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# Checkpoint Dependent Inhibition of DNA Replication Initiation by SId3 and Dbf4 Phosphorylation

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# Summary

The initiation of eukaryotic DNA replication is regulated by three protein kinase classes: cyclin dependent kinases (CDK), Dbf4-dependent kinase (DDK) and the DNA damage checkpoint kinases1. CDK phosphorylation of two key initiation factors, Sld2 and Sld3, promotes essential interactions with Dpb112-4 whilst DDK acts by phosphorylating subunits of the Mcm2-7 helicase5. CDK plays an additional role in replication by preventing the re-loading of Mcm2-7 during S, G2 and M phases6, thus preventing origin re-firing and re-replication. During G1-phase, both CDK and DDK are down-regulated, which allows origin licensing and prevents premature replication initiation3. Origin firing is also inhibited during S-phase when DNA damage or replication fork stalling activates the checkpoint kinases7-10. Here we show that, analogous to the situation in G1 phase, the checkpoint kinase Rad53 inhibits both CDK- and DDK-dependent pathways, which acts redundantly to block further origin firing. Rad53 acts on DDK directly by phosphorylating Dbf4, whereas the CDK pathway is blocked by Rad53 phosphorylation of the downstream CDK substrate, Sld3. This allows CDK to remain active during S phase in the presence of DNA damage, which is crucial to prevent reloading of Mcm2-7 onto origins that have already fired6. Our results explain how checkpoints regulate origin firing and demonstrate that the slowing of S phase by the 'intra-S checkpoint' is primarily due to the inhibition of origin firing.

> Activation of the DNA damage checkpoint kinases in S-phase regulates genomic replication in at least two ways: firstly by protecting stalled replication forks11-14 and secondly by blocking further origin firing7-10. To determine whether the DNA replication machinery is directly regulated by checkpoints, we set out to identify Rad53 substrates *in vivo* in the budding yeast, Saccharomyces cerevisiae. A library of tagged replication proteins was screened for Rad53-dependent reduction in mobility in SDS-PAGE after treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU) (Supp. Table 1). Two essential replication factors exhibited HU- and Rad53-dependent shift in mobility (Figure 1a,b): Dbf4, a previously characterised Rad53 substrate15, and Sld32,3. Phosphatase treatment of Sld3 purified from HU-treated yeast cells converted the slower migrating bands into a single, faster migrating band (Figure 1b) indicating that Sld3 is phosphorylated in an HUand Rad53-dependent manner. Phosphorylation of Sld3 also occurred in cells entering S phase in the presence of the DNA alkylating agent methyl methanesulfonate (MMS), but did not occur in an unperturbed S-phase (Supp. Figure 1a). Sld3 phosphorylation did not require Dun1 (Supp. Figure 1a), a checkpoint kinase downstream of Rad53. These data indicate that, like Dbf4, Sld3 is a target of Rad53 in vivo.

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The magnitude of the shift and the multitude of bands seen in SDS-PAGE (Figure 1b) indicated that the serine/threonine-rich Sld3 protein (Figure 1c) is multiply phosphorylated after checkpoint activation. We used purified Rad53 to phosphorylate a series of Sld3 fragments (Figure 1c) in vitro. Figure 1d (and Supp Figure 1b) shows that Rad53 predominately phosphorylates Sld3 in two C-terminal fragments. To compare this to phosphorylation in vivo, we expressed and purified a version of Sld3 in which the N- and Cterminal domains were tagged and could be separated by cleavage with TEV protease (Figure 1c top). Figure 1e shows that only the C-terminal domain of Sld3 exhibited a  $\lambda$ phosphatase-sensitive shift in SDS PAGE after HU treatment in vivo. To identify individual Rad53 sites in Sld3 in vitro we phosphorylated arrays of peptides corresponding to the entire Sld3 amino acid sequence attached to a cellulose membrane. Consistent with Figure 1d, most of the phosphorylated peptides occurred within the C-terminal domain of Sld3 (Supp. Figure 1c). Because of the extensive overlap in the peptides on the array (Supp. Figure 2a-d) most sites could be identified unambiguously. All 38 potential serine and threonine phosphorylation sites were mutated to alanine (Figure 1c and Supp. Table 2). Compared to the wild type protein, this allele of Sld3 (sld3-A) shifted only slightly in SDS-PAGE after HU treatment (Figure 1f). A variety of sld3 mutants containing subsets of the 38 sites mutated to alanine all show reduced phosphorylation shift indicating that many or most of the sites contribute to the full phosphorylation shift and Sld3 inhibition *in vivo* (Supp. Figure 2e,f). The residual shift in *sld3-A* may be due to additional sites missed in our analysis or may due to be cryptic sites only phosphorylated when the stronger sites in the wild type protein are absent. Yeast strains expressing *sld3-A* as the sole copy of Sld3 showed no sensitivity to HU or DNA damaging agents and did not exhibit synthetic growth defects with several conditional alleles of essential replication proteins (Supp. Figure 3) arguing that the Sld3-A protein is functional for DNA replication.

These Rad53 sites are primarily in the C-terminal portion of Sld3, where the essential CDK sites (Thr600, Ser622) are found (Figure 1c). Figure 2a shows that, whilst CDK phosphorylation of the C-terminus of Sld3 in vitro allows direct binding to Dpb11 but not to a Dpb11 truncation lacking the first BRCT repeat ( $\Delta N$ ); however, subsequent Rad53 phosphorylation of Sld3 inhibits interaction with Dpb11. Mutation of the strongest Rad53 sites in the C-terminus of Sld3 to aspartate residues (*sld3-12D* – Figure 1c, Supp. Table 2), to mimic constitutive phosphorylation, also blocks interaction with Dpb11 in vitro (Figure 2b) without blocking CDK phosphorylation (Supp. Figure 4) and is unable to support growth in vivo (Figure 2c). The CDK-dependent interaction between Sld3 and Dpb11 can be bypassed in vivo by direct covalent fusion of these proteins3. Figure 2c shows that fusion of the Sld3-12D mutant protein to Dpb11 restored its ability to support growth. This argues that Sld3 phosphorylation by Rad53 inhibits its ability to interact with Dpb11 in vitro and in vivo. Two additional Rad53 phosphorylation sites (Ser306, Ser310) that contribute to the Rad53 phosphorylation-dependent shift of Sld3 in SDS-PAGE (Supp. Fig. 2e) lie considerably further upstream of the Dpb11 interaction domain. Mutation of these two sites to aspartate in the *sld3-12D* background also produced a protein (Sld3-14D – Figure 1c) that could not interact with Dpb11 (Figure 2b) and could not support growth (Figure 2c). In contrast to *sld3-12D*, however, *sld3-14D* could not support growth after fusion to Dpb11 (Figure 2c). Previous work has shown that Sld3 also interacts with Cdc45 and the GINS subunit Psf1 in a two hybrid assay16. A mutant protein in which Ser306 and Ser310 were changed to aspartate (Sld3-2D) interacted with both Dpb11 and Psf1 in a two hybrid assay (Figure 2d). However, compared to wild type, Sld3-2D showed a reduced interaction with Cdc45. Consistent with this weakened interaction, over-expression of Cdc45 allowed the Sld3-14D-Dpb11 fusion to support growth (Figure 2e). These results indicate that Sld3 phosphorylation by Rad53 inhibits interactions with both Dpb11 and Cdc45. A mutant of Sld3 in which 34 Rad53 sites were converted to aspartate (sld3-34D - Figure 1c) did not support growth, even when fused to Dpb11 (Figure 2c) in the presence of over-expressed

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Cdc45 (data not shown). This suggests that additional functions of Sld3 may be inhibited in the fully phosphorylated molecule.

Since Sld3 is essential for replication initiation but not elongation17 and interactions with Cdc45 and Dpb11 are essential for its function, we hypothesised that phosphorylation of Sld3 by Rad53 may act to prevent late origin firing. Mutation of these sites to alanine might, therefore bypass the ability of Rad53 to block late origin firing. Figure 3a lanes 1-4 (bottom panel) shows, however, that the sld3-A allele by itself was not sufficient to allow accumulation of replication intermediates (RI) from the late origin ARS501 in HU. Since Dbf4 is also a substrate of Rad53 (Figure 1a), we next tested the possibility that Rad53 inhibits both Sld3 and Dbf4. We combined the *sld3-A* allele with the *mcm5-bob1* mutant, which bypasses the requirement for Cdc7-Dbf4 in vivo18. Similar to sld3-A, the mcm5*bob1* mutant by itself did not allow firing of ARS501 in HU (Figure 3A, lanes 5-8). However, Figure 3a shows that the combination of *sld3-A* with *mcm5-bob1* allowed ARS501 firing in HU (Figure 3a, lanes 9-12). The timing and extent of Rad53 activation as judged by its hyperphosphorylation was similar in these strains (Figure 3b), indicating that late origin firing in the double mutant is not due to a defect in checkpoint activation. Indeed, the extent of Rad53 phosphorylation appears greater in the double mutant, consistent with an increased number of origins being fired and, hence, an increased number of stalled replication forks (Ref  $^{19}$  – see also Figure 3d and Supp Figure 6,7).

We used the peptide array approach described above to identify 19 Rad53 phosphorylation sites in Dbf4 (Supp Figure 5a and Supp Table 3). Mutation of all these sites to alanine (*dbf4-19A*) significantly reduced the shift of Dbf4 in HU (Figure 3c). Combination of *dbf4-19A* with *sld3-A* resulted in the activation of several late origins in HU as shown by the accumulation of replication intermediates both by 1D electrophoresis (Figure 3d and Supp. Figure 5b,c) and 2D electrophoresis (Figure 3e). Rad53 activation was similar in the single and double mutant strains (Figure 3d, lower panel).

Strains expressing *dbf4-19A*, however, were unable to grow at 37°C (Supp. Figure 6a). We employed two approaches to exclude that the deregulation of late origin firing was linked to this temperature sensitivity. Firstly, we identified four serine residues in Dbf4 which, when changed to alanine, generated a mutant (dbf4-4A) that allowed origin firing in HU when combined with *sld3-A* but was no longer temperature sensitive (Supp. Figure 6b). Secondly, we reasoned that if deregulation of late origin firing was linked to loss of Dbf4 function, then both temperature sensitivity and late origin firing should be recessive; however, if dbf4-19A contributes to late origin firing in HU because it is refractory to Rad53 inhibition, it should be dominant. We introduced the *sld3-A* and *dbf4-19A* alleles as second copies into cells containing wild type DBF4 and SLD3. This strain is no longer temperature sensitive (Supp. Figure 6b), and the late origin ARS501 fires in HU (Fig. 3f). Therefore, deregulation of late origin firing in HU is not due to a recessive defect in the Sld3/Dbf4 alanine mutants. This also argues that Rad53 must inhibit the entire cellular pools of Sld3 and Dbf4 to completely block origin firing, which is consistent with the fact that the entire population of both Sld3 and Dbf4 appears phosphorylated in HU (Figure 1b and Figure 3c). Taken together, these results show that Rad53 prevents origin firing redundantly by phosphorylating both Sld3 and Dbf4. As is the case for both  $rad53\Delta$  and  $mrc1\Delta$ cells8,20,21, the order of origin firing in *dbf4-19A*, *sld3-A* appears unaffected since the late origins ARS501, ARS603 and ARS1412 fire after the early origins, ARS305, ARS607 and ARS315 in HU (Figure 3d and Supp. Figure 5b) suggesting that the temporal order of origin firing may remain intact.

In contrast to wild type cells, *rad53* mutant cells traverse S phase quickly in the presence of MMS22 at least partly because of global derepression of origin firing11. This 'intra-S

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checkpoint' is lost in the dbf4-4A, sld3-A double mutant because its S phase in MMS is at least as fast as that of the  $rad53\Delta$  sml1 $\Delta$  strain. Rad53 activation occurred normally in the dbf4-4A, sld3-A double mutant (Figure 4b). Therefore, origin firing appears to be globally derepressed in this double mutant. The dbf4-4A, sld3-A double mutant actually traverses S phase slightly faster than the rad53 mutant in MMS. Rad53 is also required to stabilise forks that have stalled11-14,19. rad53 mutants do not resume DNA synthesis efficiently after an HU arrest; however, the dbf4-4A, sld3-A double mutant is capable of resuming replication with similar kinetics to the wild type strain (Supp Figure 7a). Consistent with this, the dbf4-4A, sld3-A double mutant retained high viability during this rapid S phase in the presence of MMS (Figure 4c) as well as in the presence of high concentrations of hydroxyurea (Supp. Figure 7b). Under both conditions the rad53 mutant lost viability. This provides additional evidence that the primary role of Rad53 in maintaining cell viability after DNA damage correlates with the protection of replication forks. The checkpointdependent block to late origin firing appears to make only a minor contribution to cell viability after genotoxic stress. It will be interesting to determine whether it contributes quantitatively to genome stability.

Checkpoint-dependent inhibition of origin firing is a conserved feature of eukaryotic DNA replication. Here we have shown that the checkpoint inhibits origin firing in budding yeast by interfering with both the CDK- and DDK-dependent activation of origins: by phosphorylation of Sld3, which prevents interaction with Cdc45 and Dpb11, and by phosphorylation of Dbf4. How DDK is inhibited by Dbf4 phosphorylation is presently unclear. Inhibition of late origin firing by Rad53 is analogous to the inhibition of replication during G1 phase (Figure 4d) when initiation is prevented by the inhibition of both CDK and DDK. CDK cannot be inhibited during S phase in budding yeast because it is required to prevent the re-licensing of early-firing origins6. In higher eukaryotes, the DNA replication checkpoint may inhibit CDK activity, for example through phosphorylation and degradation of the Cdc25 phosphatases23, since there are CDK-independent pathways that also prevent re-licensing, such as geminin6 and PCNA-dependent Cdt1 degradation24. However, Cdc25 degradation is unlikely to represent the sole mechanism by which origin firing is inhibited in mammalian cells25,26. It is likely that the checkpoint in higher eukaryotes also targets replication initiation factors directly and Dbf4 has indeed been shown to be a checkpoint substrate in other organisms15. An orthologue of Sld3, Treslin/ticrr has recently been identified in metazoans27-29 and it will be interesting to see if they are also targets of checkpoint kinases. With the identification of these key Rad53 substrates in yeast, together with recent work identifying the key CDK and DDK substrates, we now have a detailed understanding of how protein kinases regulate the initiation of DNA replication.

## **Experimental Procedures**

#### Yeast strains and methods

All yeast strains were based on W303-1a (MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100) and are described in Supplementary Material. Mutagenesis of Sld3 at the endogenous locus was done as described3. Cell growth and cell cycle blocks, samples for flow-cytometric analysis and yeast protein extracts were collected and processed as described previously3.

## Kinase assays and peptide arrays

Recombinant Rad53 was purified from *E.coli* and assayed *in vitro* as previously described30. Peptide arrays were synthesised directly onto cellulose membranes using an Intavis Autospot.

#### **Detection of replication intermediates**

1 and 2D gels were performed as previously described with minor modifications – see supplementary methods.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Figure 1.

Sld3 is novel target of Rad53 *in vivo*. A) Western blot after release of alpha factor arrested cells (G1) into 200mM HU. B) Anti-myc western blot of purified His/Myc-tagged Sld3 from yeast cells arrested as in A). C) schematic representation of yeast Sld3. All serine/threonine residues are marked by black bars (top), with the position of the identified Rad53 sites in either the Sld3 alanine mutant (open circles) or aspartic acid mutants (closed circles) below. The essential CDK sites (600/622) and the position of the TEV protease site for figure 1e are shown above. The position of the 5 Sld3 fragments in Figure 1d are shown below. D) *in vitro* Rad53 kinase assay with bacterially expressed Sld3 fragments 1-5. E) Western blots of purified and cleaved Sld3-TEV allele from HU arrested cells. This allele contains a myc tag at the C-terminus, HA-tag in the middle, with a TEV protease cleavage site in-between. This allele is viable as the only copy in yeast. F) Western blot of Sld3-13myc from cells arrested in G1 with alpha factor and released into HU for the indicated times.



## Figure 2.

At least two essential functions of Sld3 are inhibited by Rad53. A) Sld3 C-terminus (530-668), which was first phosphorylated with hot ATP and CDK, was incubated without (–) or with increasing amounts of Rad53 and cold ATP (+). This protein was then used in a pulldown with Dpb11-GST (1-395) (WT) or (90-395) ( $\Delta$ N). Top -autoradiogram, bottom – coomassie. B) Dpb11-GST pulldown assay as in A, with CDK phosphorylated full length Sld3 either with all the alanine mutations (38A) or with different subsets of the aspartic acid mutations (12D, 14D and 20D). C) Viability and tetrad analysis of diploids heterozygous for the corresponding Sld3 allele. Sld3 is full length, Dpb11 fusion is 253-764. D) Yeast-two-hybrid assays between the indicated baits and full length Sld3, either wild type or with S306D, T310D mutations (2D). E) Tetrad analysis on YP-galactose of a diploid heterozygous for *sld3-14D-DPB11* fusion and Gal-CDC45. Circles are the *sld3-14D-DPB11* fusion allele alone. Squares are the *sld3-14D-DPB11* fusion allele + Gal-CDC45.

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#### Figure 3.

Sld3 and Dbf4 are the minimal substrates of Rad53 for the block to origin firing. A) Top -Southern blot of alkaline gel of replication intermediates from cells arrested in G1 with alpha factor and released into HU for the indicated times and probed for specific origins as indicated. –B) Rad53 western blot of experiment in a). C) Western blot of Dbf4 (top) and Rad53 (bottom) after G1 arrest with alpha factor ( $\alpha$ ) and release into 200mM HU for the indicated times. The rad53 $\Delta$  strain is also sml1 $\Delta$ . \* indicates a non-specific band. D) as in A) bottom – Rad53 western blot. E) 2D neutral/neutral electrophoresis of replication intermediates from cells arrested for 90 mins in HU after release from G1 phase. The Msc1 digest was probed first for ARS501 and then ARS315. F) as in A). For lanes 1-12 the allele dbf4-19A, sld3-38A or both are present as second copies in haploid yeast. Zegerman and Diffley



#### Figure 4.

Separation of function analysis of the "intra-S checkpoint". A) Flow cytometry of cells blocked in G1 phase with alpha factor and then either released into nocodazole (dotted lines) or into 0.033% MMS (grey). B) Rad53 western blot from experiment in A). C) Viability of cells plated from experiment in A, error bars are standard error of the mean, n=4. D) Model of inhibition of CDK and DDK in G1 phase by the APC/C or in S-phase by Rad53.