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S-phase checkpoint activity and function throughout the cell cycle

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Summary

S-phase checkpoint activity and function throughout the cell cycle

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DNA damage or replication stress during S-phase can activate the S-phase checkpoint which executes a variety of responses, such as the inhibition of origin firing and replication fork stabilisation. Deregulation of the S-phase checkpoint leads to genomic instability, which has been implicated in diseases such as cancer. In this thesis, I aimed to address whether the S-phase checkpoint is regulated outside of S-phase, and how the S-phase checkpoint targets its substrates in budding yeast.

Although this checkpoint has thus far been associated exclusively with S-phase, it remains unknown whether its responses such as inhibition of origin firing can also occur in other phases of the cell cycle. To investigate this, the targets of the S-phase checkpoint for the inhibition of origin firing were analysed outside of S-phase upon DNA damage. Interestingly, I showed that the S-phase checkpoint effector kinase Rad53 phosphorylates its targets to inhibit origin firing outside of S-phase upon DNA damage when there is no replication. I then set out to test whether inhibition of origin firing by Rad53 outside of S-phase might be important for faithful DNA replication.

Having shown that the checkpoint response is not specific for any cell cycle phases, I then tested how the specificity of Rad53 for its substrates might be determined. After demonstrating that the essential replication protein Cdc45 is required for Rad53 to phosphorylate the initiation factor Sld3, the key residues of Cdc45 necessary for Rad53 interaction were identified. A Cdc45 allele was produced by mutating the identified residues. This allele of Cdc45 is a separation-of-function mutant which prevents Sld3 phosphorylation upon DNA damage, but retains its function in DNA replication. Because Cdc45 travels with the replication fork, it is possible that Cdc45 also targets Rad53 to the replication fork to stabilise it upon replication stress. Overall, this thesis provides evidence that the S-phase checkpoint can function throughout the cell cycle and that Cdc45 targets Rad53 to some of its substrates, and possibly plays a role in replication fork stabilisation.

Declaration of Authorship

I, Geylani Can, declare that this thesis titled, ‘S-phase checkpoint activity and function throughout the cell cycle’ and the work presented within it are my own. I confirm that:

- This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.
- It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text
- This dissertation does not exceed the word limit of 60,000 prescribed by the Degree Committee for the Faculty of Biology.

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Contents

Abbreviations

2D	**2**-**d**imensional
4-NQO	**4**-**N**itro**q**uinoline-1-**o**xide
5-FOA	**5**-**F**luoro**o**rotic **a**cid
APS	**A**mmonium **p**er**s**ulfate
ARS	**A**utonomously **R**eplicating **S**equence
ATR	**A**taxia- **t**elangiectasia mutated and **R**ad3 related
BSA	**B**ovine **s**erum **a**lbumin
bp	**B**ase **p**air
CDK	**C**yclin-**d**ependent **k**inase
DNA	**D**eoxyribo**n**ucleic **a**cid
dATP	**D**eoxy**a**denosine **t**ri**p**hosphate
dNTPs	**D**eoxyribo**n**ucleosid **t**ri**p**hosphate
DSB	**D**ouble **s**trand **b**reak
dsDNA	**D**ouble-**s**tranded **D**NA
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
FACS	**F**luorescence-**a**ctivated **c**ell **s**orting
g	Earth **g**ravity constant (9.81m/s2)
h	**H**our(s)
HCl	**H**ydro**c**hloric acid
HU	**H**ydroxy**u**rea
IB	**I**mmuno**b**lotting
kb	**K**ilo**b**ase
LB	**L**yosgeny **b**roth
M	**M**olar
MCM	**M**ini chromsome **m**aintenance
min	**M**inute(s)
MMS	**M**ethyl **m**ethane**s**ulphonate
NHEJ	**N**on-**h**omologous **e**nd **j**oining
OD600nm	**O**ptical **d**ensitiy at a wavelength of **600nm**
ORC	**O**rigin **r**ecognition **c**omplex
PBS	**P**hosphate-**b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
pH	Negative logarithm of the **h**ydrogen ion concentration
pre-IC	**P**re**i**nitiation **c**omplex
pre-RC	**P**re-**r**eplication **c**omplex

QFA	**Q**uantitative **f**itness **a**nalysis
rDNA	**R**ibosomal **D**NA
RNA	**R**ibo**n**ucleic **a**cid
Rpm	**R**evolutions **p**er **m**inute
RT	**R**oom **t**emperature
S. cerevisiae	***S****accharomyces* ***c****erevisiae*
SDS	**S**odium **d**odecyl **s**ulphate
SDS-PAGE	**S**DS **p**olyacrylamide **g**el **e**lectrophoresis
Sec	**S**econd(s)
S. pombe	***S****accharomyces* ***p****ombe*
ssDNA	**S**ingle-**s**tranded DNA
TBS-T	**T**ris-**b**uffered **s**aline containing 1% **T**ween-20
TEMED	N,N,N',N'-**t**etra**m**ethyl**e**thane-1,2-**d**iamine
Tris	**T**ris(hydroxymethyl)aminomethane
U	**U**nits
UV	**U**ltra**v**iolet radiation
w/v	**W**eight per unit **v**olume
YNB	**Y**east **N**itrogen **B**ase
YPD	**Y**east-Extract-**P**eptone-**D**extrose

Chapter 1

Introduction

1.1. The cell cycle

The process of cell division results in the production of two daughter cells, and is tightly regulated through a series of events termed the cell cycle. The cell cycle consists of three fundamental steps in all living organisms: growth, genome duplication, and division. In contrast with bacteria, where genome duplication and division take place simultaneously, the eukaryotic cell cycle has four distinct phases: gap 1 (G1) phase, synthesis (S) phase, gap 2 (G2) phase and mitosis (M phase). Cells grow during G1 phase and prepare for the transition to S-phase, during which nuclear DNA is replicated. Following a specific checkpoint in G1, cells are no longer able to halt this process until the completion of the cell cycle. In G2 phase, cells grow further to prepare for mitosis, when genomic information is dispensed into two daughter cells. Cells can also exit the cell cycle and enter a resting state known as the G0 phase, usually in

response to entering an unfavourable environment or following differentiation (Alberts *et al*., 1997).

This cell cycle duration differs heavily depending on the eukaryotic cell being studied. In *S. cerevisiae,* the budding yeast, the cell cycle is completed in approximately 90 minutes (Forsburg & Nurse, 1991). This speed is in marked contrast to the cycle in many animal cells, which can occur over up to 24 hours in some human cell types. However, there remains considerable variation between cell types, and the stage of development can also play a role in cycle length.

1.1.1. Cell cycle control

To ensure that each daughter cell contains a full and high-fidelity copy of the genome, it is necessary for the transition between cell cycle phases to be robustly regulated. Without this regulation, genomic instability can accrue, increasing the risk of cancer development (Shen, 2011). The main eukaryotic regulators of cell cycle progression are cyclins, and the cyclin-dependent kinases (CDK). These CDKs dimerise with cyclin, and the resultant activity of the complex is responsible for many of the cellular alterations in each cycle stage (Bloom & Cross, 2007; Harashima *et al*., 2013; Morgan, 1995; Pines, 1995). CDKs are often categorised by the cell cycle phase during which they are active, resulting in three sub-categories: G1-CDKs, S-CDKs, and M-CDKs. Whilst in the mammalian systems studied there are numerous CDKs which associate with many cyclins, resulting in a highly complex regulatory network, in *S. cerevisiae* Cdc28 is the main CDK, with its changing role in the cell cycle deriving from its association with different cyclins at each phase (Bloom & Cross, 2007). These are Cln1-3 in G1, Clb5/6 in S-phase, and Clb1-4 in M phase (Bloom & Cross, 2007). CDKs are themselves regulated through numerous mechanisms including phosphorylation, inhibitor binding, as well

as through the regulation of cyclin levels at both the transcriptional and post-transcriptional stages.

Progression through the cell cycle is also controlled by a number of checkpoints, ensuring this only occurs once the previous stage has been completed successfully. Two crucial checkpoints in *S. cerevisiae* are the S-phase checkpoint, which determines whether replication was successful, and the spindle assembly checkpoint during M phase that ensures sister chromatids are faithfully segregated into the daughter cells (Hartwell & Weinert, 1989).

1.1.2. Cancer and disease

It has previously been suggested that all cancer cells possess a series of unique identifiers. These include the avoidance of apoptosis, the maintenance of growth signalling, resistance to anti-proliferative signalling, angiogenesis, metastasis and tissue invasion, and the ability to replicate without encountering the Hayflick limit on cell division (Hanahan & Weinberg, 2000). Subsequent additions to this list have included the ability of cancerous cells to avoid targeting by the immune system, and the possession of an abnormal metabolome (Hanahan & Weinberg, 2011). This list of identifiers continues to be modified as new information on cancer cells is discovered. Recently, genomic instability has been suggested as an additional hallmark (Kroemer & Pouyssegur, 2008; Negrini *et al*., 2010), reflecting its presence in all cancers regardless of stage and progression (Hills & Diffley, 2014). Genomic instability, which is present in all studied cancers, is therefore important to fully understand. However, whilst genomic instability has been previously shown to be a key hallmark of cancer cells, it has not yet been determined whether this genomic instability plays an active role in tumour progression or whether it is only a product of other aberrant functions in cancerous cells.

The role that replication stress plays in tumorigenesis appears to be complex and multifaceted, and it has been proposed that replicative stress can both halt or induce tumour progression (Lecona & Fernández-Capetillo, 2014). Replication stress, and subsequent genomic instability, are found in early-stage tumours and can be induced through oncogene disruption (Halazonetis *et al*., 2008), such as through the overexpression of the S-phase CDK modulator cyclin E (Ekholm-Reed *et al*., 2004; Jones *et al*., 2013) or slowing of replication rates through Chk1 overexpression (López-Contreras *et al*., 2012). However, replication stresses can also prove lethal or otherwise deleterious to cancer cells due to their potentially disruptive effect on mitosis (Lecona & Fernández-Capetillo, 2014). In addition, buffering replication stress and genomic instability through maintenance of high nucleotide concentrations is a known consequence of Myc oncogene activation, suggesting that a disruption of Myc function would induce genomic instability and potentially provide a mechanism for tumorigenesis (Bester *et al*., 2011). Replication stress can also function as an anti-tumorigenic mechanism due to its activation of DNA damage checkpoints, which can result in cell cycle arrest or apoptosis. The development of subsequent mutations allowing the cell to bypass these checkpoints can lead to tumour development in addition to further genomic instability. Therefore, the role of replicative stress and genomic instability in tumorigenesis appears to be dynamic and context-dependent, and may provide insight into potential novel cancer treatments.

1.2. Budding yeast as a model organism

The budding yeast *Saccharomyces cerevisiae* is a single-celled fungus with a relatively small and simple genome. Due to their rapid growth rate and the evolutionary conservation of many of their cellular processes, *S. cerevisiae* is a well-established model organism in the field of molecular genetics, and has a strong knowledge base facilitating further research such as a sequenced and well-annotated genome. *S. cerevisiae* is approximately 3-5 μm in diameter, and is protected by a tough cell wall. The cell cycle of *S. cerevisiae* begins with the formation

of a small bud which grows continuously throughout S and M phases, culminating in the distribution of one set of chromosomes into the daughter cell at the end of mitosis. *S. cerevisiae* has a long G1 during the cell cycle, followed by a relatively short S phase. However, there is no clearly-defined G2 between S phase and M phase.

S. cerevisiae can proliferate in both haploid and diploid states. Haploid cells can be two mating types, called a and α. These mating types secrete the specific pheromones a-factor and α-factor, respectively. These pheromones bind to cell-surface receptors on cells of the opposite mating type, activating signal cascades which result in G1 arrest and the synthesis of a wide variety of proteins. The morphology of these arrested cells then changes into a shape called a shmoo before fusing to form a diploid cell. The ability to arrest cells in G1 using pheromones provides a unique opportunity for the study of early cell cycle events in synchronized cells.

S. cerevisiae diploids cells also sporulate following extreme environmental conditions. Sporulation begins with meiosis in order to produce haploid cells. Four haploid cells produced after this meiosis are enclosed in a protective shell called the ascus. When these environmental conditions change, the spores germinate and mate again to produce two diploid cells. Since yeast cells can proliferate in haploid states as well, this allows for research to be undertaken on a simplified genome. More importantly, mating haploid cells with dedicated mutant yeast libraries provides the ability to perform genetic screens, through which several important genes and pathways have been identified thus far.

1.3. The molecular basis of DNA replication

1.3.1. Replication initiation

1.3.1.1. Origin of replication

In eukaryotic organisms, replication initiation occurs at hundreds to thousands of sites throughout the genome, the number of which is species-dependent (Nieduszynski *et al*., 2007; Raghuraman *et al*., 2001; Wyrick *et al*., 2001; Yabuki *et al*., 2002). The origin sites of budding yeast are best characterised, having been identified as autonomous replicating sequences (ARS) due to their ability to replicate plasmids (Dhar *et al*., 2012; Stinchcomb *et al*., 1979; Vallet *et al*., 1984). ARS sequences are approximately 200bp in length, with a strongly conserved consensus sequence rich in A and T (Nieduszynski *et al*., 2007). This consensus, termed the ACS, is either 11 or 17bp in length, and allows ORC complex binding to occur (Bell & Dutta, 2002; Theis & Newlon, 1997). The consensus sequence is found between the B1, B2, and B3 elements when present, which play respective roles in ORC binding, Mcm2-7 helicase binding and the binding of the transcription-associated ARS binding factor 1 (Abf1) (Miyake *et al*., 2002; Rao & Stillman, 1995; Rowley *et al*., 1995; Wilmes & Bell, 2002). Despite the presence of around 12,000 such consensus sequences throughout the *S. cerevisiae* genome, only 400 of these are utilised during replication, suggesting that factors beyond the presence of the ACS, such as the presence of B elements or nearby chromatin status, also play a role in determining ARS usage (Eaton *et al*., 2010; Nieduszynski *et al*., 2007). Numerous studies have been undertaken to determine which of these origins are utilised in both *S. cerevisiae* and *S. pombe*, using diverse methods such as chromatin immunoprecipitation to determine ORC or MCM2-7 complex binding regions, ssDNA analysis, copy number analysis, Okazaki fragment analysis via sequencing and a bioinformatic analysis of origin sequence conservation

(Feng *et al*., 2006; McGuffee *et al*., 2013; Müller *et al*., 2013; Nieduszynski *et al*., 2006; Wyrick *et al*., 2001; Yabuki *et al*., 2002).

In contrast to budding yeast, however, other eukaryotic species do not appear to use specific sequences for origins. Whilst an examination of fission yeast determined that origins were most likely to occur in AT-rich sequences, human studies seeking to identify sequence-specific factors mediating ORC binding and initiation have been unsuccessful (Dai *et al*., 2005; Segurado *et al*., 2003). Furthermore, although the possibility has been raised that GC-rich sequences are more often utilised in humans, drawing a parallel with fission yeast, it remains likely that origins are determined through chromatin environment as opposed to sequence specificity (Fragkos *et al*., 2015; Leonard & Méchali, 2013).

1.3.1.2. Loading of inactive replicative helicase onto DNA

In late mitosis and early G1-phase, an inactive replicative helicase is loaded onto double stranded DNA. This process is also called origin licensing, which is a stepwise process (**Figure 1-1**). Recent biochemical and structural analyses have identified many of the steps in the loading of inactive replicative helicase onto DNA. There are 3 main steps in this process, which are the binding of the Origin-Recognition Complex (ORC) to DNA, binding of CDC6 to ORCs, and finally the loading of the heptameric protein MCM•CDT1 onto dsDNA.

The first step in the loading of the MCM helicase is the binding of ORCs to dsDNA. 5 of the 6 ORC subunits (Orc1,-2,-3,-4,-5) possess sequence homology with AAA+ proteins, but only the Orc1 and Orc5 components bind ATP. It has been shown that ORC associates with chromatin during the cell cycle in *S. cerevisiae*, however this function is inhibited by CDK during S and G2 phases (Liang & Stillman, 1997).

ORC binding to DNA is sequence-specific in *S. cerevisiae*, however there is no such sequence specificity in metazoans (Leonard & Méchali, 2013). The binding of ORCs to DNA is followed by the recruitment of Cdc6, which is indispensable for the loading of MCM•Cdt1 proteins to the DNA. Cdc6 binding is ATP-dependent and highly transient, only being detected when ATP hydrolysis is inhibited (Speck *et al*., 2005). The *in vivo* and *in vitro* removal of Cdt1 does not hinder the binding of Cdc6 to ORC, however the association of MCM•Cdt1 with ORC requires Cdc6.

Once ORC and CDC6 bind to DNA, the Minichromosome Maintenance Complex (MCM2-7) is loaded onto dsDNA as an inactive double hexamer forming the inactive replicative helicase. Recent works using single molecule microscopy suggest that a single MCM•Cdt1 is loaded onto DNA as an intermediate step, followed by the loading of another MCM•Cdt1 to assemble an inactive double MCM 2-7 helicase with a head-to-head orientation (Ticau *et al*., 2015). After the loading of the first MCM, Cdc6 and Cdt1 are released from the intermediate complex and another Cdc6 is required for the loading of the second MCM•CDT1. The single MCM bound with ORC and Cdc6 has also been observed using cryo-EM analysis (Sun *et al*., 2013), which supports the stepwise loading of MCM. The ring structure of MCM2-7 must be opened in order to load dsDNA into the central channel of the ring, and closed afterwards before DNA melting occurs. An elegant work indicated that the Mcm2-Mcm5 gate has to be opened in order to load DNA into the central channel of the MCM, and forcing the gate to stay closed prevents MCM loading *in vitro* and is lethal *in vivo* (Samel *et al*., 2014). It has been shown in several organisms that multiple inactive MCM2-7 helicases are loaded onto DNA (Dimitrova *et al*., 1999; Donovan *et al*., 1997; Krude *et al*., 1996). However, only 1-10% of these are activated during replication (Hyrien *et al*., 2003). This inactive helicase, and its associated licensing factors, are collectively known also as the Pre-Replication Complex(Pre-RC).

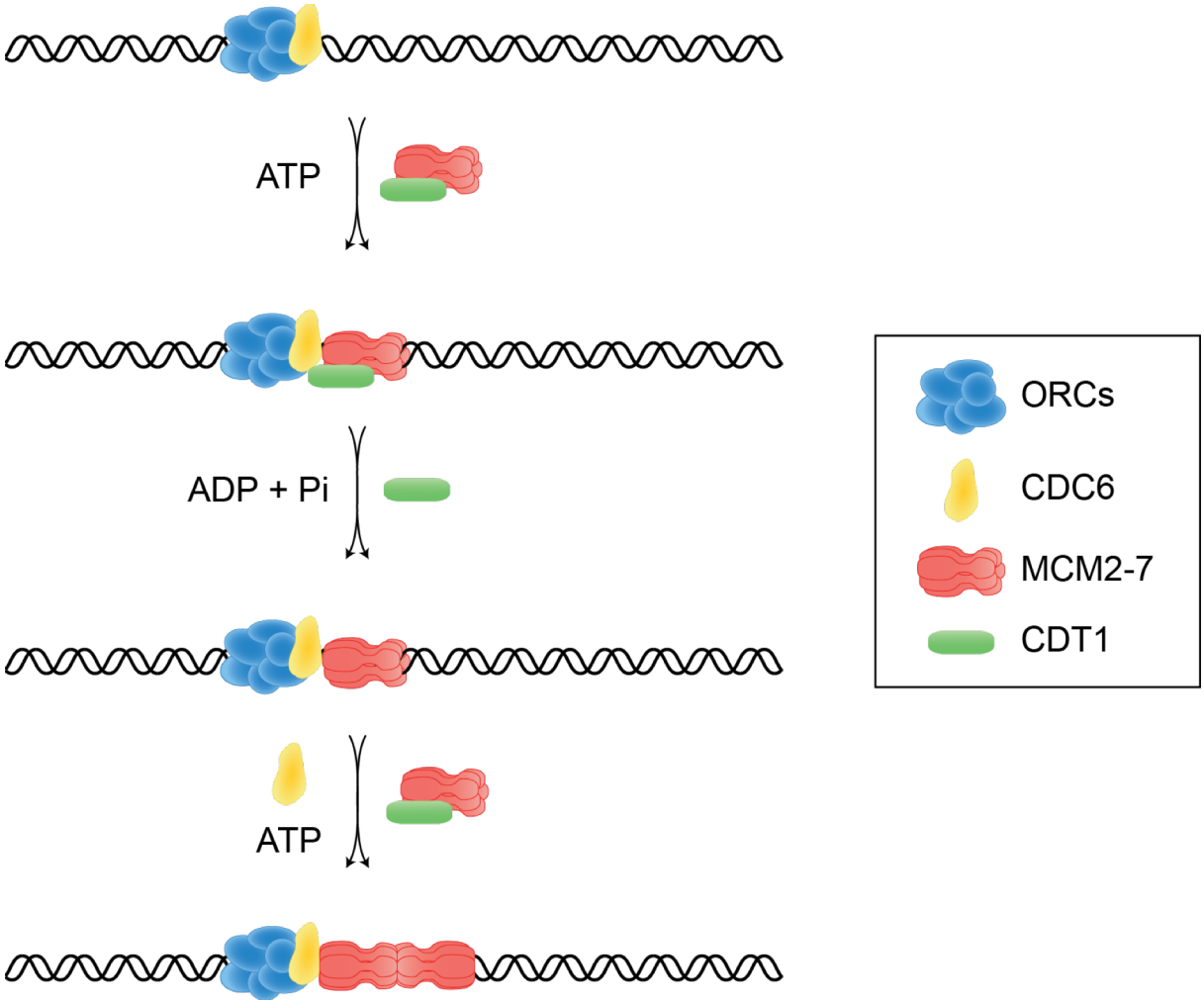


Figure 1-1Loading of MCM double hexamers. Head-to-head double hexamers are loaded in a stepwise procces. Binding of Cdc6 on ORC recruits a single MCM•Cdt1. The Mcm2-Mcm5 gate then opens and encircles dsDNA. The second MCM•Cdt1 requires a new Cdc6.

1.3.1.3. DNA origin firing

The inactive MCM double hexamer has to be activated in order for DNA replication to begin. This involves the formation of the CMG complex, which contains Cdc45, MCM and GINS proteins. This process is tightly regulated through the activity of two essential kinases, DDK and CDK. For the initiation of DNA replication, a number of steps must occur: 1) DNA melting, 2) localisation of ssDNA into the central ring of a single MCM hexamer, 3) recruitment of other

replication fork factors, 4) activation of MCM. The precise order of those events is still not well understood. Recent *in vitro works* have also indicated that 9 replication factors, which are DDK, CDK, Cdc45, Sld3, Dpb11, Sld2, GINS, Mcm10 and DNA Polymerase ε (Pol ε), are necessary and sufficient for the activation of the MCM (Yeeles *et al*., 2015).

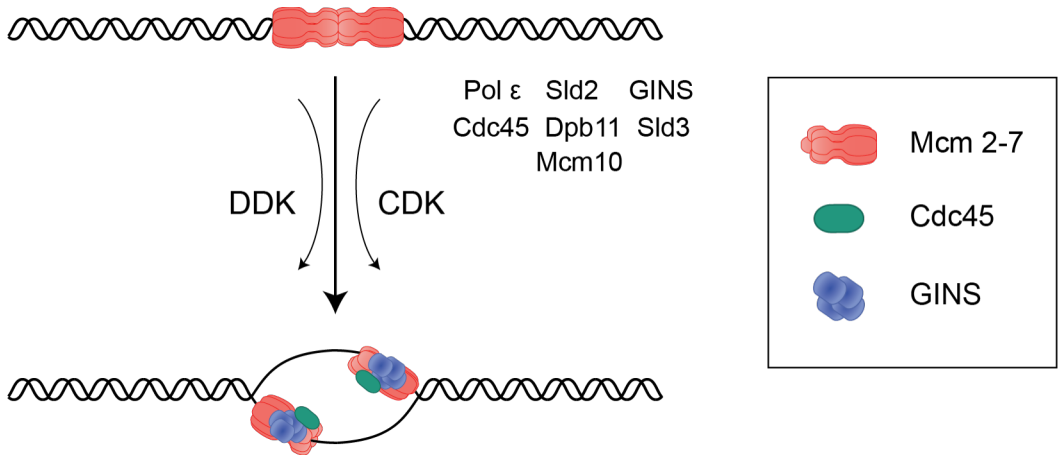


Figure 1-2 The formation of CMG complex in *S. cerevisiae*. The factors necessary for CMG formation are listed in the figure.

Different *in vitro* and *in vivo* studies indicated that DDK phosphorylates MCM (Randell *et al*., 2010; Y J Sheu & Stillman, 2010) which is necessary for the recruitment of Sld3/7 and Cdc45 on the MCM (Heller *et al*., 2011; Tanaka *et al*., 2011). Cdc45 and Sld3 are mutually dependent on one another for their association with the origins, and these proteins are recruited to the origin with Sld7. Because the role of DDK can be bypassed with the *mcm5-bob1* allele, it suggests that MCMs are the only targets of DDK (Hardy *et al*., 1997). Further studies have shown that several MCM subunits are phosphorylated by DDK (Francis *et al*., 2009; Lei *et al*., 1997; Yi Jun Sheu & Stillman, 2006). However, the effect of MCM phosphorylation by DDK on Cdc45 and GINS recruitment is still unclear. On the other hand, it has been established that CDK phosphorylation of Sld2 and Sld3 results in their binding to DPpb11 followed by the recruitment of GINS (Tanaka *et al*., 2007; Zegerman & Diffley, 2007). There are 12 predicted CDK phosphorylation sites (S/TP) in Sld3, two of which (T600 and T622) are essential. Alanine substitutions of Sld3-T600 cells grow very slowly, while T600A, T622A double mutants are not

viable indicating that phosphorylation of Sld3-T600 and T622 requires CDK, and that this phosphorylation is essential for DNA replication. Biochemical studies indicate that phosphorylated Sld3 binds to N-terminal BRCT repeats of Dpb11 (Zegerman & Diffley, 2007). Although it is not possible to bypass CDK phosphorylation of Sld3 using phosphomimetics, a sld3-dpb11 fusion protein can fulfil the CDK requirement (Zegerman & Diffley, 2007). On the other hand, there are 11 CDK phosphorylation consensus sequences in Sld2, however only one of these (T84) is essential and CDK phosphorylation of this residue can be bypassed using phosphomimetic mutants (Zegerman & Diffley, 2007). It has been previously shown that phosphorylation of Sld2 by CDK results in binding of the C-terminal BRCT repeats of Dpb11 (Zegerman & Diffley, 2007). Taken together, this suggests that Sld2 and Sld3 are the minimal set of CDK targets required for DNA replication initiation.

It has been suggested that binding of Sld2 to Dpb11 via CDK phosphorylation results in the formation of a very fragile pre-loading complex (preLC) consisting of Sld2, Dpb11, GINS and Polε (Muramatsu *et al*., 2010). The assembly of the preLC requires CDK activity but no other replication proteins. As described above, Sld3 is associated with the MCM and phosphorylated by CDK. Thus, it has been suggested that CDK phosphorylation of Sld3 brings the preLC to origins, resulting in the assembly of the preIC (Tanaka & Araki, 2013). A recent work by Yeeles *et. al.* (2015) used purified proteins to demonstrate that, although DDK and CDK activities are indispensable for helicase activation and origin firing, the sequential activities of DDK and CDK are not necessary. These two events result in the assembly of the CMG complex and the release of Sld3, Sld2, Dbp11 and Sld7 from origins.

1.3.2. Elongation

Before DNA synthesis can begin, the recruitment of polymerase α is required. Polymerase α functions as an RNA polymerase, synthesising RNA primers of approximately 10bp which are

utilised by Pol ε and Pol δ to begin synthesis and elongation (Langston & O'Donnell, 2006; Tanaka & Araki, 2013). As the leading strand polymerase, only one such priming event is required for the function of Pol ε, in contrast to the lagging strand polymerase Pol δ which relies on the presence of such RNA primers every 1-2 kb (Leman & Noguchi, 2013). This lagging strand synthesis therefore does not produce a continuous DNA strand, in contrast to leading strand synthesis, and instead creates a series of 100-200 bp DNA products known as Okazaki fragments. The Polα-dependent displacement of these RNA primers, the filling of gaps between Okazaki fragments, and the fusion of fragments through the action of DNA ligase, is therefore required to produce a continuous DNA lagging strand (Waga & Stillman, 1998). Throughout this elongation process, the dsDNA is unwound by the active replicative helicase and the resulting ssDNA is bound by Replication Protein A (RPA), which ensures that no secondary structures form which could disrupt the replication process (Wold, 1997).

The characterisation of the replication fork has shown that, during elongation, a number of subsidiary proteins can bind to facilitate DNA replication. An example of this is the Csm3-Tof1-Mrc1 complex, which is believed to help stabilise the replication fork (Bando *et al*., 2009). Given that Mrc1 interacts with MCM6 and Pol ε (Komata *et al*., 2009; Lou *et al*., 2008), and Csm3-Tof1 interacts with MCM2 (Bando *et al*., 2009), it has been hypothesised that Csm3-Tof1-Mrc1 links the helicase to Pol ε. In addition, the homologous recombination-associated repair proteins Rad51 and Rad52 have been shown to be associated with replication forks in *S. cerevisiae* (González-Prieto *et al*., 2013).

The type IB topoisomerase Top1 is also known to be associated with the replication fork (Bermejo *et al*., 2007). Type IB topoisomerases create ssDNA breaks to regulate supercoiling (Wang, 1996). This removal of supercoiling is supplemented through the action of the type II isomerase Top2, which removes supercoils by creating dsDNA. Given that the replication fork can induce supercoiling in nearby DNA, and that the product of DNA replication is a pair of sister chromatids which requires unravelling before mitosis can occur, the joint action of Top1

and Top2 allows both for the resolution of positive supercoils, and for the requisite chromatid separation through Top2 function. Together, therefore, these topoisomerases ensure chromosomal stability during and subsequent to DNA replication (Jeppsson *et al*., 2014; Wang, 2002).

1.4. DNA replication control

DNA must be replicated once and only once in every cell cycle, and any additional replication during the cell cycle results in gene duplication, polyploidy or chromosomal re-arrangements which are a hallmark of several diseases such as cancer (B. M. Green *et al*., 2010). The initiation of replication in eukaryotic cells is a two-step process. The first step occurs during either G1 phase or late in mitosis, and entails the loading of an inactive Mcm2-7 helicase complex onto dsDNA at sites known as origins of replication. During the second step, in S-phase, this helicase is activated to form the CMG complex. As both of these steps take place at mutually exclusive times in the cell cycle, it is essentially guaranteed that each origin of replication is initiated only once per cycle. Thus, the temporally separated two-step nature of this process provides a further level of fidelity to the replication process.

The initiation process is regulated through differential expression, and therefore activity, of the antagonistic factors CDK and APC/C (King *et al*., 1995). The E3 ubiquitin ligase APC/C targets a number of proteins involved in cell cycle control such as Cyclins and targets them for proteasome-dependent degradation (Visintin *et al*., 1997), whilst CDK affects cell cycle factors through phosphorylation. At the start of G1, CDK exhibits little activity, but increases over the course of the phase and maintains a strong activity from thereon. This is contrasted by APC/C, which accumulates throughout both anaphase and telophase and maintains this level until the end of G1, when it is inactivated by CDK. This system is further maintained through the action

of CDK inhibitors (CKIs), which exhibit an activity profile similar to APC/C and are degraded at the end of G1 following phosphorylation by CDK (E Schwob *et al.*, 1994).

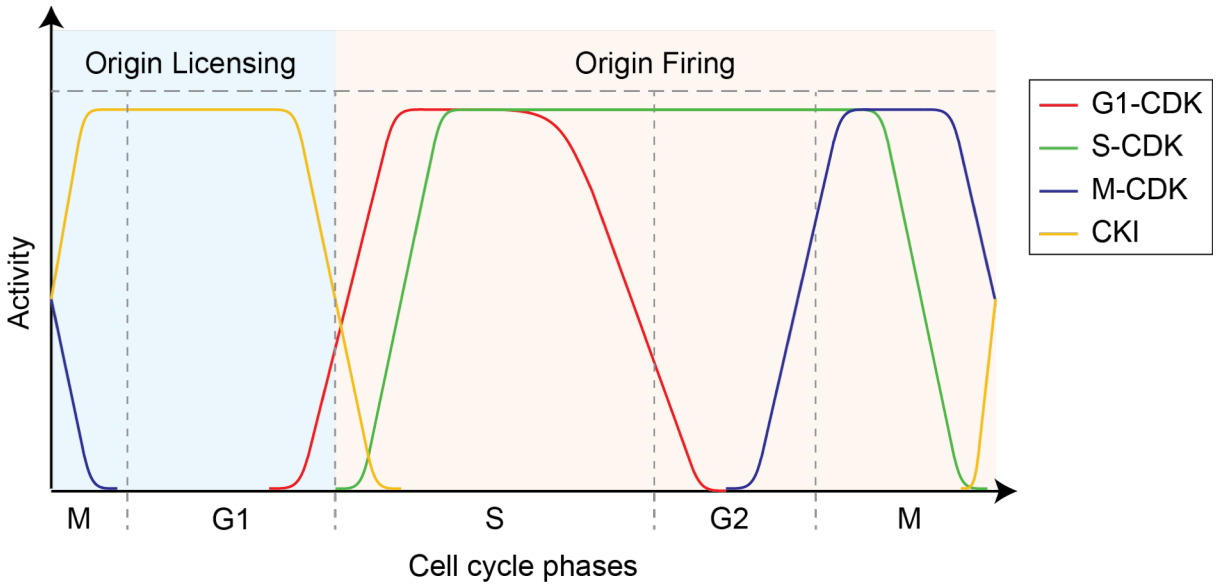


Figure 1-3 The control of cell cycle. The cell cycle is controlled by the activity of different CDKs.

Studies in *S. cerevisiae* have been successful in elucidating the mechanisms through which CDK activity in S-phase inhibits the assembly of the pre-RC, consistent with the observed roles for CDK as a safeguard against re-replication, and the simultaneous modification of CDK targets results in multiple re-replication events during S or G-2 phase (For details see next section).

This CDK-dependent regulation is not the only level of control ensuring re-replication does not occur, as regulation of DDK has also been show to play a role. DDK is a conserved protein kinase composed of catalytic (Cdc7) and regulatory (Dbf4) subunits. Given that DDK phosphorylates the Mcm2-7 complex during S-phase replication initiation, the lack of Dbf4 during both mitosis and G1 ensures that Mcm2-7 can only be activated at the correct stage of the cell cycle.

This temporal separation of licensing and activation is conserved in metazoans, whilst additional levels of control have also been identified. The first relies on geminin, a nuclear protein which inhibits Cdt1 binding to chromatin in both the S and M phases, whilst being degraded following APC/C targeting during G1 (C. Lee *et al*., 2004; Wohlschlegel *et al*., 2000). The second is the phosphorylation of any Cdt1 bound to chromatin by CDK in S-phase, which results in Cdt1 degradation through SCF^{CRL4} ubiquitin ligase activity (Senga *et al*., 2006; Zhong *et al*., 2003).

1.4.1. Cell cycle regulation of preRC formation

To prevent re-replication, loading of the replicative helicase – termed origin licensing - is temporally separated from helicase activation and replisome assembly. Specifically, origin licensing is exclusively allowed in late Mitosis and G1 phase, whereas helicase activation and replisome assembly is allowed in S-phase.

Although they can vary between different organisms, several molecular mechanisms have evolved to prevent origin licensing during S and G2 phases. In *S. cerevisiae,* modification of preRC components by CDK inhibits the loading of the MCM2-7 complex onto dsDNA outside of G1 phase (**Figure 1-4**). CDK can also phosphorylate Orc2 and Orc6, which prevents Cdt1-mediated MCM2-7 loading. The ORC6 has four CDK consensus sites which are 106, 116, 123, and 146, whereas ORC2 has six which are 16, 24, 70, 174, 188, and 206 (Nguyen *et al*., 2001). The Serine/Threonine substitutions of those residues with Alanine can bypass the regulation by CDK. On the other hand, CDC6 is degraded in a SCF^{CDC4}-dependent manner and the degradation of CDC6 can be prevented by removing the N-terminal part of Cdc6 (CDC6ΔNT) (Drury *et al*., 1997). Finally, MCM2-7•Cdt1 can be excluded from the nucleus, and forcing the localisation of Mcm7 via the addition of a Nuclear Localization Signal (NLS) results in the constitutive localisation of Mcm2-7•Cdt1 to the nucleus (Nguyen *et al*., 2000). Introducing

these mutations and modifications bypasses the control of CDK on origin licensing, and can induce re-replication in G2 phase (Nguyen *et al*., 2001).

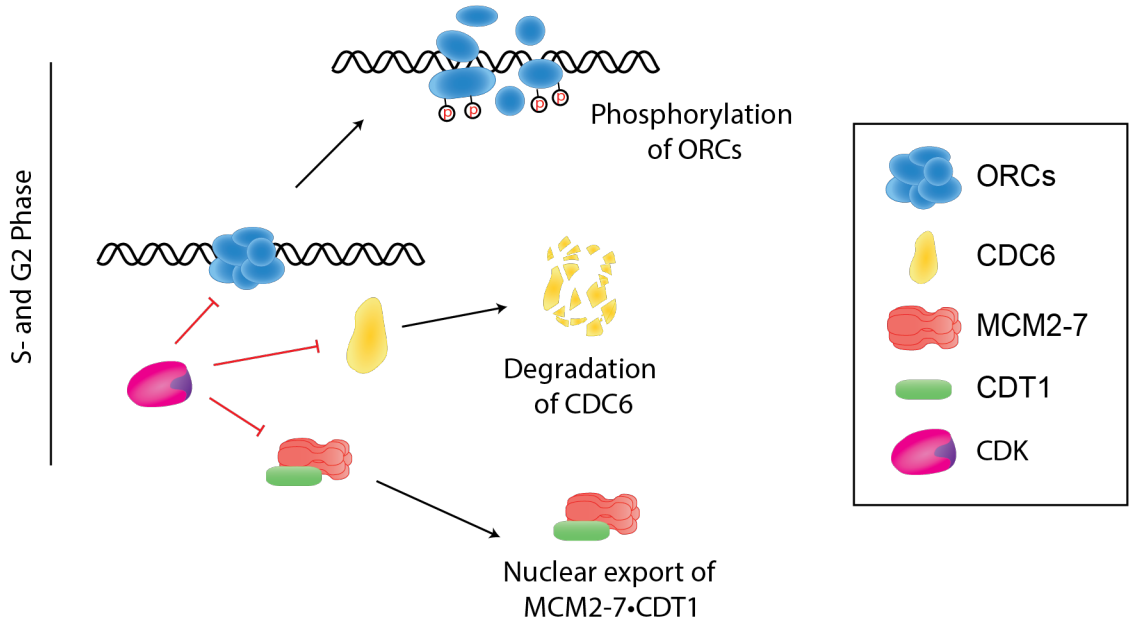


Figure 1-4 Prevention of re-replication by CDK in *S. cerevisiae*. The diagram shows the targets of CDK to prevent origin licensing in S and G2 phases. 1) CDK phosphorylates Orc2 and Orc6 which prevents the loading of MCM•Cdt1. 2) CDK regulates the degradation of Cdc6. 3) CDK control the nuclear export of MCM•Cdt1

Unlike *S. cerevisiae,* CDT1 and the *S. pombe* CDC6 homolog CDC18 are degraded during S and G2 phase, and the overexpression of CDT1 and CDC18ΔNT are enough to bypass the control of origin licensing and result in re-replication in *S. Pombe* (Nishitani *et al*., 2000). The *S. pombe* CDT1 is degraded in a Cul4–Ddb1– Cdt2 dependent manner (Ralph *et al*., 2006). The degradation of CDT1 is a conserved mechanism for controlling origin licensing among other eukaryotes. It has been shown that redundant pathways degrade CDT1 in a cell cycle-dependent manner. Chromatin-bound CDT1 is degraded in a Cul4-DBP1-Cdt2 E3 ligase-dependent manner and is mediated by PCNA, ensuring that degradation of chromatin-bound CDT1 only occurs during S-phase (Senga *et al*., 2006). On the other hand, the degradation of CDT1 by SCF-Skp2 occurs in both S- and G2-phase. The function of CDT1 is also mediated

by the inhibitor Geminin. The overexpression of CDT1 in Human, *Xenopus* and *C. elegans* also results in re-replication (Mech, 2014; Melixetian *et al*., 2004; Vaziri *et al*., 2003).

1.4.2. Cell cycle control of origin firing

Prevention of re-replication is not only regulated via origin licensing, but also through the temporal separation of origin firing. Origin firing requires the activity of two kinases: DDK and S-CDK. S-CDK phosphorylates Sld2 and Sld3, which allows their binding to the amino and carboxy terminals of Dpb11 (Tanaka *et al*., 2007; Zegerman & Diffley, 2007). Yeast cells expressing the phosphomimetic mutant of Sld2 and Sld3-Dpb11 fusion protein can initiate replication in the absence of CDK activity, suggesting that Sld2 and Sld3 are the only CDK targets for replication initiation (Zegerman & Diffley, 2007). Because G1-CDK cannot phosphorylate Sld2 and Sld3 during G1 phase, replication initiation can only occur in S-phase. DDK phosphorylates different subunits of MCM2-7 in S-phase (Francis *et al*., 2009; Lei *et al*., 1997; Yi Jun Sheu & Stillman, 2006). Dbf4 - the regulatory subunit of DDK- level oscillates heavily throughout the cell cycle, with its highest expression occurring during S-phase, before its APC/C mediated degradation in mitosis (Godinho *et al*., 2000; Oshiro *et al*., 1999; Weinreich & Stillman, 1999). This degradation ablates Dbf4 levels during G1 and ensures that DDK is only activated in S-phase (Godinho *et al*., 2000).

1.4.3. Temporal programme of replication

DNA replication is a heavily regulated process in eukaryotic cells, and the asynchronous timing of S-phase origin firing is a crucial step of this process. During the transition from G1 to S phase, some origins are immediately activated by the CDK and DDK kinases, whilst others are only activated during later stages of replication. A further subset, known as dormant origins,

are not activated at all, instead being replicated passively following origin firing at other sites (Santocanale *et al*., 1999). Early origins start to be fired at the beginning of S phase, with other later origins continue to fire throughout S phase at different times. In both fission and budding yeasts, and higher organisms, certain areas of the genome replicate on average at early or late stages using multiple origins to do so (Heichinger *et al*., 2006; McCune *et al*., 2008; Zink *et al*., 1999).

The specific timing underlying origin firing, in addition to origin sequence and location, exhibit strong conservation amongst members of the *Saccharomyces* genus (Mueller & Nieduszynski, 2012). Furthermore, though the sequence of origins is not conserved, the broad patterns of replication timing found across the chromosome are conserved both in *S. pombe* and in higher organisms (Ryba *et al*., 2010; Xu *et al*., 2012; Yaffe *et al*., 2010). This suggests that the timing of replication is not controlled by factors which delineate origin location, such as sequences, but is determined through other factors. Recent works in *S. cerevisiae* and *X. laevis* showed that some replication factors become limited during replication, and an increase in the abundance of those factors leads to cellular and developmental defects (Collart *et al*., 2013; Mantiero *et al*., 2011).

1.5. Causes and consequences of DNA replication stress

1.5.1. Obstacles to DNA replication

A number of endogenous and exogenous obstacles may halt replisome progression. These factors act either to hamper the action of helicases or polymerases or both, disrupting the integrity of the replication fork (Cortez, 2005; Zegerman & Diffley, 2009). Endogenous barriers

which can stall replication prior to completion are rDNA repeats, centromeres, tRNA sites, HMR and HML loci, G-quadruplexes, and Ty long-terminal repeats. These blocks are most likely able to stall replication due to their ability to form disruptive secondary structures, or due to associated DNA-binding proteins acting as a barrier to further replication (Durkin & Glover, 2007; Hansen *et al*., 1993; H. Kim & Livingston, 2009; J. Lu *et al*., 1996). However, whilst some barriers are likely unintentional, several have been identified which are programmed. An example of this is the polar replication barriers in yeast rDNA repeats, which act to ensure that replication and transcription occur in the same direction, thus preventing the collision of the two complexes (Brewer & Fangman, 1988; Krings & Bastia, 2005; Linskens & Huberman, 1988; Sacher M.nchez-Gorostiaga *et al*., 2004). These sites often undergo recombination, and bind Fob1 at the fork barrier to pause replication fork progression (J. Huang & Moazed, 2003; Kobayashi, 2003; Kobayashi & Horiuchi, 1996). However, it is likely that other factors also play a contributing role in recombination beyond the pausing of the replication fork at these repeat units (Labib & Hodgson, 2007; Ward *et al*., 2000). In order for successful fork pausing to occur, the Tof1-Csm3-Mrc1 complex associates with the replication fork in *S. cerevisiae* (Katou *et al*., 2003; K Labib & Hodgson, 2007; Tourrière *et al*., 2005). Given that both Mrc1 and Tof1 mutants are associated with a decreased stability of DNA repeats, it is possible that this complex binding acts to prevent recombination events at stalled forks, providing added stability to the recombination process (Shishkin *et al*., 2009; Voineagu *et al*., 2009). Sites which are prone to fork collapse upon replication fork stall are known as fragile sites, and are increasingly being implicated in human disease (Aguilera & Gomez-Gonzalez, 2008; Cha & Kleckner, 2002; Lemoine *et al*., 2005; Mirkin, 2007; Raveendranathan *et al*., 2006).

The replication fork often stalls at regions which are transcribed by RNA polymerase II and at sites of rRNA transcription, and stalling also occurs at tRNA sites when the tRNA is undergoing transcription (Azvolinsky *et al*., 2009; A. M. Deshpande & Newlon, 1996; Prado & Aguilera, 2005). Together, these results suggest that replication and transcription perturb the activities of one anothers' machinery, likely through the action of supercoiling inhibiting the efficiency of

replication, or through direct complex collision (Helmrich *et al*., 2013). When collision occurs, an RNA-DNA hybrid known as an R-loop forms, with their overabundance or increased length being correlated with genomic instability (Helmrich *et al*., 2013; Huertas & Aguilera, 2003) . Given that both transcription and replication induce positive supercoiling of the DNA , it is likely that the joint action of both machineries creates an additive effect which can lead to fork arrest (Brill *et al*., 1987; Lin & Pasero, 2012; L. F. Liu & Wang, 1987; Osborne & Guarente, 1988).

Many chemotherapeutic drugs also induce replication fork stalling, and consequent cell cycle arrest, through their genotoxic action (Huls & ten Bokkel Huinink, 2003; Lanzkron *et al*., 2008; Ulukan & Swaan, 2002) (see **Table 1-1**). These drugs can function through a variety of mechanisms, and understanding these mechanisms is crucial to developing future therapies for widespread disorders such as cancer. One such mechanism for genotoxicity is through the depletion of nucleotides via inhibition of ribonucleotide reductase, resulting in a halting of dNTP formation (Yarbro, 1992). Through the deprivation of new nucleotides, the replication fork is forced to stall, resulting in an uncoupling of the helicase-polymerase complex and cell cycle halting through the S-phase checkpoint. Whilst one response to this checkpoint is the upregulation of nucleotide production, counteracting the action of genotoxic drugs such as hydroxyurea (HU) which deplete the nucleotide pool, the result is a shifted equilibrium wherein cells replicate at a significantly reduced speed. Other replication-associated proteins can also be targeted by drugs to impede the replication process. Topoisomerase 1 is the target of the chemotherapeutic camptothecin, which covalently links Top1 to DNA and creates ssDNA breaks (Hsiang *et al*., 1985). This anchorage of Top1 to DNA creates dsDNA breaks when encountered by replication forks, but checkpoint activation is not as potent as with HU since minor ssDNA is produced upon replication fork stalling. The covalent modification of bases can also stall replication, as evidenced by the action of methyl methanesulfonate (MMS), which methylates purines (Beranek, 1990). The subsequent base mispairing halts fork progression, with a corresponding fall in the rate of S-phase progression by as much as 10-fold (Tercero & Diffley, 2001; Tercero *et al*., 2003). Other drugs, such as phleomycin, induce single or double

strand breaks through their intercalation in DNA. These lesions hinder progression of the replisome, again reducing cell cycle progression. These breaks in the DNA can also occur following exposure to ionising radiation, whilst UV radiation as well as 4-NQO produce adducts that block polymerase progression and activate the S-phase checkpoint as a result.

Types of Stress	Result	Main responders
Hydroxyurea (HU)	dNTP pools depletion	Mec1, Mrc1, Sgs1
Camptothecin (CPT)	Topoisomerase I inhibitor	Mec1/Tel1, Rad9
Methylemethanesulfonate (MMS)		Mec1, Rad9
Ultraviolet light (UV)	DNA adducts	Mec1, Rad9, Mrc1
4-Nitroquinoline 1-oxide (4-NQO)	DNA adducts	Mec1, Rad9, Mrc1
Ionizin irradiation (IR)	Single and double strand breaks	Mec1/Tel1, Rad9
Phleomycin	Single and double strand breaks	Mec1/Tel1, Rad9

Table 1-1 Sources of DNA damage. The table show different sources of DNA damage and the responses of *S. Cerevisiae.*

1.5.2. S-phase checkpoint

Historically, the S phase checkpoint has been characterized as being responsible for the maintenance of genomic integrity following detection of stalled replication forks or damaged DNA during replication. Different types of lesions can activate the S phase checkpoint, such as excessive single-stranded DNA (ssDNA), single strand-double strand junctions (ss-dsDNA), single strand DNA breaks, as well as double strand DNA breaks. The formation of such lesions are detected by different sensor proteins, which results in the activation of the master kinases Mec1/Tel1 in yeast and ATM/ATR in humans. Mec1/Tel1 transmit the signal to the effector kinases Chk1/Rad53 in yeast and Chk1/Chk2 in humans. Activation of these effectors initiates and regulates the following responses: 1) inhibition of late origin firing, 2)

stabilization the stalled replication forks until stalling can be reversed, 3) regulation of transcription 4) mitotic delay. Many factors involved in this checkpoint are oncogenic, and thus the characterisation of this checkpoint is important for the development of novel cancer therapies.

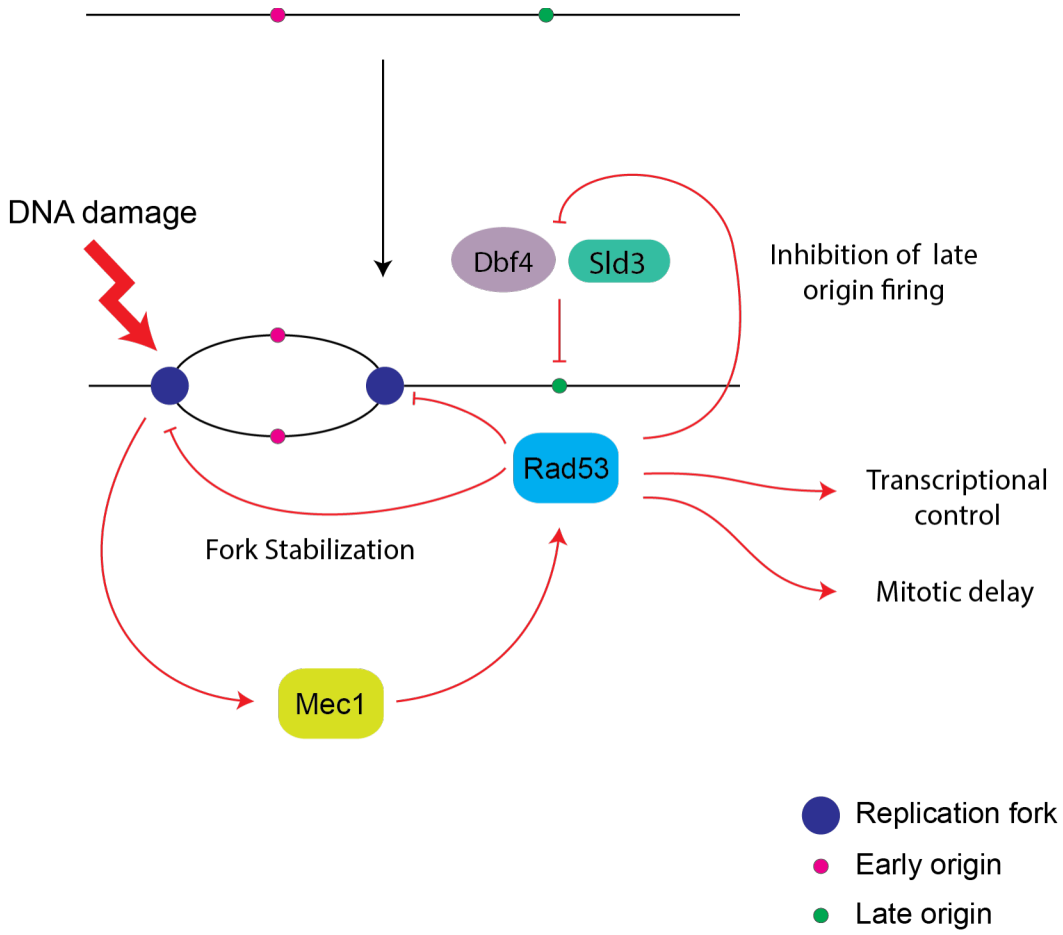


Figure 1-5 The function of the S-phase checkpoint. A simplified diagram showig the roles of Rad53 in S-phase checkpoint. Activation of Rad53 by Mec1 inhibitis origin firing, controls transcription, stablizes stalled forks and delays mitosis.

1.5.2.1. Activation of the S-phase checkpoint

Single-stranded DNA often forms as a result of replication fork stalling, as a consequence of the uncoupling and subsequent functional loss of components of the replication machinery such as the helicases and polymerases (Aguilera & Gomez-Gonzalez, 2008; Branzei & Foiani, 2007; Segurado & Tercero, 2009). The protein RPA firstly binds to ssDNA, which then binds the PI3K-like kinase (PIKK) Mec1, through the action of its Ddc2 regulatory subunit (Paciotti *et al*., 2000; Zou & Elledge, 2003). The stalled forks also create ss-dsDNA junctions which are then recognized by Rad24-RFC and results in the loading Ddc1-Rad17-Mec3 checkpoint clamp on the junction (9-1-1 complex in Humans) (Majka & Burgers, 2003). The Mec1-dependent phosphorylation of Ddc1 allows the recruitment of Dpb11, which amplifies Mec1 activation (Puddu *et al*., 2008).

On the other hand, dsDNA breaks, which can be induced by the persistence of replication fork stalling, are recognized initially by Mre11-Rad50-Xrs2 and result in the resection of one strand (D'Amours & Jackson, 2001). The resected DNA forms both an ssDNA and also an ss-dsDNA junction which are then both recognized by RPA and Rad24-RFC. Overall, the formation of lesions which halt the replication fork progression activates the S-phase checkpoint in multiple ways. However, it appears that the abundance of stalled forks is proportional to the degree of checkpoint activation (Lupardus *et al*., 2002; Stokes *et al*., 2002; Tercero *et al*., 2003). Mec1 and Tel1 activation can also occur in G2 and G1 phase if DNA damage occurs, but usually to a lesser extent (Zegerman & Diffley, 2009).

Once, Mec1/Tel1 is activated, the signal has to be transmitted to the effector kinase Rad53 (functional homolog of human Chk1). The activation of Rad53 requires priming by Mec1/Tel1 and is followed by its auto-phosphorylation (Hustedt *et al*., 2013). Several studies indicated that the Mec1-dependent phosphorylation of Rad53 requires the help of the mediator proteins Mrc1, Rad9 or Sgs1. In this context, the phosphorylation of the replisome factor Mrc1 by Mec1

results in the activation of Rad53 (Alcasabas *et al*., 2001; Osborn & Elledge, 2003). However, whether this event occurs on the replication fork or not is not yet clear. Rad53 activation can also be mediated by Rad9. The phosphorylation of the H2A histone by Mec1/Tel1 or methylation of the H3 histone by Dot1 recruits Rad9 on chromatin. Rad9 is phosphorylated by Mec1/Tel1 to which Rad53 can bind and become activated. Sgs1 is also proposed to be another mediator for Rad53 activation (Hustedt *et al*., 2013). The Human WRN homolog of Sgs1 interacts with DNA Isomerase Top3l and its deletion leads to increased chromosome mis-segregation (Watt *et al*., 1995). Rad53 can bind to Sgs1 both *in vivo* and *in vitro* (Bjergbaek *et al*., 2005; Hegnauer *et al*., 2012). Given that the deletion of Sgs1 does not change the activation of Rad53 during HU-induced fork stalling (Bjergbaek *et al*., 2005), the full function of Sgs1 in Rad53 activation remains elusive.

1.5.2.2. Function of the S-phase checkpoint

1.5.2.2.1. Inhibition of origin firing

The observation that patients with the Ataxia telangiectasia (AT) disease synthesised new DNA following ionising radiation, where their non-mutant counterparts did not, lead to the identification of the ATM (Ataxia Telangiectasia Mutated) gene, and resulted in the conclusion that ATM inhibits DNA replication after ionising radiation exposure (Painter & Young, 1980; Painter, 1977). Upon further study, this was found to be due to a failure of inhibition of origin firing in S-phase (Larner *et al*., 1999; Lee *et al*., 1997). Rad53 can also block the initiation of late origins following DNA damage or replication stalling through treatment of budding yeast cells with HU or MMS (Paulovich & Hartwell, 1995; Santocanale & Diffley, 1998; Santocanale *et al*., 1999; Shirahige *et al*., 1998), through its phosphorylation-dependent inhibition of Sld3 and Dbf4 (Duch *et al*., 2011; Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). This phosphorylation prevents interactions between Sld3 and the replication factors Dpb11 and

Cdc45, blocking the activation of pre-RCs. This mechanism is corroborated by the observation that Sld3 and Dbf4 alleles lacking RAD53 phosphorylation sites still show replication occurring following DNA damage and fires late origins (Duch *et al*., 2011; Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). The consequence of this action is that any further origins remain inhibited and do not fire upon checkpoint activation, reducing the rate of S-phase (Zegerman & Diffley, 2010).

In *S. pombe,* whilst the S-phase slows down in response to genotoxic treatment (Kim & Huberman, 2001; Marchetti *et al*., 2002), this may not be due to inhibition of origin firing given that checkpoint-proficient cells show only a limited repression of origin firing (Mickle *et al*., 2007). It is possible that this slowing of replication is due to a slowing of fork speed itself, though this remains unconfirmed.

In higher eukaryotes, Chk1 – a functional homolog of Rad53 – again slows replication through origin firing inhibition, potentially by preventing the loading of Cdc45 onto origins of replication, stopping the assembly of CMG complex and any resultant further replication (Ge & Blow, 2010; Maya-Mendoza *et al*., 2007; Petermann, Woodcock, *et al*., 2010). It has been proposed that this occurs by inactivating S-CDK Cdk2 through the Chk1-dependent degradation of the Cdk2 activator Cdc25 (Falck *et al*., 2001; Mailand *et al*., 2000). Chk1 may also inhibit origins by regulating DDK activity, which is required for loading of Cdc45 onto chromatin and therefore activating the helicase (Costanzo *et al*., 2003; Heffernan *et al*., 2007). It is also possible that the association of Treslin and TopBP1 - the yeast homolog of Sld3 with the homolog of Dbp11, respectively - is inhibited following activation of the S-phase checkpoint, potentially through the phosphorylation of Treslin (Boos *et al*., 2011). This proposal is supported by the recent observation that Chk1 can regulate origin firing by phosphorylating, and therefore inhibiting, Treslin during normal S-phase (Guo *et al*., 2015).

1.5.2.2.1. Replication fork stabilisation and restart

Given that the completion of DNA replication following a temporary genotoxic drug treatment does not require any further origin firing, it appears that stalled forks can restart after stalling (Bousset & Diffley, 1998). The completion of replication following replication fork stalling relies on both Mec1 and Rad53, as cells lacking these kinases arrest in S-phase and are unable to recover from replication blockage as stalled forks eventually collapse (Desany *et al*., 1998; Lopes *et al*., 2001; Tercero & Diffley, 2001). The checkpoint-dependent phosphorylation of several fork components such as Mrc1, Psf1, Pol3, Pol31 has been observed previously (S. Chen *et al*., 2010; De Piccoli *et al*., 2012; Osborn & Elledge, 2003; Smolka *et al*., 2007) . In addition, since the ectopic expression of Rad53 cannot rescue the stalled fork after temporary HU treatment in Rad53-null cells, Rad53 stabilizes these forks possibly through the phosphorylation of fork components, though the exact functional relevance of this phosphorylation remains unknown (Tercero *et al*., 2003). On the other hand, analysis of fork proteins showed that replication fork components still associate with chromatin in the absence of Rad53 activity upon fork stalling. This indicates that Rad53-dependent phosphorylation is crucial for the maintenance of fork functionality and prevents irreversible inactivation of the fork (De Piccoli *et al*., 2012). Given that replication forks cannot restart following long exposures to HU – in contrast to short exposure - in human cells when the checkpoint is functional (Petermann *et al*., 2010), it is likely that checkpoint-dependent fork stabilisation must occur within a specific window of time if the cell cycle is to restart.

Several lines of evidence indicate that replication forks tend to produce aberrant DNA structures such as four-way junctions (also known as chicken foot structures) at stalled replication forks in checkpoint-deficient cells (Lopes *et al*., 2001; Sogo *et al*., 2002). The pertinent studies indicate that Rad53 prevents the formation of aberrant DNA structures at stalled forks (Lopes *et al*., 2001; Sogo *et al*., 2002). Exonuclease Exo1 is recruited to stalled

replication forks in Rad53-deficient cells, and the deletion of Exo1 prevents irreversible fork collapse after fork stalling (Cotta-Ramusino *et al*., 2005; Segurado & Diffley, 2008). Although Exo1 is phosphorylated by Rad53 upon HU treatment, the exact mechanism of the regulation of Exo1 by Rad53 is still unknown. Given that Exo1 deletion does not suppress the HU sensitivity of Rad53 deficient cells, stabilisation of the stalled replication fork appears to require other actions of Rad53.

Given that Exo1 deletion cannot rescue the stalled forks from collapse in *mec1Δ* cells after HU treatment (Segurado & Diffley, 2008), Mec1 must have other roles beyond that of Rad53 activation. Although the main function of Mec1 is to activate effector kinases, Mec1 can also specifically phosphorylate some of its substrates close to stalled replication forks, such as RPA or Rtt107 (Liu *et al*., 2006; Rouse, 2004). Replication fork stabilisation therefore appears to be a complex and multifaceted process.

1.5.2.2.2. Mitotic Delay

In addition to its role in the inhibition of DNA replication initiation, the S-phase checkpoint also plays roles in delaying mitosis. A number of processes have been identified that block cells at the transition between metaphase and anaphase if DNA damage is detected. Three pathways have been identified thus far which lead to the stabilisation of Pds1, a securin known to inhibit separases. This inhibition of separases results in the increased stability of cohesins, which hold sister chromatids together, placing a block on mitosis at the metaphase-anaphase transition.

One pathway again relies on Rad53, which has been shown to block interactions between the APC/C activator Cdc20 and Pds1, which normally results in Pds1 degradation (Agarwal *et al*., 2003). While the exact mechanism responsible for this interaction blockage remains unknown,

the result is the inability for chromosomes to separate, and mitotic arrest. Chk1, the second checkpoint effector kinase, also plays roles in the stabilisation of Pds1 (Sanchez *et al*., 1999). The third mechanism relies on the activation of the Spindle Assembly Checkpoint (SAC) by the phosphorylation of Mad1, Mad2, Mad3, Bub1, and Bub3 by Mec1 and Tel1, resulting in Cdc20 inhibition and Pds1 stabilization (Kim & Burke, 2008). In addition to these three pathways resulting in Pds1 stabilization, Rad53 can also block mitosis by inhibiting Cdc5, resulting in the activation of Bub2/Bfa1 (Hu *et al*., 2001).

It is also possible that mitosis can be prevented through the protein kinase activity of the CDK inhibitor Swe1. Swe1 is known to regulate the transition from G2 to M phase through its CDK inhibitory activity, which is controlled through the balance between phosphorylation of the conserved Y19 residue of Cdc28 by Swe1 and dephosphorylation by Mih1 (Booher *et al*., 1993; Harvey *et al*., 2005). This mitotic delay is dependent on cell size, and can be triggered by checkpoints which detect anomalies in cell or bud (Harvey & Kellogg, 2003; Lew & Reed, 1995; Lew, 2003). The mammalian homolog of Swe1 is Wee1, which can delay mitosis in response to replicative stress or DNA damage (Jin *et al*., 1996). However, the role of Swe1 in DNA damage-induced mitotic delay remains poorly understood in *S. cerevisiae.* Whilst Swe1 is known to accumulate in response to DNA damage response (Liu & Wang, 2006), it has not been confirmed that this accumulation is a result of checkpoint activation (Bastos de Oliveira *et al*., 2012; Keaton *et al*., 2007). However, work by Magiera *et al*. (2014) suggests that Swe1 is constitutively active in wild type cells, and therefore the checkpoint could be active to a limited extent under normal conditions. Finally, a recent work demonstrated that Ndd1, the transcriptional activator for the CLB2 cluster, is inactivated by the joint action of Mec1 and Swe1 in response to DNA damage, suggesting that Swe1 may well play additional roles in the DNA damage response (Edenberg *et al*., 2015).

1.5.2.2.3. Transcriptional control

A major result of checkpoint activation is the upregulation of several hundred genes via two distinct mechanisms. The first mechanism is the Rad53-dependent phosphorylation of Rad53 related kinase Dun1 (Chen *et al*., 2007; Zhou & Elledge, 1993), which then acts to inhibit the function of the transcriptional repressor Crt1 (Huang *et al*., 1998), resulting in the upregulation of several genes including those involved in ribonucleotides synthesis and repair of DNA. This is corroborated by the observations that both Mec1 and Rad53 function to upregulate production of dNTPs for the repair of DNA damage during S-phase, and that cells which lack either of these proteins can be restored to viability through the activation of other mechanisms to upregulate dNTP production (Huang *et al*., 1998; Zhao *et al*., 2001). The second mechanism is the phosphorylation of Swi6, a subunit of the transcriptional activators MBF and SBF, by Rad53, resulting in an upregulation of other genes involved in DNA repair (Sidorova & Breeden, 1997).

1.5.2.3. Checkpoint recovery

After DNA damage has been repaired, or obstacles to fork progression have been removed, it is necessary for cells to inactivate the checkpoint in order for the cell cycle to continue. This recovery occurs alongside the dephosphorylation of Rad53, resulting in its inactivation. The phosphatases Ptc2, Ptc3, and Pph3 have all been shown to play a role in checkpoint recovery (Guillemain *et al*., 2007; Keogh *et al*., 2006; Leroy *et al*., 2003). These phosphatases function during recovery from different stresses, with Ptc2 and Pph3 required during the MMS response, whilst Glc7 is required during the HU response (Bazzi *et al*., 2010; O'Neill *et al*., 2007; Szyjka *et al*., 2008). Other recovery pathways do not rely on the action of phosphatases, such as the dampening of sensor kinase activity via Srs2 or Sae2, however the molecular mechanisms underlying this dampening remains broadly unknown (Krejci *et al*., 2003; Veaute

et al., 2003; Yeung & Durocher, 2011). Checkpoint recovery appears to be a multifaceted process, involving several layers affecting different proteins depending on the nature of the DNA damage or replication stress which first activated the checkpoint.

1.6. Work Presented in this thesis

DNA damage checkpoints have been proposed to act as a barrier for the suppression of cancer development (Bartkova *et al*., 2005, 2006; Gorgoulis *et al*., 2005). Indeed, checkpoint kinases are often deregulated in many cancers, as well as in other diseases, which makes them important targets for effective treatments (Chen *et al*., 2012). In addition, this checkpoint is crucial for the development of in metazoans in midblastula transition (MBT) (Budirahardja & Gönczy, 2009; Collart *et al*., 2013). Despite the fact that they play vital roles in organisms, the mechanisms underlying the DNA damage checkpoints are poorly understood.

The work described in this thesis aims to investigate temporal and substrate specificity of the S phase checkpoint in budding yeast. I mainly focus on a particular response of the S phase checkpoint termed the inhibition of origin firing. In the first results chapter, chapter 3, I investigate whether the checkpoint dependent inhibition of origin firing is targeted outside of S phase. Interestingly, the DNA damage checkpoint effector kinase Rad53 targets its substrates Sld3 and Dbf4 outside of S phase upon DNA damage. I then investigate the biological significance of the checkpoint-dependent inhibition of origin firing outside of S phase in chapter 4. Having shown that the checkpoint response is not specific for any stage of the cell cycle, I examined how Rad53 targets its substrates in chapter 5. I found that Rad53 requires the disordered loop of Cdc45 to phosphorylate Sld3 upon DNA damage. Given that Cdc45 is also required for the DNA replication forks in addition to replication initiation, in chapter 6, I investigate whether Cdc45 targets Rad53 to replication fork factors in S phase.

The results presented in this thesis are divided into 4 separate chapters (Chapter 3-6). Each of these chapters has a brief introduction that explains the rationale behind the experiments, followed by detailed results and a subsequent discussion. The results presented underlie the temporal as well as substrate specificity of the S Phase checkpoint after DNA damage, and suggest that the S phase checkpoint functions through the cell cycle and Cdc45 is required for S phase checkpoint responses.

Chapter 2

Materials and Methods

2.1. Yeast-related methods

2.1.1. Yeast strains used in this study

Yeast strains used in this study are based on W303 *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3, 112 can1-100* and are listed in Table 2.1.

Strain	Relevant genotype	Source
PZ1	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 (W303)*	PZ Lab
PZ229	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 (W303 alpha)*	PZ Lab
PZ13	*W303 a sml1Δ::URA3 rad53Δ::LEU2*	PZ Lab
PZ1223	*W303 a SLD3-10his-13myc::KanMX bar1Δ::hisG*	PZ Lab
PZ1317	*W303 a DBF4-13myc::KanMx bar1::hisG*	PZ Lab
PZ1523	*W303 a sml1Δ::URA3 rad53Δ::LEU2 SLD3-10his-13myc::KanMX bar1Δ::hisG*	This study
PZ1319	*W303 a sld3-37A-10his13myc::KanmX bar1::hisG*	PZ Lab
PZ1593	*W303 a dbf4∆::TRP1 his3::PDBF4-dbf4-4A-13myc::KanMx::HIS3 bar1∆::hisG*	This study
PZ125	*W303 a DBF4-13myc::KanMx*	PZ Lab
PZ52	*W303 a SLD3-10his-13myc::KanMX*	PZ Lab
PZ89	*W303 a sml1Δ::URA3 rad53Δ::LEU2 SLD3-13myc::KanMX*	PZ Lab
PZ228	*W303 a sml1Δ::URA3 rad53Δ::LEU2 DBF4-13myc::KanMX*	PZ Lab
PZ126	*W303 a dbf4∆::TRP1 his3::PDBF4-dbf4-4A-13myc::KanMx::HIS3*	PZ Lab
PZ2	*W303 a sld3-37A-10his13myc ::KanmX*	PZ Lab
PZ1657	*W303 a orc2-6A URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA*	This study
PZ1658	*W303 a orc2-6A URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA his3::3ld3-A-PGAL1-10-dbf4-4A::HIS3*	This study
PZ1659	*W303 a orc6-4A URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA*	This study
PZ1660	*W303 a orc6-4A URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA his3::3ld3-A-PGAL1-10-dbf4-4A::HIS3*	This study
PZ1661	*W303 a URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA*	This study
PZ1662	*W303 a URA3::PGAL-CDC6-13myc::ura3 mcm-2xNLS::ABA his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1663	*W303 a orc2-6A orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3*	This study
PZ1664	*W303 a orc2-6A orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1665	*W303 alpharc2-6 URA3::P_{GAL}-CDC6-13myc::ura3*	This study
PZ1666	*W303 a orc2-6A URA3::P_{GAL}-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1667	*W303 a orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3*	This study
PZ1668	*W303 a orc6-4A orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study

PZ1669	*W303 a orc6-4A URA3::PGAL-CDC6-13myc::ura3*	This study
PZ1670	*W303 a orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1671	*W303 a URA3::P_{GAL}-CDC6-13myc::ura3*	This study
PZ1672	*W303 a URA3::P_{GA}L-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1673	*W303 a orc2-6A orc6-4A mcm-2xNLS::ABA*	This study
PZ1674	*W303 a orc2-6A orc6-4A mcm-2xNLS::ABA his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1675	*W303 a orc2-6A mcm-2xNLS::ABA*	This study
PZ1676	*W303 a orc2-6A mcm-2xNLS::ABA his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1677	*W303 alpha orc6-4A mcm-2xNLS::ABA*	This study
PZ1678	*W303 alpha orc6-4A mcm-2xNLS::ABA his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1679	*W303 alpha mcm-2xNLS::ABA*	This study
PZ1680	*W303 alpha mcm-2xNLS::ABA his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1681	*W303 alpha orc2-6Aorc6-4A*	This study
PZ1682	*W303 a orc2-6A orc6-4A his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1683	*W303 a orc2-6A*	This study
PZ1684	*W303 a orc2-6A his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1685	*W303 a orc6-4A*	This study
PZ1686	*W303 a orc6-4A his3::3ld3-A-PGAL1-10-dbf4-4A::HIS3*	This study
PZ1687	*W303 a orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3*	This study
PZ1688	*W303 a orc6-4A URA3::P_{GAL}-CDC6-13myc::ura3 his3::3ld3-A-$P_{GAL1-10}$-dbf4-4A::HIS3*	This study
PZ1449	*W303 a orc2-6A orc6-4A his3::pRS303::HIS3*	PZ Lab
PZ1450	*W303 alpha orc2-6A orc6-4A his3::pRS303::HIS3*	PZ Lab
PZ1453	*W303 alpha orc6-4A his3::pRS303::HIS3*	PZ Lab
PZ1454	*W303 alpha orc2-6A his3::pRS303::HIS3*	PZ Lab
PZ1455	*W303 a orc6-4A his3::pRS303::HIS3*	PZ Lab
PZ1456	*W303 alpha orc2-6A his3::pRS303::HIS3*	PZ Lab
PZ1721	*W303 a URA3::P_{GAL}-Cdc6-13myc*	This study
PZ2008	*W303 a URA3::P_{GAL}l-Cdc6-13mychis3:: sld3-A-$P_{GAL1-10}$-dbf4-A::HIS3*	This study
PZ1739	*W303 a sml1Δ::URA3 cdc28-as1*	This study
PZ1741	*W303 a sml1Δ::URA3 rad53Δ::LEU2 cdc28-as1*	This study
PZ1742	*W303 a sml1Δ::URA3 mec1Δ::LEU2 cdc28-as1*	This study

PZ1767	*W303 a cdc28-as1 dbf4-4A::HIS3 sld3-37A-10his-13myc ::KanNX*	This study
PZ1922	*W303 a bar1∆::TRP1*	This study
PZ1923	*W303 a bar1∆::TRP1 trp1::SLD3-$P_{GAL1-10}$-DBF4::TRP1*	This study
PZ1924	*W303 a bar1∆::TRP1 trp1::sld3-37A-$P_{GAL1-10}$-dbf4-4A::TRP1*	This study
PZ1925	*W303 a bar1∆::TRP1 his3::P_{GAL}-sld2-T84D::HIS3*	This study
PZ1926	*W303 a bar1∆::TRP1 trp1::SLD3-$_{PGAL1-10}$-DBF4::TRP1 his3::PGAL-sld2-T84D::HIS3*	This study
PZ1927	*W303 a bar1∆::TRP1 trp1::sld3-37A-$P_{GAL1-10}$-dbf4-4A::TRP1 his3::PGAL-sld2-T84D::HIS3*	This study
PZ1928	*W303 a bar1∆::TRP1 his3::P_{GAL}-sld2-T84D::HIS3 ura3::P_{CDC6}-cdc6∆NT::URA3*	This study
PZ1929	*W303 a bar1∆::TRP1 trp1::SLD3-$P_{GAL1-10}$-DBF4::TRP1 his3::P_{GAL}-sld2-T84D::HIS3 ura3::P_{CDC6}-cdc6∆NT::URA3*	This study
PZ1930	*W303 a bar1∆::TRP1 trp1::sld3-37A-$P_{GAL1-10}$-dbf4-4A::TRP1 his3::P_{GAL}-sld2-T84D::HIS3 ura3::P_{CDC6}-cdc6∆NT::URA3*	This study
PZ1891	*W303 a CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMx*	PZ Lab
PZ2167	*W303 a CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMx URA3::PCDC45-CDC45::ura3*	This study
PZ171	*W303 a sld3-2D-10his-13myc::KanMX*	PZ Lab
PZ2400	*W303 a dpb11-1::KanMX SLD3-10his-13myc::KanMX*	
PZ519	*W303 a sml1Δ::URA3 DBF4-13myc::KanMX*	PZ Lab
PZ228	*W303 a sml1Δ::URA3 rad53Δ::LEU2 DBF4-13myc::KanMX*	PZ Lab
PZ660	*W303 a sml1Δ::URA3 DBF4-13myc::KanMx CDC45-3HA::HIS3*	PZ Lab
PZ2168	*W303 a CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX URA3::PCDC45-cdc45-T189A::ura3*	This study
PZ2169	*W303 a CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX URA3::PCDC45-cdc45-T189D::ura3*	This study
PZ2170	*W303 a CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX URA3::PCDC45-cdc45-T189E::ura3*	This study
PZ2198	*W303 a SLD3-10his-13myc::KanMX cdc45-T189A*	This study
PZ2199	*W303 a SLD3-10his-13myc::KanMX cdc45-T189D*	This study
PZ2200	*W303 a SLD3-10his-13myc::KanMX cdc45-T189E*	This study

PZ2286	*W303 alpha CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX TRP1::PCDC45-cdc45-2A::trp1*	This study
PZ2287	*W303 alpha CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX TRP1::PCDC45-cdc45-2D::trp1*	This study
PZ2288	*W303 alpha CDC45::cdc45-td::TRP1 ubr1Δ::PGAL-UBR1::HIS3 SLD3-10his-13myc::KanMX TRP1::PCDC45-cdc45-2E::trp1*	This study
PZ2226	*W303 a SLD3-10his-13myc::KanMX cdc45-2A*	This study
PZ2336	*W303 a dbf4-4A::HIS3 cdc45-2A*	This study
PZ355	*W303 a mrc1Δ::URA3*	PZ Lab
PZ2313	*W303 a mrc1Δ::URA3 cdc45-2A*	This study
PZ2325	*W303 a ddc1Δ::KanMX*	This study
PZ2332	*W303 a ddc1Δ::KanMX cdc45-2A*	This study
PZ1535	*W303 a rad17Δ::URA3*	PZ Lab
PZ2446	*W303 a rad17Δ::URA3 cdc45-2A*	This study
PZ1545	*W303 a sgs1Δ ::HPH*	PZ Lab
PZ2412	*W303 a sgs1Δ ::HPH cdc45-2A*	This study
PZ246	*W303 a rad9∆::TRP1*	PZ Lab
PZ2444	*W303 a rad9∆::TRP1 cdc45-2A*	This study
PZ2448	*W303 a mec3Δ::KanMX*	This study
PZ2451	*W303 alpha mec3Δ::KanMX cdc45-2A*	This study
PZ2452	*W303 a sgs1∆::HPH ddc1∆::KanMX*	This study
PZ2453	*W303 a sgs1∆::HPH ddc1∆::KanMX cdc45-2A*	This study
PZ2485	*W303 alpha ddc1Δ::KanMX rad9∆::TRP1*	This study
PZ2486	*W303 a ddc1Δ::KanMX rad9∆::TRP1 cdc45-2A*	This study
PZ2360	*W303 a mrc1∆::HIS3 ddc1∆::KanMX sml1∆::URA3*	This study
PZ2361	*W303 a mrc1∆::HIS3 ddc1∆::KanMX sml1∆::URA3 cdc45-2A*	This study
PZ2707	*W303 a RAD53-3HA::HIS3*	This study
PZ2489	*W303 a RAD53-3HA::HIS3 cdc45-2A*	This study
PZ2560	*W303 a RAD53-3HA::HIS3 mrc1Δ::URA3*	This study
PZ2561	*W303 a RAD53-3HA::HIS3 mrc1Δ::URA3cdc45-2A*	This study
PZ2565	*W303 a 3HA-RAD53::KanMX*	This study
PZ2567	*W303 a 3HA-RAD53::KanMX cdc45-2A*	This study
PZ2334	*W303 a sml1Δ::URA3 MRC1-13myc::KanMX cdc45-2A*	This study
PZ2227	*W303 a sml1Δ::URA3 MRC1-13myc::KanMX*	This study
PZ2228	*W303 a sml1Δ::URA3 rad53Δ::LEU2 MRC1-13myc::KanMX*	This study

PZ568	*W303 a sml1Δ::URA3 SGS1-13myc::KanMX*	PZ Lab
PZ2407	*W303 a sml1Δ::URA3 EXO1-13myc::KanMX*	This study
PZ2410	*W303 a sml1Δ::URA3 RRM3-13myc::KanMX*	This study
PZ2246	*W303 a sml1Δ::URA3 SRS2-13myc::KanMX*	This study
PZ2601	*W303 a sml1Δ::URA3 SGS1-13myc::KanMX cdc45-2A*	This study
PZ2603	*W303 a sml1Δ::URA3 Rrm3-13myc::KanMX cdc45-2A*	This study
PZ2607	*W303 a sml1Δ::URA3 SRS2-13myc::KanMx cdc45-2A*	This study
PZ2615	*W303 a sml1Δ::URA3 EXO1-13myc::KanMX cdc45-2A*	This study

Table 2-1 Yeast strains used in this study.

2.1.2. Yeast media

The medium used to grow yeast was YP medium, autoclaved prior to use and supplemented with 2% glucose unless stated to the contrary in the appropriate text. In order to select for marker genes, such as TRP1, HIS3, LEU2 or URA3, SC medium or minimal medium were used, without the relevant amino acid or nucleic acid. Saturated cultures were mixed with 15% glycerol prior to long-term storage at -80^{o}C where necessary. Yeast plates were maintained at 4^{o}C for short-term storage.

2.1.3. Liquid culture and synchronisation

MATa yeast was grown until the mid-log phase of growth (1x10^{7} cells/ml) at 30^{o}C. Cell counting was performed through the use of either a haemocytometer, or through the measurement of optical density at 600nm using the NanoDrop 2000c spectrophotometer. Synchronisation in G1 was performed through the addition of 5μg/ml alpha factor for 100min to the appropriate cultures except 5ng/ml alpha factor was used for bar1Δ cells. Successful arrest was confirmed by determining the ratio of budded and unbudded cells, and arrest was released by washing two times in YPD at room temperature and resuspending cells in the required medium.

Budding index was determined by taking yeast samples at specific times indicated in the relevant figures, sonicating them, and counting the number of budding cells using a microscope. M phase synchronisation was performed through the treatment of cultures with 10µg/ml nocodazole (Roche) for 90 minutes.

2.1.4. Mating and tetrad dissection

To produce new combinations of genes, relevant MATα and MATa strains were crossed by mixing two cultures with one another on a non-selective plate and incubating for at least 4h to create a new diploid strain. Diploid cells were selected using diploid-specific marker genes. These diploid strains were grown on rich sporulation medium (RSM) for 3 or more days. Tetrad presence was confirmed using a microscope, and were digested using 900U of glucoronidase in 300µl at 37^{o}C for 45min. These tetrads were then dissected under a tetrad dissection microscope, and their genotype determined through either PCR or marker selection. Mating type was determined by crossing the new strain with the tester strains DC14 and DC17 and replica plating onto minimal medium.

2.1.5. Growth assays

Yeast was grown to saturation overnight, then diluted to 10^{7} cells/ml. Yeast was then serially diluted by 3 and spotted on plates. Pictures of plates were taken using a scanner at the appropriate time points, and colony images taken using a table top microscope with a connected Leica camera.

2.1.6. Yeast transformation

50ml of $2x10^7$ cells/ml mid-log phase yeast were washed with ddH_2O, then resuspended in 1ml ddH_2O. The transformation mix (240µl 50% PEG3500, 36µl 1M lithium acetate, 10µl salmon sperm DNA (10mg/ml), 1µg DNA in 74µl ddH_2O) was then added to 100µl of yeast cells, and the mixture incubated for 40min at 42°C followed by resuspension in 0.5ml ddH_2O and plating of 250µl on the appropriate plate. If aminoglycoside antibiotics were utilised as selective markers, cells were grown in YPD for at least 3h prior to plating. Centrifugation was carried out at low speed for 2min.

2.1.7. Yeast genomic DNA extraction

This protocol is derived from that of Rose *et al*. (1990). Cells were pelleted, and 0.3ml of 0.45mm diameter glass beads were added along with 0.2ml lysis buffer (10mM Tris pH 8, 1mM EDTA, 100mM NaCl, 1% SDS, 2% Triton X-100) and 0.2ml phenol/chloroform pH 8 (1:1). Tubes were vortexed for 2min prior to the addition of 0.2ml TE, then vortexed again. Cells were then centrifuged for 5min at room temperature at maximum speed, and the aqueous layer was transferred to a new Eppendorf tube. 2 volumes of 100% ethanol were then added, and samples were centrifuged for 3min after mixing. After discarding the supernatant, the pellet was washed with 0.5ml cold 70% ethanol and briefly centrifuged. The removal of supernatant was followed by pellet air drying and resuspension in 50µl TE buffer (10mM Tris, 1mM EDTA pH 8) containing 50µg/ml RNase A. Samples were then incubated at 37°C for 1h.

2.1.8. Preparation of trichloroacetic acid (TCA) whole cell extract

Following collection of 5ml 2x10^7 cells/ml yeast culture using centrifugation, cells were resuspended in 200µl 20% TCA. Following the addition of 400µl of glass beads, samples were vortexed for 1min. 400µl of 5% TCA was then added, and the mixture centrifuged at maximum speed for 2min. After discarding the supernatant, the protein pellet was resuspended in 200µl Laemmli buffer (250mM Tris pH 6.8, 2% SDS, 40% glycerol, 20% β-mercaptoethanol and bromophenol blue). 50µl of 1M Tris base was added, and samples were centrifuged at maximum speed for 2min following boiling for 6min. Samples were stored at -20^{o}C until use, or loaded onto polyacrylamide gels.

2.2. Bacteria-related methods

2.2.1. Bacterial media, growth and strains

E. coli was liquid cultured in 25g/L LBM-Broth Miller (Formedium) at 37^{o}C while shaking at 225 rpm. LB medium supplemented with 15g/L agar was used for bacterial growth on plates. To select for bacteria containing resistance markers, LB medium was supplemented with 50µl/ml ampicillin. Competent DH5α bacteria were used for transforming and amplifying shuttle vectors.

2.2.2. Bacterial transformation

100µl of competent bacteria was thawed on ice, and mixed with added plasmid DNA by pipetting, before incubation on ice for 30min. Bacteria were then heat-shocked at 42°C for 45sec, then kept on ice for 2min. 200µl of LB medium was then added, and bacteria incubated at 37°C for 1h prior to plating on the appropriate LB resistance plates.

2.3. DNA methods

2.3.1. Plasmids

Plasmid	Description	Source
PZ848	pRS316-P_{GAL}-CDC45	this study
PZ849	pRS306-P_{CDC45}-cdc45-T189A	this study
PZ850	pRS306-P_{CDC45}-cdc45-T189D	this study
PZ851	pRS306-P_{CDC45}-cdc45-T189E	this study
PZ860	pRS306-P_{CDC45}-cdc45-T189A-3'UTR	this study
PZ861	pRS304-P_{CDC45}-cdc45-T189D-3'UTR	this study
PZ862	pRS304-P_{CDC45}-cdc45-T189E-3'UTR	this study
PZ953	pRS304-P_{CDC45}-cdc45-T189A-T195A-3'UTR	this study
PZ954	pRS304-P_{CDC45}-cdc45-T189D-T195D-3'UTR	this study
PZ955	pRS304-P_{CDC45}-cdc45-T189E-T195E-3'UTR	this study
PZ1021	pFA6a KanMx6-P_{RAD53}-3XHA	this study
PZ243	pFA6a-3HA-KanMX6	PZ lab
PZ194	pFA6a-3HA-HIS3MX	PZ lab

Table 2-2 Plasmid used in this study.

2.3.2. Polymerase chain reaction (PCR)

PCR was performed using the Phusion High Fidelity DNA polymerase (NEB) in a 50µl reaction mixture of 5x Phusion HF Buffer, 1µl 10mM dNTP mixture, 2.5µl of both the forward and reverse primers diluted to 10µM, and template DNA. Reactions were then carried out using a peySTAR 96x Universal gradient apparatus (PE-QLAB), following the standard protocol: 98^{o}C, 3min; 98^{o}C, 1 min, primer-dependent annealing temperature, 1 min, 72^{o}C, 45sec/kb of product (30 cycles); 72^{o}C, 10 min, final elongation. PCR products were visualised following agarose gel electrophoresis and, where necessary, were purified using the QIAquick PCR purification kit (Qiagen) or the QIAquick gel extraction kit (Qiagen).

2.3.3. Agarose gel electrophoresis

1% agarose gels were made using 1x TAE (40mM Tris, 20mM acetic acid, 1mM EDTA) containing 1µg/ml ethidium bromide. Samples were diluted in 6x loading buffer (0.03% w/v bromophenol blue, 60% glycerol, 10mM Tris pH 8, 60mM EDTA pH 8). Electrophoresis apparatus was run at 80-100V, and DNA bands were then visualised using a UV transilluminator, with their size estimated against a DNA ladder.

2.3.4. Restriction digestion

Plasmid DNA, or PCR products, were digested in a 40µl digestion mixture containing 4µl 10x digestion buffer (NEB), 5µl 10x BSA, and 5U restriction enzyme. This mixture was incubated at 37^{o}C for 2h. For digestion of vectors, 1U of calf intestinal alkaline phosphatase (CIP, NEB)

was added after 1.5h and after 1.75h to dephosphorylate the 5' end. Digested DNA was then run on a gel and purified using the QIAquick gel extraction kit.

2.3.5. DNA ligation

Digested PCR products were mixed with digested, phosphatase-treated vectors in a ratio of 3:1. 2µl 10x T4 DNA ligase buffer (NEB) and 1µl T4 DNA ligase (NEB) were then added, and the total volume brought to 20µl. The reaction mixture was incubated for 2h at room temperature, or incubated overnight at 16^{o}C.

2.3.6. DNA cloning

PCR products from plasmid or yeast genomic DNA templates were generated using oligonucleotides containing appropriate 5' restriction sites. These oligonucleotide restriction sites were designed to allow for unidirectional insertion. The PCR product was electrophoresed on an agarose gel, and purified using the QIAquick gel extraction kit. For product digestion, the total volume was used. Plasmid DNA was obtained from bacterial stocks with the QIAprep spin miniprep kit (Qiagen), with approximately 500ng DNA used for digestion. After being run on an agarose gel, the PCR product and target vector were purified using the above gel extraction kit. Following ligation, 10µl of ligation reaction mixture was used for transformation into competent bacteria.

2.3.6.1. Sanger sequencing and sequence analysis

DNA was sequenced by the Department of Biochemistry, University of Cambridge DNA Sequencing Facility.

2.4. Preparative and analytical biochemistry

2.4.1. SDS-PAGE

Polyacrylamide gels were composed of a 7.5% resolving gel containing 3.27ml 40% acrylamide, 1.9ml 2% bis-acrylamide, 7.5ml 1M Tris-HCl pH 8.8, 100µl 20% SDS, 6.76ml ddH_2O, 25µl of tetramethylethylenediamine (TEMED) and 100µl 10% ammonium persulfate (APS). Additionally, a 15% resolving gel is also an essential component of polyacrylamide gels, and is composed of 7.6ml 40% acrylamide, 0.88ml 2% bis-acrylamide, 7.5ml 1M Tris-HCl pH 8.8, 100µl 20% SDS, 4ml ddH_2O, 25µl TEMED, 100µl 10% APS. The stacking gels were composed of 1.28ml 40% acrylamide, 0.7ml 2% bis-acrylamide, 1.25ml 1M Tris-HCl pH 6.8, 50µl 20% SDS, 6.76ml ddH_2O, 40µl TEMED, and 80µl 10% APS. The PerfectBlue Dual Gel System Twin ExW S (VWR) was used to separate samples. Running buffer was composed of 3g/l Tris base, 14.4g/l glycine and 5g/l 20% SDS. Samples were run through the application of 80V. Precision plus protein dual marker (Bio-Rad) was used as a reference. Additionally, a 15% resolving gel is also an essential component of polyacrylamide gels, and is composed of 7.6ml 40% acrylamide, 0.88ml 2% bis-acrylamide, 7.5ml 1M Tris-HCl ph 8.8, 100µl 20% SDS, 4ml ddH_2O, 25µl TEMED, 100µl 10% APS.

2.4.2. Phos-Tag-SDS-PAGE

Polyacrylamide-Agarose gels were composed of a 4% resolving gel containing 484ul 40% acrylamide, 334ul 2% bis-acrylamide, 1875µl 1M Tris-HCl pH 8.8, 25µl 20% SDS, 1666µl 1.5% Agarose, 20 µl 5mM Phos-Tag (Wako-Chem), 17.5µl 10 mM $MnCl_2$, 555µl ddH_2O, 5µl of tetramethylethylenediamine (TEMED) and 100µl 10% ammonium persulfate (APS). Agarose

was boiled in ddH_2O and immediately added into the gel mixture. The 3% stacking gels were composed of 145µl 40% acrylamide, 100µl 2% bis-acrylamide, 250µl 1M Tris-HCl 6.8, 1493µl ddH_2O, 2µl TEMED, and 10µl 10% APS.

2.4.3. Protein transfer and immunoblotting

Proteins were then blotted onto Whatman Protran Nitrocellulose transfer membrane for 45min at 500mA using a semi-dry system (Thermo Scientific, owl hep-1) in transfer buffer (48mM Trizma base, 0.0375% SDS, 20% methanol, 39mM glycine). Following transfer, membranes were blocked in 5% milk powder diluted in 0.1% TBS with 0.1% Tween-20 (TBS-T). The primary antibody, diluted in 5% milk powder in TBS-T was added to the membrane and incubated for either 2h at RT or overnight at 4^{o}C. The membrane was subsequently washed three times in TBS-T, for 5 mins per wash, and the HRP-conjugated secondary antibody diluted in 5% milk powder in TBS-T was added. The Amersham ECL Western Blotting Detection Reagents from GE Healthcare were used to detect protein bands.

2.4.4. Antibodies

Antigen	Species	Source	Concentration
Rad53	Rabbit	Abcam, ab104232	1:5000
Orc6	Mouse	Stillman Laboratory	1:5000
c-Myc	Mouse	Roche, clone 9e10	1:5000
Dpb11	Rabbit	Diffley Laboratory	1:1000
Mouse-IgG	Horse	Vectorlabs, PI-2000	1:10000
Rabbit-IgG	Donkey	Abcam, ab16284	1:10000

Table 2-3 Antibodies used in this study.

2.4.5. Purification of Cdc45 antibody

2.4.5.1. Purification of Cdc45 (1-238) fragment

BL21 DE3 pLysS cells carrying pGEX 2TKP Cdc45 (1-238)-6His plasmid were cultured in 2TY media containing Ampicillin and Chloramphenicol overnight at 37^{o}C. Next day, the saturated bacterial culture was diluted in 1:100 into 2 litres of 2TY media containing Ampicillin and Chloramphenicol and cultured again for approximately 4h at 37^{o}C until the OD600 reached 0.4-0.5. The expression of Cdc45 (1-238) fragment was induced by adding a final concentration of 0.5 mM IPTG and incubating for 3 hours at 37^{o}C. Cells were harvested by centrifugation at 4000 rpm for 10min at 4^{o}C. The cell pellet was suspended in 10ml of ice cold lysis buffer (8M Urea, 20mM NaPhosphate buffer pH7.4, 300mM NaCl, 20mM Imidazole pH 8.0) and sonicated 2 times for 30sec (40%) with a 30 sec break in between. The cell lysate was spun down at 9500rpm for 10 minutes at 4^{o}C and the supernatant transferred to a Ni-Sepharose (GE-healthcare) column. The beads were washed once with 5ml lysis buffer containing 70 mM Imdizadole and once with 3ml lysis buffer containing 90 mM Imidazole. The Cdc45 (1-238) fragment was eluted with 8ml lysis buffer containing 175mM imidazole. To precipitate Cdc45 (1-238) fragments, 4 volumes of ice cold acetone were added to the elution and incubated overnight at -20^{o}C. Protein precipitates were collected by centrifugation at 4000 rpm for 20min at room temperature and washed twice with acetone to remove Urea crystals. Excess acetone was removed by evaporation at room temperature and Cdc45 (1-238) fragments were dissolved in 500µl 8M Urea pH 9.0 and mixed with an equal volume of 2X coupling buffer (0.2 M $NaCO_3$, 1M NaCl at pH9). Protein concentration was measured and diluted to 5 mg/ml with 1X coupling buffer containing 4M Urea.

2.4.5.2. Coupling the Cdc45 (1-238) fragment to CN-Br sepharose beads

0.25g of CN-BR sepharose beads were swollen in 5ml of 1mM HCl for 15 minutes at room temperature and spun down at 700x g for 2min. After removing the supernatant, beads were washed twice with 1 ml of 1mM HCl and once in 10 ml coupling buffer (0.1 M $NaCO_3$, 0.5 M NaCl at pH9). 1 ml of 5mg/ml Cdc45 (1-238) fragment which was dissolved in coupling buffer containing 4M Urea, added to beads, and rotated of 2 hours at room temperature. Beads were then washed twice with 5ml of blocking buffer (coupling buffer+1M Ethanolamine) and incubated with 10ml blocking buffer for 2h at room temperature. After incubation with blocking buffer, beads were washed 2 times with 10ml low pH buffer (0.1M acetic acid, 0.5M NaCl), 2 times with 10ml coupling buffer, and 1 once 10 ml 1X PBS.

2.4.5.3. Affinity purification of Cdc45 polyclonal antibody

To purify the Cdc45 specific polyclonal antibody, 2 rabbits were immunized with the Cdc45 (1-238) fragment (BioGenes GmbH). 100µl of immunized rabbit serum and 900µl of PBS were added to Cdc45 (1-238)-Sepharose beads and rotated overnight at 4^{o}C. Beads were washed 3 times with 1ml of PBS and antibodies were eluted with 500µl of 200mM glycine pH 2.8. This elution step was repeated 4 times and each fraction was neutralized with 50µl 1M Tris pH 8.0.

Chapter 3

Activation of the S-phase checkpoint outside of S-phase

3.1. Introduction

Endogenous or exogenous stress can modify or damage DNA. Depending on the type of DNA damage, lesions are recognized by different sensor proteins which lead to the activation of two master kinases called ATM and ATR in humans (Mec1 and Tel1 in yeast). Activation of the master kinases transduces the signal to the effector kinases Chk1 and Chk2 in humans (Rad53 and Chk1 in yeast) which leads to the execution of several responses. The activation of the

DNA damage checkpoint in G1 ensures that the transition to a new SPhase does not occur before the damage is repaired, whereas in G2 phase it prevents cells from entering mitosis (Siede *et al*., 1994; Weinert & Hartwell, 1988). On the other hand, different responses of the DNA damage checkpoint have been observed when it is activated in S phase, including the stabilization of stalled replication forks, inhibition of origin firing and transcriptional control.

Because different responses are observed depending on the cell cycle stages, the DNA damage checkpoint has been categorized into three groups as G1, S and G2 checkpoints (Niida & Nakanishi, 2006). However, it is still not clear whether there is a separation of the DNA damage checkpoints between the different phases of the cell cycle. Indeed, the same effector kinase - Rad53 - is activated independently of the cell cycle phase in yeast. But, to investigate the temporal specificity of the checkpoint, these responses have never been tested outside of S-phase, as the substrates of checkpoint kinases are not well understood.

Recently it has been shown in yeast that, in S-phase, the checkpoint effector kinase Rad53 phosphorylates two replication factors upon DNA damage to inhibit origin firing (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). These 2 factors are Sld3 and the DDK regulatory subunit Dbf4 (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). In order to address whether DNA damage checkpoint responses are temporally separated, I decided to investigate if Rad53 targets origin firing outside of S-phase. To do so, I analysed the phosphorylation of Sld3 and Dbf4 upon DNA damage in G1 and G2 phase (**Figure 3-1**).

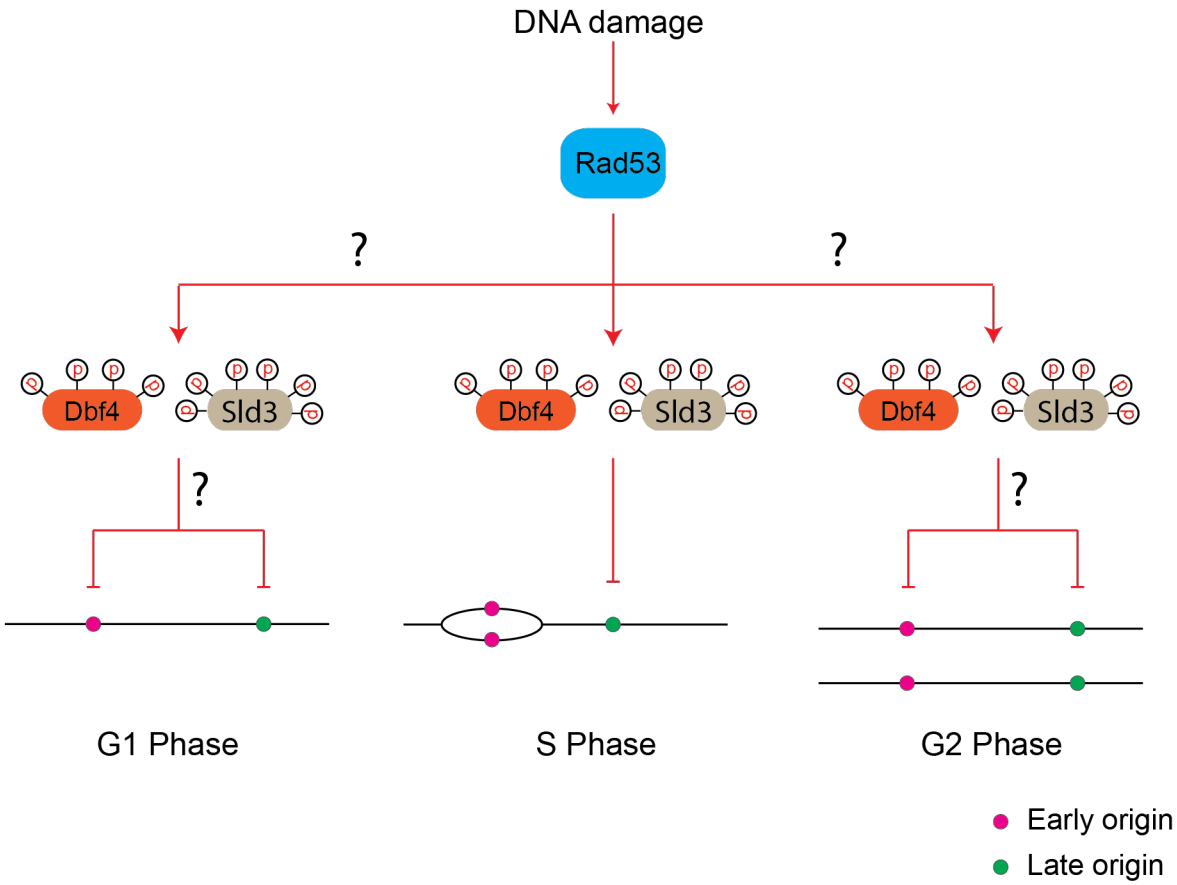


Figure 3-1 Does Rad53 phosphorylate Sld3 and Dbf4 in G1 and G2 phase?

3.2. Analysis of Rad53 target phosphorylation through the cell cycle

To establish whether Sld3 is phosphorylated in G1 phase upon DNA damage, I compared the phosphorylation status of Sld3 with or without DNA damage in G1 phase. To arrest the cells in G1 phase, the mating pheromone α-factor was used. To be sure that cells cannot escape from G1 arrest in the presence of α-factor, I decided to use *bar1Δ* cells. Bar1 is a protease which cleaves α-factor and *bar1Δ* cells are hyper-sensitive to α-factor (Chan & Otte, 1982). Once cells were arrested with α-factor in G1 phase the culture was divided into two: one group of

cells was kept without treatment, and in the second group DNA damage was induced using 4-NQO which mimics the effect of ultraviolet light. Flow cytometry analysis indicated that cells were effectively arrested in G1 phase during the experiment (**Figure 3-2**.A). Next, I wanted to analyse whether DNA damage was induced successfully by 10μg/ml 4-NQO. For the rest of the experiments same concentration of 4-NQO was used unless indicated. Activation of DNA damage signalling results in the phosphorylation of the checkpoint effector kinase Rad53, which can be monitored by the mobility shift in Western-blot analysis. As can be seen in **Figure 3-2**.B, induction of DNA damage by 4-NQO resulted in the mobility shift of Rad53 in the treated cells, suggesting that DNA damage was induced.

Next, I decided to analyse the phosphorylation status of Sld3. It has been shown that activation of the DNA damage checkpoint in S-phase causes Rad53-dependent phosphorylation of Sld3 (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). Although Rad53-dependent Sld3 phosphorylation can be monitored through the standard western blot protocol, it does not allow for the assessment of all phosphorylation events (**Figure 3-2.B.•).** In order to increase the resolution of Sld3 western blots, I decided to optimise the SDS-Page protocol used in the lab for the separation of the proteins. I performed several optimization experiments with the Phos-Tag reagent, which is a chemical that binds to the phosphate groups of proteins and slows down their relative speed compared to non-phosphorylated ones in the SDS-page gel (Kinoshita *et al*., 2009). The western blot analysis with the optimized protocol showed that Sld3 was hyper-phosphorylated in G1 phase upon DNA damage whereas no mobility shift was observed in the non-treated group (**Figure 3-2.B.♦),** suggesting that Sld3 is phosphorylated in G1 upon DNA damage.

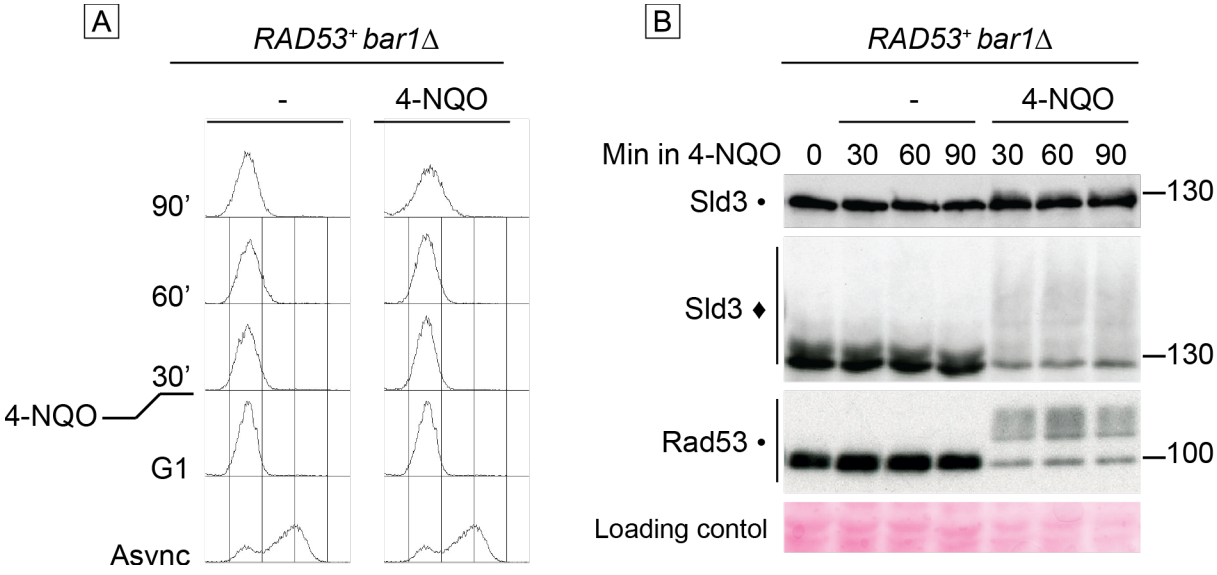


Figure 3-2 Analysis of Sld3 phosphorylation upon DNA damage in G1 phase. Cells were arrested in G1 phase with α-factor and divided in to two populations, without treatment (-) or with 4-NQO A) Flow cytometry analysis of the cells. B) Western-blot analysis of Sld3 and Rad53. Sld3 proteins were resolved either with standard SDS-Page (•) or Phos-Tag-SDS-Page (♦) protocol. Sld3 was tagged with Myc-Tag and Anti-Myc antibody was used for Sld3 detection. Anti-Rad53 antibody was used for Rad53 detection. Ponceau S staining was used as a loading control.

Given that Dbf4 is another checkpoint substrate and phosphorylated upon DNA damage in order to inhibit late origin firing in S-phase (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010), I investigated whether it is also phosphorylated in G1 phase. To do this, the phosphorylation status of Dbf4 was analysed upon DNA damage in G1 phase as in **Figure 3-2**. A slow-running subset of Dbf4 was observed in western blot analysis upon DNA damage whereas this subset was not observed in non-treated cells suggesting that Dbf4 is phosphorylated in G1 phase upon DNA damage (**Figure 3-3**).

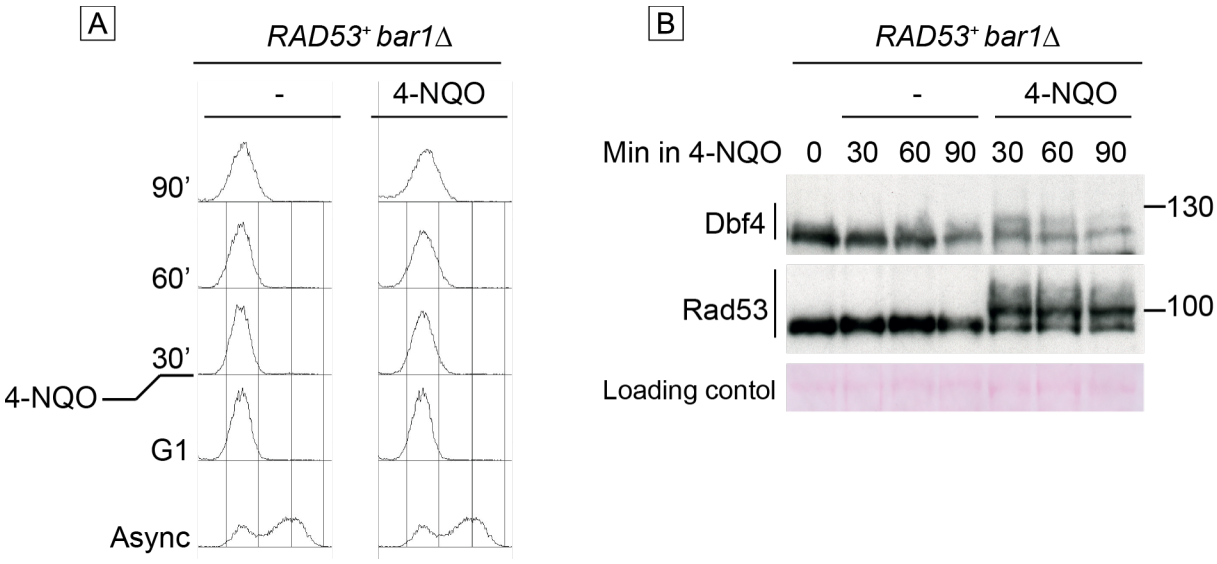


Figure 3-3 Analysis of Dbf4 phosphorylation upon DNA damage in G1 phase. Cells were arrested in G1 phase with α-factor and divided into two, without treatment (-) or with 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of Dbf4 and Rad53. Dbf4 was tagged with Myc-Tag and Anti-Myc antibody was used for Dbf4 detection. Anti-Rad53 antibody was used for Rad53 detection. Ponceau S staining was used as a loading control.

Next, I wondered whether the phosphorylation of Sld3 upon DNA damage observed in G1 phase is Rad53-dependent. To test this, I decided to analyse the phosphorylation of Sld3 upon DNA damage in the absence of Rad53. To do so, I compared the phosphorylation of Sld3 in *sml1Δ bar1Δ and rad53Δ sml1Δ bar1Δ* cells as in (**Figure 3-2**). The mobility shift of Sld3 was greatly reduced in *rad53Δ sml1Δ bar1Δ* cells after addition of 4-NQO compared to *sml1Δ bar1Δ* cells (**Figure 3-4**), suggesting that the phosphorylation of Sld3 is Rad53-dependent.

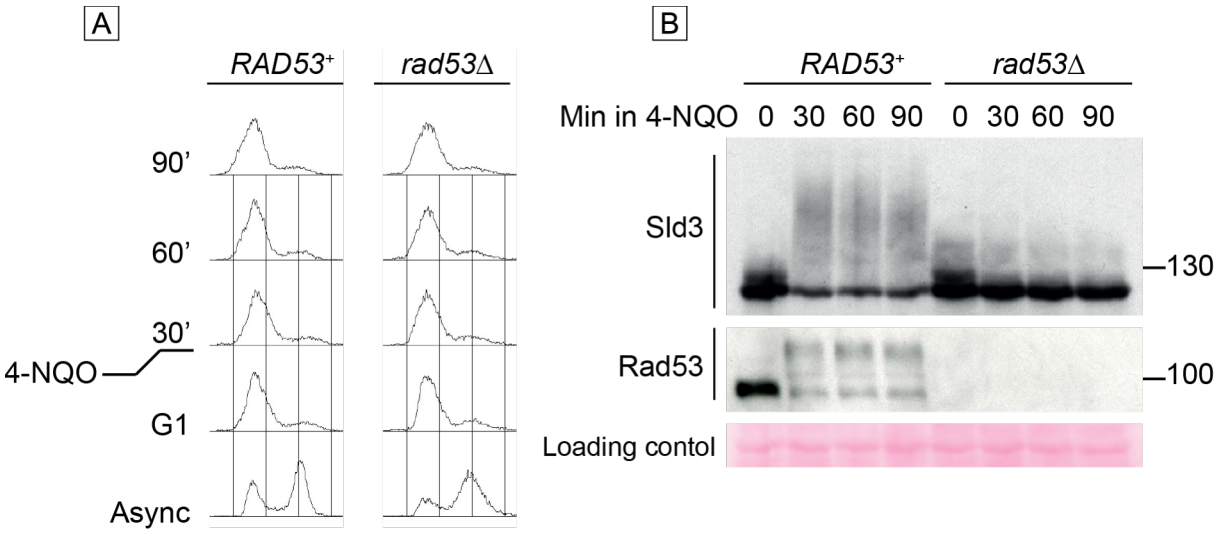


Figure 3-4 Analysis of Sld3 phosphorylation by Rad53 upon DNA damage in G1 phase. Cells were arrested in G1 phase with α-factor and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of the cells. Sld3 was endogenously tagged with the Myc tag. Anti-Myc antibody was used to detect Sld3. For Rad53 detection, an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control. Cells were isogenic for *sml1Δ.*

Next, I wondered whether the phosphorylation of Sld3 and Dbf4 by Rad53 occurs at the previously described sites (Zegerman & Diffley, 2010). It has been shown that the *sld3-A* (S306A, T310A, S421A, S434A, T435A, S438A, T442A, T445A, S450A, T451A, S452A, S456A, S458A, S459A, S479A, S482A, T507A, S509A, S514A, S519A, S521A, S524A, S529R T540A, T541A, S546A, S547A, T548A, T550A, S556A, S558A, T559A, T565A, S569A, T582A, T607A, S653A and S654A) and *dbf4-4A* (S518A, S521A, S526A and S528A) alleles in which their Rad53-dependent phosphorylation sites were replaced with alanine can indeed no longer be inhibited by Rad53 and can overcome the inhibition of origin firing in S-phase (Zegerman & Diffley, 2010). I hypothesised that, if Sld3 and Dbf4 are phosphorylated in G1 phase at the previously described sites, then the *sld3-A* and *dbf4-4A* alleles should not be phosphorylated in G1 phase upon DNA damage. To test this, I compared the phosphorylation of *sld3-A* and *dbf4-4A* with their wildtype counterparts as in **Figure 3-4**. Contrary to wildtype cells*, neither sld3-A* nor *dbf4-A* cells showed any mobility shift in the western blot analysis

(**Figure 3-5**,**Figure 3-6**). From these experiments, I concluded that Rad53 phosphorylates Sld3 and Dbf4 at the previously described residues in G1 phase upon DNA damage.

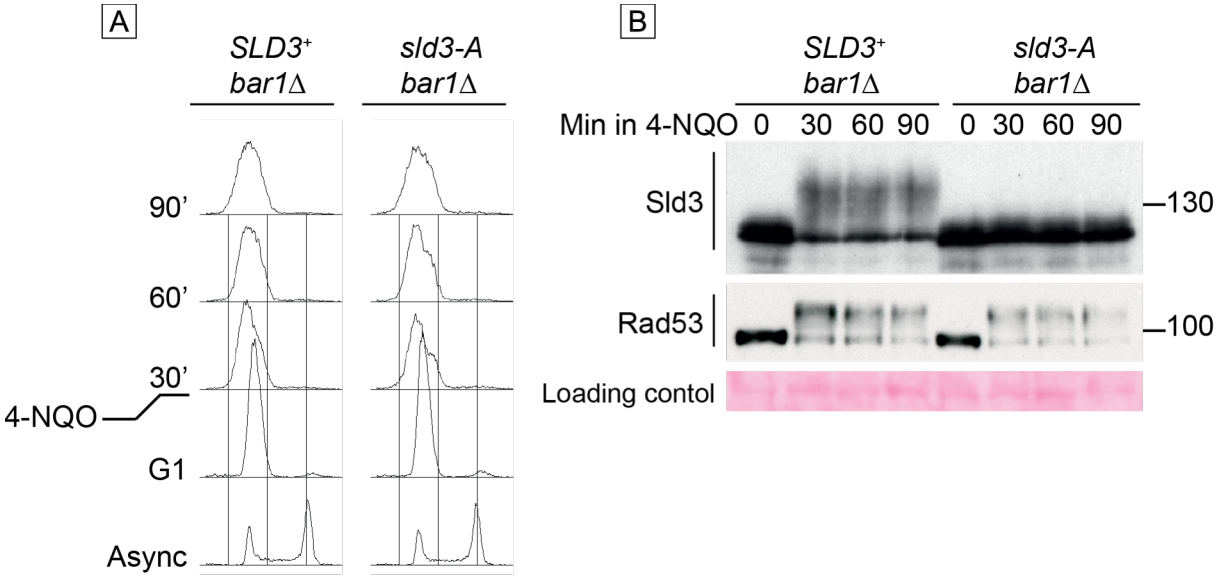


Figure 3-5 Phosphorylation status of previously described phosphorylation sites of Sld3 upon DNA damage in G1 phase. Cells were arrested in G1 phase with α-factor and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of the cells. Sld3 was endogenously tagged with myc tag. Anti-Myc antibody was used to detect Sld3. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control.

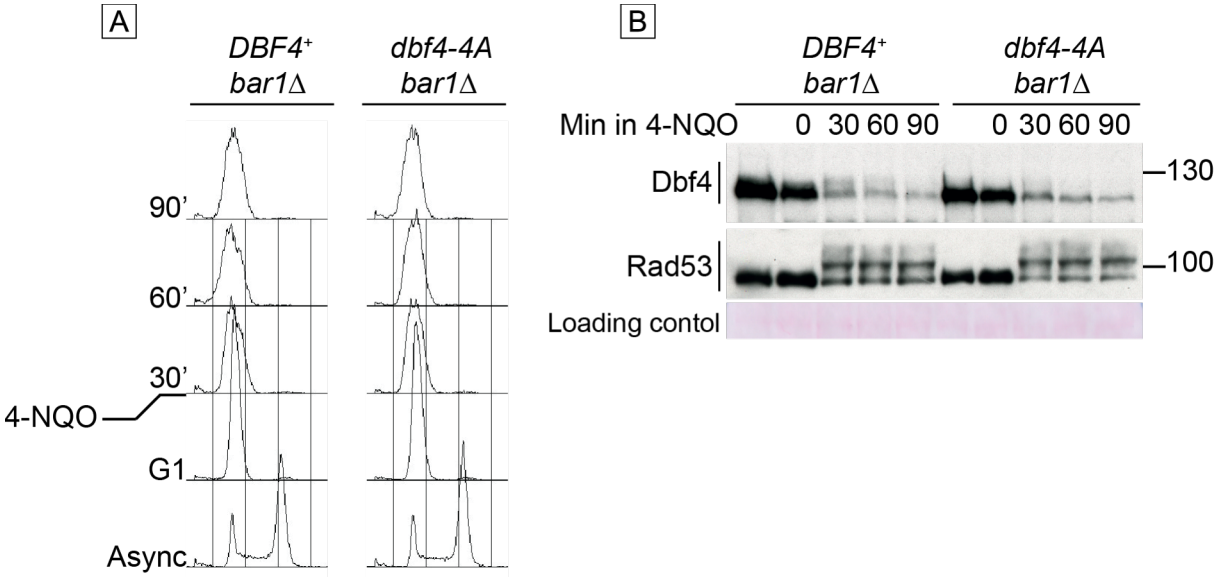


Figure 3-6 Phosphorylation status of previously described phosphorylation sites of Dbf4 upon DNA damage in G1 phase. Cells were arrested in G1 phase with α-factor and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western-blot analysis of the cells. Dbf4 was endogenously tagged with myc tag. Anti-Myc antibody was used to detect Dbf4. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control.

Since the Rad53-dependent phosphorylation of Sld3 and Dbf4 was observed in G1 phase, I wondered if the same response occurs in the G2 phase. To test this, the Rad53-dependent phosphorylation of Sld3 and Dbf4 was tested as in **Figure 3-4**, except cells were arrested in G2 phase with Nocodazole. The Western blot analysis of Sld3 in G2 phase showed 2 distinct bands before the addition of 4-NQO, possibly due to CDK phosphorylation in G2 phase. However, upon addition of 4-NQO, Sld3 became a high molecular weight smear (**Figure 3-7**) which was not present in the absence of Rad53. Dbf4 also showed 2 distinct bands in G2 phase, possibly due to CDK phosphorylation (Holt *et al*., 2009; D. Lu *et al*., 2014; Ubersax *et al*., 2003) and the mobility shift of Dbf4 increased after addition of 4-NQ. The mobility shift of Dbf4 was Rad53-dependent after DNA damage (**Figure 3-8**). These results suggest that Sld3 and Dbf4 are phosphorylated in a Rad53-dependent manner upon DNA damage in G2 phase.

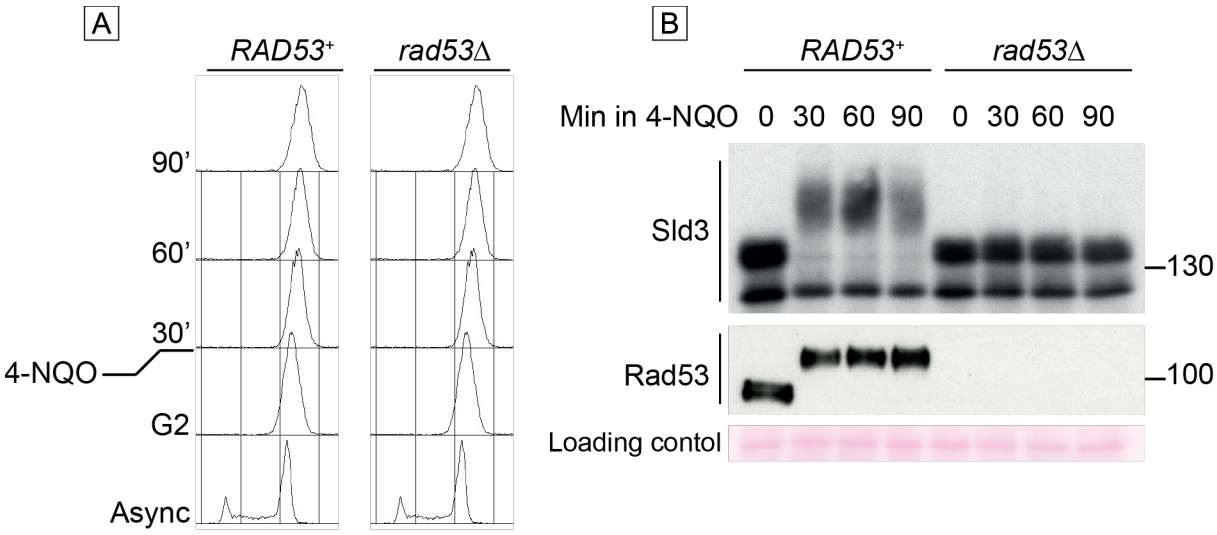


Figure 3-7 Analysis of Sld3 phosphorylation by Rad53 upon DNA damage in G2 phase. Cells were arrested in G2 phase with Nocodazole and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of the cells. Sld3 was endogenously tagged with a myc tag. Anti-Myc antibody was used to detect Sld3. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control. All strains are *sml1Δ.*

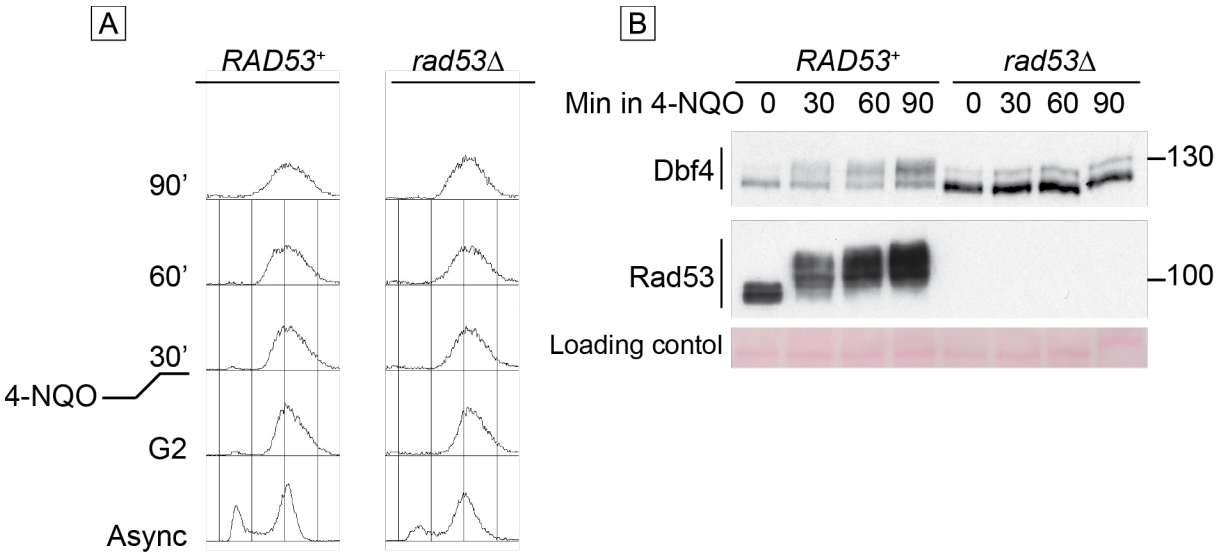


Figure 3-8 Analysis of Dbf4 phosphorylation by Rad53 upon DNA damage in G2 phase. Cells were arrested in G2 phase with Nocodazole and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of the cells. *Dbf4* was endogenously tagged with a myc tag. Anti-Myc antibody was used to detect Sld3. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control. Cells were isogenic for *sml1Δ.*

In order to understand if the Rad53-dependent phosphorylation of Sld3 and Dbf4 occurs also in G2 phase at the previously described residues, the phosphorylation status of *sld3-A dbf4-4A* cells was analysed as in **Figure 3-5**, except cells were arrested in G2 phase with Nocodazole. The hyperphosphorylation of Sld3 was greatly reduced in *sld3-A* cells upon DNA damage, compared to wildtype cells, suggesting that the phosphorylation of Sld3 occurs at the previously described residues (**Figure 3-9**). On the other hand, the phosphorylation profile of Dbf4 in wildtype and *dbf4-4A* cells showed only minor differences. As a result, it was not clear whether dbf4-4A was phosphorylated or not (**Figure 3-10**).

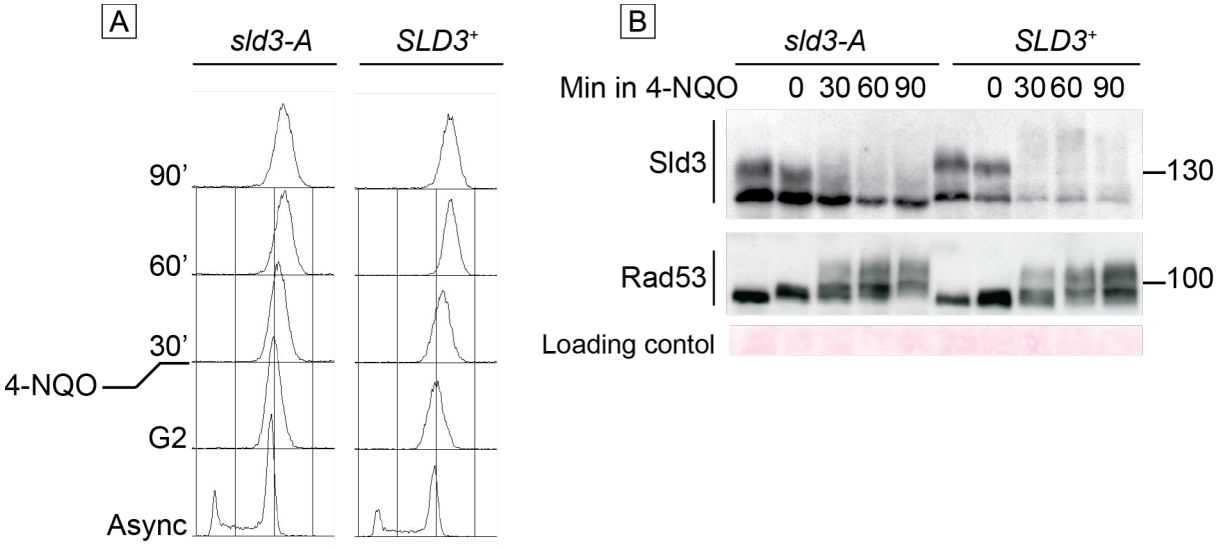


Figure 3-9 Phosphorylation status of previously described phosphorylation sites of Sld3 upon DNA damage in G2 phase. Cells were arrested in G1 phase with α-factor and DNA damage was induced by 4-NQO A) Flow cytometry analysis of the cells. B) Western blot analysis of the cells. Sld3 was endogenously tagged with a myc tag. Anti-Myc antibody was used to detect Sld3. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control.

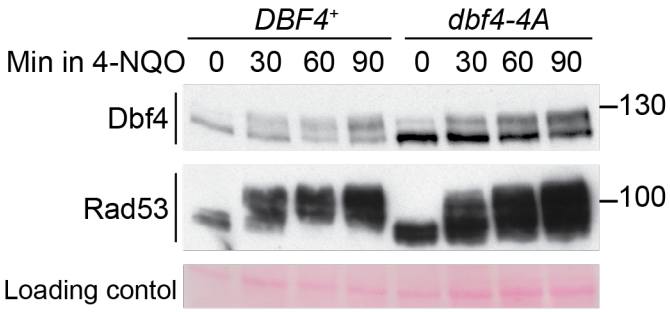


Figure 3-10 Phosphorylation status of previously described phosphorylation sites of Dbf4 upon DNA damage in G2 phase. Cells were arrested in G2 phase with Nocodazole and DNA damage was induced by 4-NQO. Western blot analysis of the cells. Dbf4 was endogenously tagged with a myc tag. Anti-Myc antibody was used to detect Dbf4. For Rad53 detection an anti-Rad53 antibody was used. Ponceau S staining was used as a loading control.

3.3. Discussion

In this chapter, I investigated the activation of the S-phase checkpoint outside of S-phase. To do so, I analysed one of the well-characterized checkpoint responses; the inhibition of origin firing. It has previously been shown that the checkpoint effector kinase Rad53 phosphorylates Sld3 and Dbf4 to inhibit late origin firing upon DNA damage in S-phase in *S. cerevisiae* (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010)

I first investigated the phosphorylation of Sld3 and Dbf4 upon DNA damage by Western-blot analysis. Because the Sld3 profile in conventional western blot analysis had a low resolution, I optimised an SDS-PAGE protocol by using the Phos-Tag reagent which specifically binds to the phosphate group of proteins and slows their relative migration (Kinoshita *et al*., 2006). This new protocol greatly improved the resolution of Sld3 compared to the standard protocol used in the Zegerman Laboratory (**Figure 3-2.B**). After the optimisation of the Western blot protocol, I compared the phosphorylation of Sld3 and Dbf4 upon DNA damage. This showed that Sld3 and Dbf4 are both phosphorylated upon DNA damage in G1 phase (**Figure 3-2.B and Figure**

3-3.B)in a Rad53-dependent manner. Next, I wondered if the phosphorylation of Sld3 and Dbf4 occurred at previously mapped sites. To answer this question, I used the *sld3-A and dbf4-4A* alleles, in which their Rad53 phosphorylation sites in S-phase are substituted with alanine. This western blot analysis indicated that both Sld3 and Dbf4 were phosphorylated at previously described sites in G1 phase (**Figure 3-5**.B, **Figure 3-6**.B). Although I did not show directly that Dbf4 is not phosphorylated in Rad53 null cells, the lack of phosphorylation in *dbf4-4A* cells strongly suggests that Rad53 is the responsible kinase for the Dbf4 phosphorylation in G1 phase upon DNA damage.

Since I showed that both Sld3 and Dbf4 were phosphorylated in G1 in a similar way to in S-phase upon DNA damage, I wondered if it also was similar in G2 phase. First, I showed that both Sld3 and Dbf4 are phosphorylated in a Rad53-dependent manner in G2 phase upon DNA damage (**Figure 3-7**, **Figure 3-8**)**.** I also showed that the phosphorylation of Sld3 occurs at previously described residues (**Figure 3-9.B**). However, it was not possible to investigate the phosphorylation sites of Dbf4 upon DNA damage in G2 phase due to the low resolution of the analysis (**Figure 3-10.B**), which could be solved by using more sensitive techniques such as Phos-Tag SDS-PAGE. In summary, Rad53 targets Sld3 and Dbf4 in G1 and G2 phase upon DNA damage (**Figure 3-11**).

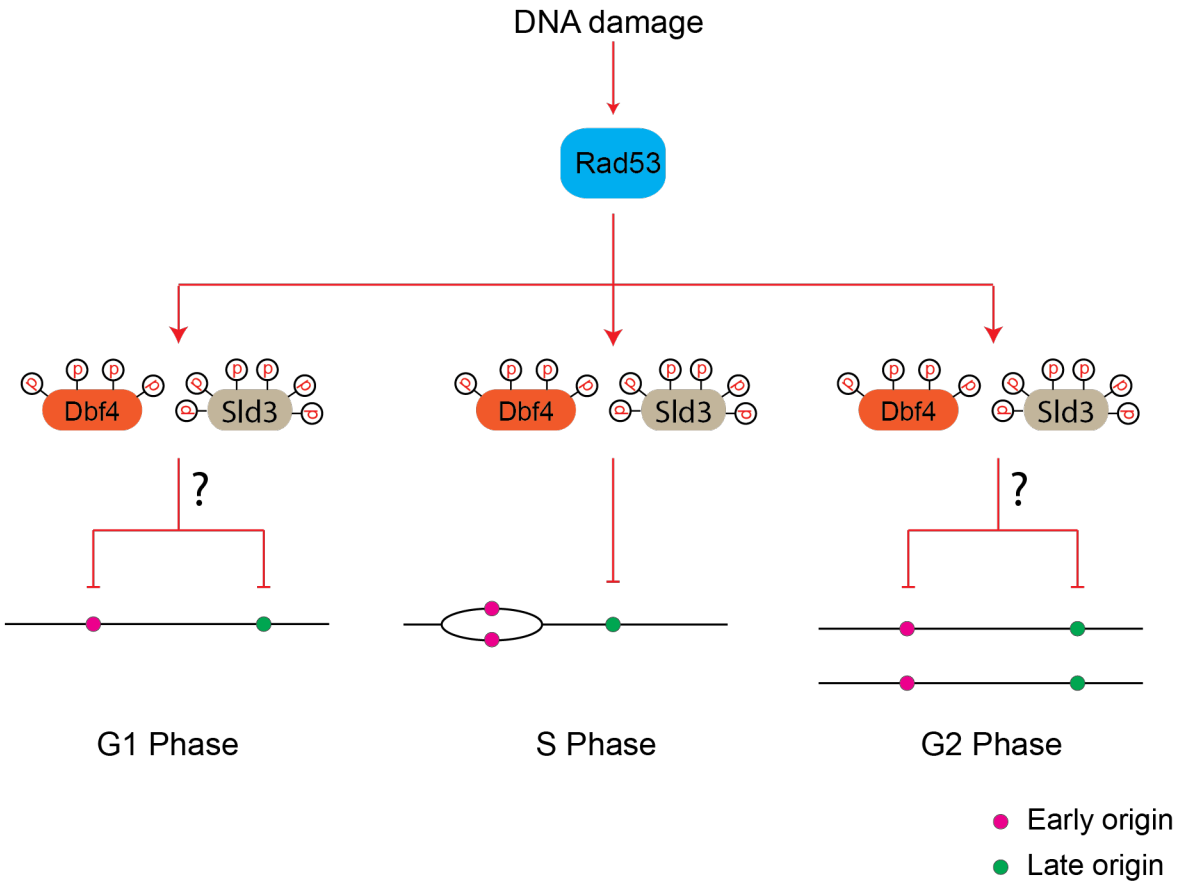


Figure 3-11 The model proposed in this chapter.

3.3.1. What is the function of inhibition of origin firing outside of S-phase?

Although this chapter shows that Rad53 targets Sld3 and Dbf4 upon DNA damage outside of S-phase, it does not show whether there is a physiological significance to this phosphorylation. Three different hypotheses could explain this observation: 1) independent of their roles in replication initiation, Sld3 and Dbf4 might be required for other cellular event(s) outside of S-phase which have to be inhibited by phosphorylation upon DNA damage, 2) the phosphorylation of Sld3 and Dbf4 might be not intended outside of S-phase, 3) the inhibition

of origin firing by checkpoint could be a safeguard mechanism to prevent possible unscheduled origin firing outside of S-phase.

Besides its function in replication initiation, DDK interacts with yeast Polo-like kinase Cdc5 via the N-terminal segment of Dbf4 (Chen & Weinreich, 2010). This interaction has been proposed to control the mitotic exit. However, whether this function of Dbf4 is targeted by checkpoint factors is not yet known. In contrast, the only known role of Sld3 is its requirement in replication initiation. It is possible to examine the other possible roles of Sld3 by its targeted degradation outside of S-phase by using the temperature degron system which would allow us to address the first two hypotheses. Given that deregulation of the cell cycle could possibly result in unscheduled replication initiation, targeting Sld3 and Dbf4 to prevent origin firing could be a safeguard mechanism to prevent further damage. This possibility is examined in Chapter 4 in detail.

On the other hand, DNA damage in G1 phase delays the entry into S-phase by activation of Rad53 through the mediator protein Rad9 (Siede *et al*., 1994). It has been proposed that the activation of Rad53 results in deregulation of the transcription cofactor Swi6, which results in the repression of several proteins required for the G1/S transition including the G1 cyclins Cln1 and Cln2 (Sidorova & Breeden, 1997). However, ectopic modulation of the G1/S transcription does not fully suppress the delay upon MMS treatment, suggesting that other targets of the checkpoint might delay the cell cycle. Given that Sld3 and Dbf4 are phosphorylated in G1 phase, their phosphorylation upon DNA damage could be another mechanism to ensure that S-phase entry is delayed.

3.3.2. Is there an S-phase checkpoint?

The initial observation about the checkpoint-dependent inhibition of origin firing came from cells obtained from Ataxia telangiectasia patients which were able to replicate their DNA after ionising radiation treatment, in contrast to cells obtained from healthy people (Painter & Young, 1975, 1980). This led to the characterisation of one of the DNA damage checkpoint master kinases - Ataxia Telangiectasia Mutated (ATM) (Savitsky *et al*., 1995). Later works in yeast suggested that the DNA damage response requires different proteins in S- and G2 phase, which led to the use of the term 'S-phase checkpoint' (Weinert, 1992). Given that the Mec1-Rad53 axis drastically slows down the S-phase rate upon DNA damage (Paulovich & Hartwell, 1995) by inhibiting new replication initiations (Santocanale & Diffley, 1998; Shirahige *et al*., 1998), inhibition of origin firing has been considered an S-phase checkpoint response (Longhese *et al*., 2003). By elucidating the function of other checkpoint proteins in perturbed S-phase, other responses of the S-phase checkpoint were described. For instance, it was found that replication forks cannot restart in the absence of checkpoint kinases after fork stalling (Tercero & Diffley, 2001), as checkpoint kinases stabilise the stalled replication forks to protect them from irreversible collapse (Lopes *et al*., 2001).

Recently, it has been shown that the inhibition of origin firing in S-phase is achieved by the inhibitory phosphorylation of 2 replication factors, Sld3 and Dbf4 (Zegerman & Diffley, 2010). Through this finding, the molecular mechanism behind the inhibition of origin firing has been completed in yeast. Of course, it would be unexpected to question the inhibition of origin firing outside of S-phase where there is no replication. In addition, because the molecular mechanism behind the inhibition of origin firing is unknown, it was not possible examine this response through the cell cycle.

However, independent of its physiological significance, based on this chapter, there is no temporal regulation of the phosphorylation of Rad53 targets for the inhibition of origin firing through the cell cycle. Considering that the inhibition of origin firing is one of the major responses of the S-phase checkpoint (Karim Labib & De Piccoli, 2011), it is important to question the term 'S-phase checkpoint'. Whether there is a distinct DNA damage checkpoint for S-phase has to be carefully examined in order to define the S-phase checkpoint as a temporally separated checkpoint. Gaining more insight into the molecular mechanisms of the other responses of the S-phase checkpoint would help to define its temporal specificity. It is known that the transcriptional control of RNR genes also occurs in G2 phase upon DNA damage (Mazumder *et al*., 2013). However, the molecular details of how stalled replication forks are stabilised remains unknown (Karim Labib & De Piccoli, 2011). It would be very interesting to explore the S-phase checkpoint targets for the stabilisation of replication fork and examine them outside of S-phase. This would produce a clearer picture of the responses of the DNA damage checkpoint through the cell cycle. However, it appears that the inhibition of origin firing is not a unique response of the S-phase checkpoint in yeast. Whether this is a conserved mechanism in higher eukaryotes is not clear. Given that the molecular mechanisms underlying the inhibition of origin firing in higher eukaryotes are not yet fully understood, it is not possible to address this question in other organisms at this point.

Chapter 4

Investigating the role of checkpoint-dependent inhibition of origin firing outside of S-phase

4.1. Introduction

DNA must be replicated once and only once in every cell cycle for the faithful transmission of the genetic information to the daughter cells. This is achieved through the temporal separation of origin licensing (loading of inactive helicase) and replication initiation during the cell cycle, which is accomplished by the coordinated actions of 2 enzymatic complexes: CDK and APC/C. In the absence of CDK activity, origins can be licensed in late mitosis and G1 phase. On the other hand, origins can only be fired in S-phase in the presence of CDK. Losing this control of

CDK results in re-replication (Nguyen *et al*., 2001), which leads to several genetic abnormalities (Davidson *et al*., 2006) such as gene amplification and gross chromosomal re-arrangements (Green *et al*., 2010; Truong & Wu, 2011).

CDK prevents the formation of the preRC in S and G2 phase by multiple mechanisms. In budding yeast, the inhibitory phosphorylation on Orc2 and Orc6 by CDK prevents the loading of inactive MCM helicase (Nguyen *et al*., 2001). It has also been shown that the binding of CDK on Orc6 prevents the function of ORCs (Wilmes *et al*., 2004). In addition, CDK promotes the nuclear exclusion of MCM•Cdt1 (Karim Labib *et al*., 1999; Nguyen *et al*., 2000). CDK also inhibits the function of Cdc6 via phosphorylation, promoting its degradation as well as direct binding (Drury *et al*., 1997; Mimura *et al*., 2004). On the other hand, replication initiation can only occur in S-phase through the activity of 2 kinases: CDK and DDK. CDK phosphorylates Sld2 and Sld3 which allows their binding to Dpb11, which is essential for the recruitment of the other replication factors to the inactive helicase (Zegerman & Diffley, 2007), whereas DDK phosphorylates other subunits of the helicase which are required for helicase activation (Francis *et al*., 2009; Lei *et al*., 1997; Yi Jun Sheu & Stillman, 2006).

It is possible to bypass the control of CDK on preRC formation or replication initiation by deregulating those CDK targets simultaneously. This results in unscheduled origin firing re-replication (Nguyen *et al*., 2001). However, bypassing the control of CDK only leads to limited re-replication in *S. cerevisiae*, giving rise to the hypothesis that multiple non-redundant mechanisms work together to prevent re-replication (Green *et al*., 2006). One possible mechanism in addition to CDK control could be the DNA damage checkpoint, since it is activated upon induction of re-replication (Davidson *et al*., 2006; Green & Li, 2005; Melixetian *et al*., 2004; Mihaylov *et al*., 2002). Given that in the previous chapter I showed that Rad53 phosphorylates the replication initiation factor Sld3 and the regulatory subunit of DDK Dbf4 to prevent origin firing not only in S-phase, but also in G1 and G2 phase upon DNA damage, I

hypothesised that Rad53 targets Sld3 and Dbf4 outside of S-phase as a contingency mechanism to prevent unscheduled DNA replication in the case of CDK misregulation.

More specifically, in this chapter, I seek to answer the following questions: 1) Does Rad53 activation inhibit unscheduled origin firing through Sld3 and Dbf4 phosphorylation, 2) Does Rad53 limit the extent of re-replication by inhibiting origin firing via Sld3 and Dbf4 phosphorylation outside of S-phase phase (**Figure 4-1**)?

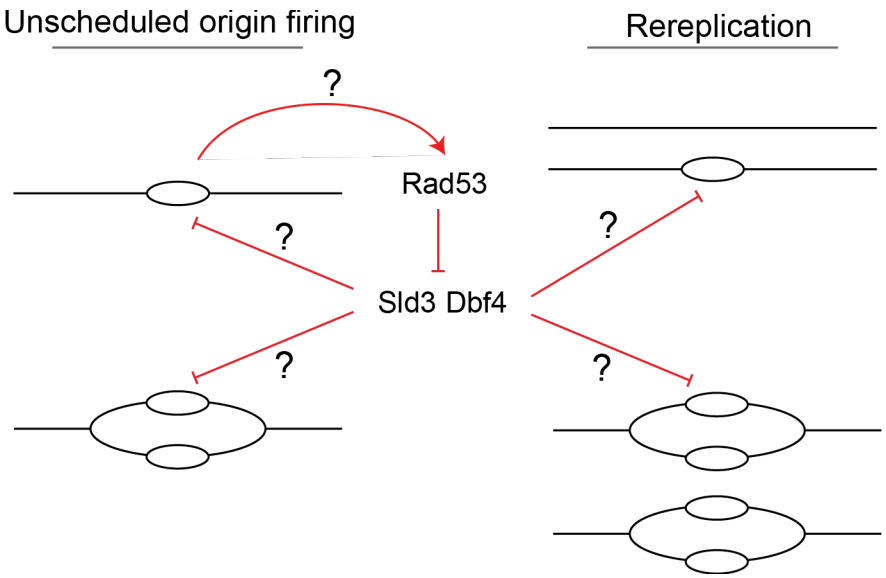


Figure 4-1 Is the Rad53-dependent inhibition of origin firing required for the prevention of unscheduled replication initiation and/or re-replication in G1 and G2 phase?

To obtain answers for the questions mentioned above, I used three different approaches to induce unscheduled replication initiation and analysed the contribution of thecheckpoint-dependent inhibition of the origin firing by bypassing the control of Sld3 and Dbf4 by Rad53.

In the first approach, replication initiation was achieved by bypassing the control of CDK on origin licensing via the deregulation of CDK targets. It has been shown before that the simultaneous introduction of the following modifications bypasses the control of CDK on preRC formation: alanine substitution of the CDK phosphorylation sites on Orc2 and Orc6,

overexpression of N-terminally truncated Cdc6 resulting in partial stabilisiation, and constitutive localisation of MCM7 to the nucleus via a Nuclear Localisation Signal (NLS) (Nguyen *et al*., 2001). For the purpose of clarity, these modifications shall be referred to as Origin Licensing Mutants (OMLs) and individual modifications shall be called mcm-NLS, orc2-A, orc6-A, and cdc6ΔNT.

In the second approach to induce unscheduled origin firing, the CDK targets for replication initiation were bypassed. Sld2 and Sld3 are the only essential targets of CDK for replication initiation. CDK phosphorylates Sld2 and Sld3 which allows them to bind Dbp11 (Zegerman & Diffley, 2007). The regulation of CDK on replication initiation can be bypassed by phosphomimetic mutations of CDK targets on Sld2 (sld2-D), whereas the regulation on Sld3 can be bypassed using an sld3-dpb11 fusion protein in which the N-terminal segment of Sld3 is covalently fused with the C-terminal segment of Dpb11 (Zegerman & Diffley, 2007). It has been shown before that the simultaneous expression of these two factors results in replication initiation in the absence of CDK activity (Zegerman & Diffley, 2007). Given that the absence of CDK activity allows preRC formation, re-replication has also been observed.

In the last approach, instead of modifying the CDK targets, I directly impaired the CDK activity by specifically inhibiting the catalytic subunit of CDK *Cdc28.* To do so, I took advantage of the *Cdc28* allele *cdc28-as1* (Bishop *et al*., 2000). The ATP-binding pocket *cdc28-as1* is modified which allows the binding of the ATP analog 1-NM-PP1 that prevents its function (Bishop *et al*., 2000). By using all these approaches, I addressed the contribution of checkpoint dependent inhibition of origin firing during unscheduled replication initiation.

4.2. Deregulated origin firing does not increase the extent of re-replication

As mentioned in section 4.1, the contribution of the Rad53-dependent inhibition of origin firing was tested when the CDK targets (Orc2 Orc6 Mcm7 Cdc6) were deregulated. Re-replication causes deleterious genomic instability which exhibits decreased cell growth and increased cell death. Therefore, to address whether Rad53 is involved in preventing further re-replication as observed in OLMs through the inhibition of origin firing, I compared the growth of cells that are re-replicating in the presence or absence of Rad53 control on the inhibition of origin firing. Rad53 phosphorylates Sld3 and Dbf4 upon DNA damage to inhibit origin firing. In order to bypass the control of Rad53 on the inhibition of origin firing, I used the *sld3-A* and *dbf4-4A* alleles in which the Rad53 phosphorylation site is replaced with alanine (Zegerman & Diffley, 2010).

Since cells carrying origin licensing modifications simultaneously are not viable because of excessive genomic instability, it is necessary to develop a system which allows the assessment of the contribution of the inhibition of origin firing during re-replication. Although it had been proposed initially that all four modifications are simultaneously required for effective re-replication in G2 phase (Nguyen *et al*., 2001), it has since been shown that only the mcm7-NLS and the overexpression of cdc6ΔNT is enough to induce re-replication (Green *et al*., 2006). Hence, the systematic analysis of modifications which lead to re-replication can provide a valuable insight into the importance of each modification.

Taking advantage of the fact that all the modifications mentioned before (expression of Cdc6, *mcm7-NLS, orc2-A, orc6-A, sld3-A, dbf-4A*) are dominant, I analysed the growth of diploid cells that were heterozygous for each mutation with one exception. P_{GAL}-*CDC6* was used instead of P_{GAL}-*cdc6ΔNT* to decrease the efficacy of preRC formation, in order to increase the probability

of cell growth. To bypass the control of Rad53 on the inhibition of origin firing, the *sld3-A dbf4-4A* alleles were used as a second copy under a galactose-inducible promoter (P_{GAL}). To obtain diploid cells by mating, equal numbers of MATa and MATα haploid cells carrying different mutations were mixed in liquid YPD media and incubated for 8 hours (**Figure 4-2**). Next, cells were plated on to different types of plates using a 48 Pin Multi-Blot replicator. Since both haploid and diploid cells grow on YPD, this media was used to control for starting cell culture densities. Plates lacking histidine and uracil were used to evaluate the efficiency of mating as only diploid cells would survive. Finally, Sc+galactose-URA-HIS plates were used to induce the genes under the Gal1-10 promoter (P_{GAL}) to analyse the effect of genes under the inducible promoter.

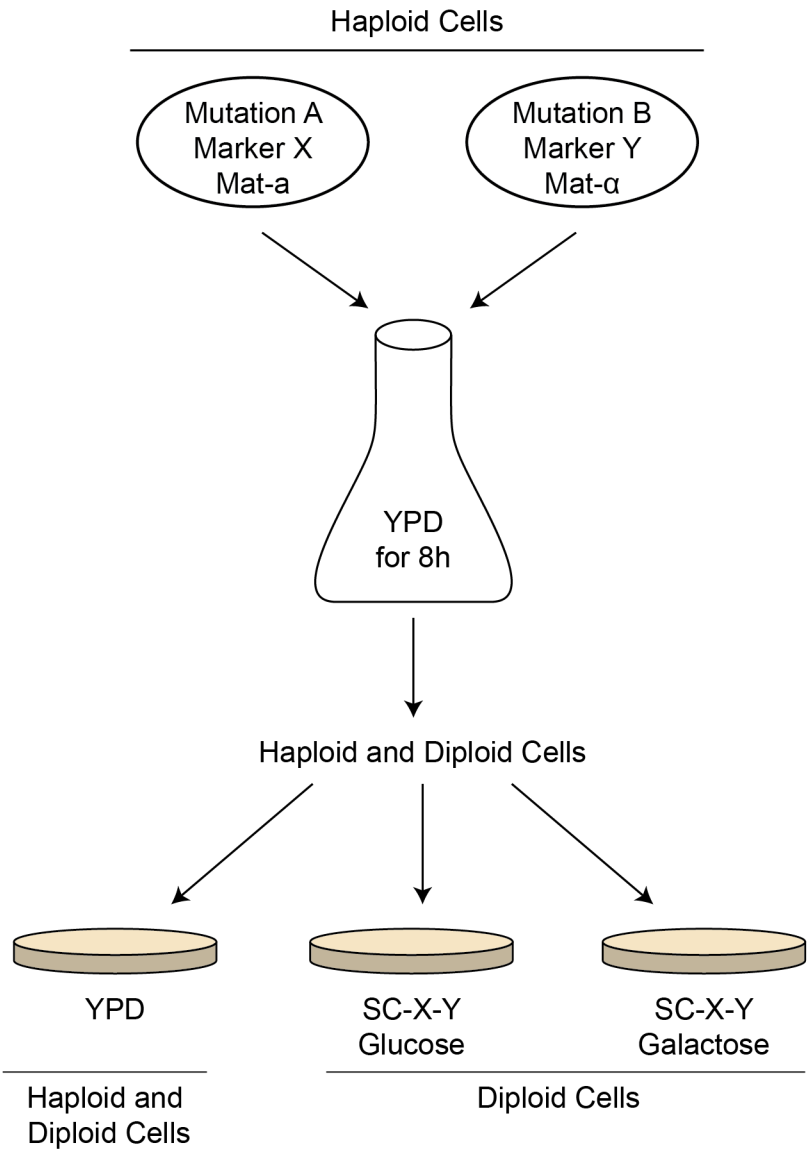


Figure 4-2 Diagram showing the experimental procedure for obtaining diploid cells carrying different combinations of origin licensing mutations.

From these crosses, it was possible to obtain most of the combinations containing P_{GAL}-*CDC6* which were indicated in **Figure 4-3**. Growth on YPD plates indicated that a similar amount of cells were used for each mating (**Figure 4-3**). Growth on glucose plates indicated that similar amounts of diploid cells were obtained after each mating. However, no cell growth was observed on galactose plates. As a result, it was not possible to analyse the effect of the inhibition of the origin firing by Rad53 on cell viability when CDK targets for preRC formation were deregulated in this experimental set up.

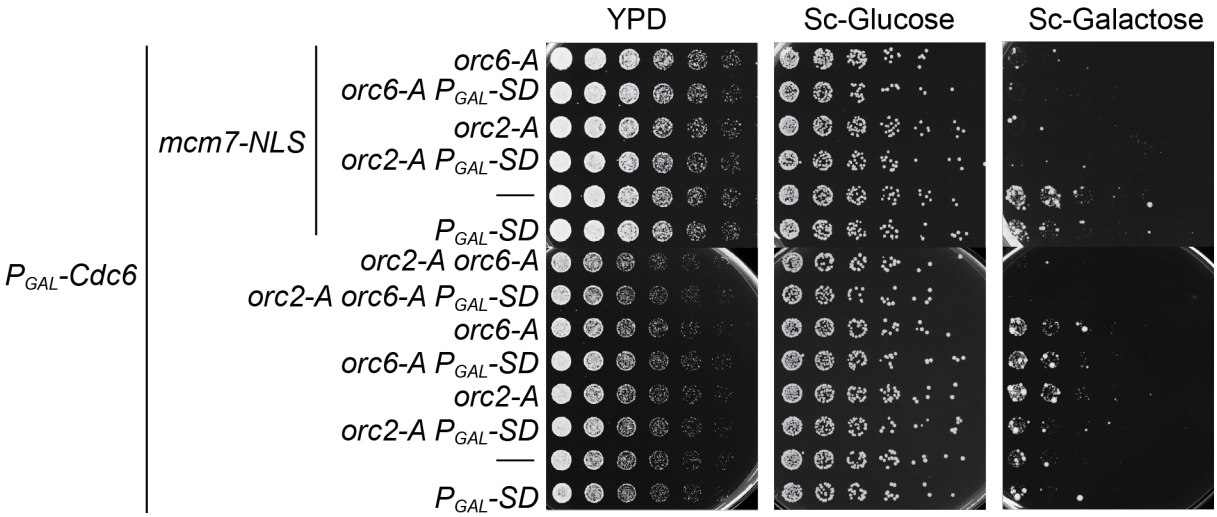


Figure 4-3 The effect of overexpression of *sld3-A dbf4-4A* on the growth of different origin licensing mutants: Cells carrying different origin licensing mutants. Heterozygous diploid cells with the indicated genotypes were created through mating of haploid cells. YPD plates were used as a control for cell density. Sc plates do not contain uracil and histidine, in order to select for diploid cells. SD indicates *sld3-A dbf4-4A*.

Given that all tested combinations of origin licensing mutants carried the P_{GAL}-*CDC6,* introducing another gene under P_{GAL} (in this case P_{GAL}-*sld3-A dbf4-4A*) could potentially dilute the pool of transcription factors available for P_{GAL}-*CDC6.* To overcome this possible dilution of *CDC6* transcription, a very similar experiment was conducted, with the exception that endogenously mutated *sld3-A dbf4-4A* were used instead of a second copy of *sld3-A dbf4-4A* under P_{GAL}. Diploid cells were obtained as mentioned above (**Figure 4-2**) and plated on YPD, and selective plates containing either glucose or galactose using a 48 Pin Multi-Blot replicator. However, in contrast to the previous experiment, all possible combinations of origin licensing mutants were obtained with or without endogenous *sld3-A dbf4-4A* in this experiment. Similar amounts of growth on YPD plates indicated that an equal amount of cells were mixed for individual mating. Selective plates containing glucose were used to analyse the origin licensing mutant combinations without Cdc6 (although cells carried P_{GAL}-*CDC6,* they did not express it because of the lack of galactose in the media), whereas selective plates containing galactose

were used to analyse the origin licensing mutant combinations with overexpression of Cdc6. The *orc2-A orc6-A mcm7-NLS* P_{GAL}*-Cdc6, orc6-A mcm7-NLS* P_{GAL}*-Cdc6,* and *orc2-A mcm7-NLS* P_{GAL}*-Cdc6* cells with or without *sld3-A dbf4-4A* did not grow on selective plates containing galactose, suggesting that the level of genomic instability due to re-replication in those cells was lethal. On the other hand, no growth differences were observed between cells on selective plates containing galactose, suggesting that *sld3-A dbf4-4A* did not exacerbate the extent of re-replication in that cells. In addition, all tested combinations, which did not have Cdc6 overexpression grew similarly compared to the same mutations combined with *sld3-A dbf4-4A.* In conclusion, deregulating the control of Rad53 over the inhibition of the origin firing exhibited no growth phenotype on the different combinations of origin licensing mutants in diploid cells that were viable.

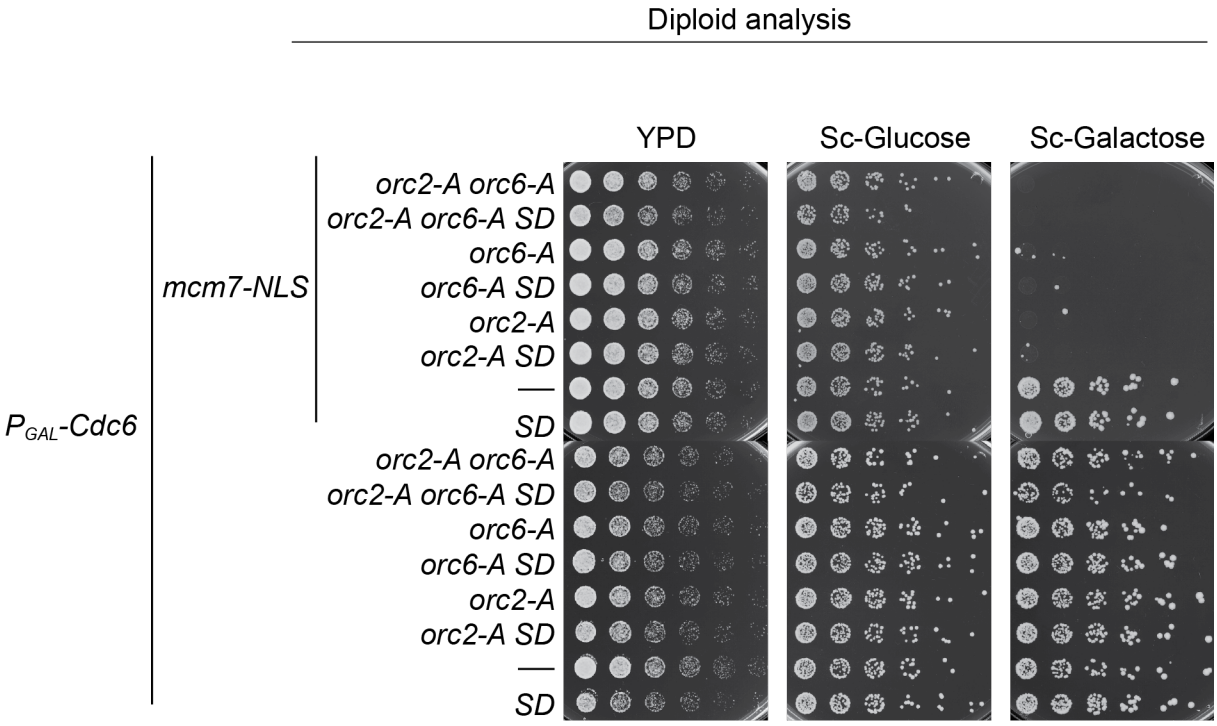


Figure 4-4 The effect of endogenous *sld3-A dbf4-4A* on the growth of different origin licensing mutants: Cells carrying different origin licensing mutations. Heterozygous diploid cells with indicated genotypes were produced through mating of haploid cells. YPD plates were used as a control for cell density. Sc plates do not contain uracil and histidine in order to select diploid cells. SD indicates *sld3-A dbf4-4A.*

To verify the results obtained above, a similar experiment was conducted in haploid cells. The growth of all possible combinations of origin licensing mutants were compared with corresponding mutants which overexpress *sld3-A dbf4-4A* under P_{GAL}. To do so, exponentially growing cells were plated on selective plates containing either galactose or glucose. As can be seen in **Figure 4-5**, all cells except *mcm7-NLS orc2-A orc6-A* P_{GAL}*-CDC6* and *mcm7-NLS orc2-A orc6-A* grew normally on selective plates containing glucose, meaning that similar densities of cells were used for the experiment. The *mcm7-NLS orc2-A orc6-A, mcm7-NLS orc6-A, mcm7-NLS orc2-A, orc2-A orc6-A* and *mcm7-NLS* cells did not grow when Cdc6 was overexpressed. On the other hand*, orc6-A* P_{GAL}*-CDC6, orc2-A* P_{GAL}*-CDC6* and P_{GAL}*-CDC6* grew on selective plates containing galactose, however the overexpression of *sld3-A dbf4-4A* did not cause any growth defect in these cells.

For cells which did not overexpress *CDC6* (**Figure 4-5** lower panel) the overexpression of *sld3-A dbf4-4A* resulted in some growth defects in the *mcm7-NLS orc6-A orc2-A, mcm7-NLS orc6-A, mcm7-NLS orc2-A, orc6-A orc2-A and orc6-A* cells. However, this effect was not observed in *mcm7-NLS* and *orc2-A* cells*.* Overall, bypassing the Rad53-dependent inhibition of origin firing decreased the growth of some origin licensing mutant combinations in haploid cells, suggesting that inhibition of origin firing by Rad53 might decrease the extent of unscheduled replication initiation and rereplication.

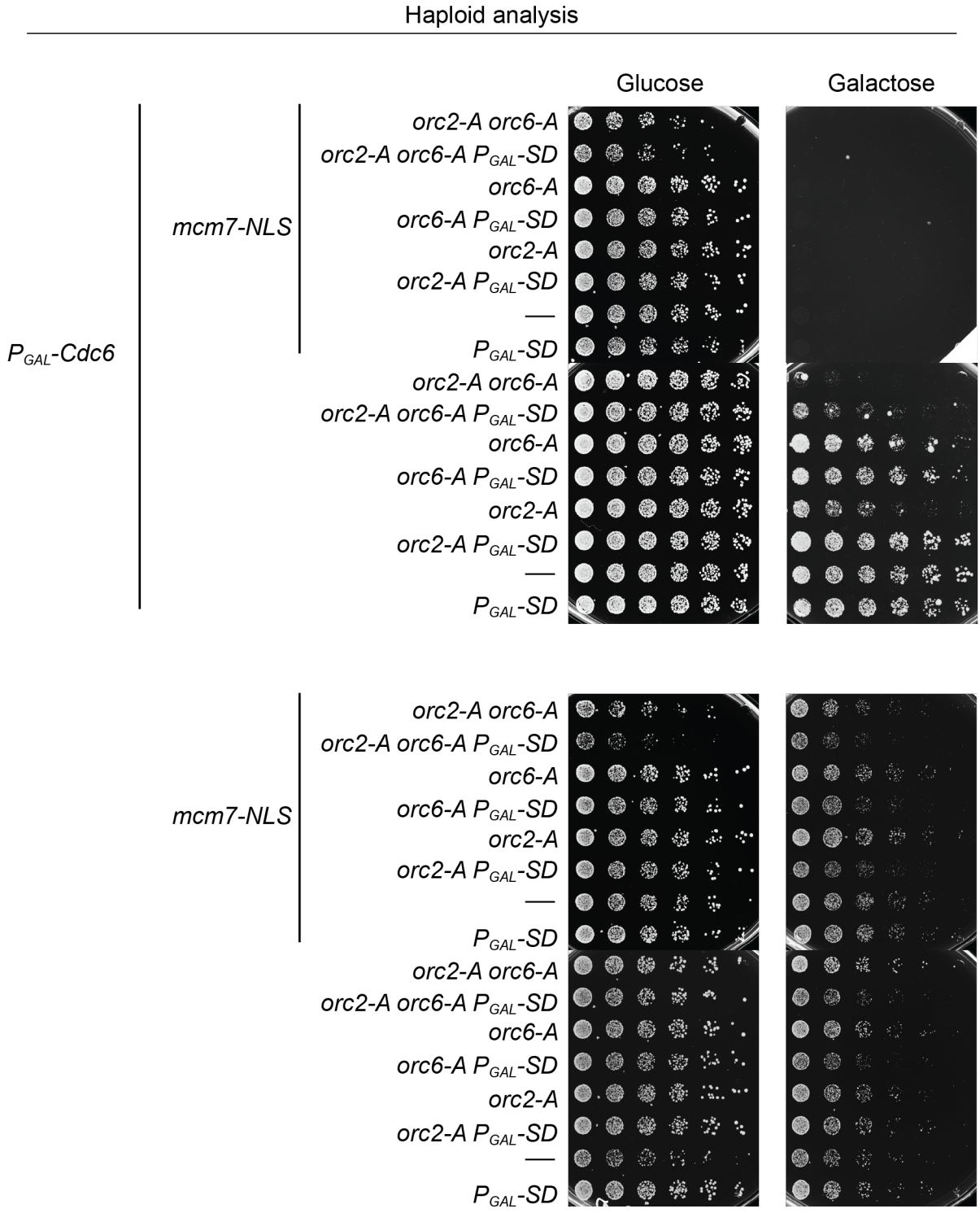


Figure 4-5 The effect of overexpression of *sld3-A dbf4-4A* on the growth of different origin licensing mutants: Haploid cells carrying different origin licensing mutants. SD indicates *sld3-A dbf4-4A.*

In the previous experiment, bypassing Rad53 control of the inhibition of origin firing resulted in growth defects in some combinations of origin licensing mutants. However, *sld3-A and dbf4-*

4A were overexpressed in those cells. In order to rule out whether the growth defects that were observed were due to the overexpression of *sld3-A and dbf4-4A,* a similar experiment was performed to ask whether *sld3-A dbf4-4A* alleles as the only copy at their endogenous locus caused the same phenotype. Cells carrying origin licensing mutations indicated in **Figure 4-6** were plated both on glucose and galactose selective plates. The glucose plates do not overexpress *CDC6*, whereas galactose plates induce the overexpression of *CDC6* as in **Figure 4-4**.

As can be seen in **Figure 4-6**, the *mcm7-NLS orc2-A orc6-A, mcm7-NLS orc6-A*, *mcm7-NLS orc2-A, mcm7-NLS and orc2-A orc6-A* cells were dead when *CDC6* was overexpressed. Interestingly, endogenous *sld3-A dbf4-4A* caused a slight growth defect in *orc6-A* P_{GAL}*-CDC6 and* P_{GAL}*-CDC6* cells when Cdc6 was overexpressed. However, these combinations were different than the previously observed combinations (**Figure 4-5**). On the other hand, endogenous *sld3-A dbf4-4A* did not cause a significant growth defect in the cells that were not overexpressing Cdc6. Overall, by passing the control of Rad53 on the inhibition of origin firing by endogenous *sld3-A dbf4-4A* alleles, the sickness of cells carrying different origin licensing mutants was exacerbated. This suggests that inhibition of origin firing by Rad53 might contribute to the extent of unscheduled replication initiation and rereplication.

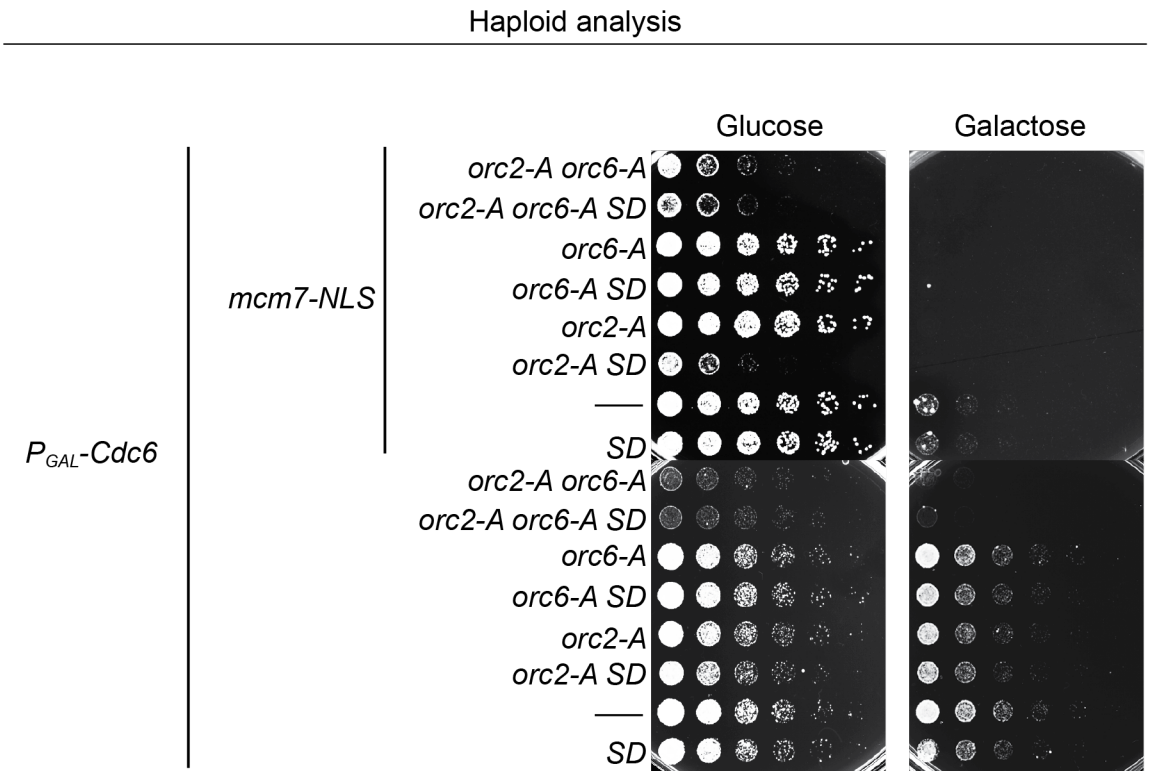
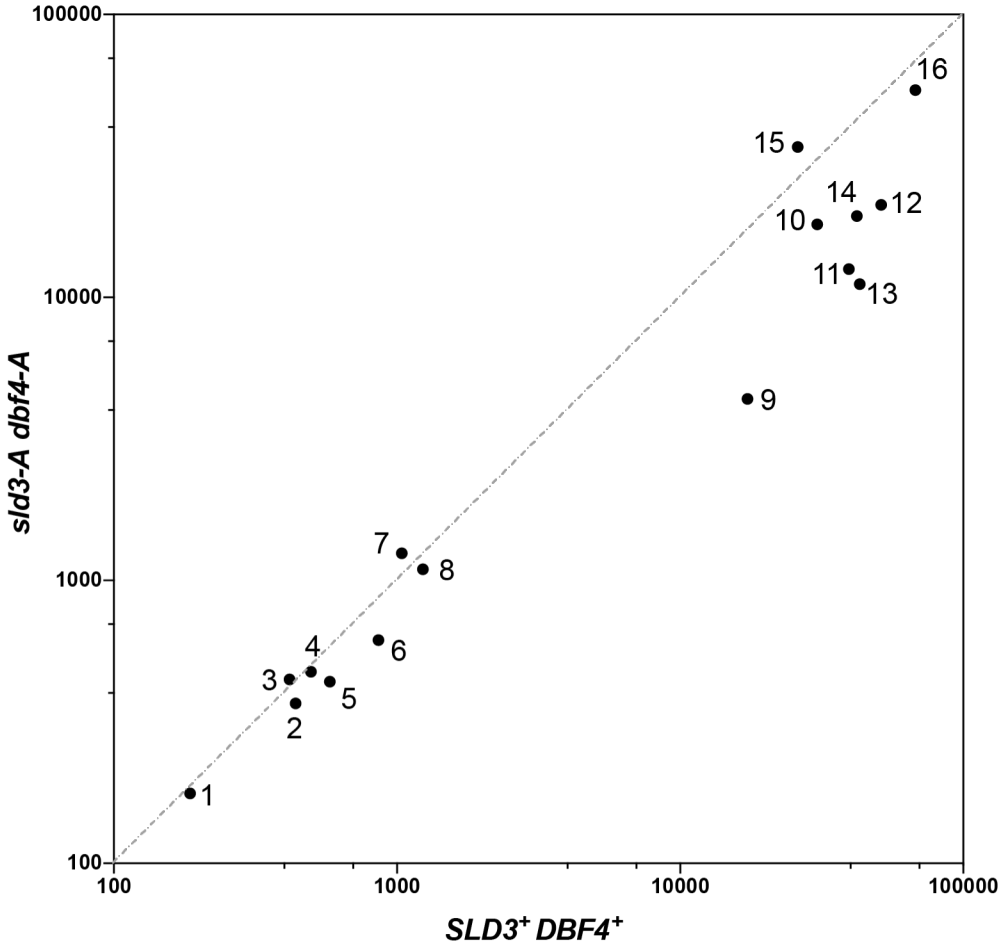


Figure 4-6 The effect of endogenous *sld3-A dbf4-4A* on the growth of different origin licensing mutants: Haploid cells carrying different origin licensing mutants. SD indicates *sld3-A dbf4-4A*.

Because only major differences can be observed using drop-test analysis and micro colonies could not be observed, the size of colonies originating from single cells was also measured in order to increase sensitivity. For this, I imaged growing colonies by using a camera attached to a microscope, and measured their size using image processing software ImageJ. Since the overexpression of *CDC6* was synthetic lethal with some origin licensing mutants, the concentration of galactose was reduced in the media to decrease the expression from P_{GAL}-*CDC6*. Haploid cells with indicated mutations were plated on selective plates containing 0.5% galactose and 1.5% sucrose plates and colonies were examined after 39h. As seen in **Figure 4-7**, overexpression of CDC6 affected the cell growth drastically even though the overall percentage of galactose was reduced in the growth media. However, deregulated origin firing via endogenous *sld3-A dbf4-4A* alleles did not exacerbate the sickness of re-replication

mutants overexpressing Cdc6. On the other hand, endogenous *sld3-A dbf4-4A* did affect the growth of *mcm7-NLS orc2-A orc6-A, mcm7-NLS orc6-A, mcm7-NLS orc2-A, orc6-A* and *orc2-A* but not in *orc2-A orc6-A* and *mcm7-NLS* cells compared to controls (**Figure 4-7**) which was in line with **Figure 4-5**.



1 *mcm7-NLS orc2-A orc6-A* P_{GAL}*-CDC6*
2 *orc2-A orc6-A* P_{GAL}*-CDC6*
3 *mcm7-NLS orc6-A* P_{GAL}*-CDC6*
4 *mcm7-NLS orc2-A* P_{GAL}*-CDC6*
5 *orc6-A* P_{GAL}*-CDC6*
6 *orc2-A* P_{GAL}*-CDC6*
7 *mcm7-NLS* P_{GAL}*-CDC6*
8 P_{GAL}*-CDC6*
9 *mcm7-NLS orc2-A orc6-A*
10 *orc2-A orc6-A*
11 *mcm7-NLS orc6-A*
12 *mcm7-NLS orc2-A*
13 *orc6-A*
14 *orc2-A*
15 *mcm7-NLS*
16 *URA3*$^{+}$

Figure 4-7 The colony size analysis of origin licensing mutants. The effect of *sld3-A dbf4-4A* overexpression on the average colony size of the origin licensing mutants. Corresponding mutations are indicated above. Pictures were taken 39-hour post-plating. SC+1.5% Sucrose+0.5% galactose plates were used.

To verify previous results, and produce time course growth kinetics, quantitative fitness analysis (QFA) was performed in collaboration with Prof. Dr. David Lydall and Dr. Peter Banks at Newcastle University. QFA is as workflow that compares the fitness of microbial cultures in parallel (Banks *et al*., 2012). The growth fitness of the origin licensing mutants indicated in **Figure 4-8** were compared with the same mutations plus *sld3-A dbf4-4A*. In addition, wildtype and *sld3-A dbf4-4A* cells were used as controls. To do so, each strain was spotted in at least 4 biological replicates, grown for several days and photographed every 4 hours. Next, each image was processed and the growth curve for each strain was calculated. From each growth curve, 2 parameters were obtained. The first was the maximum doubling rate, and the second was the maximum doubling potential. The fitness of each strain was calculated by multiplying these 2 parameters together. Finally, the fitness of individual origin licensing mutants was compared to their *sld3-A dbf4-4A* counterparts. The fitness of control strains was assayed and mutants were plotted against a relative control. As shown in **Figure 4-8**, although different combinations of origin licensing mutants exhibited differential fitness levels, endogenous *sld3-A dbf4-4A* did not decrease the fitness of origin licensing mutants. Very similar experiments were also performed on plates containing different genotoxic drugs in order to activate the DNA damage checkpoint, however the fitness of origin licensing mutants carrying the *sld3-A dbf4-4A* allele were similar to their $SLD3^{+}$ $DBF4^{+}$ counterparts, suggesting that Rad53 control on the inhibition of origin firing does not contribute to the fitness of these mutants.

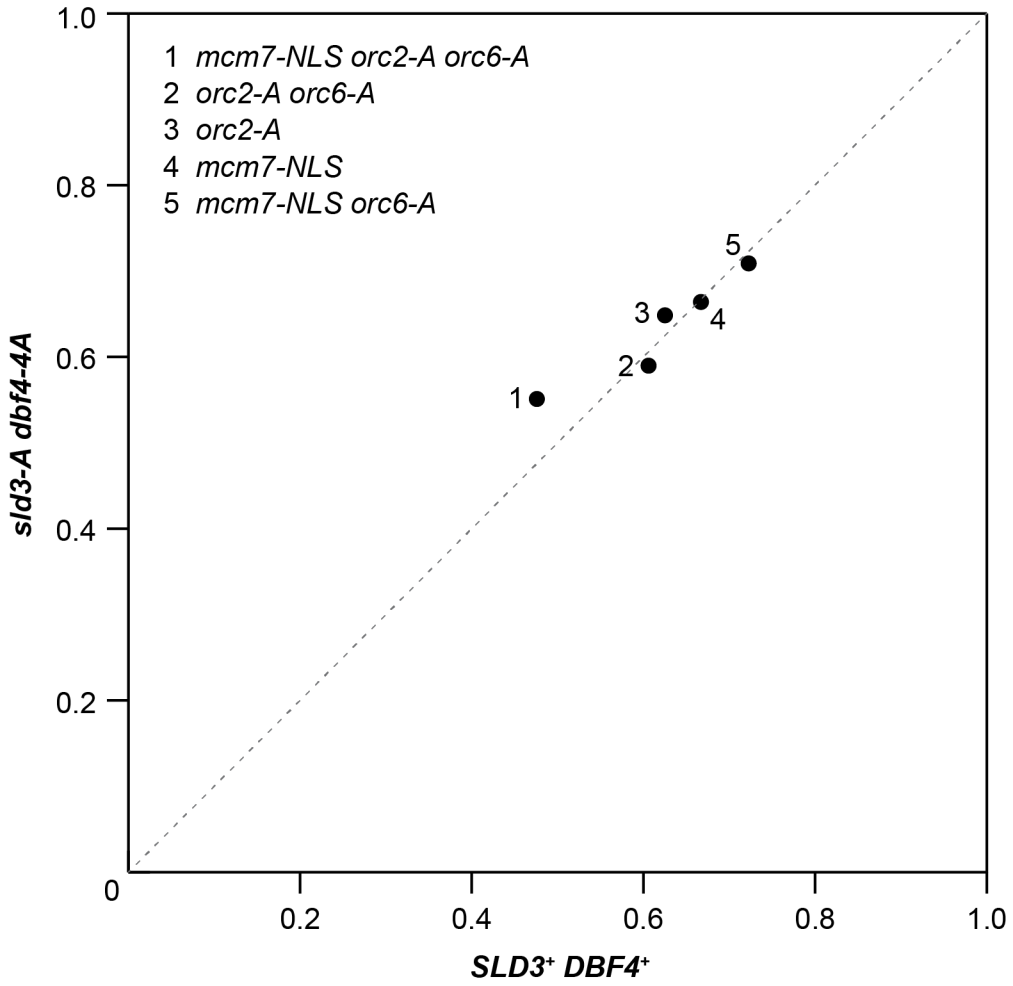


Figure 4-8 The effect of *sld3-A dbf4-4A* on the fitness of origin licensing mutants. Scatter plot showing the fitness of origin licensing mutants and corresponding mutations plus endogenous *sld3-A dbf4-4A.* Each dot represents a different origin licensing mutant indicated in the plot.

Next, I wanted to test whether the checkpoint-dependent inhibition of origin firing prevents the extent of unscheduled replication initiation in G1 phase. To test this, a system which can bypass the requirement of CDK for replication initiation was necessary. DNA replication can be initiated in the absence of CDK activity in G1 phase by bypassing the CDK targets for origin firing and overexpressing DDK subunit Dbf4. Replication initiation requires the CDK-dependent phosphorylation of 2 essential replication factors Sld2 and Sld3 in S-phase, which allow them to bind the BRCT domains of Dpb11. It has previously been shown that the simultaneous expression of Dbf4, a phosphomimetic mutant of CDK sites on Sld2 sld2-D, and the sld3-dbp11

fusion which consists of the N-terminal part of Sld3 and C-terminal part of Dbp11 can bypass the requirement of CDK, resulting in replication initiation in the absence of CDK activity in G1 phase (Zegerman & Diffley, 2007). However, contrary to the sld3-dpb11 phusion protein, the sld3-**D**-dpb11 fusion protein in which the Rad53 phosphorylation sites on the sld3 fragment are replaced with aspartate is not viable in sld3Δ dbp11Δ, indicating that Rad53 could possibly prevent origin firing by phosphorylating the sld3-dpb11 fusion protein upon DNA damage. One way to bypass the control of Rad53 on sld3-dbp11 fusion protein would be by replacing Rad53 phosphorylation sites of sld3 fragment with Alanine. Instead, I decided to overexpress Sld3, attempting to force it to interact with Dpb11 in G1 phase. In addition, in order to support re-licensing after forced origin firing, the N-terminally truncated *CDC6 allele* cdc6ΔNT - which is partially stabilised - was used.

To test if any of the manipulations above would result in unscheduled origin firing and if the deregulated Rad53 targets dbf-A and sld3-A would increase the extent of unscheduled origin firing, the strains indicated in **Figure 4-9** were made and growth analysis was performed. Given that some genes were expressed under $P_{GAL,}$ exponentially growing cells were plated on either YPD as a control or YPGal to induce genes under P_{GAL}. As indicated in **Figure 4-9**, overexpressing sld3-A and dbf-4A significantly decreased growth of the cells. However, overexpressing Sld3 and Dbf4 exhibited no growth differences compare to wild type cells. Contrarily, overexpression of sld2-D with Sld3 and Dbf4 reduced the growth of the cells, whereas overexpression of sld2-D alone or together with sld3-A and dbf-4A had no effect on the cell growth. On the other hand, cdc6Δnt decreased the growth of sld2-D sld3-A dbf4-4A overexpressing cells, while it did not affect the growth of the cells overexpressing sld2-D alone or together with Sld3 and Dbf4 (**Figure 4-9**). Overall, although overexpression of sld3-A and dbf4-4A showed marginal growth defects, these were not execrated by the combinations with other mutants, suggesting that the growth defects were not due to genomic instability caused by unscheduled origin firing.

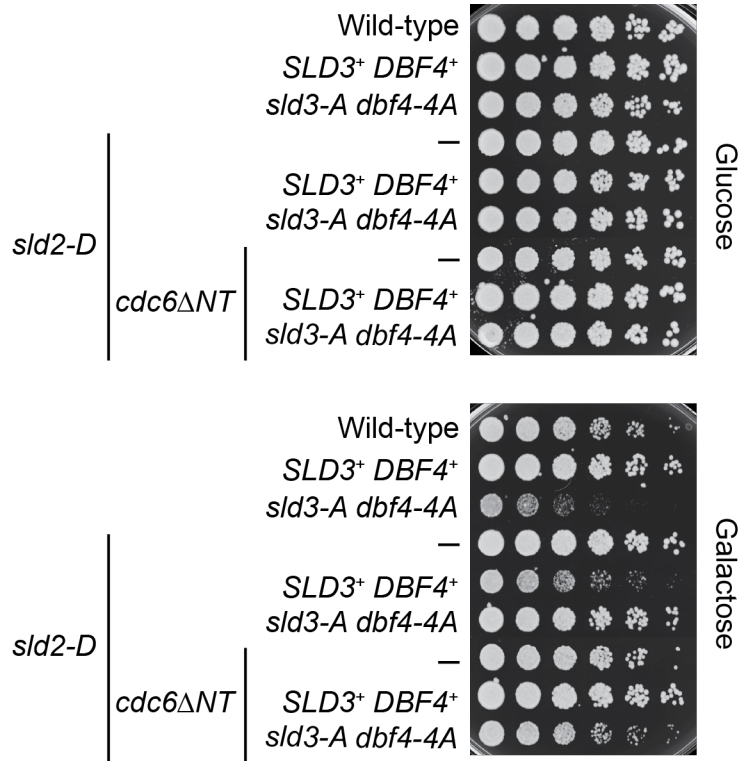


Figure 4-9 Forcing the replication initiation in G1 phase. YPD plates were used as a control. The *sld2-D, DBF4*$^{+}$*, SLD3*$^{+}$*, sld3-A* and *dbf4-4A* were overexpressed under P_{GAL}.

Because the growth analyses so far are only a readout for the indirect growth effects of unscheduled replication initiation, I decided to perform an assay which gives a direct readout for unscheduled replication initiation. There are different approaches to assess if DNA replication is initiated, such as 2D gel electrophoresis, whole genome sequencing or flow cytometry analysis. Although flow cytometry analysis is less sensitive than other techniques mentioned, it is more feasible to screen multiple candidates. For these reasons, I analysed the increase in bulk DNA amount in G1 or G2 phase when DNA replication was forced to initiate. To do so, first the increase in the bulk DNA amount in G2 phase was tested using cells carrying different origin licensing mutations. Because glucose is a favoured carbon source in yeast, the addition of galactose to cells growing in glucose-containing media did not activate the transcription from Gal promoter. To efficiently induce the Gal promoter, cells must grow in a media containing a less favourable carbon source than galactose, such as raffinose. For this purpose, cells were grown in YP+Raffinose (YPRaff). Exponentially growing cells were

arrested in YPRaff at 30°C in G2 phase with nocodazole for 90 minutes and 2% galactose added to induce the expression of genes under the Gal promoter. Samples were collected every hour following the addition of galactose. Flow cytometry analysis indicated that *orc6-A mcm7-NLS* P_{GAL}*-Cdc6* cells could re-replicate after addition of galactose in G2 phase. However, overexpression of *sld3-A dbf4-4A* did not increase the extent of re-replication (**Figure 4-10**). In fact, *orc6-A mcm7-NLS* P_{GAL}*-Cdc6* P_{GAL}*-sld3-A-dbf4-4A* showed less replication than *orc6-A mcm7-NLS* P_{GAL}*-Cdc6*, possibly due to the dilution of transcription factors for each P_{GAL}. In addition, the rest of the tested cells stayed did not show any increase in bulk DNA amount after the addition of galactose, suggesting that bulk DNA replication did not begin in these cells in G2 phase. Overall, this experiment suggested that either flow cytometry was not sensitive enough to monitor re-replication or no additional re-replication is started in G2 phase by overexpression of sld3-A dbf4-A.

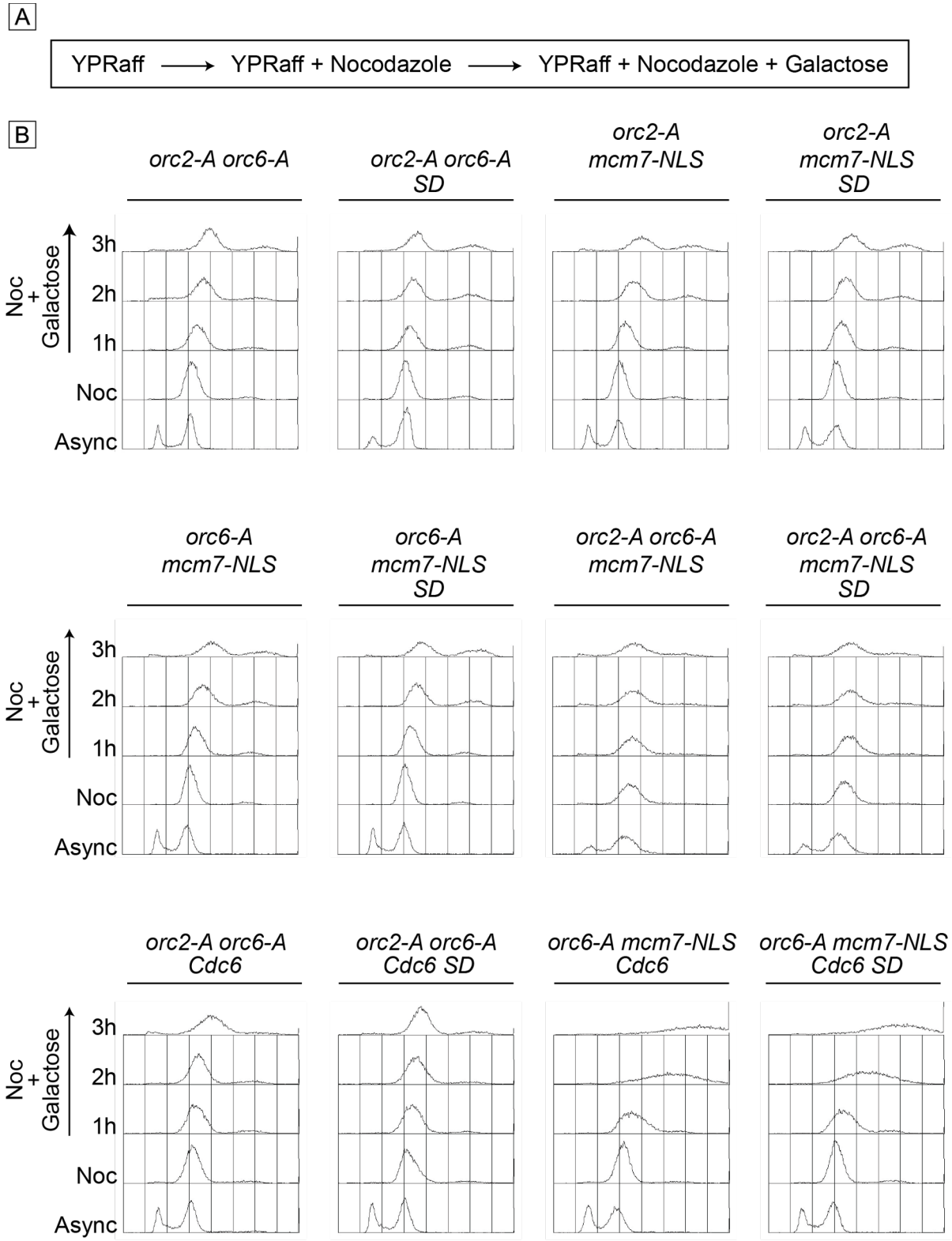


Figure 4-10 Flow cytometry analysis cells carrying different origin licensing mutations in G2 phase. A) schematic representation of the experimental steps. B) flow cytometry profiles of the cells. SD indicates *sld3-A dbf4-4A. CDC6* and *sld3-A dbf4-4A* were overexpressed under P_{GAL} in the cases indicated.

Next, to test if the Rad53-dependent inhibition of origin firing decreased the extent of replication initiation, the bulk DNA amount of the cells that can potentially fire replication origins in G1 phase were analysed by flow cytometry. In order to prevent the release from G1 arrest during the experiment, all cells used in this experiment were lacking the Bar1 protease which degrades α-factor. Since CDK phosphorylates Orc6 in S but not in G1 phase, the phosphorylation of Orc6 was used as an indicator of G1 arrest. Western blot analysis of Orc6 indicated that Orc6 was not phosphorylated, meaning that cells were arrested and stayed in G1 phase during the experiment (**Figure 4-11.C**). Flow cytometry analysis showed that wild-type, *SLD3*$^{+}$*DBF4*$^{+}$, *sld3-A dbf4-4A,* and *sld2-D cdc6-ΔNT* cells did not show significant DNA replication after the addition of galactose (**Figure 4-11.B**). However, overexpression of either Sld3 and Dbf4 or sld3-A dbf4-4A in a *sld2-D cdc6ΔNT* background was sufficient to initiate DNA replication in G1 phase. Although the bulk DNA mount for those cell was similar at the last time point, their progression patterns were different. The replication of *sld3-A dbf4-4A sld2-D cdc6ΔNT* cells progressed slower, whereas *SLD3*$^{+}$*DBF4*$^{+}$ *sld2-D cdc6ΔNT* progressed faster at the beginning but then almost stopped.

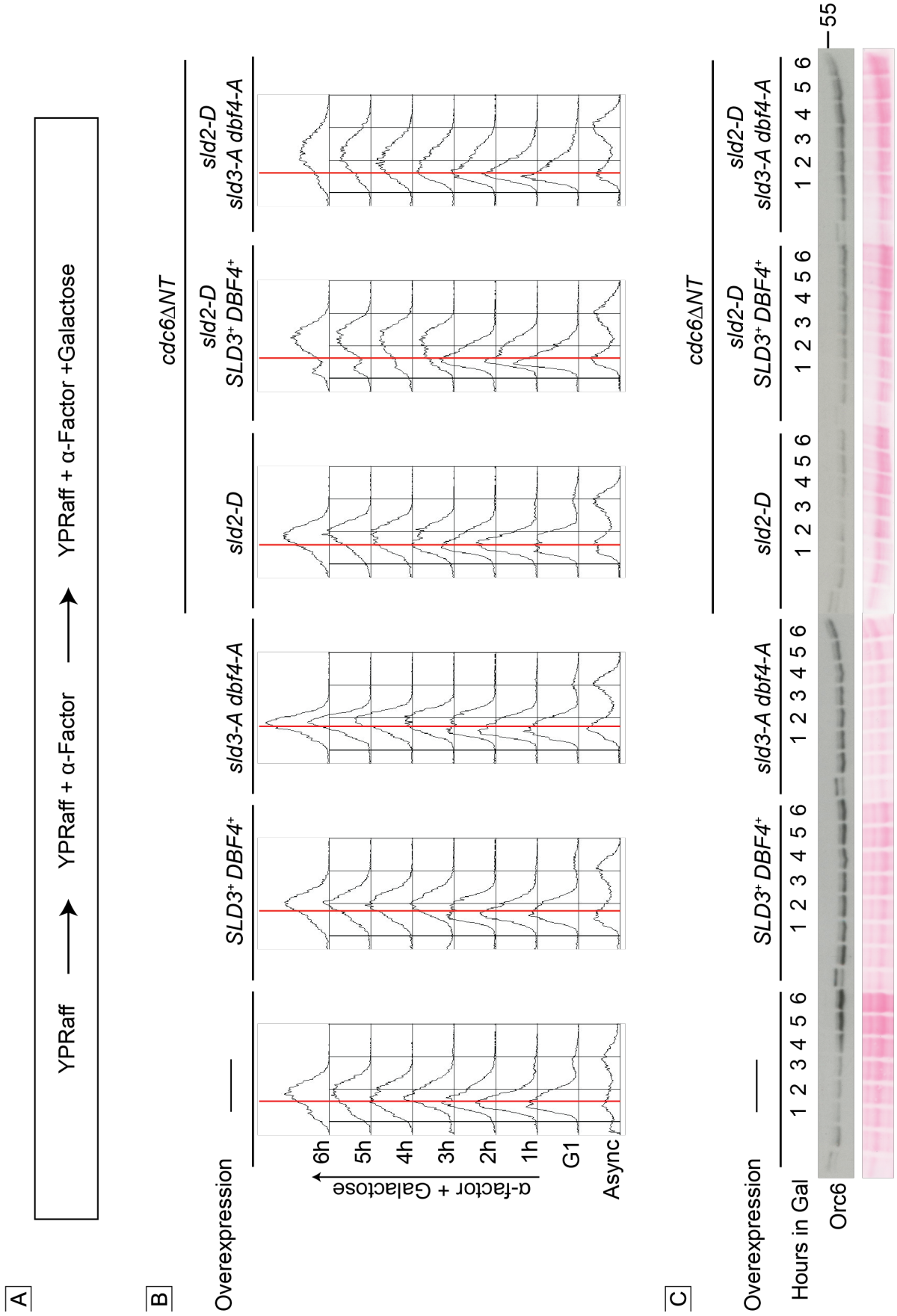


Figure 4-11 The replication initiation analysis in G1 phase. All cells were isogenic for *bar1Δ.* A) schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Orc6. Anti-Orc6 antibody was used for probing.

4.3. Deregulated origin firing decreases the viability of cells when CDK activity is impaired

Although the activity of CDK is well-regulated throughout the cell cycle, it is possible that it may fluctuate during S or G2 phases or during transitions between phases. This might occur due to the inefficient degradation of CDK inhibitors (CKIs) during G1-S transition, or misregulation of Cyclins in S- or G2 phase as observed in many cancers (Deshpande *et al*., 2005). The lack of the yeast CKI Sic1 results in significant genomic instability due to unscheduled origin firing, such as double strand breaks and gross chromosomal rearrangements (Lengronne & Schwob, 2002), however *sic1Δ* cells are viable, suggesting that the DNA damage checkpoint compensates the severity of the damage. As a result, it is interesting to assess the contribution of the DNA damage checkpoint in the case of CDK misregulation. So far, I have described an analysis of re-replication after deregulation of individual DCDK targets. Following this, I wondered whether checkpoint-dependent inhibition of origin firing is important when CDK activity is impaired. To impair CDK activity, I used the *CDC28* allele *cdc28-as1*, whose activity can be ectopically diminished by an ATP analogue 1-NMPP1 which can only bind to the modified Cdc28 ATP-binding cassette (Bishop *et al*., 2000). It has previously been shown that impaired CDK activity via *cdc28-as1* sensitises yeast cells to DNA damaging agents (Enserink *et al*., 2009).

In order to establish if the Rad53-dependent inhibition of origin is important when CDK activity was impaired, I analysed the effect of impaired CDK activity when inhibition of origin firing was deregulated by *sld3-A dbf4-4A* alleles cells. To do so, exponentially growing cells were plated on YPD plates containing different concentrations of 1-NM-PP1. As can be seen in **Figure 4-12** the growth of *cdc28-as1* cells slowed down on 10 nM 1-NM-PP1 containing YPD and did not grow on 50 nM 1-NM-PP1 plates, whereas *sld3-A dbf4-4A* cells grew similarly on all tested conditions. Interestingly, although *sld3-A dbf4-4A cdc28-as1* grew similarly to *cdc28-as1* cells

up to 10 nM 1-NM-PP1, their growth was affected more than *cdc28-as1* cells on plates containing 20 to 50 nM 1-NM-PP1. In conclusion, impaired CDK activity negatively affected the growth of *sld3-A dbf4-4A* cells more than *SLD3*$^{+}$ *DBF4*$^{+}$ cells, suggesting that Rad53-dependent inhibition of origin firing is important when CDK is misregulated.

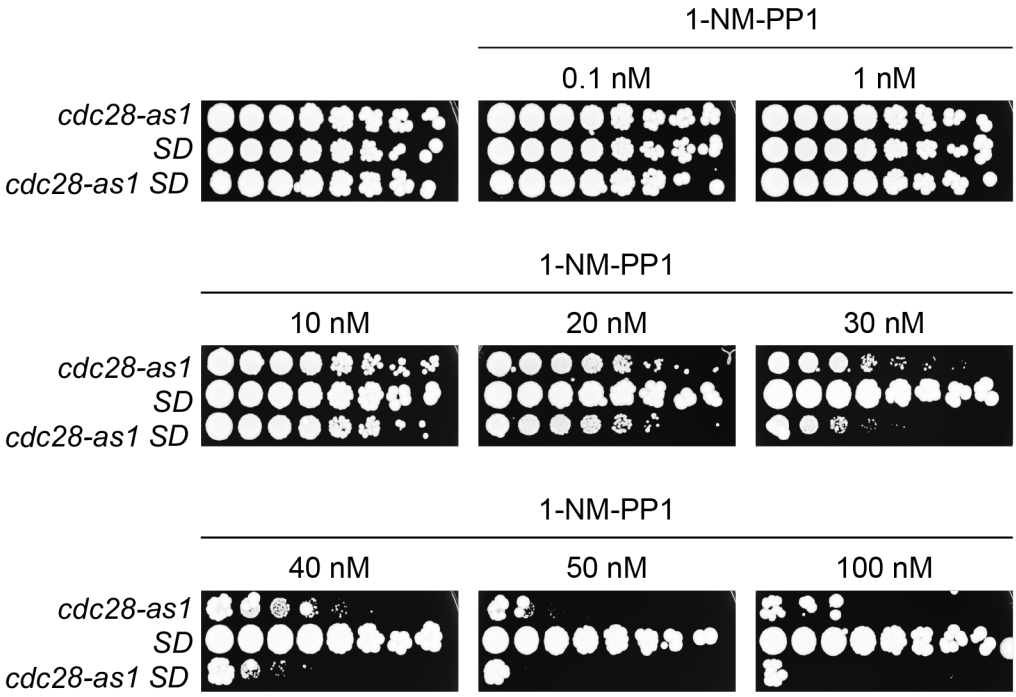


Figure 4-12 The effect of de-regulated origin firing during impaired CDK activity. Cells were plated on YPD plates containing indicated concentrations of 1-NM-PP1. *SD* indicates *sld3-A dbf4-4A.*

Next, I wondered if the activation of the checkpoint by small amounts of DNA damage would increase the growth defect of *sld3-A dbf4-4A* cells when CDK activity is impaired. To test this, exponentially growing cells were plated on plates containing low concentrations of different types of DNA-damaging drugs including 30 or 40 nM 1-NM-PP1. Although *sld3-A dbf4-4A cdc28-as1* cells grew slower than *cdc28-as1* when CDK activity was decreased by 30 or 40 nM 1-NM-PP1, this effect was not exacerbated by inducing DNA damage through low concentrations of DNA damaging agents (**Figure 4-13**).

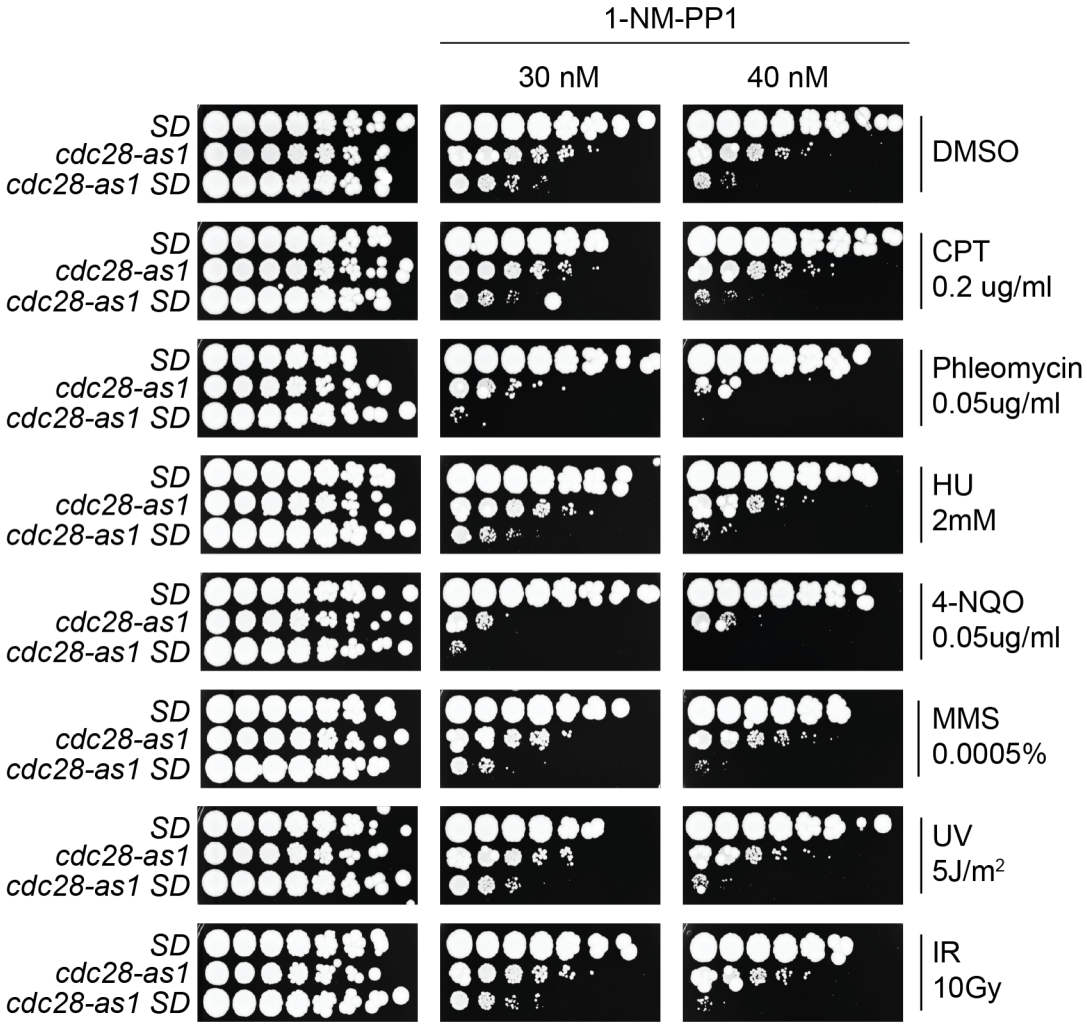


Figure 4-13 The effect of de-regulated origin firing during impaired CDK activity. Cells were plated on YPD plates containing indicated concentrations of DNA damaging drugs and 1-NM-PP1. *SD* indicates *sld3-A dbf4-4A.*

Given that *sld3-A dbf4-4A* cells exhibited a decreased growth phenotype when CDK activity was deregulated, one possible explanation is that losing the control on the inhibition of origin firing resulted in more unscheduled origin firing which generated more genomic instability. To test this, I decided to analyse the increase in bulk DNA amount when CDK activity was impaired by flow cytometry analysis. It is important to note that replication initiation in G2 phase would first require preRC formation, then origin firing. To find a window for CDK activity that can no longer prevent preRC formation but can still fire origins, cells were tested in different concentrations of 1-NM-PP1. As can be seen in **Figure 4-14**, the inhibition of CDK activity via

different concentrations of 1-NM-PP1 did not result in any replication in G2 phase, as *cdc28-as1* cells did not show a different pattern than *sld3-A dbf4-4A* cells in flow cytometry analysis. In addition, *cdc28-as1 sld3-A dbf4-4A* cells were also similar to both *sld3-A dbf4-4A* and *cdc28-as1 sld3-A dbf4-4A*, suggesting that either replication initiation does not occur via this approach or that the level of replication initiation is under the detection limit of flow cytometry analysis.

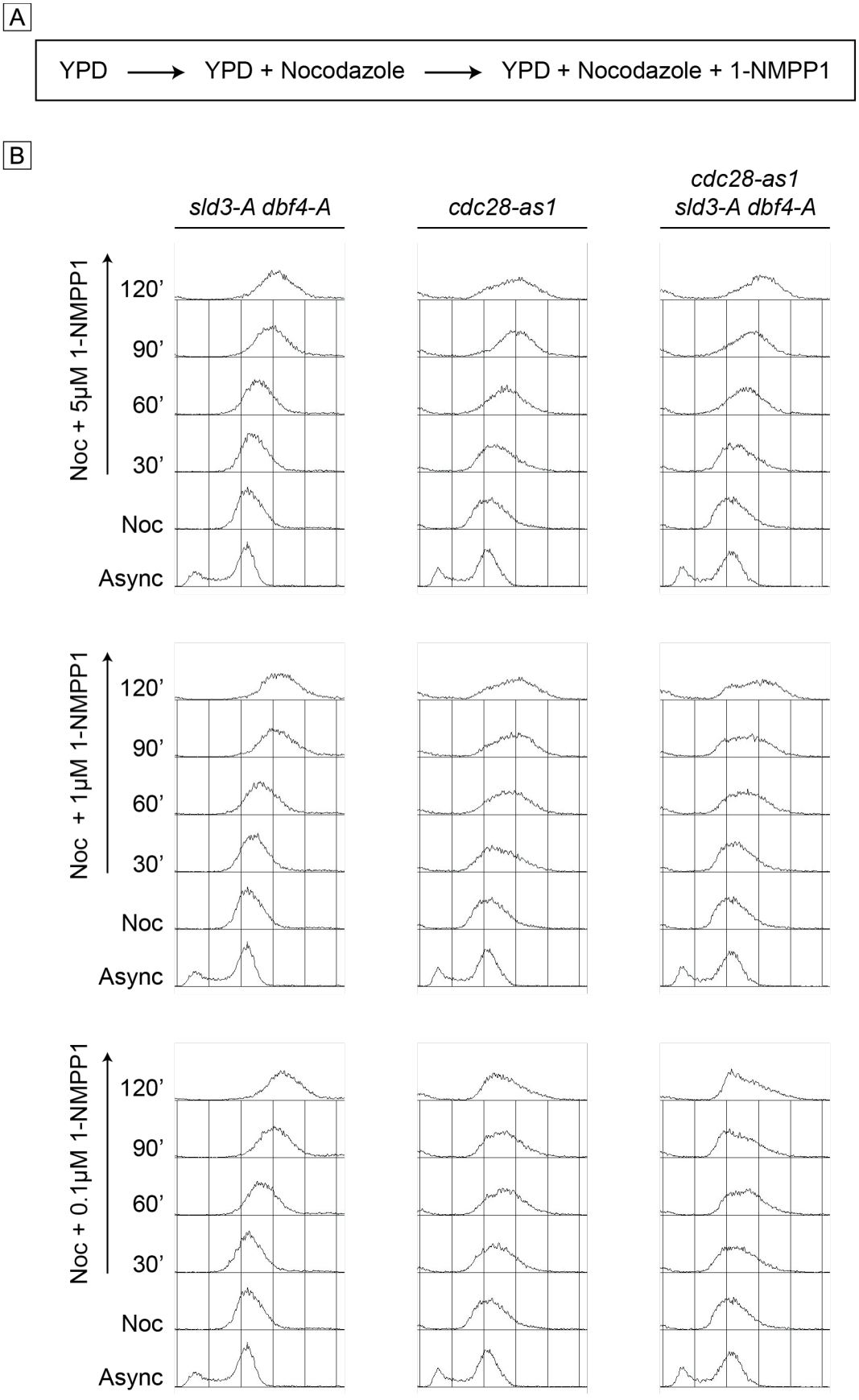


Figure 4-14 The effect of de-regulated origin firing on replication during impaired CDK activity in G2 phase. A) schematic representation of the experimental steps. B) flow cytometry profiles of the cells.

4.4. Discussion

The regulation of the cell cycle is tightly controlled by multiple mechanisms and misregulation of these controls could result in unscheduled replication initiation as well as rereplication. Different experimental systems are developed for addressing the possible source and implication of unscheduled origin firing and re-replication from yeast to human. However, the extent of unscheduled replication initiation and re-replication are always limited in these experimental systems (Green *et al*., 2006; Melixetian *et al*., 2004; Nguyen *et al*., 2000). Given that the activation of the DNA damage checkpoint upon re-replication is observed in different organisims (Davidson *et al*., 2006; Green *et al*., 2006; Green & Li, 2005; McGarry, 2002; Melixetian *et al*., 2004), the DNA replication stress checkpoint might prevent the occurrence of excessive unscheduled replication initiation and rereplication. However, the DNA replication stress checkpoint has multiple responses such as inhibition of origin firing, replication fork stabilization, mitotic delay and transcriptional control, and which of these responses is involved during re-replication is not well understood. Considering that the checkpoint-dependent inhibition of origin firing occurs not only in S-phase but also in G1 and G2, the limited re-replication that was observed before may be due to the inhibition of origin firing by the DNA damage checkpoint. To understand this, I investigated the involvement of checkpoint-dependent inhibition of origin firing during unscheduled replication initiation.

First, I systematically investigated the contribution of each origin licensing mutants which bypass the CDK control on re-replication on the unscheduled S-phase entry. I started with growth analysis of the different origin licensing mutants with both haploid and diploid cells. The drop test analysis indicated that *mcm7-NLS orc2-A orc6-A, mcm-NLS orc6-A, mcm7-NLS orc2-A, orc6-A orc2-A and mcm7-NLS* cells are not viable when *CDC6*$^{+}$ is overexpressed (**Figure 4-5** and **Figure 4-6**), possibly due to excessive genomic instability. The rest of the combinations were viable, and deregulation of the inhibition of origin firing by *sld3-A dbf4-4A*

alleles affected the growth of some viable origin licensing mutants. In order to be more precise and receive a quantitative output, the size of colonies carrying different origin licensing mutations was measured. Interestingly, the colony size analysis showed that deregulating the checkpoint control on inhibition of origin firing by overexpressing sld3-A dbf4-4A exacerbated the growth defect of *mcm7-NLS orc2-A orc6-A, mcm7-NLS orc6-A, mcm7-NLS orc2-A, orc2-A* and *orc6-A* cells (**Figure 4-7**), although it was not possible to validate this result with quantitative fitness assays.

Considering all these growth assays, inconsistencies between the experiments is a major issue. One explanation could be that accumulation of the several secondary mutations leads to increased or decreased growth phenotypes. Given that *mcm7-NLS orc2-A orc6-A* cells grew slower than the single or double mutants, some degree of mutations might have been introduced while preparing the cells or before the experiments as cells were originated from a diploid cell that was heterozygous for all the mentioned mutations. This could be ruled out by cloning all these mutants under an inducible promoter and only activating prior to the experiment.

On the other hand, whether checkpoint-dependent inhibition of origin firing is required for the prevention of excessive re-replication was not clear from the growth analysis. One possible explanation would be that the experimental setup was not suitable to address the contribution of the checkpoint. Because cells were either completely dead in some combinations or showed no significant growth defect, this could mean that either some combinations produced excessive levels of re-replication and/or other detrimental consequences or other combinations produced no re-replication. This could be ruled out by using other systems such as different Orc6 mutants. CDK both phosphorylates and binds Orc6 to prevent MCM loading. It has been shown before that instead of mutating phosphorylation site to Orc6, removing the CDK binding sites (RXL) on Orc6 (orc6-RXL) partially deregulates Orc6 but not as significantly as the *orc6-A* mutant (Archambault & Ikui, 2005). Using *orc6-RXL* instead of *orc6-A* might give a milder

initiation of unscheduled replication that would be suitable for assessing the contribution of checkpoint dependent inhibition of origin firing.

The growth analysis also indicated that phosphorylation of Orc2 or Orc6 alone is not sufficient to disrupt the function of ORC completely. Cells carrying either *orc2-A* or *orc6-A* were not viable when MCM7 was constitutively localised in the nucleus and Cdc6 was overexpressed (**Figure 4-4**, **Figure 4-5** and **Figure 4-6**). This observation is further validated by measuring the bulk DNA amount by flow cytometry analysis (**Figure 4-10**) in which bulk DNA was increased in *orc6-A mcm7-NLS* cells after Cdc6 overexpression. However, this experiment did not show whether this increase was due to reinitiation or rereplication. Given that FACS analysis only shows bulk DNA replication, analysing the re-firing of origins requires different assays such as 2D gel electrophoresis or high-throughput sequencing for copy number variations. However, since deregulation of Rad53 targets did not increase the bulk DNA amount in the tested cells, those assays were not used.

In addition, it has been previously proposed that full-length Cdc6 does not efficiently increase the bulk DNA amount of *mcm7-NLS orc2-A orc6-A* cells (Nguyen *et al*., 2001) in which the more stable Cdc6 cdc6ΔNT is used. However, this work showed that even in *mcm7-NLS orc6-A* cells the bulk DNA amount increased significantly when full length Cdc6 was overexpressed in flow cytometry analysis (**Figure 4-10**). It is important to note that different *S. cerevisiae* strains were used in the current study. It would be very interesting to compare genetic differences between these strains, which might enlighten another non-redundant mechanism that prevents re-replication.

Although CDK targets at least 4 different factors in yeast (Orc2 Orc6 Mcm7 and Cdc6) to prevent preRC formation, and deregulating Mcm7 Orc6 Cdc6 was enough to initiate re-replication (this study), re-replication only requires the deregulation of the Cdt1 inhibitor geminin in *X. laevis* and humans (Melixetian *et al*., 2004; Mihaylov *et al*., 2002). It is still

unknown why less stringent control of preRC formation evolved in higher eukaryotes. However, one possible explanation could be allowing more efficient endoreplication during development.

As explained before, one way to induce unscheduled replication initiation is to bypass the control of CDK on preRC formation. However, bypassing the CDK control on origin firing would also allow unscheduled replication initiation in G1 phase. To address whether the inhibition of origin firing by the checkpoint has any role in the case of unscheduled replication initiation in G1 phase, I established a system that can initiate replication in G1 phase in the absence of CDK activity. I showed that the overexpression of *sld2-D SLD3*$^{+}$ *and DBF4*$^{+}$ *in* a *cdc6ΔNT* background is enough to initiate detectable levels of replication by flow cytometry in G1 phase (**Figure 4-11**). However, the progression of DNA replication significantly slowed down 2h after the of overexpression of *sld2-D SLD3*$^{+}$ *DBF4*$^{+}$*.* This could be due to another checkpoint-dependent response or some factors becoming limited after 2h of induction, or the absence of G1/S transcription factors. On the other hand, the *sld2-D sld3-A dbf4-4A cdc6ΔNT* cells progressed slower at the beginning, however showed a similar amount of bulk DNA after 6h.

It is important to note that flow cytometry analysis only measures the bulk DNA amount. One can imagine that multiple rounds of preRC formation and firing at the same origin resulted in re-replication in *sld2-D sld3-A dbf4-4A cdc6ΔNT* cells which then lead to replication fork collapse and slowed progression of replication. On the contrary, DNA replication was initiated once in *sld2-D SLD3*$^{+}$ *and DBF4*$^{+}$ *cdc6ΔNT* cells, but then the checkpoint inhibited further initiation which allowed the fast progression of ongoing forks. However, this hypothesis has to be tested with more informative assays such as 2D gel electrophoresis. Given that cells showed different progression patterns but the same bulk DNA amount at the end of experiment, it is very hard to conclude whether checkpoint-dependent inhibition of origin firing is required for the prevention of re-replication or not.

Other explanations could be proposed for why bypassing the checkpoint-dependent inhibition of origin firing did not show more bulk DNA amount at the end of the experiment. It is possible that this system did not produce enough initiation events. It would be worth investing time in optimising this system in order to generate more replication initiation. Given that cells carrying *DPB11* on a high-copy plasmid show significant replication initiation upon overexpression of sld2-D in G1 phase (Tanaka *et al*., 2007), the overexpression of Dpb11 could be used instead of Sld3 overexpression, or the extra copy of *Dpb11* could be added to the current system to increase the efficiency of replication initiation.

Because CDK inhibits preRC formation and initiates DNA replication in S-phase, it is possible to deregulate origin firing by impairing the CDK activity. There are different approaches to impairing CDK activity. It has been shown before that transient inhibition of CDK via its inhibitor *SIC1* results in a complete round of re-replication by reseting the cell cycle in G2 phase (Dahmann *et al*., 1995), before the completion of the previous cell cycle. To avoid total cell cycle reset, I decided to decrease but not totally inhibit the CDK activity. I took advantage of the *cdc28-as1* allele, which can be inhibited using the ATP analog 1-NM-PP1 in a concentration-dependent manner (Bishop *et al*., 2000).

It has previously been observed that *cdc28-as1* is a hypomorphic allele that increases the viability of *rad53Δ and mec1Δ* cells (Manfrini *et al*., 2012). Interestingly, deregulating the single function of checkpoint –inhibition of origin firing- by *sld3-A dbf4-4A* alleles had a drastic effect on cell growth when CDK was impaired (**Figure 4-12**). However, it is important to note that different concentrations of 1-NM-PP1 were used in these experiments. It is possible that a slight reduction in CDK activity only delays the cell cycle, whereas decreasing further leads to preRC formation and unscheduled origin firing. However, *sld3-A dbf4-4A* cells did not show more bulk DNA replication in G2 phase compared to *SLD3 DBF4* cells in flow cytometry analysis when CDK activity was decreased. It is also important to note that even the lowest concentration of 1-NM-PP1 (100 nM) that was used for flow cytometry analysis was higher

than the growth analysis concentration (40 nM). It is possible that at 100 nM, CDK was no longer able to fire origins. On the other hand, given that flow cytometry analysis could only measure drastic changes in bulk DNA amount, there could still be some replication initiation below the detection threshold. Whether impairing CDK activity leads to unscheduled origin firing or not should be tested with more sensitive techniques such as high-throughput sequencing for copy number variations.

It is known that the deletion of the yeast CDK inhibitor Sic1 causes genomic instability due to unscheduled origin firing in late G1 (Lengronne & Schwob, 2002). Although *sic1Δ* cells exhibit a slower cell cycle rate, deletion of DNA damage checkpoint master kinase Mec1 in *sic1Δ* cells results in an even slower cell cycle rate suggesting that the extent of DNA damage caused by Sic1 knockout somehow decreased the DNA damage checkpoint in *sic1Δ* cells (Lengronne & Schwob, 2002). Given that *sic1Δ* cells fire fewer origins in S-phase then wild type cells, it is possible that further replication initiations are prevented by the checkpoint-dependent phosphorylation of Sld3 and Dbf4. It would be very interesting to test whether *sic1Δ* cells exhibit more origin firing in addition to more DNA damage when the checkpoint-dependent inhibition of origin firing is deregulated by *sld3-A dbf4-A* alleles. This would give an idea of the importance of the checkpoint-dependent inhibition of origin firing in the case of unscheduled replication initiation during the G1/S transition.

Different types of mutations could result in deregulation of replication initiation in mammals. Deregulated replication initiation could have 3 different outcomes, including origin under-usage, origin over-usage or origin re-usage. These outcomes would result in genomic instability which could possibly drive the formation of dysplasia or progression from dysplasia to carcinoma. Several lines of evidence suggest that replication initiation is deregulated in different cancers (Hills & Diffley, 2014). In most cancers, numerous oncogenes are found to be mutated, which could be linked to deregulated replication initiation (Hanahan & Weinberg, 2011). For example, Cyclin D1, a G1 cyclin in humans, is heavily deregulated in different types

of cancers which is possibility associated with defective replication initiation (Cheung *et al*., 2001; Dickson *et al*., 1995; Vielba *et al*., 2003). Although the overexpression of Cyclin D1 is observed in the earliest stages of cancers but not in premalignant lesions, it is not clear whether Cyclin D1 is required for malignant transformation or required for the progression of cancers. However, because overexpression of Cyclin D1 results in re-replication and also increases gene amplification in cancer cells (Aggarwal *et al*., 2007; Sherr, 1996), it is possible to hypothesise that overexpression of Cyclin D1 increases the heterogeneity of cancer by gene amplification which might increase their survival capacity. However, whether the checkpoint-dependent inhibition of origin firing decreases the number of initiation events when replication initiation is deregulated is not clear.

In summary, I investigated the function of the inhibition of origin firing in G1 and G2 phase by allowing unscheduled replication initiation. I tested several approaches to initiate DNA replication outside of S-phase and obtained valuable information about vital cellular process such as origin licensing. Given that bypassing the checkpoint regulation produced a significant growth phenotype when CDK activity was impaired, checkpoint substrates for the inhibition of origin firing in human could be a good candidate for the treatment of different disease such as cancer. Because replication initiation is deregulated in different cancers, bypassing the checkpoint control on the inhibition of replication initiation by small molecules might increase genomic instability that would lead to cell death.

Chapter 5

Cdc45 is required for the S-phase checkpoint

5.1. Introduction

Endogenous or exogenous stress may halt DNA replication, activating the S-phase checkpoint. The S-phase checkpoint starts with the recognition of DNA lesions by sensor proteins which trigger the activation of 2 phosphatidylinositol 3' kinase-like kinases (PIKK) ATM and ATR in humans (Mec1 and Tel1 homolog of yeast) (Hustedt *et al*., 2013). Activation of these master kinases transduces the signal to the effector kinases Chk1 and Chk2 (functional homolog of Rad53 and Chk1 in yeast) which leads to the execution of different responses such as transcriptional control, the inhibition of late origin firing, and replication fork stabilization and mitotic delay.

Several lines of evidence indicate that misregulation of DNA damage checkpoints results in genomic instability which has been implicated in several diseases such as cancer (Barlow *et al*., 1996; Bartkova *et al*., 2005; Gorgoulis *et al*., 2005). The importance of the DNA damage checkpoint makes it one of the central target for the treatment of numerous diseases such as cancer (Chen *et al*., 2012). However, its essential roles during development as well as cellular homeostasis prevent its direct deregulation, which makes it necessary to deregulate its individual responses (de Klein *et al*., 2000; Lee *et al*., 2000). Therefore, understanding the checkpoint pathways is of great importance if specific responses are to be targeted instead of complete deregulation. However, very few checkpoint substrates have been identified so far, such as Sld3 and Dbf4 in yeast (Zegerman & Diffley, 2010). Although some high-throughput screens from yeast to humans have suggested several candidates, most of them have never been validated, possibly due to the high false positive rate of the applied approaches (Blasius *et al*., 2011; Chen *et al*., 2010; Smolka *et al*., 2007). In addition to that, some already known checkpoint substrates have never been observed in those studies. Together, different approaches are required to delineate the substrates of checkpoint.

The evolutionary conservation of the S-phase checkpoint from yeast to human, as well as its advanced properties described in chapter 1.2, makes budding yeast an excellent organism for identifying novel checkpoint substrates and their functions. Rad53, the yeast functional homolog of human Chk1, is the main effector kinase in the S-phase checkpoint which is responsible for the responses explained above. However, current knowledge about its substrates is limited. Because, Rad53 is one of the least specific yeast kinases *in vitro* (Mok *et al*., 2010), it is hard to decipher its substrates by high-throughput screens. Thus, the discovery of new Rad53 substrates requires targeted approaches. One way to explore novel substrates would be by understanding the mechanisms of substrate-kinase interaction.

The aim of this chapter was to understand how Rad53 targets its substrates *in vivo*, which might help to identify novel substrates. To do so, I investigated how Rad53 targets its

substrates for the inhibition of origin firing. Rad53 targets Sld3 and Dbf4 upon DNA damage to prevent origin firing (Lopez-Mosqueda *et al*., 2010; Zegerman & Diffley, 2010). It has been shown that Rad53 binds to the N-terminal portion of Dbf4 directly and phosphorylates it (Chen *et al*., 2013). However, how Rad53 targets Sld3 is unknown. Recently, it has been observed in the Zegerman Lab that C-terminally HA-tagged *CDC45* (*cdc45-HA*) abrogates Rad53 dependent phosphorylation of Sld3 upon DNA damage (Dr P Zegerman, and C Kleinert, data not shown). Cdc45 interacts with Sld3 (Yoichiro Kamimura *et al*., 2001) and this interaction is essential for replication initiation (Zegerman & Diffley, 2010). Based on this observation, this chapter is mostly focused on the role of Cdc45 in the checkpoint to understand how Rad53 ensures its target specificity, more precisely, the function of Cdc45 on Rad53 dependent Sld3 phosphorylation(**Figure 5-1**).

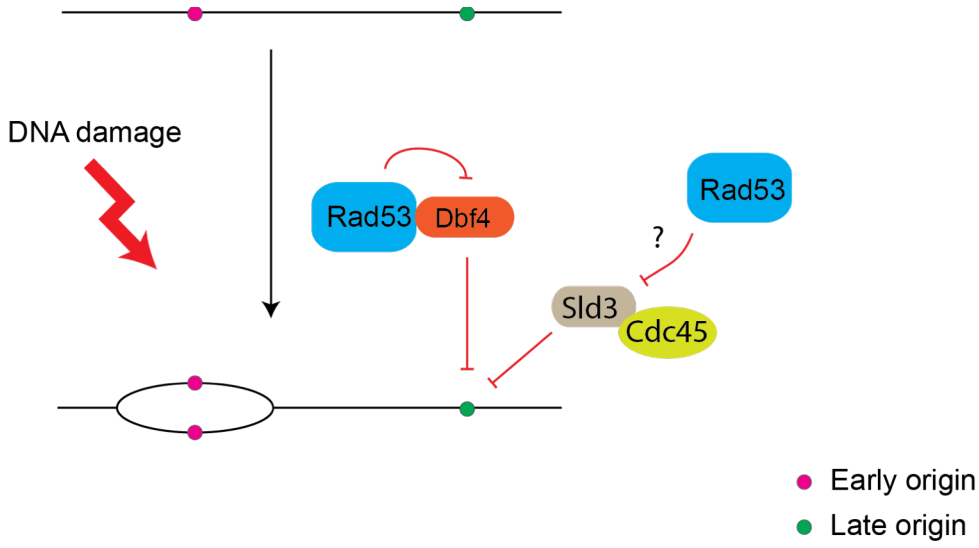


Figure 5-1 How does Rad53 target its substrates?

5.2. Cdc45 is required for Rad53-dependent Sld3 phosphorylation

Given that *cdc45-HA* prevents Rad53-dependent Sld3 phosphorylation (personal communication with Dr. P Zegerman), I wondered whether Cdc45 itself is required for Sld3 phosphorylation, or whether an HA tag somehow prevents Sld3 phosphorylation. To rule this out, I decided to test the requirement for Cdc45 in Rad53-dependent Sld3 phosphorylation. Because *CDC45* is an essential replication factor, it is not possible to create *CDC45* null cells for functional studies. To circumvent this issue, I used a powerful genetic tool called the temperature-activated degron method. With this technique, the desired protein can be degraded rapidly above a threshold temperature. Furthermore, because *cdc45-td* cells have an extra copy of *Ubr1* under a Gal1-10 promoter, addition of galactose into the media enhances the degradation of the degron fusion. I used an endogenously modified temperature-activated degron *CDC45* mutant *cdc45-td* (Tercero *et al*., 2000). As a control, I used a wild type copy of *Cdc45* under its promoter in another locus, thus rescuing Cdc45 degradation upon temperature shift.

Cdc45 is an essential replication factor and travels with the replication fork (Tercero *et al*., 2000). In order to address its requirement in the checkpoint, the absence of Cdc45 has to be analysed independently from its function in replication. Since Rad53 phosphorylates Sld3 not only in S-phase but also in G1 and G2 phase upon DNA damage (Chapter 3), I conducted the experiment in G2 phase where Cdc45 would not be required for replication. I arrested *cdc45-td and cdc45-td CDC45*$^{+}$ strains in YPRaff at permissive temperature (24°C) in G2 using nocodazole and then shifted cultures to the restrictive temperature (37°C) to degrade Cdc45. One hour after incubation at 37°C, the DNA damaging agent 4-NQO was added and samples were collected every 30 minutes. The flow cytometry profiles showed that cells were arrested in G2 phase during the experiment (**Figure 5-2.C**). Western blot analysis showed a

phosphorylation-dependent mobility shift of Rad53, indicating that the DNA damage checkpoint is activated in G2 phase equally in both strains (**Figure 5-2.B**). While Sld3 phosphorylation occurred in the strain with wildtype Cdc45, I did not observe any mobility shift of Sld3 in *cdc45-td* cells. This suggests that Cdc45 is required for the Rad53-dependent phosphorylation of Sld3 upon DNA damage, and its function is prevented by the HA tag in *cdc45-HA* cells.

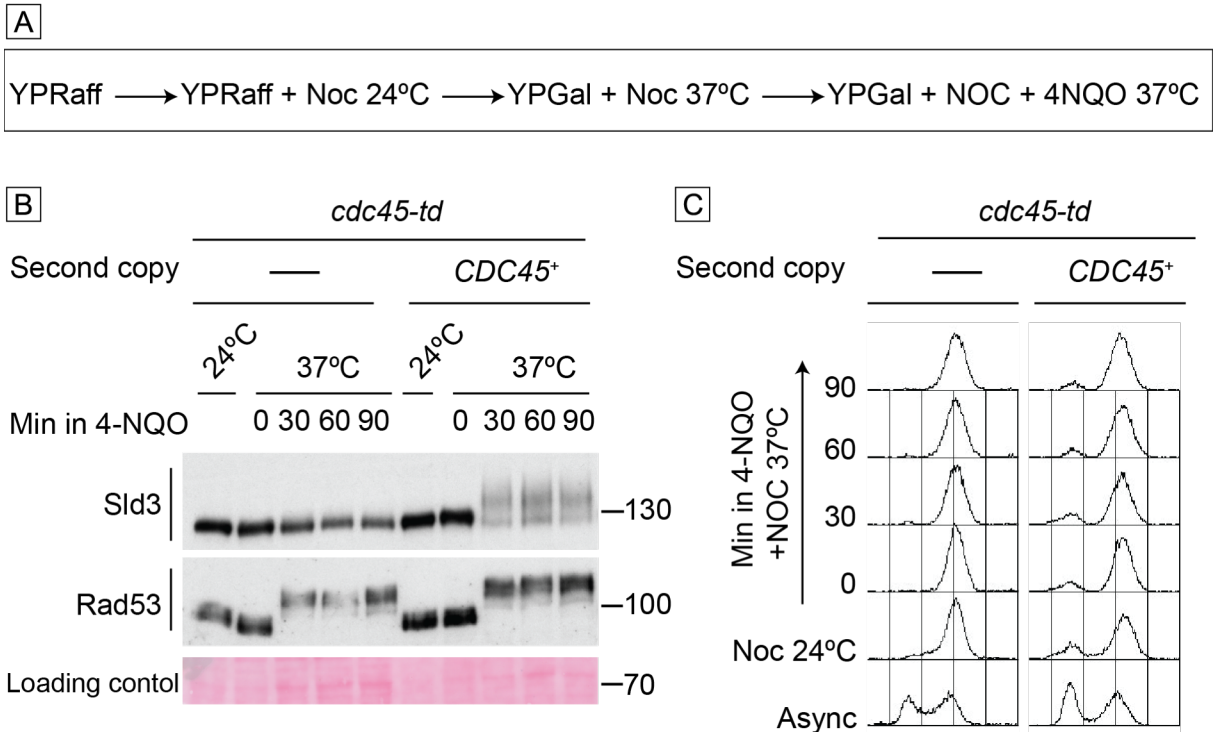


Figure 5-2: Cdc45 is required for Rad53 dependent Sld3 phosphorylation. A) Schematic representation of the experimental steps. B) Western blot analysis of Rad53 and Sld3. An anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control. C) Flow cyctometry analysis of the cells.

There are several possible explanations for the requirement of Cdc45 for Rad53-dependent Sld3 phosphorylation. It is possible that Cdc45 has a role in Rad53 regulation, or Cdc45 could

target Rad53 to Sld3 for Sld3 phosphorylation. Cdc45 interacts with Sld3, which is essential for replication initiation (Zegerman & Diffley, 2010). Given that Rad53 phosphorylation still occurred in the absence of Cdc45 upon DNA damage, it is possible that Cdc45 is not required for Rad53 regulation. However, if the second explanation is correct, interrupting the Cdc45-Sld3 interaction would have an effect on Sld3 phosphorylation. To test this hypothesis, I used the *sld3-2D* mutant which has a reduced interaction with wild type Cdc45 (Zegerman & Diffley, 2010). I arrested wild type and *sld3-2D* cells in G2 using nocodazole and induced DNA damage through 4-NQO to analyze whether reduced Sld3-Cdc45 interaction affects the phosphorylation of Sld3 by Rad53. The flow cytometry analysis showed that both strains were arrested in G2 phase before addition of 4-NQO (**Figure 5-3.B**). The phospho-dependent mobility shift of Rad53 indicated that the DNA damage checkpoint was activated by 4-NQO (**Figure 5-3.C**). In contrast to wild type cells, *sld3-2D* showed a significantly reduced mobility shift. Given that the sld3-2D mutant still interacts with Cdc45 but to a lesser extent, this small degree of interaction might explain the residual Sld3 phosphorylation. From this experiment I conclude that the Sld3-Cdc45 interaction is necessary for Rad53-dependent phosphorylation of Sld3 upon DNA damage, which would explain the result in the Cdc45 degron strain (**Figure 5-2**).

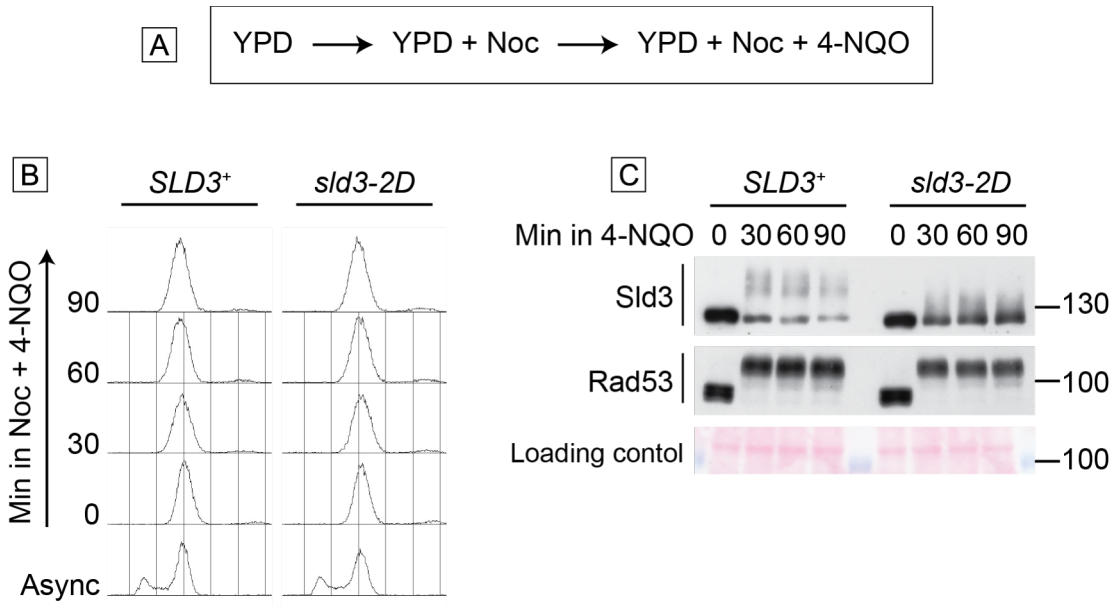


Figure 5-3: Cdc45-Sld3 interaction is required for Rad53-dependent Sld3 phosphorylation. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Sld3. An anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control.

Next, I wondered whether the effect of Cdc45 on Sld3 phosphorylation by Rad53 was specific, or whether other proteins that bind to Sld3 are also required. To test this, I used a hypomorphic Dbb11 allele called *dpb11-1* which is temperature sensitive at 37°C (Kamimura *et al*., 1998) and performed the experiment in G2 phase as before. Following this, I added 4-NQO to induce DNA damage. Flow cytometry analysis showed that cells were entirely arrested in G2 phase (**Figure 5-4**.B). Next, I proceeded with Western blot analysis of Dpb11. A DNA damage-dependent shift of Dbp11 was observed in wild type cells, corroborating previous work (Puddu *et al*., 2008). As expected, mobility shifts of Rad53 after addition of 4-NQO in both strains were indicators of checkpoint activation. Finally, I did not observe any change in the magnitude of the shift of Sld3. I therefore concluded that Rad53-dependent phosphorylation of Sld3 does not require Dpb11 interaction.

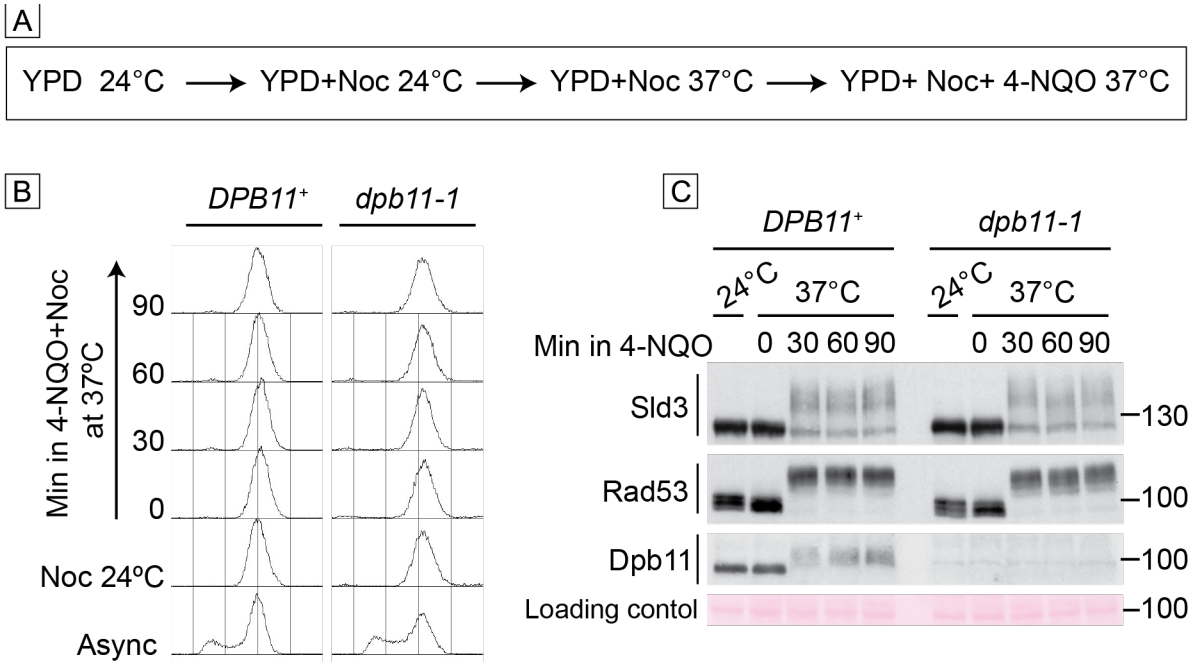


Figure 5-4: Cdc45, but not other Sld3 interacting proteins, is required for Rad53-dependent Sld3 phosphorylation. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53, Sld3 and Dpb11. An anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control.

Next, I considered whether the requirement of Cdc45 for Rad53 dependent phosphorylation is specific for Sld3, or whether other Rad53 substrates also require Cdc45 for Rad53-dependent phosphorylation upon DNA damage. To test this, I decided to assess the phosphorylation of Dbf4 upon DNA damage in G2 phase. Given that cdc45-HA prevents Rad53-dependent Sld3 phosphorylation (P Zegerman, personal communication). I used *cdc45*-HA to investigate the effect of Cdc45. Taking into account several previous studies suggesting that Dbf4 is phosphorylated by Cdk (Holt *et al*., 2009; D. Lu *et al*., 2014; Ubersax *et al*., 2003), in order to be able to discriminate between the Rad53-dependent and independent phosphorylation subset of Dbf4, I used *rad53Δ* cells as a control.

I arrested cells at 30°C in G2 Phase with nocodazole and added 4-NQO to induce DNA damage. As expected, the flow cytometry profile of cells showed that cells were arrested in G2 phase (**Figure 5-5.B**). Since cdc45-HA mutants did not affect the phosphorylation pattern of Dbf4 (unlike the phosphorylation of Sld3 - Dr P Zegerman and C Kleinert, data not shown), I conclude that Cdc45 is only required for Sld3 but not for Dbf4 phosphorylation (**Figure 5-5**). This observation is in agreement with previous work which proposes that Rad53 directly binds Dbf4 through the N-terminal domain of Dbf4 (Chen *et al*., 2013). However, since Dbf4 is modified in G2 phase cells even in the absence of Rad53, it is difficult to rule out whether Dbf4 is fully phosphorylated by Rad53.

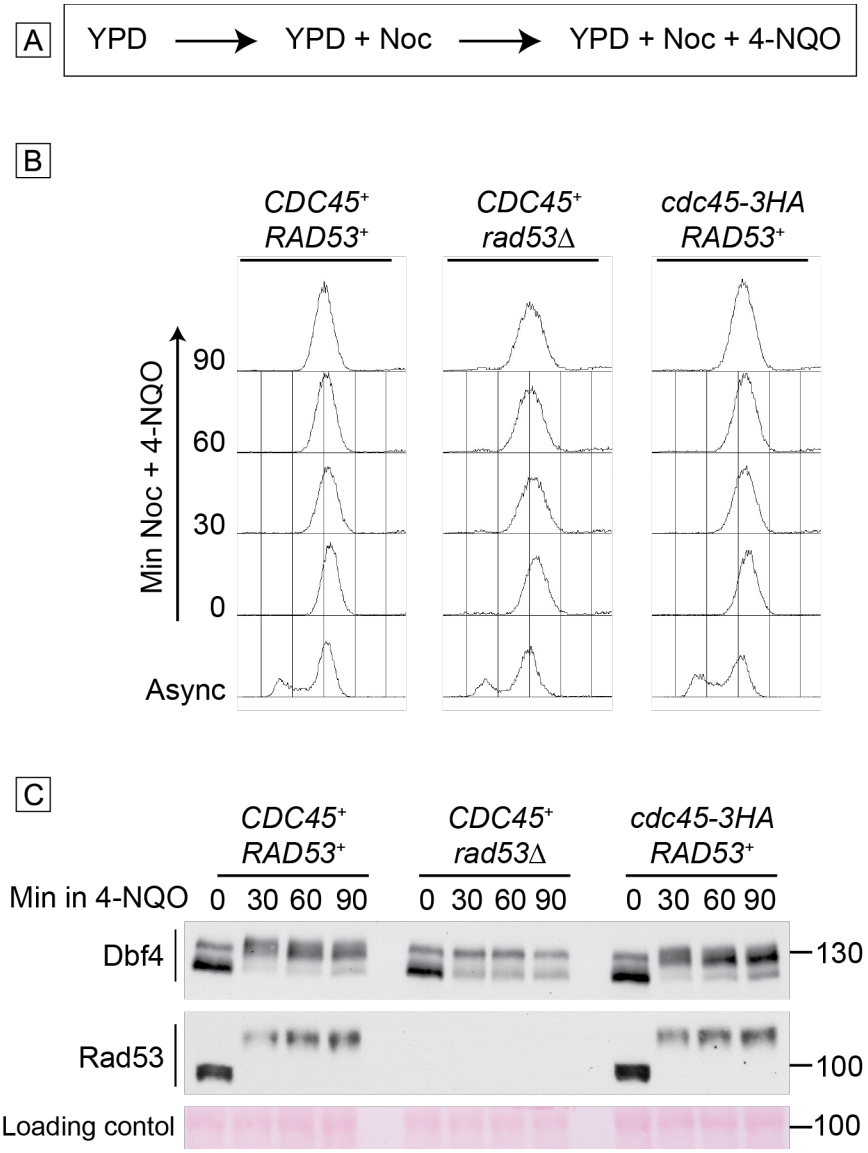


Figure 5-5: Cdc45-HA does not interfere with Rad53-dependent Dbf4 phosphorylation. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Dbf4. An anti-Myc antibody was used for Dbf4 detection. Ponceau-S staining of the membrane was used as a loading control. All strains were isogenic for *sml1Δ*.

5.3. A disordered loop of Cdc45 is the binding site of Rad53

Considering that the experiment explained above showed the requirement of Cdc45 for Rad53 dependent Sld3 phosphorylation, I next wondered how Cdc45 targets Rad53 to Sld3. Rad53

contains a Serine/Threonine kinase domain which is flanked by two forkhead homology-associated (FHA1 and FHA2) domains (Wybenga-Groot *et al*., 2014). These FHA domains are described as phospho-peptide recognition domains, which are specific for phosphothreonine (pT)-containing epitopes. The FHA1 domain of Rad53 binds to *pTXXD* motifs with high affinity *in vitro* (Liao *et al*., 2000). Cdc45 contains 5 *TXXD* motifs (T147, T189, T195, T278 and T438), two of which (T189 and T195) are located in a unstructured loop (**Figure 5-6**). Aucher *et al.* have shown that the Rad53 FHA1 domain co-precipitates with Cdc45 (Aucher *et al*., 2010). Moreover, the FHA1 domain of Rad53 interacts with a Cdc45 (154-270) fragment by yeast two-hybrid assays, and this interaction is decreased drastically following a T189A substitution of the Cdc45 (154-270) fragment (Aucher *et al*., 2010). I therefore considered whether the disordered loop of Cdc45 is the binding site of Rad53 required for Rad53 targeting to Sld3 upon DNA damage. To test this, I decided to replace the T189 residue of Cdc45 with alanine to abrogate possible interactions with Rad53, and also replaced the same residue with aspartic acid and glutamic acid to mimic possible phosphorylation events, to determine whether phosphorylation is required for this interaction.

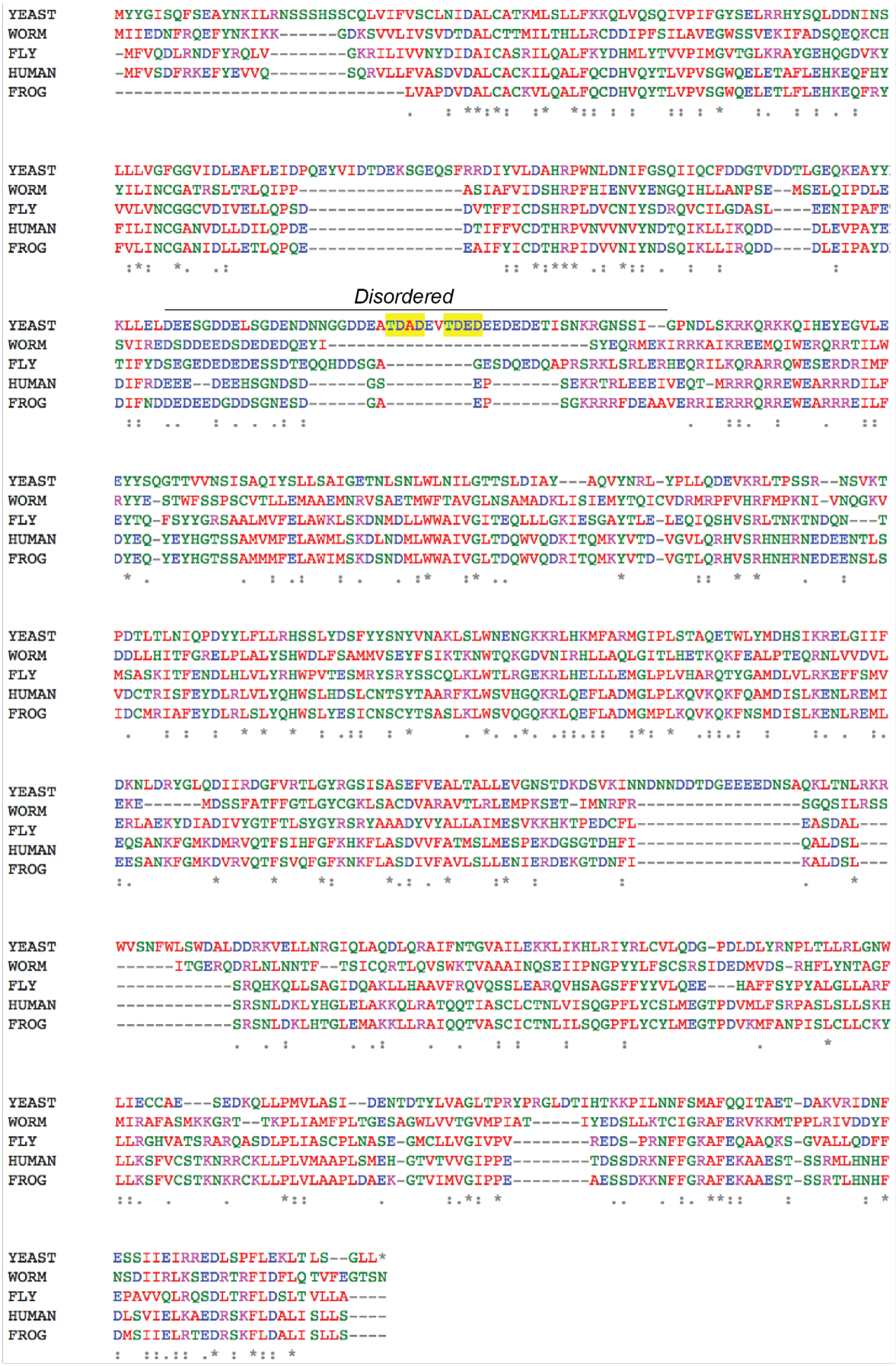
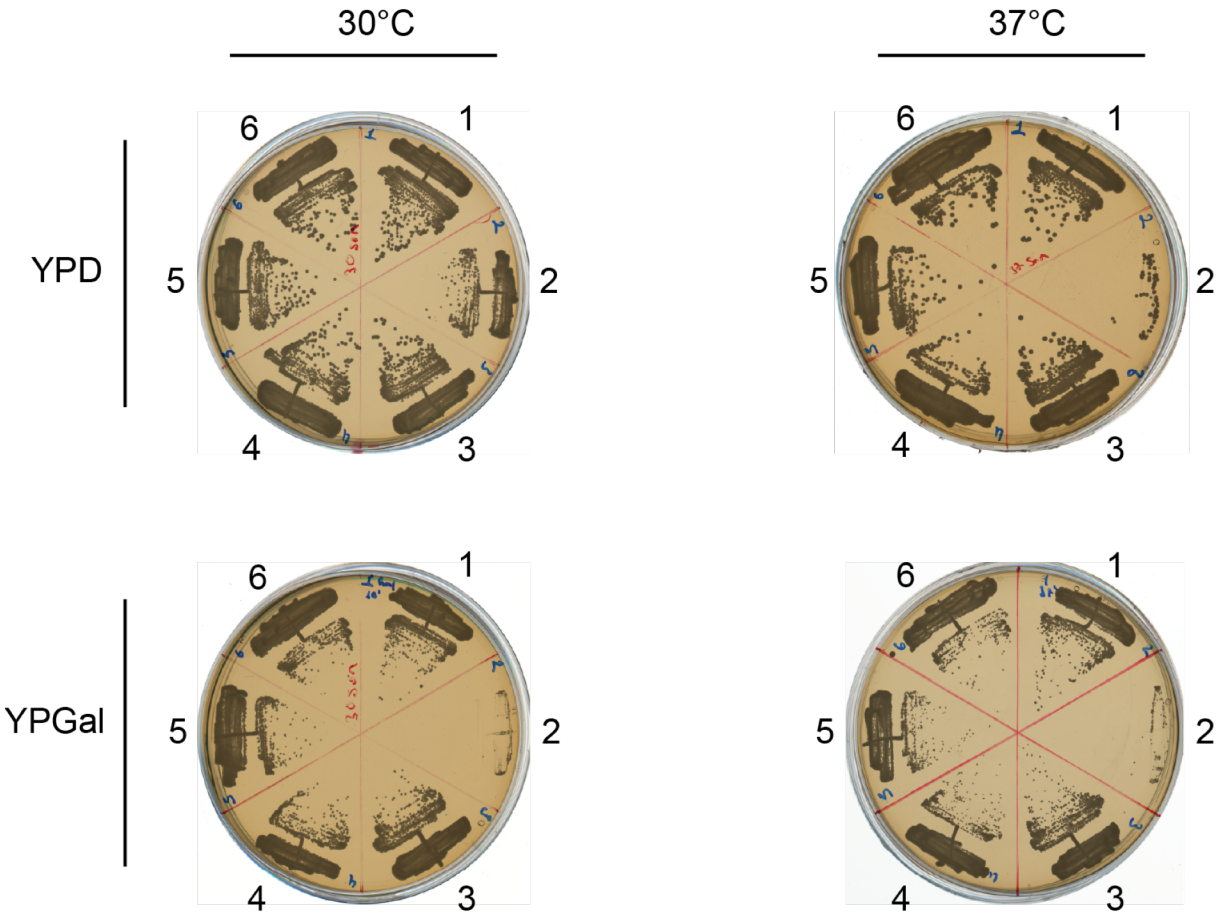


Figure 5-6 The multiple sequence alignment of Cdc45. The disordered loop of Cdc45 predicted for Human Cdc45 (Simon et al., 2016). Yellow boxes indicate TxxD domains on the disordered loop.

Since Cdc45 is an essential replication factor, any loss of function induced through the introduction of a mutation can be lethal. Hence, I decided to introduce *CDC45*$^{+}$, *cdc45-189A*, *cdc45-189D* and *cdc45-189E* into *cdc45-td* cells as a second copy under the *CDC45* promoter. To avoid confusion, I have called these mutants *cdc45-1A, cdc45-1D and cdc45-1E.* In order to analyse if the mutations affected the function of Cdc45, I tested their growth at the non-permissive temperature where the cdc45-td was degraded (**Figure 5-7**). As expected, wild-type cells (1) grew on both YPD and YPGal plates at all tested temperatures. The *cdc45-td* cells (2) grew on both YPD but not on YPGal at 30°C. On the other hand, *cdc45-1A,1D,1E* grew on all plates. This suggests that the substitution of Cdc45-T189 to A, D or E does not have any significant effect on the essential functions of Cdc45.



1	Wild-type
2	—
3	P_{CDC45} - *Cdc45*$^{+}$
4	P_{CDC45} - *cdc45-T189A*
5	P_{CDC45} - *cdc45-T189D*
6	P_{CDC45} - *cdc45-T189E*

cdc45-td

Figure 5-7: Cdc45 T189A-D-E mutants are viable. Growth analysis of *CDC45* mutants. A second copy of either wild type or mutant Cdc45 mutants were tested in the *cdc45-td* background. Corresponding genotypes are indicated in the grey box.

I decided to analyse whether these Cdc45 mutants affect the Sld3 phosphorylation after DNA damage in G2 phase as in **Figure 5-2**. The mobility shift of Rad53 after 4-NQO addition indicated that the DNA damage checkpoint was activated in all strains (**Figure 5-8**.C). I then checked the mobility shift of Sld3 by western blot analysis. There was no visible mobility shift in *cdc45-td* after the addition of 4-NQO, showing the requirement of Cdc45 for Sld3 phosphorylation by Rad53 as in **Figure 5-2**. The mobility shift of Sld3 was restored in *cdc45-td CDC45*$^{+}$ cells upon DNA damage due to the presence of a wild type copy of *CDC45*. Some mobility shift of Sld3 was observed in *cdc45-td cdc45-1A* cells, however the magnitude of this shift was greatly reduced compare to *cdc45-td CDC45*$^{+}$ cells. This indicated that T189 is an important residue for Rad53-dependent Sld3 phosphorylation. Although the use of D and E are supposed to mimic phosphorylated residues, they both showed the same level of phosphorylation as *cdc45-1A*, *s*uggesting that either the phosphorylation of this residue is not required or that D or E substitutions were unable to mimic phosphorylation.

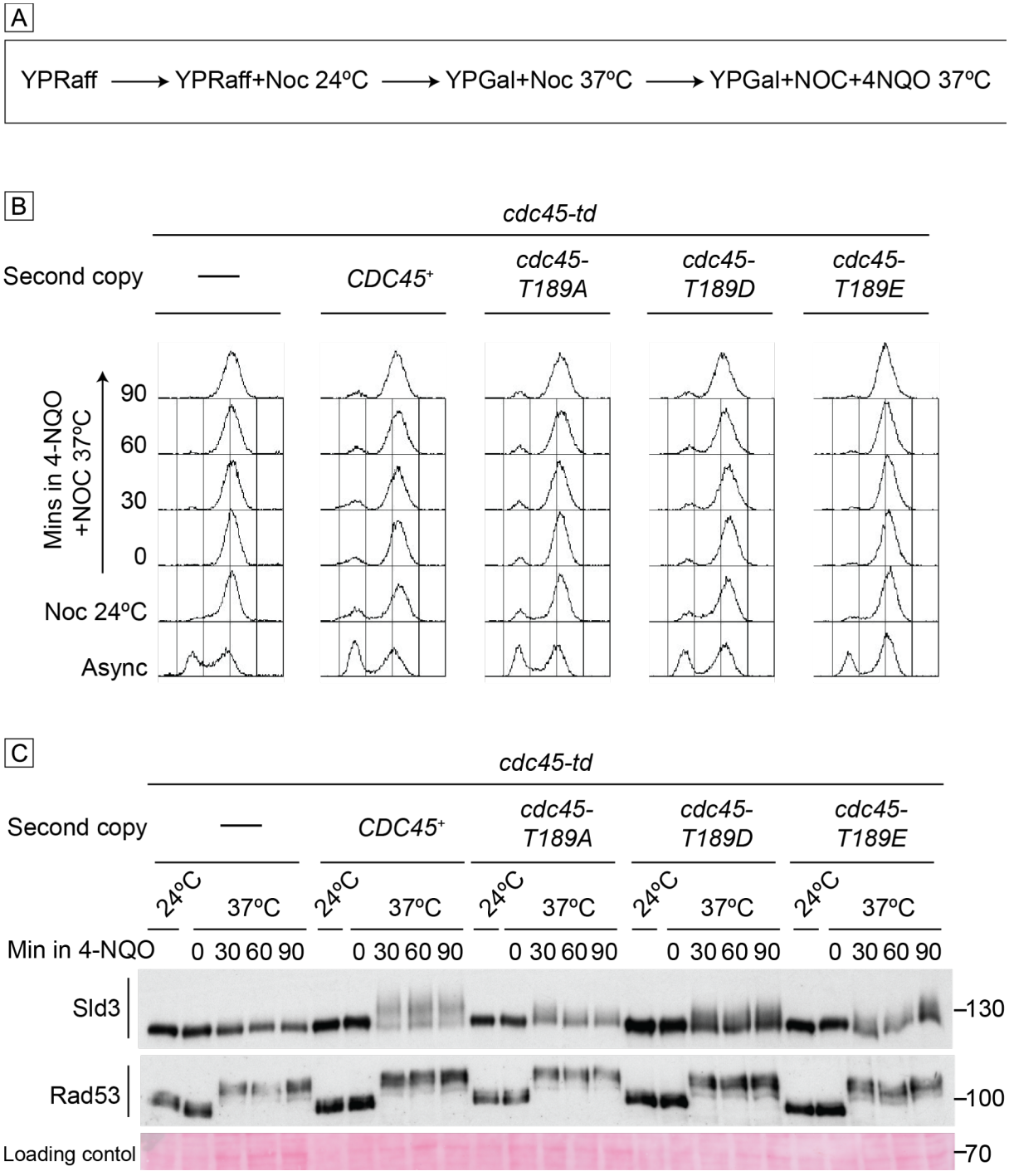


Figure 5-8 Cdc45 T189 residue is required for Rad53 dependent Sld3 phosphorylation in *cdc45-td cells*. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Sld3. Anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control. All strains were isogenic P_{GAL}-*UBR1*. The expression of second copy genes was under P_{CDC45}.

The temperature-activated degron method is a robust strategy for assessing the effect of mutations, but is not the most optimal experimental setup since the experiments must be

performed at 37°C in G2 phase and the desired protein must be expressed at a distal locus. After determining that cdc45-T189A, T189D and T189E substitutions are not lethal (**Figure 5-7**), I mutated endogenous *CDC45* to analyze the effect of 189A, D or E substitutions. I replaced endogenous *CDC45*$^{+}$ with *cdc45-T189A*, *T189D* and *T189E* mutants respectively (**Figure 5-9**). To do so, I used a modified 2-step gene replacement protocol. At the first step, I cloned the coding sequence of *CDC45*$^{+}$ with the Gal1-10 into an episomal plasmid containing *URA3*$^{+}$ (pRS316). I transformed this plasmid into *CDC45*$^{+}$/*cdc45Δ::KanMX* diploid cells and then isolated *cdc45Δ* strains carrying the episomal P_{GAL}-*CDC45*$^{+}$ plasmid by tetrad dissection of sporulated diploids. These cells were *URA3*$^{+}$ *KAN*$^{+}$ and grown using only galactose-containing media. I then transformed these haploid cells with a PCR product of *cdc45-T189A, T189D and T189E* which carried the *5'* and *3' UTR*s of *CDC45*$^{+}$ in order to replace the *KAN*$^{+}$ gene at the Cdc45 locus by homologous recombination. I grew these cells on YPD plates in order to halt the expression of Cdc45 from the *URA3*$^{+}$ episomal plasmid, and replica plated on 5-FOA plates in order to select cells which lost the episomal plasmid. I picked 8 different colonies per insert and streaked them on YPD, YPD+Kanamycin, and Minimal Medium-Uracil in order to control the replacement and 5-FOA plates to control the plasmid loss. All 8 colonies per insert which were picked grew on YPD and 5-FOA plates but did not grow on uracil plates, suggesting that these cells lost the episomal plasmid and therefore expressed mutant Cdc45. As they did not grow also on Kanamycin plates, this replacement occurred in the *CDC45* locus. To be completely sure that this integration occurred successfully, I verified insertions by PCR analysis and sequenced the full *CDC45* locus by Sanger sequencing. PCR analysis showed that mutant *cdc45* integrations occurred correctly and Sanger sequencing showed that *cdc45-T189A, T189D and T189E* were integrated successfully into the Cdc45 locus.

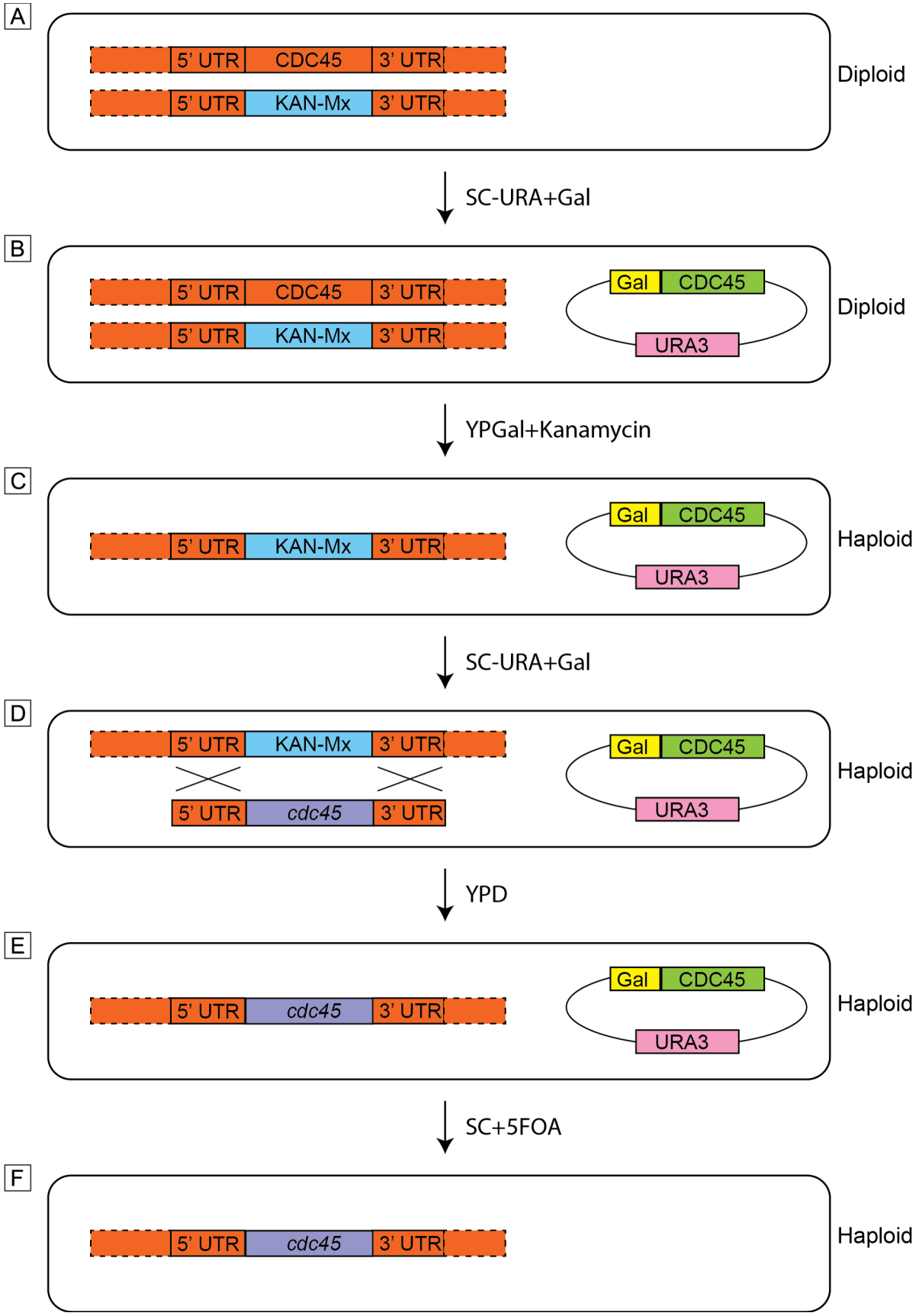


Figure 5-9 Schematic representation of integration of mutant Cdc45 at the endogenous locus.

Next, I wondered whether these mutations had any effect on growth. After 36 hours of incubation, the growth of all mutated strains was similar to wild-type at all temperatures tested. This result demonstrates that endogenous replacement of *CDC45*$^{+}$ *with cdc45-1A, 1D* and *1E* does not have any effect on cell growth on its own (**Figure 5-10**).

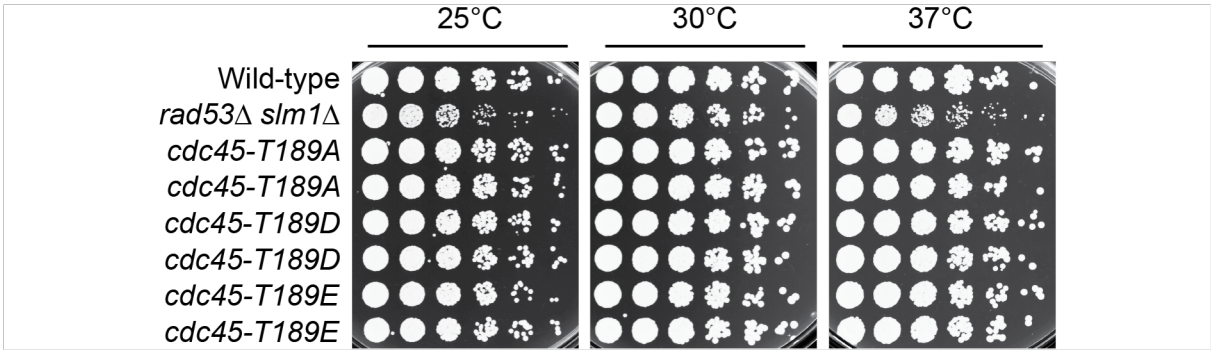


Figure 5-10 Cdc45-T189A, D or E substitutions does not exhibit a growth phenotype. Exponentially growing cells were counted and 3-fold serial dilutions from initial 0.4x10^{7}cells were spotted onto YPD plates. 2 different isolates *of cdc45 T189A, D or E* cells were used. Pictures were taken after 36h incubation.

To test the effect of the Cdc45 mutants as the only copy of Cdc45 on Sld3 phosphorylation, I arrested these mutants in G1 phase using α-factor, released them synchronously into S-phase in the presence of 200mM HU, and collected samples every 30 minutes. As expected, flow cytometry analysis showed that cells were arrested in G1 phase and cells did not progress after release due to the inhibition of replication by HU (**Figure 5-11.B**). The mobility shift of Rad53 indicated that the checkpoint was activated equally in all tested strains. In later time points, a subset of Rad53 appeared dephosphorylated, which is an indication of adaptation to HU. The mobility shift of Sld3 was observed in all strains but the magnitude of shift was reduced in *cdc45-1A*, *cdc45-1D* and *cdc45-1E* cells, consistent with the *cdc45-td* data (**Figure 5-8**).

Together with **Figure 5-8**, this experiment suggests that the disorganized loop of Cdc45 is important for Rad53-dependent Sld3 phosphorylation.

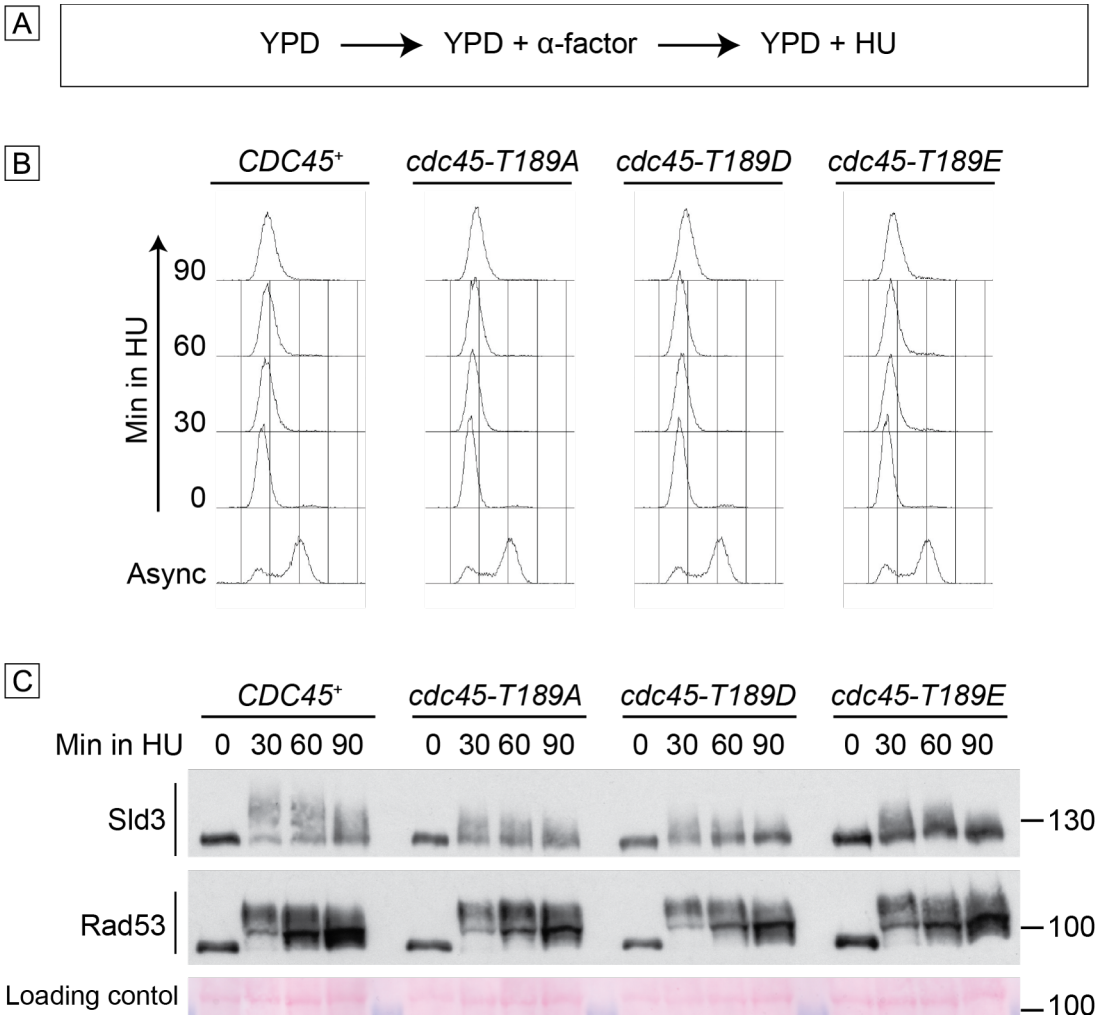


Figure 5-11 Cdc45 T189 residue is required for Rad53-dependent Sld3 phosphorylation in S-phase. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Sld3. An an nti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control.

Since the effect of the Cdc45 189 mutation was partial, I wondered if Rad53 might be able to be recruited to Cdc45 via other sites. Interestingly, there are two *TXXD* motifs in the disorganised loop of Cdc45. I wondered if I could completely abrogate Sld3 phosphorylation by mutating both motifs. To answer this question, I began with the temperature degron strain again, as I did not know the effect of double mutation on the function of Cdc45. I mutated both

Cdc45 T189 and T195 residues to alanine, glutamic acid or aspartic and transformed into the degron strain as in **Figure 5-7**. To prevent any confusion, these double mutants are referred to *cdc45-2A, cdc45-2D or cdc45-2E.* To test whether these mutations affect the function of *cdc45*, I performed a growth assay. After incubation for 48 hours, *cdc45-td* did not grow on YPD at 37°C as expected, whereas P_{CDC45}-*CDC45*$^{+}$ rescued the phenotype as they grew at a similar rate to wild-type cells (**Figure 5-12 3rd row**). In addition, I did not observe any growth defect in 2A, 2D or 2E cells, which also grew similarly to wild-type, suggesting that the substitution of T189 and T195 of Cdc45 with alanine, glutamic acid or aspartic acid probably does not affect its function during replication.

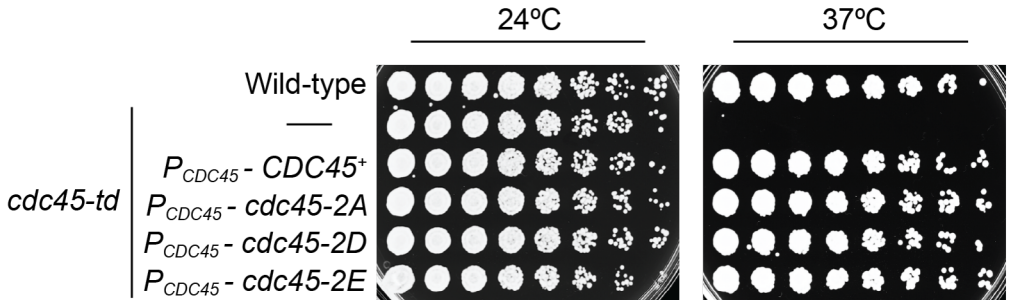


Figure 5-12 Cdc45 T189, T195 A, D or E double mutants are viable. Growth analysis of Cdc45 mutants. A second copy of either wild type or mutant Cdc45 mutants were tested in a cdc45-td background. Corresponding genotypes are indicated in the box.

Although the growth analysis did not show any growth differences between wildtype and mutant Cdc45, it is still possible that these mutations affected the expression of Cdc45. For this reason, I wanted to verify the expression of wild-type and mutant Cdc45. Because, there is no commercial yeast Cdc45 antibody, I purified a polyclonal Cdc45 antibody for the rest of experiments (**Figure 5-13**). To validate if the purification yielded that was specific for Cdc45, *cdc45-td* cells were used as a control, because *cdc45-td* cells only have a fusion protein which is approximately 20 KDa bigger than full length Cdc45. Western blot analysis indicated that

serum has non-specific binding to a band very close to wildtype Cdc45 proteins (**Figure 5-13.A**). However, this nonspecific binding disappeared after purification, suggesting that this antibody could be used for probing Cdc45 (**Figure 5-13.B**). This antibody also detected cdc45-td fusion proteins, however purified antibody still had nonspecific binding very close to cdc45-td.

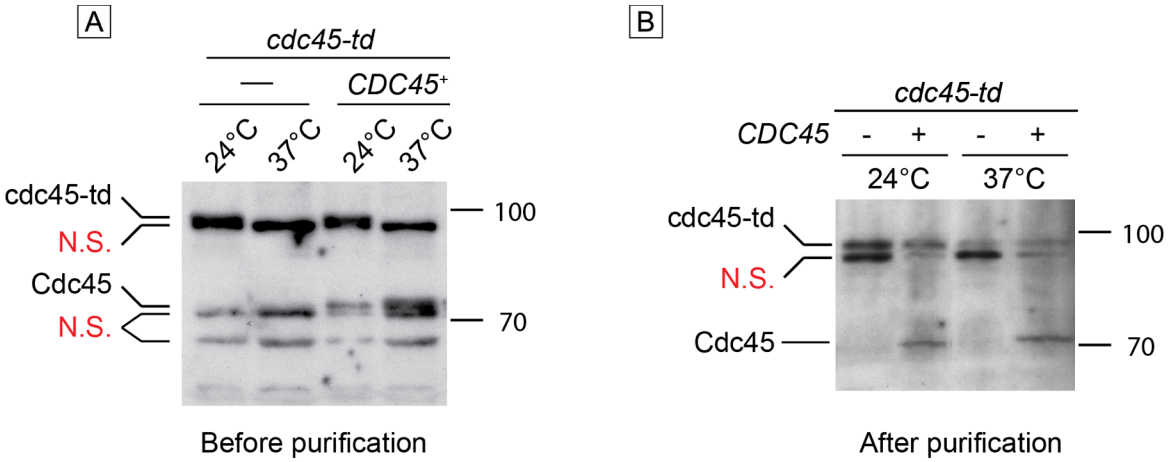


Figure 5-13 Purification of specific antibody for Cdc45. Membranes were probed with non-purified serum A) or purified antibody B).

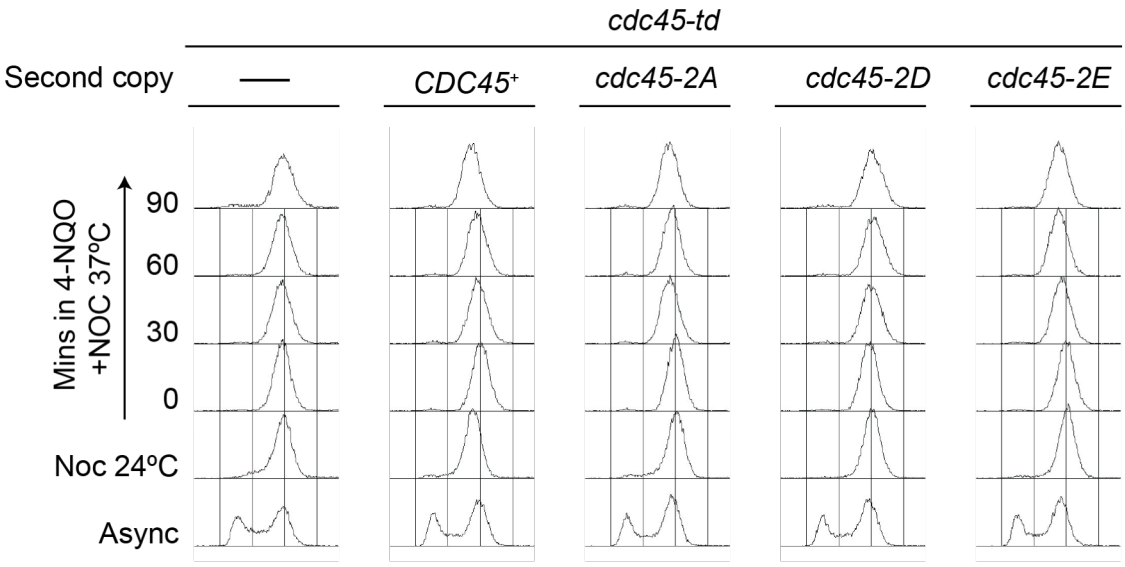
After purification of the Cdc45 antibody, I analysed the effect of 2A, 2D, 2E mutations on the expression level of Cdc45. **Figure 5-14.C** shows that these substitutions did not affect the stability of Cdc45 as their expression were similar to wildtype Cdc45. I next analysed the effect of Cdc45 double mutants on Sld3 phosphorylation following DNA damage as in **Figure 5-8**. Western blot analysis of second copy Cdc45 showed that wild-type and mutant proteins were expressed similarly to each other in *trp1-1* locus and there was no wild-type expression in the *cdc45-td* strain (**Figure 5-14.C**). In addition, the majority of cdc45-td protein was degraded following a temperature shift. Finally, I analysed the mobility shift of Sld3 by western blot. As expected, the mobility shift of Sld3 was greatly reduced compared to control cells after the addition of 4-NQO. The minor shift of Sld3 could be due to the inefficient degradation of cdc45-td in this experiment. Sld3 phosphorylation in the Cdc45 2A, 2D and 2E cells was also abrogated, as in *cdc45-td* cells (**Figure 5-14.C**). The main conclusion of this experiment is that

the two *TXXD* motifs in the disorganized loop of Cdc45 are required for Rad53-dependent Sld3 phosphorylation upon DNA damage. Considering the work of Aucher *et al.* (2010) in which an *in vitro* interaction of a Cdc45 (154-270) fragment with the FHA1 domain of Rad53 was shown, it is likely that Rad53 binds to the disorganized loop of Cdc45 for Sld3 phosphorylation *in vivo*.

A

YPRaff ⟶ YPRaff+Noc 24ºC ⟶ YPGal+Noc 37ºC ⟶ YPGal+NOC+4NQO 37ºC

B



C

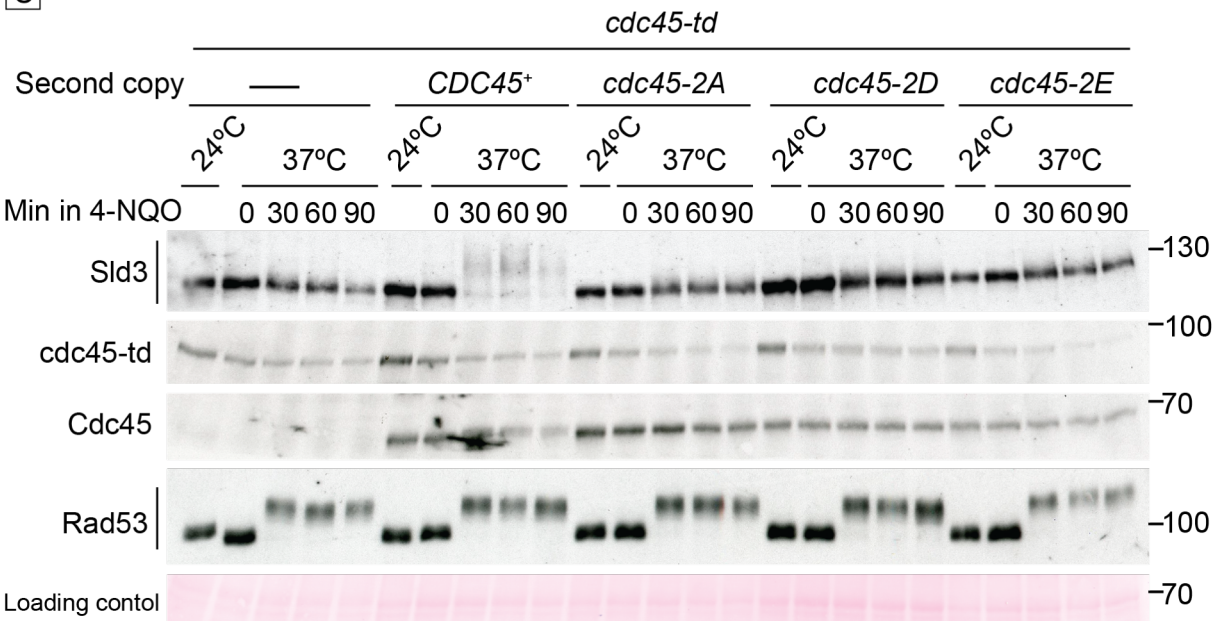


Figure 5-14 Cdc45 T189, T195 residues are essential for Rad53 dependent Sld3 phosphorylation in cdc45-td cells in G-2 phase. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Sld3. An anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control. All strains were isogenic P_{GAL}-*UBR1*. The expression of the second copy of Cdc45 was under its own promoter (P_{CDC45}).

As for the single mutants of Cdc45, I mutated endogenous *CDC45* with *cdc45-2A* by the modified 2-step replacement strategy as I described before in **Figure 5-9**. After verifying the endogenous *cdc45-2A* strain with Sanger sequencing, I compared the phosphorylation of Sld3 in wild-type, *cdc45-1A* and *cdc45-2A* cells in S-phase upon DNA damage as in **Figure 5-11**. The mobility shift of Sld3 after DNA damage was reduced in a *cdc45-1A* background compared to wild type cells as in **Figure 5-11**, however, the mobility shift of Sld3 was even further reduced in the *cdc45-2A* mutant upon DNA damage (**Figure 5-15.C**). It is important to note that, although hyper-phosphorylation of Rad53 decreased over time in both the wild type and *cdc45-1A* background, the hyper-phosphorylation of Rad53 did not change. In summary, I have demonstrated that the TXXD motifs in the disorganized loop of Cdc45 are required for the phosphorylation of Sld3 by Rad53 upon fork stalling.

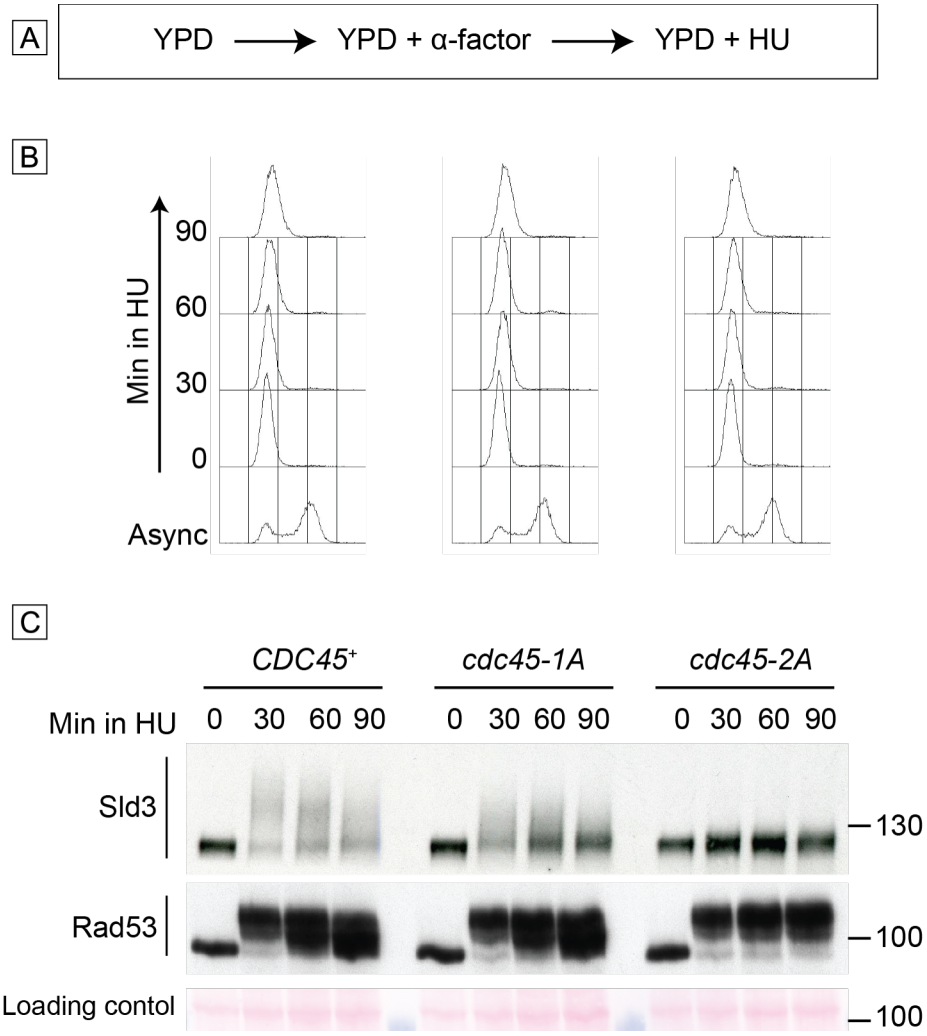


Figure 5-15 Cdc45 T189, T195 residues are essential for Rad53 dependent Sld3 phosphorylation in S-phase. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells. C) Western blot analysis of Rad53 and Sld3. An anti-Myc antibody was used for Sld3 detection. Ponceau-S staining of the membrane was used as a loading control.

5.4. Cdc45 is phosphorylated in a Rad53-dependent manner upon DNA damage

Although *in vitro* studies indicate that the FHA1 domain of Rad53 binds preferentially to phosphorylated TXXD-containing peptides, the phosphomimetic approach did not show constitutive Cdc45-Rad53 interaction (**Figure 5-14**). There are two potential explanations for this result. Either the phosphorylation of T198 and T195 are not required for binding of Rad53

to Cdc45, or D or E substitutions were not able to mimic phosphorylation successfully. To test the first possibility, I wanted to investigate whether Cdc45 is phosphorylated upon DNA damage. To do so, I compared the phosphorylation status of Cdc45 in wildtype*, rad53Δ sml1Δ* and *cdc45-2A* cells upon DNA damage. Because checkpoint-dependent Cdc45 phosphorylation has not been observed with the standard SDS-Page protocol upon DNA damage (**Figure 5-14**), the Phos-TAG-SDS-PAGE protocol was used to resolve Cdc45. Interestingly, a distinct slow running subset of Cdc45 was observed in wild type cells treated with hydroxyurea, indicating that Cdc45 was phosphorylated upon replication stress. Importantly, this was significantly reduced in *rad53Δ sml1Δ* cells which suggests that Cdc45 may also be a Rad53 target. However, whether this phosphorylation was performed directly by Rad53 or another Rad53-dependent kinase such as Dun1 is unknown. In addition, a slow running subset of Cdc45 was not observed in *cdc45-2A* cells, suggesting that either the disordered loop of Cdc45 is phosphorylated by Rad53 or that Rad53 binds to the disordered loop of Cdc45 (which was destroyed in *cdc45-2A* cells) and phosphorylates some residues on it.

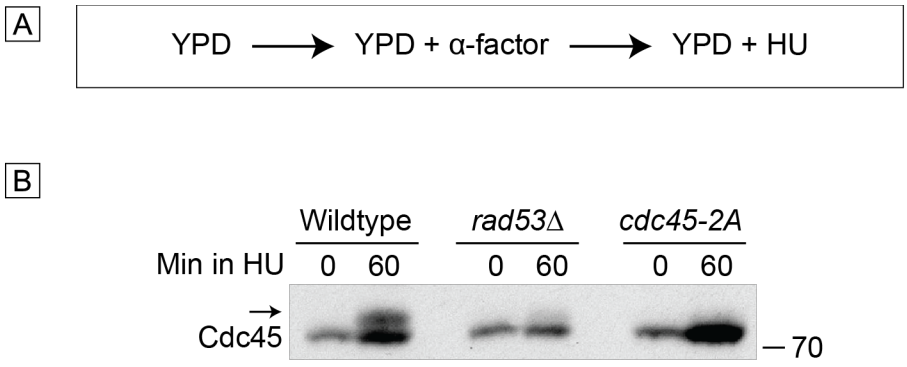


Figure 5-16 Rad53-dependent phosphorylation of Cdc45. A) Schematic representation of the experimental steps. B) Western blot analysis of Cdc45. Samples separated with Phos-Tag SDS-Page gel. An anti-Cdc45 antibody was used for Cdc45 detection.

5.5. Epistasis analysis between cdc45-2A and Rad53

Cdc45 is an essential DNA replication factor, and it is possible that the observed phenotype of *cdc45-2A* in S-phase was due to inefficient DNA replication. It was therefore crucial to confirm that *cdc45-2A* was a true separation of function mutant and its replication function was intact.

Hypomorphic replication mutants are synthetic lethal with checkpoint mutants. In order to test if *cdc45-2A* was a hypomorphic mutant for DNA replication, the synthetic lethality of *cdc45-2A* with *rad53Δ* was tested. To do so, tetrad analysis of a diploid strain which was heterozygous for *sml1Δ, rad53Δ and cdc45-2A* was performed (**Figure 5-17**). No synthetic lethality was observed between *rad53*Δ and *cdc45-2A* alleles, as *rad53*Δ *cdc45-2A sml1*Δ spores were viable. Synthetic sickness was also not observed and the size of *rad53*Δ *cdc45-2A sml1*Δ *and rad53*Δ *sml1*Δ spores were similar. Tetrad analysis of yeast exhibits a Mendelian distribution and the distribution of genotypes can be quantified. In summary, *cdc45-2A* was epistatic with *rad53Δ* which suggests that its replication function was intact.

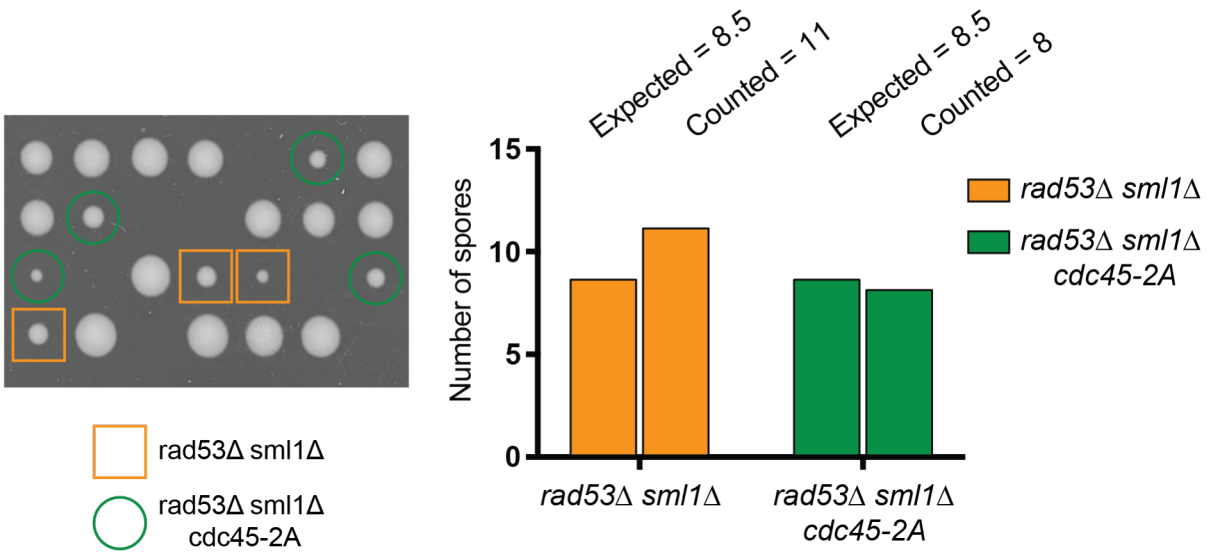


Figure 5-17 *cdc45-2A* is not a hypomorhic replication mutant. Tetrad analysis of heterozygous *cdc45-2A rad53Δ sml1Δ* diploid cells. A total of 68 isolates were analyzed.

5.6. cdc45-2A bypasses the function of Sld3 in the inhibition of late-origin firing

The regulation of DNA replication by checkpoint kinases upon DNA damage occurs through two distinct mechanisms: the first is stabilisation of active replication forks, and the second is blocking late origin firing (Zegerman & Diffley, 2010). It has been shown before that Sld3 and Dbf4 are the minimal substrates of Rad53 for the blockage of late origin firing (Zegerman & Diffley, 2010). The *sld3-A dbf4-4A* double mutants in which Rad53 phosphorylation sites are replaced with alanine can bypass the checkpoint-dependent inhibition of late origin firing (Zegerman & Diffley, 2010). Although the Rad53-dependent mobility shift of Sld3 was significantly reduced in *cdc45-2A* cells, Sld3 could still be phosphorylated to some extent. Rad53-dependent Sld3 phosphorylation occurs at multiple residues and requires 37 serine to alanine substitutions in order to bypass the inhibition of late-origin firing. To understand the extend of the inhibition of Sld3 phosphorylation in *cdc45-2A* cells, I wanted to use a functional assay that can monitor the inhibition of late origin firing. Because wildtype cells cannot fire late origins upon DNA damage (Zegerman & Diffley, 2010), their replication slows down drastically which can be monitored by flow cytometry analysis. Therefore, I decided to test if *cdc45-2A dbf4-4A* double mutants can bypass the inhibition of late origin firing upon DNA damage. Given that cdc45-2A prevents Rad53-dependent Sld3 phosphorylation, I wondered if *cdc45-2A dbf4-4A* could prevent the checkpoint-dependent block of origin firing.

To test this, I arrested *dbf4-4A, cdc45-2A and cdc45-2A dbf4-4A* cells in G1 phase with α-factor, released them into S-phase in the presence of 0.05% MMS and collected samples every 20 minutes. MMS is used in this experiment as a DNA-damaging agent because cells can still replicate upon MMS treatment (Tercero & Diffley, 2001). However, the progression of replication is very limited due to the inhibition of late origin firing (Tercero & Diffley, 2001). The *dbf4-4A* cells were used as a control because the late origin firing would be inhibited by Sld3 phosphorylation (Zegerman & Diffley, 2010). Mobility shift of Rad53 in western blot analysis

showed that the checkpoint was activated normally in all tested strains. As expected, minor DNA replication was observed in *dbf4-4A* and *cdc45-2A* cells even after 2 hours of incubation as assessed by the flow cytometry profile (**Figure 5-18**.**B**). On the other hand, *cdc45-2A dbf4-4A* double mutants traversed the S-phase in contrast to *dbf4-4A* or *cdc45-2A* cells and their bulk DNA amount seemed to double, most probably due the global derepression of late origin firing. In conclusion, preventing Sld3 phosphorylation by *cdc45-2A* is efficient to bypass the regulation of Sld3 by Rad53 for the block to late origin firing.

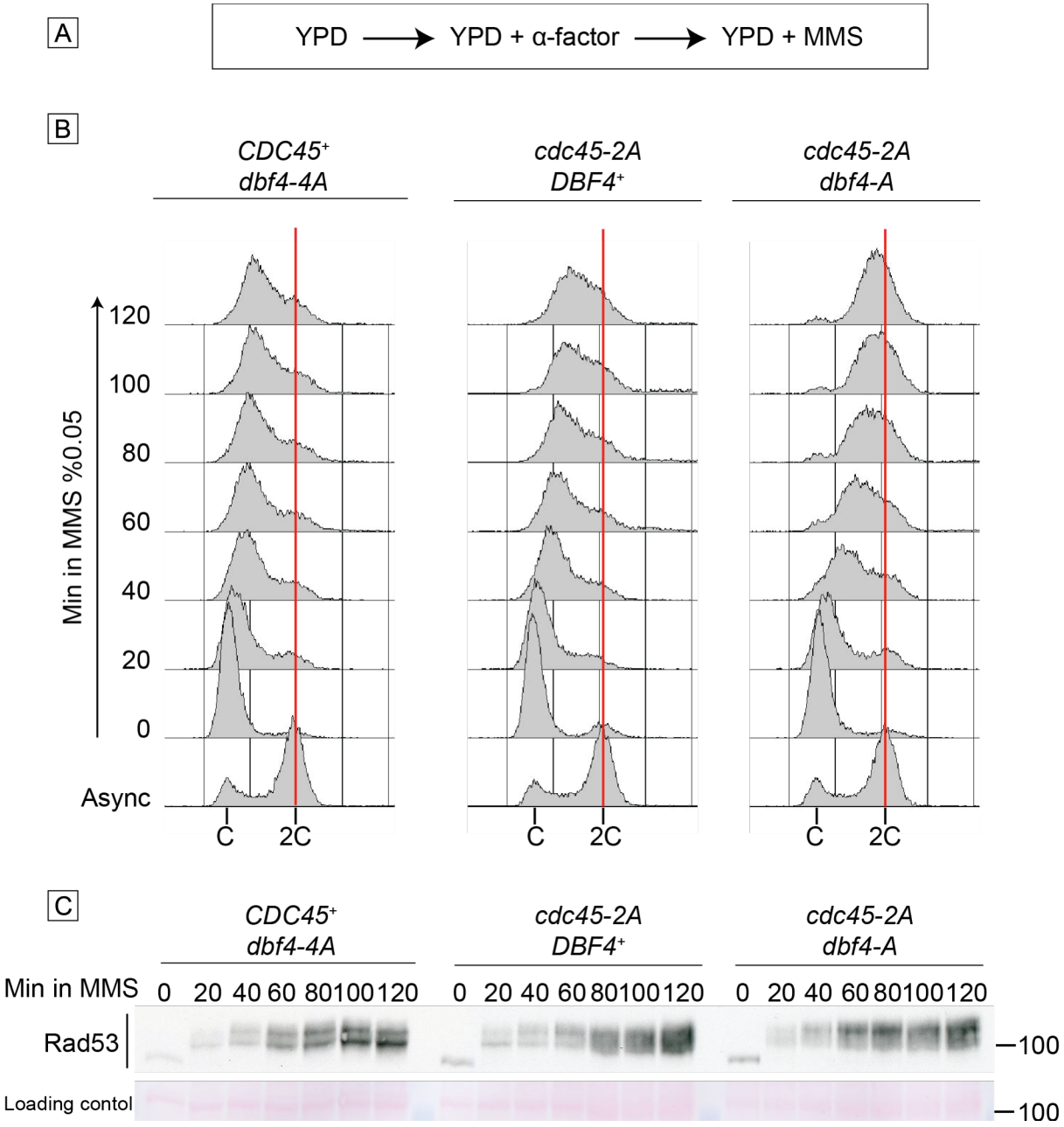


Figure 5-18 Analysis of late origin firing upon DNA damage. A) Schematic representation of the experimental steps. B) Flow cytometry analysis of the cells.

5.7. Discussion

Although Rad53 is a very important effector kinase in DNA damage checkpoint, very little is known about how Rad53 targets its substrates. In this chapter, I have shown that a solvent accessible flexible loop of Cdc45 is required for Rad53-dependent Sld3 phosphorylation upon DNA damage.

Dr. Zegerman observed that Sld3 is not phosphorylated by Rad53 upon DNA damage in *cdc45-HA* cells, in which endogenous CDC45 is C-terminally HA tagged (data not shown). Based on this observation, I have shown that Cdc45 is required for Rad53-dependent Sld3 phosphorylation by taking advantage of *cdc45-td* cells (**Figure 5-2**). Given that reduced Sld3 and Cdc45 interaction decreased Sld3 phosphorylation (**Figure 5-3**), I concluded that Cdc45-Sld3 interaction is necessary for Rad53 dependent Sld3 phosphorylation. Further investigations with *dpb11-1* cells indicated that Cdc45, but not Dpb11 is required for Sld3 phosphorylation (**Figure 5-4**). In addition, Cdc45 is required for Sld3 but not Dbf4 phosphorylation (**Figure 5-5**).

At least two different hypotheses could explain the requirement of Cdc45 for the phosphorylation of Sld3 by Rad53. Either the Cdc45-Sld3 interaction changes the 3D conformation of Sld3 and exposes a binding domain for Rad53, or the binding of Rad53 to Cdc45 is required for Sld3 phosphorylation. Although Rad53 is one of the least specific yeast kinases (Mok *et al*., 2010), the FHA1 domain of Rad53 has binding specificity to the phospho-peptide *p*TXXD *in vitro* (Liao *et al*., 2000). As, it has been previously suggested that Cdc45 (154-270) fragment interacts with Rad53 FHA1 and two TXXD domains (T189 and T195) of Cdc45 are located in this fragment (Aucher *et al*., 2010), I wondered whether these domains were required for Sld3 phosphorylation. Given that, these residues are also aligning with the disordered loop of human Cdc45 (Simon *et al*., 2016), I hypothesized that they might be accessible by Rad53. Interestingly, I have shown with different approaches that although the

alanine substitution of Cdc45-T189 (*cdc45-1A*) significantly decreases Sld3 phosphorylation (**Figure 5-8** and **Figure 5-11**), double alanine substitution of T189 and T195 (*cdc45-2A*) almost fully abrogated the phosphorylation of Sld3 upon DNA damage (**Figure 5-15**). I concluded from these experiments that the TXXD domains on Cdc45 are required for the Rad53 dependent Sld3 phosphorylation. However, because T195A alone have never been tested, it is not known whether T189 and T195 together or T195 alone is required for Sld3 phosphorylation.

Given that the FHA1 domain of Rad53 has a binding preference for phosphorylated TXXD peptides, I used a phosphomimetic approach to test if it is possible to create a constitutive interaction between flexible loop of Cdc45 and Rad53. However, both single T189 and double T189 T195 substitutions with glutamic acid or aspartic acid showed similar Sld3 phosphorylation pattern with alanine substitutions suggesting that phosphomimetic approach was not successful. There are different explanations for the failure of this phosphomimetic approach: 1) the glutamic acid or aspartic acid substitutions are not enough to mimic *p*T, 2) phosphorylation of Cdc45 is not required for Rad53 binding. Given that Cdc45 was phosphorylated in a Rad53-dependent manner (**Figure 5-16**), mapping the phosphorylation sites on Cdc45 would allow us to understand why the phosphomimetic approach on the flexible loop of Cdc45 did not work.

Although this chapter provides evidence that mutating the potential FHA1 biding domain on the disordered loop of Cdc45 is sufficient to prevent Sld3 phosphorylation, it does not show direct binding of Rad53 on Cdc45. But given that Aucher *et al.* has shown that the Cdc45 (154-270) fragment interacts with the FHA1 domain of Rad53 *in vitro*, it is possible that Rad53 interacts via its FHA1 domain with the flexible loop of Cdc45 *in vivo*. On the other hand, whether the FHA2 domain of Rad53 has biological significance for the Sld3 phosphorylation is unknown. The significance of FHA2 domain of Rad53 could be addressed by using a *rad53-11* allele, in which the FHA2 domain cannot bind its substrates. The phosphorylation status of

Sld3 upon DNA damage in *rad53-11* cells could potentially reveal the significance of the FHA2 domain on the Sld3 phosphorylation.

At least two different models could explain the mechanism of Rad53-dependent Sld3 phosphorylation based on the evidence obtained in this chapter and the literature. Either the FHA1 domain binds to the flexible loop of Cdc45 which is then handed over to Sld3 (Model 1) or Rad53 binds to the flexible loop of Cdc45 and phosphorylates Sld3 (Model 2) (**Figure 5-19**). These 2 models could be tested by different biochemical tools such as Chemically Induced Dimerization (CID) (Fegan *et al*., 2010). With this technique, the flexible loop of Cdc45 could be forced to dimerize with the FHA1 domain of Rad53. In such a case, If Sld3 is phosphorylated upon DNA damage, then the second model would probably be true. However, if Sld3 is not phosphorylated after the constitutive dimerization of the flexible loop of Cdc45 with Rad53, then the first model would probably be true. On the other hand, whether there is a function of Rad53-dependent Cdc45 phosphorylation in these models is not yet known. Mapping the Rad53-dependent phosphorylation sites of Cdc45 using biochemical assays such as mass spectrometry analysis would determine the direction of the research.

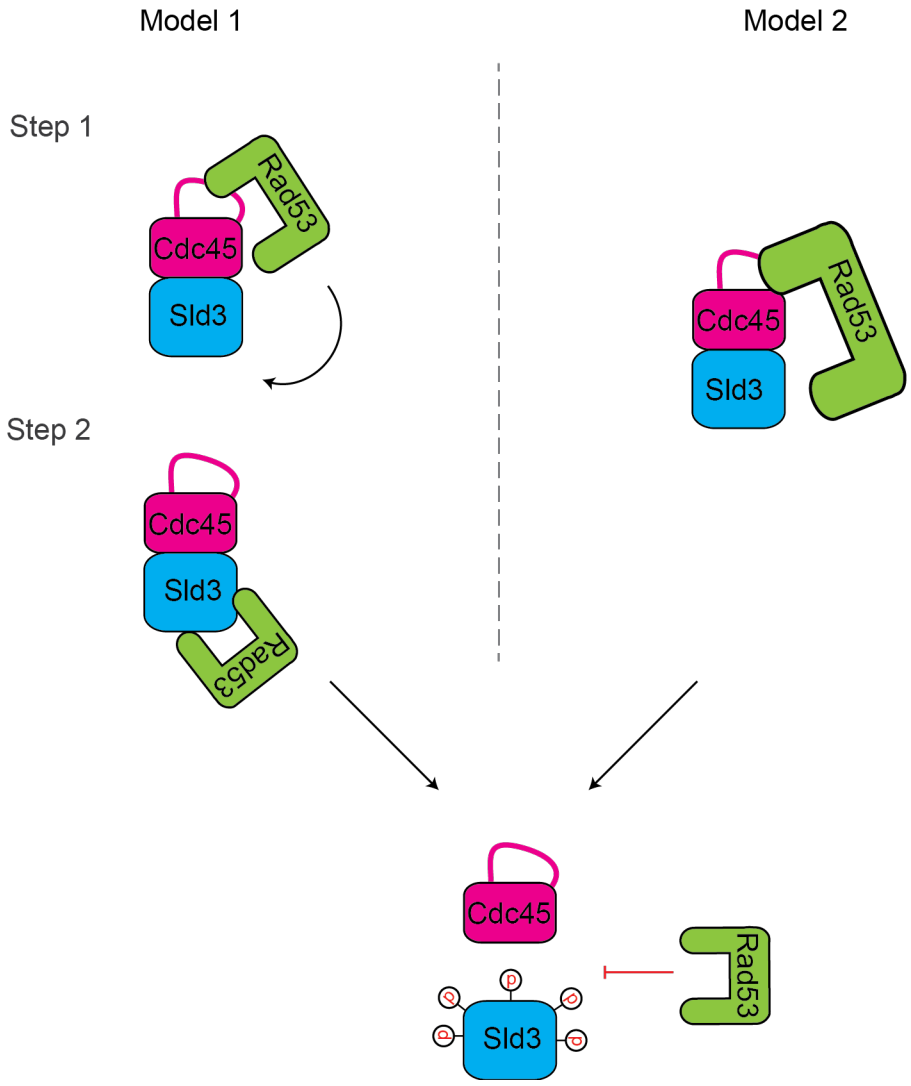


Figure 5-19 Possible models for the mechanism of Rad53 dependent Sld3 phosphorylation.

While cdc45-2A prevents Sld3 phosphorylation, it could still be a hypomorphic allele for its functions for replication. Cells carrying hypomorphic replication alleles are usually synthetic lethal with *rad53Δ* which has been observed for *cdc45*-HA (personal communication with Dr Zegerman). Interestingly, epistasis analysis with *rad53Δ* suggested that the function of cdc45-2A is probably intact during replication, because *rad53Δ sml1Δ cdc45-2A* cells were viable. However, more detailed research could be performed through DNA fiber analysis with double staining. With this technique both the number of replication initiations and the speed of the

replication fork could be determined which would allow us to understand the possible small degree of loss of function in cdc45-2A (Etienne Schwob *et al*., 2009).

On the other hand, since Sld3 is phosphorylated at multiple residues (Zegerman & Diffley, 2010), there could be still some phosphorylation which was not observed by western blot analysis in *cdc45-2A* cells. In the case of some residual phosphorylation in *cdc45-2A* cells upon DNA damage, it would be expected to inhibition late origin firing. To rule out this possibility, a functional experiment was carried out in which the inhibition of late origin firing observed by monitoring the progression of DNA replication upon DNA damage. The flow cytometry analysis indicated that, although *dbf4-4A* cells did not fire late origins after MMS treatment, the bulk DNA amount increased in *cdc45-2A dbf4-4A* cells (**Figure 5-18**). This experiment proved that the inhibition of late origin firing cannot happen through the phosphorylation of Sld3 in *cdc45-2A* cells. Unexpectedly, *cdc45-2A* showed a sign of late origin firing. Initial research that identified the Sld3 as a target for the inhibition of late origin firing suggested that the *sld3-A* mutant cannot fire late origin firing alone (Zegerman & Diffley, 2010). However, this could be due to incomplete mapping of phosphorylation sites of Sld3. Although this experiment had enough evidence to suggest that cdc45-2A dbf4-4A mutants prevent late origin firing, it could be further validated by more sensitive techniques such as 2D gel electrophoresis or replication intermediate analysis in order to show directly the replication initiations from the late origins. Together, I conclude that, *cdc45-2A* is a separation of function mutant that prevents Sld3 phosphorylation by Rad53 upon DNA damage but does not affect its function during DNA replication.

Nucleotide depletion via HU or other nucleotide analogues results in decreased polymerase activity and increased amount of ssDNA which activates S-phase checkpoint (Hustedt *et al*., 2013). Rad53 activation by DNA damage result in the regulation the nucleotide pool via transcriptional control of ribonucleotide reductase (RNR) (Huang *et al*., 1998) and the degradation RNR inhibitors by Dun1 (Lee *et al*., 2008; Zhao & Rothstein, 2002). Further

production of dNTPs results in increased dNTP level as well as polymerase activity and decreased ssDNA. Because of this adaptation, dephosphorylation of Rad53 was observed in wildtype cells after 90 minutes HU treatment (**Figure 5-15**). However, dephosphorylation of Rad53 was not observed in *cdc45-2A* cells suggesting that either more replication origins fire in *cdc45-2A* cells upon HU treatment which results in more damage, or Cdc45 is also involved in dephosphorylation of Rad53 after the recovery from DNA damage. Because some degree of late origin firing was observed in *cdc45-2A* cells after MMS treatment (**Figure 5-18**), the lack of Rad53 dephosphorylation after 90 minutes HU treatment is possibly due to an increased number of origin firing. However, the observation that *cdc45-2A* fires some late origins upon DNA damage should be tested with the more precise techniques mentioned above.

This chapter provides some evidence that Cdc45 is not required for the phosphorylation of Dbf4 upon DNA damage, which is in line with the previous work showing that Rad53 directly binds to and phosphorylates Dbf4 (Chen *et al.*, 2013). However, as mentioned above, in addition to its function in replication initiation, Cdc45 also travels with the replication fork which suggests that Cdc45 might be required for the phosphorylation of the other replication fork components. The next chapter (6) attempts to address this question in detail.

The regulation of the Sld3 human homolog Treslin by checkpoint kinases is still not clear. Gao *et al.* (2015) recently proposed that Chk1 associates with the C-terminal domain of Treslin which is not present in yeast Sld3. Although the flexible loop of Cdc45 is conserved among the different organisms but TXXDs are not (**Figure 5-6**), the requirement of Cdc45 for Chk1-dependent Treslin phosphorylation might be lost during evolution. However, this loop could still be required for the phosphorylation of other factors on the replication fork.

According to the cryo-EM structure of yeast CMG complex, there is another solvent accessible loop on yeast Cdc45 between 436-461 and a TXXD (T437) domain is located in this region (Yuan *et al.*, 2016). Whether this domain is also important for Rad53-dependent

phosphorylation of replication factors is unknown. Considering *cdc45-2A* bypasses the Rad53 dependent Sld3 phosphorylation, this putative domain is not required for Sld3 phosphorylation. However, this domain might have another function during replication. Surprisingly, this loop is also conserved in human Cdc45 (E384-D395), suggesting that possible roles of this second loop during replication could also be conserved.

Considering previous studies and this chapter, solvent accessible TXXD domains could be used for the prediction of the possible Rad53 interaction sites which might help to find novel Rad53 substrates. For instance, Sld5 the yeast GINS subunit has a solvent accessible TXXD domain at T259 which could be a possible binding site for Rad53. However, it is important to keep in mind that this approach relies on the available protein structures which could be a bottleneck for further research.

Chapter 6

Cdc45 is involved in the stabilisation of stalled fork

6.1. Introduction

The inhibition of the progression of polymerase or helicase by endogenous or exogenous sources (see chapter 1.5.1) results in replication fork stalling which leads to activation of the DNA damage checkpoint in S-phase. Stalled replication forks produce aberrant DNA structures and their detection by sensor proteins promote the activation of two master checkpoint kinases, ATM and ATR in humans (Mec1 and Tel1 in yeast). These master kinases transduce the signal to the effector kinases Chk1 and Chk2 in humans (Rad53 and Chk1 in yeast) to execute several responses including the stabilization of stalled replication forks.

Stalled forks could produce aberrant DNA structures such as excessive single strand DNA (ssDNA), single strand-double strand DNA junctions (ss-dsDNA), double stand breaks (DBSs) or single strand breaks (SSBs) (Hustedt *et al*., 2013). For instance, small DNA base adducts created by MMS or dNTP depletion with HU can stop the progression of the polymerase which generates long tracks of ssDNA due to the continuous DNA unwinding by the helicase (Poli *et al*., 2012; Tercero & Diffley, 2001). These long tracks of ssDNA are rapidly coated by replication protein A (RPA) (Alani *et al*., 1992). Mec1 and Ddc2 is recruited to ssDNA track by RPA and results in the activation of Mec1 (Zou & Elledge, 2003). However, in Xenopus, ssDNA is not sufficient to activate the checkpoint (MacDougall *et al*., 2007). Free 5' or 3' ends are compulsory for the effective checkpoint activation (MacDougall *et al*., 2007). The free 5' end can be generated during lagging strand synthesis whereas free 3' ends can be generated during both lagging and leading strand synthesis. Free 5' and 3' ends create ss-dsDNA junctions in which the PCNA-like loading clamp 9-1-1 complex (Ddc1-Rad17-Mec3) is loaded by Rad24-RFC (Majka & Burgers, 2003). Mec1 phosphorylates Ddc1 on 9-1-1 complex which further stimulates Mec1 activation by the recruitment of Dpb11 (Puddu *et al*., 2008). On the other hand, DSBs are recognized by the MRX complex (Mre11-Rad50-Xrs2) which promotes resection of a single strand (D'Amours & Jackson, 2001). This resection creates an ss-dsDNA junction which then is loaded onto the 9-1-1 complex as well as RPA and promotes Mec1 activation.

Activation of the checkpoint response kinase Rad53 upon replication fork stalling requires different mediator proteins such Mrc1, Rad9 and Sgs1 in yeast (Hustedt *et al*., 2013). Mrc1 is a replication fork component and travels with the fork during replication (Katou *et al*., 2003). DNA replication slows down in the absence of Mrc1 during unperturbed replication, suggesting that Mrc1 is required for the maintenance of the replication fork (Alcasabas *et al*., 2001). In addition, Mrc1 is the key mediator protein upon replication fork stalling in which its

phosphorylation by Mec1 results in the activation of Rad53 (Alcasabas *et al*., 2001). It also interacts with Tof1-Csm3 to form a stable pausing complex upon fork stalling (Bando *et al*., 2009; Katou *et al*., 2003). Rad53 activation can be mediated also by Rad9 (Sanchez *et al*., 1999). Phosphorylation of Rad9 by Mec1/Tel1 upon DNA damage results in the recruitment and activation of Rad53 (Emili, 1998). Finally, RecQ family helicase Sgs1 can also activate Rad53 (Frei & Gasser, 2000). Sgs1 constitutively associates with the replication fork and is phosphorylated in a Mec1-dependent manner upon DNA damage (Frei & Gasser, 2000). Double deletion of Sgs1 and Rad24 has been shown to reduce Rad53 activation, suggesting that Sgs1 is required for Rad53 activation (Bjergbaek *et al*., 2005).

The replication forks cannot continue to synthesise DNA after transient fork stalling in checkpoint-deficient yeast cells (Tercero *et al*., 2003). Checkpoint kinases are mandatory for the stabilisation of stalled forks in order for their restart after removal of the block (Tercero *et al*., 2003). Forks are stabilized by the checkpoint kinases Mec1 and Rad53 to prevent irreversible fork collapse in yeast upon stalling (Lopes *et al*., 2001). Because replication fork factors cannot be reloaded upon helicase activation (Labib *et al*., 2000), collapsed forks could be rescued by a fork fired from an adjacent origin (McIntosh & Blow, 2012). However, if two converging forks irreversibly collapse, the DNA between these two forks cannot be replicated which could generate genomic instability (Blow *et al*., 2011). For this reason, the stabilisation of stalled replication forks must be achieved by a robust mechanism to ensure that stalled forks do not collapse. As the stabilisation of the replication fork is of vital importance to the faithful duplication of genomic DNA, understanding the molecular mechanisms underlying replication fork stabilisation could have potential implications in the treatment of several diseases.

Stalled replication fork components still associate with chromatin in *rad53Δ* cells (De Piccoli *et al*., 2012). In addition, some replication proteins are phosphorylated in a Rad53-dependent manner such as Mrc1, Sgs1, Srs2, Rrm3 and Exo1 (Alcasabas *et al*., 2001; Hegnauer *et al*., 2012; Liberi *et al*., 2000; Morin *et al*., 2008; Rossi *et al*., 2015). These two observations suggest

that Rad53 could be required for the modification of replication fork components to keep stalled forks functional until the removal of the block. Although Rad53 associates with stalled replication forks (Katou *et al.*, 2003), exactly how it targets stalled forks is unknown. Understanding how Rad53 targets stalled replication forks might allow for the discovery of mechanisms of fork stabilisation.

In chapter 5, I showed that Cdc45 is required for Rad53-dependent Sld3 phosphorylation. Cdc45 is required for both replication initiation and elongation (Tercero *et al.*, 2000). Given that Cdc45 associates with chromatin in G1 phase (Aparicio *et al.*, 1999) and travels with the replication forks during S-phase (Moyer *et al.*, 2006), the flexible loop of Cdc45 might serve as a binding site for Rad53 to target and phosphorylate stalled replication fork components. In this chapter, I investigated whether the flexible loop of Cdc45 targets Rad53 to stalled replication forks for their stabilisation (**Figure 6-1**).

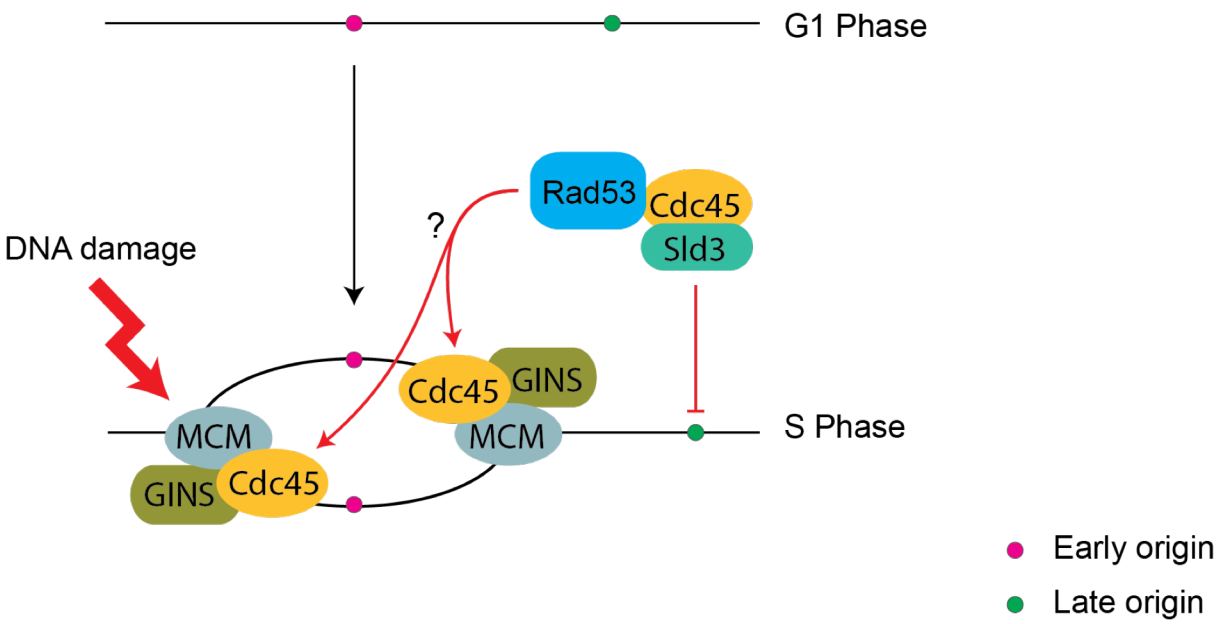


Figure 6-1 How is Rad53 recruited to stalled replication forks?

6.2. Checkpoint mediators do not a have synthetic growth phenotype in cdc45-2A mutants

If Cdc45 is required for the binding of Rad53 to replication forks upon DNA damage, then the cdc45-2A mutant, which cannot bind to Rad53, could prevent the recruitment of Rad53 to the stalled forks. This would lead to inefficient fork stabilization and growth defects. To test this, the effect of mutated Cdc45 (cdc45-2A) upon DNA damage on cell growth was tested on different drug plates. As expected, wild type cells grew on all tested conditions and *rad53Δ sml1Δ* died on drug-containing plates. However, no growth deficiency was observed in *cdc45-2A* cells on different types of drug plates compare to wild type cells, suggesting that either mutating the disordered loop of Cdc45 alone does not sufficiently affect cell viability or the flexible loop of Cdc45 is not required for the stabilization of stalled replication forks (**Figure 6-2**).

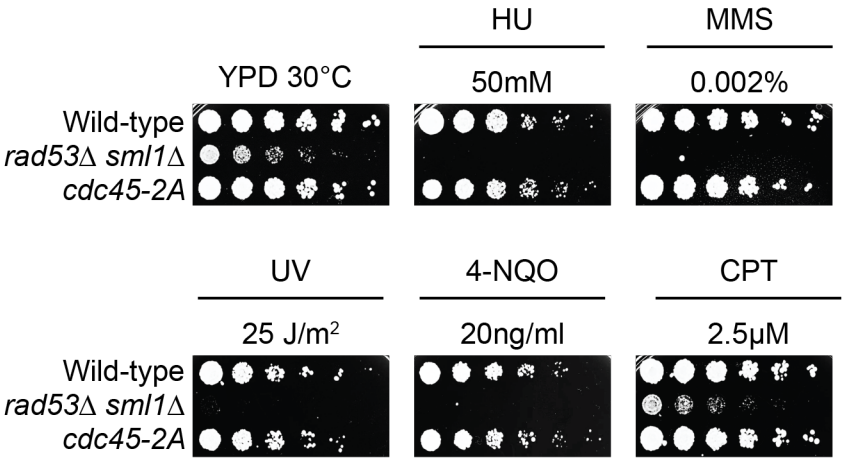


Figure 6-2 *cdc45-2A* does not have growth phenotype upon DNA damage. Exponentially growing cells were counted and 3-fold serial dilutions spotted onto plates containing the indicated DNA damaging agents.

Although the disordered loop of Cdc45 could act as a binding site for Rad53 to stabilize the replication fork upon fork stalling, it does not necessarily mean it is the only binding site for Rad53. It is possible that the stabilization of replication fork requires other possible Rad53

binding sites, which might compensate the lack of Rad53 binding to Cdc45. I hypothesized that if other possible Rad53 substrates on the stalled replication forks are involved in the fork stabilization, then decreasing the local Rad53 concentration around the stalled forks could exacerbate the effect of Rad53 binding on Cdc45. Given that checkpoint mediators possibly increase the local concentration of Rad53 around replication forks (Hustedt *et al*., 2013), lack of those mediators might decrease the concentration of Rad53 around stalled forks, resulting in growth defects. To test this hypothesis, I analyzed the growth of individual null mutants of *MRC1, SGS1, RAD9 or* 9-1-1 subunits *DDC1, MEC3,* or *RAD17* with or without *cdc45-2A* on different drug plates (**Figure 6-3,4,5,6**). The *cdc45-2A* cells grew similarly to wild type cells in all tested conditions, in agreement with previous observations (**Figure 6-2**). However, cdc45-2A did not exacerbate the growth defect of any of the null mutants tested on different drug plates suggesting that *cdc45-2A* is not synthetic lethal with *mrc1Δ, rad9Δ, sgs1Δ, mec3Δ, rad17Δ* or *ddc1Δ* upon DNA damage.

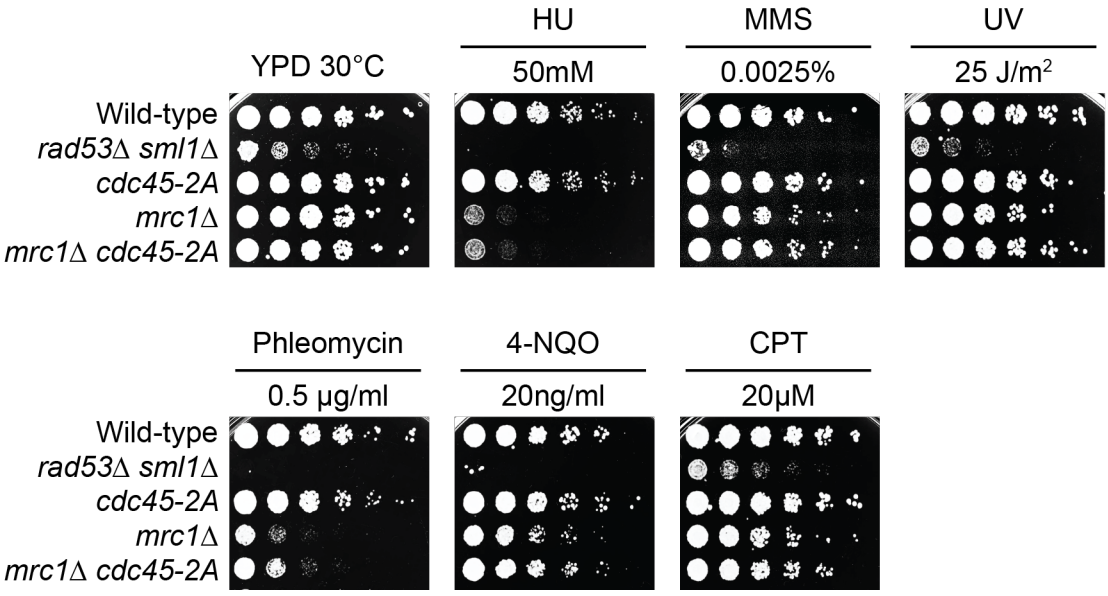


Figure 6-3 *cdc45-2A* is not synthetic lethal with *mrc1Δ* on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

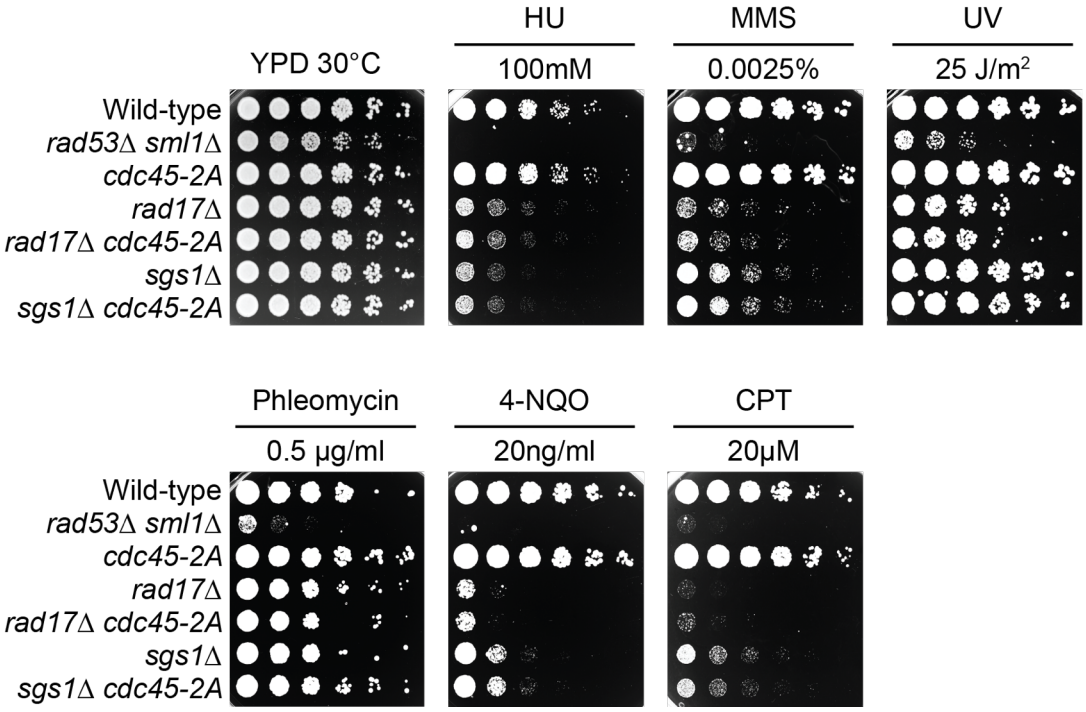


Figure 6-4 *cdc45-2A* is not synthetic lethal with *rad17Δ or sgs1Δ* on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

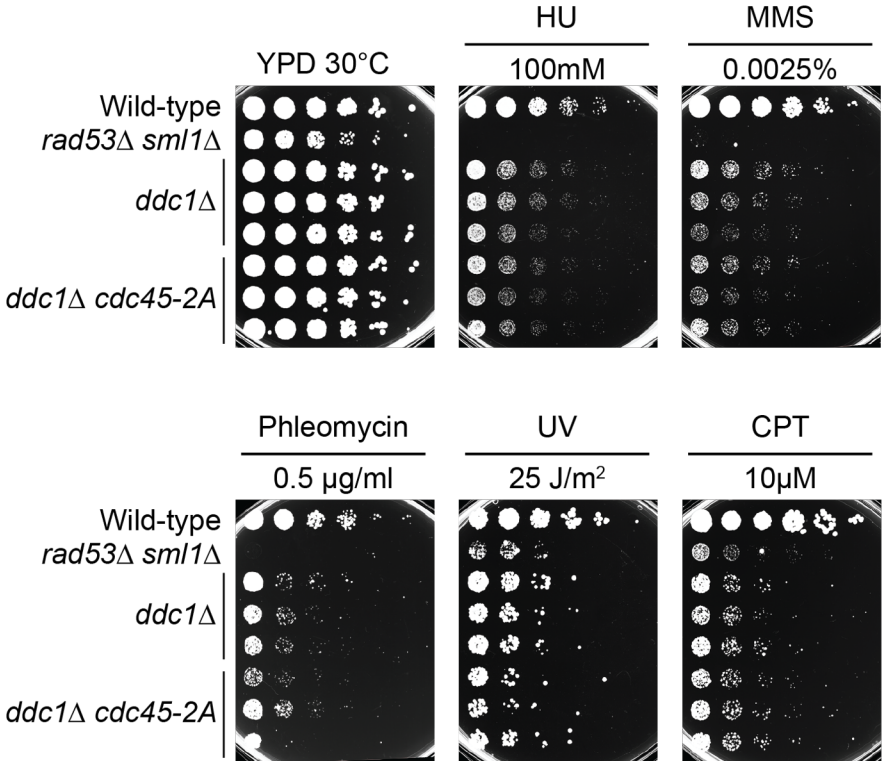


Figure 6-5 *cdc45-2A* is not synthetic lethal with *ddc1Δ* on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

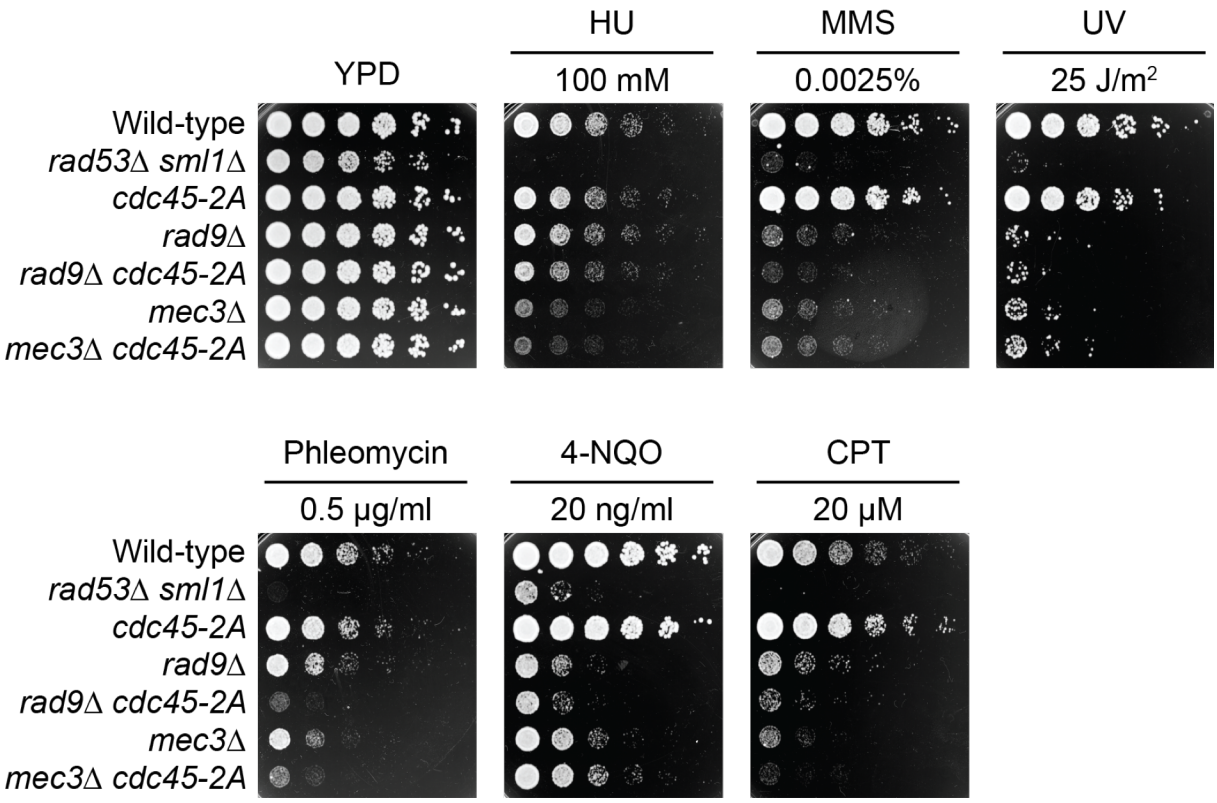


Figure 6-6 *cdc45-2A* is not synthetic lethal with *rad9Δ or mec3Δ* on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

Although, cdc45-2A did not exacerbate the growth defect of any of the single checkpoint mediator null mutants, it is possible that the availability of other checkpoint mediators might increase the local concentration of Rad53, which could be enough to stabilize the stalled replication forks. In order to decrease the Rad53 concentration around the stalled forks even further, I decided to test the effect of cdc45-2A on double checkpoint mediator null mutants. To do so, I analyzed the growth of *ddc1Δ sgs1Δ, rad9Δddc1Δ* and *ddc1Δ mrc1Δ* on different drugs with wild type Cdc45 or with cdc45-2A (**Figure 6-7**, **Figure 6-8** and **Figure 6-9**). Although wild type and single null cells grew on drug plates, cells carrying the combination of different double knockout genes were not viable on drug plates. The *rad9Δ ddc1Δ* cells grew on 50 mM HU but cdc45-2A did not exacerbate the growth defect of *rad9Δ ddc1Δ* cells (**Figure 6-9**). Decreasing the concentration of DNA damaging drugs allowed some growth in *ddc1Δ mrc1Δ* cells (**Figure 6-8**). However, cdc45-2A did not affect the growth of *mrc1Δ ddc1Δ* cells. Given

that almost all double checkpoint cells were not viable on drug plates possibly due to the ineffective checkpoint activation, it was not possible to observe the effect of inhibited Cdc45-Rad53 interaction.

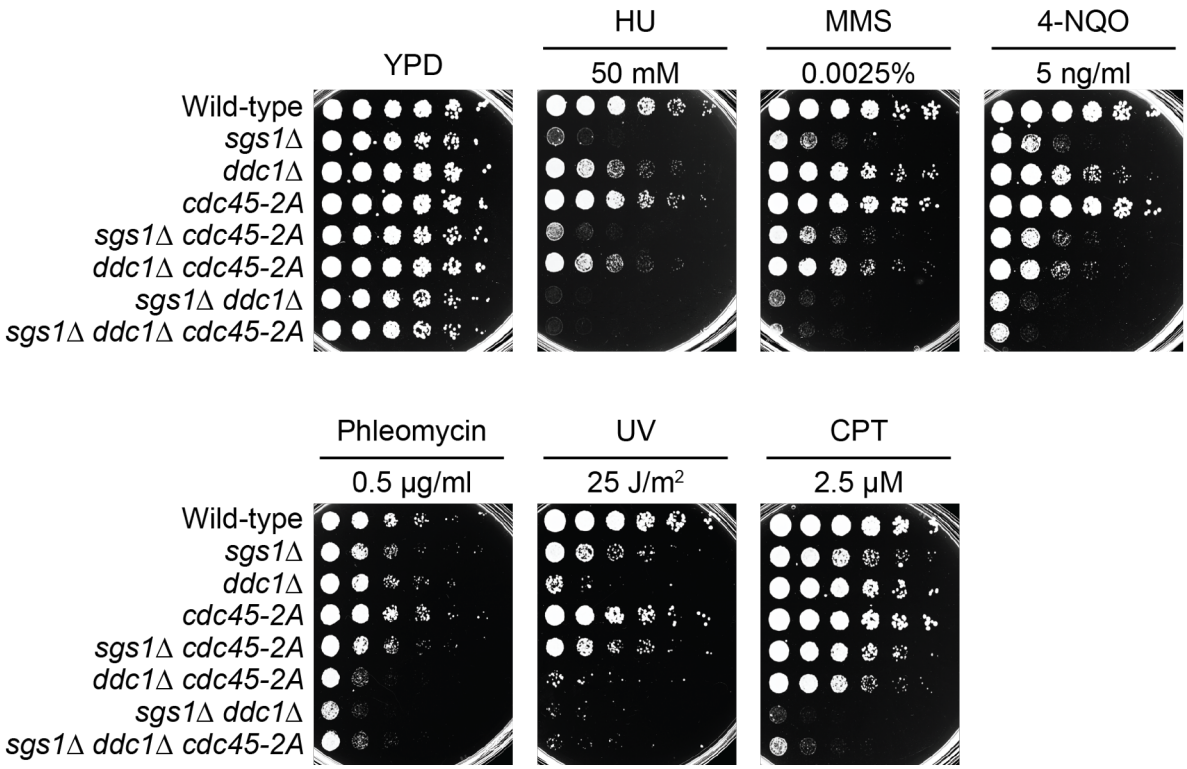


Figure 6-7 *cdc45-2A* is not synthetic lethal with *ddc1Δ sgs1Δ* double knouckouts on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

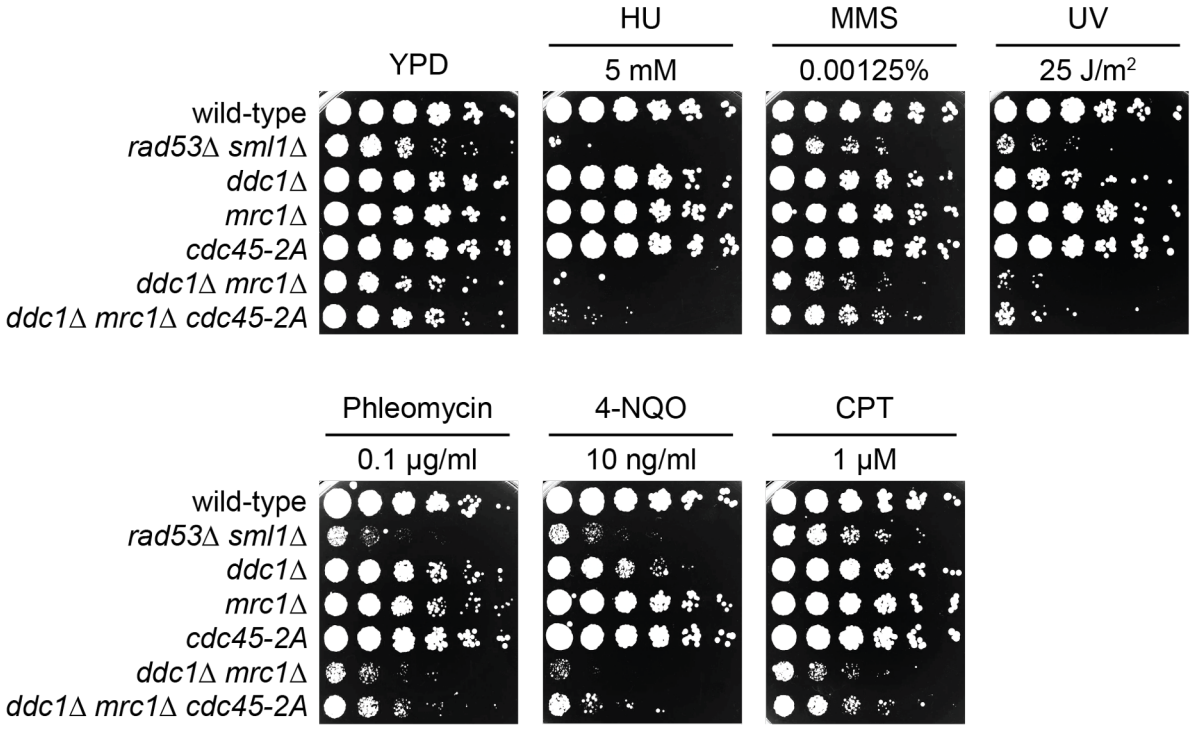


Figure 6-8 *cdc45-2A* is not synthetic lethal with *ddc19Δ mrc19Δ* double knouckouts on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

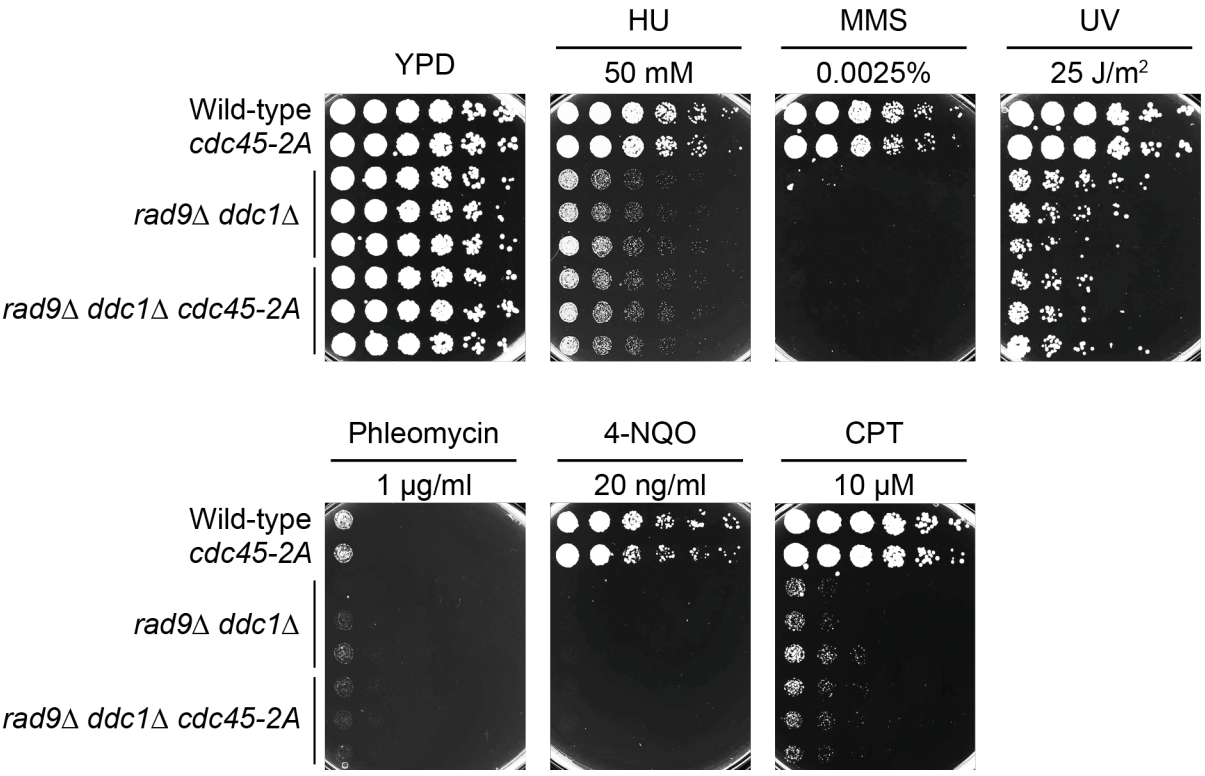


Figure 6-9 *cdc45-2A* is not synthetic lethal with *ddc19Δ rad9Δ* double knouckouts on drug plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated DNA damaging agents.

Because decreasing Rad53 concentration around the replication fork by removing checkpoint mediators was either ineffective (single knockout cells) or lethal (double knockout cells), I decided to decrease the total Rad53 in cells in order to address whether decreasing Rad53 around stalled replication forks would exacerbate the effect of cdc45-2A. It has been shown before that C-terminally tagged Rad53 (rad53-HA) has reduced expression compared to wild type (Cordón-Preciado *et al*., 2006). This information was used to address if the reduction of Rad53 levels sensitizes *cdc45-2A* cells to HU. In addition, since Mrc1 is an important mediator for Rad53 activation around stalled replication forks (Alcasabas *et al*., 2001), an Mrc1 null mutant was used to decrease the local concentration of Rad53 around stalled replication forks. In order to verify the previous work (Cordón-Preciado *et al*., 2006), first the expression level of Rad53-HA was verified by western blot analysis of Rad53. Western blot analysis of Rad53 indicated that Rad53-HA was expressed less than wild type Rad53 (**Figure 6-10**). Since the level of Rad53 was very low, a long blot exposure was required to get a Rad53 signal from *rad53-HA* cells. However, even after a very long exposure, phosphorylated rad53-HA signals were not detected.

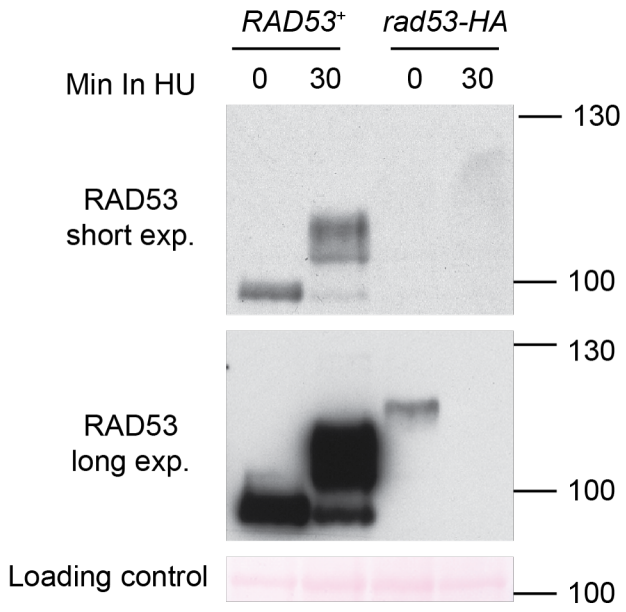


Figure 6-10 The protein expression of rad53-HA. Western-blot analysis wild-type and rad53-HA cells. Cells were arrested in G1 and relaesed into YPD media contaning 200 mM HU. The blot was probed with an anti-Rad53 antibody.

Because it was verified that *rad53-HA* had reduced Rad53 expression, I wanted to test whether *cdc45-2A* cells had any growth defect when Rad53 expression was lower than the physiological level. To do so, cells were subjected to growth analysis on YPD plates containing different concentrations of HU. Growth analysis of the cells indicated that wild-type and *cdc45-2A* cells grew at all tested HU concentrations similar to YPD plates (**Figure 6-11**). In addition, *rad53-HA, mrc1Δ, rad53-HA cdc45-2A, mrc1Δ cdc45-2A* cells grew like wild type cells up to 15 mM HU and their growth gradually decreased with increasing concentration of HU. On the other hand, *mrc1Δ rad53-HA* cells exhibited a decreased growth phenotype from 15mM HU onwards and were completely dead on 50 mM HU. However, *mrc1Δ rad53-HA cdc45-2A* cells showed a decreased growth phenotype from 5 mM and were completely dead on 10 mM HU. This experiment indicated that *mrc1Δ rad53-HA* and *cdc45-2A* are synthetic lethal at very low concentrations of HU.

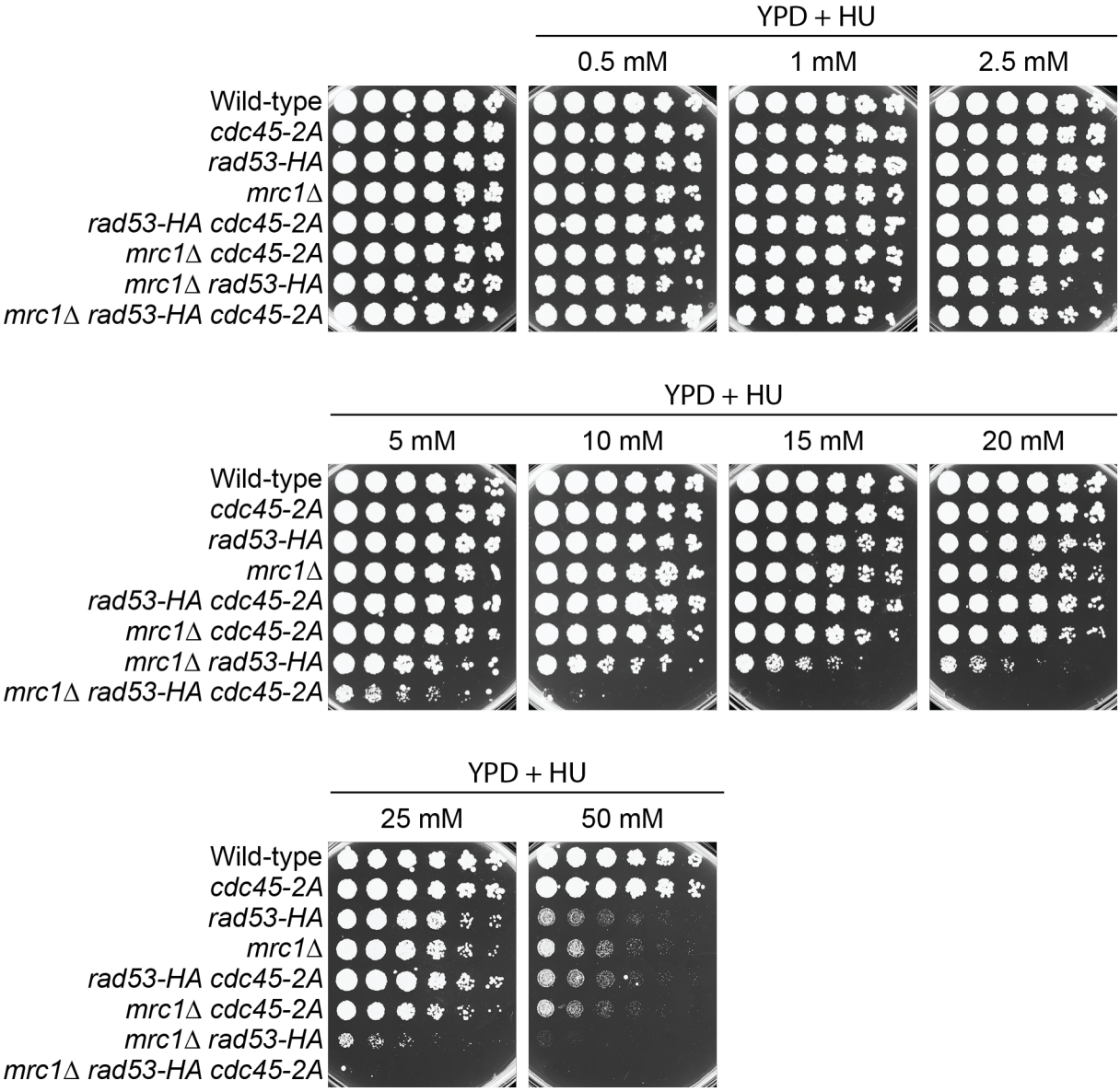


Figure 6-11 *cdc45-2A* is synthetic lethal with *mrc1Δ rad53-HA* on HU plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated concentration of HU.

To test whether the HA tag prevents the function of Rad53, I made an N-terminally HA tagged Rad53 (HA-rad53). Western-blot analysis of Rad53 showed that *HA-rad53* cells had lower expression than wild type cells as well (**Figure 6-12.A**). However, it seemed that the expression level of HA-rad53 was higher than rad53-HA. In addition, the hyper-phosphorylation of HA-rad53 was also observed in G2 phase upon DNA damage suggesting that the HA tag did not affect the activation of Rad53 upon DNA damage (**Figure 6-12.B**).

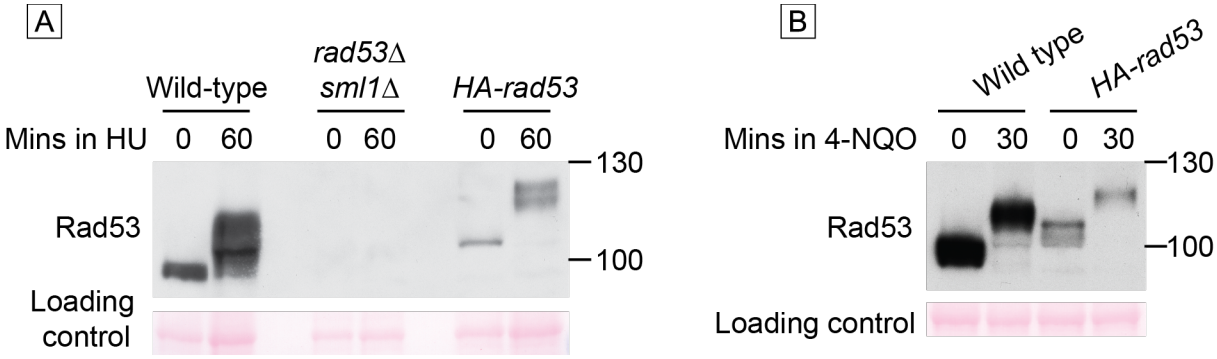


Figure 6-12 Western blot analysis wild-type and HA-rad53 cells. A) Cells were arrested in G1 and released into YPD media contaning 200 mM HU. B) cells were arrested in G2 with nocodazole and 10μg/ml 4-NQO was added. The blot was probed with an anti-Rad53 antibody.

After the verification of the expression and hyper-phosphorylation of HA-rad53, cells were subjected to growth analysis, in order to test whether decreased Rad53 expression would increase the genomic instability when the Rad53-Cdc45 interaction was inhibited by cdc45-2A upon fork stalling. Because HA-rad53 expression was greater than rad53-HA, higher concentrations of HU were used. Growth analysis showed that *HA-rad53* cells exhibited a decreased growth phenotype on 200 mM HU plates compare to wild type and *cdc45-2A* cells (**Figure 6-13**). Furthermore, *HA-rad53 cdc45-2A* cells were almost completely dead on 200 mM HU. In conclusion, *cdc45-2A* is synthetic lethal with *HA-rad53* upon replication fork stall by HU.

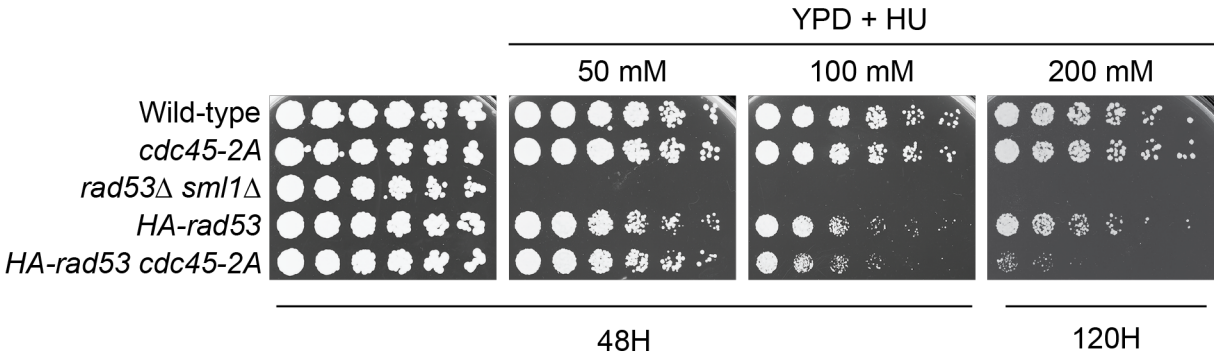


Figure 6-13 *cdc45-2A* is synthetic lethal with *HA-rad55* on HU plates. Exponentially growing cells were counted and 3-fold serial dilutions were spotted onto plates containing indicated concentration of HU.

The growth phenotype that was observed in the *cdc45-2A* background when rad53 expression was low could be due to fork collapse generated by inefficient replication fork stabilization upon fork stalling. I hypothesized that, if the replication fork stabilization is not efficient in these cells, then their stalled replication forks should not restart when the block is relieved. To investigate this possibility, *HA-rad53 and HA-rad53 cdc45-2A* were subjected to replication fork restart analysis. To do so, cells were arrested in G1 phase with α-factor and released into YPD containing 200 mM HU. After 1h incubation with HU, cells were released into YPD and the DNA replication was analyzed after HU was removed. Western blot analysis indicated that Rad53 was hyper phosphorylated in wild type, *cdc45-2A, HA-rad53* and *HA-rad53 cdc45-2A* cells (**Figure 6-14.C**). Rad53 was immediately dephosphorylated after washing away of HU in wild type cells. However, it took longer for *cdc45-2A* cells since Rad53 was mostly phosphorylated 20 minutes after HU removal. It took even longer for *HA-rad53* cells with dephosphorylation starting approximately 1h after HU removal. Dephosphorylation took longest for *HA-rad53 cdc45-2A* cells, with Rad53 still fully hyper phosphorylated 1h after HU removal and mostly hyperphosphorylated after 100 minutes.

Flow cytometry analysis indicated that wild type cells continued DNA replication after the removal of HU and their bulk DNA amount doubled 40 minutes after HU removal (**Figure 6-14.B**). Although it has been before shown that *rad53Δ sml1Δ* cells cannot continue replication after 180 minutes fork stalling with HU (Lopes *et al*., 2001), slight replication was observed in *rad53Δ sml1Δ* after the removal of HU (**Figure 6-14.B**) but replication occurred very slowly. The replication progression of *cdc45-2A* cells was similar to wild type cells. On the other hand, *HA-rad53 and HA-rad53 cdc45-2A* cells restarted replication later than wild type cells and their bulk DNA amount was doubled 80 minutes following HU removal. In addition, some *HA-rad53* cells started a new cell cycle as the G1 population appeared 150 minutes after HU removal, but all *HA-rad53 cdc45-2A* cells were retained in G2 until the end of the experiment suggesting that *HA-rad53 cdc45-2A* cells might have more genomic instability to repair, delaying the entry into mitosis.

Although cells progressed to S-phase after HU removal in flow-cytometry analysis, the increase in bulk DNA could be due to the activation of new replication forks after HU removal from late or dormant origins. However, given that stalled but not stabilized forks could create genomic instability, it is expected that viability would decrease if forks are not stabilized. To understand if stalled forks were restarted properly and did not create any DNA damage upon fork stalling, the viability of the cells was investigated by colony formation assay. To do so, a known number of cells were collected at the 2 different time points – after α-factor arrest and 1 hour after HU removal - and spread onto YPD plates and incubated at 30°C for 48 hours. The colony formation assay indicated that wild type and *cdc45-2A* cells were completely rescued after HU treatment and were viable. Although *rad53Δ sml1Δ* cells replicated slightly after HU arrest, cells were not able to survive after fork stalling. 60% cell viability was observed in *HA-rad53* cells and this dropped to 38% in *HA-rad53 cdc45-2A* cells suggesting that the stalled forks collapsed more in *HA-rad53 cdc45-2A* cells, possibly due to inefficient stabilization (**Figure 6-14.D**).

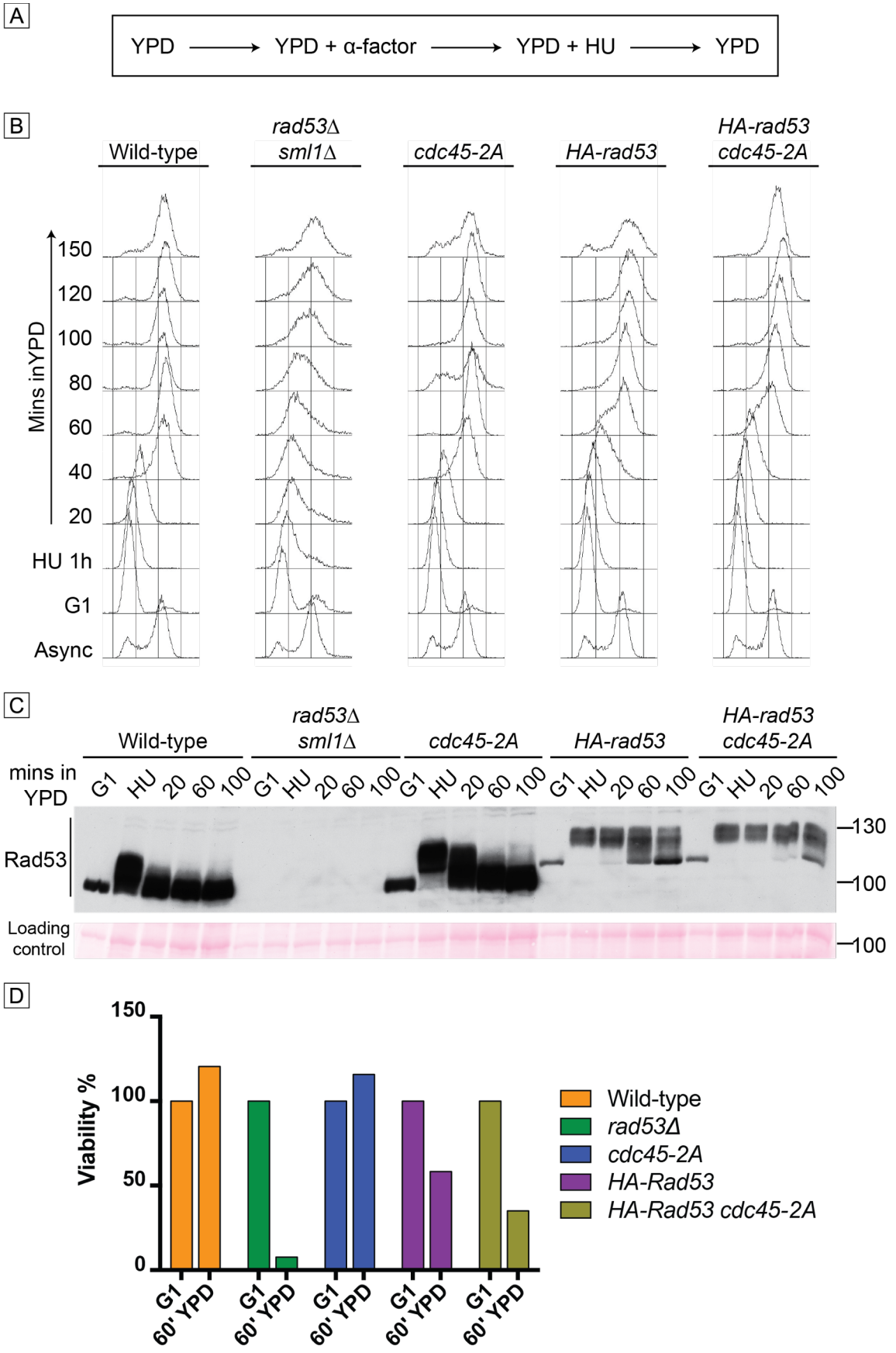


Figure 6-14 Fork restart analysis. A) Diagram showing experimental design. B) Flow cytometry analysis of the cells. C) western blot analysis of the cells. The blot was probed with anti-Rad53 antibody. D) Viability assay of the cells. 3X100 cells were spread on YPD plates at indicated time points.

6.3. Cdc45 does not regulate the phosphorylation of the other replication fork components by Rad53

As Cdc45 is required for Rad53 dependent phosphorylation of Sld3 (Chapter 5), and Cdc45 travels with replication forks during DNA replication (Moyer *et al*., 2006), it might also be required for the phosphorylation of replication fork components by Rad53. Considering that fewer *HA-rad53 cdc45-2A* cells survived after transient replication fork stalling (**Figure 6-14.D**), this could be due to the lack of Rad53-dependent phosphorylation of other replication fork factors. It has been shown before that Rad53 phosphorylates some replication factors upon DNA damage such as Mrc1, Sgs1, Srs2, Rrm3 and Exo1 (Alcasabas *et al*., 2001; Hegnauer *et al*., 2012; Liberi *et al*., 2000; Morin *et al*., 2008; Rossi *et al*., 2015). Given that Mrc1 also travels with the CMG complex (Gambus *et al*., 2006) and Rad53 phosphorylates Mrc1 upon DNA damage (Alcasabas *et al*., 2001), I hypothesized that Cdc45 could be required for the Rad53-dependent Mrc1 phosphorylation upon DNA damage. To do so, the phosphorylation status of Mrc1 was analyzed in *cdc45-2A* cells. Because it is not possible to resolve Rad53-dependent phosphorylation of Mrc1 with conventional SDS-Page analysis, the Phos-Tag-SDS-PAGE protocol was used (Chapter 3). In addition, as Mrc1 is both phosphorylated by Rad53 and Mec1 (Alcasabas *et al*., 2001), *rad53Δ* cells were used to distinguish Mec1- and Rad53-dependent phosphorylation. The mobility shift of Mrc1 in wildtype cells - but not in *rad53Δ* cells - indicated that Rad53 phosphorylated Mrc1 upon DNA damage (**Figure 6-15.C**). However, the mobility shift of Mrc1 in *cdc45-2A* showed a very similar pattern to wild type cells, suggesting that the flexible loop of Cdc45 is not required for Rad53-dependent Mrc1 phosphorylation.

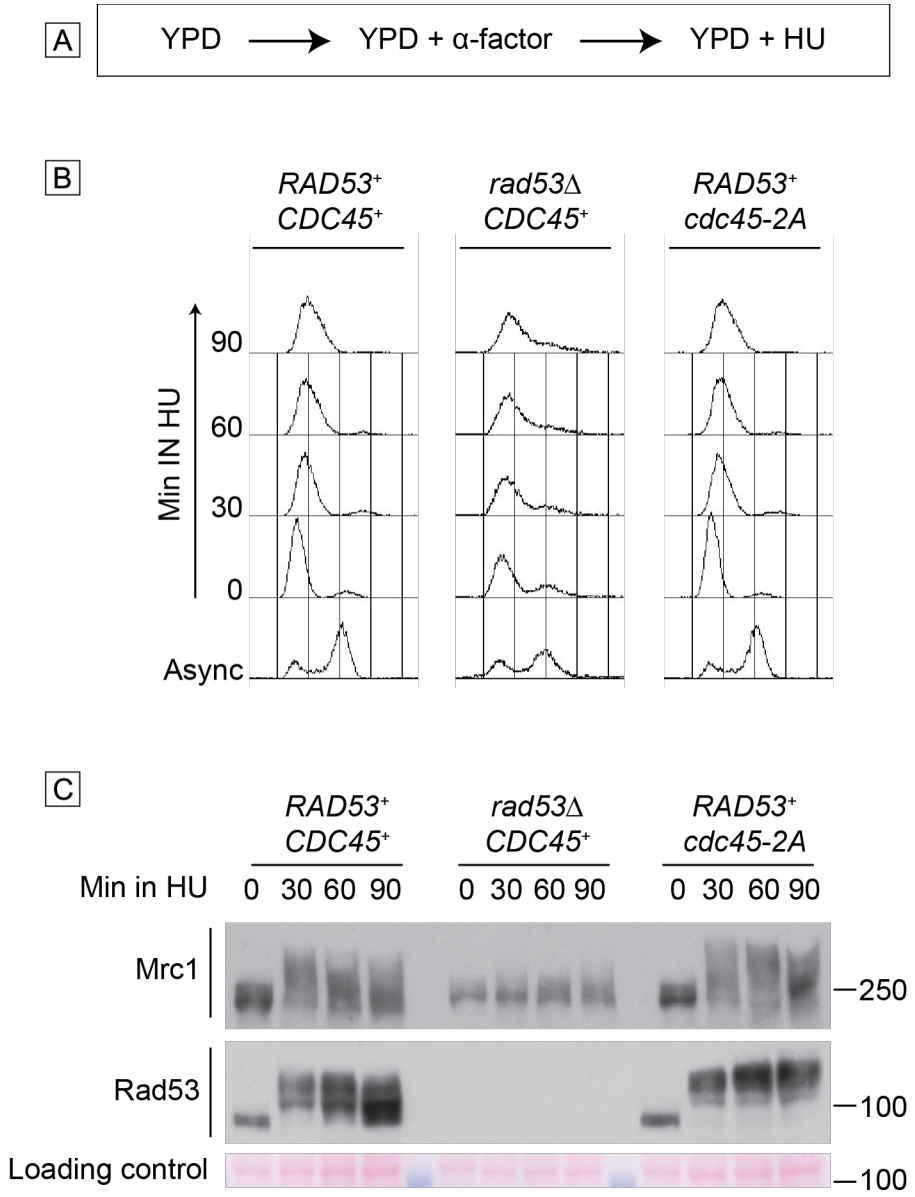


Figure 6-15 Western blot analysis of wild type and cdc45-2A cells. Cells were arrested in G1 and released into YPD media containing 200 mM HU. 13-myc tagged A) TOF1, B) DNA2 C) SGS1 D), SRS2 cells. Flow cytometry analysis performed to control G1 arrest. Blots were probed with anti-Rad53 and anti-Myc antibodies.

As Mrc1 is not the only known Rad53 substrate during replication fork stalling, I decided to check the phosphorylation status of other known Rad53 substrates on the replication fork in order to investigate whether the flexible loop of Cdc45 is required for their Rad53 dependent phosphorylation upon DNA damage. For this, the phosphorylation status of Rrm3, Sgs1, Srs2 and Exo1 were analyzed in a *cdc45-2A* background by using the Phos-Tag-SDS-PAGE

protocol. The mobility shift of Rrm3, Srs2, Exo1, Sgs1 showed no differences in *cdc45-2A* cells compared to wild type cells, suggesting that the flexible loop of Cdc45 is not required for the Rad53-dependent phosphorylation of Rrm3, Srs2, Exo1, Sgs1 (**Figure 6-16**).

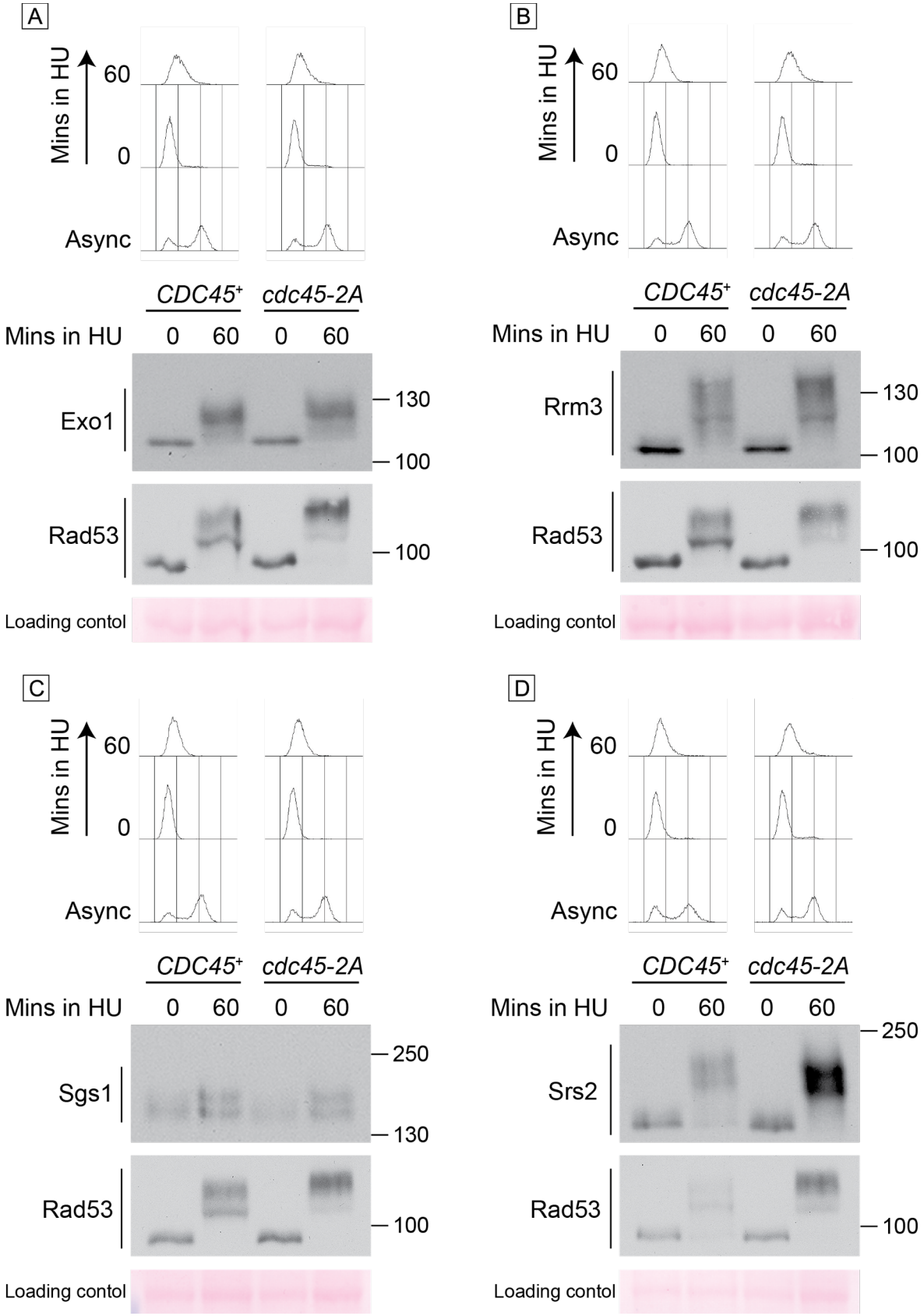


Figure 6-16 Western blot analysis wild-type and cdc45-2A cells. Cells were arrested in G1 and relaesed into YPD media contaning 200 mM HU. 13-myc tagged A) Exo1, B) Rrm3 C) SGS1 D), SRS2 cells. Flow cytometry analysis was performed to control G1 arrest. Blots was probed with anti-Rad53 and anti-Myc antibodies.

6.4. Discussion

Cdc45 is an essential protein which is required for replication initiation and travels with the DNA replication fork (Aparicio *et al*., 1999; Moyer *et al*., 2006). In Chapter 5, I have shown that the disordered loop of Cdc45 is required for Rad53-dependent Sld3 phosphorylation. Because Cdc45 also travels with the replication fork, I hypothesised that the disordered loop of Cdc45 might be a binding site for Rad53 upon DNA damage in order to stabilize the stalled replication fork. I have shown in this chapter that the flexible loop of Cdc45 has a role in replication fork stabilisation.

First, I hypothesized that if the flexible loop of Cdc45 is required for the stabilisation of replication fork by Rad53 upon DNA damage, mutating this loop would fail to stabilize the stalled replication forks and would cause cell death. However, the *cdc45-2A* cells did not show any growth defect on DNA damaging drug-containing plates, suggesting that this loop on its own is not sufficient for the stabilisation of stalled forks and perhaps other regulatory mechanisms compensate for the lack of Cdc45-dependent Rad53 recruitment (**Figure 6-2**).

Although the flexible loop of Cdc45 is a possible binding site for Rad53, Rad53 might also bind to the other factors on the stalled replication fork. To rule out this possibility, I hypothesised that decreasing the local concentration of Rad53 around the replication forks would result in inefficient replication fork stabilization and eliminating a possible binding site directly by cdc45-2A would result in even fewer stabilized forks upon stalling. The checkpoint mediators are assumed to increase the local concentration of active Rad53 around the stalled or damaged replication forks (Hustedt *et al*., 2013). To decrease the amount of Rad53 around the stalled replication forks, I used different checkpoint mediator knockouts (*mrc1Δ, sgs1Δ, ddc1Δ, mec3Δ, rad17Δ and rad9Δ*). However, cdc45-2A did not exacerbate the growth defect of none of the tested knockout cells compared to wild type Cdc45 on drug plates, possibly as removal

of single checkpoint mediators was not enough to decrease the local concentration of Rad53 (**Figure 6-3,Figure 6-4**, **Figure 6-5 and Figure 6-6**). To decrease the local Rad53 concentration even more, I decided to use double checkpoint mediator-knockouts. However, double mutants (*ddc1Δ rad9Δ, ddc1Δ mrc1Δ, ddc1Δ sgs1Δ*) that were tested showed limited growth on drug plates, possibly due to the inefficient checkpoint activation (**Figure 6-7, Figure 6-8 and and Figure 6-9**).

Because decreasing the local concentration of Rad53 by removing mediators did not decrease the fitness of *cdc45-2A* cells on drug plates, I decided to decrease the total Rad53 amount in the cell. Although, both C- (rad53-HA) and N- (HA-rad53) terminal HA tagging decreased the expression of Rad53, the expression of HA-rad53 was higher than rad53-HA (**Figure 6-10 and Figure 6-12**). The growth analysis of *rad53-HA cdc45-2A* cells did not show significant any difference compared to *rad53-HA* on HU plates, however *mrc1Δ rad53-HA cdc45-2A* cells were more sensitive to low concentrations of HU compared to *mrc1Δ rad53-HA* (**Figure 6-11**). Interestingly, *HA-rad53 cdc45-2A* cells were almost dead upon exposure to high concentrations of HU whereas *HA-rad53* showed minor growth defect (**Figure 6-13**). These results suggest that either decreased levels of Rad53 is synthetic lethal with *cdc45-2A* or that HA-Rad53 is hypomorphic and shows synthetic lethality with cdc45-2A upon DNA damage.

It could be hypothesized that if replication fork stabilization is abrogated in *HA-rad53 cdc45-2A* cells, then stalled replication fork cannot restart. To test this, I decided to perform replication fork restart analysis (**Figure 6-14**). However, some experimental difficulties did not allow me to establish a complete answer. The main problem of this analysis was that the amount of DNA in *rad53Δ sml1Δ* cells increased after the removal of HU. It is possible that not all origins were fired during the 60 minutes of HU treatment, and that the remaining origins were fired after removal of HU which resulted in DNA replication.

However, that experiment still provided some useful information. Dephosphorylation of Rad53 took longer in *HA-rad53 cdc45-2A* cells compared to *HA-rad53* after the removal of HU (**Figure 6-14.C**). This could be explained by two ways. Either more origins are fired – possibly late origins - in *HA-rad53 cdc45-2A* cells than in *HA-rad53* cells, which increase the number of fork collapse or because of inefficient stabilization, more replication forks collapsed in *HA-rad53 cdc45*-2A which took a longer time to repair. However, it is important to note that these explanations are not mutually exclusive. It is possible that inefficient fork stabilization in *HA-rad53 cdc45-2A* cells required more Rad53 around the collapsed forks to repair, leaving less Rad53 for inhibition of origin firing and other responses. It is possible to rule out whether the difference in the viability that was observered between *HA-rad53 cdc45-2A* and *HA-rad53* cells is due to more origin firing. Given that *sld3-A dbf4-A* alleles in which Rad53-dependent phosphorylation sites were replaced with alanine can fire late origins upon replication fork stalling (Zegerman & Diffley, 2010), these alleles could be introduced to *HA-rad53 cdc45-2A and HA-rad53* cells in order to fire all origins upon replication fork stalling. Because the number of active forks would be very similar in this condition for both cells, the requirement of Cdc45 could be tested. If *HA-rad53 cdc45-2A sld3-A dbf4-A* cells showed less viability than *HA-rad53 sld3-A dbf4-A* cells, then the requirement of the disordered loop of Cdc45 would be supported.

Although, the growth assay (**Figure 6-13**) and the viability assay (**Figure 6-14.D**) indicate that the flexible loop of Cdc45 has a role in the stabilisation of the replication fork, they do not reveal its function. Two different hypotheses could explain its role. Either the Rad53 dependent-phosphorylation of Cdc45 (**Figure-5.16**) has a function for replication fork stabilisation, or Rad53 binds on the flexible loop of Cdc45 for the phosphorylation of other replication fork factors. Given that Cdc45 is located on the Mcm2-Mcm5 gate (Yuan *et al*., 2016), the phosphorylation of Cdc45 might prevent the escape of ssDNA from Mcm2-Mcm5 gate upon fork stalling. On the other hand, it is known that some replication factors are phosphorylated by Rad53 upon DNA damage such as Mrc1, Exo1, Rrm3, Srs2 and Sgs1. I hypothesized that the flexible loop of Cdc45 could be required for the phosphorylation of these factors. However,

cdc45-2A did not prevent the Rad53 dependent phosphorylation of Mrc1, Exo1, Rrm3, Srs2 and Sgs1 upon fork stall (**Figure 6-15** and **Figure 6-16**). However, because Rad53 substrates on the replication fork have not been fully characterized yet, this result does not necessarily mean that Cdc45 is not required for the phosphorylation of stalled fork components. It could be very interesting explore all Rad53 targets and test whether the flexible loop of Cdc45 is required for their phosphorylation. The binding of Rad53 on the flexible loop of Cdc45 could still be tested by other biochemical techniques such as chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-Seq). If less DNA was enriched after Rad53 immunoprecipitation in *cdc45-2A* cells compare to wild type cells upon replication fork stalling, it would provide evidence for the binding of Rad53 on the flexible loop of Cdc45 on the replication fork.

Given that there is no consensus sequence for Chk1 binding (Rad53 functional homolog in humans), it is difficult to address Chk1 targets in human. However, considering the conservation of the flexible loop of Cdc45 among eukaryotes, it could be possible to specifically investigate Chk1 targets on the replication fork. Understanding the mechanisms of fork stabilization could have potential implications such as in the treatment of cancers. As drug resistance is one of the biggest problems facing cancer treatment, preventing the stabilization of the replication fork could be a potential target. Inhibition of fork stabilization by a small molecule could increase the efficacy of chemotherapeutic drugs which target DNA replication - such as methotrexate or 5-fluorouracil – due to increased genomic instability, and could lead to cell death before the drug resistance occurs.

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