Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks

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The Cdc45 protein is crucial for the initiation of chromosome replication in eukaryotic cells, as it allows the activation of prereplication complexes (pre-RCs) that contain the MCM helicase. This causes the unwinding of origins and the establishment of DNA replication forks. The incorporation of Cdc45 at nascent forks is a highly regulated and poorly understood process that requires, in budding yeast, the Sld3 protein and the GINS complex. Previous studies suggested that Sld3 is also important for the progression of DNA replication forks after the initiation step, as are Cdc45 and GINS. In contrast, we show here that Sld3 does not move with DNA replication forks and only associates with MCM in an unstable manner before initiation. After the establishment of DNA replication forks from early origins, Sld3 is no longer essential for the completion of chromosome replication. Unlike Sld3, GINS is not required for the initial recruitment of Cdc45 to origins and instead is necessary for stable engagement of Cdc45 with the nascent replisome. Like Cdc45, GINS then associates stably with MCM during S-phase.

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Introduction

Chromosome replication in eukaryotic cells is regulated in a highly complex fashion in order to maintain the integrity of the genome from one generation to the next. DNA replication forks are established at different moments in time during S-phase from multiple origins on each chromosome, yet initiation at each origin can occur just once, so that a single copy of the genome is generated in each round of a typical cell cycle (Blow and Dutta, 2005). This is achieved by dividing the cell cycle into a period when prereplication complexes (pre-RCs) of proteins essential for initiation are assembled at origins but cannot be activated, and a subsequent and mutually exclusive period when pre-RCs can be activated but can no longer form (Diffley *et al*, 1994). At each

origin, the pre-RC is lost during initiation, so that each region of the chromosome is replicated precisely once during each cell cycle.

The key event in the formation of pre-RCs—also known as the ‘licensing’ of origins—is the loading of the hetero-hexameric MCM2–7 complex (MCM) (Chong *et al*, 1995; Kubota *et al*, 1995; Aparicio *et al*, 1997; Tanaka *et al*, 1997). The MCM complex is essential for unwinding of the origin and for the establishment of DNA replication forks during initiation (reviewed by Forsburg, 2004). MCM then moves away as part of the replisome—this step is associated with loss of the pre-RC at the origin—and is essential for progression of DNA replication forks and continued unwinding of DNA during elongation (Aparicio *et al*, 1997; Labib *et al*, 2000; Pacek and Walter, 2004; Shechter *et al*, 2004). A subcomplex of MCM4/6/7 can unwind double-strand DNA and prefers forked substrates (Ishimi, 1997; Lee and Hurwitz, 2001; Kaplan *et al*, 2003); the MCM2–7 complex thus represents the best candidate for the essential ‘replicative helicase’ in eukaryotic cells, though efficient helicase activity of the endogenous heterohexamer has yet to be observed *in vitro*. This may reflect the complex regulation of the eukaryotic MCM complex at DNA replication forks, and it is notable that homologues of MCM in archaea form hexameric rings that act *in vitro* as processive DNA helicases (Kelman *et al*, 1999; Chong *et al*, 2000; Shechter *et al*, 2000; McGeoch *et al*, 2005).

Activation of the pre-RC at origins is poorly understood but requires the action of two kinases, Cyclin-dependent kinase (CDK) and Cdc7. As cells enter S-phase, CDK and Cdc7 allow MCM to form a stable complex with another protein, Cdc45 (Jares and Blow, 2000; Walter, 2000; Zou and Stillman, 2000; Masuda *et al*, 2003). Just like MCM, Cdc45 is essential for the establishment and progression of DNA replication forks (Hopwood and Dalton, 1996; Owens *et al*, 1997; Mimura and Takisawa, 1998; Miyake and Yamashita, 1998; Mimura *et al*, 2000; Tercero *et al*, 2000; Walter and Newport, 2000), and for the unwinding of the parental DNA duplex (Pacek and Walter, 2004; Nedelcheva *et al*, 2005). Cdc45 only associates stably with chromatin during S-phase when it binds tightly to MCM (Mimura and Takisawa, 1998; Miyake and Yamashita, 1998; Mimura *et al*, 2000; Walter and Newport, 2000; Gregan *et al*, 2003). But two previous studies with budding yeast used formaldehyde crosslinking and chromatin immunoprecipitation (ChIP) to show that the initial loading of Cdc45 at early origins can occur during G1-phase (Aparicio *et al*, 1999; Kamimura *et al*, 2001), suggesting that the incorporation of Cdc45 into the replisome is a complex process that can occur in several stages.

A variety of proteins including Sld3, Dpb11/Cut5, Mcm10 and GINS are all required for Cdc45 to be loaded stably onto S-phase chromatin, although the contribution of each protein to the initiation process has previously been unclear (reviewed by Mendez and Stillman, 2003). Of these, Sld3 is

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of particular interest, as it interacts with Cdc45 in both budding yeast and fission yeast, and is required for the MCM–Cdc45 complex to form (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002). In *Saccharomyces cerevisiae*, Sld3 and Cdc45 behave in a strikingly similar manner: they initially associate with early origins of DNA replication before initiation—and before a stable MCM–Cdc45 complex has formed—yet at later origins both proteins are only loaded during S-phase around the time of initiation (Kamimura *et al.*, 2001). Sld3 is required for loading of Cdc45 during initiation in both budding and fission yeast (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002); in budding yeast the reverse is also true (Kamimura *et al.*, 2001), although fission yeast Sld3 associates with origins independently of Cdc45 (Yamada *et al.*, 2004).

Although the molecular role of Sld3 during chromosome replication is unknown, it has been suggested that Sld3 is required, like Cdc45, for both the establishment and subsequent progression of DNA replication forks (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002), perhaps acting as part of the MCM helicase. But previous studies have not determined whether Sld3 does indeed associate with the replisome as a component of DNA replication forks, and the potential role of Sld3 during elongation remains unclear.

Budding yeast Sld3 is also required to load the four-protein GINS complex at origins during initiation, and Sld3 and GINS were found to interact with each other in a two-hybrid assay (Takayama *et al.*, 2003). The role of GINS during initiation has been unclear until now, but it appears to be required at an early stage (Kanemaki *et al.*, 2003; Kubota *et al.*, 2003; Takayama *et al.*, 2003), and the association of Cdc45 with S-phase chromatin is compromised in the absence of GINS in budding yeast or extracts of *Xenopus* eggs (Kubota *et al.*, 2003; Takayama *et al.*, 2003). It has been suggested that Sld3, Cdc45 and GINS may load at origins in a mutually dependent fashion (Takayama *et al.*, 2003), and studies with budding yeast have shown that GINS moves with DNA replication forks (Takayama *et al.*, 2003; Calzada *et al.*, 2005) and is required for normal progression of the replisome (Kanemaki *et al.*, 2003).

Here we identify important differences between Sld3 and GINS, indicating that they play distinct but complementary roles during the establishment of eukaryotic DNA replication forks.

Results

Sld3 does not move away from origins with DNA replication forks

We used a quantitative ChIP assay (Cobb *et al.*, 2003; Calzada *et al.*, 2005) to compare the behaviour of Sld3, Cdc45 and GINS during assembly and activation of the replisome at the budding yeast early origin *ARS305* on chromosome 3 (similar results were also obtained for the neighbouring origin *ARS306*). We synchronised a culture of cells in G1 phase at 24°C by addition of alpha factor mating pheromone to the culture medium, and then washed cells into fresh medium and took samples every 15 min. For each sample, we added formaldehyde to crosslink proteins and DNA, and then used chromatin IPs to study the association of specific DNA sequences with particular target proteins as described in Materials and methods.

We began by examining Cdc45, and found that the DNA fragment comprising *ARS305* was strongly enriched in the chromatin immunoprecipitate immediately after release from G1 arrest (Figure 1Aa, Cdc45-18Myc 0'). We confirmed this result in five independent experiments and found that as cells subsequently entered S-phase, the enrichment of the origin in the Cdc45 IP increased further to reach 1.5–2.5 × the initial value immediately after release from the arrest in G1 phase (Figure 1Aa and Ab, Cdc45-18Myc 30'), consistent with previous observations (Aparicio *et al.*, 1999; Zou and Stillman, 2000). At the same time, Cdc45 associated transiently with sequences away from the origin as the newly formed DNA replication fork passed through the region (Figure 1Aa, Cdc45-18Myc 30'), equivalent to the behaviour of other replisome components such as Pol2 (Calzada *et al.*, 2005). The observed enrichment at forks is lower than at the origin for at least two reasons: firstly, the origin signal actually comprises two nascent forks that are established during initiation of bi-directional replication; secondly, progression of the fork away from the origin is very rapid and synchrony between cells quickly decreases. At later times, the specific enrichment decreased to background levels throughout the region as replication was completed (Figure 1Aa and Ab, Cdc45-18Myc, 45' and 60').

We then examined Sld3 under identical conditions, and again found that the origin was strongly enriched in the specific immunoprecipitate immediately after release from G1-arrest (Figure 1Aa, Sld3-18Myc 0'). In striking contrast to Cdc45, however, the Sld3 signal at the origin simply decreased as chromosome replication proceeded (Figure 1Aa and Ab, Sld3-18Myc, 30'–60'). Most importantly, Sld3 did not associate with sequences away from the origin at any stage of the experiment, indicating that the protein was displaced from the origin during initiation and did not migrate with the newly formed DNA replication fork.

Finally, we examined the behaviour of the Psf2/Cdc102 protein, a component of the GINS complex (Kanemaki *et al.*, 2003; Takayama *et al.*, 2003). Unlike Cdc45 and Sld3, Psf2 did not associate with the origin immediately after release from G1-arrest (Figure 1Aa, Psf2-9Myc 0'). Subsequently, however, Psf2 associated with both origin and fork with similar kinetics to Cdc45 (Figure 1Aa, Psf2-9Myc 30'–60').

These experiments highlight important differences between Sld3, Cdc45 and GINS. Sld3 associates with the origin during G1-phase, and is then displaced without being incorporated into the replisome. Cdc45 initially behaves like Sld3, but the origin signal then increases as cells enter S-phase and the protein moves away as part of the replisome. GINS loads at origins around the time that the Cdc45 signal increases, and then moves away with forks.

Initial association of Sld3 and Cdc45 with early origins does not require CDK activation

To confirm that Sld3 is loaded at early origins before initiation and then displaced after the activation of S-CDK, we examined cells that were synchronised in G1-phase as before and then released in the presence of a stable version of the CDK-inhibitor Sic1 (Drury *et al.*, 1997; Desdouets *et al.*, 1998). In the absence of S-CDK activity, cells budded but did not enter S-phase (Figure 1Bb, Sld3-18Myc). Notably, Sld3 associated with *ARS305* throughout the experiment and was not displaced (Figure 1Ba, Sld3-18Myc). We also found that

Figure 1 Sld3 does not move with DNA replication forks away from origins. (A, B) The indicated strains were grown at 24°C in YPRaff medium and then synchronised in G1-phase with mating pheromone. The cells were then transferred to YPGal medium containing mating pheromone for 50', washed twice in YPGal medium, and samples taken at the indicated times for (a) ChIP analysis and (b) flow cytometry. The strains in (B) expressed a stable form of the CDK inhibitor Sic1 (Sic1ΔNT) under the control of the *GAL1,10* promoter.

Cdc45 behaved in a similar fashion—note that inhibition of S-CDK prevented the 1.5–2.5 × increase in the Cdc45 signal normally observed upon entry into S-phase (Figure 1B, Cdc45-18Myc). Finally, we confirmed that CDK inhibition prevented the association of GINS with the origin (Figure 1B, Psf2-9Myc), supporting our previous observations with a nonquantitative version of the ChIP assay (Kanemaki *et al.*, 2003). We thus conclude that Sld3 and Cdc45, unlike GINS, are initially able to interact with early origins during G1-phase. Following the activation of S-CDK, both Cdc45 and GINS are incorporated into the replisome at nascent forks, whereas Sld3 is displaced from the origin.

Sld3 does not interact stably with MCM and is only detected in MCM IPs from crosslinked cells before initiation

Both Sld3 and Cdc45 are thought to function in association with the MCM complex that first assembles into pre-RCs at origins at the end of mitosis and then moves with forks and is essential for progression of the replisome (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002). Although Sld3 and Cdc45 initially interact with early origins during G1-phase, previous studies have not determined whether they associate

with MCM at this stage (this would presumably be an unstable interaction), or whether they only bind to MCM subsequently at nascent forks. Based on the above data, we predicted that Sld3 might interact with MCM before initiation but would not do so at forks. We thus compared the interaction of Sld3 with Mcm4 under two conditions: firstly when cells were arrested in G1-phase (Sld3–Cdc45 at early origins, but no forks); secondly when cells were arrested in early S-phase by addition of the drug hydroxyurea (slow forks from early origins cause the 'origin-firing checkpoint' to block association of Cdc45 and thus presumably Sld3 with later origins (Santocanale and Diffley, 1998; Aparicio *et al.*, 1999)). In the absence of a crosslinking agent, we could not detect stable interaction of Sld3 with Mcm4 either before initiation (Figure 2Aa, Sld3, G1–X-linker), or in cells containing forks (Figure 2Aa, Sld3, HU–X-linker). We then repeated the experiment using cells that had been treated with formaldehyde in order to trap weak interactions as in the ChIP assay. Notably, we found that Sld3 associated with Mcm4 before initiation (Figure 2Aa, Sld3, G1 + X-linker) but not in cells containing only forks (Figure 2Aa, Sld3, HU + X-linker).

These experiments indicate that Sld3 can interact unstably or indirectly with MCM before establishment of

Figure 2 Sld3 and Cdc45 interact unstably (or indirectly) with MCM before initiation; GINS and Cdc45 interact stably with MCM after initiation (also see Supplementary Figure 4). (A) (a) YMK456 was grown at 24°C in YPD medium and then synchronised in G1-phase with alpha factor (G1), before releasing into fresh medium containing 0.2 M hydroxyurea (HU) for 90'. At each stage of the experiment, aliquots of cells were incubated in the presence or absence of the crosslinking agent formaldehyde, and cell extracts were prepared and then processed as for ChIP. After immunoprecipitation of Mcm4-5FLAG, tagged proteins were detected by immunoblotting with specific monoclonal antibodies; and (b) a similar experiment was performed with YMK487. (B) (a) YMK301 and (b) YMK425 were synchronised in G1-phase at 24°C in YPRaff medium as above and a sample was then processed for ChIP (24°C). Cells were then washed twice with YPGal medium containing mating pheromone and incubated at 24°C for 40' to induce expression of *GAL-UBR1*, before shifting the culture to 36°C for 60' to deplete Mcm4-td protein in YMK425, and taking another ChIP sample (36°C). (c) Cell extracts were prepared and used to confirm by immunoblotting that Cdc45-18Myc was not affected by degradation of Mcm4-td in YMK425 at 36°C.

a DNA replication fork. Once the fork is established, this interaction is lost.

GINS is not detected in MCM IPs from crosslinked cells before initiation but interacts stably with MCM after initiation

We then examined the interaction of the Psf2 subunit of GINS with MCM in similar experiments. Unlike Sld3, Psf2 was not

observed in Mcm4 IPs before initiation, even in extracts of crosslinked cells (Figure 2Ab G1 + X-linker), consistent with the ChIP data (Kanemaki *et al.*, 2003; Takayama *et al.*, 2003; Figure 1). In contrast, Psf2 was found to associate with Mcm4 after initiation even in cells that had not been treated with formaldehyde (Figure 2Ab HU–X-linker), in agreement with the findings of previous experiments involving *Xenopus* egg extracts (Kubota *et al.*, 2003). We thus conclude that GINS behaves differently to Sld3 and interacts with MCM (and perhaps during) initiation.

Cdc45 is present in MCM IPs from crosslinked cells before initiation and also interacts stably with MCM after initiation

As previously reported (Zou and Stillman, 2000), we found that Cdc45 also formed a stable complex with Mcm4 after assembly of the replisome (Figure 2Aa, Cdc45, HU–X-linker), just like GINS. In extracts of crosslinked cells, however, Cdc45 associated with Mcm4 both before and after initiation (Figure 2Aa, Cdc45, G1 and HU + X-linker), in agreement with the ChIP data.

If Cdc45 interacts with MCM at origins before initiation, then the maintenance of Cdc45 at early origins during G1-phase should require the continued presence of the MCM complex. To test this prediction, we used a strain in which the chromosomal locus of the *MCM4* gene had been modified so that the encoded protein carried the 'heat-inducible degron cassette' at the amino-terminus (Dohmen *et al.*, 1994), allowing us to deplete Mcm4 rapidly by raising the temperature from 24 to 37°C after induction of the Ubr1 ubiquitin ligase that specifically recognises the degron (Labib *et al.*, 2000).

Firstly, we synchronised control cells in G1-phase at 24°C, and then raised the temperature to 36°C for 45', maintaining the G1-arrest throughout. We used ChIP to show that Cdc45 associated at both 24 and 36°C with the two early origins ARS305 and ARS306 (Figure 2Ba). We then repeated the same experiment with the *mcm4-td* strain ('td' = temperature-sensitive degron). Cdc45 associated with both ARS305 and ARS306 during G1-phase at 24°C, but rapid depletion of Mcm4-td at 36°C caused the displacement of Cdc45 from both origins (Figure 2Bb). As a control, we confirmed that Cdc45 was still present in cells after degradation of Mcm4-td (Figure 2Bc). Mcm4 is essential, therefore, for Cdc45 to remain at early origins during G1-phase.

Putting these data together with previous findings (Kamimura *et al.*, 2001), we conclude that budding yeast Sld3 and Cdc45 can associate with MCM at early origins even before initiation during the G1-phase of the cell cycle. At this stage, the interaction between MCM and Sld3–Cdc45 is unstable or indirect and can only be detected in the presence of a crosslinking agent. Subsequently, CDK and Cdc7 cause assembly of an active replisome within which MCM interacts stably with Cdc45 and GINS; Sld3 is displaced from the origin during this process.

Sld3 is not required to complete chromosome replication after initiation

Our data indicate that Sld3 does not move with DNA replication forks or associate stably with MCM during S-phase, in contrast to Cdc45 and GINS. We thus predicted that Sld3 would not be required for progression of DNA replication forks. Previous experiments showed that cells containing the

sld3-5 temperature-sensitive mutation are not able to complete the cell cycle if arrested in HU at 24°C and then released from HU at 37°C (Kamimura *et al.*, 2001). Chromosome replication still proceeds slowly at 37°C in *sld3-5* cells, however, even when the protein is inactivated prior to replication during G1 phase (Kamimura *et al.*, 2001). In addition, initiation is markedly defective in these cells even at lower temperatures (Kamimura *et al.*, 2001), so that *sld3-5* grows slowly at 24°C and requires the Rad9 checkpoint protein for normal viability (Supplementary Figure 2A and B). This suggests that *sld3-5* cells accumulate DNA damage due to the usage of fewer origins, probably explaining why these cells fail to complete the cell cycle at high temperatures. To assess the role of the Sld3 protein after initiation, we generated a new allele of *SLD3*—*sld3-7td* (see Supplementary Methods)—that blocks chromosome replication very tightly at 37°C (Figure 3A), but grows very well at 24°C even without Rad9 (Supplementary Figure 2).

To test whether Sld3 is essential for the progression of DNA replication forks during chromosome replication, we investigated whether the *sld3-7td* strain is able to complete chromosome replication when the protein is inactivated after early origins of replication have already fired. We grew asynchronous cultures of four strains at 24°C: a control strain, *sld3-7td*, *cdc45-td* that is defective in both initiation and elongation (Tercero *et al.*, 2000), and *cdc7-1* that is defective in initiation but not elongation (Bousset and Diffley, 1998; Donaldson *et al.*, 1998). We synchronised cells in G1 phase at 24°C, allowed early origins to fire by releasing cells into HU medium at 24°C for 60 min, and then inactivated the mutated proteins at 37°C before washing into fresh medium lacking HU so that replication could potentially resume. After a further incubation of 45 min, we added mating pheromone so that any cells that completed the cell cycle would arrest in the subsequent G1 phase with a 1C DNA content. As shown in Figure 3B, the control strain rapidly completed replication and subsequently underwent cell division before blocking in the next G1 phase. Consistent with previous findings (Tercero *et al.*, 2000), *cdc45-td* did not complete replication and cells remained with a DNA content of less than 2C at the end of the experiment. The *cdc7-1* strain completed replication slowly, but most cells did not subsequently undergo cell division, in accord with a prior report (Bousset and Diffley, 1998). Importantly, the *sld3-7td* strain resumed replication upon release from HU arrest and reached a 2C DNA content with similar kinetics to *cdc7-1* (Figure 3B). Subsequently, most cells passed through mitosis and cell division, showing that chromosome replication had indeed been completed (we also confirmed that the viability of *sld3-7td* cells remained high at the end of the experiment, in comparison with *cdc45-td* and *cdc7-1*). We thus conclude that Sld3 is not required for the completion of chromosome replication after the firing of early origins. This indicates that it is not essential for the progression of DNA replication forks, consistent with our finding that the Sld3 protein is not associated with the replisome and is instead displaced from origins during the initiation process.

Recruitment of Cdc45 to early origins of DNA replication in the absence of GINS

GINS is required for Cdc45 to associate with S-phase chromatin in budding yeast and extracts of *Xenopus* eggs (Kubota *et al.*, 2003; Takayama *et al.*, 2003). In budding yeast,

Figure 3 Sld3 is not required for completion of chromosome replication after release from HU arrest. **(A)** YMK302 (*control*), YBH42 (*cdc45-td*), YKL55 (*cdc7-1*) and YMK517 (*sld3-7td*) were grown as for the experiment in Figure 2B, and then released from G1 arrest at 37°C for the indicated times. After confirming that budding had occurred in all cultures, mating pheromone was added once again at the 45' timepoint, so that any cells completing mitosis and cell division would arrest in the subsequent G1 phase. **(B)** An equivalent experiment was performed in which cells were first released from G1 arrest for 60' into YPRaff medium containing 0.2 M HU at 24°C, before induction of *GAL-UBR1* in YPGal + HU medium at 24°C for 35', transfer to YPGal + HU medium at 37°C for 50', and finally release into YPGal medium without HU for the indicated times. As before, mating pheromone was added again 45' after release from HU arrest.

however, GINS is recruited to early origins after the initial recruitment of Cdc45 (Kanemaki *et al.*, 2003; Takayama *et al.*, 2003). We thus used ChIP to examine the assembly of Cdc45 at early origins either in the presence or the absence of GINS, by comparing a control strain with another in which the degron cassette was fused to Psf2/Cdc102 (Kanemaki *et al.*, 2003). We synchronised cells in G2–M phase at 24°C by addition of nocodazole to the culture medium, induced expression of Ubr1, and then raised the temperature to 36°C for 45' to degrade Psf2/Cdc102 in the *cdc102-td* strain. As shown in Figure 4A, Cdc45 was not observed at *ARS305* or *ARS306* in either strain at this point in the experiment. We then washed cells into fresh medium at 36°C containing mating pheromone. Both strains completed mitosis and cytokinesis before arresting in the subsequent G1 phase, showing that GINS is not required for these processes in budding yeast (Figure 4B, G1 36°C). Significantly, Cdc45 was recruited to both *ARS305* and *ARS306* regardless of the presence or absence of GINS (Figure 4A). To confirm that we had efficiently inactivated GINS function, we then washed cells into fresh medium and continued incubation at 36°C: the control cells completed chromosome replication within

Figure 4 Recruitment of Cdc45 to origins of DNA replication without GINS. (A) YMK301 (*CDC45-18MYC*) and YMK292 (*cdc102-td CDC45-18MYC*) were grown in YP Raff medium at 24°C and then synchronised in G2/M phase with nocodazole, before inducing expression of *GAL-UBR1* for 35'. Cells were shifted to 36°C for 50' to deplete Psf2/Cdc102-td in YMK292 and a sample processed for ChIP (G2/M). Cells were then washed twice in fresh medium containing alpha factor and incubation was continued at 36°C for 90' until cell division was complete and cells arrested in the next G1-phase, before processing another ChIP sample (G1). (B) DNA content was measured by flow cytometry throughout the experiment; cells were also released from G1-arrest at the end of the experiment for the indicated times, to confirm that chromosome replication did not occur after depletion of Psf2/Cdc102-td.

60 min, but replication was blocked even after 80 min in the absence of GINS (Figure 4B). We conclude, therefore, that GINS—in contrast to Sld3 (Kamimura *et al*, 2001)—is not required for initial recruitment of Cdc45 to origins before CDK activation.

After the activation of S-CDK, an apparent increase in the association of Cdc45 with early origins is detected by ChIP (Figure 1Aa Cdc45-18Myc 30'), although at present we do not know if this represents the recruitment of additional molecules of Cdc45 to the origin, or else represents altered binding that improves the efficiency of crosslinking. As GINS is loaded around the same time, we tested whether GINS is required for this increased enrichment of Cdc45 at early origins, after the initial recruitment of Cdc45 to the pre-RC. We grew control and *cdc102-td* strains at 24°C, arrested cells in G1-phase with mating pheromone, and then increased the temperature to 36°C for 45' to degrade Psf2/Cdc102 in the *cdc102-td* strain. We then washed cells into fresh medium at 36°C and monitored chromosome replication by flow cytometry, and the association of Cdc45 with ARS305 by ChIP. As before, we observed initial recruitment of Cdc45 to the origin

during G1-phase in both strains (Figure 5Aa). Crucially, we found that the Cdc45 signal increased and then decreased with similar kinetics after release from G1-arrest regardless of the presence of GINS (Figure 5Aa), although chromosome replication only occurred in the presence of GINS (Figure 5Ab). Repeats of this experiment showed that the increase and decrease of Cdc45 at the origin was comparable in both strains, although the very rapid kinetics of origin activation at 36°C means that the signal observed at a particular time will vary slightly from one experiment to another. We conclude, therefore, that GINS is not required for initial recruitment of Cdc45 to early origins either during G1-phase (Figure 4), or subsequently after S-CDK activation (Figure 5A). It is also apparent that Cdc45 is then lost from the origin after entry into S-phase, both in the presence and absence of GINS.

GINS is essential for progression of Cdc45 away from origins and for stable association of Cdc45 with the pre-RC after S-CDK activation

In the presence of GINS, the loss of Cdc45 from the origin during initiation reflects the assembly of an active Cdc45–MCM complex that subsequently moves away from the origin with the DNA replication fork (Aparicio *et al*, 1997). We therefore wanted to determine whether Cdc45 also moves away from ARS305 in the absence of GINS, as Cdc45 is also lost from the origin in the absence of GINS, after the initial increase (Figure 5A). A failure to detect such movement would imply that Cdc45 falls off the origin when cells enter S-phase without GINS, and would thus indicate that GINS is specifically required for stable incorporation of Cdc45 into the nascent replisome. As before, we synchronised cultures of control and *cdc102-td* cells at 24°C before rapidly depleting Psf2/Cdc102 in the *cdc102-td* strain and then washing cells into fresh medium at 36°C (Figure 5B). Progression of the replisome is very rapid at high temperatures and has previously only been observed by ChIP when cells have been treated with HU to reduce fork speed. Using the quantitative ChIP assay, however, we found that by analysing samples every 5 min after release from G1-arrest we could detect progression of the replisome at 36°C even in the absence of HU. A representative experiment is shown in Figure 5Ba; association of Cdc45 with ARS305 and the neighbouring region peaked around 20 min after release from G1-arrest and then started to decrease. Importantly, Cdc45 did not enter this region in the absence of GINS (Figure 5Bb).

These experiments support two conclusions: firstly, that GINS is essential for progression of Cdc45 away from origins as part of an active replisome; secondly, that GINS is essential for stable association of Cdc45 with origins after activation of S-CDK, so that Cdc45 falls-off the origin after CDK activation in the absence of GINS.

We then examined the fate of MCM and Sld3 in the absence of GINS. We first confirmed that both proteins were still present in cells after degrading Psf2/Cdc102-td (not shown). Assembly and progression of the replisome is normally associated with loss of Mcm4 from the origin (Figure 6A +GINS 60'). In the absence of GINS, however, Mcm4 remained at ARS305 even 60' after release from G1-arrest at 36°C (Figure 6A –GINS 60'), when chromosome replication would normally have finished (Figure 6A +GINS 60'); by 90' the enrichment of ARS305 in the Mcm4 immunoprecipitate was still 50% of the value immediately after

release from G1-phase (Figure 6A –GINS 90'). This indicates that MCM stays at the origin for a greatly extended period when Cdc45 is displaced in the absence of GINS.

Figure 6 Mcm4 remains at ARS305 when cells enter S-phase in the absence of GINS, and Sld3 is slowly lost from the origin. (A) Experiments equivalent to those shown in Figure 5A were performed with YMK423 (*MCM4-FLAG*) and YMK424 (*MCM4-FLAG cdc102-td*). ChIP and flow cytometry data are presented as before. Nocodazole was added to the medium 50' after release from G1 arrest, to prevent any cells that had completed chromosome replication from passing through mitosis. (B) Identical experiments were performed with YMK297 (*SLD3-MYC*) and YMK296 (*SLD3-MYC cdc102-td*).

Figure 5 GINS is essential for incorporation of Cdc45 into an active replisome. (A) YMK301 (*CDC45-18MYC*) and YMK292 (*cdc102-td CDC45-18MYC*) were synchronised in G1 phase as above, before induction of *GAL-UBR1* for 45' and depletion of Psf2/Cdc102-td in YMK292 at 36°C. Cells were then released from G1-arrest at 36°C and samples processed for ChIP (a) and flow cytometry (b) at the indicated times. (B) A similar experiment was performed with shorter timepoints, to facilitate analysis of fork progression through the region adjacent to ARS305. In (a) and (b), the data corresponding to the ARS305 proximal region are duplicated in an enlarged form below the main graph for greater clarity.

We then investigated whether loss of Cdc45 in the absence of GINS was a specific effect or else could be explained by a concomitant loss of Sld3. In the presence of GINS, enrichment of Sld3 at the origin was reduced after 20' to 47% of the initial value during G1-phase, and by 60' to 1% (Figure 6B +GINS). In the absence of GINS, however, association of Sld3 with ARS305 was unchanged after 20', and remained at 45% of the initial value even after 60' (Figure 6B—GINS). It is clear, therefore, that Sld3 is lost much more slowly from the origin when assembly of the replisome fails in the absence of GINS. This contrasts with the behaviour of Cdc45, and suggests that loss of Sld3 is not the primary reason why Cdc45 is displaced in the absence of GINS. Instead, GINS is crucial for maintenance of Cdc45 at origins after the activation of S-CDK.

GINS is essential for activation of the pre-RC

The preceding experiments show that Cdc45 is displaced from early origins if cells enter S-phase in the absence of GINS. In contrast, Sld3 is lost more slowly, and MCM remains for an extended period. The resolution of the ChIP assay is limited to about 500 bp, however, and so these experiments do not show whether the pre-RC remains intact under such conditions, or else is transformed. We therefore used genomic footprinting to examine protein–DNA complexes with nucleotide resolution at ARS305 as cells were released from G1-arrest in the presence (control) or absence (*cdc102-td*) of GINS. During most of the cell cycle, ARS305 exists in a 'postreplicative state' that is characterised by three ORC-induced hypersensitive sites in the DNaseI footprint (Figure 7Aa G2/M (Perkins and Diffley, 1998)). When the pre-RC is assembled during G1-phase, however, the three hypersensitive sites are protected together with an adjacent region (Figure 7Aa G1). We previously showed that MCM is essential for the formation and maintenance of this pre-Replicative footprint (Labib *et al.*, 2001).

As control cells were released from G1-arrest at 36°C in the presence of GINS, protection of the three hypersensitive sites was gradually removed, reflecting loss of the pre-RC during initiation (Figure 7Ab and B, Control). In the absence of GINS, protection of these sites was largely maintained (Figure 7Ab and B, *cdc102-td*), showing that the pre-RC was retained. Consistent with this fact, we observed by ChIP that GINS is required for loading of RPA and DNA polymerase epsilon during initiation (Supplementary Figure 3). We thus conclude that GINS plays an essential role in activation of the pre-RC during the initiation of eukaryotic chromosome replication.

Discussion

We have shown that Sld3 does not move away from origins with DNA replication forks, and is not associated with MCM when cells are arrested in S-phase, in contrast to GINS and Cdc45 (Figures 1 and 2). In a parallel study, we found that GINS is required for stable association of Cdc45 and other proteins with MCM during S-phase, to form large 'Replisome Progression Complexes', and once again it is striking that such complexes do not contain Sld3 (Gambus *et al.*, 2006). Instead, we show here that Sld3 is displaced from origins during the initiation process, and is not required for the completion of replication after the firing of early origins. Considering our data together with previous studies

Figure 7 GINS is essential for activation of the pre-RC. (A) (a) Control cells (YMK302) were synchronised in G1-phase or G2/M phase as before and protein–DNA complexes at ARS305 (indicated by the black bar) were then examined by genomic footprinting. The asterisks mark three ORC-induced hypersensitive sites that are suppressed when pre-RC formation occurs during G1-phase. (b) Control (YMK302) and *cdc102-td* cells (YMK458) were synchronised in G1-phase as above and then Psf2/Cdc102-td was depleted in YMK458 for 45' before both strains were released into fresh medium at 36°C. Samples were taken at the indicated times. (B) The region of the gel that corresponds to the three hypersensitive sites in (A) (b) was quantified for the 0' and 60' samples as described in Materials and methods.

(Kamimura *et al.*, 2001; Nakajima and Masukata, 2002; Takayama *et al.*, 2003), we suggest that Sld3 is required specifically during initiation for the loading of GINS and Cdc45.

We have shown that GINS is essential for activation of the pre-RC and thus for progression of Cdc45 away from early origins as part of an active DNA replication fork. Moreover, GINS is required for maintenance of Cdc45 at ARS305 after S-CDK activation (Figure 5), consistent with the previous finding that Cdc45 is not stably bound to chromatin during S phase in the absence of GINS (Kubota *et al*, 2003; Takayama *et al*, 2003). This suggests that GINS is needed for stable association of Cdc45 with origins during S-phase, in contrast to Sld3 that is required for initial recruitment of Cdc45 and GINS (Kamimura *et al*, 2001). As Sld3 only associates with later origins during S-phase (Kamimura *et al*, 2001), this means that the distinction between the function of Sld3 and GINS is harder to observe at these origins. Nevertheless, we suggest that the same mechanism will apply, with Sld3 principally required for recruitment and GINS for stable incorporation of Cdc45 into an active replisome.

As the association of Sld3 with each origin is a prerequisite for initiation, but Sld3 is not incorporated into the replisome, we suggest that the presence of Sld3 at an origin is a key feature of the preinitiation complex (pre-IC), distinguishing it from both the pre-RC and the nascent replisome (Figure 8). The strength of the various protein–protein interactions within the pre-IC may vary between species, so that in budding yeast the Sld3–Cdc45–MCM interaction is unstable but still detectable during G1-phase even without GINS, whereas this may not be the case in fission yeast (Yamada *et al*, 2004). We suggest that the underlying mechanism in both species will similarly involve recruitment of GINS and Cdc45 by Sld3, followed by stable formation of the GINS–MCM–Cdc45 complex with consequent exclusion of Sld3 from the origin.

In higher eukaryotes, an orthologue of Sld3 has yet to be reported. As noted previously (Takayama *et al*, 2003), however, it appears that proteins which control particular steps of chromosome replication such as initiation may have diverged greatly in primary sequence during the course of evolution, in response to differing needs for regulation. Examples include the orthologues of Dpb11/Cut5 in higher eukaryotes (Yamane *et al*, 1997; Van Hatten *et al*, 2002; Hashimoto and Takisawa,

2003) and the recently described homology between RTS protein (Rothmund–Thomson Syndrome) and budding yeast Sld2 (Sangrithi *et al*, 2005). In contrast, core components of the replisome such as MCM, Cdc45 and GINS are more highly conserved in all eukaryotes. We note that MCM–Cdc45–GINS are more closely related even between budding and fission yeasts, in comparison with other factors such as Sld3, Dpb11, Cdc6, or Cdt1 (Supplementary Table 1).

Figure 8 A model for the loading of Cdc45 at origins by Sld3 and GINS during the initiation of chromosome replication. MCM is assembled into pre-RCs at origins during G1-phase (for simplicity other proteins such as the Origin Recognition Complex are not shown). Following activation of CDK and Cdc7, Sld3 recruits GINS and Cdc45 to MCM, converting the pre-RC into a pre-IC. The order of assembly may vary in different species or at different origins; thus, at early origins in budding yeast the initial recruitment of Cdc45 occurs during G1-phase. GINS then allows stable incorporation of Cdc45 into an active replisome, so that MCM–Cdc45–GINS move away with the newly formed DNA replication forks. Sld3 is displaced during the initiation reaction.

Table I Strains used in this study

Strain	Genotype
W303-1a	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>
YMK286	<i>SLD3-18MYC (K.I.TRP1)</i>
YMK369	<i>CDC45-18MYC (K.I.TRP1)</i>
YMK158	<i>PSF2/CDC102-9MYC (K.I.TRP1)</i>
YMK388	<i>SLD3-18MYC (K.I.TRP1) sic1Δ::GAL-SIC1 (URA3)</i>
YMK379	<i>CDC45-18MYC (K.I.TRP1) sic1Δ::GAL-SIC1 (URA3)</i>
YMK190	<i>PSF2/CDC102-9MYC (K.I.TRP1) sic1Δ::GAL-SIC1 (URA3)</i>
YMK456	<i>MCM4-5FLAG (hphNT) CDC45-3HA (TRP1) SLD3-9MYC (LEU2) pep4Δ::URA3 ADE2</i>
YMK487	<i>MCM4-5FLAG (hphNT) PSF2/CDC102-9MYC (K.I.TRP1) pep4Δ::URA3 ADE2</i>
YMK301	<i>CDC45-18MYC (K.I.TRP1) ubr1Δ::GAL-UBR1 (HIS3)</i>
YMK425	<i>CDC45-18MYC (K.I.TRP1) ubr1Δ::GAL-UBR1 (HIS3) mcm4-td (kanMX)</i>
YMK302	<i>ubr1Δ::GAL-UBR1 (HIS3)</i>
YBH42	<i>cdc45-td (kanMX) ubr1Δ::GAL-UBR1 (HIS3)</i>
YKL55	<i>cdc7-1</i>
YMK517	<i>sld3-7td (kanMX) ubr1Δ::GAL-UBR1 (HIS3)</i>
YMK292	<i>CDC45-18MYC (K.I.TRP1) ubr1Δ::GAL-UBR1 (HIS3) cdc102-td (kanMX)</i>
YMK297	<i>SLD3-18myc (K.I.TRP1) ubr1Δ::GAL-UBR1 (HIS3)</i>
YMK296	<i>SLD3-18myc (K.I.TRP1) ubr1Δ::GAL-UBR1 (HIS3) cdc102-td (kanMX)</i>
YMK423	<i>MCM4-5FLAG (hphNT) ubr1Δ::GAL-UBR1 (HIS3)</i>
YMK424	<i>MCM4-5FLAG (hphNT) ubr1Δ::GAL-UBR1 (HIS3) cdc102-td (kanMX)</i>
YMK458	<i>ubr1Δ::GAL-UBR1 (HIS3) cdc102-td (kanMX)</i>

W303-1a is the parental strain for all the others.

It is possible, therefore, that the putative homologue of Sld3 in higher eukaryotes has diverged considerably from its yeast equivalent, consistent with our finding that budding yeast Sld3 does not form part of the replisome but instead acts at origins during initiation.

Materials and methods

Yeast strains

The yeast strains used in this study are listed in Table I and Supplementary Table 2. Details of growth conditions are given in Supplementary Methods.

Chromatin immunoprecipitation

We performed quantitative ChIP experiments essentially as described previously (Calzada *et al.*, 2005). Briefly, we performed ChIP from extracts of crosslinked cells, with specific and nonspecific antibodies. After purification of associated DNA fragments, we used a 'real-time' PCR machine (ABI 7900) to calculate the enrichment of particular sequences in the specific IP. Details of the oligos and probes are given in Supplementary Figure 1. The nonspecific IP serves as an internal control for each extract, which reduces the effects of any variation in extract concentration between samples. This approach thus provides a robust method for analysing in a quantitative fashion the changes in specific protein-DNA interactions between samples.

For the experiments in Figures 2A, Mcm4 was isolated by immunoprecipitation from the cell extracts; the IPs were washed

as for the ChIP assay and the final eluate was then mixed with $3 \times$ Laemmli buffer before incubation at 95°C for 30 min to reverse the crosslinking and denature the eluted proteins.

Detection of tagged proteins

Cell extracts to be used for immunoblots only were prepared using trichloroacetic acid as described (Foiani *et al.*, 1994). The c-Myc, HA and 'FLAG' epitopes were detected using the mouse monoclonal antibodies 9E10 (final concentration 5 µg/ml), 12CA5 (final concentration 5 µg/ml) and M2 (Sigma F3165, final concentration 1 µg/ml) respectively.

Genomic footprinting

Genomic footprinting of ARS305 was performed as described previously (Perkins and Diffley, 1998; Noton and Diffley, 2000). The samples were run in a 5% denaturing polyacrylamide gel and radiolabelled products detected using a Storm 860 phosphorimager (Molecular Dynamics) and 'ImageQuant 5.1' software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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