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Damage-dependent Inhibition of Late Origin Activation in *Saccharomyces cerevisiae*

by

Jaime Lopez Mosqueda

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

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by

Jaime Lopez Mosqueda

Esta tesis está dedicada a mi madre y mi padre,

quienes apoyan sin reservas y sin duda todos

mis esfuerzos académicos, y me inculcaron

sus valores.

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CONTRIBUTIONS TO PRESENTED WORK

All work described in this thesis was performed under the direct supervision and guidance of Dr. David Toczyski. Additional contributions to specific chapters will be described below.

Chapter 3 was published as Lopez-Mosqueda J, Maas N, Wolschlegel J, DeFazio Eli LG, Toczyski DP (2010). Damage-dependent Phosphorylation of Sld3 is important to Block Late Origin Firing. Nature . The manuscript is reproduced here in accordance with the policies of the Nature Publishing Group. I performed most of the experiments presented in the figures as well as all the quantifications described in the text. I also prepared all the figures and wrote the manuscript together with David Toczyski. Nancy Maas, a former lab technician, spent considerable effort in building yeast strains and in helping me with experiments. Lisa De Fazio Eli identified Sld3 as a possible Rad53 substrate in her laborious Western blot screen. Zophonia Johnsson and James Wolschlegel used mass spectrometry to identify all the damage-induced phosphorylation sites on Sld3 and Dbf4.

Chapter 4 is a manuscript that will be submitted for publication in the journal Cell Cycle. This manuscript reflects a broader perspective to the work completed in Chapter 3 but was not referenced due to space constraints.

Chapter 5 was published as Lopez-Mosqueda J, Vidanes G. and Toczyski DP. The manuscript is reproduced here in accordance with the policies of the journal Cell Cycle. I performed the experiments presented in the figure, which was a proof-of-principle experiment stemming from the dissertation project of Genevieve Vidanes. Genevieve Vidanes and David Toczyski wrote the manuscript.

Damage-dependent Inhibition of Late Origin Activation in *Saccharomyces cerevisiae*

By Jaime Lopez Mosqueda

ABSTRACT

Origins of replication are activated throughout the S phase of the cell cycle such that some origins fire early and others fire late to ensure that each chromosome is completely replicated in a timely fashion. However, in response to DNA damage or replication fork stalling, eukaryotic cells block activation of unfired origins. Human cells derived from patients with ataxia telangiectasia are deficient in this process due to the lack of a functional ataxia telangiectasia mutated (ATM) kinase and elicit radioresistant DNA synthesis (Larner et al., 1994; Painter and Young, 1980; Young and Painter, 1989) after g-irradiation (Young and Painter, 1989). This effect is conserved in budding yeast, as yeast cells lacking the related kinase Mec1 (ATM and Rad3-related (ATR in humans)) also fail to inhibit DNA synthesis in the presence of DNA damage (Paulovich and Hartwell, 1995). This intra-S-phase checkpoint actively regulates DNA synthesis by inhibiting the firing of late replicating origins, and this inhibition requires both Mec1 and the downstream checkpoint kinase Rad53 (Chk2 in humans) (Santocanale and Diffley, 1998; Shirahige et al., 1998). However, the Rad53 substrate(s) whose phosphorylation is required to mediate this function has remained unknown. Here we show that the replication initiation protein Sld3 is phosphorylated

by Rad53, and that this phosphorylation, along with phosphorylation of the Cdc7 kinase regulatory subunit Dbf4, blocks late origin firing in *Saccharomyces cerevisiae*. Upon exposure to DNA damaging agents, cells expressing non-phosphorylatable alleles of SLD3 and DBF4 (*SLD3-m25* and *dbf4-m25*, respectively) proceed through the S phase faster than wild-type cells by inappropriately firing late origins of replication. *SLD3-m25 dbf4-m25* cells grow poorly in the presence of the replication inhibitor hydroxyurea and accumulate multiple Rad52 foci. Moreover, *SLD3-m25 dbf4-m25* cells are delayed in recovering from transient blocks to replication and subsequently arrest at the DNA damage checkpoint. These data indicate that the intra-S-phase checkpoint functions to block late origin firing in adverse conditions to prevent genomic instability and maximize cell survival.

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Introduction

In order for eukaryotic cells to divide they must first replicate their entire genomes every cell-cycle during the S-phase. This process is highly regulated to facilitate the precise and timely duplication of each chromosome while at the same time ensuring that no region on any chromosome undergoes re-replication. The regulatory mechanisms that control DNA replication are redundant and failure to properly regulate this process can result in aberrant chromosomal structures that activate the DNA damage response and can contribute to genomic instability.

In order to achieve the timely replication of the entire genome, DNA replication initiates from hundreds of distinct loci, known as origins of replication. These origins are located throughout each chromosome and at the onset of each S-phase, initiate DNA replication in a temporal program such that some origins are activated early while others are activated late. The underlying mechanism that controls the temporal program of origin activation remains unknown. It is clear, however, that origins of replication fire throughout the entire duration of S-phase.

When replication forks stall or encounter lesions, the DNA damage response is activated and slows S-phase progression. This response involves a signaling cascade that relies on phosphorylation to relay the signal of stalled replication forks or double strand breaks (DSB). Two important functions of the DNA damage checkpoint in S-phase are the stabilization of stalled replication forks and the inhibition of late and/or dormant origins of replication. The inhibition of late origin firing is what effectively slows S-phase progression as chromosomes will now be entirely replicated from early origins.

While the mechanism responsible for the stabilization of stalled forks remains a mystery, we now understand the molecular mechanism responsible to the inhibition of late origins of replication. The research work I performed during my doctoral training in the Toczyski lab, combined with the work from John Diffley and colleagues, uncovered the process by which late origins are maintained in a pre-RC state when replication forks stall or encounter lesions. This dissertation thesis describes my findings.

CHAPTER 1

DNA Replication

Origins

DNA replication is best understood in the model organism *Saccharomyces cerevisiae,* as most of discoveries in the DNA replication field have been made utilizing this model system. However, early observations pertaining to DNA replication were first made in simpler model systems such as prokaryotes. It was first observed in bacteria and viruses that short stretches of DNA sequences serve as replicator sequences. Moreover, initiator proteins bound to these so-called replicator sequences and these DNA-protein complexes were sufficient for promoting the assembly of DNA polymerases (Stillman, 1994). A major obstacle in the field of DNA replication was removed with the discovery of the *Autonomously Replicating Sequence* elements (ARS elements; Figure 1) (Stinchcomb et al., 1979). The evidence to prove that ARS element served as binding sites for the assembly of replication machinery was predicted to soon follow (Zakian et al., 1979). ARS elements were cloned into plasmids with selectable markers and were stably maintained in yeast. Later, two-dimensional gel electrophoresis of the ARS-containing plasmids showed bubble and y-arc structures originated from the ARS element (Brewer and Fangman, 1987; Huberman et al., 1987). Subsequent sequence analysis of ARS elements revealed an A/T rich 11bp consensus sequence: (ACS) 5'-(A/T)TTTAT (A/G)TTT(A/T-3')(Broach et al., 1983). The identification of an initiator sequence paved the road to the identification of initiator binding proteins.

The Origin Recognition Complex

The origin recognition complex (ORC) is a six-subunit complex that is highly conserved throughout evolution. The best understood ORC activity is the ability to bind,

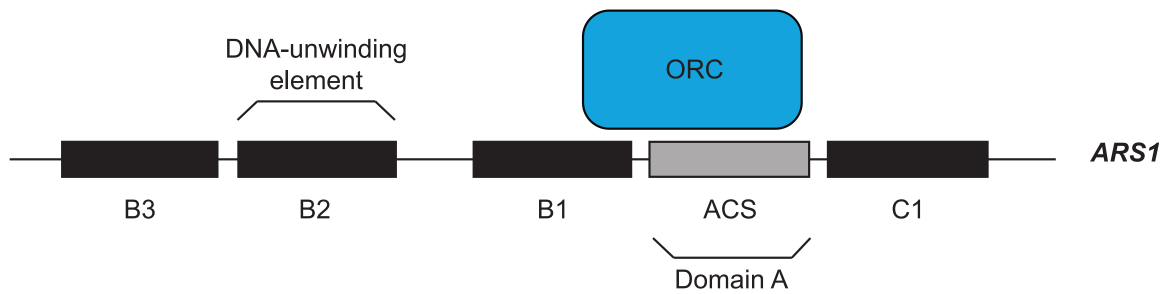


Figure 1. **Schematic illustration of an Autonomous Replication Sequence and regulatory elements**. The ORC complex, shown in blue, binds to the ACS domain and to the B1 regulatory unit.

directly, to origins of replication via the ACS and B1 elements and this binding is an absolute requirement for subsequent initiation of DNA replication at eukaryotic origins. Of the six ORC components, the largest five subunits (ORC1-5) are required to bind DNA. While Orc6 does not directly make contact with DNA and is not required for ORC binding, this subunit remains essential for DNA replication and viability (Li and Herskowitz, 1993). In addition, ORC binding to the ACS requires ATP and Orc1 is endowed with the ability to bind and hydrolyze ATP (Austin et al., 1999; Bell and Stillman, 1992). While the ATP binding of Orc1 is essential for ORC binding to DNA, it must be noted that ATP hydrolysis is not an absolute requirement for ORC binding to DNA (Chesnokov et al., 2001; Klemm et al., 1997). *In vitro* work has unequivocally shown that ORC can bind to DNA, in an ARS-dependent manner, while in the presence of non-hydrolysable ATP (ATP-γS). Interestingly, ORC protein levels or function is not cell-cycle regulated in the budding yeast. ORC has been found to bind constitutively to origins of replication *in vivo* throughout the cell-cycle (Aparicio et al., 1997; Santocanale and Diffley, 1996; Tanaka et al., 1997).

CDC6

Origin bound ORC sets the stage for the subsequent loading of other replicator proteins, namely Cdc6. Initially identified in the screen for cell division cycle mutants (Hartwell et al., 1973), Cdc6 is member of the AAA+ ATPase family. Cdc6, like Orc1 binds and hydrolyses ATP. Genetic evidence suggests that ATP hydrolysis is necessary for its association with ORC at origins and that Cdc6 functions in a step after ORC but before MCM loading (Aparicio et al., 1997). Thus the critical role of Cdc6 is the ability

to recruit the minichromosome maintenance complex (MCM2-7). Unlike ORC, Cdc6 protein levels are cell cycle regulated. As cells enter S-phase, Cdc6 is ubiquitylated by the SCF^{Cdc4} E3 ligase and subsequently targeted for proteosome-mediated destruction (Drury et al., 1997; Perkins et al., 2001; Piatti et al., 1996). However, in order for Cdc6 to be recognized by the SCF, it must first be phosphorylated by Clb5-CDK (Elsasser et al., 1999). Several CDK consensus sites are located in the N-terminus of Cdc6 and deletion of the N-terminus stabilizes Cdc6 in S-phase confirming the CDK requirement for destruction (Calzada et al., 2000; Drury et al., 2000). Although over-expression of Cdc6 (or the stabilized mutant form) is not sufficient to induce re-replication, recent studies have unequivocally shown that there are multiple redundant pathways that prevent re-replication in budding yeast (Nguyen et al., 2001).

Cdt1

Cdt1 is the last know component of the pre-RCs. First identified in the fission yeast, *Schizosaccharomyces pombe,* Cdt1 is conserved from yeast to man (Hofmann and Beach, 1994). Budding yeast Cdt1 interacts with both Cdc6 and MCMs. From these interactions, Cdt1 was thought to function in the recruitment of the MCMs by forming a bridge between Cdc6 and the MCMs (Chen and Bell, 2011; Chen et al., 2007a). However, recently John Diffley and colleagues showed, for the first time, that ORC and Cdc6 load Cdt1-MCMs heptamers into pre-RCs as head-to-head double hexamers. The requirement of Cdt1 in the loading of the MCMs could not be addressed as Cdt1 was found to be an integral component of the MCMs (Remus et al., 2009). Therefore, more work will be required to address the contribution of Cdt1 to MCM loading.

Replication Licensing

Initiation of DNA replication is a two-step process. In the first step, replication components are assembled on origins of replication (also known as licensing). In the second step, during S-phase, origins of replication are activated. Interestingly, in the G1-phase, assembled origins of replication cannot be activated, while in S-phase, origins of replication cannot be assembled. This begs the obvious question of what regulates the mutually exclusive steps of origin assembly and activation. Early experiments performed in budding yeast showed that an S-phase specific activity allowed for the activation of origins but concomitantly inhibit their assembly. The converse was also true. Extracts from G1 arrested cells lacked this activity and, therefore, the extracts were competent for the assembly of origins but incompetent for their activation. We now understand that S-CDK is the activity responsible for activating origins. Origins of replication are assembled in a low CDK state, immediately proceeding anaphase. The complexes of replication proteins that assemble in G1 are collectively referred to as the pre-Recognition complex (pre-RC). Pre-RC assembly includes the stepwise recruitment of ORC, Cdc6, Cdt1 and the MCMS to every ARS. During the G1/S transition, clb5-CDK (the major S-phase CDK) triggers the transformation of pre-RCs to the pre-Initiation complex (pre-IC). The first proteins identified to load to pre-RC are Sld3 (*S*ynthetic *l*ethal with *dpb11-1*), Cdc45, Sld2, Dpb11, the GINS (Sld5, Psf1; *P*artner of *S*ld5-*1*: Psf2, Psf3) as well as the DNA polymerases ε and α.

The MCM2-7 Protein Complex

The minichromosome maintenance complex (MCMs) was identified in several genetic screens. One of these screens was designed to identify mutants that failed to maintain plasmids (Maine et al., 1984). Since then, the six-subunit complex, Mcm2-7, has long been suspected to function as the DNA replicative helicase that unwinds double stranded DNA. However, direct evidence for this precise role of the MCMs is lacking as purified MCM2-7 complex has undetectable helicase activity *in vitro.* Interestingly, purified Mcm4/6/7 from *S. pombe*, has limited helicase activity but no detectable ATPase activity. A complex containing all six MCM subunits contains robust ATPase activity and each subunit has a distinct protein structure that is highly conserved among all eukaryotes. Each subunit contains a conserved ATPase domain at the C-terminus and varies in structure among individual subunits within the N-terminus.

Mcm2 and Mcm3 contain nuclear localization signals (NLS) in their N-termini. Cell-cycle localization analysis has shown that the MCMs are nuclear during the G1 and S-phase and are largely cytoplasmic during mitosis. MCM protein complexes that are not loaded unto pre-RCs are excluded from the nucleus during S and G2/mitosis. In budding yeast, nuclear export of the MCMs is an active process that is promoted by CDK (Labib et al., 1999; Nguyen et al., 2001) and exportation occurs as early as the G1/S transition. It is important to note that chromatin loaded MCM is protected from nuclear exportation probably due, in part, to the physical encirclement of double stranded DNA. In support of this hypothesis, loaded MCMs can be exported after the completion of S-phase.

For years, the lack of detectable helicase activity from purified MCM complex, from any species, was seen as a conundrum in the DNA replication field. However, this

apparent problem was finally resolved. *In vitro* helicase activity has been detected in MCMs isolated from *Drosophila melanogaster.* In this purification, however, MCMs co-purified with Cdc45 and the GINS complex; a complex coined CMG for Cdc45-MCMs-GINS (Moyer et al., 2006). Surprisingly, the association of Cdc45 and GINS components with the MCMs, but not the association of PCNA or DNA polymerases, was sufficient to displace 40 base pairs of DNA in an *in vitro* reaction.

MCM10

The *MCM10/DNA43* allele has been shown to have an essential role in DNA replication. Two genetic screens identified the same allele: Mcm10 was identified in the same screen that identified the MCM2-7 complex (Maine et al., 1984), while *DNA43* was found in a temperature sensitive screen for mutants with defects in DNA replication (Solomon et al., 1992). Although an essential gene, its precise role in DNA replication remains obscure. A number of studies, however, have provided clues to support a role in DNA replication. For example, the *mcm10-1* allele revealed that Mcm10 is required for the efficient initiation of DNA replication (Merchant et al., 1997). In addition, Mcm10 activity is also required throughout the duration of S-phase and is especially important in the recovery from a hydroxyurea arrest (Kawasaki et al., 2000). A variety of mutant *MCM10* alleles display synthetic interactions with alleles of DNA polymerase ε and δ, *MCM7* and *CDC45*, further bolstering the hypothesis that Mcm10 functions in DNA replication. Despite all the genetic evidence implicating Mcm10 with a role in initiation of DNA replication, it remains unknown when Mcm10 associates with chromatin and the MCMs.

CDC45

CDC45 was identified in the original cell division cycle screen and has emerged as one of the most critical factors in the initiation and elongation of DNA replication. In the budding yeast, Cdc45 physically interacts with ORC, the MCMs, GINS, RPA, as well as with DNA polymerase α and ε. Chromatin immuno-precipitation analysis has shown that Cdc45 associates at origins of replication before the DNA unwinding step. Moreover Cdc45 activity is required for subsequent polymerase loading onto origins (Aparicio et al., 1999; Mimura and Takisawa, 1998). Complete inactivation of Cdc45 prior to DNA unwinding prevents the loading of polymerases and thus prevents the initiation of DNA replication. Similarly, complete Cdc45 inactivation after initiation of DNA replication results in fork stalling and interestingly, restoring Cdc45 activity allows the resumption of fork progression. Like the MCMs, Cdc45 is a component of the replication fork machinery but unlike the MCMs, Cdc45 can be continuously reloaded on origins of replication. Lastly, cell-cycle analysis of Cdc45 loading correlates well with the onset of S-CDK activation of origins. Although some reports claim that Cdc45 associates with early origins prior to S-CDK activity, it is clear that the bulk of Cdc45 is strongly chromatin associated after elevated S-CDK activity. The biological significance of Cdc45 association to early origins is currently unknown.

Dpb11

Dpb11 is an essential protein that functions in the initiation of DNA replication. It was originally identified as a multi-copy suppressor of DNA polymerase ε mutant

(Araki et al., 1995). The *dpb11-1* allele is synthetic lethal with other Polymerase ε subunits. Dpb11 associates with origins of replication and physically interacts with DNA polymerases suggesting a role in polymerase loading. Consistent with this role, cells expressing the temperature sensitive *dpb11-1* allele, at the non-permissive temperature, fail to initiate DNA synthesis. In addition to its role in DNA replication, recently, Dpb11 has been shown to be involved in the activation of the DNA damage response. Early work in budding yeast cells expressing *dbp11* mutants were checkpoint defects. Budding yeast cells with mutant *DPB11* alleles are sensitive to treatment with DNA damaging agents and fail to activate Rad53. For these reasons, Dpb11 has long been suspected to play a role in checkpoint activation. Recently, Dpb11 was shown to have a direct role in the activation of Mec1 kinase (Ohouo et al., 2010).

SLDs

In order to discover more precise roles for Dpb11 in DNA replication, Hiroyuki Araki and colleagues designed a genetic screen to identify genes that are synthetic lethal with the *dpb11-1* allele. Seven genes were identified and were called *SLD1-7* (Kamimura et al., 2001). *SLD1* is identical to *DPB3*, *SLD2* is identical to *DRC1*, *SLD3* was an uncharacterized open reading frame, *SLD4* is identical to *CDC45*, *SLD5* is identical to *CDC105*, *SLD6* is identical to *RAD53* and *SLD7* is an non-essential gene whose identity has not yet been revealed. Like Cdc45, the Sld proteins all appear to function at the initiation step of DNA replication. Of these proteins, Sld2 and Sld3 have emerged as the minimal S-CDK substrates. Clb5-CDK phosphorylates Sld2 at the N-terminus and Sld3 is phosphorylated at the C-terminus (Masumoto et al., 2002; Tak et al., 2006; Tanaka et

al., 2007; Zegerman and Diffley, 2010). Phosphorylated Sld2 and Sld3 interact with Dpb11 through its BRCT domains. The Sld2-Dpb11-Sld3 interaction is absolutely required to initiate DNA replication although the precise molecular mechanism remains to be elucidated.

In addition, Sld3 physically interacts with Cdc45 and their mutual association at origins of replication is required (Kamimura et al., 2001). Like Cdc45, Sld3 can also be detected at early origins of replication in G1 arrested cells and similarly, the function of this precocious recruitment at origins is unknown (Kamimura et al., 2001). However, unlike Cdc45, Sld3 does not move with replication fork machinery nor is not required for elongation of replication forks (Kanemaki and Labib, 2006). Thus, Sld3's essential function appears to be required in the initiation step and not the elongation step.

The GINS complex

The GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese) complex was independently identified in budding yeast by two different groups (Kanemaki et al., 2003; Takayama et al., 2003). GINS, a heterotetrameric protein complex consisting of Sld5, Psf1 (Partner of Sld5-1), Psf2, and Psf3 and functions in the initiation and elongation steps of DNA replication. The GINS complex is conserved in all eukaryotes and evidence suggests that it is a functional member of the replicative helicase. This evidence stems from the fact that Drosophila GINS complex co-purifies with Cdc45 and the MCMs. In addition, purified GINS-Cdc45-Mcm complex has significant helicase activity *in vitro*.

Two S-phase Kinases Activate Origins

The concerted actions of two S-phase kinases are required to trigger origin activation: cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). At the onset of S-phase, concomitant with an increase in clb5-CDK and DDK activities, pre-RCs are converted into pre-ICs. Pre-IC formation entails the sequential loading of Sld3, Cdc45, Sld2, Dpb11, the GINS complex, as well as the DNA polymerases ε and α. As previously mentioned above, S-CDK phosphorylates at least Sld2 and Sld3. While it is known that phosphorylation of these two proteins is essential for viability, we do not yet understand how these phosphorylations leads to loading of replication proteins and subsequent origin firing. Although Cdc45 is not a CDK substrate, we understand that Cdc45 becomes more stably associated with origins, which may, in part, be a result of Sld3 phosphorylation since Sld3 and Cdc45 physically interact.

The second S-phase kinase required for origin activation is Cdc7. Active Cdc7 kinase requires the regulatory subunit, Dbf4 and it is often referred to as DDK (Dbf4-dependent kinase). Dbf4 protein levels rise at the G1/S transition, peak during S-phase and sharply decrease after mitosis (Jackson et al., 1993) and Cdc7 activity mirrors cyclical Dbf4 expression. Unlike CDK, DDK activity is required throughout the entire duration of S-phase for timely DNA replication. Previous work has clearly established that DDK is required to fire each origin individually (Bousset and Diffley, 1998; Donaldson et al., 1998). This finding stems from experiments where a prolong S-phase ensues after conditional inactivation of either Dbf4 or Cdc7. Importantly, late origins of

replication remain in a pre-RC state, as judged by DNA footprinting, when Cdc7 is conditionally inactivated (Bousset and Diffley, 1998). The MCMs appear to be the most relevant DDK substrates. All the MCM subunits, except for Mcm5, are phosphorylated by DDK *in vitro*, suggesting that they may be phosphorylated by DDK *in vivo*. Of the MCMs, Mcm2, 4 and 6 are the best characterized DDK substrates but a clear functional role still remains to be discovered. It is certainly possible that DDK phosphorylation of the MCMs creates binding sites for GINS and Cdc45. Alternatively, DDK dependent phosphorylation of MCMs can potentially lead to structural changes that promote GINS and Cdc45 binding. Direct evidence for this hypothesis comes from the *mcm5-bob1* (bypass of block1) allele (Hardy et al., 1997). Interestingly, this mutation suppresses the lethality of *CDC7* or *DBF4* deletion. A single point mutation in *MCM5* (P83L) results in a modest structural change that perhaps resembles what normally happens with phosphorylated MCMs (Fletcher et al., 2003).

Chapter 2

The DNA Damage Response

DNA Damage Checkpoints

The DNA damage checkpoint is a genome surveillance mechanism that responds to DNA damage and perturbations to DNA replication. This mechanism coordinates cell-cycle progression with DNA repair and is critical for cell survival. The response to DNA damage was first identified in budding yeast with the realization that yeast cells arrest at G2/M after X-ray irradiation. This damage-induced arrest was dependent on the *RAD9* gene and delaying the onset of mitosis reduced the damage sensitivity of *rad9* mutants (Weinert T, Hartwell L 1988). These experiments led to the view that the DNA damage checkpoint system functioned to arrest cell-cycle progression to provide time for DNA repair to occur. In budding yeast, there are three main checkpoints: (1) the DNA damage checkpoint (2) the Replication checkpoint and (3) the intra-S-phase checkpoint. The main concepts underlying these molecular mechanisms in response to a DNA damage signal is a system of sensors that relay the damage signal to signal transducers which in turn, amplify the signal to numerous downstream proteins.

The DNA Damage Checkpoint

The key components of the DNA damage checkpoint pathway are the phosphoinositol-3-kinase-related kinase (PIKK) family members. Budding yeast have two PIKK proteins, Mec1 and Tel1, which are endowed with the function of both damage sensors and transducers. Mec1 kinase is the homolog of human ataxia-telangiectasia and Rad3-related (ATR) while Tel1 kinase is the homolog of human ataxia-telangiectasia mutated (ATM). These two kinases do not recognize DNA double strand breaks, per se, but are instead recruited to break sites independently. Double stranded breaks are rapidly

processed to give rise to single stranded DNA (ssDNA) providing a common substrate for recognition by the DNA damage system. For instance, Mec1 is recruited to breaks by Ddc2, the homolog of ATR interacting protein (ATRIP), which itself binds to RPA-coated ssDNA. The Mre11-Rad50-Xrs2 complex, on the other hand, recruits Tel1, independently, to ssDNA-dsDNA junctions. The PCNA-like Ddc1-Mec3-Rad17 complex is an additional damage sensor that influences the activation of Mec1 and Tel1. Exactly how Mec1 and Tel1 are activated has been an area of intense research. Recent work has shown that forced-colocalization of Mec1-Ddc2 and Ddc1-Mec3-Rad17 to tandem LacI arrays in the absence of physical DNA damage results in checkpoint activation (Bonilla et al., 2008). Interestingly, localization of Mec1-Ddc2 and Ddc1 alone (in a *mec3*Δ and *rad17*Δ background), is sufficient to activate Mec1 suggesting that either Ddc1 or an Ddc1-interacting protein influences Mec1 activation. Dpb11 interacts with Ddc1, both physically and genetically, and functions to stimulate Mec1 activity *in vitro* (Mordes et al., 2008b). *In vivo* results have shown that Dpb11 mediated Mec1 activation is cell-cycle regulated. Specifically, Dpb11 together with the 9-1-1 complex activates Mec1 in S-phase and mitosis. However, Dpb11 is dispensible for Mec1 activation in the G1 phase (Navadgi-Patil and Burgers, 2008). Once activated Mec1 and Tel1 preferentially phosphorylate serines and threonines preceding a glutamine amino acid on numerous substrates. One of the earliest substrates phosphorylated by Mec1 and Tel1 is histone H2A at serine 129 (Downs et al., 2000). Although the phosphorylation of histone H2A is not required for Mec1-dependent cell-cycle arrest, it is necessary for efficient repair of a DNA double strand break by non-homologous end joining.

In addition to phosphorylating downstream substrates, Mec1 and Tel1 also phosphorylate other kinases such as Chk1 and Rad53. In metazoans, Chk1 phosphorylation is required for the checkpoint-dependent inhibition of CDK. In budding yeast, however, attenuation of CDK activity in response to checkpoint activation is not required (Amon et al., 1992) (Sorger and Murray, 1992). Instead, phosphorylated Chk1 phosphorylates Pds1. The destruction of Pds1 by the APC/C ubiquitin ligase triggers chromosome segregation in anaphase. However, upon checkpoint activation, Chk1-mediated Pds1 phosphorylation inhibits its destruction and is the primary role of the checkpoint response (Sanchez et al., 1999). Damage-induce Pds1 stabilization is further enhanced by the Rad53-dependent inhibition of Cdc20 (APC/C) recruitment (Agarwal et al., 2003). Lastly, both Chk1 and Rad53 inhibit late mitotic events by interfering with both early and late nucleolar Cdc14 releases (FEAR and MEN pathways) (Liang and Wang, 2007). The release of the phosphatase, Cdc14, from the nucleolus marks the end of mitosis as Cdc14 dephosphorylates key CDK substrates (Visintin et al., 1998).

The Mec1 and Tel1-dependent activation of Rad53 require the phosphorylation of the scaffold Rad9. Phosphorylated Rad9, will in turn, multimerized using its tandem BRCT domains and binds to phosphorylated histone H2A (Soulier and Lowndes, 1999). In addition, Rad9 contains tandem Tudor domains that are utilized to interact with methylated histones (Grenon et al., 2007). Once recruited to chromatin, Rad9 can interact with Rad53 via two forkhead-associated (FHA) domains on Rad53 (Schwartz et al., 2002). Interestingly, *rad9* mutants are sensitive to DNA damage and cannot activate Rad53. However, in *rad9*Δ cells, both the sensitivity to DNA damage and Rad53

activation can be rescued by directly fusing Ddc2 to Rad53 (Ddc2-Rad53) arguing that Rad9 functions, in part, to recruit Mec1 to Rad53 (Lee et al., 2004).

Activated Rad53 phosphorylates Dun1 kinase on its T-loop, thereby activating Dun1 (Chen et al., 2007b). In addition, Rad53 can physically interact to Dun1 through FHA domains on Dun1 (Bashkirov et al., 2003). Once Dun1 activation is achieved, Dun1 phosphorylates several downstream targets. Sml1, a ribonucleotide reductase (RNR) inhibitor, is the best-characterized Dun1 target.

The Replication and Intra-S-phase checkpoints

When replication forks stall or encounter lesions, the replication checkpoint is activated to arrest cells in mitosis and allow time for repair and the completion of DNA replication. Like the DNA damage checkpoint mentioned above, the replication checkpoint also depends on Mec1 and Rad53 kinases. However, there are some noteworthy differences between the two systems. The signal that recruits Mec1 to replication forks is the accumulation of replication protein A (RPA) to single stranded DNA. Paradoxically, RPA is a constitutive component of the replication fork machinery and functions to keep unwound DNA from re-annealing. An RPA threshold, therefore, appears to be the key activating damage signal during S-phase. It is estimated that RPA normally coats approximately 300 base pairs (bp) of DNA during an unperturbed S-phase. In contrast, during replication fork stalling, for instance, RPA has been detected to coat up to 3 kilobases (Kb) of single stranded DNA and this accumulation highly correlates with checkpoint activation. Once recruited to replication forks, Mec1 is primed to phosphorylate the replication specific adaptor Mrc1. Mrc1 is a constitutive

component of the replisome and functions during elongation to couple polymerases with the MCMs. Mrc1 is not an essential gene, in budding yeast, but *mrc1*Δ mutants are checkpoint deficient and extremely sensitive to DNA damaging agents. The adaptor functions of Mrc1 are analogous to that of Rad9, in that they both serve to recruit the effector kinase Rad53. At this stage, Rad53 auto-phosphorylates and can subsequently phosphorylate proteins to stabilize replication forks and delay S-phase progression. In the absence of Rad53, replication forks slow the rate of DNA synthesis with rates that are undistinguishable to those of wild-type cells. Thus, DNA damage during S-phase slows the rate of replication fork progression in a checkpoint-independent manner. However, the DNA damage induced S-phase delay is entirely due to the intra-S-phase checkpoint. Once Rad53 is activated, specifically in S-phase, Rad53 serves two important functions: stabilization of replication forks and inhibition of late origins of replication. Therefore, it is the inhibition of late origins of replication that significantly delays S-phase progression. Consequently, chromosomes duplication will be exclusively from those early origins that were activated prior to intra-S-phase checkpoint activation since late origins are inhibited by Rad53 and will not be utilized until the subsequent cell-cycle provided no intra-S-phase checkpoint activation occurs. How Rad53 stabilizes stalled replication forks in currently unknown and an area of intensive research. Evidence for this function of Rad53 stems from what occurs to replication forks and their components in *rad53*Δ cells. Rad53 mutant cells are extremely sensitive to very low levels of DNA damaging agents and replication inhibitors. While wild-type cells efficiently recover from transient treatments with DNA damaging agents, *rad53*Δ cells cannot recover and elicit a dramatic viability loss. Chromatin immuno-precipitation (ChIP) of replication

fork components appears to be less abundant in checkpoint mutants compared to wild-type counterparts. In addition, 2D gel electrophoretic analyses of replication intermediates clearly reveal the presence of "cone-shape" signals at early replicating origins and are widely accepted as evidence of replication fork collapse (Lopes et al., 2001; Tercero and Diffley, 2001).

Chapter 3

Damage-dependent Phosphorylation of SLD3 is Important to Block Late Origin Activation

Origins of replication are activated throughout the S phase of the cell cycle such that some origins fire early and others fire late to ensure that each chromosome is completely replicated in a timely fashion. However, in response to DNA damage or replication fork stalling, eukaryotic cells block activation of unfired origins. Human cells derived from patients with ataxia telangiectasia are deficient in this process due to the lack of a functional ataxia telangiectasia mutated (ATM) kinase and elicit radioresistant DNA synthesis (Larner et al., 1994; Painter and Young, 1980; Young and Painter, 1989) after γ-irradiation (Young and Painter, 1989). This effect is conserved in budding yeast, as yeast cells lacking the related kinase Mec1 (ATM and Rad3-related (ATR in humans)) also fail to inhibit DNA synthesis in the presence of DNA damage (Paulovich and Hartwell, 1995). This intra-S-phase checkpoint actively regulates DNA synthesis by inhibiting the firing of late replicating origins, and this inhibition requires both Mec1 and the downstream checkpoint kinase Rad53 (Chk2 in humans) (Santocanale and Diffley, 1998; Shirahige et al., 1998). However, the Rad53 substrate(s) whose phosphorylation is required to mediate this function has remained unknown. Here we show that the replication initiation protein Sld3 is phosphorylated by Rad53, and that this phosphorylation, along with phosphorylation of the Cdc7 kinase regulatory subunit Dbf4, blocks late origin firing in *Saccharomyces cerevisiae*. Upon exposure to DNA damaging agents, cells expressing non-phosphorylatable alleles of SLD3 and DBF4 (*SLD3-m25* and *dbf4-m25*, respectively) proceed through the S phase faster than wild-type cells by inappropriately firing late origins of replication. *SLD3-m25 dbf4-m25* cells grow poorly in the presence of the replication inhibitor hydroxyurea and accumulate multiple Rad52 foci. Moreover, *SLD3-m25 dbf4-m25* cells are delayed in recovering from transient

blocks to replication and subsequently arrest at the DNA damage checkpoint. These data indicate that the intra-S-phase checkpoint functions to block late origin firing in adverse conditions to prevent genomic instability and maximize cell survival.

In a whole-genome screen for proteins whose electrophoretic mobility was altered by DNA damage (L.G.D.E. and D.P.T., unpublished results), we found that Sld3, an essential protein required for the initiation of DNA replication in budding yeast (Kamimura et al., 2001), becomes substantially modified in response to checkpoint activation. Sld3 was hyper-phosphorylated in cells treated with genotoxic drugs, but not in cells arrested at the spindle checkpoint with nocodazole (Fig. 1a, b). The Sld3 damage-induced phosphorylation depends on Mec1 and Rad53 and is compromised in mutants with reduced Rad53 activation (Fig. 1c). DNA-damage-induced Sld3 phosphorylation is not cell-cycle regulated, as cells blocked in G1 or mitosis and subsequently treated with 4-nitroquinoline 1-oxide (4NQO) or zeocin contained phosphorylated Sld3 (Fig. 1d). Origin firing requires both the phosphorylation of Sld3 by the S-phase cyclin-dependent kinase (CDK) (Tanaka et al., 2007; Zegerman and Diffley, 2007) and the activity of the Dbf4-dependent kinase (DDK) (Bousset and Diffley, 1998; Donaldson et al., 1998). The S-phase CDK phosphorylation of Sld3 allows binding to Dpb11 and promotes origin firing (Tanaka et al., 2007; Zegerman and Diffley, 2007). *In vitro* binding assays using recombinant full-length Sld3 and immobilized Dpb11 revealed that S-phase CDK phosphorylation of Sld3 is required to bind Dpb11, as previously seen (Tanaka et al., 2007; Zegerman and Diffley, 2007). However, subsequent Rad53 phosphorylation of S-phase-CDK-phosphorylated Sld3 eliminated binding to Dpb11 (Fig. 1e). This indicates that Sld3 is a Rad53 substrate and suggests that, by antagonizing Sld3 binding to Dpb11,

Rad53 can inhibit late origin firing.

To address this hypothesis directly, we constructed an unphosphorylatable *SLD3* allele. Using mass spectrometry, 28 phosphorylation sites were identified (Supplementary Table 1a). Twenty-five potential phosphorylation sites were mutated to generate the *SLD3-m25* allele. When *SLD3-m25* was introduced into cells, damage-induced Sld3 phosphorylation was not detected (Fig. 2a and Supplementary Fig. 2). *In vitro* kinase assays revealed that activated Rad53, but not a catalytic mutant (Rad53(K227A)), phosphorylated Sld3 strongly, but phosphorylated the Sld3-m25 mutant less efficiently (Fig. 2b). Additional mass spectrometry analysis of Sld3 phosphorylated *in vitro* by Rad53 identified nine phosphorylation sites, five of which were mutated in the *SLD3-m25* allele (Supplementary Table 1a).

Intra-S-phase checkpoint-defective mutants, such as *rad53*Δ, fail to prevent late origin firing in the presence of DNA damage and consequently proceed through the S phase faster than wild-type cells (Santocanale and Diffley, 1998; Shirahige et al., 1998). Cells expressing SLD3-m25 in the presence of methyl methanesulphonate (MMS) proceeded through the S phase faster than wild-type cells (Fig. 2c, d), although they progressed slower than *rad53*Δ cells. Like Sld3, Dbf4 functions to promote firing of all origins of replication and is phosphorylated in response to checkpoint activation in a Rad53-dependent manner (Duncker et al., 2002; Weinreich and Stillman, 1999) (Figs. 1b and 2a). Dbf4 is a periodically expressed activator of the Cdc7 kinase, which is known to

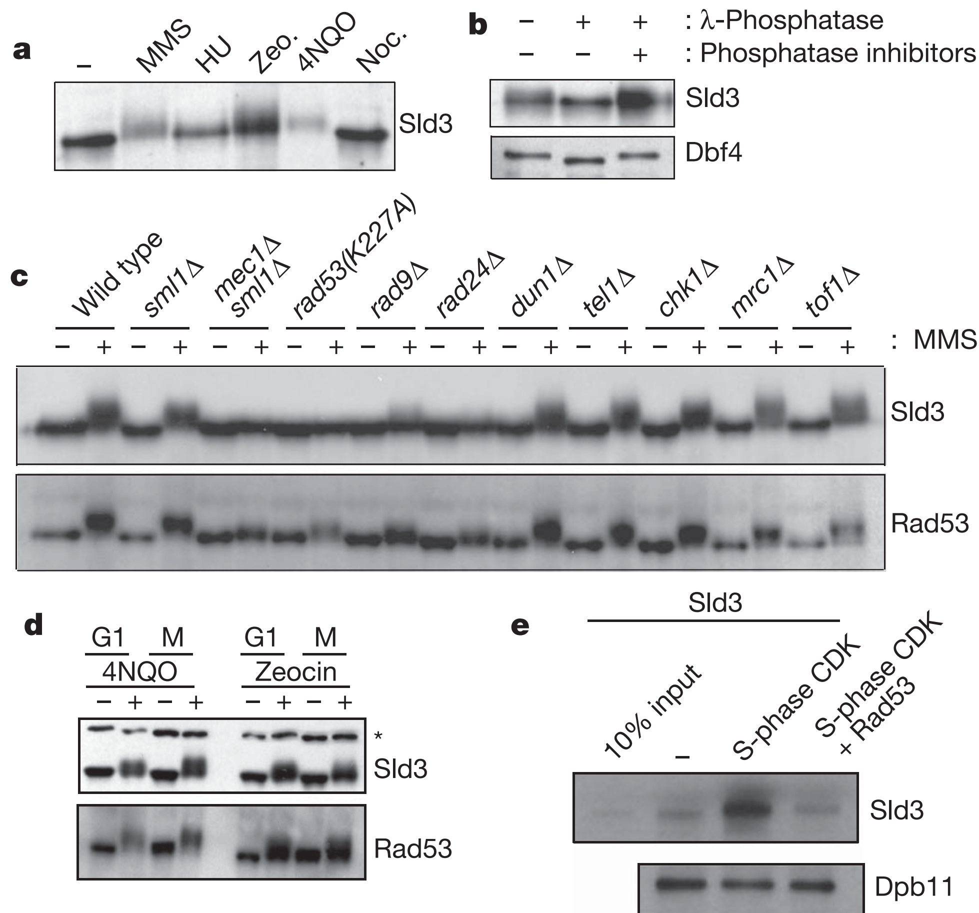


Figure 1. **Rad53-dependent phosphorylation of Sld3. a**, Immunoblotting of asynchronous cells expressing Sld3-3Flag in the absence (-) or presence of MMS (alkylating agent-0.05%), HU (ribonucleoside reductase inhibitor-200 mM), zeocin (radiation mimetic-200 mg/mL), 4NQO (UV-mimetic-2 mg/mL), or nocodazole (10 mg/mL) for 90 minutes. **b**, Phosphatase assays of Sld3-3Flag and Dbf4-TAP purified from MMS-treated cells. **c**, Immunoblot of checkpoint deletion mutants expressing Sld3-9myc. Samples were taken from cells arrested in G1 with a-factor and released in the presence or absence of MMS for 180 minutes. **d**, Immunoblot of cells expressing Sld3-9myc arrested in G1 with a-factor or metaphase with nocodazole for 120 minutes and then treated with either 4NQO or Zeocin, as in **a**, for 60 minutes. * Denotes a background band. **e**, *In vitro* binding assay. Recombinant Sld3-3Flag mock phosphorylated (-), Clb5-TAP CDK phosphorylated or Clb5-TAP CDK phosphorylated followed by Rad53 phosphorylation. 10% of the untreated Sld3-3Flag is shown as input. Immobilized Dpb11-TAP was used to pull down the differentially phosphorylated Sld3 species.

a

SLD3			SLD3-m25
sites mapped in vivo	*sites mutated*	*sites mapped in vitro*	*sites mapped in vitro*
		T14	
			T88
			T92
Y153	Y153		
Y154	Y154		
S233			
Y298			
		T310	
S370	S370		
T373	T373		
		S421	
S439			
			T445
Y446			
T451	T451		
S456	S456		
			S458
			S459
S463	S463		
S466	S466		
S467			
			S479
			S482
T487			
S493	S493	S493	
T495	T495		
S497	S497	S497	
S505	S505		
T507	T507		
		S514	S514
S521	S521	S521	
S534	S534	S534	
			T553
			T559
T582	T582		
S591	S591		
	T607		
	T609		
	S618	S618	
S622			
	T631		
	T638		
S639	S639		
T642	T642		
			S643
T644			
T646	T646		
			S653

b

DBF4
sites mutated
T5
T16
T18
S29
T30
T31
S40
T50
T51
S53
S59
S84
S92
T95
T105
T114
S124
T131
Y139
T171
T175
S235
S356
T473
S632

Supplemental Table 1a and b. **Damage-induced phosphorylation sites.** **a**, Identification of damage-induced Sld3 and Dbf4 phosphorylation sites *in vivo* and in vitro. Rad53 phosphorylation sites on recombinant Sld3 and Sld3-m25 by mass spectrometry. Sld3 sites T607, T609, T631 and T638 were not mapped. In **b**, sites in bold were mapped *in vivo*.

phosphorylate the replication helicases (MCMs) (Francis et al., 2009; Jiang et al., 1999; Lei et al., 1997; Sheu and Stillman, 2010). We reasoned that Dbf4 may also be inhibited by Rad53 to block late origin firing. Using a similar mass-spectrometry approach, six phosphorylation sites were identified on Dbf4 (Supplementary Table 1b). The six phosphorylation sites were mutated to generate the *dbf4-m6* allele; however, the Dbf4 damage-induced phosphorylation shift was intact when *dbf4-m6* cells were treated with MMS (data not shown). Previous studies have shown that a Dbf4 mutant lacking the non-essential amino terminus (1–109) is not phosphorylated in damage-treated cells (Gabrielse et al., 2006). For this reason, we mutated all serine and threonine residues in this non-essential domain of Dbf4 (except those matching the CDK consensus sequence), in addition to the six sites identified by mass spectrometry, to generate the *dbf4-m25* allele. Damage-induced Dbf4-m25 phosphorylation was reduced, but not eliminated (Fig. 2a), indicating that not all damage-induced phosphorylation sites were identified or that Dbf4 is also phosphorylated by another damage-responsive kinase(s).

Cells expressing *SLD3-m25*, *dbf4-m25* or both mutant alleles were viable and had no growth defects at any temperature tested (Supplementary Figs 3 and 4a). Cells expressing *dbf4-m25* alone seem to be intra-S-phase checkpoint competent (Fig. 2c, d). However, cells expressing *SLD3-m25* and *dbf4-m25* progressed faster through the S phase in the presence of MMS than cells expressing either allele alone (Fig. 2c, d and Supplementary Fig. 5), suggesting that Sld3 and Dbf4 are both important for the block to late origin firing. The finding that *SLD3-m25 dbf4-m25* cells progressed through the S phase slower than *rad53*Δ cells can best be explained by the fact that Dbf4-m25 is still

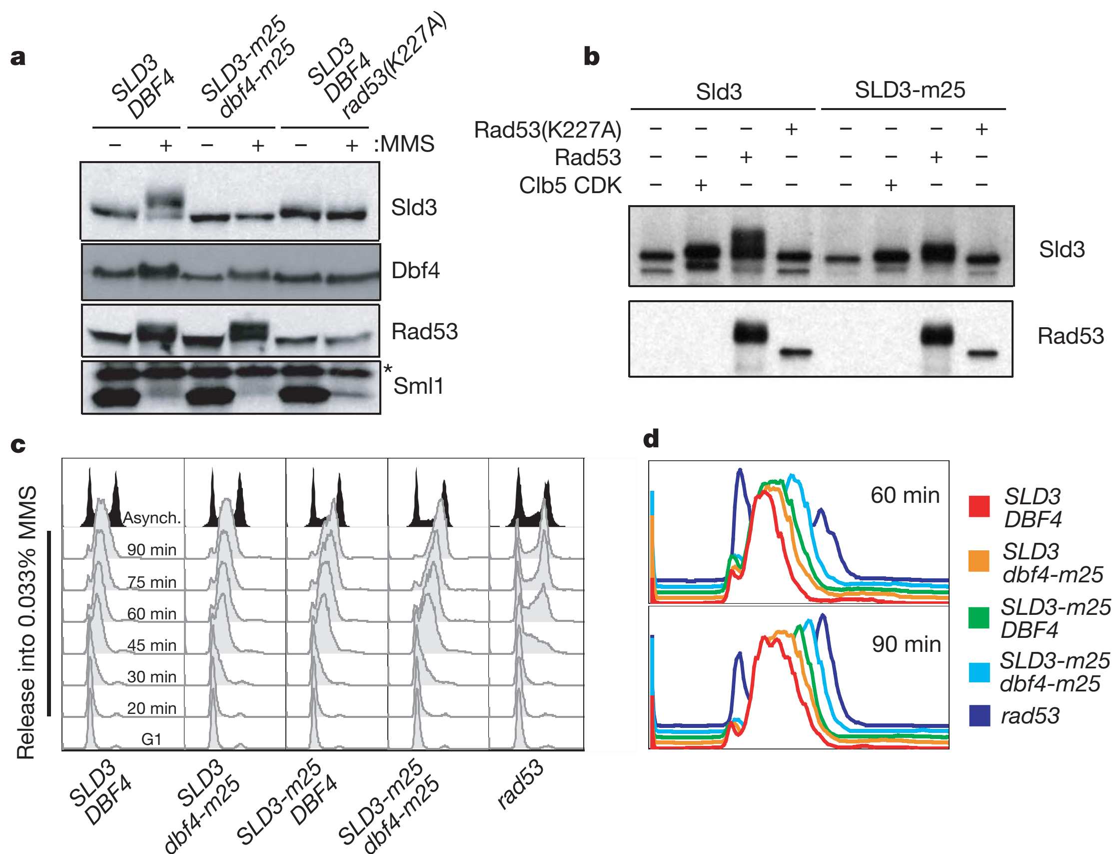
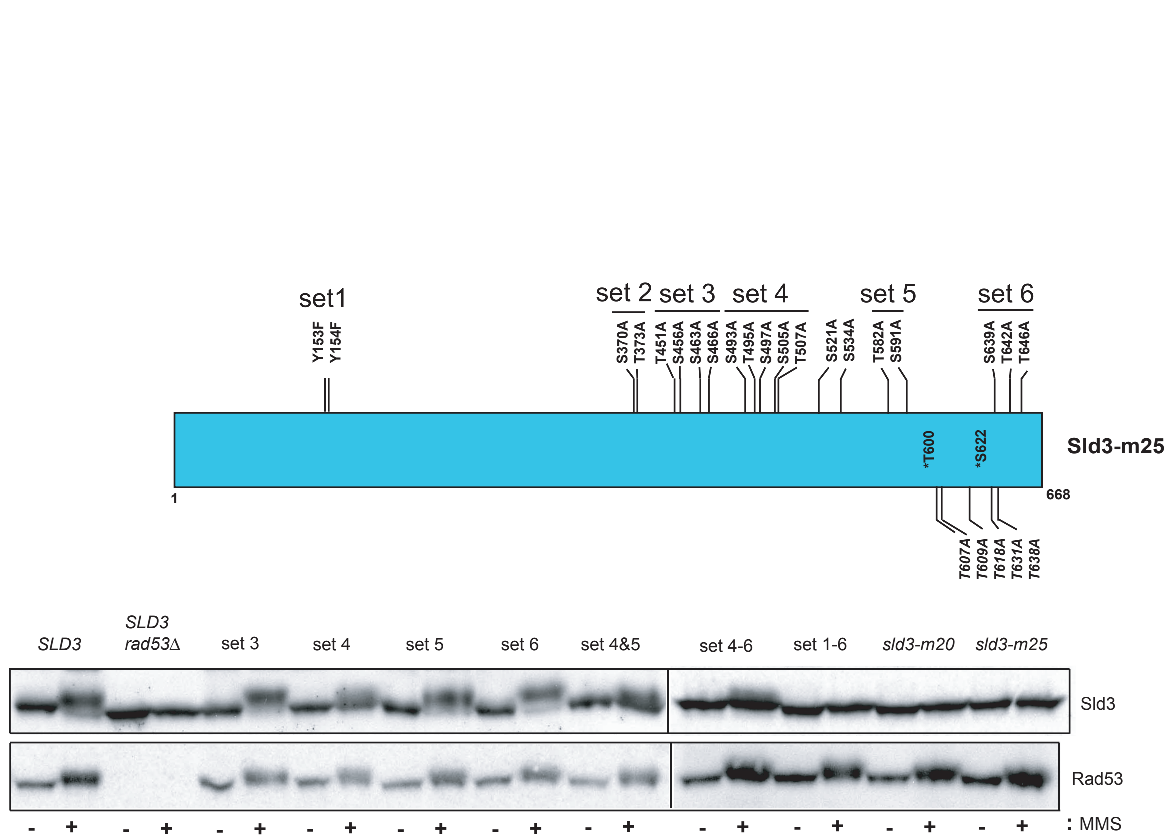


Figure 2. ***SLD3-m25 and dbf4-m25* cells are intra-S-phase checkpoint defective.** **a**, Immunoblots from asynchronous cells grown in the presence or absence of 0.05% MMS for 90 minutes. *rad53-KD (K227A)* is a hypomorphic allele that is checkpoint-defective, but able to perform the essential function rescued by Sml1 deletion. **b***,* Immunoblots of an *in vitro* kinase assay on recombinant full-length Sld3 or Sld3-m25 substrates treated with yeast clb5-TAP-CDK, recombinant Rad53 or Rad53-KD *(K227A)* **c**, Flow cytometry of wild-type, *SLD3-m25, dbf4-m25, SLD3-m25 dbf4-m25, or rad53*Δ cells synchronized in G1 with a-factor and released into 0.033% MMS at 30°C. **d**, The 60 and 90 minute profiles from **c,** displayed together.



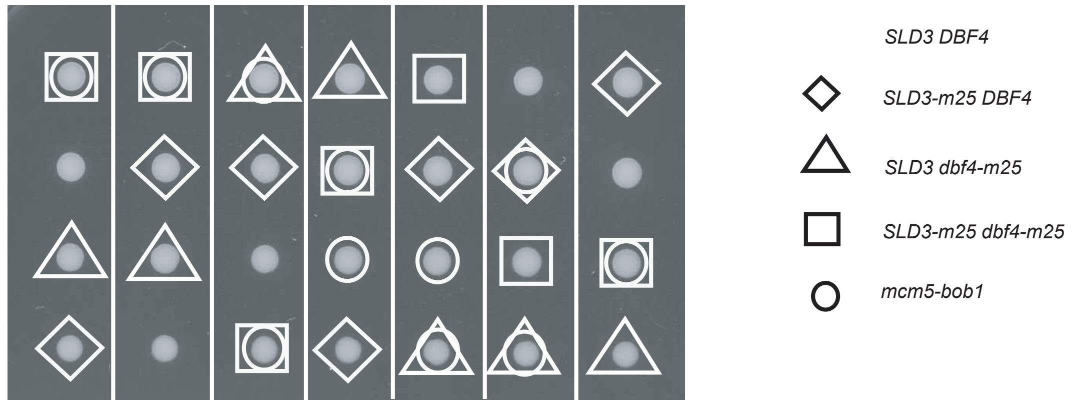
Supplemental Figure 2. **Damage-induced phosphorylation sites.**
a, Sld3 illustration indicating mutated sites (set 3: T451A, S456A, S463A, S466A; set 4: S493A, T495A, S497A, S505A, T507A; Set 5: T582A, S591A; Set 6: S639A, T642A, T646A; *sld3-m20*: set 1-6 + S521A S534A; *SLD3-m25: sld3-m20* + T607A T609A T618A T613A T638A).**b,** Immunoblot of Sld3 and Rad53 from cells expressing wild-type or mutant Sld3-3Flag in the presence or absence of 0.05% MMS for 90 minutes.

partially phosphorylated *in vivo* (Fig. 2a). Damage-induced Rad53 phosphorylation and Sml1 degradation (Zhao and Rothstein, 2002) are intact in *SLD3-m25 dbf4-m25* cells (Fig. 2a), indicating that these mutations do not compromise checkpoint activation.

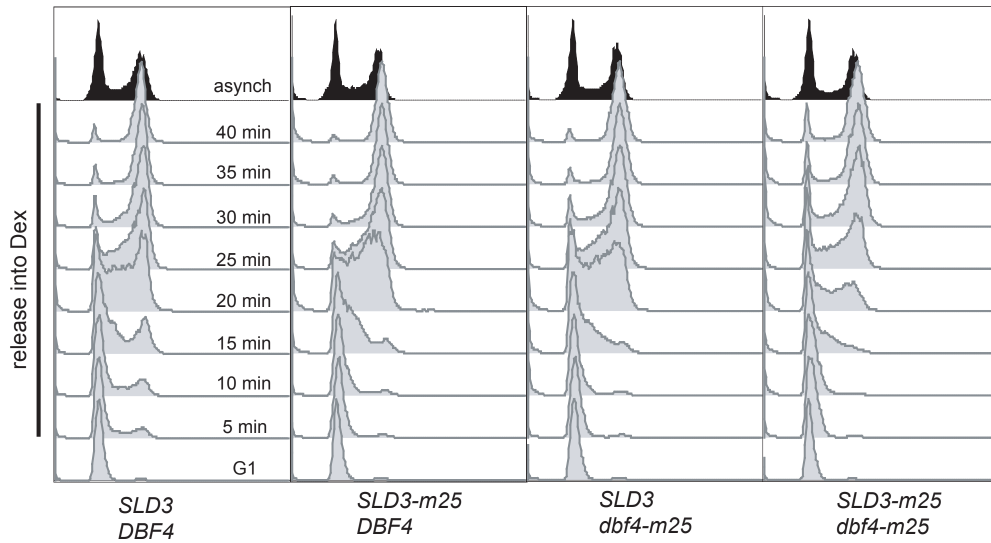
To determine unambiguously whether the fast S phase observed in *SLD3-m25 dbf4-m25* cells, in the presence of MMS, is due to the inappropriate firing of late origins of replication, firing was directly analysed by two-dimensional gel electrophoresis (Friedman and Brewer, 1995). In hydroxyurea-treated cells, early origins ARS305 and ARS607 fired efficiently and with normal kinetics in *SLD3-m25*, *dbf4-m25* and *SLD3-m25 dbf4-m25* mutants compared to wild-type cells (Fig. 3a and Supplementary Fig. 6b). This finding is consistent with early origins being refractory to intra-S-phase checkpoint regulation (Santocanale and Diffley, 1998; Shirahige et al., 1998). We next looked at several late origins: ARS501, ARS609, ARS1007 and ARS1212 (Fig. 3a, Supplementary Fig. 6c and data not shown). In hydroxyurea-treated cells, late origin firing was not detected in wild-type cells, but was evident in *rad53*Δ cells. Inappropriate late origin firing was also observed in *SLD3-m25*, *dbf4-m25* and most strongly in the *SLD3-m25 dbf4-m25* double mutant. In these mutant cells, late origins fired after early origins, confirming the finding that the temporal regulation of origin firing is not subject to intra-S-phase checkpoint regulation (Supplementary Fig. 6b, c). These results suggest that the fast S phase observed in *SLD3-m25 dbf4-m25* cells is due to the inappropriate firing of late replicating origins.

If Rad53-mediated phosphorylation of Sld3 slows the S phase by blocking late origin firing, then constitutive Rad53 phosphorylation of Sld3 would constitutively slow the S phase, even in the absence of DNA-damaging agents. To test this prediction, we

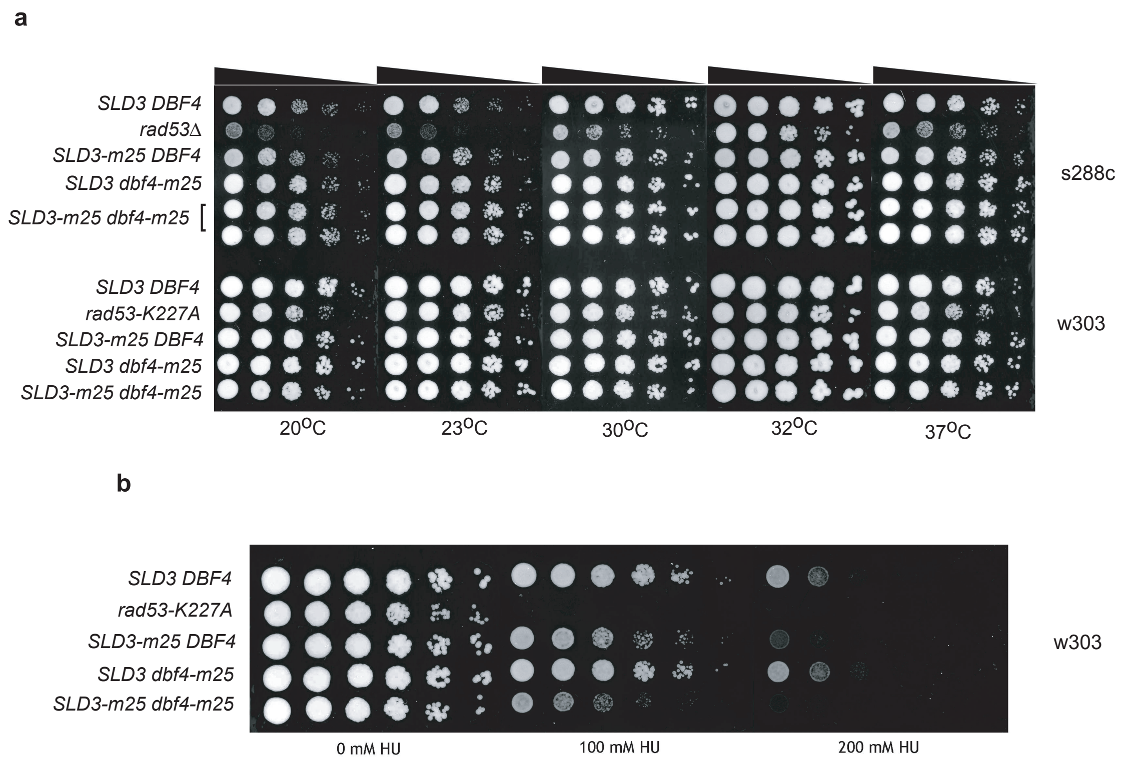
a



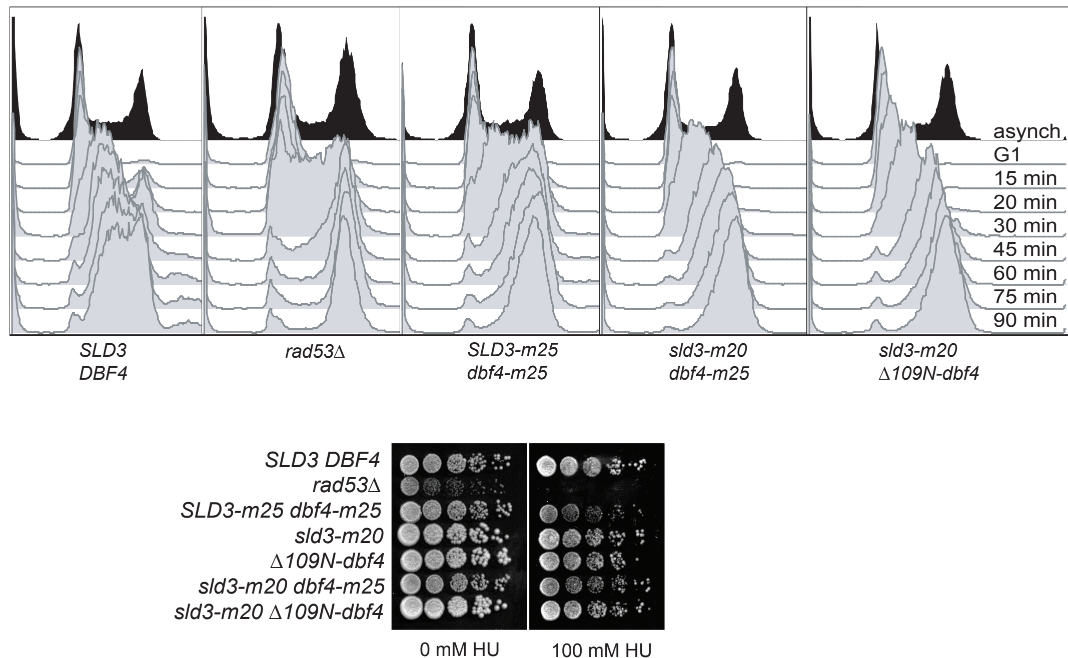
b



Supplemental Figure 3. ***SLD3-m25 dbf4-m25* cells are wild-type for growth. a**, Representative tetrads from an *SLD3/SLD3-m25 DBF4/dbf4-m25 MCM5/mcm5-bob1* heterozygote. **b**, Flow cytometry of wild-type, *SLD3-m25, dbf4-m25, and SLD3-m25 dbf4-m25* cells synchronized in G1 with a-factor and released into medium without a-factor at 30°C.



Supplemental Figure 4. **Growth assay of *SLD3-m25 dbf4-m25* cells. a**, 5-fold serial dilutions of wild-type or mutant cells in two strain backgrounds (s288c and w303) grown at 20°, 23°, 30°, 32°, and 37°C. **b**, 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 100 mM, or 200 mM HU, as in Fig. 3c, but in w303 cells. In this strain background, *SLD3-m25* cells are partially HU-sensitive.



Supplemental Figure 5. ***sld3-m20 Δ109N-dbf4* cells are intra-S-phase checkpoint deficient**. **a,** Flow cytometry of wild-type, *rad53*Δ, *SLD3-m25 dbf4-m25, sld3-m20 dbf4-m25, and sld3-m20* Δ*109N-dbf4* cells synchronized in G1 with a-factor and released into 0.033% MMS at 30°C. **b,** 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM and 100 mM.

created a phospho-mimetic allele, where 21 residues targeted by Rad53 were mutated to aspartic acids to mimic constitutive Sld3 phosphorylation (*sld3- m21D*). Cells expressing the *sld3-m21D* allele revealed a slow S phase in the absence or presence of DNA damaging agents (Fig. 3b and Supplementary Fig. 7a). The slow S phase is not a result of checkpoint activation, as *sld3-m21D* cells do not elicit Rad53 phosphorylation in the absence of damage (Supplementary Fig. 7b). In addition, *sld3-m21D* cells grow with wild-type rates, are not hydroxyurea sensitive, and fire early origins of replication with efficiencies that are indistinguishable from those observed in wild-type cells (Fig. 3c and Supplementary Fig. 7c). This suggests that Sld3 phosphorylation is sufficient to inhibit origin firing, although effects on other aspects of replication may also have a contribution (Supplementary Fig. 1).

An Sld3–Dpb11 fusion has previously been shown to bypass the requirement for S-phase CDK activity for origin firing (Zegerman and Diffley, 2007). If Rad53 phosphorylation of Sld3 functions solely to inhibit an Sld3–Dpb11 interaction, then cells expressing an *Sld3–Dpb11* fusion should be intra-S-phase checkpoint defective, akin to the *SLD3-m25* allele. However, cells expressing the *Sld3–Dpb11* fusion were neither intra-S-phase checkpoint defective (Supplementary Fig. 8) nor sensitive to growth on DNA-damaging agents (data not shown). This suggests that, although Rad53 inhibits the Sld3–Dpb11 interaction, this inhibition is not sufficient to block late origin firing.

To test if growth of *SLD3-m25 dbf4-m25* cells is impaired by DNA damaging agents, cells were spotted on hydroxyurea and MMS plates and sensitivity was compared to wild-type and *rad53*Δ cells. *SLD3-m25 dbf4-m25* cells are mildly sensitive to the replication inhibitor hydroxyurea in a dosage-dependent manner (Fig. 3c and

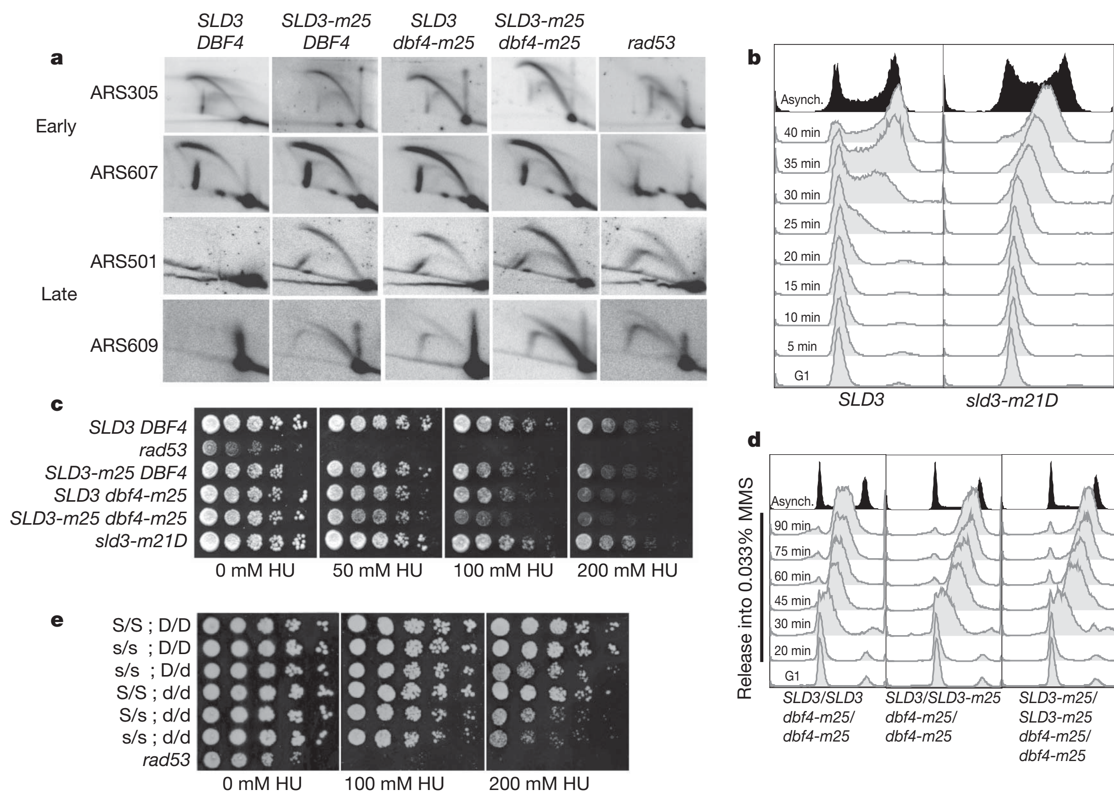
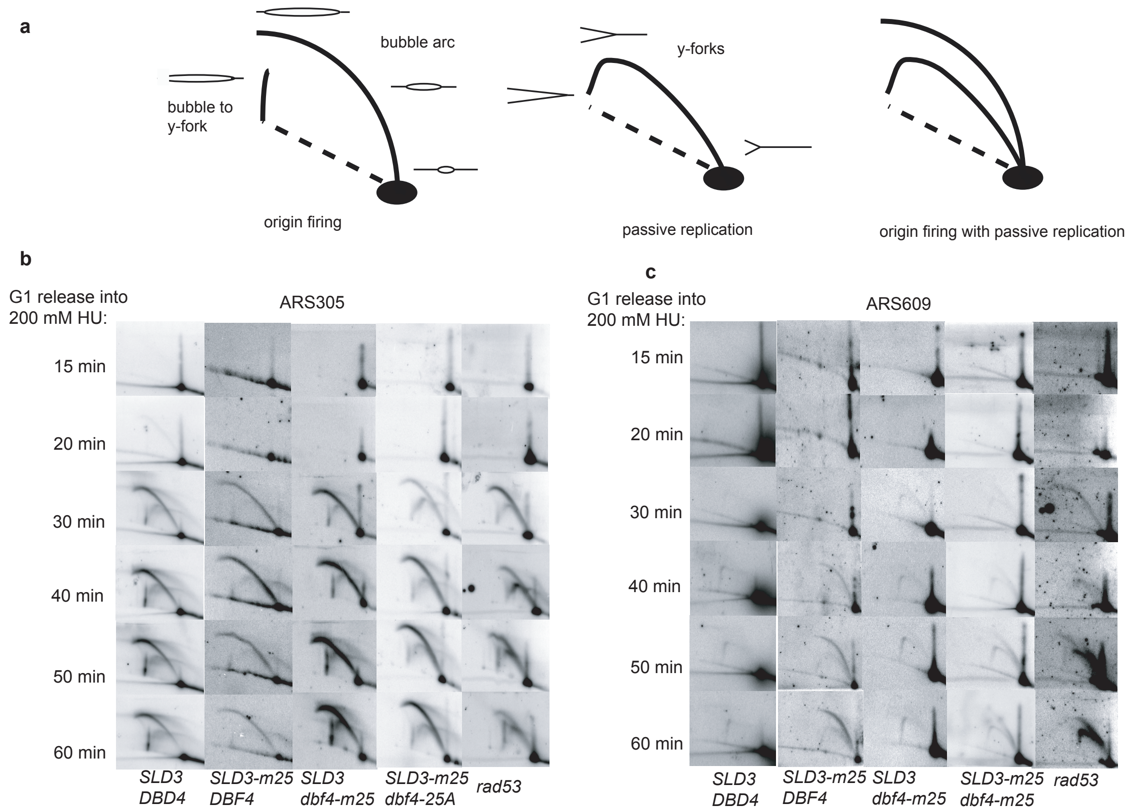


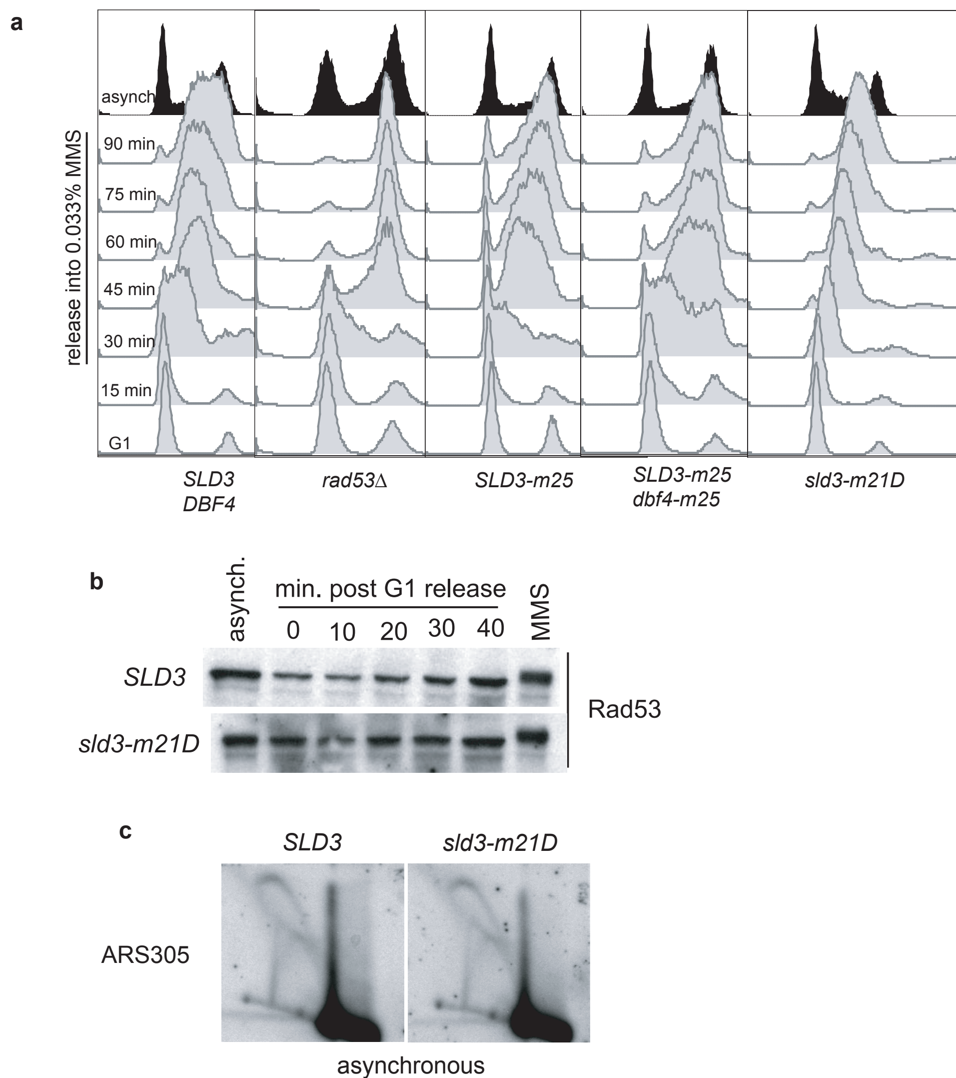
Figure 3. **Inappropriate firing of late origins in the presence of replication inhibitors.** **a**, Two-dimensional gel analysis of replication intermediates. Wild-type, *SLD3-m25, dbf4-m25, SLD3-m25 dbf4-m25 or rad53*Δ cells were synchronized with a-factor and released into 200 mM HU. Cells were collected at 30, 40, 50, 60 and 70 minutes into the HU release and pooled. 20 mg aliquots of DNA were analyzed by two-dimensional electrophoresis as previously described(Friedman and Brewer, 1995). early origins; *ARS305* and *ARS60*7. late origins; *ARS501* and *ARS609*. **b**, Flow cytometry of cells expressing *SLD3-3Flag* or a phosphorylation-mimetic allele *sld3-m21D-3Flag*. Cells were arrested in G1 with a-factor and released into media without a-factor at 30°C. **c**, 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 50 mM, 100 mM, or 200 mM HU. **d**, Flow cytometry of diploid MAT a/a cells treated as in **2c**, from *SLD3/SLD3 dbf4-m25/dbf4-m25, SLD3/SLD3-m25 dbf4-m25/dbf4-m25, and SLD3-m25/SLD3-m25 dbf4-m25/dbf4-m25* cells. **e**, 5-fold serial dilutions, as in **c**, of diploid strains to assess dominance. Capital **S** and **D** represent wild-type *SLD3* and *DBF4*, whereas lowercase **s** and **d** represent *SLD3-m25* and *dbf4-m25* respectively.



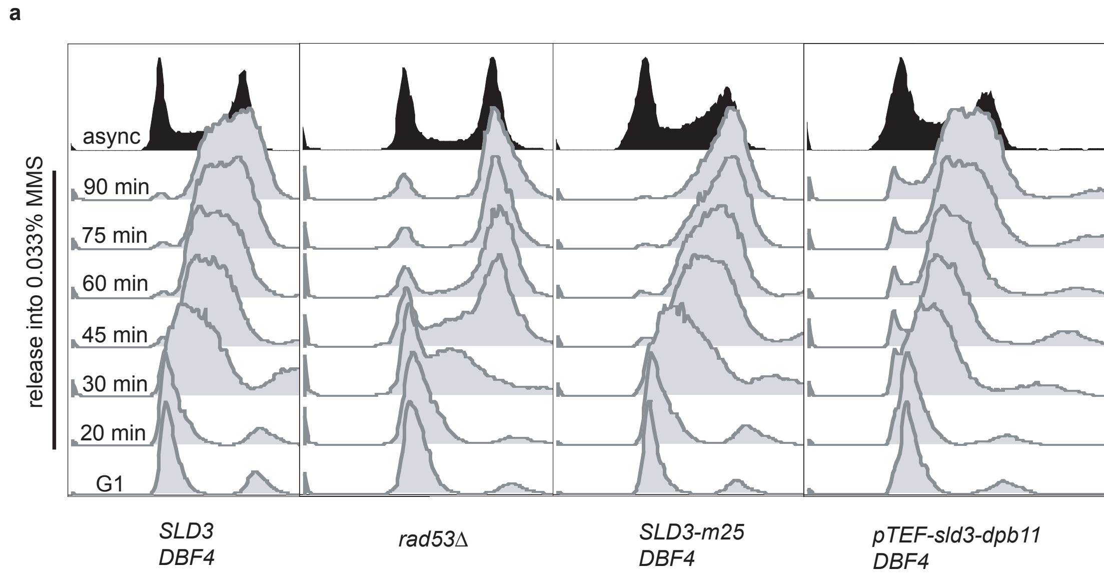
Supplemental Figure 6. **Inappropriate firing of a late origin**. **a**, Schematic illustrating relevant replication structures and their corresponding migration pattern after two-dimensional gel electrophoresis. **b** and **c**, Time-course analysis of replication intermediates from *SLD3 DBF4*, *SLD3-m25 DBF4*, *SLD3 dbf4-m25*, *SLD3-m25 dbf4-m25*, and *rad53* cells (w303) synchronously released into 200 mM HU. DNA was prepared from each time-point and probed for **b**, ARS305 (early origin) or **c**, ARS609 (late origin).

Supplementary Fig. 4b), but were not sensitive to MMS (data not shown). Notably, *mec1-100* cells, which are defective in the block to late origin firing, are also mildly hydroxyurea sensitive, but not MMS sensitive (Paciotti et al., 2001; Tercero et al., 2003). It is possible that *SLD3-m25 dbf4-m25* cells are sensitive to hydroxyurea simply because these mutant alleles are hypomorphic. Two results indicated that this was not the case. First, the checkpoint-defective *rad53-R70,R605A* allele is epistatic to *SLD3-m25 dbf4-m25*: *SLD3-m25 dbf4-m25 rad53-R70,R605A* cells were not more hydroxyurea sensitive than *rad53-R70,R605A* cells (Supplementary Fig. 9a, b). Second, *SLD3-m25* is dominant to wild-type for hydroxyurea sensitivity. In hydroxyurea-sensitivity experiments using heterozygous diploid cells (Fig. 3e), homozygous *SLD3/SLD3 dbf4-m25/dbf4-m25* diploid cells are resistant to hydroxyurea. In contrast, heterozygous *SLD3/SLD3-m25 dbf4-m25/dbf4-m25* diploid cells are mildly hydroxyurea sensitive, similarly to homozygous *SLD3-m25/SLD3-m25 dbf4-m25/dbf4-m25* diploid cells (Fig. 3e). *SLD3-m25* is similarly dominant for its checkpoint phenotype. These results strongly suggest that the hydroxyurea sensitivity seen in the *SLD3-m25 dbf4-m25* double mutant is not due to insufficient Sld3 activity in the *SLD3-m25* allele, but instead is due to it being refractory to Rad53 inhibition.

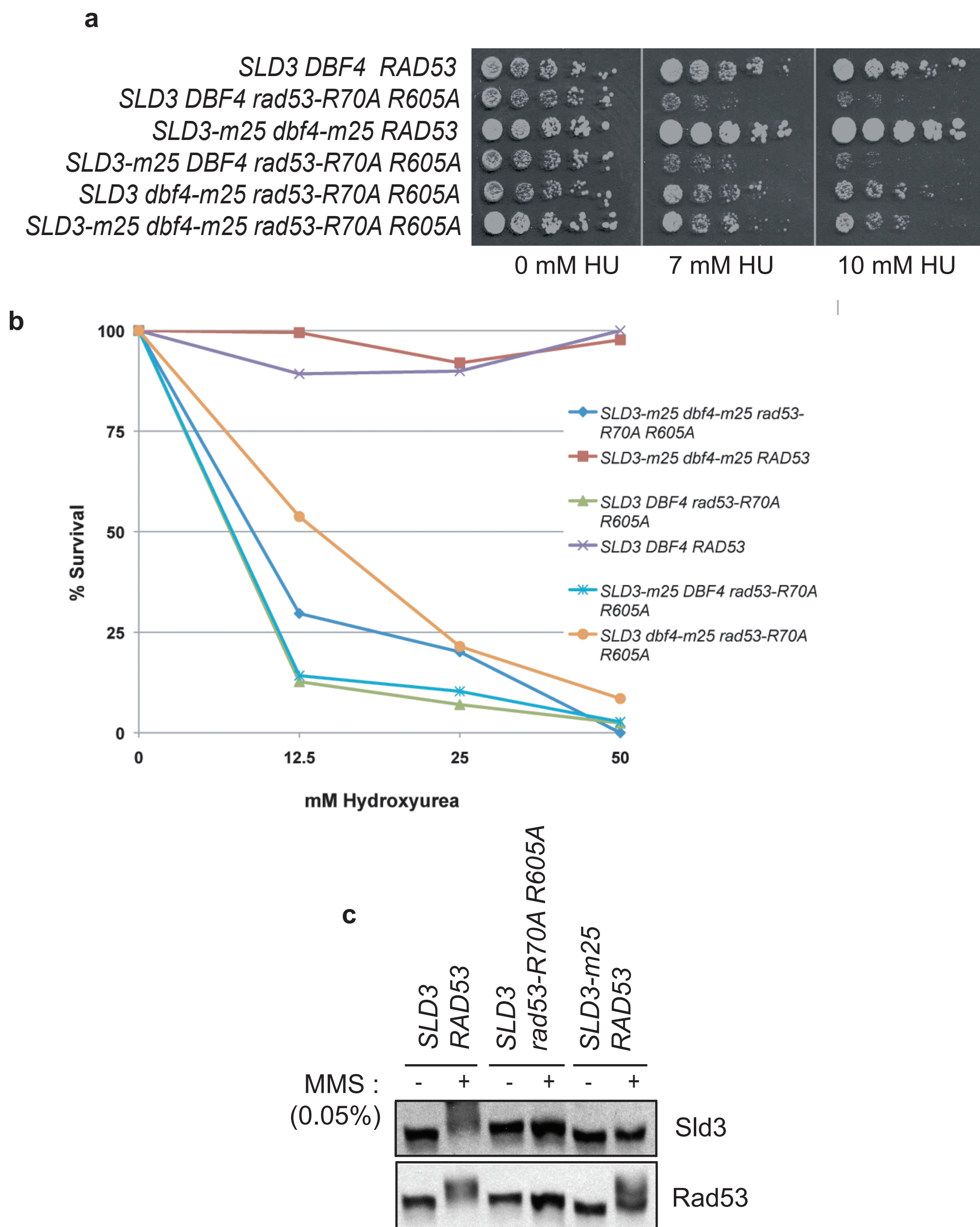
Previous studies have shown that wild-type cells can efficiently recover from hydroxyurea arrest, whereas *rad53*Δ cells cannot (Desany et al., 1998; Lopes et al., 2001). We asked if *SLD3-m25 dbf4-m25* cells could recover from transient blocks to DNA replication. Wild-type, *SLD3-m25* and *dbf4-m25* single mutant cells recovered from hydroxyurea arrest and divided, entering the next cell cycle by 120 min (Fig. 4a).



Supplemental Figure 7. **An Sld3 phosphorylation mimic has a slow S-phase.**
a. Flow cytometry of *SLD3 DBF4, rad53Δ, SLD3-m25, SLD3-m25 dbf4-m25* cells or the phosphorylation mimic *sld3-m21D* synchronously released into medium containing 0.033% MMS. **b**, Immunoblot of samples from Fig. 3b, probed for Rad53. "MMS" are control samples from cells treated with 0.05% MMS for 90 minutes. **c**, Two-dimensional gel electrophoresis of asynchronous cells expressing either *SLD3-3Flag* or the phosphorylation mimic, *sld3-m21D.* DNA was digested with EcoR1 and probed for the early origin ARS305.



Supplemental Figure 8**. Sld3-Dpb11 fusion cells are intra-S-phase checkpoint competent.** Flow cytometry of wild-type, *rad53*Δ, *SLD3-m25*, and *sld3-dpb11* fusion cells synchronized in G1 with a-factor and released into 0.033% MMS at 30°C.



Supplemental Figure 9. ***SLD3-m25* is epistatic to *rad53* mutant cells. a,** 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 7 mM, 10 mM HU for epistasis analysis. The *rad53-R70,R605* allele has mutations in the FHA1 and FHA2 domains, respectively, and lacks all checkpoint function, but is proficient for Rad53's checkpoint-independent role in DNA replication (Dohrmann et al., 1999). (See Supplemental methods) **b,** Growth curves of wild-type or mutant strains grown asynchronously in the presence of 0 mM, 12.5 mM, 25 mM, or 50 mM HU for three hours at 30°C. For each concentration, ~200 cells were plated and grown for 3 days at 30°C. **c,** Immunoblots from asynchronous cells grown in the presence or absence of 0.05% MMS for 90 minutes.

However, only a fraction of *SLD3-m25 dbf4-m25* cells appeared to recover from hydroxyurea arrest by 120 min; the majority remained arrested in mitosis, indicating that these cells harboured DNA damage (Fig. 4a). We then examined Rad53 de-phosphorylation kinetics. In wild-type, *SLD3-m25* and *dbf4-m25* cells, Rad53 became phosphorylated during the hydroxyurea arrest and was de-phosphorylated after the hydroxyurea release. In contrast, *SLD3-m25 dbf4-m25* cells retained Rad53 phosphorylation for 120 min after hydroxyurea release (Fig. 4b). Although it is likely that inappropriate firing of late origins leads to a proportional increase in fork collapse, Sld3 phosphorylation probably provides additional hydroxyurea resistance through other uncharacterized means.

Rad52 functions in recombinational repair and can potentially repair or restart broken or stalled replication forks. Rad52–GFP foci have been shown to form in response to replication fork collapse during the S phase in several checkpoint mutants (Barlow and Rothstein, 2009; Lisby et al., 2004). In wild-type cells, however, foci were not observed when cells are treated with either hydroxyurea or MMS (Alabert et al., 2009; Barlow and Rothstein, 2009). Consistently, we observed Rad52–GFP foci in 3% of wild-type and 53% of *rad53*Δ cells treated with hydroxyurea. Interestingly, 32% of *SLD3-m25 dbf4-m25* cells treated with hydroxyurea formed Rad52–GFP foci (Fig. 4c), with some cells containing multiple foci (Supplementary Figs 10–12). We also observed that *SLD3-m25 dbf4-m25* double mutant cells exhibited a 50% viability loss after a 4-h hydroxyurea treatment (Fig. 4d). Consistent with the observed increase in Rad52 foci, the mild viability loss seen in *SLD3-m25 dbf4-m25* cells after transient hydroxyurea treatment is exacerbated when *RAD52* is also deleted. This indicates that in hydroxyurea, *SLD3-m25*

dbf4-m25 cells repair DNA damage by homologous recombination to survive. As expected, *rad53*Δ cells show a severe viability loss after hydroxyurea treatment due the additional roles of Rad53 in fork stabilization (Desany et al., 1998; Lopes et al., 2001). Taken together, these data indicate that failure to block late origins in the presence of replication inhibitors results in an increased number of stalled replication forks, and perhaps increases the propensity of replication fork collapse. Rad52-dependent pathways may function to resolve or restart these stalled forks and its loss probably generates problems at the fork that ultimately contribute to cell lethality.

DNA replication and the intra-S-phase checkpoint machinery are conserved in organisms ranging from yeast to human. Recently several vertebrate candidate Sld3 orthologues have been identified, including Treslin (also called ticrr), GEMC1 and DUE-B (Labib, 2010). Of these candidates, Treslin shares significant domain conservation with Sld3 (Sanchez-Pulido et al., 2010). It remains to be seen if ATM/ATR or Chk2 also inhibit any of these new DNA replication factors upon DNA damage.

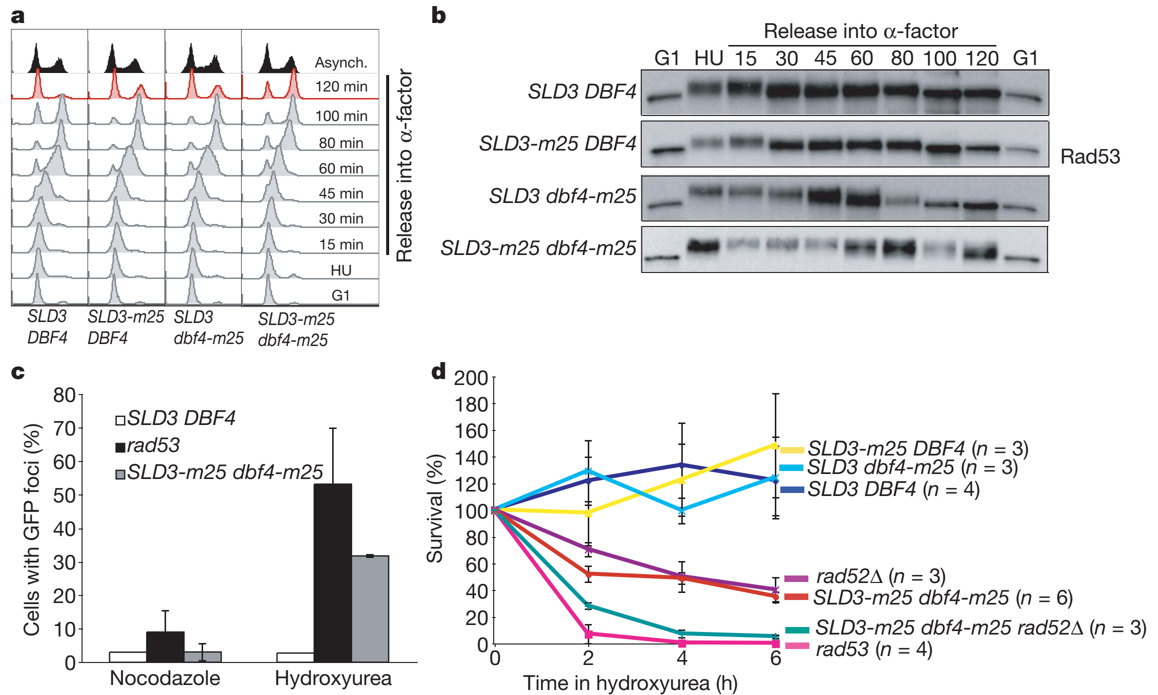
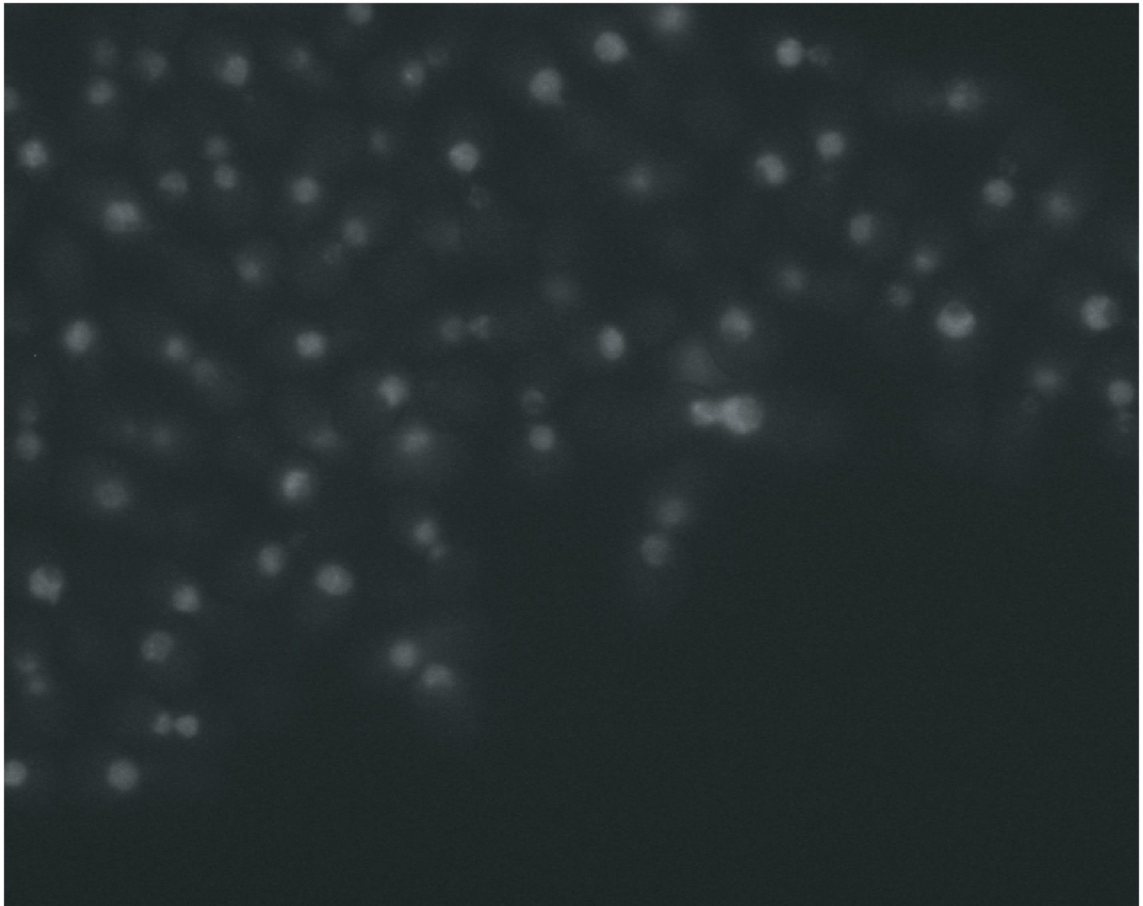
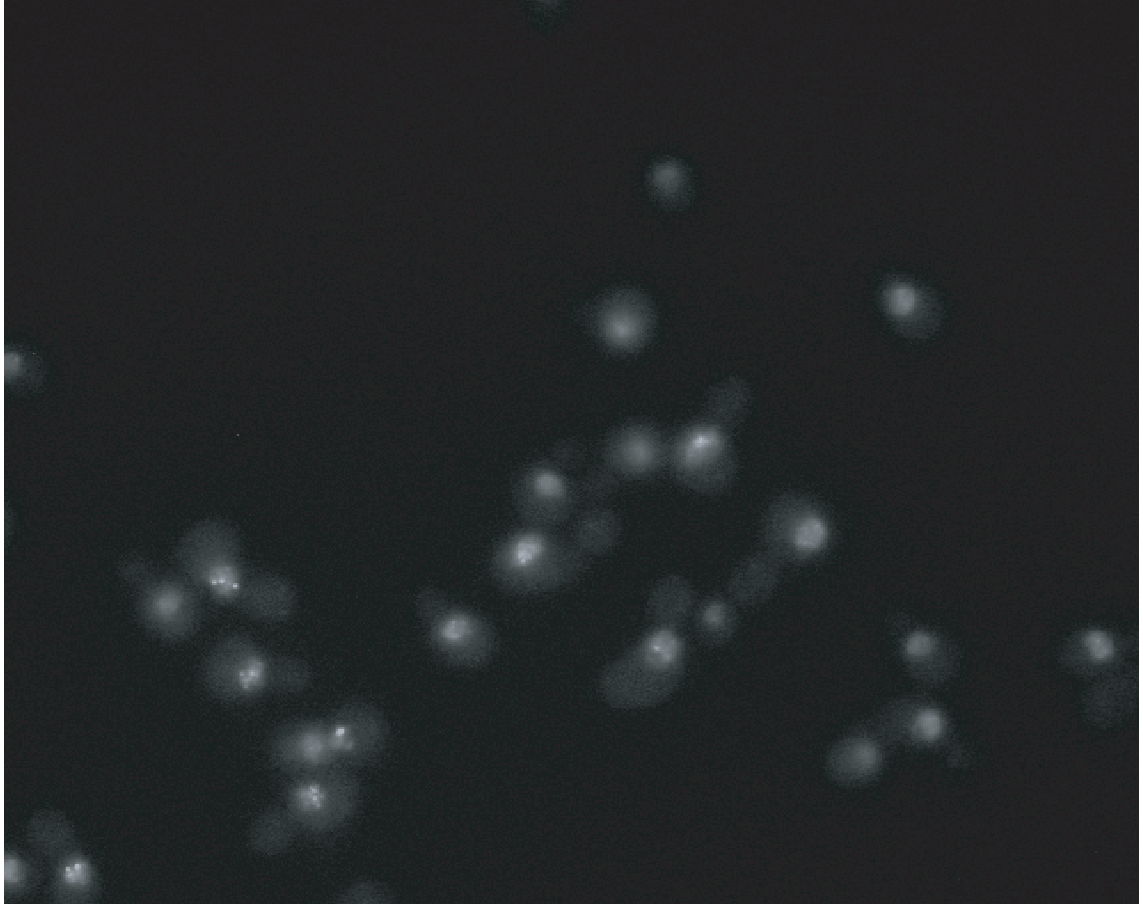


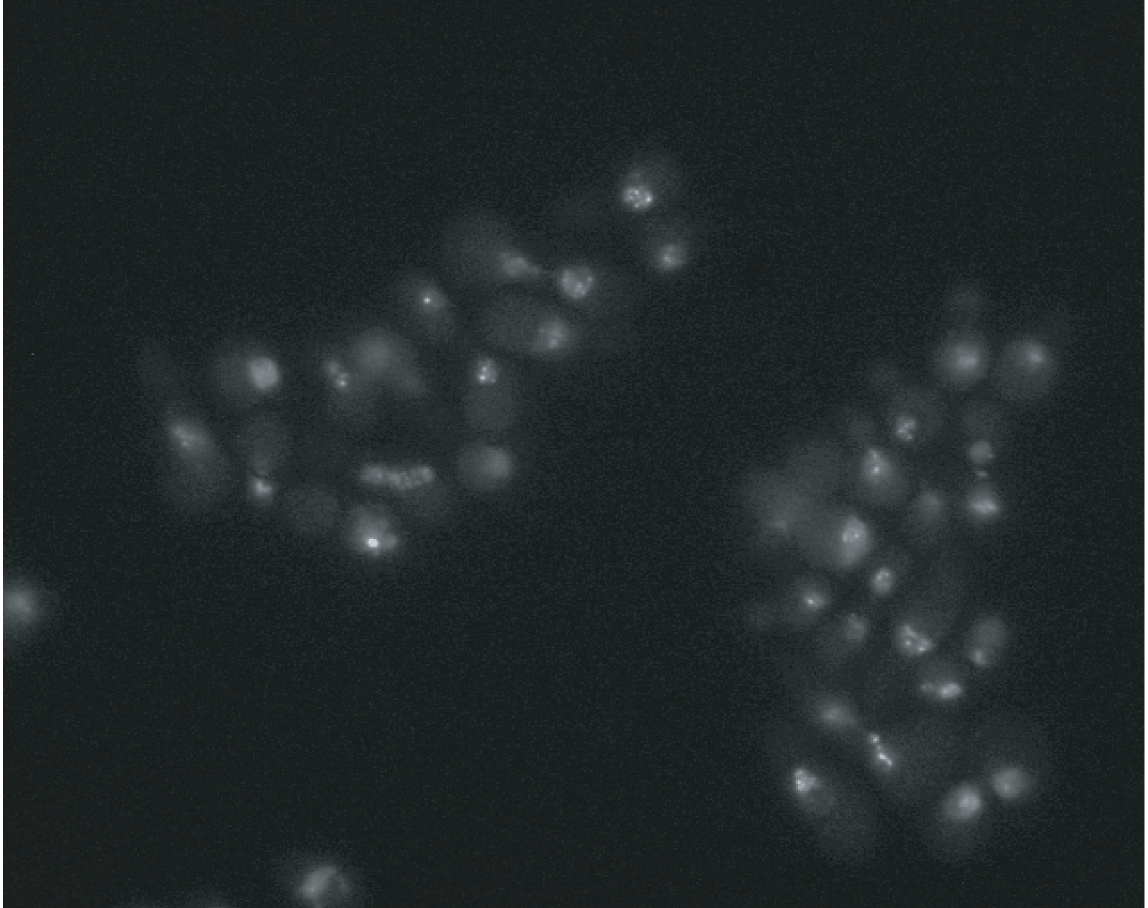
Figure 4. **Inappropriate firing of late origins elicits DNA damage.** **a**, Flow cytometry of wild-type or mutant strains synchronized in G1 with a-factor, released into a 200 mM HU block for 2 hours, then released into medium with a-factor. **b**, Immunoblots of samples from **a**, taken at indicated time points and probed for Rad53. **c**, Quantification of cells with one or more Rad52-GFP foci after incubating for 90 minutes in the presence of nocodazole or 200 mM HU. **d**, Survival assay of strains synchronized in G1 and released into 200 mM HU for 2, 4 or 6 hours. Error bars represent s.e.m.



Supplemental Figure 10. **Rad52-GFP Foci.** Representative cells from *in vivo* fluorescent microscopy of Rad52-GFP foci in wild-type strains arrest in G1 with a-factor and released into 200 mM HU for 90 minutes.



Supplemental Figure 11. **Rad52-GFP Foci.** Representative cells from *in vivo* fluorescent microscopy of Rad52-GFP foci in *SLD3-m25 dbf4-m25* strains arrest in G1 with a-factor and released into 200 mM HU for 90 minutes.



Supplemental Figure 12. **Rad52-GFP Foci.** Representative cells from *in vivo* fluorescent microscopy of Rad52-GFP foci in *rad53*Δ strains arrest in G1 with a-factor and released into 200 mM HU for 90 minutes.

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Author contributions

L.G.D.E. identified Sld3 in initial proteomic screen. Z.O.J. and J.W. performed mass spectrometry analysis. J.L.M. performed all other experiments with assistance from N.L.M. J.L.M and D.P.T. designed experiments and wrote the manuscript.

Strain	Genotype	source
yJLO18	*SLD3-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO23	*pGAL1-10-URA3-SLD3-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO20	*SLD3-3Flag::HygroR rad53Δ::KanMx sml1Δ::LEU2his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yNLM133	*SLD3-m25-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO92	*mcm5-bob1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO110	*SLD3/SLD3-m25-3Flag::HygroR DBF4/dbf4Δ::KanMx MCM5/mcm5-bob1*	this study
yJLO119	*SLD3/SLD3-m25-3Flag::HygroR DBF4/dbf4-m25::LEU2::dbf4Δ::KanMx MCM5/mcm5-bob1*	this study
yJLO123	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO124	*dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO155	*Rad52-GFP::HIS3 SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO156	*Rad52-GFP::HIS3 SLD3-m25-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO157	*Rad52-GFP::HIS3 dbf4-m25::LEU2::dbf4Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO158	*Rad52-GFP::HIS3 rad53Δ::KanMx sml1Δ::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO159	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx rad52Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO163	*SLD3-m25-3Flag::HygroR/SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx/dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO164	*SLD3-3xFlag::Hyg/SLD3-3xFlag::Hyg dbf4-25A::LEU2::dbf4Δ::KanMx/dbf4-25A::LEU2::dbf4Δ::KanMx*	this study
yJLO165	*SLD3-3xFLAG/SLD3-m25-3xFlag::HyG dbf4-25A::LEU2::dbf4Δ::KanMx/dbf4-25A::LEU2::dbf4Δ::KanMx*	this study
yJLO70	*HygroR::pTEF-sld3dpb11::DPB11 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yNLM91	*sld3-m4-3Flag::HygroR (set3: T451A S456A S463A S466)*	this study
yNLM93	*sld3-m5-3Flag::HygroR (set4: S493A T495A S497A S505A T507A*	this study
yNLM95	*sld3-m2-3Flag::HygroR (set5: T582A S591A)*	this study
yNLM97	*sld3-m4-3Flag::HygroR (set6: S639A T642A T646A)*	this study
yNLM99	*sld3-m7-3Flag::HygroR (set 4&5: S493A T495A S497A S505A T507A T582A S591A)*	this study
yNLM101	*sld3-m10-3Flag::HygroR (Set4-6: S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A*	this study
yNLM107	*sld3-m18-3Flag::HygroR (set1-6: Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A*	this study
yNLM147	*sld3-m20-3Flag::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A S639A T642A T646A)*	this study
yNLM133	*SLD3-m25-3Flag::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*	this study
yJLO180	*SLD3-3Flag::HygroR DUN1-TAP::HISMx*	this study
yJLO181	*SLD3-3Flag::HygroR rad53Δ::KanMx sml1Δ::LEU2 DUN1-TAP::HISMx*	this study
yJLO182	*SLD3-m25::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx DUN1-TAP::HISMx*	this study
yJLO187	*SLD3-3Flag::HygroR DBF4-9Myc::TRP*	this study
yJLO188	*SLD3-m25-3Flag::HygroR dbf4-m25-9Myc::TRP*	this study
yJLO189	*SLD3-3Flag::HygroR DBF4-9Myc::TRP rad53-K227A*	this study
yJLO190	*sld3-m21D3FLAG::HygroR (T451D S456D S463D S466D S493D T495D S497D S505D T507D S521D S534D T582D S591D T607D T609D S618D T631D T638D S639D T642D T646D)*	this study
yJLO192	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx rad53Δ::KanMx sml1::LEU2*	this study

yJLO193	*CDC7-TAP::HIS3 SLD3-3Flag::HygroR DBF4-9Myc::TRP*	this study
yJLO194	*CDC7-TAP::HIS3 SLD3-3Flag::HygroR dbf4-m25-9Myc::TRP*	this study
yJLO260	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO261	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [RAD53::URA3]*	this study
yJLO262	*SLD3-3Flag::HygroR DBF4 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO263	*SLD3-3Flag::HygroR DBF4 rad53Δ::HIS3 [RAD53::URA3]*	this study
yJLO264	*SLD3-m25-3Flag::HygroR DBF4 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO265	*SLD3-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
	rad52Δ::KanMx	Deletion Library
	pGal1-10-URA3-DBF4-Morf::URA3 [2m]	Open Biosystems
	DPB11-TAP::HIS3Mx	TAP Library (Weissman&O'Shea)

Supplemental Table 2. **Yeast Strains**. Strains and corresponding genotypes that were used in this study.

Plasmid name	Relevant genotype
pJLO2	*SLD3-3Flag::HygroR (tagging construct)*
pJLO3	*sld3-3Flag::HygroR (set3: T451A S456A S463A S466A)*
pJLO4	*sld3-3Flag::HygroR (set4: S493A T495A S497A S505A T507A)*
pJLO5	*sld3-3Flag::HygroR (set6: S639A T642A T646A)*
pNM10	*sld3-m2::HygroR (set5: T582A S591A)*
pNM11	*sld3-m7::HygroR (set4&5: S493A T495A S497A S505A T507A T582A S591A)*
pNM12	*sld3-m10::HygroR (set4-6: S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM13	*sld3-m14::HygroR (set3-6: T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM15	*sld3-m16::HygroR (set2-6: T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM16	*sld3-m18::HygroR (set1-6: Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM19	*sld3-m21::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A T607A T609A S618A S639A T642A T646A)*
pNM21	*sld3-m20::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A S639A T642A T646A)*
pNM22	*sld3-m23::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*
pNM23	*SLD3-m25::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*
pNM25	*Δ109N-Dbf4::LEU2 (pRS305)*
pJLO12	*DBF4::LEU2 (pRS305)*
pJLO13	*dbf4-m2::LEU2 (T171A T175A)*
pNM28	*dbf4-m1::LEU2 (S84A)*
pNM29	*dbf4-m2::LEU2 (S84A S235A)*
pNM30	*dbf4-m3::LEU2 (S84A S235A T473A)*
pNM33	*dbf4-m4::LEU2 (S84A S235A S356A T473A)*
pJLO15	*dbf4-m6::LEU2 (S84A Y139F S235A S356A T473A S632A)*
pJLO16	*dbf4-m27::LEU2 (S3A T5A S11A T16A T18A S29A T30A T31A S40A T50A T51A S53A S59A S84A S92A T95A T105A T114A S124A T131A Y139F T171A T175A S235A S356A T473A S632A)SacII/BsaBI fragment from pUC57 subcloned into pJLO15 SacII/BsaBI*
pJLO17	*dbf4-m25::LEU2 (T5A T16A T18A S29A T30A T31A S40A T50A T51A S53A S59A S84A S92A T95A T105A T114A S124A T131A Y139F T171A T175A S235A S356A T473A S632A)*
pJLO28	*GST-Sld3-3Flag pDEST15*
pJLO29	*GST-Sld3-m25-3Flag pDEST15*
pJLO31	*GST-dbf4-6xHis pDEST15*

pJLO32	*GST-dbf4-m25-6xHis* *pDEST15*
pJL033	*sld3-m21D-3Flag::HygroR (T451D S456D S463D S466D S493D T495D S497D S505D T507D S521D S534D T582D S591D T607D T609D S618D T631D T638D S639D T642D T646D)*
pGal-HO	*pGal-HO::URA3 (cen)*
rad53-FHA1FHA2	*rad53 R70A R605A:URA3 (cen)*

Supplemental Table 3. **Plasmids**. Plasmids and corresponding information used to make yeast strains.

Methods

All yeast strains used in this study are of the s288c strain background unless otherwise noted and were created using standard yeast techniques. All constructs were sequence verified.

Epitope tagging of Sld3 at the endogenous locus was done by integration as follows: the 3Flag::HygroR cassette(Gelbart et al., 2001) was targeted to the *SLD3* ORF to yield *SLD3- 3Flag::HygMx* (yJLO18). *SLD3-3Flag::HygMx* was PCR amplified from yJLO18 and cloned into pGEM-T-easy (Promega) to generate pJLO2. Mutagenesis of Sld3 at the endogenous locus was done by integration as follows: pJLO2 was mutated using QuikChange site-directed mutagenesis (Stratagene) following the manufacturer's instructions. Resultant plasmids were then digested to release constructs used to transform diploid strains (for example, (yNLM133) *SLD3- m25-3Flag::HygMx*; pNM23 was digested to release a StuI/BstZ17I fragment used to target SLD3). T607A, T609A, T631A and T638A were not identified by mass spectrometry but were mutated because these sites are present in a peptide shown to bind Dpb11 *in vitro* (Tanaka et al., 2007; Zegerman and Diffley, 2007). To create *sld3-m21D–3Flag*, a DNA fragment including amino acids corresponding to the last 21 mutations in the *SLD3-m25–3Flag* allele was synthesized, such that each of these 21 phosphorylation sites were now mutated to aspartic acids, and cloned into pJ201 (DNA 2.0). An EcoN1/BamH1 fragment (857 bp) was subcloned into pNM14 digested with EcoN1/BamH1. The resulting plasmid, pJLO18, was then digested with EcoN1 and Bstz17I to integrate it at the endogenous *SLD3* locus in diploid cells. Diploids were subsequently sporulated to obtain haploid

cells.

Dbf4 was cloned into pGEM-T-easy to generate pJLO12. Mutations identified by mass spectrometry were mutated using QuikChange mutagenesis as described above to generate pJLO15. Mutations in the Dbf4 N-terminal domain were synthesized and cloned into pUC57 (Bio-Basic). A SacII/BsaBI fragment from pUC57 was subcloned into pJLO15 to generate pJLO16. pJLO16 was modified by QuikChange mutagenesis to revert mutations (A3S A11S) to generate pJLO17. pJLO17 was digested with SacII to integrate into yJLO110 at the *dbf4Δ::KanMx* locus.

Rad53-R70A,R605A (FHA1 FHA2 mutants). A heterozygous diploid *SLD3/ SLD3-m25 RAD53/rad53Δ DBF4/dbf4-m25 SML1/sml1-1* was transformed with a CEN plasmid harbouring *rad53-R70A,R605A::URA3*. Resultant transformants were sporulated and dissected. Previous work has shown that Rad53 has an additional direct role in DNA replication leading to synthetic lethal interactions with *cdc7* or *dbf4* alleles(Dohrmann et al., 1999). This interaction is checkpoint-independent, as *RAD53* alleles with mutations in both FHA domains, which lack all checkpoint functions (Pike et al., 2004; Tam et al., 2008), are fully competent for this checkpoint-independent function. To avoid complications arising from this, we have used this *rad53* allele.

MAT a/a diploids were created by transforming a/a diploid cells with a CEN plasmid containing *pGal-HO::URA3*. Mating-type switching was induced with 2% galactose for 30 min. Diploids were then plated on 5-FOA to lose the plasmid. Resulting diploids were then tested for the ability to arrest in G1 with α-factor. Phosphatase assay. Sld3–3Flag or Dbf4–TAP was overexpressed and immuno-precipitated using anti-M2 Flag sepharose (Sigma) or IgG sepharose (GE Healthcare) from cells treated with 0.05%

MMS or 2μg ml^{-1} 4NQO. Immunoprecipitates were washed twice in λ-phosphatase buffer and treated with 100 units of λ -phosphatase (NEB) for 30 min at 30 °C in the presence or absence of phosphatase inhibitors (ZnCl2, 1 mM NaF, 1 mM Na-orthovanadate).

Flow cytometry.

Yeast cells were fixed with 70% ethanol and stored at 4 °C. Cells were then sonicated, treated with 0.25 mg ml^{-1} RNase A for 1 h at 50 °C, followed by digestion with 0.125 mg ml^{-1} proteinase K for 1 h at 50 °C and labelling with 1 μM Sytox Green (Invitrogen). Data were collected using a FACSCalibur (Becton Dickinson) and analysed with FlowJo software (Tree Star, Inc.).

Two-dimensional gel electrophoresis.

Asynchronous cultures were harvested at an optical density at 600 nm (OD600) of ~1. Synchronized cultures were held in G1 with α-factor until 90% of cells were unbudded. α-Factor was washed out and cells were re-suspended in media pre-warmed to 30 °C containing 200 mM hydroxyurea. At the indicated time points, aliquots were collected and all samples were processed as previously described(Friedman and Brewer, 1995). DNA was digested with Pst1 for ARS607, Xba1 for ARS501, and EcoR1 for ARS305 and ARS609. DNA probes were labelled using the Prime-it II kit (Stratagene).

Spot assays.

Mid-log cultures were diluted to OD600 = 0.5. Cultures were subjected to fivefold serial dilution and 5 μl were spotted on plates containing various hydroxyurea concentrations. Plates were subsequently incubated at 30 °C for 18–36 h. Hydroxyurea survival assay. Overnight cultures were diluted to OD600 = 0.3 and incubated at 30 °C

for 2 h before synchronizing in G1 with α-factor for an additional 2 h. α-factor was washed out and cells were re-suspended in pre-warmed media containing 200 mM hydroxyurea. At the indicated timepoints, 200 μl of each culture was diluted 5,000-fold in media and 200 μl of cells were plated and subsequently grown at 30 ^{o}C for 36 h. For asynchronous cultures, cells were diluted back to OD600 = 0.3 for 2 h before treating with 0 mM, 12.5 mM, 25 mM, or 50 mM hydroxyurea for 3 h. Cells were counted, diluted to 200 cells ml^{-1}, plated and grown at 30 ^{o}C for 36 h. Colonies were scored as per cent of untreated.

GFP microscopy.

Images of Rad52–GFP foci were obtained on a Leica DMRXA fluorescence microscope with a X100, 1.4 NA PlanApo Olympus Oil Immersion objective. GFP fluorescence was detected using a Chroma FITC filter set (excitation 485/20nm, emission 515/30nm). Images were taken with a Hamamatsu C4742-95 CCD camera. Data were analysed with OpenLab software (Improvision). Black and white images were all captured for the same exposure times, and contrast enhancement was performed equivalently on all panels.

Western blotting.

An equivalent of six OD600 cells were pelleted, washed with cold water, and re-suspended in pre-heated SDS sample buffer (50mM Tris pH7.5, 5mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.05% bromophenol blue, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 mg ml^{-1} PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate). Cells were lysed by bead beating for 3 min in a Mini BeadBeater (Biospec) using 100 μl glass beads. Samples

were then centrifuged at 15,000 r.p.m. and heated to 95 ^{o}C for 5 min and clarified by centrifugation. Extracts were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and probed with antibodies against Flag (Clone M2, Sigma), Rad53 (rabbit anti-Rad53 provided by D. Durocher), Myc (9E10) and the TAP tag (rabbit anti- CBP, provided by J. Weissman).

Protein preparations.

Eighteen liters of yeast cells were grown to an OD600 = 1.0 and Sld3–3Flag was induced for 6 h with 2% galactose and treated with 0.05% MMS or left untreated. Cells were re-suspended in 50 ml lysis buffer (25mM HEPES KOH pH 7.5, 150 mM KCl, 0.1 mM EDTA, 0.5 mM EGTA, 2mM MgCl2, 20% glycerol, 0.02% NP40, 1 mM DTT, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 mg ml^{-1} PMSF, 1 mM NaF, 1 mM Na-orthovanadate, 80 mM β-glycerolphosphate). Cells were lysed by five sequential passes through a microfluidizer (Microfluidics) with coil on iced water. Crude extracts were clarified by centrifugation and clarified extracts were incubated with 500 ml M2-Flag resin (Sigma) for 4 h at 4 ^{o}C. Beads were washed 3X with 10 ml lysis buffer supplemented with 300 mM KCl. Sld3 was eluted from beads with five 500- μl elutions using 150 ng ml^{-1} 3xFlag peptide.

Dbf4–MORF (movable ORF; Thermo Scientific) was overexpressed from 6 l of yeast in synthetic complete-Uracil drop-out medium plus 2% galactose for 6 h and treated with 0.05% MMS. Cells were lysed by bead beating in lysis buffer (25 mM HEPES HCl pH8.0, 300mM NaCl, 0.1% NP40, 1mM EDTA, 33mM EGTA, 1 mM PMSF, 50 mM NaF, 80 mM β-glycerolphosphate, 1 mM Na-orthovanadate, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine). Crude extracts were clarified by centrifugation and

clarified extracts were incubated with 200 μl IgG sepharose (GE Healthcare) for 2 h at 4 ^{o}C. Beads were washed 3X in 10 ml wash buffer (25 mM HEPES HCl pH 8.0, 300 mM NaCl, 0.1% NP40). Dbf4 was eluted from beads in sample buffer and separated by SDS–PAGE. Dbf4 was excised from silver-stained gel for mass spectrometry.

GST–SLD3–3Flag and GST–SLD3-m25–3Flag were expressed and purified from Rosetta (DE3) pLysS cells (Novagen). Expression was induced with 0.5 mM IPTG at 19 ^{o}C for 15 h and lysed in 1x PBS pH 7.4 supplemented with 1% Triton X-100, 1 mM EDTA, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 mg ml^{-1} PMSF, 10 μg ml^{-1} RNase A, 20 U DNase. Crude extracts were centrifuged at 10,000 g for 30 min at 4 ^{o}C (Beckman JA25.50). Clarified extracts were then incubated with 500 μl M2-Flag resin (Sigma) for 2.5 h at 4 ^{o}C. Resin was washed five times in 10 ml 1x PBS 1 mM EDTA. Proteins were eluted five times with 500 ml 3xFlag peptide (150 ng ml^{-1}) in 1x PBS. Proteins were then dialysed overnight against 1x PBS 10% glycerol at 4 ^{o}C before being frozen.

CDK Clb5–TAP was overexpressed in 3 l of yeast for 6 h in synthetic complete-Uracil drop-out medium plus 2% galactose. Cells were harvested and washed once with cold water and frozen in liquid nitrogen. Cells were lysed by bead beating in 40 ml lysis buffer (25 mM HEPES HCl pH 8.0, 300 mM NaCl, 0.1% NP40, 1 mM EDTA, 33 mM EGTA, 1 mM PMSF, 50 mM NaF, 80 mM β-glycerolphosphate, 1 mM Na-orthovanadate, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine). Crude extracts were centrifuged at 15,000 r.p.m. for 30 min at 4 ^{o}C. Cleared extracts were then incubated with 200 μl IgG-sepharose (50% slurry: GE Healthcare) for 2 h at 4 ^{o}C. Resin was washed five times with wash buffer (25 mM HEPES HCl pH 8.0, 300 mM NaCl,

0.1% NP40) followed by two washes with TEV cleavage buffer (25 mM HEPES HCl pH 8.0, 300 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT, 10% glycerol). CDK Clb5 was cleaved off resin in 200 μl TEV buffer supplemented with 10 μl TEV protease.

6xHis–Rad53 and 6xHis–Rad53(K227A) (plasmids were provided by D. Durocher) were induced in 1 l Rosetta(DE3)pLysS cells (Novagen) with 1 mM IPTG for 2 h. Cells were harvested and lysed in 20 ml lysis buffer (50 mM HEPES HCl pH 7.4, 300 mM NaCl, 5 mM imidazole, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 mg ml^{-1} PMSF). Clarified extract was incubated with 200 μl Ni-NTA resin (50% slurry: Qiagen) for 2 h at 4 ^{o}C. Resin was washed three times in 10 ml wash buffer (50 mM HEPES HCl pH 7.4, 300 mM NaCl, 20 mM imidazole). Proteins were eluted twice with two-column volumes elution buffer (50 mM HEPES HCl pH 7.4, 300 mM NaCl, 250 mM imidazole). Eluted proteins were dialysed overnight against 20 mM HEPES HCl pH 7.5, 10% glycerol, aliquoted and frozen.

***In vitro* kinase and binding assay.**

Kinase reactions were done in 15 μl kinase buffer (20 mM Tris-HCl pH 7.5, 20 mM MgCl2, 2 mM MnCl2, 1 mM DTT, 1 mM ATP) for 30min at room temperature using ~ 150 nM substrate and ~ 40 nM Rad53 or 25 nM Clb5 CDK. Reactions were stopped by adding sample buffer and reactions were separated by SDS–PAGE. Binding reactions were done essentially as described (Tanaka et al., 2007).

Mass spectrometry.

TCA precipitates were re-suspended in digestion buffer (100mM Tris-HCl, pH8.5, 8M urea), digested by the sequential addition of lys-C and trypsin proteases, and analysed using shotgun proteomics methods on an LTQ-Orbitrap mass spectrometer

(Thermofisher) as previously described(Florens et al., 2006; Washburn et al., 2001; Wohlschlegel, 2009; Wolters et al., 2001). SEQUEST and DTASelect algorithms were used to identify peptide sequences from tandem mass spectra (Eng, 1994; Tabb et al., 2002). Phosphopeptides were identified using a differential modification search that considered a mass shift of 179.9663 on serines, threonines and tyrosines. If a phosphopeptide contained multiple phosphorylatable residues that prevented the confident identification of the exact residue that was phosphorylated, all potential phosphoacceptor residues in the peptide were considered as candidates for mutagenesis.

Chapter 4

Checking in on Origins of Replication

Every S-phase, eukaryotic cells are faced with the tremendous challenge of faithfully replicating their genomes to completion. The physical process of DNA replication is a vulnerable stage in a cell's life as numerous sources, both endogenous and exogenous, can directly cause DNA lesions and, when left unchecked, can contribute to genomic instability and cancer. Eukaryotic cells, however, have evolved intricate DNA surveillance systems termed DNA damage checkpoints that detect lesions, modulate cell-cycle progression and coordinate DNA repair. The intra-S-phase checkpoint is a well-characterized signaling network that responds to DNA lesions and fork stalling during the S-phase. One attribute of the intra-S-phase checkpoint is the ability to inhibit DNA replication by regulating origin activation (Santocanale and Diffley, 1998; Shirahige et al., 1998). This physiological response was first characterized in cells from patients with ataxia telangiectasia (Painter and Young, 1980). Because these cells harbor a dysfunctional ATM kinase, they cannot respond to double strand breaks and thus are incapable of inhibiting DNA synthesis (Young and Painter, 1989). PI3 kinase-related kinases, ATM/Tel1 and ATR/Mec1, are the most upstream members of this signaling pathway and these kinases are recruited to double strand breaks or stalled replication forks, respectively. Once recruited, activated ATR/Mec1 will in turn activate the effector kinase Chk2/Rad53 to phosphorylate downstream targets.

Many important advances in our understanding of both DNA damage checkpoint regulation and DNA replication have been made utilizing the model system, *Saccharomyces cerevisiae*. Origin activation requires the ordered recruitment of many replication factors onto specific DNA sequences that will serve as origins of replication. The origin recognition complex (ORC) serves as a platform for subsequent loading of

Cdc6 and Cdt1. The Cdc6/Cdt1 complex is first to load at origins of replication, and this in turn facilitates the loading of the mini-chromosome maintenance (MCM) complex, the key component of the replicative helicase. This establishes what is collectively known as the pre-replication complex (pre-RC). In most eukaryotic organisms, origin activation follows a temporal program where origins are activated throughout the duration of S-phase. The molecular mechanism responsible for the temporal program of origin activation is currently not understood. In order to achieve origin activation, transformation of pre-RCs into pre-initiation complexes (pre-ICs) must occur upon entering S-phase. This task is achieved by the combined action of two kinases- CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase). In budding yeast, CDK phosphorylates at least the two essential replication factors: Sld3 and Sld2 (Tanaka et al., 2007; Zegerman and Diffley, 2007). CDK-dependent phosphorylated forms of Sld3 and Sld2 simultaneously bind to Dpb11. DDK, on the other hand, targets the MCMs by phosphorylating several of its subunits. Recently Mcm4 emerged as being a critical *in vivo* DDK target. DDK-dependent phosphorylation of Mcm4 alleviates an intrinsic inhibitory function of Mcm4 and thereby contributes to the net activation of the MCMs (Sheu and Stillman, 2010). By an unknown mechanism, the Sld3-Dpb11-Sld2 complex, together with phosphorylated MCMs, supports recruitment of additional replication factors, including Cdc45, MCM10, the GINS and DNA polymerases. Therefore, the concomitant activities of both CDK and DDK are strictly required to trigger activation of early and late origins of replication.

In response to checkpoint activation, metazoans inhibit origin firing by at least two different methods. Cells elicit a strong G1 checkpoint that prevents cells from

entering S-phase. This G1 checkpoint is achieved by the Chk2 dependent phosphorylation of Cdc25A, a phosphatase that removes inhibitory tyrosine phosphorylations on CDK2. Cdc25A, following Chk2 phosphorylation, is subsequently destroyed by the ubiquitin-proteasome system (Falck et al., 2002). Thus CDK2 remains inhibited and cells cannot proceed past the G1-S transition. The second method is by inhibiting origin activation directly. A hallmark of this response is the inhibition or reduction of Cdc45 loading onto chromatin in DNA damage treated cells.

In the budding yeast, the intra-S-phase checkpoint also functions to inhibit origin activation (Paulovich and Hartwell, 1995). While the DNA damage checkpoint aatenuates CDK activity in metazoans, the same does not occur in budding yeast. High CDK activity is a regulatory mechanism that prevents re-activation of fired origins and thus lowering CDK activity in S-phase would allow yeast cells to incur the detrimental consequences of re-replication. Instead, the intra-S-phase checkpoint circumvents lowering CDK activity by inhibiting essential CDK substrates. Recently, we, and others, showed that the kinase Rad53 blocks pre-IC formation at late origins of replication upon intra-S-phase checkpoint activation by phosphorylating Sld3 and Dbf4, (Duch et al., 2010; Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Rad53 phosphorylates Sld3 *in vitro*, suggesting that Rad53 directly phosphorylates Sld3 *in vivo*. Importantly, the Rad53-dependent phosphorylation of Sld3 prevents its S-CDK mediated binding to Dpb11 (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010), and also inhibits Sld3-Cdc45 interactions (Zegerman and Diffley, 2010). The intra-S-phase checkpoint also targets Dbf4, however, the precise role of these phosphorylations is not entirely understood. Several studies have suggested that this phosphorylation reduces DDK

activity and/or displaces it from origins (Pasero et al., 1999; Weinreich and Stillman, 1999). Since DDK activity is required throughout S-phase to activate each origin, it is interesting to speculate whether the inhibitory function of Mcm4, at late/unfired origins, as a direct result of Rad53-dependent DDK inhibition.

Although the molecular players involved in origin activation are presumed to be the same for all origins, only late activating origins of replication are inhibited by the intra-S-phase checkpoint. This begs the question of why and how early origins escape checkpoint inhibition. It is generally thought that early origins of replication are refractory to intra-S-phase checkpoint inhibition. At least two groups have detected the presence of Sld3 and Cdc45 at early origins, but not late origins, in G1 arrested cells (Aparicio et al., 1999; Kamimura et al., 2001; Kanemaki and Labib, 2006). While the biological significance of this preferential association is unknown, it was possible that Sld3, once bound to early origins, was resistant to checkpoint inhibition and thereby allowed early origins to escape checkpoint inhibition. However, two lines of evidence argue against this model. First, yeast cells expressing an *SLD3* phospho-mimetic allele (*SLD3-m21D:* 21 serines and threonines targeted by Rad53 are mutated to aspartic acids) do not arrest at the G1/S transition. If Sld3 molecules that were already associated with preRCs in G1 were merely refractory to Rad53, the phosphomimic would nonetheless be able to inhibit these origins. Instead, cells harboring the *SLD3-m21D* allele proceed slowly through S-phase, without concomitant Rad53 activation (Lopez-Mosqueda et al., 2010), and appear to fire early origins at wild-type levels. Secondly, damage-induced Sld3 phosphorylation in anaphase-arrested cells does not result in a block to DNA replication in the subsequent cell cycle (*unpublished results*). Given that pre-Rc

formation occurs immediately following anaphase onset, we reasoned that if damage-induced Sld3 phosphorylation was achieved before pre-RC formation that in the next S-phase, phosphorylated Sld3 forms would, in theory, prevent activation of early origins. To test this hypothesis, we utilized a temperature sensitive *cdc15-2* allele to block cells after chromosome segregation (at the restrictive temperature) and subsequently treated arrested cells with zeocin to cause DNA damage. We then removed cells from zeocin and released them into medium containing BrdU at the permissive temperature while simultaneously over-expressing separase to bypass securin inhibition (Tinker-Kulberg and Morgan, 1999). We found that damage treated cells incorporated BrdU and progressed into S-phase despite the presence of phosphorylated Sld3 and Rad53. Taken together, these observations suggest that while Rad53-dependent phosphorylation of Sld3 may be required, in part, to block the pre-RC to pre-IC transformation at late replicating origins, the same is not the case for early replicating origins. Coincidently, Dbf4 cannot be inhibited in G1 arrested cells as Dbf4 is a target of the APC/C and thus is not physically present to be inhibited by Rad53 (Ferreira et al., 2000; Weinreich and Stillman, 1999).

Origins of replication are activated throughout an unperturbed S-phase. While we now understand that by phosphorylating Sld3 and Dbf4 upon intra-S-phase checkpoint activation cells block late origin firing to effectively slow S-phase progression, we do not understand the temporal regulation of origin activation. Perhaps once we learn the precise mechanisms that govern the temporal activation of origins we might also learn why and how early origins are refractory to Rad53 dependent regulation.

Chapter 5

Cdc5 Blocks In Vivo Rad53 Activity, But Not In Situ Activity (ISA)

DNA damage promotes the activation of a signal transduction cascade referred to as the DNA damage checkpoint. This pathway initiates with the Mec1/ATR kinase, which then phosphorylates the Rad53/Chk2 kinase. Mec1 phosphorylation of Rad53 is then thought to promote Rad53 auto-phosphorylation, ultimately leading to a fully active Rad53 molecule that can go on to phosphorylate substrates important for DNA damage resistance. In the absence of DNA repair, this checkpoint is eventually down-regulated in a Cdc5-dependent process referred to as checkpoint adaptation. Recently, we showed that over-expression of Cdc5 leads to checkpoint inactivation and loss of the strong electrophoretic shift associated with Rad53 inactivation. Interestingly, this same over-expression did not strongly inhibit Rad53 auto-phosphorylation activity as measured by the in situ assay (ISA). The ISA involves incubating the re-natured Rad53 protein with γ-32P labeled ATP after electrophoresis and Western blotting. Using a newly identified Rad53 target, we show that despite strong ISA activity, Rad53 does not maintain phosphorylation of this substrate. We hypothesize that, during adaptation, Rad53 may be in a unique state in which it maintains some Mec1 phosphorylation, but does not have the auto-phosphorylations required for full activity towards exogenous substrates.

The signaling network that responds to DNA double stranded breaks (DSBs) has been well characterized. Two upstream PI3 kinase-related kinases, called Mec1/ ATR and Tel1/ATM, are recruited to the DNA damage site, resulting in their activation. In the case of Mec1/ATR, this activation is mediated, at least in part, by the co-recruitment of a PCNA-related complex called the 9-1-1 complex (Bonilla et al., 2008; Kondo et al., 1999; Majka et al., 2006; Melo et al., 2001; Navadgi-Patil and Burgers, 2009). This 9-1-1 complex both directly and indirectly (through the Dpb11 protein) activates

Mec1(Kumagai et al., 2006; Mordes et al., 2008a; Navadgi-Patil and Burgers, 2009). Mec1 (and Tel1) then phosphorylates an adaptor molecule, Rad9, which is also recruited to the damage site (Emili, 1998; Sun et al., 1998; Vialard et al., 1998). Rad9 phosphorylation promotes its association with the FHA domains of the Rad53 kinase, which is subsequently primed by Mec1/Tel1 phosphorylation. In a poorly defined process, this Rad9-associated and primed Rad53 is then competent to undergo auto-phosphorylation, resulting in a fully activated kinase (Gilbert et al., 2001).

Previously, we have shown that, in *Saccharomyces cerevisiae*, irreparable DSBs do not lead to permanent activation, but instead result in attenuation of the checkpoint signal after approximately six hours (Lee et al., 1998; Sandell and Zakian, 1993; Toczyski et al., 1997). A loss of function allele of the essential polo kinase CDC5, called *cdc5- ad*, was found to disable this process, leading to persistent checkpoint signaling in response to irreparable breaks (Toczyski et al., 1997). Conversely, over-expression of Cdc5, but not *cdc5-ad*, leads to premature loss of checkpoint signaling (Vidanes et al., 2010). We have suggested that Cdc5-mediated checkpoint inactivation specifically inactivates the step of Rad53 auto-phosphorylation, since Rad9 phosphorylation and its binding to Rad53 are maintained (Vidanes et al., 2010). While it was not possible to uniquely examine Mec1 priming in this scenario, several observations suggested it was maintained. First, Mec1 phosphorylation of Rad9 was largely intact, suggesting that Mec1 kinase activity was not significantly compromised. Second, Rad53 retained a basal electrophoretic shift, consistent with Mec1 priming. Finally, Rad53 activity in vitro, as measured by the in situ assay (ISA), was also largely present, despite the fact that the strong electrophoretic shift associated with Rad53 auto-phosphorylation was lost. *CDC5* over-expression was

presumed to inhibit Rad53 activity *in vivo* based upon both the loss of an electrophoretic shift and the observation that the checkpoint arrest itself was compromised. However, this was difficult to examine directly, since we lacked a direct Rad53 substrate showing a robust (and therefore tractable) electrophoretic phospho-shift.

We now examine Rad53 activity *in vivo* by examining the Rad53-dependent electrophoretic shift of Sld3. Sld3 is an essential protein required for origin firing (Kamimura et al., 2001). Our laboratory and the Diffley laboratory recently showed that Sld3 is phosphorylated upon DNA damage in a Rad53-dependent manner (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Moreover, Rad53 directly phosphorylates Sld3 *in vitro*, suggesting that it is a direct Rad53 target *in vivo*. The phosphorylation of Sld3 inhibited the ability of Sld3 to promote origin firing. To determine whether Sld3 phosphorylation is lost upon *CDC5* over-expression, we examined both Rad53 and Sld3 during a time course of *CDC5* over-expression. Cells were damaged for two hours before *CDC5* expression was initiated by the addition of galactose. Nocodazole was added with galactose to maintain the G2/M arrest, such that our results were not confounded by the cell cycle differences between the *CDC5* over-expressing cells (which adapt to the arrest) and control cells lacking the *GAL-CDC5* construct. In control samples, both Sld3 and Rad53 phosphorylation was maintained throughout the time course (Fig. 1). In contrast, both Sld3 and Rad53 lost their hyper-phosphorylation one hour after galactose addition (the three hr time point). As seen in our previous experiments, Rad53 ISA activity remained high, despite loss of the hyper-phosphorylation normally associated with high Rad53 ISA activity. In fact, Rad53

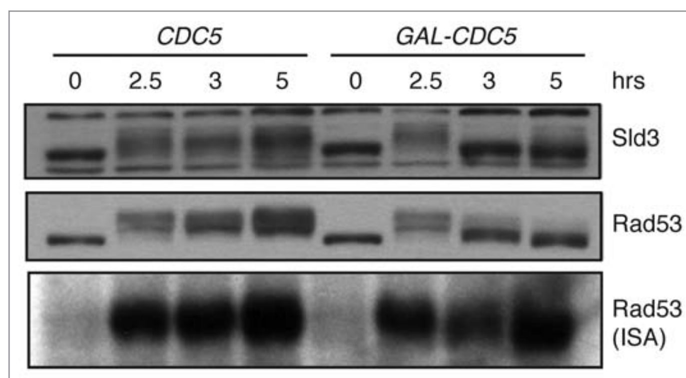

Figure 1. **In vivo inhibition of Rad53 activity by *CDC5* over-expression**. DNA damage was induced at the zero time point in strain yDPT195-1 (*cdc13-1 SLD3-3xFLAG::Hyg*) and yDPT196-1 (*cdc13-1 SLD3-3xFLAG::Hyg URA3::pGal-CDC5*). After overnight growth in rich media with 2% raffinose, culture were shifted to 32°C, the non-permissive temperature of the *cdc13-1* allele. After two hours, 10 μg/mL nocodazole and 2% galactose, to induce *CDC5*, were added to the cultures. The samples were collected at the indiciated time points and analyzed by western blot and ISA. Sld3 and Rad53 were detected in the Western blot with α-FLAG and α-Rad53 (DAB001, gift from the Durocher lab) antibodies, respectively.

activity was observed in the faster mobility band that runs similarly to the mobility of the unphosphorylated, inactive Rad53 protein (Vidanes et al., 2010). Thus, loss of Rad53 activity in vivo does not necessarily correlate with loss of ISA activity.

The ISA assay was developed to measure Rad53 autophosphorylation activity (Pellicioli et al., 1999). Rad53 isolated from DNA-damaged cells has significantly more ISA activity than Rad53 isolated from un-damaged cells (Pellicioli et al., 1999). Despite the fact that the ISA is widely used, it is unclear what it is actually measuring. Exactly which biological aspects of Rad53 activation are required for this activity is not known, nor is it clear what the phosphorylated substrate is. During this procedure, total protein lysates are renatured on a PVDF membrane. The membrane is subsequently blocked with BSA and incubated with γ-32P labeled ATP for the kinase reaction. It is possible that this BSA serves as a heterologous substrate for the activated kinase. Alternatively, Rad53 could serve as both kinase and substrate. Recombinant Rad53 becomes extensively phosphorylated in bacteria (even more so than in yeast cells after DNA damage), and this material is active against histone H1 in vitro. However, it is only poorly active in an ISA unless it is first partially dephosphorylated *in vitro*, suggesting that Rad53 is indeed both substrate and enzyme in the ISA and that the hyper-phosphorylated Rad53 has no available sites for further auto-phosphorylation (Ma et al., 2006). Importantly, bacterially expressed Rad53 is reactive with antibodies against the Mec1/Tel1-phosphorylated residues ([S/T]Q), suggesting that at extremely high concentrations, Rad53 can ectopically hit these sites; therefore, ISA activity of recombinant Rad53 may still require priming phosphorylation of Mec1-sites on Rad53 (Ma et al., 2006). In this sense, the ISA would reflect the second, auto-phosphorylation step of checkpoint activation. A priori, it

must be the case that primed but un-autophosphorylated Rad53 is competent, at some level, to phosphorylate another Rad53 molecule, since, after a DSB, the first activated Rad53 molecule has not, by definition, already been Rad53-phosphorylated. Rad53 trans-phosphorylation may simply require basal Rad53 activity coupled to direct juxta-position of two Rad53 molecules. This could be mediated by multiple Rad53 molecules binding Rad9, followed by direct Rad53-Rad53 interactions. Mec1 phosphorylation of the N-terminus of Rad53 promotes Rad53-Rad53 interactions through Rad53's FHA1 domain (Lee et al., 2003). Thus, Mec1 priming phosphorylation of Rad53 may be the critical modification required for ISA activity. Mec1 (or Tel1) activity is, in fact, required for ISA activity. However, in the absence of these upstream kinases, Rad53 auto-phosphorylation does not occur, and thus it is impossible to distinguish whether it is the Mec1 phosphorylations, per se, that are required for the ISA. It should be noted that one of the Rad53 auto-activation sites that has been mapped corresponds to the Rad53 activation loop (Sweeney et al., 2005). Activation loop phosphorylation increases the activity of many kinases, suggesting that auto-activation may also contribute to ISA activity (Nolen et al., 2004).

While we do not know the exact method by which Cdc5 inactivates Rad53, our data are consistent with a model in which Cdc5 phosphorylation of either Rad53 or Rad9 eliminates the ability of Rad9 to promote auto-phosphorylation of Rad53. This model is consistent with our observation that the ISA activity of Rad53 isolated from Cdc5 over-expressing cells is high, despite the fact that it is neither hyper-phosphorylated nor competent to promote Sld3 phosphorylation in vivo. Alternatively, it may be that the hypo-phosphorylated Rad53 that is observed upon Cdc5 over-expression still retains a

subset of the auto-phosphorylation sites. These sites may be required for in vivo activity, by localizing Rad53 or promoting substrate interactions, but may not be required for in vitro activity. Interestingly, the human homolog of Cdc5, Plk1, also negatively regulates the mammalian checkpoint pathway (van Vugt and Medema, 2005). This regulation may also target 53BP1 and Chk2, the mammalian equivalents of Rad9 and Rad53 respectively, suggesting that this mode of inhibition is likely conserved (van Vugt et al., 2010).

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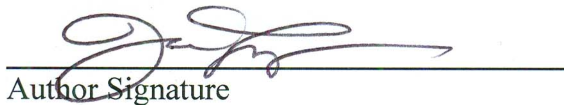

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