc2.1w allele indicating that the observed genetic interaction between Cdc2 and Rqh1 is due to elevated kinase activity and not due to changes in cell cycle distribution.

3.1.2.6 Inactivation of Srs2 DNA Helicase strongly increases the sensitivity to CPT in the absence of Tdp1.

Despite the ability of Tdp1 to cleave the DNA in the vicinity of the Topoisomerase-DNA complex (Pouliot et al. 1999), *S. pombe* cells devoid of this enzyme are only sensitive to high concentrations of CPT. Since *S. cerevisiae* Srs2 DNA helicase facilitates the cleavage of blocked 3'-ssDNA ends by the Rad16 ($Rad1^{Sc}$) 3'-flap endonuclease in the presence of CPT (Vance and Wilson 2002), a *srs2-d tdp1-d* and a *rad16-d tdp1-d* double mutant was constructed. Deletion of Srs2 helicase renders cells devoid of Tdp1 very sensitive to CPT, even under acute conditions, showing that Srs2 performs an important repair function in the absence of Tdp1 (Figure 3-13). Since Srs2 DNA helicase cannot remove covalently bound Topoisomerase I on its own from DNA, this alternative repair pathway has to depend on a nuclease. It is very likely that this nuclease is Rad16-Swi10 because the *rad16-d tdp1-d* double mutant is not viable (not shown) indicating that *S. pombe* cells become heavily dependent on this 3'-flap endonuclease when Tdp1 function is impaired. This conclusion is consistent with the functional interdependence of Srs2 and Rad16 ($Rad1^{Sc}$) in *S. cerevisiae* (Vance and Wilson 2002). Exonuclease I, which acts on DNA double-strand breaks in parallel to Rqh1 (Mimitou and Symington 2008) is unlikely to act in conjunction with Srs2 helicase because deletion of *exo1* does not increase the CPT sensitivity of *tdp1-d* cells (not shown).

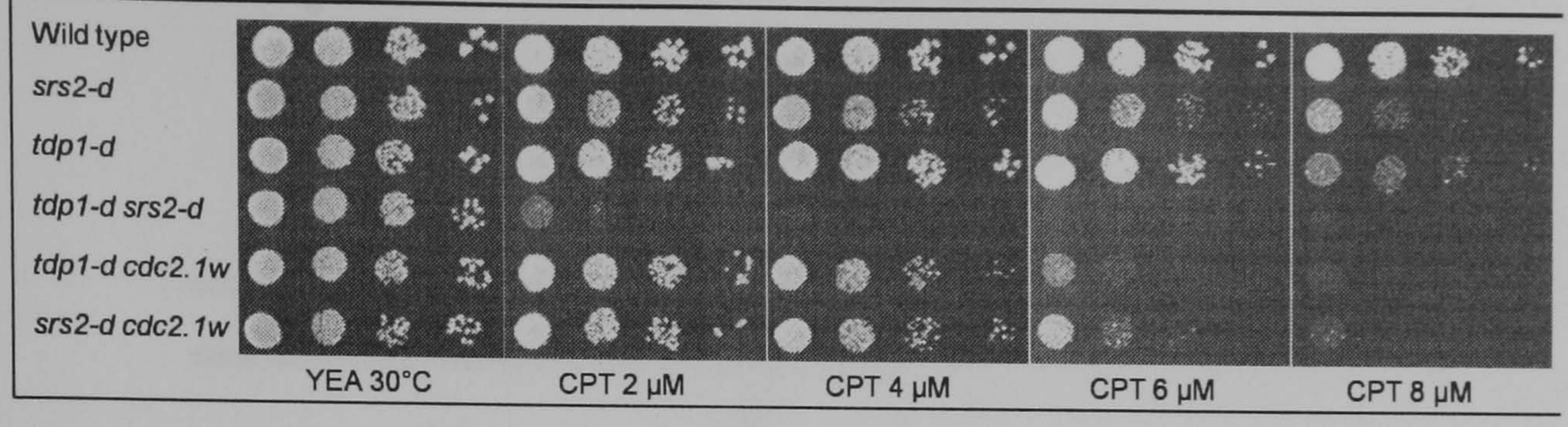


Figure 3-13. Deletion of Srs2 helicase renders cells devoid of Tdp1 very sensitive to CPT,

A) Serial dilutions of indicated strains were spotted onto YEA plates with CPT concentrations between 0 – 8µM. Cells were incubated at 30°C for 4 days. B) Acute survival of the indicated strains at 30°C in the presence of 40µM CPT. Samples were taken at indicated times and plated on YEA followed by 4 days incubation at 30°C.

Taken together, these data suggests that Cdc2 seems to regulate three CPT repair pathways depending on Mus81 and Rad16 in association with Srs2 DNA helicase (Figure 3-14). Interestingly, Rqh1 DNA helicase is not part of this novel regulatory network which may coordinate the response to CPT in G2.

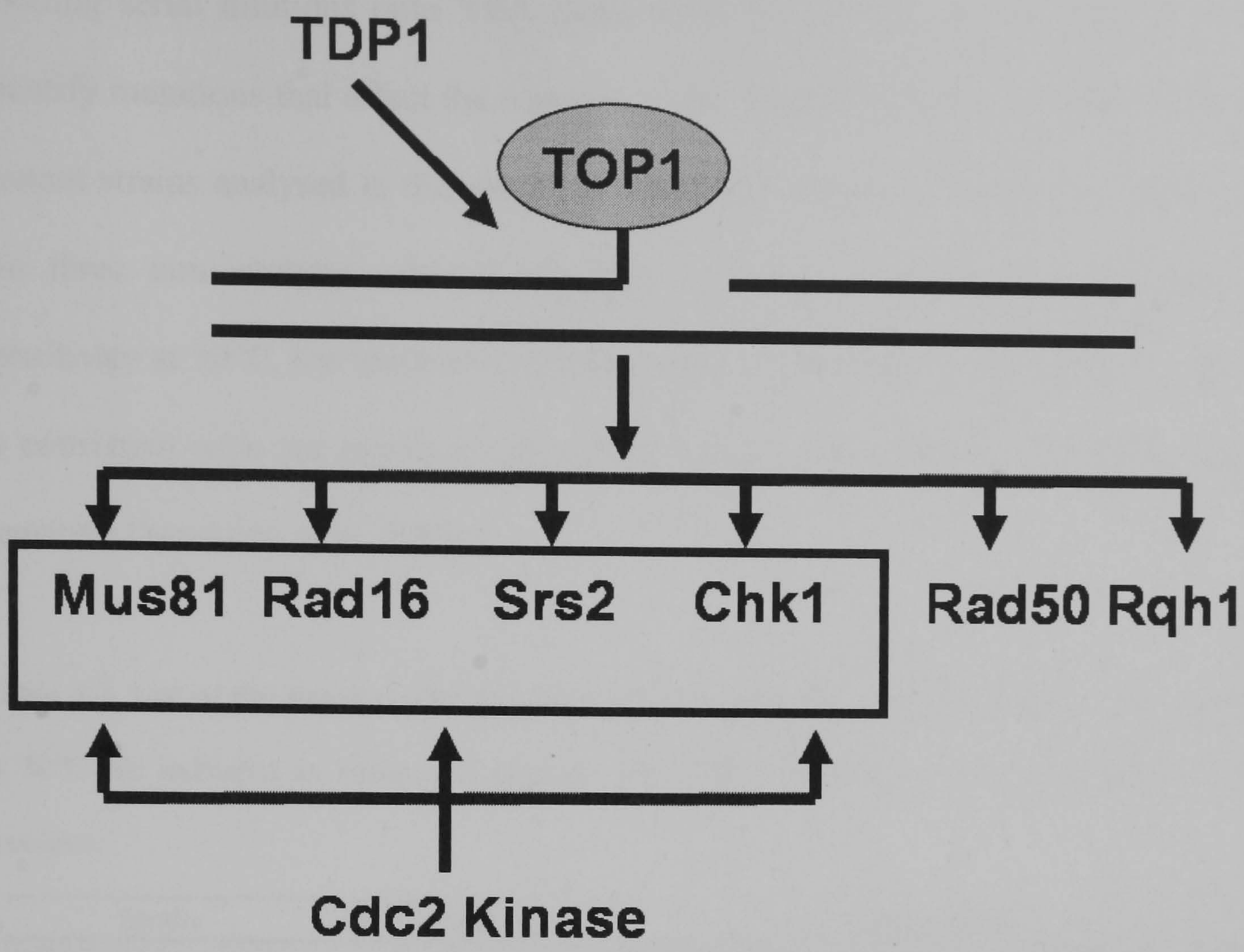


Figure 3-14. Schematic of a working model.

In response to CPT-caused DNA double-strand breaks, Cdc2 seems to regulate the repair pathways depending on Mus81, Rad16 and Srs2 DNA helicase but independent of Rqh1 and Rad50.

3.2 Cdc2 activity and the DNA replication stress caused by mutations in replication proteins

There is evidence in the literature suggesting that *S. pombe* Wee1 kinase regulates Cdc1, one of the three non-catalytic subunits of the replication polymerase delta (Fantes et al. 1991). Strains containing temperature sensitive mutations in different subunits of DNA polymerases alpha, delta and epsilon (α, δ, ε, respectively) were tested at the semi-permissive temperature of 30°C for their CPT sensitivities by

spotting serial dilutions onto YEA plates with increasing concentrations of CPT to identify mutations that affect the response to this replication poison. Table 3-2 lists all mutant strains analysed in this assay. Interestingly, a mutant defective in only one of the three non-catalytic subunits of Polδ, *cdc1-P13*, possesses a significant CPT sensitivity at 30°C, but mutants in neither of the other subunits do (Figure 3-15). This is consistent with the previous report showing that *cdc1* mutants are MMS and HU sensitive (MacNeill et al. 1996).

Table 3-2. List of the tested replication mutant strains and their CPT sensitivity. CPT sensitivities at 30°C are indicated as follow: (-) resistant, (+) mildly sensitive, (++) sensitive and (+++) very sensitive.

Strain	CPT sensitivity	Description
swi7.H4	-	Catalytic subunit of Pol α - synthesis of the first, short stretch of DNA utilising the RNA primer synthesised by primase
cdc20-M10	-	Catalytic subunit of Pol ε - leading strand synthesis
cdc6.23	-	Catalytic subunit of Pol δ - lagging strand synthesis
cdm1-SP212	-	Non-catalytic subunit of Pol δ
cdc1-P13	+++	Non-catalytic subunit of Pol δ
cdc27-P11	-	Non-catalytic subunit of Pol δ
cdc27-P11 cdc2.1w	+	
rad2-d	+	FEN1 endonuclease
rad2-d cdc2.1w	+	
cdc22-M45	+	Ribonucleotide reducatse
cdc22-M45 cdc2.1w	+	
cdc18-K46	-	MCM helicase loader
cdc18 –K46 cdc2.1w	+++	

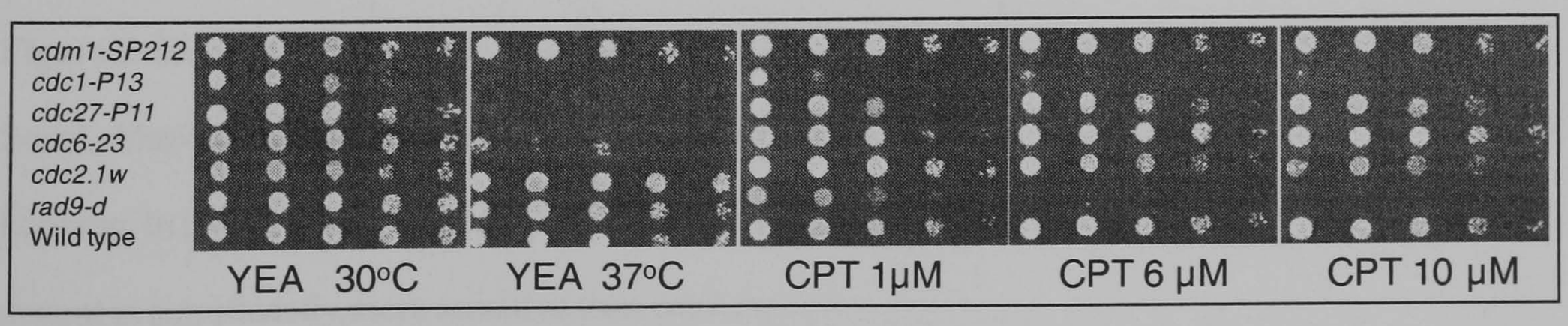


Figure 3-15. Comparison of the CPT sensitivity of the catalytic subunit of Polymerase δ (*cdc6*) with its non-catalytic subunits (*cdc27*, *cdm1* and *cdc1*).

Polymerase δ consists of one big catalytic subunit *cdc6* and three non-catalytic subunits *cdc27*, *cdm1* and *cdc1* (Bermudez et al. 2002). Serial dilutions of cells with temperature-sensitive point mutations in

each of the Pol δ subunits were spotted onto YEA plates with increasing CPT concentrations and incubated at the semi-permissive temperature of 30°C for 4 days. Only *cdc1-P13* possesses CPT sensitivity at 30°C suggesting a link between Cdc2 and Cdc1.

In addition to mutants in DNA replication polymerases, other replication mutants were tested for their CPT sensitivity, both with normal and with elevated Cdc2 activity (again see Table 3-2 for the strain list). Cdc18 loads the MCM DNA helicase at the start of DNA replication. The interesting observation was that the *cdc18.K46* mutant on its own is not CPT sensitive but the *cdc2.1w cdc18.K46* double mutant is significantly more sensitive than *cdc2.1w* (Figure 3-16). This may indicate that the DNA repair pathways regulated by Cdc2 becomes important when loading of the MCM helicase is impaired and it could point towards a link between Cdc18 and either Tdp1 or Rqh1. The latter is suggested by the increase in CPT sensitivity of the *tdp1-d cdc2.1w* and *rqh1-d cdc2.1w* mutants (Figure 3-8; Figure 3-12).

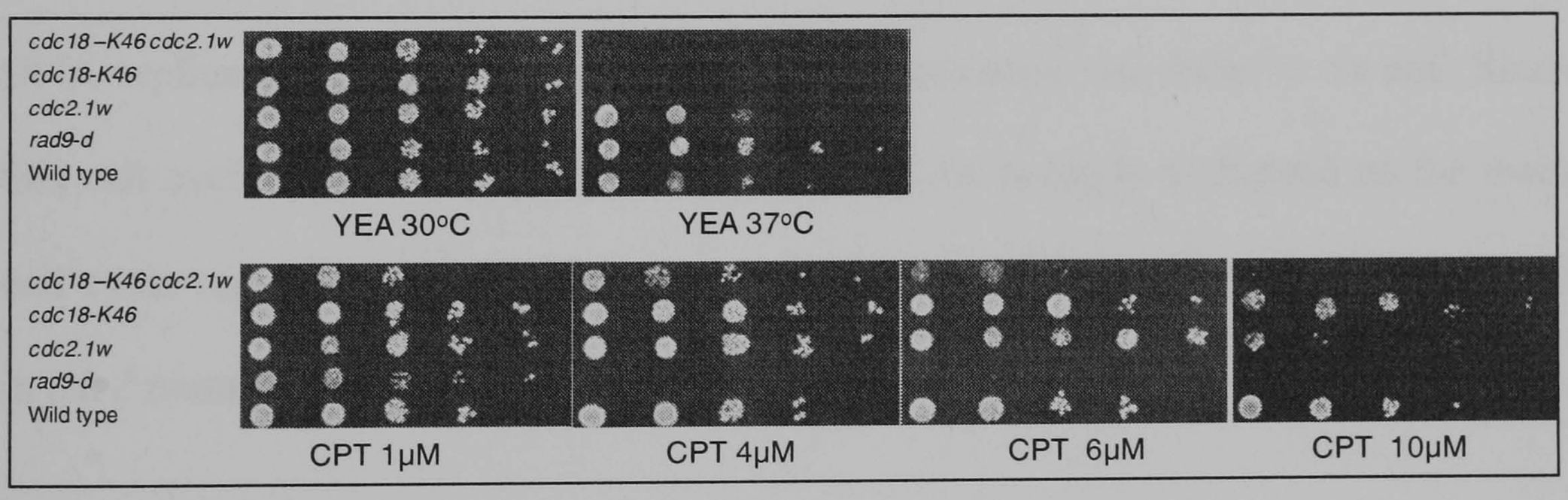


Figure 3-16. The MCM helicase may be involved in CPT repair when Cdc2 activity is elevated.

Serial dilutions of indicated strains were spotted onto YEA agar plates with CPT of concentration between 0-10μM. The *cdc18.K46* mutant is temperature sensitive. The *cdc2.1w cdc18.K46* double mutant is significantly more sensitive than *cdc2.1w*.

3.3 Cdc2 activity regulates inter-sister recombination in mitotic cells

One of the earliest signs of cancer development is the accumulation of broken chromosomes in response to DNA replication stress (Bartkova et al. 2005). Aberrant DNA replication caused, for example, by activation of oncogenes can result in single-stranded DNA (ssDNA) gaps behind the replication fork which, if processed by nucleases, can cause a chromosome to break. How do cells repair these ssDNA regions? Although these ssDNA gaps are a very good substrate for DNA recombination, experimental evidence suggests that cells actively suppress DNA recombination in S phase to avoid interference with DNA replication processes. For example, *S. pombe* recombination proteins travel with the MCM DNA helicase along chromosomes during DNA replication, but they are not required for the replication process per se (Bailis et al. 2008). It seems that DNA lesions that escape repair during DNA replication are repaired in G2 once bulk replication has come to an end. Since this cell cycle-dependent regulation of recombination is likely to depend on the main cell cycle regulator Cdc2, spontaneous mitotic recombination rates were investigated in *cdc2* mutant strains with *in vivo* elevated kinase activity.

3.3.1 The Recombination Assay

Mitotic recombination frequencies were determined by using strains containing an intra-chromosomal recombination substrate consisting of non-tandem direct repeats of *ade6*$^{-}$ heteroalleles separated by a functional *ura4*$^{+}$ gene (Figure 3-17). Frequencies of

spontaneous recombination between the *ade6*$^{-}$ heteroalleles were determined by recovery of Ade^{+} recombinants as described in (Osman et al. 2000). The *ade6*$^{-}$ heteroalleles can recombine with each other to generate a wild-type *ade6*$^{+}$ gene either by gene conversion, that is by transfer of wild-type information from one heteroallele to the other thereby retaining the *ura4*$^{+}$ gene, or by an intra-chromatid loop-out event resulting in the loss of *ura4*$^{+}$ (i.e. deletion type). The conversion type recombinants would exhibit a genotype *ade6*$^{+}$*ura4*$^{+}$, while the deletion genotype would be *ade6*$^{+}$*ura4*$^{-}$ (Osman et al. 2000).

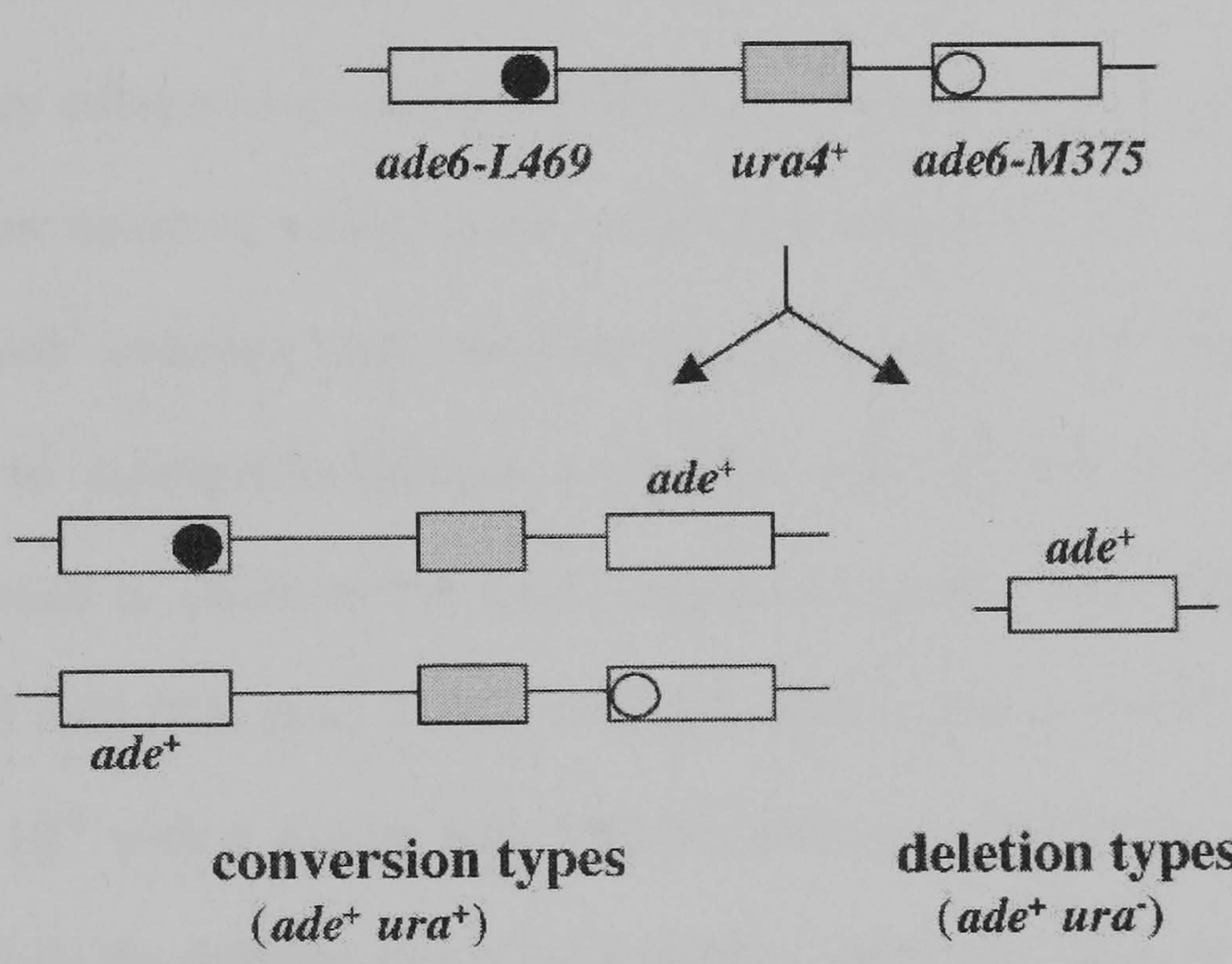


Figure 3-17. Schematic of the spontaneous intra-chromosomal recombination assay.

Non-tandem direct repeats of *ade6*$^{-}$ heteroalleles are flanking a functional *ura4*$^{+}$ gene. Recombination events at the *ade6* duplication can generate *ade*$^{+}$ recombinants. The *ura4*$^{+}$ marker gene enables distinction between conversion and deletion types of recombinants (*ade6*$^{+}$*ura4*$^{+}$ and *ade6*$^{+}$*ura4*$^{-}$ genotypes respectively). Solid and open circles represent the *ade6-L469* and *ade6-M375* mutations respectively. Figure adapted from (Kai et al. 2005).

3.3.2 Recombination rates in Cdc2 point mutants with *in vivo* elevated kinase activity

3.3.2.1 *In vivo* elevated Cdc2 activity increases the spontaneous gene conversion rate

Cells containing either the *cdc2.1w* (Booher and Beach 1986) or the *cdc2.3w* (Basi and Enoch 1996) mutation were crossed into a genetic background containing the recombination substrate (Figure 3-17). Single colonies were taken from selective plates and grown in rich medium at 30°C until they entered stationary phase. Dilutions of the stationary cultures were plated on rich medium plates to determine the total cell number and on selective plates minus adenine to select for *ade6*$^{+}$ recombination events. All *ade6*$^{+}$ colonies were subsequently replica-plated onto selective medium minus uracil to distinguish between conversion and deletion events. Fluctuation analysis was used to calculate the recombination frequency (recombination rate per generation and cell) (Kai et al. 2005). Wild type cells show an overall recombination rate of 1.1 x 10^{-4} with a similar split between gene conversion and deletion events (Table 3-3).While the deletion rate is comparable to wild type cells, both *cdc2* mutant strains possess a specific and reproducible increase in gene conversion rates, which are between 3 and 5-fold higher than in wild type cells. Conversions rates are increased from the wild type level of 0.5±0.1 x 10^{-4} to 1.5±0.5 x 10^{-4} for the *cdc2.1w* strain and 2.5±0.96 x $10^{-4}$for the *cdc2.3w* strain respectively (Table 3-3; Figure 3-18). This selective increase in conversion events suggests that elevated Cdc2 activity results in DNA replication lesions which stimulate the exchange of genetic information between the two *ade6*$^{-}$ heteroalleles located on opposite sister chromatids without loss of the connecting sequence. Such exchange of DNA information is indicative of homologous

recombination events where the invading DNA strand is being returned to its original place once the genetic information stored in the sister chromatid has been copied.

Table 3-3. Spontaneous recombination rates in wild type and Cdc2 mutant strains. In mutant strains which possess high Cdc2 activity deletion rates are comparable to wild type, whereas conversion rates are significantly increased from the wild type level of 0.5±0.1 x 10^{-4} to 1.5±0.5 x 10^{-4} for the *cdc2.1w* strain and to 2.5±0.96 x 10^{-4} for the *cdc2.3w* strain.

Strain	Deletion	Factor	Conversion	Factor	Del error	Con error
Wild type	0,6	1,0	0,5	1,0	0,3	0,1
cdc2.3w	0,5	0,83	2,5	5,0	0,2	0,96
cdc2.1w	0,7	1,15	1,5	3,0	0,12	0,5

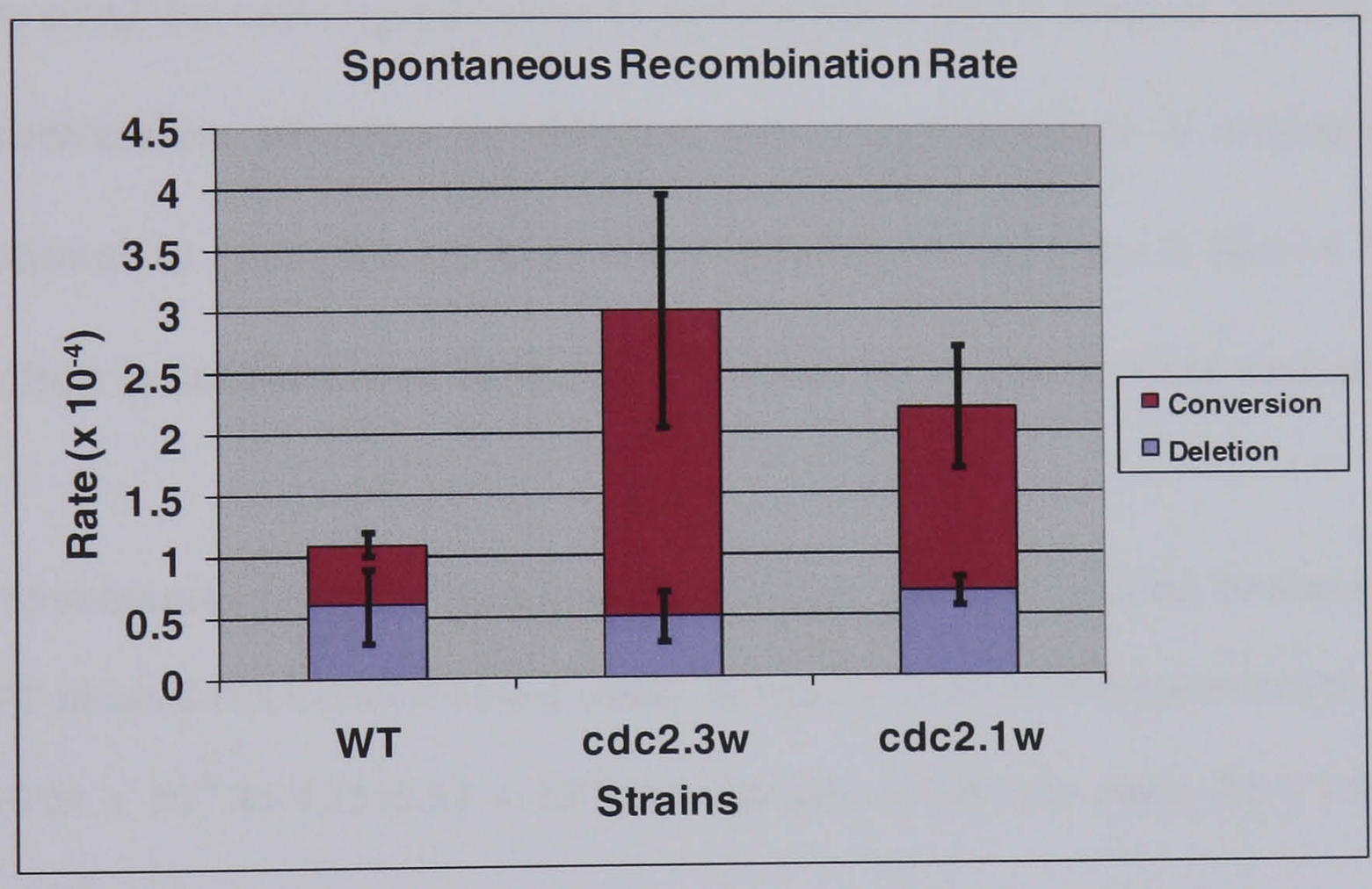


Figure 3-18. Hyperactive Cdc2 increases the spontaneous gene conversion rate.

Two different point mutations in Cdc2, which both elevate its *in vivo* kinase activity, result in an increase in spontaneous gene conversion, while the deletion rate is comparable to wild type cells. Despite the large differences observed between wild type and *cdc2.1w* and *cdc2.3w* mutant strains, these differences were not found to be statistically significant at the $p < 0.05$ level, following a one-way Analysis of Variance for either conversion ($p = 0.290$) or deletion ($p = 0.067$) rates. The error bars indicate standard errors of the mean. Recombination has been measured using a tandem repeat of two differently mutated *ade6* marker genes.

3.3.2.2 CPT sensitivity of *cdc2.1w* cells is not caused by abnormal recombination in response to CPT

To gain further insight into the underlying mechanisms of the link between DNA replication stress and recombination, the recombination rates of wild type and *cdc2.1w* cells were determined in the presence and absence of 5μM CPT. Consistent with the idea that CPT-induced DNA double-strand breaks are recombination substrates in G2, wild type cells showed a 4-5 fold increase in *ura4*$^{+}$ deletions at the sub-lethal concentration of 5μM. Since a similar increase was observed in *cdc2.1w* mutant cells (Table 3-4; Figure 3-19), elevated Cdc2 activity is unlikely to impair the recombination response in presence of CPT. This shows that the recombination increase in *cdc2.1w* cells is restricted to spontaneous DNA lesions, which are repaired by gene conversion, whereas the deletion type recombination in response to CPT is fully functional in presence of high Cdc2 activity. Moreover, it shows that aberrant recombination is not the cause of the CPT sensitivity of *wee* mutant strains.

Table 3-4. Spontaneous recombination rates in wild type and Cdc2 mutant strains in the presence of CPT. CPT induced DNA double-strand breaks increase the rate of deletion events in wild type cells from 0.76±0.09 x 10^{-4} to 4.25±0.82 x 10^{-4} and similarly in *cdc2.1w* strain from 0.8±0.3 x 10^{-4} to 3.66±1.56 x 10^{-4}.

Strain	Deletion	Factor	Conversion	Factor	Del error	Con error
Wild type	0,76	1,0	1,26	1,0	0,09	0,15
Wild type + CPT	4,25	5,6	1,65	1,3	0,82	0,56
cdc2.1w	0,8	1,0	3,6	1,0	0,3	1,4
cdc2.1w + CPT	3,66	4,6	2,78	0,8	1,56	0,74

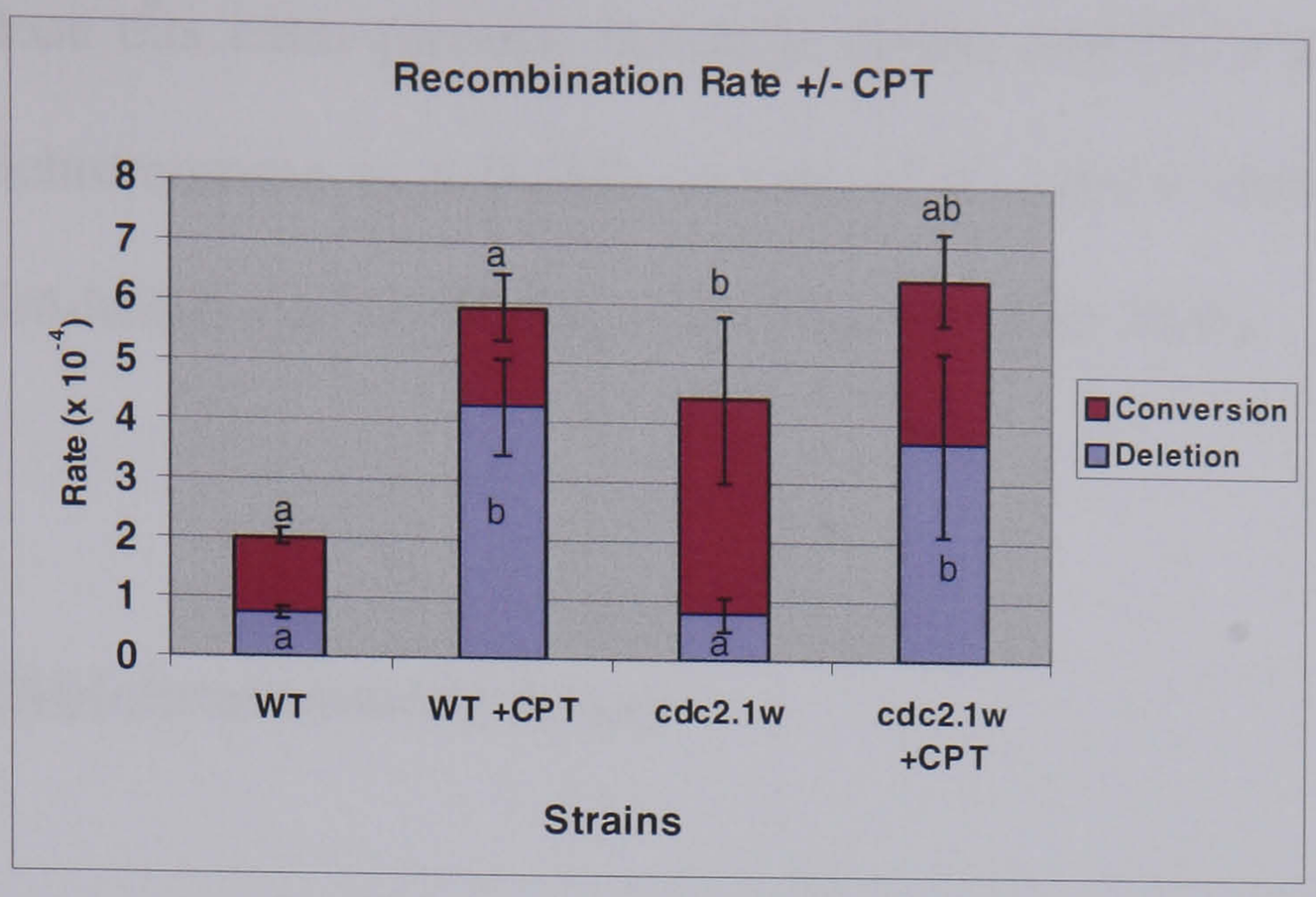


Figure 3-19. In the presence of CPT *cdc2.1w* mutant cells have an increase in the rate of deletion events similar to that of wild type cells.

In response to CPT induced DNA damage, wild type cells significantly increase the rate of deletion events. A similar increase is observed in cells with the *cdc2.1w* mutation suggesting that elevated Cdc2 activity does not impair this type of recombination response. Thus the CPT sensitivity of *cdc2.1w* cells is unlikely to occur due to abnormal recombination in response to CPT. Recombination was measured using a tandem repeat of two differently mutated *ade6* marker genes. One-way Analysis of Variance was carried out to establish if any significant differences existed between strains. Results showed that differences were statistically significant at the $p < 0.05$ level, both for conversion ($p = 0.013$) and deletion ($p = 0.001$) rates. Post-hoc tests were carried out using the Tukey LSD test to determine where significant differences lay between strains. These are indicated with different letters (a and b). The error bars indicate standard errors of the mean.

3.4 Elevated Cdc2 activity results in increased loss of a non-essential minichromosome

The elevated gene conversion rate of *cdc2.1w* cells is consistent with the presence of DNA replication stress in these cells. Since replication stress is known to increase the spontaneous loss rate of a non-essential minichromosome (Maine et al. 1984), the non-

essential Ch^{16}-MG minichromosome was used to determine the percentage of cells which would lose this extra-genomic molecule during growth in rich medium. The Ch^{16}-MG minichromosome is a 0.5Mb section of *S. pombe* chromosome III and encodes the *ade6*-M216 marker (Figure 3-20) (Prudden et al. 2003).

3.4.1 The Minichromosome Assay

The non-essential minichromosome Ch^{16}-MG is a 500kb linear fragment of *S. pombe* chromosome III (ChIII) (Niwa et al. 1986). Ch^{16}-MG carries an *ade6*-M216 point mutation, which if present with an *ade6*-M210 heteroallele on ChIII, results in an ade^+ phenotype through intragenic complementation (Leupold and Gutz 1964). The $rad21^+$ gene is located 28kb distal to the *ade6* locus on Ch^{16}-MG and contains an integrated *MATa* restriction site that is cleaved by *S. cerevisiae* HO-endonuclease (Osman et al. 1996; Prudden et al. 2003) as well as the G418 resistance marker (*kanMX6*) adjacent to the *MATa* sequence (Figure 3-20). This minichromosome assay can be used in two ways, to measure the spontaneous frequency of minichromosome loss without HO cleavage and to determine the frequency of inter-chromosome (i.e. between chromosome III and Ch^{16}-MG) recombination upon HO cleavage. Strains containing the Ch^{16}-MG chromosome and a plasmid encoding *S. cerevisiae* HO endonuclease are $ade6^+$ and G418 resistant prior to HO expression. Following HO expression, dependent on which DSB repair mechanism is engaged, different phenotypes are predicted. When the DSB is repaired by inter-chromosomal HR utilising the $rad21^+$ gene on ChIII as a template, both the *MATa* and *kanMX6* sequences are lost and cells become G418 sensitive. If the inter-chromosomal exchange is limited to the *rad21* gene region, cells stay $ade6^+$, whereas an extended exchange beyond the *rad21* gene

region would copy the *ade6-M210* information from ChIII rendering cells *ade6*$^-$. This allows for the distinction between short-tract gene conversion (*ade6*$^+$ G418^S) and long-tract gene conversion (*ade6*$^-$ G418^S). Since the latter genotype could also result from loss of the minichromosome, additional tests are required to distinguish between both events. NHEJ would be expected to rejoin the DSB within the *MATa* sequence and thus retain the G418 marker (*ade*$^+$, G418^R).

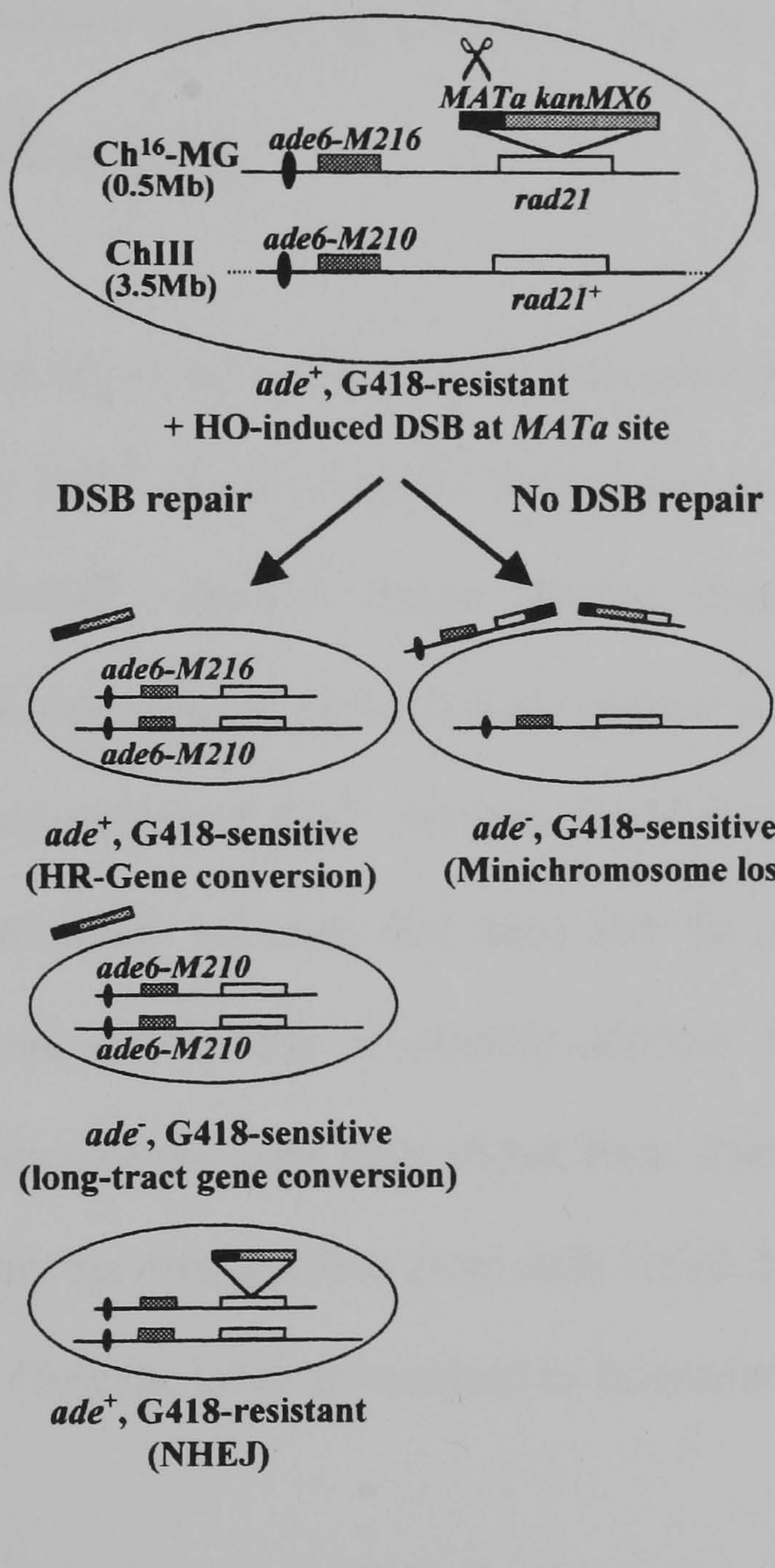


Figure 3-20. Schematic of the minichromosome assay.

The Ch^{16}-MG minichromosome is a 0.5Mb part of *S. pombe* chromosome III, which carries the *ade6*-M216 marker complementary to *ade6*-M210 heteroallele on chromosome III. Additionally, *MATa* sequence adjacent to G418 resistance marker (*kanMX6*) is integrated in the *rad21*$^+$ gene 28kb distal from *ade6* gene. *MATa* is the *S. cerevisiae* HO-endonuclease target site which allows the generation of a

DSB within the minichromosome. The DSB is produced upon expression of HO endonuclease from plasmid pREP81X-HO in the absence of the repressor thiamine. Different phenotypes are predicted from DSB repair by HR, NHEJ, long-tract gene conversion and non-repair. HR would result in *ade*$^{+}$, G418^{S} phenotype, NHEJ in *ade*$^{+}$, G418^{R}, whereas *ade*$^{-}$, G418^{S} could occur due to long-tract gene conversion or loss of minichromosome as a consequence of failure to repair. Figure adapted from (Prudden et al. 2003).

3.4.2 Minichromosome loss is significantly increased in cells with elevated Cdc2 activity

To determine the percentage of cells which would lose spontaneously the non-essential Ch^{16} minichromosome, *ade6*$^{+}$ G418^{R} colonies were grown in rich medium containing adenine until they reached stationary phase. Diluted aliquots of these stationary cultures were plated on rich medium plates lacking adenine to determine both the total cell number and the percentage of *ade6*$^{-}$ colonies. Such plates contain only a limited amount of adenine and *ade6*$^{-}$ colonies that have lost the minichromosome during growth become dark red due to lack of external adenine. Importantly, cells which would lose the minichromosome later once plated form colonies with white and red sectors and can therefore be distinguished from cells which lost the minichromosome during mitotic growth. Only the latter are counted to determine the loss frequency.

As shown in Figure 3-21 hyper-active Cdc2 causes an approximately 25-fold increase in spontaneous loss of the minichromosome from the wild type level of 0.23% to 5.95% in *cdc2.1w* cells. The percentages were calculated using 6 independent colonies for each strain. This increase is indicative of DNA replication stress and is consistent with the elevated rate of spontaneous gene conversion (Figure 3-18).

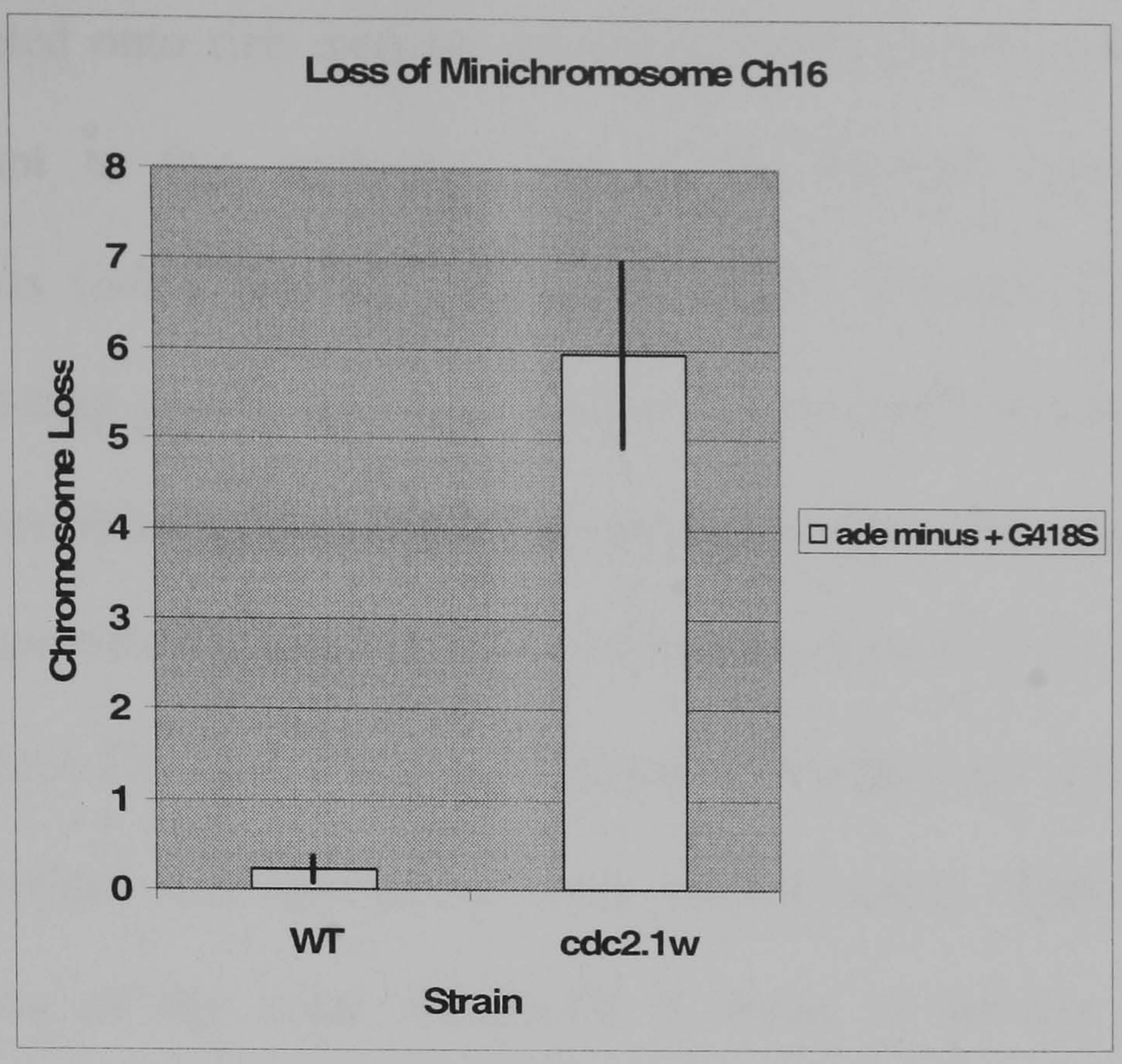


Figure 3-21. Loss of the non-essential minichromosome Ch[16].

Elevated Cdc2 activity causes an approximately 25-fold increased in spontaneous minichromosome loss from the wild type level of 0.23% to 5.95% in *cdc2.1w* cells. For each strain 6 independent colonies were scored. An independent samples t-test was conducted to determine whether statistically significant differences existed between wild type and *cdc2.1w* strains in terms of percentage of loss of the minichromosome Ch[16]. The difference between the two strains was found to be highly significant at the $p < 0.05$ level ($p = 0.002$). The error bars indicate standard errors of the mean.

3.4.3 Elevated Cdc2 activity suppresses short-tract gene conversion

Since elevated Cdc2 activity increases the rate of spontaneous inter-sister gene conversion, the HO-inducible mini-chromosome system was used to test whether elevated Cdc2 activity also affects inter-chromosome conversion events. Single *ade6*$^{+}$ G418^{R} colonies were grown for 48 hours in synthetic minimal medium with or without thiamine to either suppress or induce expression of HO-endonuclease. Diluted aliquots of these cultures were plated on rich medium plates lacking adenine to determine both the total cell number and the percentage of *ade6*$^{-}$ colonies. Subsequently, the colonies

were replica-plated onto rich medium containing G418 to determine the number of colonies resistant to this antibiotic. Due to the elevated spontaneous rate of chromosome loss (*ade6*$^{-}$ G418^{S}), only *ade6*$^{+}$ G418^{S} colonies which would have undergone short-tract gene conversion between the two *rad21* alleles were scored. In contrast to spontaneous inter-sister conversion events, the number of inter-chromosome conversions is approximately 20-fold lower in *cdc2.1w* cells compared to wild type (Figure 3-22). This difference indicates that high Cdc2 activity has opposite effects on inter-sister and inter-chromosome recombination. While Cdc2 seems to promote invasion of the sister chromatid, it seems to prevent invasion of the homologue chromosome.

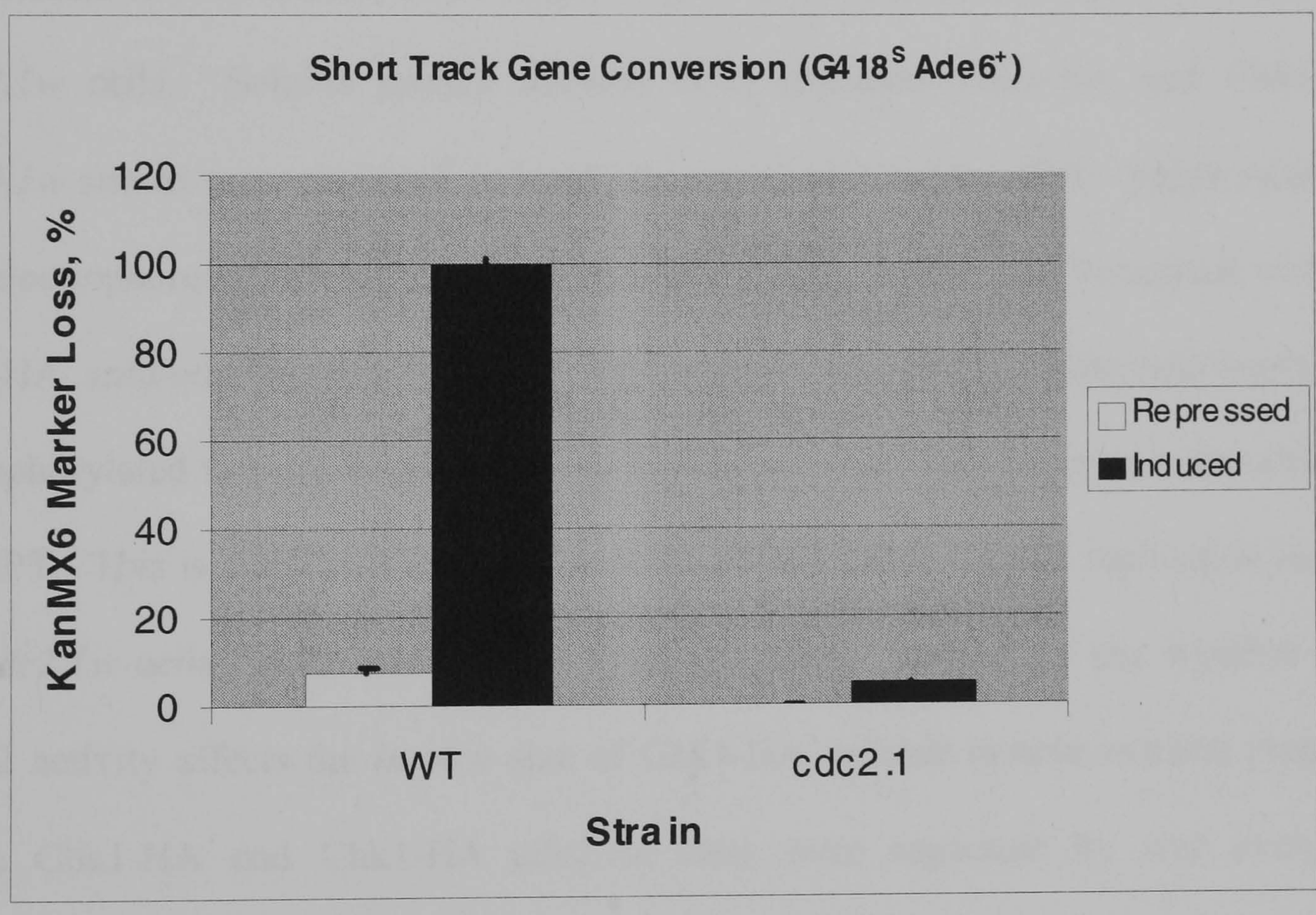


Figure 3-22. Short-tract gene conversions in wild type and *cdc2.1w* mutant cells.

Elevated *in vivo* Cdc2 activity prevents invasion of the homologue chromosome by suppressing short-tract gene conversion by approximately 20-fold comparing to wild type cells. The error bars indicate standard errors of the mean.

3.5 *S. pombe* Chk1 checkpoint kinase is constitutively activated in mutants with elevated Cdc2 activity

3.5.1 Cdc2 and Chk1 show physical interaction

To investigate the causes of DNA replication stress in *cdc2.1w* cells further it was tested whether high Cdc2 activity results in phosphorylation of Chk1 kinase. *S. pombe* Chk1 kinase becomes phosphorylated in a Rad3-dependent manner when DNA replication is challenged by CPT (Collura et al. 2005). Since this phosphorylation can be detected as a band shift, a HA-tagged *chk1* allele was introduced into wild type and *cdc2.1w* cells. Soluble protein extracts from untreated Chk1-HA and Chk1-HA *cdc2.1w* strains were prepared and proteins were separated by SDS - polyacrylamide gel electrophoresis followed by Western blotting. Chk1 kinase was visualised with the anti-HA antibody (Figure 3-23, A). This analysis revealed an additional band, the phosphorylated form of Chk1 in cells with elevated Cdc2 activity even in the absence of CPT. This is consistent with the presence of spontaneous DNA replication lesions in *cdc2.1w* cells, which activate the Rad3 checkpoint kinase. To test whether high Cdc2 activity affects the *in vivo* size of Chk1-HA, soluble protein extracts prepared from Chk1-HA and Chk1-HA *cdc2.1w* cells were separated by size exclusion chromatography on a Superdex-200 gel filtration column. The majority of Chk1-HA peaks in fractions 9-11 corresponding to an apparent molecular weight of 400-700kDa showing that Chk1 (56.5 kDa) assembles in larger protein complexes. This fractionation pattern of Chk1-HA is not affected by high Cdc2 activity, but the slower migrating, phosphorylated form was detectable in fractions 11-24 (Figure 3-23, B).

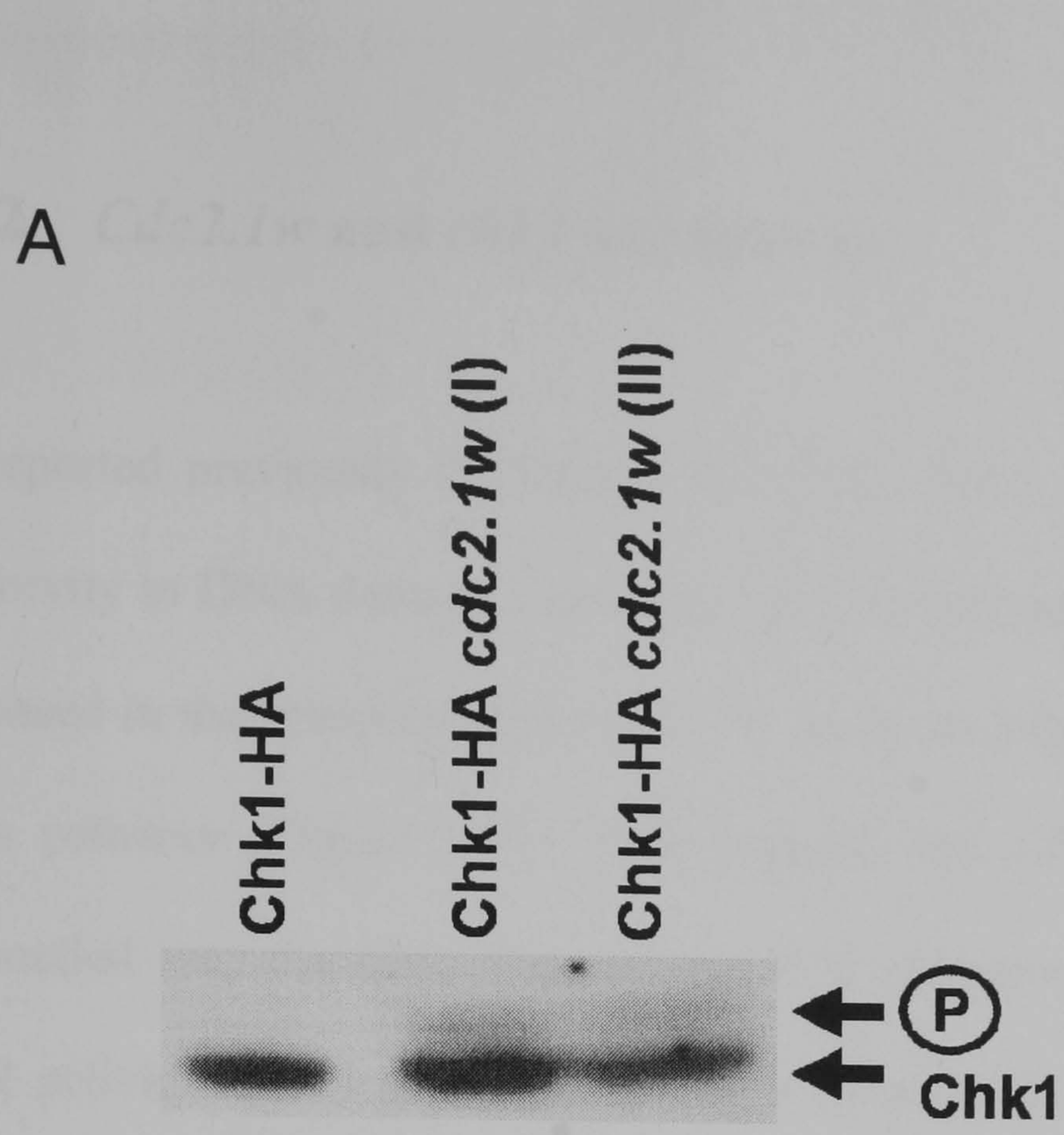


Figure 3-23. Chk1 kinase is constitutively activated in *S. pombe cdc2.1w* mutant.

A) Soluble protein extracts from wild type strain with HA-tagged Chk1 kinase and from *cdc2.1w* Chk1-HA cells, were separated by SDS – PAGE and Chk1-HA was visualised by Western blotting using anti-HA antibody. Cells with *in vivo* elevated Cdc2 activity show additional, phosphorylated form of Chk1 kinase (indicated by "P"), which suggests the presence of spontaneous DNA replication lesions in *cdc2.1w* cells. Two independent strains with high Cdc2 activity were analysed: *cdc2.1w (I)* and *cdc2.1w (II)*. **B)** Soluble protein extracts from Chk1-HA and *cdc2.1w* Chk1-HA strains were separated by protein size on a Superdex-200 gel filtration column. Fractions 8 till 17 of each strain were then further separated by electrophoresis on a 10 % SDS -polyacrylamide gel followed by Western blotting and probed with anti-HA antibody. The majority of Chk1-HA assembles in larger protein complexes in fractions 9-11 and the slower migrating, phosphorylated form was detectable in fractions 11-24.

3.5.2 *Cdc2.1w* and *chk1* are epistatic

As reported previously (Collura et al. 2005), *Chk1* deleted cells show an increased sensitivity to DNA damage caused by CPT. Interestingly this sensitivity is not further increased in the presence of the *cdc2.1w* allele showing that Cdc2 and Chk1 act in the same pathway (Figure 3-24). This suggests that CPT-dependent DNA lesions are channelled into the Chk1-dependent G2-M checkpoint in the presence of elevated Cdc2 activity. This genetic interaction is reflected by a physical interaction between Chk1 and Cdc2 as shown in chapter 3.5.1.

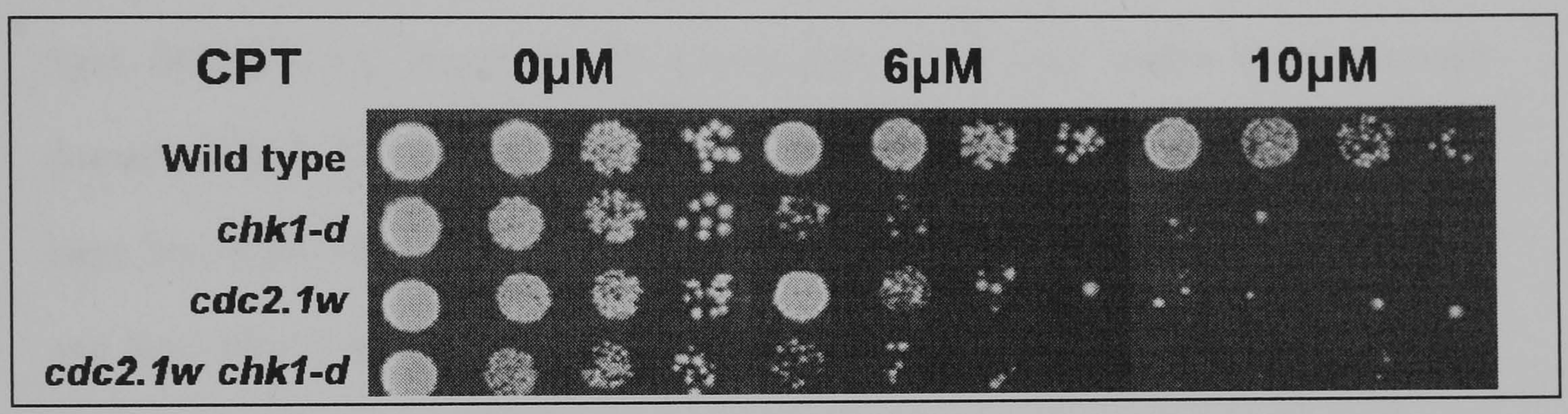


Figure 3-24. *Cdc2.1w* and *chk1* are epistatic.

Drop test analyses of indicated strains were carried out on YEA agar plates with different CPT concentration at 30°C. Cells lacking Chk1 kinase and carrying the *cdc2.1w* point mutation are not more sensitive to CPT than either single mutant.

4 Cdc2 regulates the anti-recombinogenic activity of Srs2 DNA helicase

The genetic evidence presented in chapter 3.1.2 suggests that Cdc2 regulates Mus81, Rad16 and Srs2 in the absence of Tdp1 to coordinate the Chk1-dependent response to CPT damage in G2. This chapter investigates the role of Cdc2 in the regulation of Srs2 DNA helicase. *S. pombe* Srs2 helicase consists of a N-terminal ATP Binding Domain followed by the Helicase Domain and a C-terminal domain of unknown function. Work in *S. cerevisiae* shows that Cdc28 ($Cdc2^{Sp}$) regulates the association of Srs2 with Sgs1 ($Rqh1^{Sp}$) and Mre11 by phosphorylating seven sites within the C-terminal domain of Srs2. These modifications are important to promote re-organisation of this large Srs2-Sgs1-Mre11 complex into two smaller complexes consisting of Srs2-Mre11 and Sgs1-Mre11 in response to DNA methylation by MMS (Chiolo et al. 2005). In contrast to *S. cerevisiae* Srs2, *S. pombe* Srs2 has only one potential Cdc2 phosphorylation motif within its ATP binding domain (serine 21) (Figure 4-1).

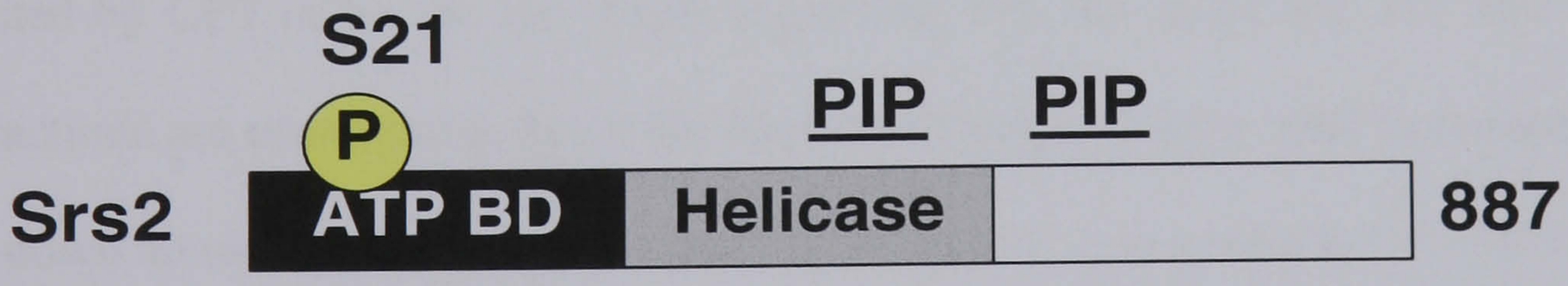


Figure 4-1. Domain organisation of Cdc2 and PCNA sites of *S. pombe* Srs2 DNA helicase.

S. pombe Srs2 DNA helicase possesses one potential Cdc2 phosphorylation site and two PCNA interaction domains (PIP boxes).

4.1 Srs2 interacts with Cdc2 and PCNA

PCNA plays an important part in the recombination avoidance pathways. In *S. cerevisiae* ubiquitinylation of PCNA initiates either translesion synthesis or template exchange depending on the number of ubiquitin molecules linked to lysine 164. During unperturbed S phase the same residue can also be attached to SUMO, which recruits Srs2 DNA helicase to stalled replication forks (Hoege et al. 2002). This SUMO-ylation has neither been found in *S. pombe* nor in human cells (Frampton et al. 2006). To test whether *S. pombe* Srs2 interacts with Cdc2 and/or PCNA, a C-terminally MYC-tagged Srs2 protein and commercial anti-Cdc2 and anti-PCNA antibodies were utilised for co-immunoprecipitation experiments from soluble protein extracts prepared from wild type cells. Both the anti-Cdc2 and the anti-PCNA antibody pulled down a sub-fraction of soluble Srs2-MYC, strongly indicating that Cdc2 and PCNA associate with Srs2 helicase in undamaged cells (Figure 4-2). The experiment was repeated using protein extracts prepared from cells arrested with hydroxyurea in S phase and from cells treated with 40μM CPT for 4 hours. Neither interaction was affected by CPT or by the HU arrest suggesting that the Srs2-Cdc2 and Srs2-PCNA interactions are constitutive. Since the intensity of the Srs2-Myc band in the anti-Cdc2 pull-down shown in Figure 3-2 B appeared to decline in the presence of HU or CPT, the experiment was repeated. The repeat experiment confirmed the initial conclusion that both interactions are constitutive (not shown). The membranes were also probed with anti-Cdc2 and anti-PCNA antibodies but no PCNA was detected in the Cdc2-pull-down and no Cdc2 was detected in the PCNA-pull-down, indicating that the Srs2-Cdc2 and Srs2-PCNA complexes are distinct.

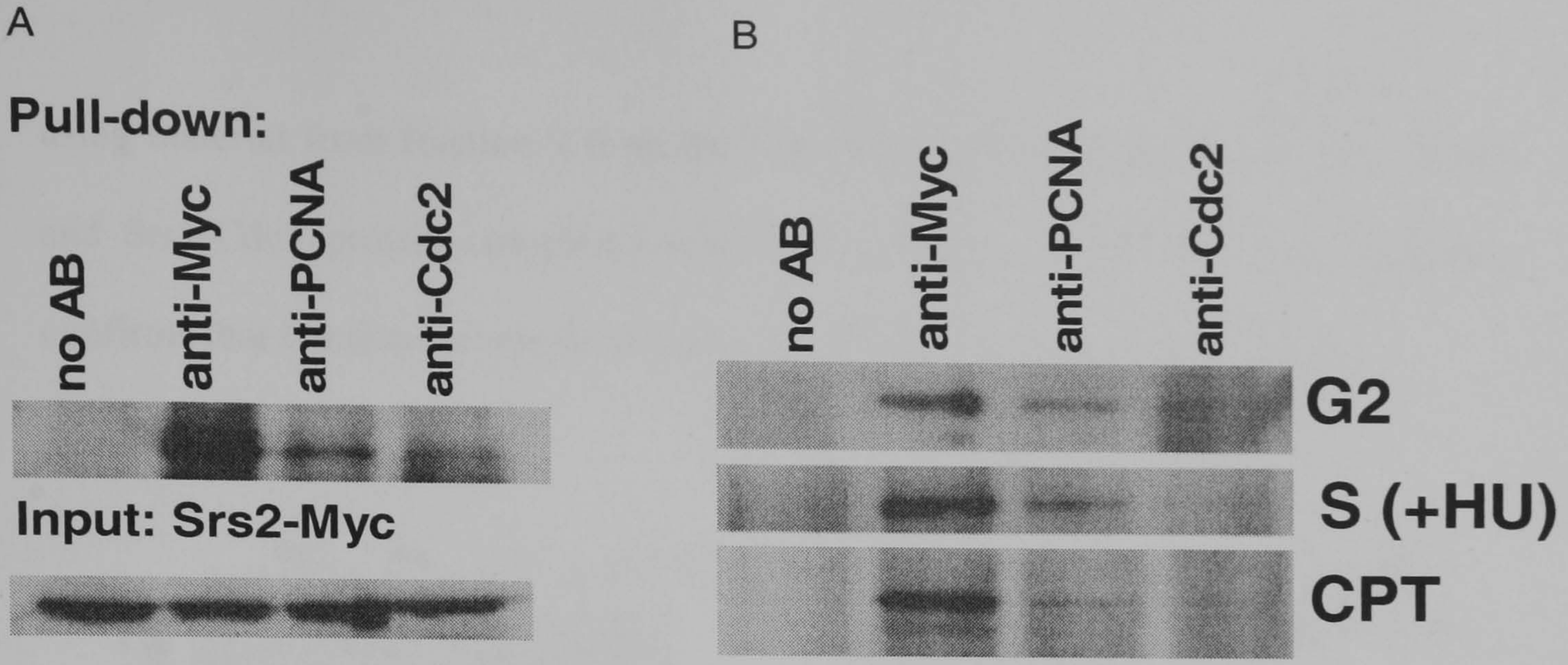


Figure 4-2. Srs2 co-immunoprecipitate with Cdc2 and PCNA from soluble protein extracts.

A) Srs2-Myc was precipitated from total soluble extracts using the indicated antibodies and the isolated material was probed for Srs2-Myc, Cdc2 and PCNA. **B)** Repeats of this experiment using protein extracts prepared from asynchronous (mainly G2) cells, cells arrested with hydroxyurea (S phase; 12mM for 3 hours) and cells treated with 40 μM camptothecin (CPT) for 4 hours.

To further establish whether Srs2 associates with Cdc2 and PCNA, a combination of size exclusion chromatography and co-immunopreciptation was used. Soluble protein extracts were prepared from untreated, asynchronous (G2) cells expressing either Srs2-HA, Srs2-Myc or Rqh1-Myc. Extracts were then separated by size on a Superdex-200 gel filtration column and analysed by Western blotting. Cdc2 was detected with an anti-Cdc2 antibody and PCNA was visualised with anti-PCNA antibody in the Srs2-HA samples (Figure 4-3, A). PCNA peaked in fraction 9 and fractions 13-16 and modified species were detected mainly in fraction 14. Cdc2 peaked in fraction 9 and fraction 13, whereas Srs2-Myc/HA and Rqh1-Myc were mainly present in fractions 8 and 9. The latter two fractions contain all protein complexes which are larger than the exclusion size of 700kDa of the Superdex-200 column. Hence co-fractionation of proteins in these two fractions could either indicate a direct interaction or the fact that both protein are simply too large to be separated. To distinguish between these two possibilities, the co-immunoprecipitation experiment (Figure 4-2, A) was repeated

using material from fraction 9 from the Srs2-Myc strain, and again both Srs2-PCNA and Srs2-Cdc2 protein complexes were detected (Figure 4-3, B). This experiment confirms that fraction 9 contains at least two different Srs2 protein complexes.

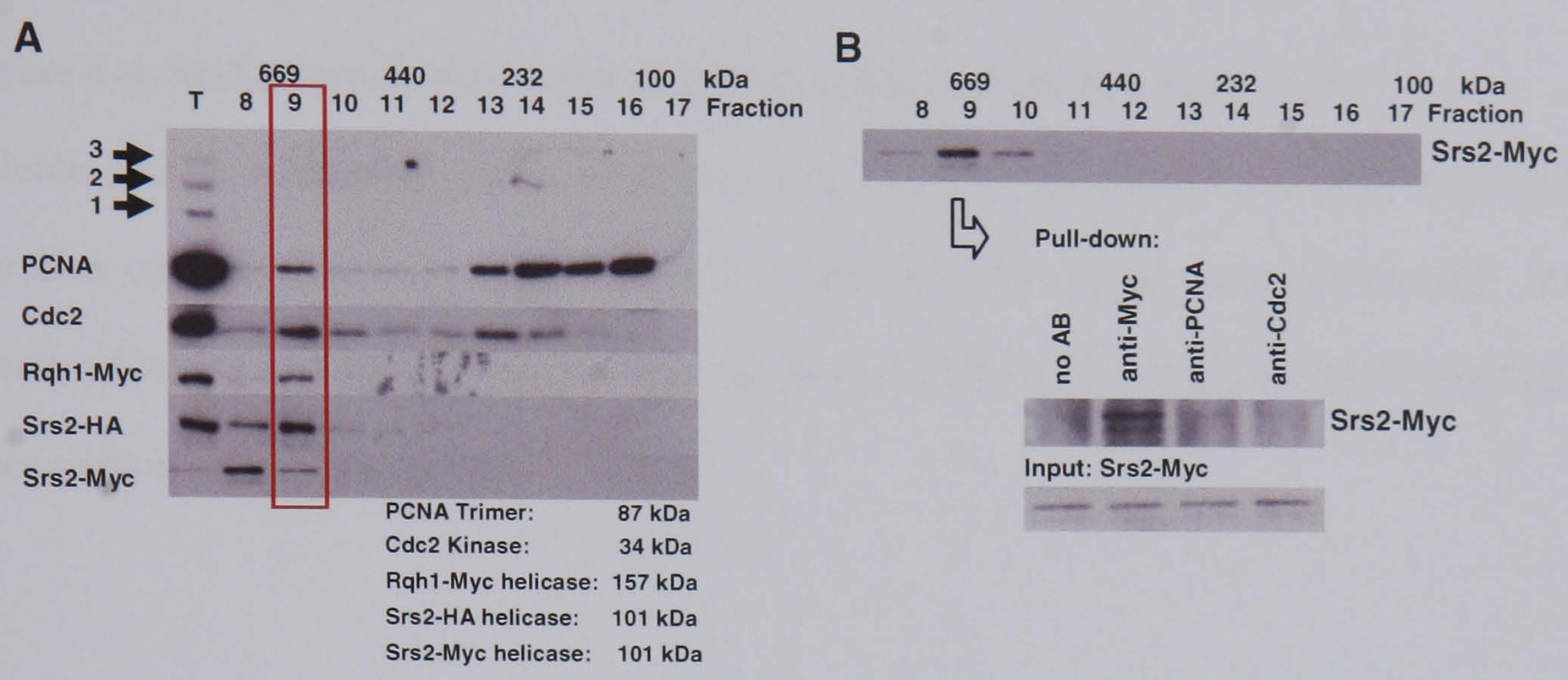


Figure 4-3. Srs2 precipitates with Cdc2 and PCNA after size exclusion chromatography.

A) Soluble protein extracts were separated by size (Superdex-200) and the indicated fractions were analysed for PCNA, Cdc2, Srs2-HA, Srs2-Myc and Rqh1-Myc. The arrows 1-3 indicate different modified forms of PCNA. **B)** The immunoprecipitation experiment was carried out using material from fraction 9 from the Srs2-Myc strain, and both Srs2-PCNA and Srs2-Cdc2 protein complexes were detected.

To test whether elevated Cdc2 activity or loss of Rqh1 DNA helicase affects the fractionation pattern of Srs2-Myc, soluble extracts from *srs2-Myc*, *srs2-Myc cdc2.1w* and *srs2-Myc rqh1-d* cells were subjected to size fractionation (Figure 4-4). High Cdc2 activity as well as deletion of *rqh1* affected the fractionation pattern of Srs2-Myc such that more protein was detected in fraction 8 as well as in fractions 11-13. This observation indicates that Srs2 interacts with Rqh1 and that Cdc2 activity affects this interaction. This conclusion would be consistent with the Cdc28 dependent changes in the interaction between Srs2 and Sgs1 ($Rqh1^{Sp}$) in *S. cerevisiae* (Chiolo et al. 2005).

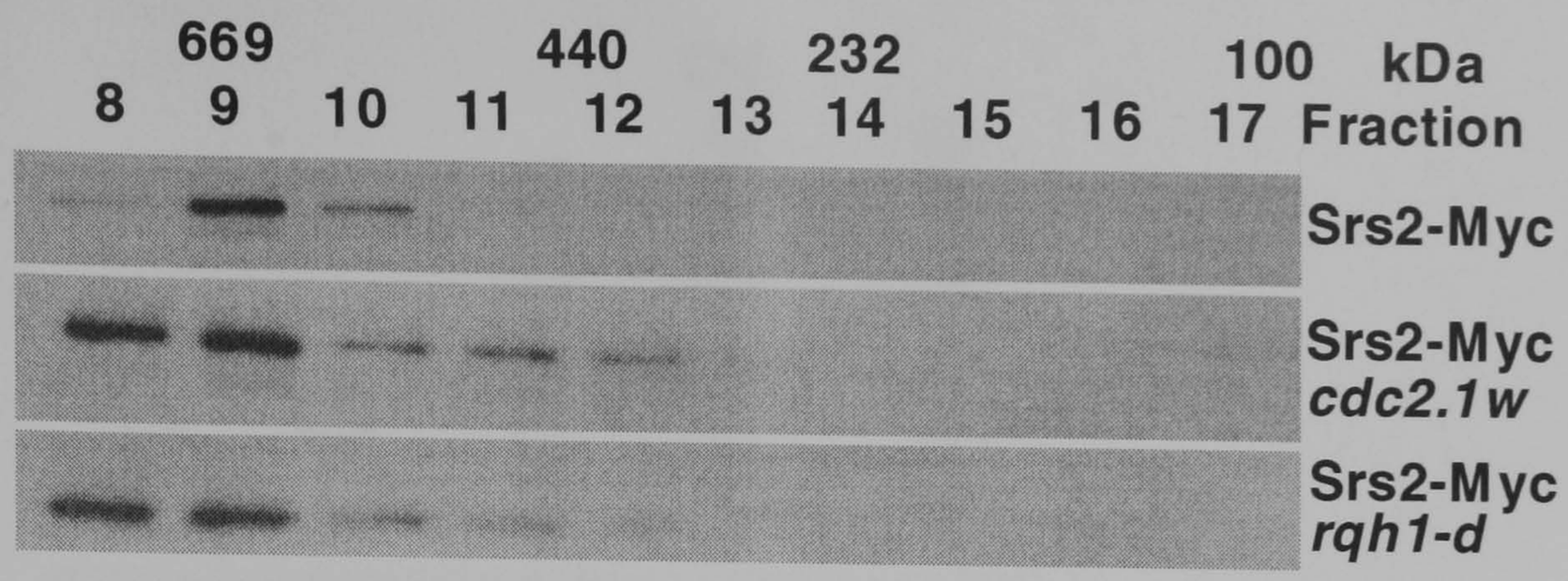


Figure 4-4. Srs2 interacts with Rqh1 and Cdc2 activity affects this interaction.

Soluble protein extracts of indicated strains were separated by protein size on a Superdex-200 gel filtration column. Fractions 8 to 17 of each strain were then further analysed by SDS -PAGE and Western blotting and probed with anti-Myc antibody. *Cdc2.1w* as well as deletion of *rqh1* affected the fractionation pattern of Srs2-Myc.

4.2 Cdc2 interacts with Chk1 but Chk1 does not interact with Srs2 helicase

Given the epistatic relationship between *cdc2.1w* and *chk1-d*, fractions 9-12 from a size fractionation experiment using protein isolated from untreated *srs2-Myc chk1-HA* cells were pooled and subjected to a pull-down experiment with anti-HA, anti-Cdc2 and anti-Myc antibodies (Figure 4-5). The aim of the experiment was to test whether Chk1-HA interacts with Srs2-Myc and/or Cdc2. As in the previous experiments (Figure 4-2; Figure 4-3), the anti-Cdc2 antibody enriched Srs2-Myc but also pulled down Chk1-HA. Since the anti-HA antibody failed to precipitate Srs2-Myc and because the anti-Myc antibody failed to precipitate Chk1-HA, Cdc2 interacts with both Srs2-Myc and Chk1-HA in two distinct protein complexes.

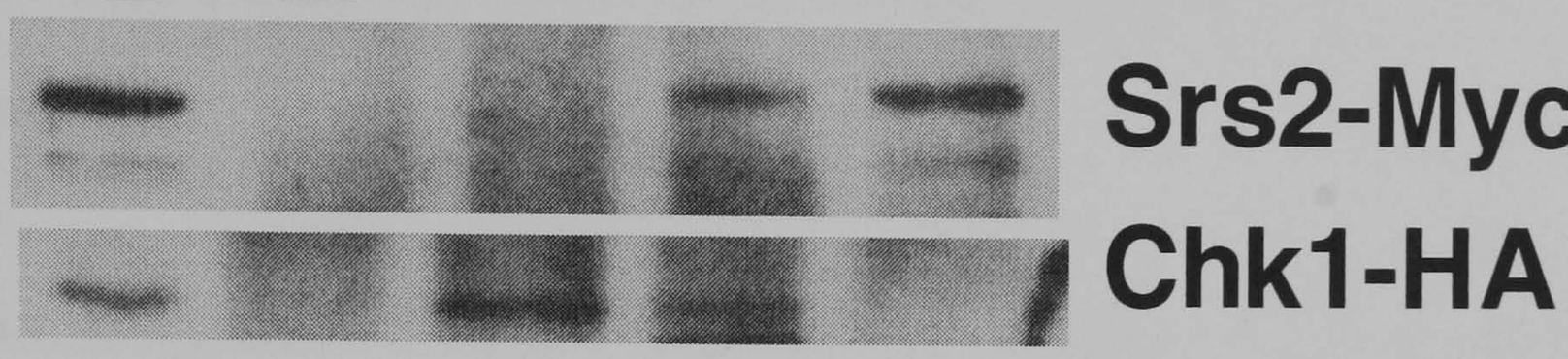


Figure 4-5. Cdc2 interacts with both Srs2-Myc and Chk1-HA in two distinct protein complexes.

Fractions 9-12 from a size fractionation experiment using protein isolated from untreated *srs2-Myc chk1-HA* cells were pooled and co-immunoprecipitation experiment with anti-HA, anti-Cdc2 and anti-Myc antibodies was carried out.

Taken together these biochemical experiments suggest the existence of at least three distinct protein complexes in soluble protein extracts of untreated cells: (i) a Srs2-Cdc2 complex, a Srs2-PCNA complex and a Chk1-Cdc2 complex.

4.3 The Cdc2-Srs2 interaction is important to prevent spontaneous DNA recombination

As shown in Figure 3-19, *cdc2.1w* cells show a 2-3 fold increase in the rate of spontaneous gene conversion events. Since a similar increase in gene conversion was reported for *S. pombe* cells lacking *srs2* (Doe and Whitby 2004), the spontaneous recombination frequencies were determined for a *cdc2.1w srs2-d* double mutant as well as for the corresponding single mutants and wild type cells (Table 4-1; Figure

4-6). Loss of Srs2 DNA helicase results in a 6-fold increase in conversion events, which are not further stimulated by the introduction of the *cdc2.1w* allele (*cdc2.1w srs2-d*). This suggests that both proteins act in the same pathway and that *in vivo* elevated Cdc2 activity may switch off the anti-recombination activity of Srs2 DNA helicase.

Table 4-1. Comparison of spontaneous recombination rates in *cdc2.1w, srs2-d* and *cdc2.1w srs2-d* mutant strains. In mutant strains with elevated Cdc2 activity (*cdc2.1w*) deletion rates are comparable to wild type, whereas conversion rates are 3.8-fold increased from the wild type level of $0.72\pm0.3 \times 10^{-4}$ to $2.66\pm1 \times 10^{-4}$. Cells lacking Srs2 helicase (*srs2-d*) have a 6-fold increase in the conversion rate ($4.45\pm1.5 \times 10^{-4}$) which is not further increased in the double mutant *cdc2.1w srs2-d* ($4.23\pm0.93 \times 10^{-4}$).

Strain	Deletion	Conversion	Del Error	Del Error	con error	con error
Wild type	0,66	0,72	0,2	0,2	0,3	0,3
cdc2.1w	0,62	2,66	0,33	0,33	1	1
srs2-d	0,46	4,45	0,12	0,12	1,5	1,5
srs2-d cdc2.1w	0,4	4,23	0,16	0,16	0,93	0,93

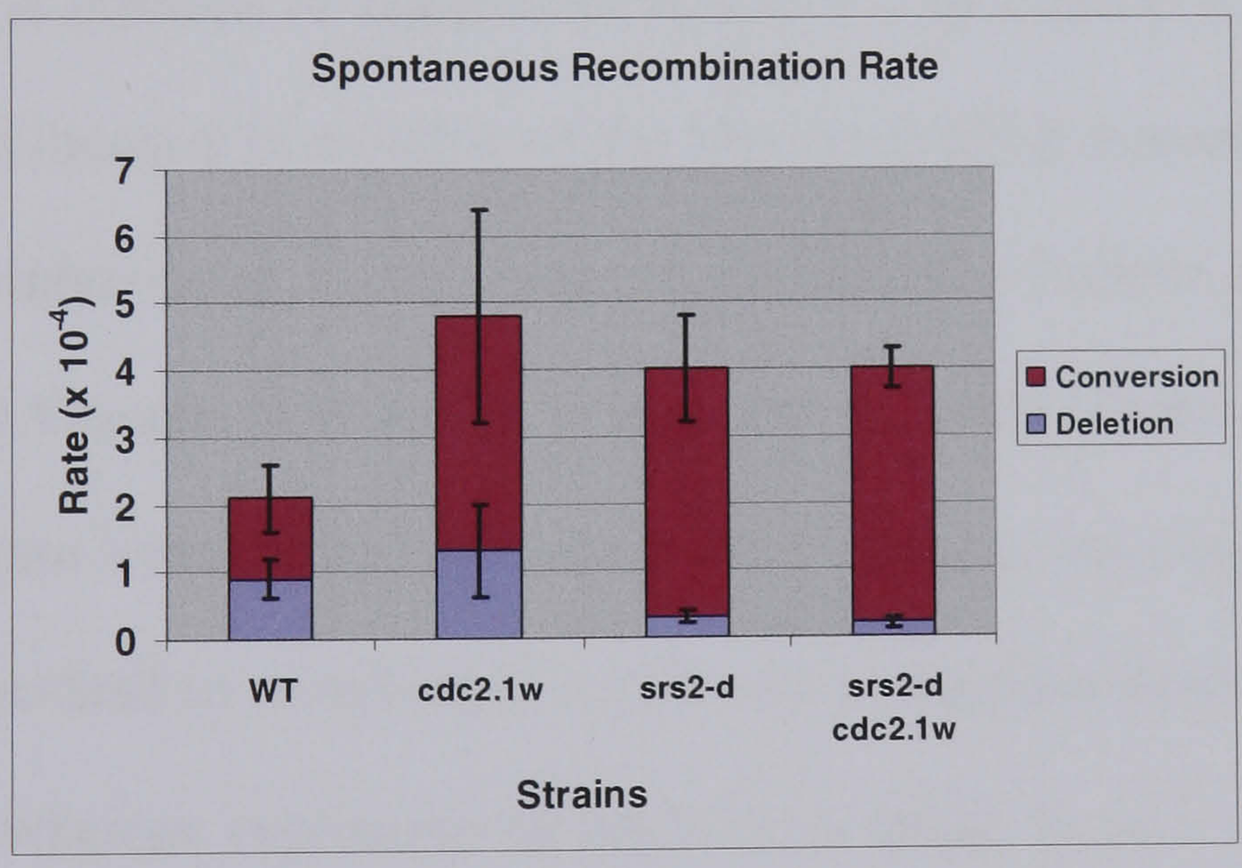


Figure 4-6. Loss of Srs2 helicase does not further increase the spontaneous recombination rate of *cdc2.1w* mutant cells.

Both deletion of Srs2 (*srs2-d*) and hyper-activation of Cdc2 (*cdc2.1w*) show a similar increase in spontaneous gene conversion, which is not further increased in the double mutant, suggesting that both proteins act in the same pathway. Recombination was measured with the spontaneous intra-chromosomal recombination assay (Figure 3-17). The error bars indicate standard errors of the mean.

4.4 Cdc2 may regulate Srs2 by phosphorylating serine 21

As shown in Figure 4-1, the potential Cdc2 phosphorylation site serine 21 was mutated in a plasmid encoding N-terminally Myc-tagged Srs2 under control of the thiamine-repressed *nmt41* promotor. Serine 21 was replaced by either an alanine residue or by a glutamate residue, which may mimic a negatively charged phosphate group. Expression of wild type Myc-Srs2, Myc-Srs2-S21A or Myc-Srs2-S21E in *srs2* deleted cells and in *cdc2.1w* in the presence of thiamine (the *nmt* promoter is leaky and permits a low basal expression level even in the presence of thiamine) revealed that an alanine in position 21 increases the electrophoretic mobility of Myc-Srs2-S21A compared to wild type Myc-Srs2, whereas a glutamate in this position leads to loss of the protein (Figure 4-7). Since small amounts of Myc-Srs2-S21E could be detected upon induction of the *nmt41* promotor in the absence of thiamine (not shown), it is very likely that a negative charge at position 21 either destabilises Srs2 or leads to a strongly increased degradation. The increase in mobility of the Myc-Srs2-S21A mutant may indicate that serine 21 is phosphorylated *in vivo*, since many phosphorylations are known to slow down movement through SDS PAGE (Caspari et al. 2000). Expression of Srs2 from the *nmt41* promotor without the Myc tag (as the N-terminal myc tag appears to impair the ability of Myc-Srs2 to complement *srs2-d* cells) complemented the CPT sensitivity of *srs2-d* cells, whereas expression of Srs2-S21A failed to do so (not shown). The latter observation indicates that Srs2-S21A is not able to fulfil the requirements of SRS2 in the presence of CPT.

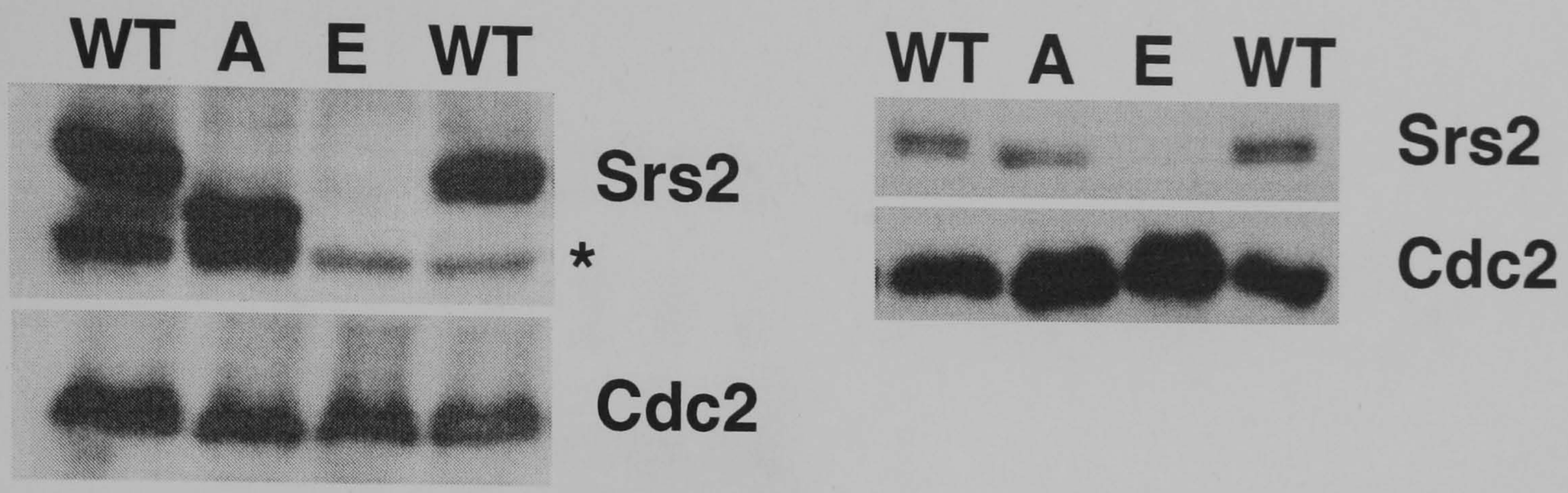


Figure 4-7. Plasmid expression of Srs2, Srs2-S21A and Srs2-S21E.

Plasmids encoding Myc-Srs2 (WT), Myc-Srs2-S21A (A) or Myc-Srs2-S21E (E) were transformed into **(A)** *srs2-d* and **(B)** *srs2-d cdc2.1w* cells and protein extracts were analysed using either the anti-Myc or the anti-Cdc2 antibody (* - unspecific band). S21A = no Cdc2 phosphorylation, S21E = mimicked phosphorylation. The S21A mutant is camptothecin sensitive.

4.5 Cdc2 interacts with PCNA

A bioinformatical analysis of *S. pombe* Cdc2 revealed the existence of a PCNA binding domain (PIP box) at the end of the C-terminal domain (Figure 4-8, B). The core element of a PIP box is the sequence QxxI/L/V followed by two hydrophobic amino acid residues which can be either F, Y, W or H (Moldovan et al. 2007) that in *S. pombe* Cdc2 is represented by **Q**NY**L**RD**FH*** (* indicates stop codon). An interaction between Cdc2 and PCNA is also supported by a previous report showing that human Cdc2 binds to PCNA (Zhang et al. 1993). To test whether *S. pombe* Cdc2 associates with PCNA, a soluble extract prepared from wild type cells was subjected to a pull-down experiment with antibodies against PCNA and Cdc2. The anti-Myc antibody was

used as a negative control. PCNA was enriched by the anti Cdc2 antibody suggesting that the PIP box in Cdc2 is functional (Figure 4-8, A).

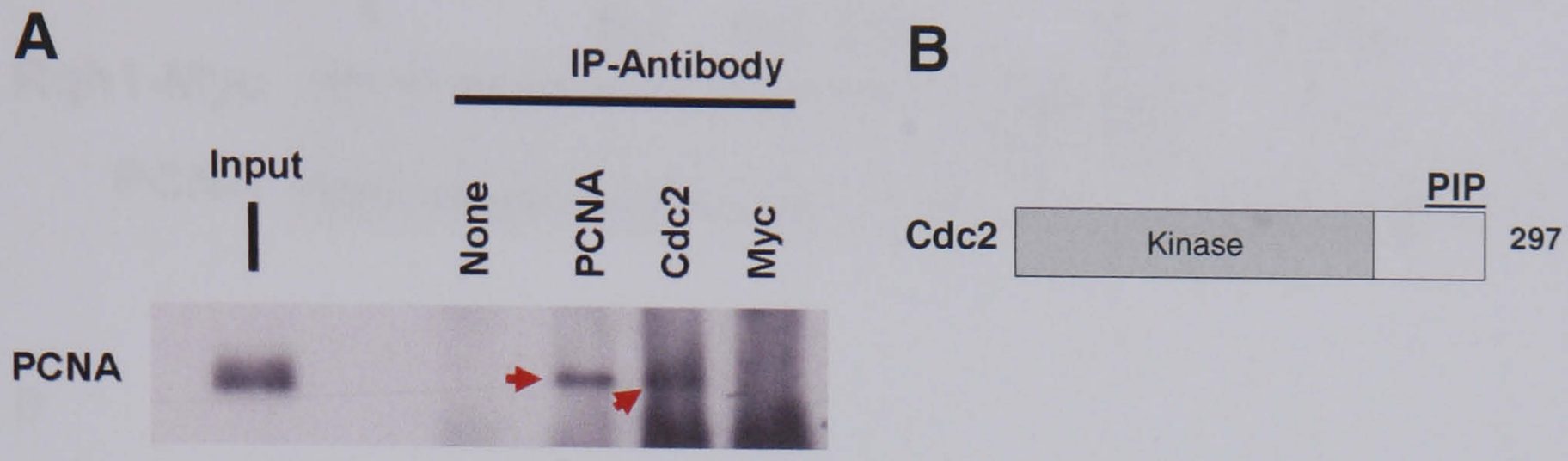


Figure 4-8. Cdc2 precipitate with PCNA from fraction 9 after size exclusion chromatography.

A) Soluble protein extract from wild type cells was run on a Superdex 200 gel filtration column and fraction 9 was used as input for co-immunoprecipitation analysis using indicated antibodies (anti-Myc was used as a control). PCNA was detected with anti-PCNA antibody. B) Schematic of localization of Cdc2 PIP box.

4.6 PCNA displays an interaction with Rqh1

Like Srs2 and Cdc2, Rqh1 DNA helicase contains a potential PCNA interaction domain (PIP box) in its C-terminal domain (Figure 4-9, B). To test whether Rqh1 interacts with PCNA, C-terminally Myc-tagged Rqh1 helicase was immuno-precipitated from soluble total cell extracts with both anti-PCNA and anti-Myc antibodies. As shown in Figure 4-9 A, the anti-myc antibody not only enriches Rqh1-Myc but also PCNA. However, the anti-PCNA antibody failed to pull-down detectable levels of Rqh1-MYC. This could indicate that the Rqh1-PCNA complex represents only a small fraction of the different PCNA protein complexes and that the anti-PCNA antibody concentration was not high enough to deplete all PCNA from the supernatant.

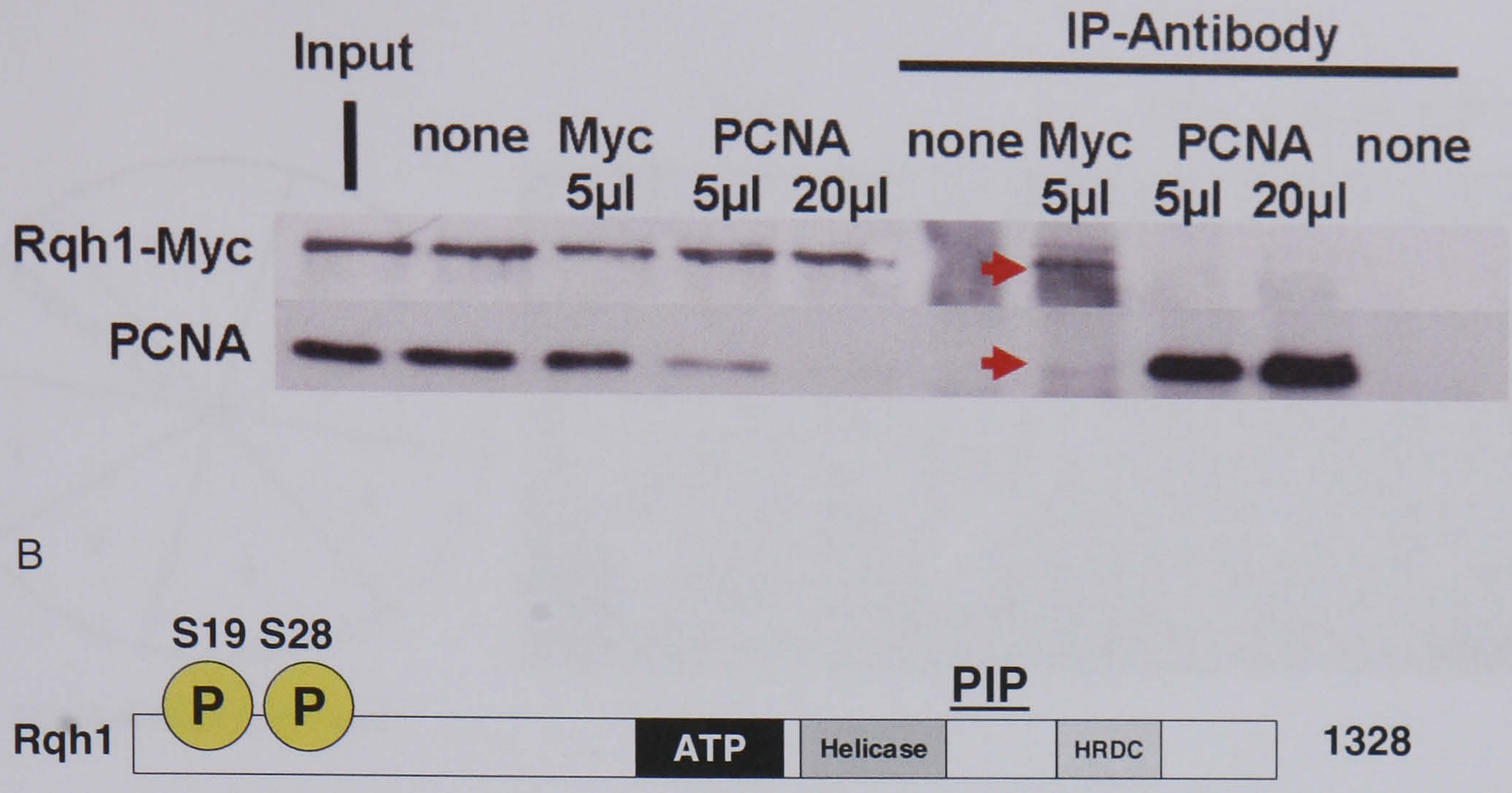


Figure 4-9. Biochemical interactions between PCNA and Rqh1 DNA helicase.

A) Co-immunoprecipitation analyses were carried out using soluble protein extract from Myc-tagged Rqh1 cells with either none, anti-MYC or anti-PCNA antibodies. Rqh1 was visualised with anti-MYC antibody and PCNA with anti-PCNA. **B)** Rqh1 possesses one PCNA interaction domain (PIP) and two potential Cdc2 phosphorylation sites, S19 and S28.

In response to a replication block caused by DNA damage, the *S. cerevisiae* PCNA lysine 164 is monoubiquitinated by Rad18-Rad6 which triggers error-prone translesion synthesis (Hoege et al. 2002). *PCNA-K164R* cells carry a mutation in which lysine 164 is mutated to arginine, and this modification results in the inactivation of TLS polymerases (Stelter and Ulrich 2003). Ubiquitinylation of lysine 164 is conserved in *S. pombe* (Frampton et al. 2006) and a *pcna-K164R rqh1-Myc* strain was constructed to test whether the inability to ubiquitinylate PCNA does interfere with the Rqh1-PCNA interaction. During strain construction, it was noticed that all *rqh1-Myc pcna-K164R* strains possess a unique sensitivity to CPT, which is not displayed by either single strain (Figure 4-10). This increase in CPT sensitivity indicates that the inability to ubiquitinylate PCNA affects either the interaction between PCNA and the C-

terminally tagged Rqh1 DNA helicase or that the tagged helicase has lost a function which becomes important for CPT resistance in the absence of PCNA ubiquitinylation.

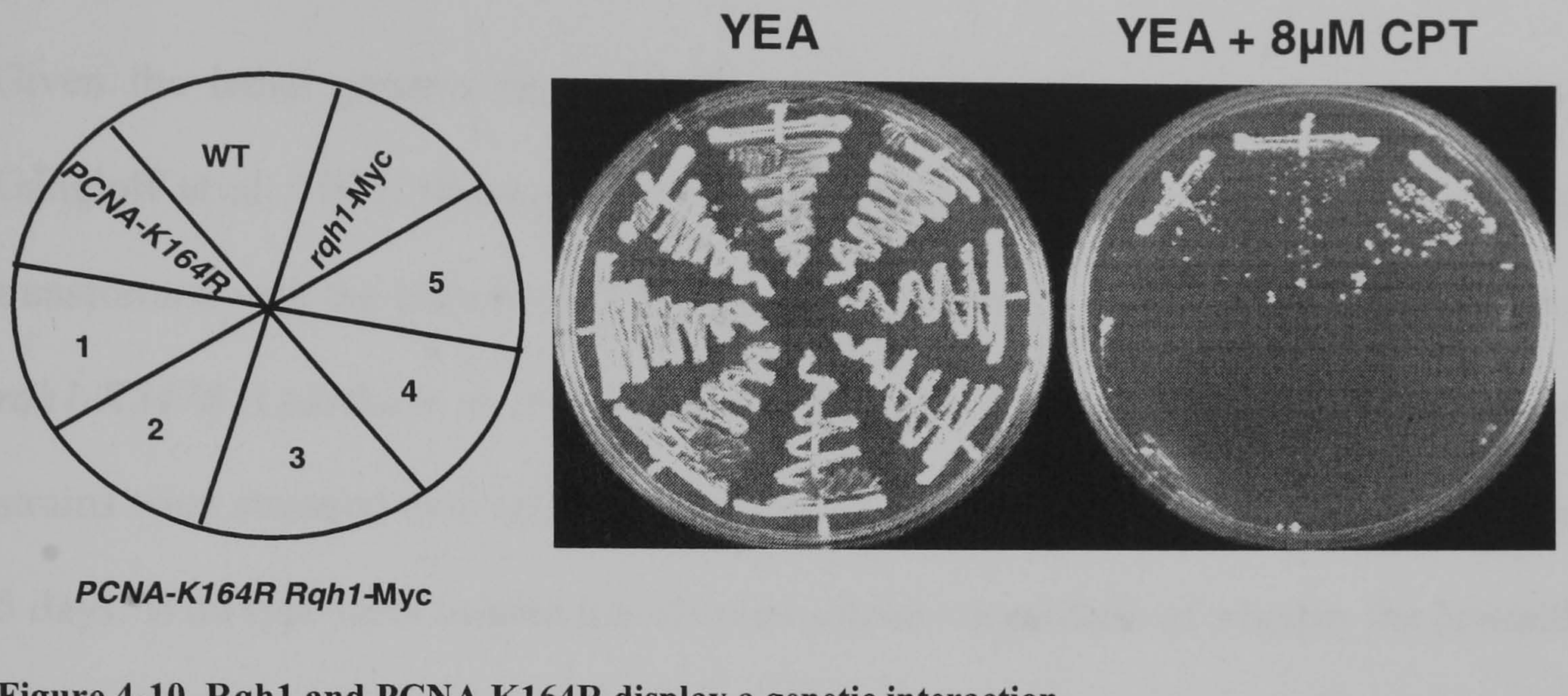


Figure 4-10. Rqh1 and PCNA K164R display a genetic interaction.

The *pcna-K164R* allele was crossed into the strain expressing Rqh1-Myc and 5 double mutant strains (indicated by 1 – 5) were grown on YEA agar plates for 3 days at 30°C and then replica plated onto YEA and YEA with 8 μM CPT followed by 3 day incubation at 30°C.

Table 4-2 summarises the interactions between different proteins identified in this study by means of co-immunoprecipitation.

Table 4-2. The summary of identified in this study different protein-protein interactions.

Interaction idetified by co-IP	Reference in text
Srs2 - Cdc2	Figure 4-2; 4-3 B
Srs2 - PCNA	Figure 4-2; 4-3 B
Cdc2 - Chk1	Figure 4-5
Cdc2 - PCNA	Figure 4-8 A
Rqh1 - PCNA	Figure 4-9 A

4.7 *Cdc2.1w* cells can not tolerate over-expression of Rqh1 DNA helicase.

Given the lethal genetic interaction between *srs2-d* and *rqh1-d* (Lee et al. 1999; Gangloff et al. 2000; Wang et al. 2001), wild type cells and *cdc2.1w* mutants were transformed with the plasmid pREP42 carrying either *rqh1* or a helicase-dead ATP site *rqh1-K547R* (Laursen et al. 2003). After transformation, wild type and *cdc2.1w* mutant strains were streaked onto selective minimal medium plates and incubated at 30°C for 5 days. Wild type cells formed transformed colonies regardless of whether the plasmid encoded wild type Rqh1 or its inactive mutant variant (the thiamine-repressed *nmt42* promotor permits a basal expression level on yeast nitrogen minimal medium). However, in the case of *cdc2.1w* cells, only the plasmid encoding inactive Rqh1 resulted in the formation of single colonies, strongly suggesting that even a moderate increase in Rqh1 DNA helicase activity is lethal in the presence of elevated Cdc2 activity (Figure 4-11).

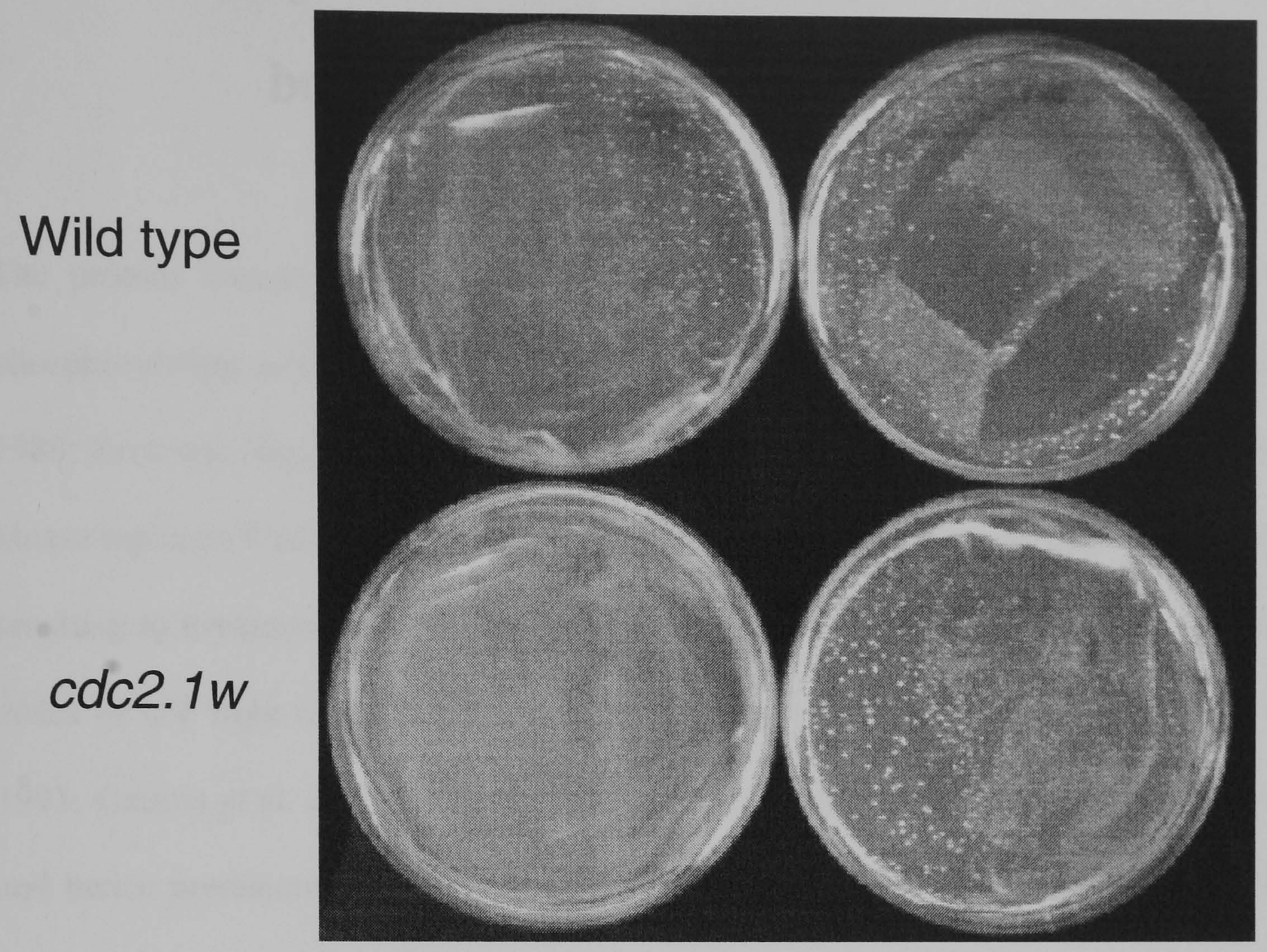


Figure 4-11. Transformation of wild type and *cdc2.1w* cells with pREP42-*rqh1* and pREP42-*rqh1-K547I.*

Cdc2.1w cells can not tolerate a moderate increase in Rqh1 DNA helicase (pREP42-*rqh1*). pRep42-*rqh1-K547I* – plasmid harbouring Rqh1 with inactive helicase activity; pREP42-*rqh1* plasmid with wild type Rqh1.

5 The *S. pombe* Cdc2 inhibitors Wee1 and Mik1 may be directly involved in DNA repair

The protein kinases Wee1 and Mik1 are both able to prevent Cdc2 activity by phosphorylating a conserved tyrosine residue, tyrosine 15 (Tyr 15) (Gould and Nurse 1989; Krek and Nigg 1991; Norbury et al. 1991). While Wee1 acts mainly in G2, Mik1 kinase replaces Wee1 during S phase (Figure 5-1). Loss of Wee1 activity renders cells sensitive to treatments that cause DNA DSBs such as ionising radiation (IR) and high doses of UV light (Alkhodairy and Carr 1992; Rowley et al. 1992; Barbet and Carr 1993; Garinis et al. 2005). Although Wee1 deletion allows premature Cdc2 activation and hence premature advance into mitosis (Gould and Nurse 1989), Barbet and Carr (Barbet and Carr 1993) clearly demonstrated that the IR sensitivity of *wee1-d* mutant cells does not originate from a shortened cell cycle arrest, which indicates that Wee1 kinase plays a role in DNA DSB repair, which may be distinct from its role in Cdc2 regulation. So far, no DNA repair function for Mik1 kinase has been reported. This chapter investigates possible roles of the main Cdc2 inhibitor Wee1 and the minor Cdc2 inhibitor Mik1 in DNA repair.

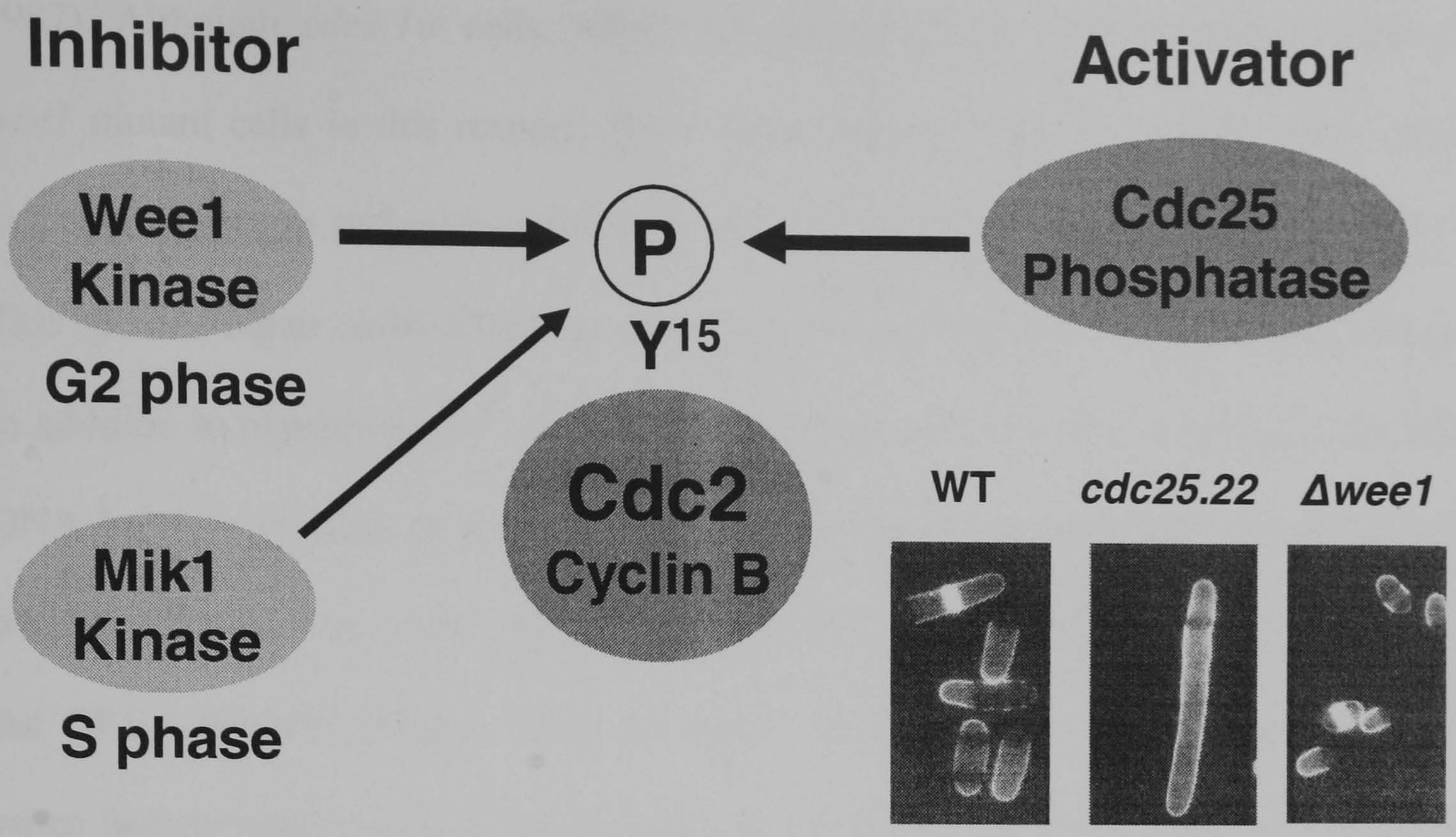


Figure 5-1. Regulation of *S. pombe* Cdc2

In fission yeast, entry into mitosis requires activation of cyclin-dependent kinase Cdc2-cyclin B. Wee1 protein kinase inhibits Cdc2 by phosphorylating a conserved tyrosine residue in the Cdc2 active site (Tyr 15). Cdc25 is a phosphatase that activates Cdc2 by removing the phosphate from the Tyr15. In addition to Wee1, fission yeast contains another, minor Cdc2 inhibitor, Mik1, which also phosphorylates the Tyr 15 residue.

5.1 The DNA repair function of *S. pombe* Wee1 is distinct from its role as a regulator of Cdc2-cyclin B

5.1.1 Wee1 might have a function in G1/G0 phase of the cell cycle in addition to its role in regulating Cdc2-cyclin B in G2.

The main consequence of loss of Wee1 activity is the increase in mitotic Cdc2 activity resulting in a shortened G2 phase and premature mitotic entry (Russell and Nurse

1987). Although *cdc2.1w* cells, which are insensitive to Wee1 inhibition, resemble *wee1* mutant cells in this respect, Wee1 inactivation results in a unique DNA repair defect in response to ionising radiation and UV irradiation (Barbet and Carr 1993). This specific repair defect indicates that Wee1 kinase performs a yet unknown function in addition to regulation of Cdc2. This DNA repair function may be linked with Rqh1 DNA helicase because loss of Wee1 suppresses the CPT sensitivity of *rqh1-d* cells, whereas introduction of the *cdc2.1w* allele in the same genetic background increases the CPT sensitivity (Figure 3-12, B). Since Wee1 may have an additional function when fission yeast cells exit the cell cycle in G1 in response to nitrogen starvation (Wu and Russell 1997), *wee1-d* wild type and *rad9-d* cells were synchronised in G1/G0 phase by transferring cells from minimal medium with ammonium sulphate into synthetic minimal medium without a nitrogen source (Fantes and Nurse 1977). After 24 hours in the absence of a nitrogen source, cells were plated on rich medium and exposed to a range of UV light between 0 and 150 J m^{-2}. After four days incubation at 30°C colonies were counted and percentage survival calculated for each strain (Figure 5-2, A). The percentages of G1/G0 and G2 cells were determined by FACS for both strains in the presence and absence of a nitrogen source. In presence of nitrogen, wild type cells spend around 70% of their cell cycle time in G2, while nitrogen starvation shifts more than 50% of cells into G1/G0. In contrast, *wee1-d* cells spend significantly longer in G1 in the presence of nitrogen to compensate for the reduction in G2. The proportion of G1 cells is further increased upon nitrogen starvation (Figure 5-2, B). Interestingly, nitrogen starvation significantly increases the UV sensitivity of *wee1-d* when compared to wild type cells. Since the amount of G1 cells is not so different in the presence and absence of nitrogen, the increased UV sensitivity may be a consequence of cell cycle exit from G1 into G0 rather than a mere reflection of G1 cells.

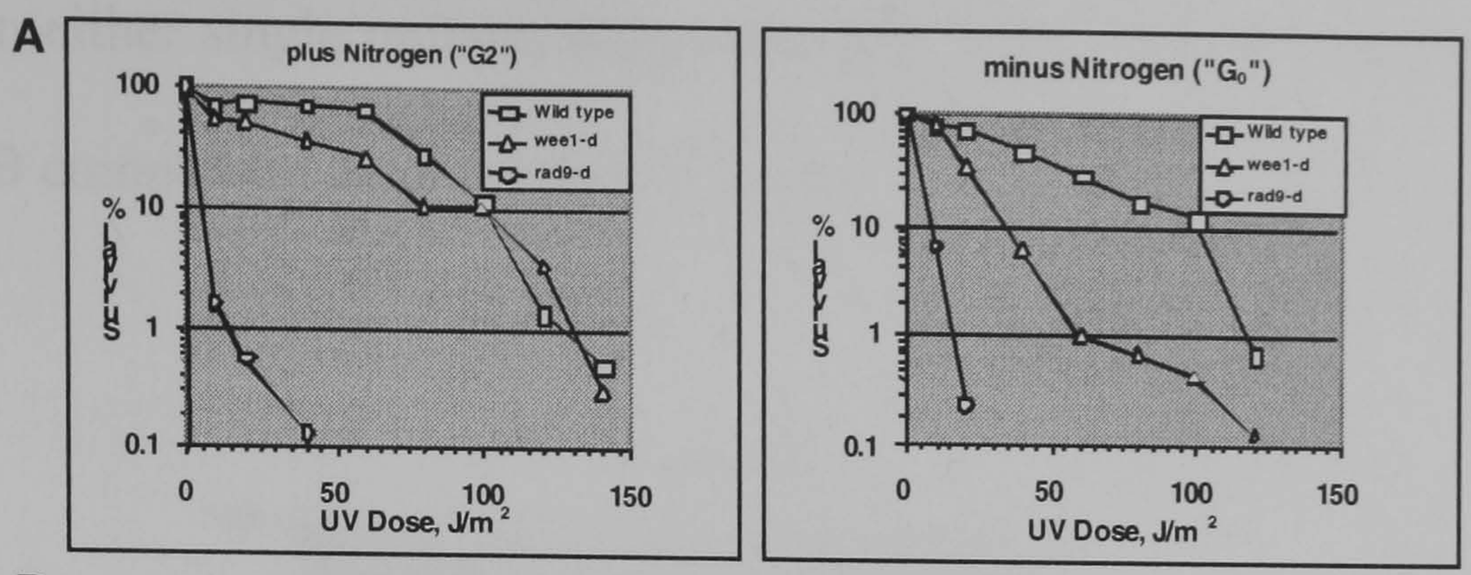


Figure 5-2. Cells lacking Wee1 kinase are more UV sensitive when starved for nitrogen.

A) Wild type cells and cell deleted for Wee1 kinase or for the checkpoint protein Rad9 were shifted for 24 hours from minimal medium with ammonium sulphate to minimal medium without a nitrogen source. The UV sensitivity of starved cells was compared between 0 and 150 J m^{-2} with cells which were grown for the same period of time in medium with nitrogen. **B)** Samples from all cultures were subjected to FACS analysis to determine the percentage of G1/G0 and G2 cells.

5.1.2 Wee1 and cyclin B are implicated in distinct UV DNA damage response pathways

If Wee1 and the Cdc2-cyclin B complex are involved in distinct DNA repair pathways, cells devoid of *wee1* and mutated in the *cdc13* gene encoding cyclin B will be expected to be more UV sensitive than either single mutant. The *cdc13-245* point mutation reduces Cdc2 activity in a temperature-dependent manner and renders cells sensitive to treatments that cause DSBs even at the permissive temperature (Caspari et al. 2002). As shown in Figure 5-3, a *wee1-d cdc13-245* mutant is indeed more UV

sensitive than either single mutant, suggesting that Wee1 can act independently of the Cdc2-cycin B complex in response to UV light.

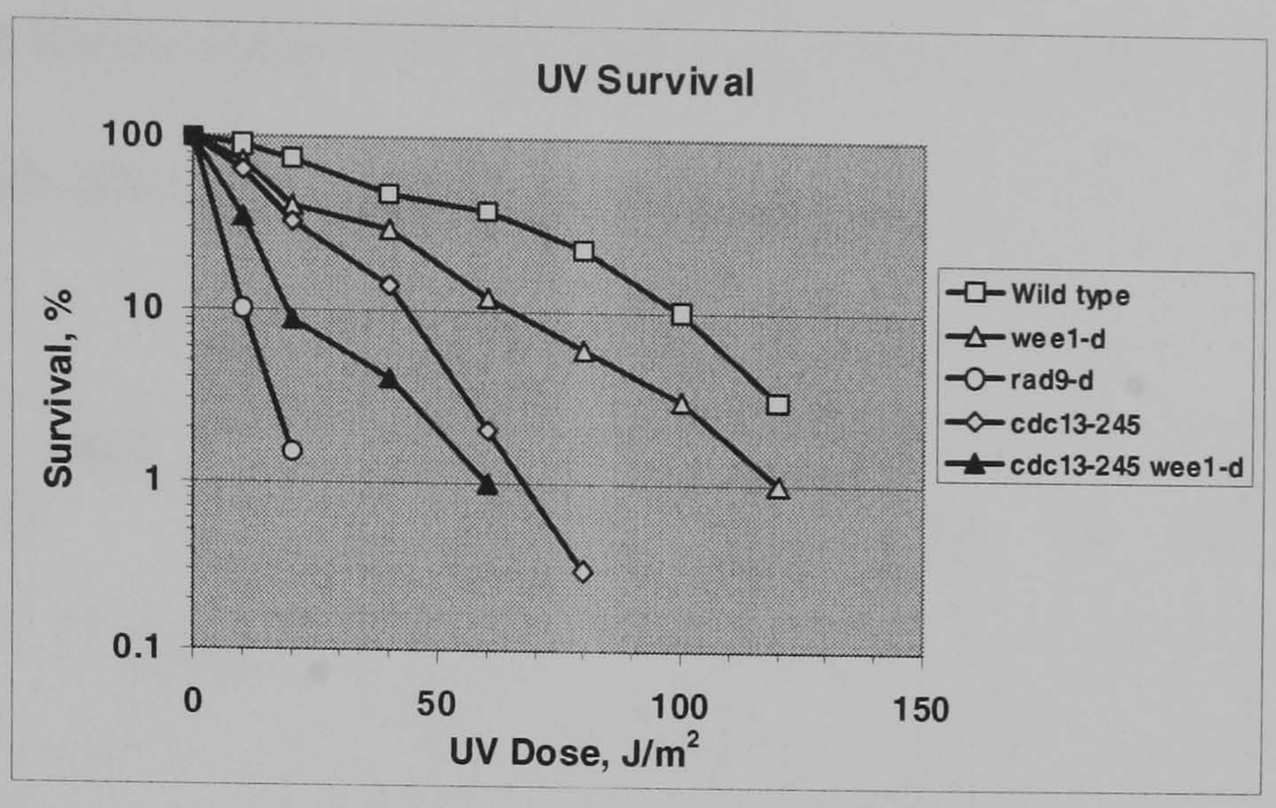


Figure 5-3. Wee1 and cyclin B are implicated in distinct UV DNA damage response pathways

The UV sensitivity of the indicated strains was tested by exposing approximately 500 cells on YEA plates to a UV dose between 0 and 120 J/m^2. The percentage of surviving cells was determined after 4 days at 30°C.

5.1.3 Cdc2 forms at least two distinct protein complexes

Since Wee1 may act independently of Cdc2-cyclin B, soluble protein extracts were prepared from *S. pombe* cells expressing N-terminally HA-tagged Wee1 kinase to analyse the size of protein complexes containing Wee1, Cdc2 and Cdc13. Soluble protein was separated by size on a Superdex 200 gel filtration column and the resulting fractions were probed with the anti-HA, anti-Cdc2 and anti-Cdc13 antibody. Consistent with the role of Wee1 as Cdc2-cyclin B regulator, all three proteins co-fractionated in fraction 9 which contains protein complexes larger than 650kDa (Figure 5-4). A second peak of both Cdc2 and Cdc13 was detected in fractions 15 and 16 which did not contain detectable HA-Wee1 suggesting that the soluble protein extract contains at least two Cdc2-Cdc13 protein complexes, a large complex

containing Wee1 and a smaller complex devoid of Wee1. The large complex may be functionally related to the cell cycle regulator complex previously identified in *S. cerevisiae* (Harvey et al. 2005). Since fraction 9 contains large protein complexes, which migrate in the exclusion volume of the gel filtration column, it may well be that Wee1 kinase binds also to other protein complexes.

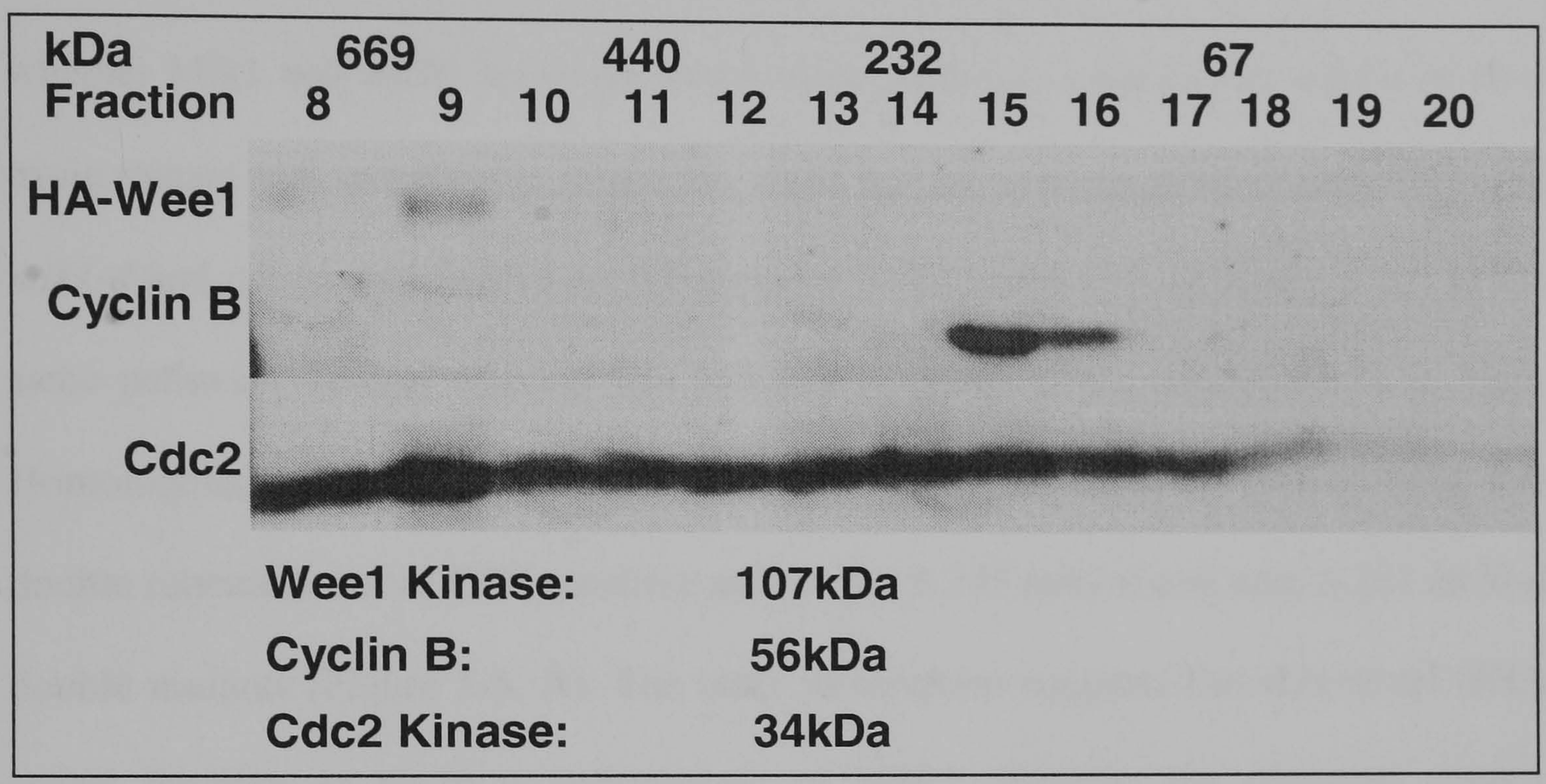


Figure 5-4. Size exclusion chromatography of Cdc2, Cdc13 cyclin and Wee1 kinase.

Cdc2 forms at least two distinct protein complexes with an apparent molecular weight of ≥650kDa and 150-200 kDa after gel filtration on a Superdex 200 column. Cdc2 was detected with an anti-Cdc2 antibody, Cdc13 with anti-cdc13 and Wee1 with an anti-HA antibody.

5.2 The Ku heterodimer but not Ligase IV may act in the same pathway as *wee* mutants.

To test the role of Mik1, which is required for the inhibition of Cdc2 specifically in S phase (Christensen et al. 2000), a *cdc13-245 mik1-d* double mutant was tested for its

sensitivity to camptothecin, the Topoisomerase poison acting in S phase (Horwitz and Horwitz 1973; Hsiang et al. 1989). Although cyclin B mutant cells are deficient in Rqh1-dependent homologous recombination (Caspari et al. 2002), *cdc13-245* cells are sensitive to CPT only at high concentrations (Figure 5-5, B). However, deletion of either *mik1* or *ku70* increases the CPT sensitivity of *cdc13-245* mutant cells (Figure 5-5, A) (deletion of *mik1* or *ku70* on its own do not cause CPT sensitivity). To test whether Mik1 and Ku70 act in the same repair pathway, a *cdc13-245 mik1-d ku70-d* triple mutant was constructed. Since this triple mutant is as sensitive as the *cdc13-245 mik1-d* and *cdc13-245 ku70-d* double mutants, Mik1 and Ku70 are likely to act in the same pathway. To test whether this pathway is linked to the role of Ku70 in Non-Homologous End-Joining, *ligase IV* was deleted in *cdc13-245* cells. Interestingly, this double mutant is not as CPT sensitive as the *cdc13-245 mik1-d* and *cdc13-245 ku70-d* double mutants (Figure 5-5, A). The latter observation suggests that this novel DNA repair function of Ku70 is independent of NHEJ. Whether the observed genetic interactions are directly dependent on Mik1 or whether loss of Mik1 activity indirectly increases Cdc2 activity is not yet clear.

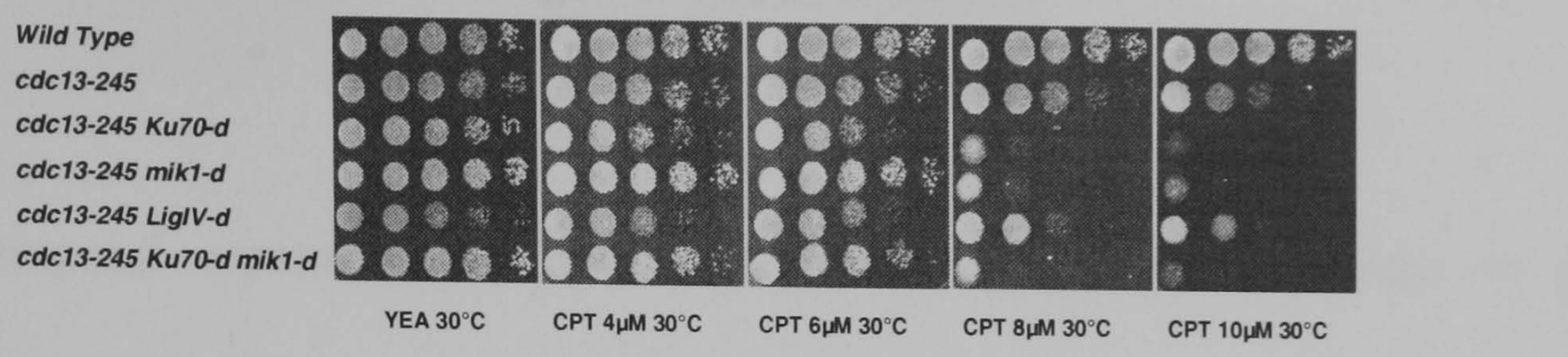


Figure 5-5. Loss of Mik1 and Ku70 specifically increases sensitivity to CPT.

Serial dilutions of indicated strains were spotted onto YEA media plates with increasing CPT concentrations. *Ku70-d,* m*ik1-d* and *ligIV-d* are not CPT sensitive under these conditions. **A)** A significant increase in CPT sensitivity was observed upon deletion of Mik1 kinase (*mik1-d)* and Ku70 (*Ku70-d*) in a strain in which HR was affected by a mutation in cyclin B (*mik1-d cdc13-245* and *Ku70-d cdc13-245*). **B)** Although *cdc13-245* mutant cells are impaired in HR, they are not very sensitive to CPT.

6 Discussion

The temporal separation of replication and recombination is crucial to prevent deleterious gross genomic rearrangements, which are frequently found in cancer cells. Eukaryotic cells postpone homologous recombination until G2 phase of cell cycle (Meister et al. 2005). Relevant to this work is the observation that camptothecin induced damage escapes the ATR (Rad3)-Chk1 (Cds1) intra-S checkpoint and is detected by the ATM (Rad3)-Chk2 (Chk1) G2-M checkpoint in G2 (Furuta et al. 2003; Pommier 2006; Takemura et al. 2006). PCNA and its covalent linkage to small protein modifiers plays an important role in the switch between anti- and pro- recombinogenic activities of DNA helicases such as SRS2 as well as in the use of Translesion DNA polymerases or template-switch mechanism. These cell cycle dependent changes in catalytic activities of DNA helicases and other repair factors are most likely to be dependent on the cell cycle regulators, the cyclin-dependent kinases. This PhD project proposes a role for the main *S. pombe* cell cycle regulator Cdc2 ($CDK1^{Hs}$, $Cdc28^{Sc}$) in the cell cycle-dependent coordination of DNA recombination in unperturbed cells as well as in response to CPT.

6.1 Summary of the main findings

The data reported in this thesis identify two novel functions for Cdc2 in the regulation of DNA repair.

During the unperturbed cell cycle, elevated Cdc2 activity (*cdc2.1w*) causes DNA replication problems, which lead to a 2-3 fold increase in spontaneous gene conversion events and the frequent loss of the non-essential mini-chromosome Ch^{16}-MG (Figure 3-18; Figure 3-21). Cdc2 physically interacts with Srs2 DNA helicase in undamaged cells (Figure 4-2; Figure 4-3) and this interaction is reflected in the similar increase in gene conversion events in *cdc2.1w*, *srs2-d* and *cdc2.1w srs2-d* cells (Figure 4-6). Since mutation of serine 21, the potential Cdc2 phosphorylation site in Srs2, changes the electrophoretic mobility of Srs2-S21A-Myc (Figure 4-7), it could be possible that Cdc2 phosphorylates Srs2 DNA helicase at serine 21 to prevent spontaneous gene conversion reactions in S phase. The nature of the DNA replication lesions caused by elevated Cdc2 activity is not yet known, but they seem to activate the Rad3-Chk1 DNA damage checkpoint pathway. This is indicated by the constitutive phosphorylation of Chk1 in undamaged *cdc2.1w* cells (Figure 3-23). An alternative explanation for this Chk1 phosphorylation is however suggested by the physical association between Chk1 and Cdc2 in unperturbed cells (Figure 4-5). Srs2 doesn't appear to be part of this complex (Figure 4-5) indicating that Cdc2 associates with DNA repair and DNA checkpoint proteins in distinct complexes. Whether the constitutive phosphorylation of Chk1 is a DNA damage checkpoint response or an indication of a direct modification by Cdc2 is not yet clear.

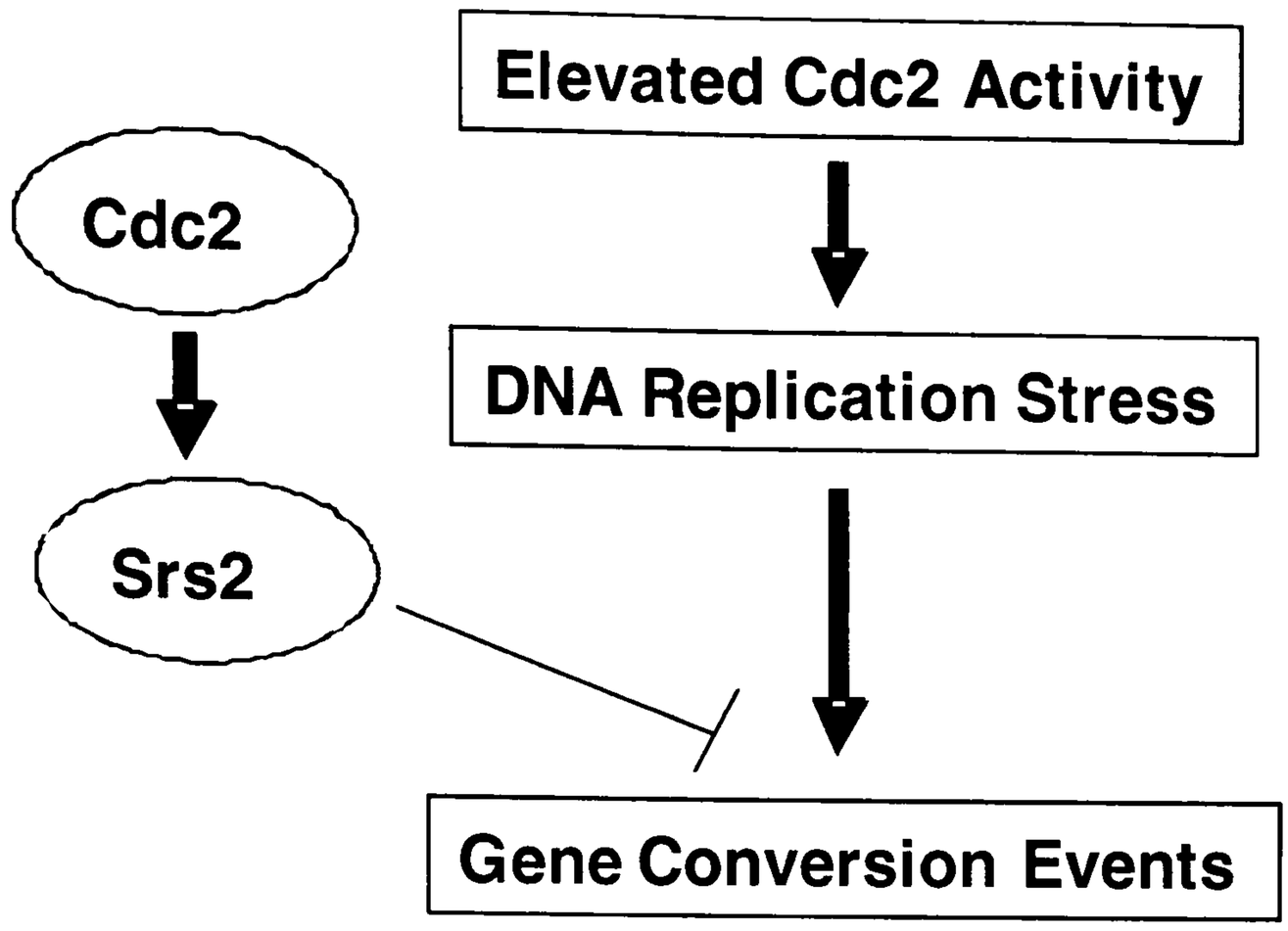


Figure 6-1. Model suggesting a role for the Cdc2 in the regulation of the anti-recombinogenic activity of Srs2 DNA helicase during unperturbed cell cycle.

In unperturbed cells elevated Cdc2 activity causes DNA replication stress, which leads to increased spontaneous gene conversion events. The model suggests that Cdc2 phosphorylates Srs2 DNA helicase to prevent spontaneous gene conversion in S phase. DNA replication lesions caused by elevated Cdc2 activity activate Chk1 kinase possibly *via* Rad3-dependent DNA damage checkpoint pathway or by direct modification by Cdc2.

The second novel requirement for Cdc2 is its specific involvement in the repair of camptothecin (CPT) induced DNA damage. CPT traps Topoisomerase I in the vicinity of the DNA replication fork in S phase leading to a broken chromosome upon collision of the fork complex with the immobilised enzyme. This DNA lesion is however not detected by the intra-S phase checkpoint but by the Chk1-dependent G2-M checkpoint (Furuta et al. 2003; Pommier 2006; Takemura et al. 2006). Tyrosyl-DNA-phosphodiesterase 1 (TDP1) can release Topoisomerase 1 by cleaving the enzyme close to its active tyrosine residue. If this does not happen, either the nucleases Rad16, Mus81 or Mre11 (Rad50 complex) cut the DNA in the vicinity of the DNA-Top1-CPT complex or the DNA helicases Srs2 and Rqh1 become involved in the repair. The *cdc2.1w* mutation is epistatic with the deletions of *mus81*, *rad16* or *srs2*, but not with the deletion of *rqh1* or the mutations *rad50S* and *rad32ND* (*mre11*), which specifically

impair CPT repair by the Mre11-Rad50-Nbs1 complex (Figure 3-9 - 3-13). This indicates that Cdc2 regulates the repair functions of Mus81, Rad16 and Srs2 but not of the Rad50 complex or Rqh1 DNA helicase. These repair functions become very important in the absence of Tdp1 because *tdp1-d cdc2.1w* are much more CPT sensitive than either single mutant (Figure 3-8). As during the unperturbed cell cycle, the kinases Cdc2 and Chk1 are functionally linked when cells have to respond to CPT induced DNA damage (Figure 3-24). Since no physical interaction has so far been detected between Cdc2 and Mus81 or between Cdc2 and Rad16, it is likely that Cdc2 indirectly regulates either repair factor.

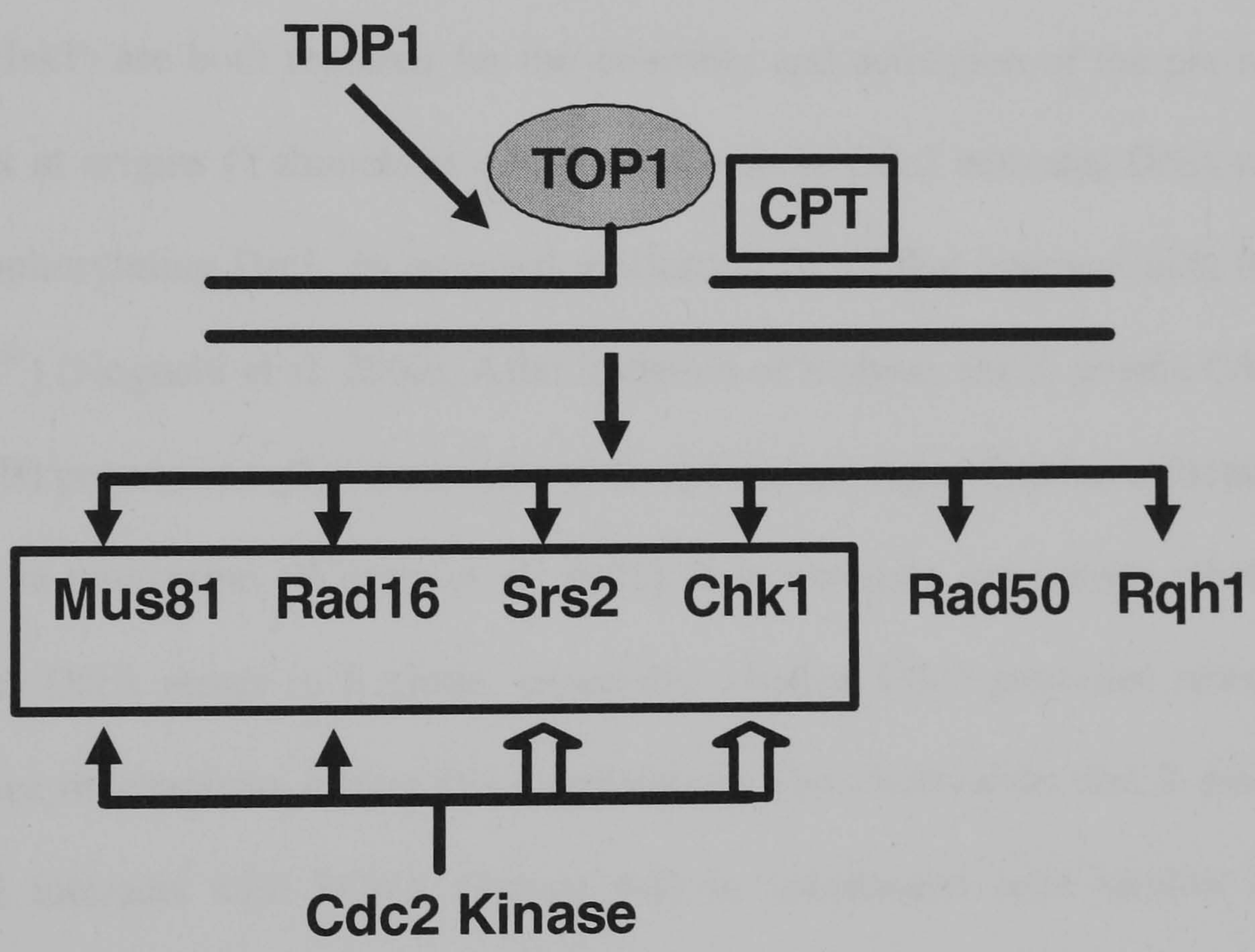


Figure 6-2. Model for the novel requirement of Cdc2 in the repair of CPT-induced DNA damage. In response to camptothecin-induced DNA double-strand breaks Cdc2 seems to regulate the repair functions of Mus81, Rad16 and Srs2 but not of Rad50 and Rqh1 and similar to the unperturbed cell cycle, Cdc2 is functionally linked with Chk1 kinase. These repair functions become very important in the absence of Tdp1 (the broad arrows indicate physical interactions, the thin arrows genetic interactions).

In addition, the data presented here propose DNA repair activities for Wee1 and perhaps Mik1 kinases, which are independent of their role as negative regulators of Cdc2-cyclin B complex.

6.2 Cdc2 regulates the anti-recombinogenic activity of Srs2 DNA helicase during unperturbed S phase

Cdc2 plays a variety of important roles in S phase. At the G1/S transition Cdc2 and Cdc7 (Hsk1) are both required for the assembly and activation of the pre-replication complex at origins (Yabuuchi et al. 2006). *S. pombe* Cdc2 activates DNA replication by phosphorylating Drc1, an essential replication factor that interacts with Rad4/Cut5 ($Dpb11^{Sc}$) (Noguchi et al. 2002). After initiation of S phase, the *S. pombe* Cdc2-Cdc13 (cyclin B) protein complex associates with replication origins that have already fired to prevent re-replication (Wuarin et al. 2002). It is currently not known whether Cdc2 regulates DNA repair in S phase, especially whether Cdc2 promotes recombination avoidance mechanisms during DNA replication. The observation that *S. pombe* Cdc2 (CDK1) interacts with PCNA (Figure 4-8) in undamaged cells implies that Cdc2 associates with the replication machinery throughout the cell cycle. A similar interaction has been reported for human CDK2-cyclin A, which phosphorylates DNA Ligase IV and DNA Polymerase lambda when bound to PCNA (Koundrioukoff et al. 2000; Frouin et al. 2005). It is currently unclear which, if any, of these Cdc2 functions is related to the 2-3 fold increase in gene conversion and the 25-fold increase in chromatin loss observed in *cdc2.1w* mutant cells (Figure 3-18; Figure 3-21). *Cdc2.1w* does not genetically interact with the DNA replication mutants tested in this thesis, but

it increases the CPT sensitivity of *cdc18-K46* cells (Figure 3-16), which are deficient in the loading of the MCM DNA helicase complex at the beginning of S phase (Nishitani and Nurse 1995). *S. pombe* Cdc1 interacts with Cdc18 (Brown et al. 1997) and it is possible that the combination of the mutated *cdc18-K46* and *cdc2.1w* alleles impair the repair response to CPT. The latter would be consistent with the link between Cdc18 and Rad3 kinase (Fersht et al. 2007), which may explain the continuous modification of Chk1 (Figure 3-23). Alternatively, Cdc18 and Cdc2 act in two redundant pathways, which when both impaired render cells sensitive to CPT.

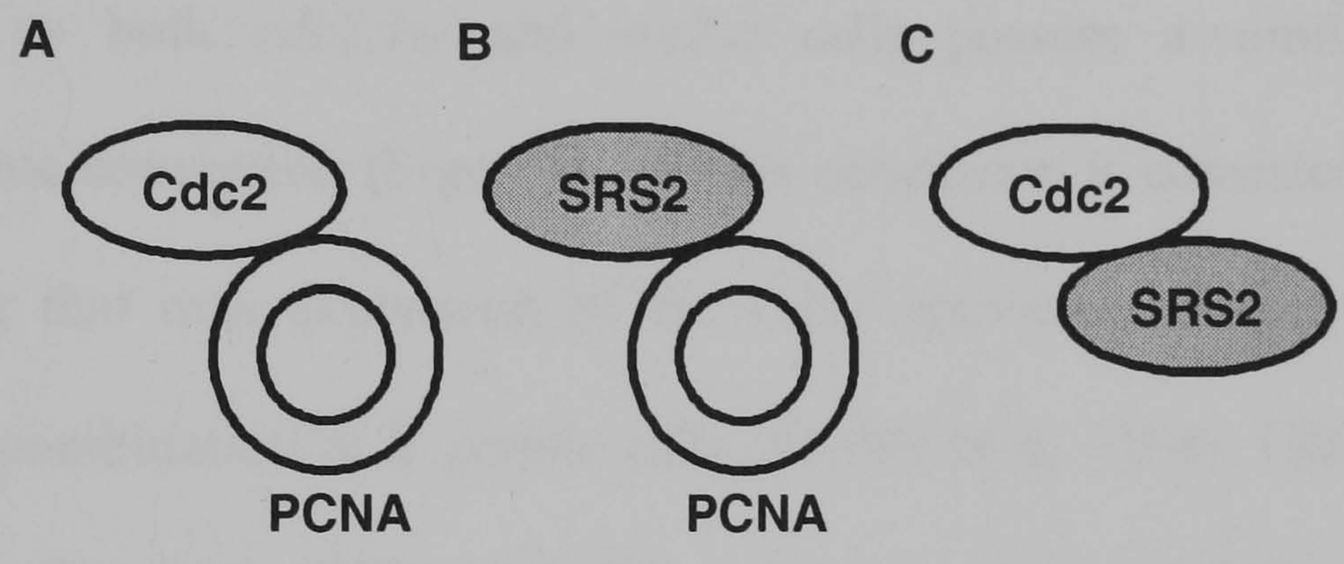


Figure 6-3. The identified biochemical protein-protein interactions.

A) Cdc2-PCNA, **B)** Srs2-PCNA and **C)** Cdc2-Srs2 protein complexes were identified in this study.

As shown in Figure 4-6, gene conversion rates are increased in *cdc2.1w* cells and this increase is not further enhanced by the loss of Srs2 DNA helicase, which itself prevents recombination. This functional relationship combined with the direct interaction between Cdc2 and Srs2 (Figure 4-2; Figure 4-3) strongly suggests a regulatory role for Cdc2 such that the kinase stimulates the anti-recombination activity of Srs2 as long as cells progress through S phase. The co-immunoprecipitation of Srs2 and PCNA from undamaged G2 cells (Figure 4-2) shows that a sub-fraction of the helicase interacts with PCNA. This interaction appears to be constitutive and did not change when cells were arrested with hydroxyurea in S phase or treated with CPT (Figure 4-2, B). This constitutive interaction may explain why SUMO-ylation, which

recruits *S. cerevisiae* Srs2 to stalled replication forks (Papouli et al. 2005; Pfander et al. 2005) is not conserved in *S. pombe* or human cells (Frampton et al. 2006).

The current experimental evidence indicates that the Cdc2-PCNA, Cdc2-Srs2 and Srs2-PCNA complexes are distinct. Since Srs2 and Cdc2 are both required to suppress gene conversion events and because the electrophoretic mobility of Srs2 changes upon mutation of the potential Cdc2 phosphorylation site serine 21 (Figure 4-7), the Cdc2-Srs2 protein complex is the most likely candidate for the regulatory function impaired by elevated Cdc2 activity in *cdc2.1w* cells. Elevated Cdc2 activity appears to inhibit Srs2 activity, as both *cdc2.1w* and *srs2-d* cells possess a similar increase in spontaneous gene conversion (Figure 4-6). This conclusion is consistent with a recent report showing that over-expression of the Cdc2 activating kinase Csk1 increases spontaneous recombination in *S. pombe* cells (Gerber et al. 2008). Other proteins that may be inhibited by high CDK1 ($Cdc2^{Sp}$) activity are human BRCA2 (Esashi et al. 2005) and *S. pombe* Crb2 (Esashi and Yanagida 1999). CDK1-dependent phosphorylation of both proteins peaks early in mitosis at the height of CDK1-cyclin B activity indicating that CDK1 inactivates homologous recombination by switching off this important mediator of recombination. In line with this idea, *S. pombe* cells expressing a Crb2 mutant protein that cannot be phosphorylated by Cdc2 (*crb2-T215A*), possess elevated recombination levels in response to DNA damage (Caspari et al. 2002). In summary, high Cdc2 activity may correlate with inactivation of homologous recombination either during S phase or in mitosis. This conclusion appears to be contradictory to the requirement of high Cdc28 (Cdc2) activity in *S. cerevisiae* for the generation of 3'-single stranded DNA at a broken chromosome (Ira et al. 2004). It is however important to remember that *S. cerevisiae* is unique in that cells arrest with high Cdc28 activity in response to DNA damage (Amon et al. 1992;

Sorger and Murray 1992; Li and Cai 1997). Consistent with an inhibitory function of Cdc2, inter-chromosome gene conversion upon chromosome cleavage by the HO endonuclease is strongly reduced by elevated Cdc2 activity in *cdc2.1w* cells (Figure 3-22).

The observation that *S. pombe* Srs2 associates with Cdc2 in a large protein complex of more than 600kDa (Figure 4-3) is consistent with data published by Chiolo and colleagues (Chiolo et al. 2005), who reported that *S. cerevisiae* Cdc28 ($Cdc2^{Sp}$) regulates the association of Srs2 with Sgs1 ($Rqh1^{Sp}$) and Mre11 by phosphorylating several sites in C-terminal domain of Srs2. Currently it is not known whether the *S. pombe* Srs2 complex (Figure 4-3) contains Rqh1 or Mre11. The data presented in this thesis indicate that *S. pombe* cells have at least two different Srs2 protein complexes, one containing PCNA and one containing Cdc2 (Figure 4-3). The presence of distinct Srs2 protein complexes is consistent with the situation in *S. cerevisiae*, where SUMO-ylated PCNA recruits Srs2 to stalled replication forks (Papouli et al. 2005; Pfander et al. 2005) and Nej1 recruits the helicase to HO-induced DSBs in a manner dependent on Dun1 kinase (Carter et al. 2009). Interestingly, neither SUMO-ylation of PCNA nor the Dun1-Nej1 pathway are conserved in *S. pombe*.

In addition to Srs2 and PCNA, Cdc2 interacts with Chk1 (Figure 4-5). A similar interaction was found between human Cdc2 (CDK1) and Chk1 (Ikegami et al. 2008). CDK1 phosphorylates Chk1 at serine 286 and serine 301 in response to DNA replication stress caused by hydroxyurea (HU) and UV light. Since CDK1 phosphorylates the same residues during mitosis (Shiromizu et al. 2006), it is possible that CDK1 down-regulates Chk1 under these conditions.

6.3 Cdc2 regulates repair of CPT-induced DNA damage

The initial observation that led to this project was the specific camptothecin sensitivity of all wee mutants. As shown in Figure 3-2, the sensitivity to chronic exposure to CPT correlates well with the level of *in vivo* elevated Cdc2 activity. This strongly suggests that high activity levels of Cdc2 inhibit repair of DNA lesions, which occur when cells grow in the presence of CPT. This conclusion is well supported by the experiment shown in Figure 3-5, in which reduction of Cdc2 activity by the introduction of the *cdc25.22* mutant allele restores resistance to CPT. The Cdc2-dependent repair pathway is predicted to act on DNA lesions, which accumulate in the absence of Tdp1 because the CPT sensitivity is greatly increased in a *tdp1-d cdc2.1w* double mutant (Figure 3-8).

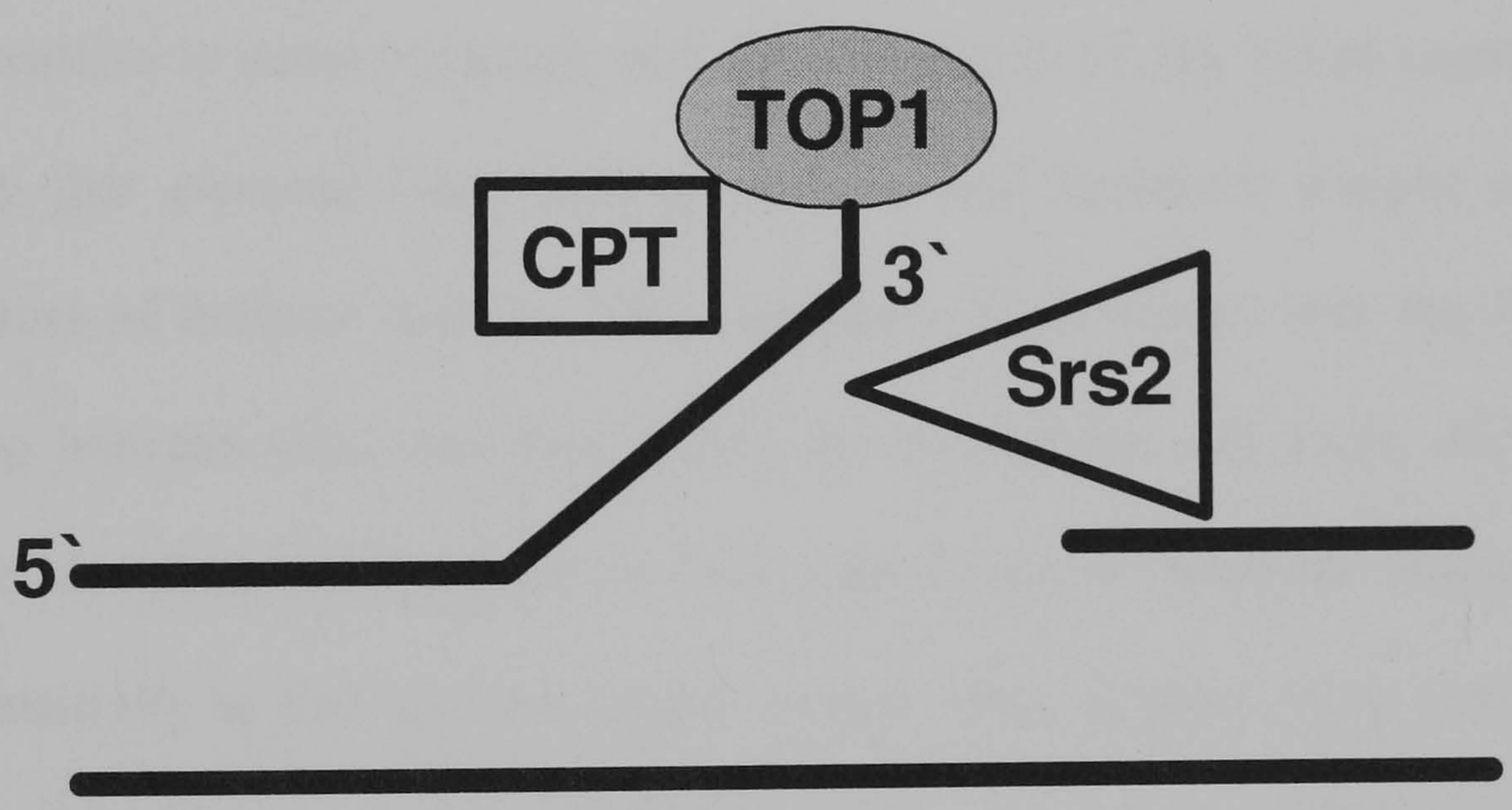


Figure 6-4. The Srs2 helicase activity may become important when CPT traps Top 1 on the DNA. When camptothecin stabilises the covalent complex between Topoisomerase I and the 3'-end of the DNA, in the absence of *tdp1*, the 3'-5' DNA helicase activity of Srs2 may be required together with an yet unidentified nuclease activity to remove the enzyme. Possible nuclease candidates are Rad16 or Mus81.

Srs2 is like Rqh1 (BLM^{Hs}) a 3'-5' DNA helicase (Krejci et al. 2003) and this activity may become important when immobilised Topoisomerase I is not cleaved off the 3`-end of the DNA by Tdp1 (Figure 6-4). Since CPT stabilises the covalent cleavage complex between Top1 and DNA, Srs2 helicase requires a nuclease activity to remove the enzyme. Srs2 can remove Rad51 proteins from single-stranded DNA, but Rad51 does not bind covalently to DNA. The nuclease activity required for the repair of immobilised Top1 could be provided by Rad16, as deletion of *rad16* in a *tdp1-d* background is lethal (not shown) and because *cdc2.1w* is epistatic with a *rad16* deletion (Figure 3-10). Another candidate is Mus81, as indicated by the observation that introduction of the *cdc2.1w* allele only very slightly increases the CPT sensitivity of *mus81-d* cells (Figure 3-11). The model shown in Figure 6-4 is also supported by the strong increase in CPT sensitivity upon deletion of *srs2* in *a tdp1-d* mutant strain (Figure 3-13, A). In contrast to the *cdc2.1w tdp1-d* strain, *srs2-d tdp1-d* cells also become sensitive to acute treatment with CPT (Figure 3-13, B). Taken together these data imply that elevated Cdc2 activity reduces Srs2 functions without causing a complete loss of helicase activity. This conclusion is consistent with the functional relationship between Cdc2 and Srs2 during the unperturbed cell cycle, the mobility shift of the Myc-Srs2-S21A protein on SDS page (Figure 4-7) and the strong increase in CPT sensitivity in the *cdc2.1w rqh1-d* mutant strain (Figure 3-12). Loss of Srs2 activity in the absence of Rqh1 DNA helicase is a lethal event (Lee et al. 1999; Gangloff et al. 2000; Wang et al. 2001) because cells have lost the two major DNA helicases removing incorrectly formed Rad51-ssDNA filaments. This synthetically lethal relationship between Srs2 and Rqh1 may explain why *cdc2.1w rqh1-d* mutant cells possess a slow growth phenotype and accumulate genetic suppressors with a high frequency. A similar increase in genetic instability was observed for the *cdc2.1w rad50-d* mutant (figure not shown) suggesting that Rad50 and Rqh1 act in the same

pathway. This conclusion is supported by the physical interaction of the Rad50-Mre11-Nbs1 complex with BLM (Rqh1) in human cells (Wang et al. 2000). It is unlikely that Cdc2 targets the Rad50 complex in the presence of CPT because the CPT sensitivity of the *rad50S cdc2.1w* mutant is higher compared to the *rad50S* single mutant (Figure 3-9).

6.4 The DNA repair function of *S. pombe* Wee1 is distinct from its role as a regulator of Cdc2-cyclin B

Findings presented in this study propose a dual role for *S. pombe* Wee1 as the negative regulator of Cdc2-cyclin B (the cell cycle regulation complex) and as a DNA repair protein. Cells devoid of *wee1* showed a specific increase in UV sensitivity in response to nitrogen starvation suggesting an additional DNA repair function for Wee1 in G1/G0 (Figure 5-2). Also, the UV sensitivity of *wee1-d cdc13-245* mutant cells was higher than those of either single mutant indicating that *wee1* and *cdc13* (cyclin B) products are implicated in distinct UV damage response pathways (Figure 5-3). The third piece of evidence is the specific suppression of the CPT sensitivity upon loss of *wee1* in the *rqh1* deletion mutant (Figure 3-12, B). Since *cdc2.1w* does not show a similar rescue effect, but rather increases the CPT sensitivity in a *rqh1-d* strain (Figure 3-12, A), Wee1 has to perform a function, which is not only different from its role as negative regulator of Cdc2 but also allows it to overcome the high CPT sensitivity of a *"wee" rqh1-d* strain (i.e. loss of Wee1 would abrogate the repair function and increase Cdc2 activity at the same time). Because the high CPT sensitivity of *cdc2.1w rqh1-d* mutant cells could be explained by a reduction in Srs2 helicase activity and because

mutations in *rad51*, rhp55 or rhp57 are known to suppress the synthetic lethality of a *srs2-d rqh1-d* double mutant (Doe et al. 2004), it is possible that loss of Wee1 impairs an early step in homologous recombination. It is however unclear how this could explain the increase in UV sensitivity when cells are starved of nitrogen.

A unique role of Wee1 in DNA repair has been reported previously by Barbet and Carr (Barbet and Carr 1993) who showed that *wee1-d* cells are sensitive to ionising radiation whereas *cdc2.1w* mutant cells are not. A role of Wee1 in homologous recombination would be consistent with the ionising radiation sensitivity of the *wee1* deletion mutant. Alternative roles of Wee1 have been published in different model systems. For example, in *Drosophila melanogaster* Wee1 associates in a complex with 5 γ-tubulin ring proteins to ensure proper positioning and activation of mitotic spindles (Stumpff et al. 2005). In *Xenopus* egg extracts, Wee1 forms a complex with several proteins that promote temperature-induced apoptosis (Smith et al. 2002). It has also been reported that the up-regulation of Wee1 protects human and HeLa cells from apoptosis in response to DNA damage (Yuan et al. 2003; Wang et al. 2005) further supporting the idea of Wee1 processing a DNA repair activity.

Consistent with a separation between cell cycle regulation and DNA repair, Cdc2 forms at least two distinct protein complexes. Cdc2 and cyclin B peak in fractions 15 and 16, corresponding to a molecular weight of approximately 200kDa (Figure 5-4). These fractions do not contain wee1, which co-fractionates with both Cdc2 and cyclin B in fraction 9. This fraction contains protein complexes larger than 650kDa. This large complex may contain the Wee1 regulators Cdr1 and Cdr2 as well as the anillin-like protein Mid1, which all co-localise with Wee1 to cortical nodes in the middle of G2 cells in *S. pombe* (Moseley et al. 2009). Since this complex links cell size to the

activation of Cdc2, it is possible that this large complex is the cell cycle regulatory complex of Wee1.

Results presented here clearly show that it is unlikely that Wee1 kinase acts in the same homologous recombination pathway as cyclin B in response to UV damage and that Wee1 DNA repair function becomes important in G1/G0 phase of the cell cycle. Taken together, this findings could be explained if Wee1 impinges on NHEJ. NHEJ, in contrast to HR, does not rely on the presence of a sister chromatid and therefore can repair broken chromosomes throughout the cell cycle and in non-cycling cells (Karathanasis and Wilson 2002). There is evidence in the literature suggesting a role for Wee1 kinase in non-cycling cells, where Wee1, but not Cdk1-cyclin B, is expressed and active in terminally differentiated neurons (Tomashevski et al. 2001). Wild type fission yeast cells devoid of NHEJ proteins such as Ku70 or DNA Ligase IV are not sensitive to IR (Manolis et al. 2001), most probably because they have a very short G1 phase and can therefore utilise HR most of the time. However *wee1* mutant cells spend more time in G1 (Figure 5-2, B) (Russell and Nurse 1987) and thus the impairment of NHEJ would have a stronger impact.

Like loss of *wee1*, deletion of *mik1* increases the DNA damage sensitivity of *cdc13-245* cells (Figure 5-5, A). A similar increase is observed upon deletion of *ku70* in *cdc13-245* cells, suggesting that loss of *mik1* or introduction of a "wee" mutation affects a pathway that depends on Ku70 (Figure 5-5, A). This conclusion is confirmed by the *mik1-d ku70-d cdc13-245* triple mutant, which is no more CPT sensitive than either double mutant (Figure 5-5, A). Since deletion of *ligase IV* fails to produce a similar CPT sensitivity it is unlikely that the Ku70 pathway defective in a *mik1-d* mutant is NHEJ. The Ku70-Ku80 heterodimer has also been implicated in DNA

replication and the G1-S transition (Rampakakis et al. 2008). Whether this requirement of Mik1 and Ku70 for the repair of CPT induced DNA damage represents a novel function of Mik1 or is a mere reflection of an increase in Cdc2 activity remains unknown.

Overall, the data presented here suggest DNA repair activities for Wee1 and perhaps Mik1, which are independent of their role as negative regulators of Cdc2-cyclin B. Wee1 is a target of the checkpoint kinase Chk1 (Oconnell et al. 1997) and it is conceivable that checkpoint activation triggers the DNA repair function of Wee1 such that homologous repair in G2 or NHEJ in G1/G0 is stimulated.

6.5 Final conclusion

Taken together this PhD project proposes two novel functions for the main *S. pombe* cell cycle regulator Cdc2 ($CDK1^{Hs,}$ $Cdc28^{Sc}$) in the coordination of DNA recombination. First, as a regulator of the anti-recombinogenic activities of Srs2 DNA helicase during unperturbed cell cycle and second, as a regulator of the repair of camptothecin-induced DNA damage. In addition, it is suggested here, that Wee1 and perhaps Mik1 have DNA repair activities that are independent of their roles as negative regulators of Cdc2-cyclin B complex.

APPENDIX

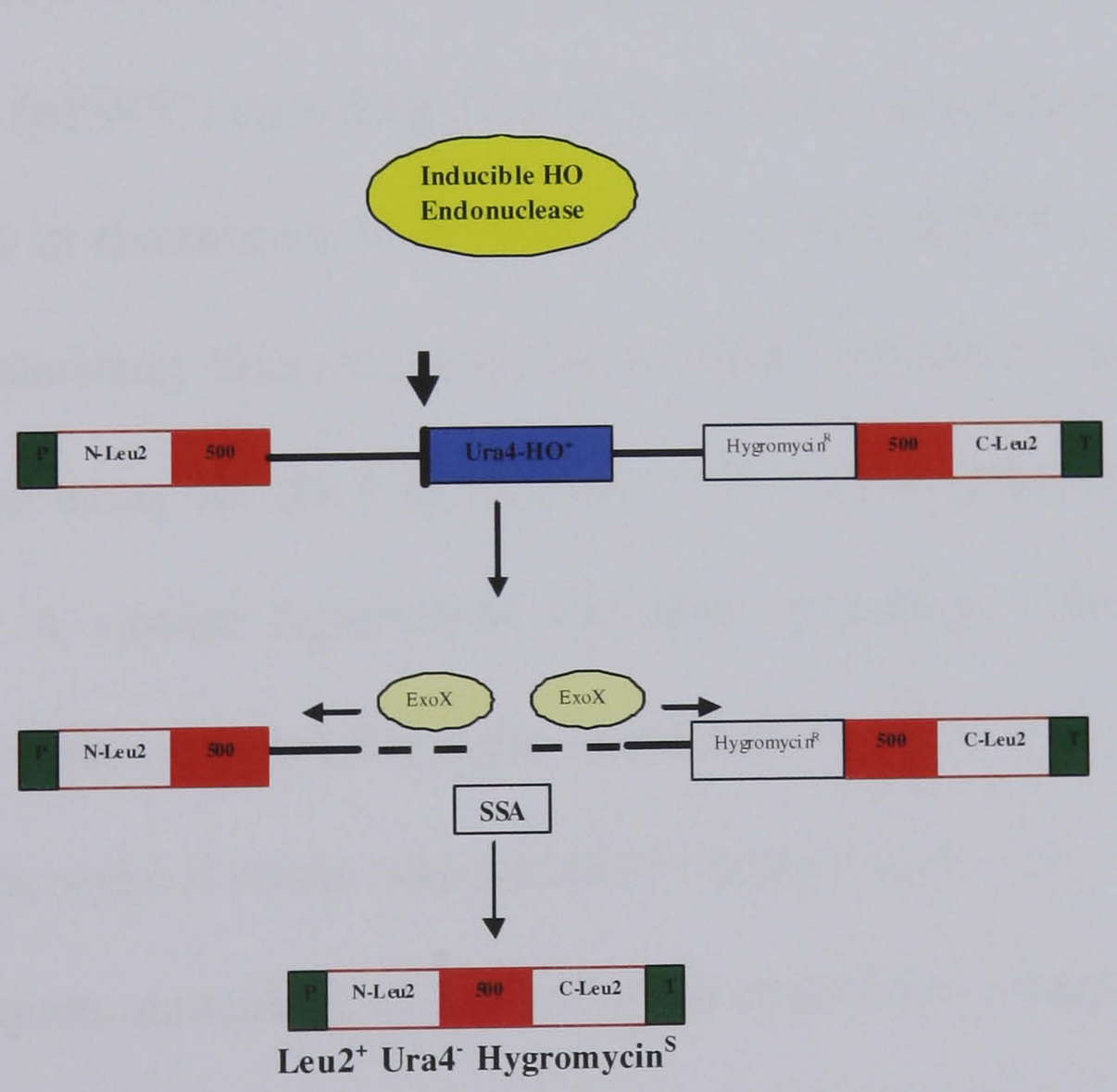
Analysis of HO endonuclease-induced DNA double-strand break repair in a cdc13-245 mutant background

Chromosomal breakage is one of the earliest signs of cancer development, and can arise at any stage of the cell cycle (Bartkova et al. 2005). A major challenge for cells is to suppress recombination in areas of the genome with repetitive DNA sequences such as the rDNA array, transposons or tRNA genes. Specific sites in the eukaryotic chromosomes are particularly sensitive to breakage and known as "fragile sites". Interestingly, screens for fragile sites in *S. cerevisiae* revealed tRNA genes and transposons as being frequently linked with chromosomal breakage (Admire et al. 2006). To assay the role of cyclin B in the regulation of DSB repair in a region with repetitive sequence motifs, we used the HOSSA system to study the repair of a single DSB in chromosome II induced by HO Endonuclease in the *ura4+* marker gene flanked by 500bp of homologous sequence (Appendix - Figure 1). Such a HO-induced break is recognised by the *S. pombe* DNA repair and checkpoint system (Du et al. 2003).

The HOSSA System

The HOSSA System (*S. cerevisiae* HO endonuclease Single Strand Annealing) (Appendix - Figure 1) was adapted for the use in *S. pombe* by Petra Werler in Prof Anthony Carr's group at the Genome Damage and Stability Centre in Brighton (Werler and Carr; 2005 unpublished) and its design is based on a system successfully used in *S. cerevisiae* (Vaze et al. 2002). The *S. cerevisiae* HO endonuclease introduces a specific

double strand break in the mating type locus initiating mating-type interconversion. The HO endonuclease recognizes and cleaves a specific 22bp target sequence (Nickoloff et al. 1990). This target sequence is present only once in the entire *S. cerevisiae* genome and not at all in the *S. pombe* genome. The HO recognition motif can be inserted at different locations outside the mating type region and the HO endonuclease will produce a DSB upon induction from a plasmid. The endonuclease will re-cut its target sequence as long as the enzyme is not degraded or the sequence is either lost or mutated. This is why faithful NHEJ cannot be monitored with this system. In *S. pombe*, the *S. cerevisiae* HO endonuclease is expressed from a plasmid under the control of thiamine-regulated *nmt41* promoter (pPW52). In contrast to the GAL system the induction from the *nmt* promoter is slow and about 60-90% of HO sites are cut in a given cell population within 23 hours (Du et al. 2003). The turnover rate of *S. cerevisiae* HO endonuclease in *S. pombe* is unknown.



Appendix - Figure 1. The Experimental HO-Endonuclease System to induce a defined DNA double-strand break in *S. pombe*.

The system is comprised of two inactive halves of the *leu2* marker gene (N-Leu2 and C-Leu2), which share 500bp of homology. This cassette was integrated in chromosome II and is separated by non-

essential DNA containing the *ura4* gene with a unique HO restriction site and the hygromycin resistance gene. The *S.cerevisiae* HO endonuclease is expressed from a plasmid under the control of thiamine-regulated *nmt41* promoter. Upon induction, the HO-endonuclease cuts at its recognition site generating a single DSB within the *ura4* gene. The main repair mechanism is Single-Strand-Annealing (SSA), a process in which unknown nucleases (ExoX) resect the chromosome until the repair system reaches the homologous sequences and restores the intact *leu2* gene resulting in the loss of both *ura4-HO* and the hygromycin resistance gene. Alternatively, a functional *leu2* gene can be restored by a loop-out event in the absence of a DSB by recombination between the two identical *leu2* regions. Such loop-out events appear to happen at a very low frequency, since the number of spontaneous *leu2+* colonies is very low. A third possible event restoring the *leu2* locus could happen by an unequal recombination between the two sister chromatids. P – promoter; T – terminator; 500 – 500bp length DNA.

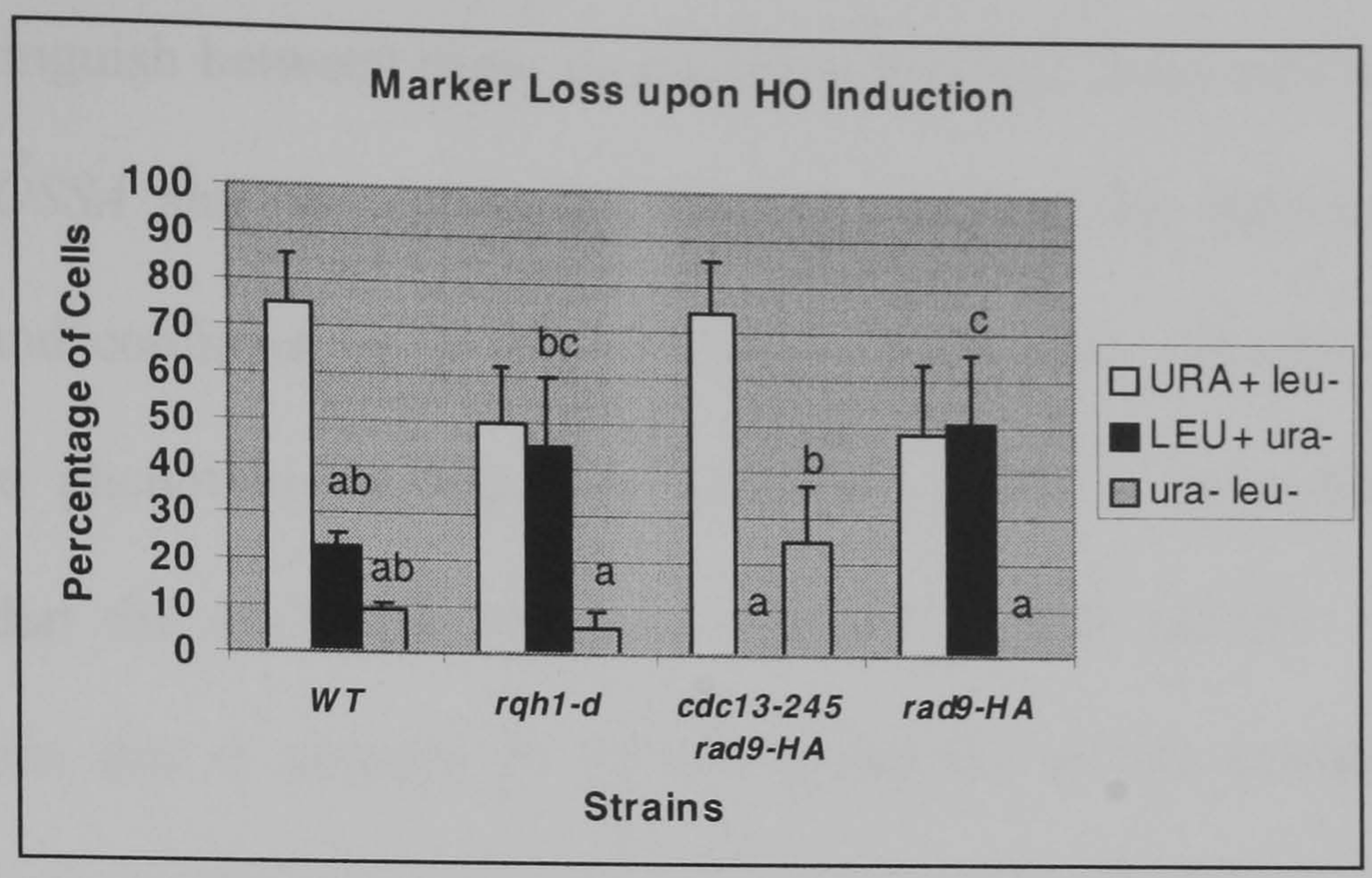
HO-induced DNA DSB repair in cdc13-245 mutant cells

To study the role of cyclin B (*cdc13*) in the process of SSA, both the wild type strain and its mutant version *cdc13-245* containing the HOSSA construct were transformed with the plasmid (pPW52) encoding *S. cerevisiae* HO endonuclease. After induction of the endonuclease in thiamine-free medium for 24 hours at 25°C, cells were plated on rich medium containing thiamine and the resulting colonies were replica-plated on selective plates to assay for the loss of *ura4+-HO* marker gene and the restoration of the *leu2+* gene. A similar experiment was also conducted with a strain expressing Rad9-HA and a strain deleted for *rqh1* encoding the 3'-5' DNA helicase related to human BLM. The *rqh1-d* strain was included because in *S. cerevisiae* the Rqh1 DNA helicase Sgs1 rejects annealing of homologous sequences during SSA in association with the mismatch repair proteins MSH2 and MSH6 if these sequences contain mutations (Sugawara et al. 2004). The Rad9-HA strain was included because the *cdc13-245* strain contained as well the *rad9-HA* allele. Between 50% and 70% of wild type and *rad9-HA* cells, which should resemble wild type cells, were still able to grow

in the absence of uracil after induction of the HO endonuclease indicating that the *ura4+* gene was intact (Appendix - Table 1; Appendix - Figure 2). Either the enzyme was not expressed in these cells or the restriction site was re-joined by NHEJ in a manner that allowed expression of *ura4+* but prevented the endonuclease from re-cutting it. Between 20% and 50% of wild type and *rad9-HA* cells regained the ability to grow in the absence of leucine indicating that the HO break was repaired by SSA. Consistent with this, these cells became dependent on uracil in the medium. A small proportion of wild type cells required both uracil and leucine indicating that the *ura4+* gene was lost but no functional *leu2+* gene was restored.

Appendix - Table 1. Phenotypic analysis of the indicated HOSSA strains after induction of the HO endonuclease. This data show that the HO endonuclease cuts with an efficiency of 20-60% and that with the exception of the *cdc13-245 HOSSA* strain no 4 all *cdc13-245* strains failed to produce a functional *leu2* gene. In contrast, wild type (WT), *rqh1-d* and *rad9-HA HOSSA* strains produced a functional *leu2* gene with a frequency between 20-70%.

Strain	Total EMM + ALUH	Ura4+ EMM + ALH	Leu2+ EMM + AUH	Ura4-Leu2-	Coments
WT (1)	100,00%	77,94%	22,06%	0,00%	
WT (2)	100,00%	65,80%	26,31%	7,89%	
WT (3)	100,00%	90,90%	0,00%	9,10%	
WT (4)	100,00%	67,65%	20,58%	11,77%	
rqh1-d (1)	100,00%	58,30%	33,30%	8,40%	
rqh1-d (2)	100,00%	37,50%	62,50%	0,00%	
rqh1-d (3)	100,00%	56,25%	40,62%	3,13%	
rqh1-d (4)	100,00%	100,00%	0,00%	0,00%	
cdc13-245 (1)	100,00%	100,00%	0,00%	0,00%	cut not induced
cdc13-245 (2)	100,00%	100,00%	0,00%	0,00%	cut not induced
cdc13-245 (3)	100,00%	72,72%	0,00%	27,28%	
cdc13-245 (4)	100,00%	60,00%	20,00%	20,00%	
cdc13-245 rad9-HA (1)	100,00%	61,54%	0,00%	38,46%	
cdc13-245 rad9-HA (2)	100,00%	67,30%	0,00%	32,70%	
cdc13-245 rad9-HA (3)	100,00%	87,87%	0,00%	12,13%	
cdc13-245 rad9-HA (4)	100,00%	82,00%	0,00%	18,00%	
rad9-HA (1)	100,00%	52,95%	47,05%	0,00%	
rad9-HA (2)	100,00%	28,57%	71,43%	0,00%	
rad9-HA (3)	100,00%	50,00%	50,00%	0,00%	
rad9-HA (4)	100,00%	64,00%	36,00%	0,00%	



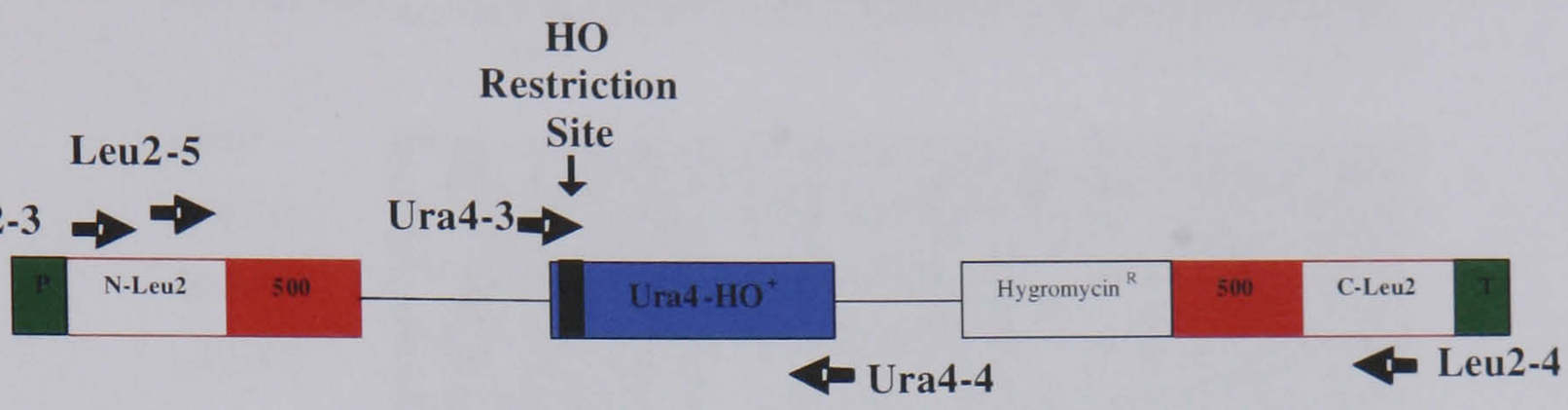
Appendix - Figure 2. Marker loss upon HO induction.

Upon HO induction, wild type (WT), *rqh1-d* and *rad9-HA HOSSA* strains produced a functional *leu2* gene with a frequency between 20-70%, whereas *cdc13-245 rad9-HA HOSSA* strains fail to restore functional *leu2* gene and show increase in *ura4*$^-$ *leu2*$^-$ phenotype. The error bars indicate standard errors of the mean. A one-way Analysis of Variance (ANOVA) was carried out to determine if there were any significant differences between indicated strains for the different phenotypes. No statistically significant differences at the $p < 0.05$ level were found between strains for phenotype Ura4+ ($p = 0.114$). However, differences between strains were found to be significant for both Leu2+ ($p = 0.001$) and for Ura4-Leu2- ($p = 0.009$). Post-hoc tests were carried out using the Tukey LSD test to determine where significant differences lay between strains. These are indicated with different letters (a, b, c).

Interestingly, no leucine prototroph (*leu2+*) colonies were recovered from the *cdc13-245* mutant strain except one (Appendix - Table 1; Appendix - Figure 2) and this mutant produced also a higher frequency (25%) of cells dependent on both uracil and leucine. This indicates that cells that experienced a HO cut failed to complete SSA successfully. Loss of Rqh1 helicase had no effect on SSA indicating that in *S. pombe* Rqh1 is not required to prevent annealing of mutated sequences during SSA. This increased frequency of *ura4*$^-$*leu2*$^-$ cells in the *cdc13-245 HOSSA* strain suggests that the *ura4-HO* gene has been cut but that the *leu2* gene is either not restored or restored in a way that results in its inactivation.

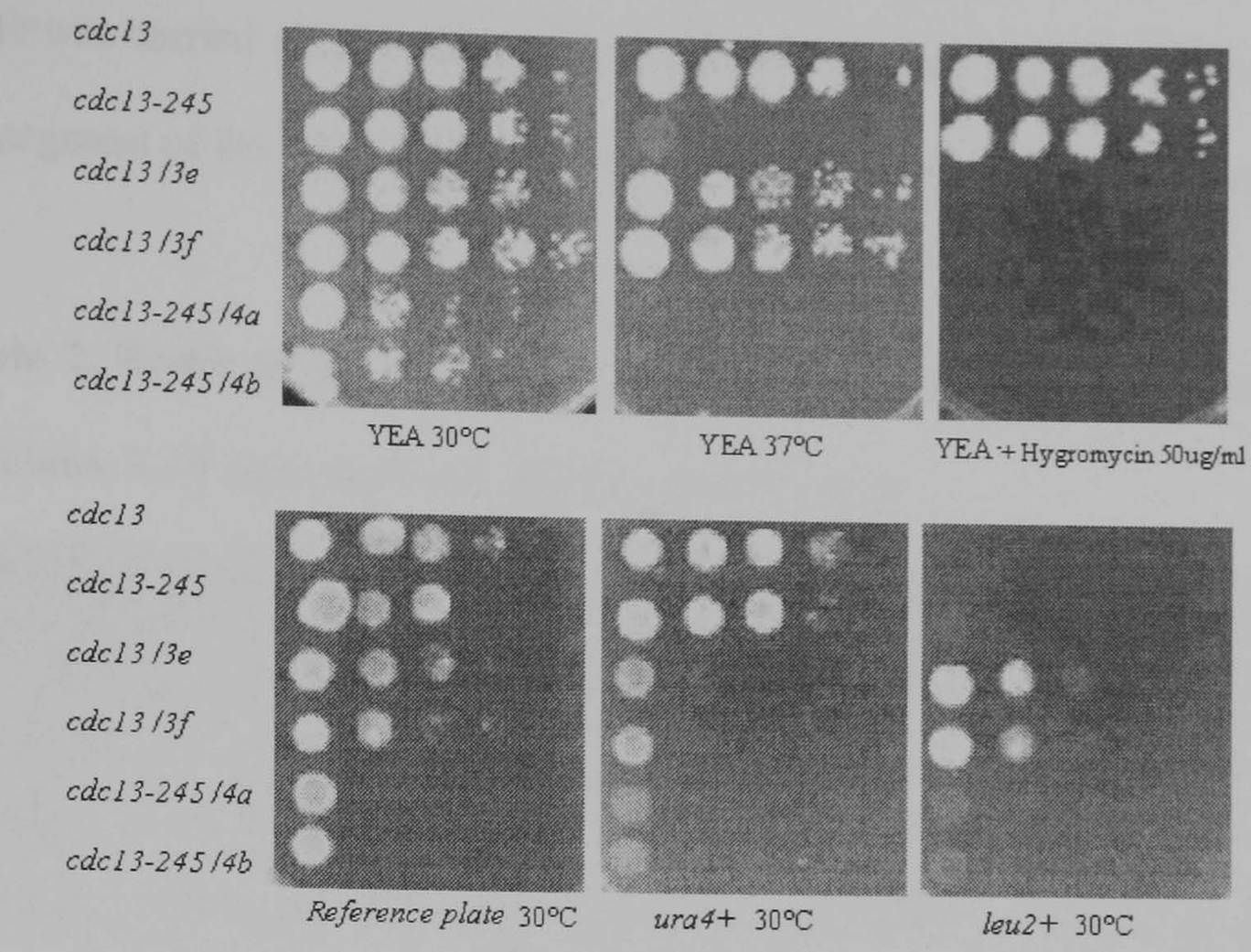
In order to distinguish between these possibilities, the *leu2* locus was amplified from two *cdc13*$^{+}$ *HOSSA* and two *cdc13-245 HOSSA* isolates after induction of the HO endonuclease and confirmation of the phenotypes (Appendix - Figure 3; Appendix - Figure 4). The phenotypic and genetic analysis shown in Appendix - Figure 4 demonstrates that the *leu2* gene has been restored in both *cdc13+* and *cdc13-245* mutant cells but that it appears to be non-functional in the mutant background. According with the restoration of the *leu2+* gene, the *ura4* marker was lost in all four strains (Appendix - Figure 4). This observation raises the interesting question why the *leu2* genes restored in the *cdc13-245 HOSSA* mutant background are not functional despite the fact that the original *leu2* sequences in the HOSSA construct contains no mutation? This finding would be consistent with a working model such that an exonuclease activity, which is required to degrade the DNA region between the identical *leu2* regions during SSA, becomes deregulated in *cdc13-245* mutant cells resulting in small deletions or mutations within the coding region of *leu2* rendering it non-functional. In order to test this model, the amplified *leu2* genes were sequenced from both wild type HOSSA and *cdc13-245 HOSSA* mutant (Appendix - Table 2). The entire ORF of the amplified *leu2* genes was sequenced to identify any mutations which may impair Leu2 expression of function. Interestingly, both *leu2* sequences obtained from the *cdc13-245 HOSSA* isolates contained mutations within the *leu2* region. The mutation in isolate 4a is a miss-sense mutation that results in the replacement of serine 322 by a glycine residue, whereas a single adenine base at position 304 has been deleted in the isolate 4b generating a premature stop codon at position 106. While the frame shift mutation results in a truncated translation product, it may be that the glycine at position 322 interferes with Leu2 function. No mutations were found in the two *leu2* genes amplified from wild type cells. Since the original HOSSA construct does not contain any mutations, the sequence changes may have

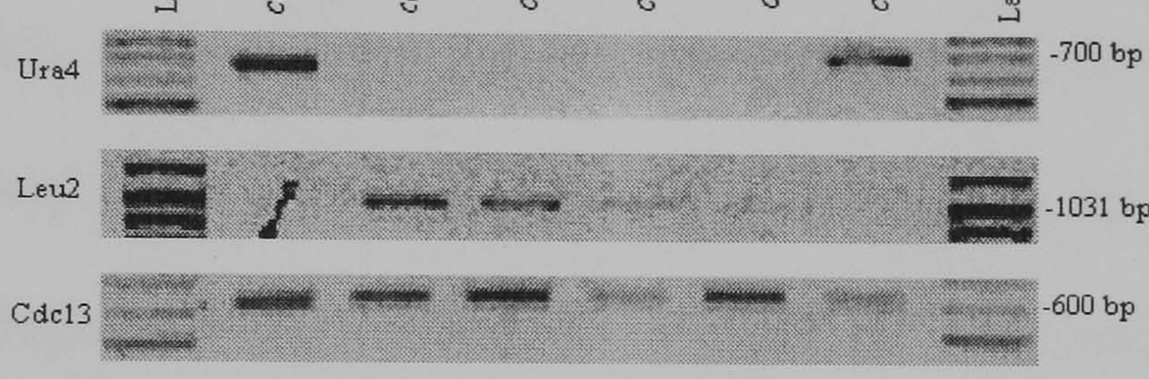
occurred either during the repair process or during the amplification of the *leu2* gene fragments.



Appendix - Figure 3. The location of the *leu2* and *ura4* primers.

Leu2-3, Leu2-4 – pair of primers used to amplify the *leu2* gene; Leu2-5 – primer used for sequencing the amplified fragment.





Appendix - Figure 4. Phenotypical and genetic analysis of different HOSSA isolates after induction of the HO endonuclease.

A) Drop test analysis of *cdc13*$^{+}$ *HOSSA* (801) and *cdc13-245 HOSSA* (813) phenotypes. The strains *cdc13 nt* and *cdc13-245 nt* in the top two rows contain the uncut HOSSA sequence (*nt* stands for not transformed with the plasmid encoding HO endonuclease), whereas the strains *cdc13*-3e-f and *cdc13-245*-4a-b are independent isolates after induction of the HO endonuclease and repair of the induced DSB. Since none of the latter strains grows on hygromycin plates or on plates lacking uracil, the DNA region between the identical *leu2* sequences containing the *ura4-HO* and *hyg*^{R} genes must haven been lost indicating that the HO endonuclease produced a DSB. As expected, the *leu2*$^{+}$ gene was functionally restored in *cdc13+ HOSSA* isolates as demonstrated by growth on selection plates lacking leucine. However, both *cdc13-245 HOSSA* isolates failed to grow on plates lacking leucine showing that the *leu2* gene is either missing or contains loss-of-function mutations. To confirm the presence of the temperature-sensitive *cdc13-245* mutations, strains were dropped on YEA plates and incubated at the

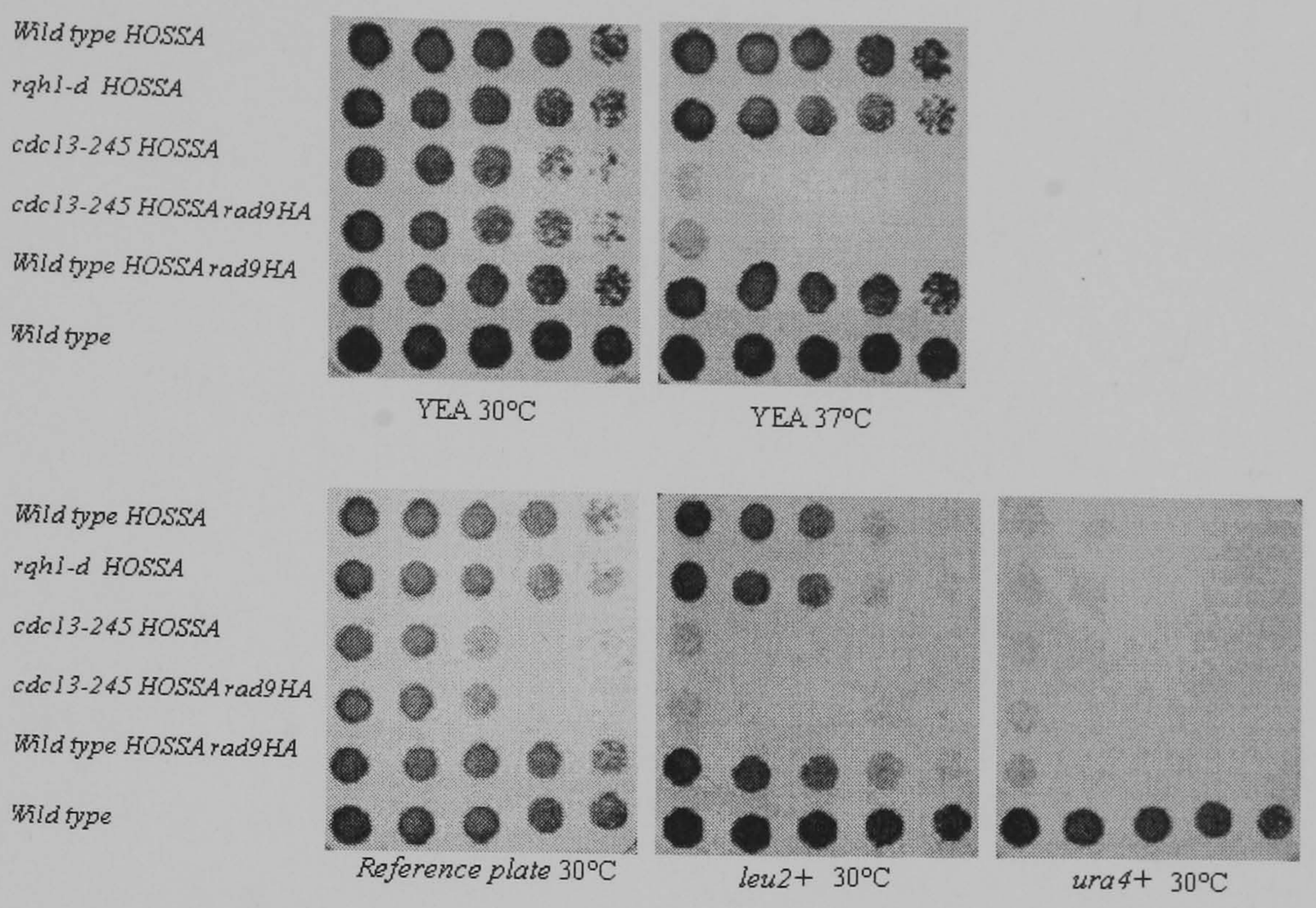
restrictive temperature of 37°C. All other plates were incubated at the permissive temperature of 27°C. **B)** Genomic PCR was carried out for the indicated strains to confirm the presence or absence of *ura4-HO* and *leu2*. A segment of the *cdc13* gene was amplified from every strain in a control experiment.

Appendix - Table 2. Result of the sequences analysis of the amplified *leu2* genes from both wild types *HOSSA* isolates 3-e/f and *cdc13-245 HOSSA* isolates 4-e/f.

Cells Type	Mutation
Wild type *cdc13 (3e)*	No mutation
Wild type *cdc13 (3f)*	No mutation
cdc13-245 (4a)	A → G missense mutation (S322 → G)
cdc13-245 (4b)	Deletion of 1 A at position 304 (new stop codon at amino acid position 106)

The experiment was repeated with new isolates and in contrast to the previous one, it failed to amplify the *leu2* fragment from the *cdc13-245* isolate (Appendix - Figure 5). In order to verify if the sequence changes shown in Appendix - Table 2 occurred as a result of impaired repair process in *cdc13-245* mutants or during the amplification of the *leu2* gene fragments, another two *leu2* fragments from different *cdc13-245 HOSSA* isolates that possess the ura4$^-$ leu2$^-$ phenotype were amplified and sequenced. The sequenced *leu2* fragments did not contain any mutations suggesting that the previously observed mutations appeared during PCR process and that the inability of those isolates to grow in the absence of leucine is NOT a result of impaired repair process but of impaired expression of *leu2* gene in *cdc13-245* mutant background. To test this, the latter *cdc13-245* temperature sensitive cells that possess ura4$^-$ leu2$^-$ phenotype and contain *leu2* gene were crossed with wild type cells (temperature resistant, *leu2*$^-$) and colonies were selected for temperature resistance and leu2$^+$ (not shown). Since the non-functional *leu2* gene in *cdc13-245* cells complemented *leu2*$^-$ wild type cells, overall, it seems that the expression of *leu2* gene is impaired in *cdc13-245* cells and

that the *cdc13-245* mutation does not impinge on the error-free repair of HO-induced DNA double-strand break.



Appendix - Figure 5. Phenotypical and genetic analysis of different HOSSA isolates after induction of the HO endonuclease.

To verify the idea that the *leu2* gene cannot be restored in *cdc13-245* mutant cells without mutations, phenotypes of the indicated strains were analysed using the drop test assay on different selective plates (**A**) and confirmed the physical presence of the *leu2* gene by colony PCR (**B**). As expected, all strains growing on selective plates without leucine contain the *leu2* gene. While the *leu2* locus was restored in

the *cdc13-245 rad9-HA HOSSA* isolate (lane 5), it failed to amplify this region from the genomic DNA isolated from the *cdc13-245 HOSSA* isolate (lane 4). Ura4, Leu2, Cdc13 – PCRs carried out using primers confirming presence of appropriate *ura4*, *leu2* and *cdc13* genes; *nt* – strains not transformed with HO plasmid.

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