

Sld7, an Sld3-associated protein required for efficient chromosomal DNA replication in budding yeast

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Genetic screening of yeast for *sld* (synthetic lethality with *dpb11*) mutations has identified replication proteins, including Sld2, -3, and -5, and clarified the molecular mechanisms underlying eukaryotic chromosomal DNA replication. Here, we report a new replication protein, Sld7, identified by rescreening of *sld* mutations. Throughout the cell cycle, Sld7 forms a complex with Sld3, which associates with replication origins in a complex with Cdc45, binds to Dpb11 when phosphorylated by cyclin-dependent kinase, and dissociates from origins once DNA replication starts. However, Sld7 does not move with the replication fork. Sld7 binds to the nonessential N-terminal portion of Sld3 and reduces its affinity for Cdc45, a component of the replication fork. Although Sld7 is not essential for cell growth, its absence reduces the level of cellular Sld3, delays the dissociation from origins of GINS, a component of the replication fork, and slows S-phase progression. These results suggest that Sld7 is required for the proper function of Sld3 at the initiation of DNA replication.

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Introduction

Eukaryotic chromosomes must be duplicated once and only once per cell cycle to maintain the integrity of their genetic information. To ensure the accurate timing of chromosomal duplication and to prevent rereplication during the cell cycle,

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the initiation of DNA replication is strictly regulated. DNA replication is initiated from specific regions called ‘origins’, which are scattered in large numbers along chromosomes. The six-subunit origin recognition complex (Orc) associates with replication origins throughout the cell cycle in budding yeast. Mcm, a DNA helicase comprising Mcm2–7, is loaded onto the Orc-bound origins to form the prereplicative complex (pre-RC). This loading requires Cdc6 and Cdt1, and occurs from late M to G₁ phase. The pre-RC-formed origins are activated by two different protein kinases, cyclin-dependent kinase (CDK) and the Dbf4-dependent Cdc7 kinase (Labib, 2010). Once an origin has been activated, the pre-RC disassembles, and its reassembly is prevented until the next cell cycle (Bell and Dutta, 2002; Ghaemmaghami *et al*, 2003; Schwob and Labib, 2006; Sivaprasad *et al*, 2006; Walter and Araki, 2006; Masai *et al*, 2010). The process by which replication origins are activated was unclear until the replication proteins involved in this step were identified and characterized.

Screening for the yeast *sld* (synthetic lethality with *dpb11-1*) mutations (Kamimura *et al*, 1998) paved the way for the clarification of the initiation step in chromosomal DNA replication after the formation of the pre-RC. This screening isolated the *sld1*, -2, -3, -4, -5, and -6 mutations, which cannot be combined with the *dpb11-1* mutation in the same cell. The *sld1* mutation occurs in the third largest subunit, Dpb3, of DNA polymerase ϵ (Pol ϵ). This polymerase synthesizes the leading strand and functions in the checkpoint at the replication fork, and in the initiation of DNA replication (Masumoto *et al*, 2000; Dohrmann and Sclafani, 2006; Lou *et al*, 2008; Pursell and Kunkel, 2008). The *DPB11* gene was originally isolated as a multicopy suppressor of mutations in Pol ϵ (Araki *et al*, 1995). The *sld2* and *sld3* mutations were identified in previously unidentified replication proteins, Sld2 and Sld3, both of which interact with Dpb11 when they are phosphorylated by CDK (Wang and Elledge, 1999; Kamimura *et al*, 1998, 2001; Masumoto *et al*, 2002; Tanaka *et al*, 2007; Zegerman and Diffley, 2007). These phosphorylation-dependent interactions are essential and represent the minimal requirement for the CDK-dependent activation of DNA replication (Tanaka *et al*, 2007; Zegerman and Diffley, 2007; Tanaka and Araki, 2010). *sld4* is a mutation in Cdc45, a protein required for the initiation and elongation steps of DNA replication. Sld3 and Sld4 (Cdc45) form a complex throughout the cell cycle, which associates in a mutually dependent manner with the pre-RC-formed origins. This association occurs with early-firing origins in G₁ and with late-firing origins in S phase (Kamimura *et al*, 2001; Kanemaki and Labib, 2006). The *sld5* mutation occurs in a subunit of GINS, which consists of Sld5, Psf1, Psf2, and Psf3. GINS functions in the initiation and elongation stages of chromosomal DNA replication (Kanemaki *et al*, 2003; Kubota *et al*, 2003; Takayama *et al*, 2003; Labib and

Gambus, 2007). Cdc45 and GINS associate with Mcm to form an active replicative helicase, the Cdc45–Mcm–GINS (CMG) complex (Gambus *et al*, 2006; Moyer *et al*, 2006; Pacek *et al*, 2006; Ilves *et al*, 2010). The *sld6* mutation occurs in Rad53 kinase, which functions at checkpoints and in the regulation of the initiation of DNA replication (Dohrmann and Sclafani, 2006).

Dpb11 has two pairs of tandem BRCT domains, known as the phosphopeptide-binding domains (Glover *et al*, 2004). The N-terminal pair of BRCT domains binds to phosphorylated Sld3, and the C-terminal pair binds to phosphorylated Sld2 (Masumoto *et al*, 2002; Tanaka *et al*, 2007; Zegerman and Diffley, 2007). Their interactions are essential for the activation of origins, but the mechanism underlying the activation of origins by these interactions is unclear. One clue comes from the identification of the preloading complex (pre-LC). This complex contains Pol ϵ , GINS, Dpb11, and Sld2, and its formation depends on the interaction between Dpb11 and Sld2, but not on the pre-RC. Thus, the phosphorylation-dependent interaction between Dpb11 and Sld2 has a role in the formation of the pre-LC. Although the role of the interaction between Dpb11 and Sld3 is unknown, it has been proposed that GINS in the pre-LC is recruited to replication origin to form the CMG complex via the interaction between Dpb11 in the pre-LC and phosphorylated Sld3 on origins (Muramatsu *et al*, 2010; Tanaka and Araki, 2010). At the initiation of DNA replication, CMG, Dpb11, Sld2, and Sld3 dissociate from origins and the CMG complex moves with the replication fork.

In this study, we screened for novel *sld* mutations using the *dpb11-24* allele. This *dpb11* allele has a mutation in the N-terminal pair of tandem BRCT domains of Dpb11, whereas the *dpb11-1* mutation used in the previous screening is located in the C-terminal BRCT domains. This new round of screening led to the isolation of the *sld7* mutation, which occurs in a previously uncharacterized gene, and other mutations in known replication genes. Although it is not essential, the *SLD7* gene has an important role in chromosomal DNA replication, as do the other *SLD* genes. The Sld7 protein forms a tight complex with Sld3 throughout the cell cycle, stabilizes Sld3, and reduces the affinity of Sld3 for Cdc45. The absence of Sld7 delays the dissociation of GINS from the origin and the progression of S phase. Based on these data, we discuss a possible function for Sld7.

Results

***SLD7* gene is not essential but is important for cell growth and chromosomal DNA replication**

To gain insight into the molecular mechanisms underlying chromosomal DNA replication, we rescreened for further *sld* mutations, which cannot be combined with the *dpb11-24* mutation in a cell, as described previously (Kamimura *et al*, 1998). We isolated six mutations from 32 000 mutagenized colonies. Five of these occurred in known genes (two in *SLD3*, and one each in *DPB2*, *MCM3*, and *MCM10*), and the remaining mutation occurred in an unknown gene (*YOR060c*, *Saccharomyces* Genome Database) (Valens *et al*, 1997). We named this gene *SLD7* (synthetic lethality with *Dpb11-24*) and characterized it further. This gene encodes a 29-kDa protein comprising 257 amino acids. The Sld7 protein has no sequence motifs that indicate its function. Using the

BLAST and FASTA homology search programs, we found possible homologues of the Sld7 protein in *Saccharomyces* yeasts and in closely related genera, such as *Zygosaccharomyces*, *Kluyveromyces*, *Eremothecium*, and *Pichia* (Supplementary Figure S1). However, a homologue of the Sld7 protein could not be found in fission yeasts or in organisms other than yeasts. The isolated mutation (*sld7-1*) involves the substitution of arginine for glycine at position 42 of the Sld7 protein. The glycine 42 residue is conserved in the Sld7 homologues found in *Zygosaccharomyces* and *Eremothecium* species (Supplementary Figure S1).

To examine whether the *SLD7* gene is essential for cell growth, one of the *SLD7* gene copies in a *leu2/leu2* diploid cell was replaced with *sld7 Δ ::LEU2*. Sporulation and tetrad dissection of the resultant *SLD7/sld7 Δ ::LEU2* cells yielded two large and two small colonies (Figure 1A). All the large colonies were Leu⁻, whereas all the small colonies were Leu⁺, indicating that the small colonies were derived from an *sld7 Δ ::LEU2* allele. Two of 36 Leu⁻ spores (wild type) and 15 of 36 Leu⁺ spores (*sld7 Δ ::LEU2*) did not form colonies. The viability of the Leu⁺ spores varied across experiments (50–90%). Microscopic observations revealed that some of these lethal spores carrying the *sld7 Δ ::LEU2* allele died without germinating, whereas others died after several divisions. The dissection of tetrads derived from *SLD7/sld7 Δ ::LEU2* cells carrying the YEp195SLD7[*URA3*] plasmid revealed that 90% of the spores formed colonies. All the clones grew on 5-fluoroorotic acid plates, which only allow the growth of cells that have lost YEp195SLD7[*URA3*], although the *sld7 Δ* (Leu⁺Ura⁻) cells grew slightly more slowly on the plates than did the wild-type cells. Therefore, the *SLD7* gene is important but not essential for cell growth. Although the null mutant of *SLD7*, constructed in a systematic high-throughput deletion study, was reported to be inviable (Winzeler *et al*, 1999), the same construct obtained from EUROSCARF was viable in our hands. The *sld7 Δ* cells grew more slowly than the wild-type cells in liquid YPD medium (doubling time for the wild type was 77.5 min and for *sld7 Δ* was 86.2 min at 30°C), as observed on the plates.

The *sld7 Δ* mutation was synthetically lethal with mutations in *DPB11* (*dpb11-1* and *dpb11-24*), *SLD* genes (*drc1-1* [*sld2*], *sld5-12*, *sld3-5*, and *cdc45-27* [*sld4*]), Pol ϵ (*pol2-11*), and GINS (*psf1-1*), but not with mutations in components of the pre-RC (*mcm2-1*, *mcm3-1*, *orc2-1*, and *orc5-1*) or in the checkpoint gene (*ddc1 Δ*). These genetic interactions suggest that Sld7, like other Sld proteins, functions in DNA replication. Therefore, we examined the chromosomal DNA replication in *sld7 Δ* cells with flow cytometry. The *sld7 Δ* cells arrested in G₁ phase with α -factor were released at 25°C, and their DNA content was measured. The DNA content of the wild-type cells increased after 30 min and reached 2C 45 min after release (Figure 1B), whereas that of the *sld7 Δ* cells increased after 40 min and reached 2C 70 min after release, taking about twice as long as the wild-type cells to complete their DNA replication. The buds appeared at the same time in both cells, suggesting that the cell-cycle events at the G₁/S boundary are normal in *sld7 Δ* cells, except for DNA replication (Figure 1C). Although the *sld7 Δ* cells transiently exposed to hydroxyurea (HU) or methyl methanesulfonate (MMS) had slightly reduced viability compared with that of the wild-type cells, like many mutants defective in DNA replication, their growth on plates was inhibited by HU

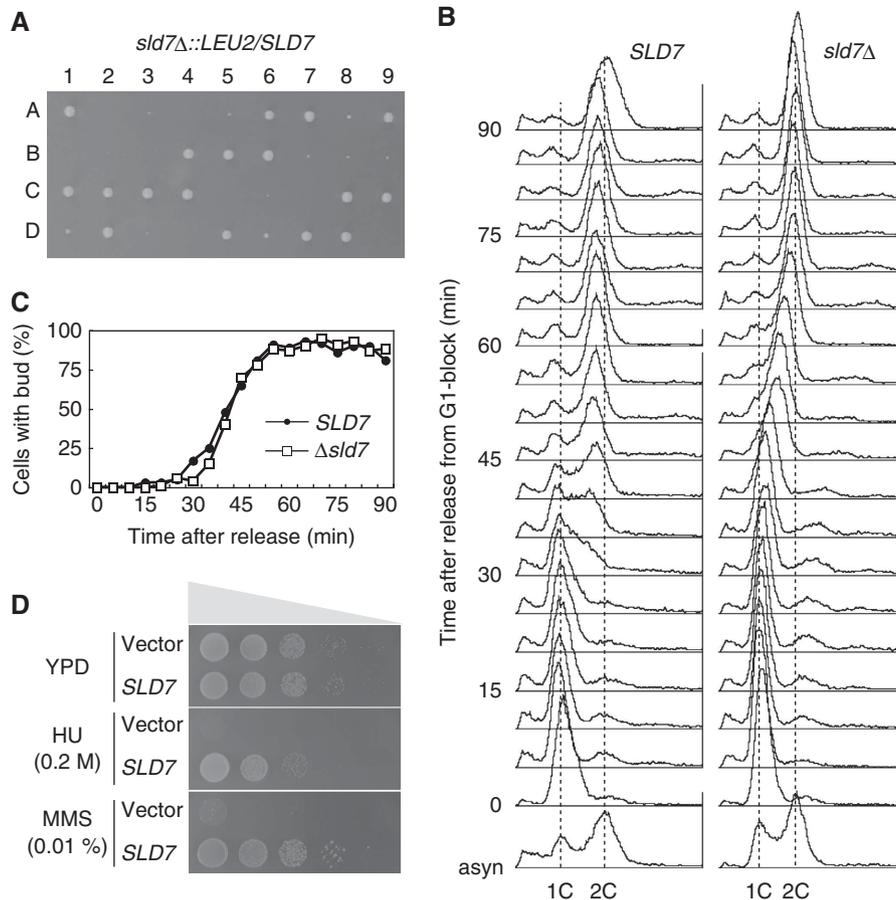


Figure 1 Important functions of Sld7 in chromosomal DNA replication. (A) *sld7Δ::LEU2/SLD7* diploid cells were sporulated and dissected. The spores were incubated on YPD plates at 30°C for 3 days. (B) DNA replication in *sld7Δ* cells. The YTT1 (*sld7Δ*) and wild-type (WT: YK9) cells were synchronized in G₁ phase with α -factor and released from G₁ arrest at 25°C. Aliquots of the cells were sampled at 5 min intervals, and their DNA content was measured by flow cytometry. (C) The population of cells with buds after release from the G₁ block in the same culture, as in (B). (D) Sensitivity of *sld7Δ* cells to HU and MMS. Plasmids with or without the *SLD7* gene were introduced into *sld7Δ* cells. Tenfold serial dilutions of the cells were spotted onto YPD plates containing 0.2 M HU or 0.01% MMS. The cells were incubated at 25°C for 4 days.

or MMS more severely than was that of the wild-type cells (Figure 1D). Therefore, Sld7 seems to function in cooperation with the Dpb11 and Sld proteins to ensure efficient chromosomal DNA replication.

Sld7 protein forms a complex with Sld3 protein

To clarify the function of Sld7, we used two-step immunoprecipitation (IP) of cells expressing Sld7 protein tandemly tagged with 3Flag-HA (Sld7-3Flag-HA) to identify proteins that bind to Sld7. The Sld7-3Flag-HA complex was immunoprecipitated from the extracts with anti-Flag M2 affinity agarose and then with an anti-HA matrix, after the proteins had been released from the M2 affinity beads with Flag peptides. A comparison with immunoprecipitates from extracts of wild-type cells showed that an 80-kDa protein coprecipitated specifically with Sld7-3Flag-HA (Figure 2A). The band containing this protein was excised from the gel after electrophoresis, digested with trypsin, and subjected to analysis by mass spectrometry. According to the spectra assigned to the peptides in this analysis, the protein was identified as Sld3. Western blots probed with anti-Sld3 antibodies confirmed that the Sld3 protein coprecipitated with the Sld7-3Flag-HA complex (Figure 2A).

To examine whether the interaction between Sld7 and Sld3 is direct, 6His-Sld3 and Sld7 proteins were coexpressed in *Escherichia coli* cells, and the 6His-Sld3 was precipitated with Ni-NTA agarose (Figure 2B). In this assay, Sld7 protein was precipitated and eluted with the 6His-Sld3 protein. Sld3-Flag and Sld7, expressed separately in and purified from *E. coli* cells, also coprecipitated and coeluted when they were mixed (Figure 2C). Therefore, Sld7 protein binds directly to Sld3 protein. We also analysed the purified proteins by glycerol gradient sedimentation. When Sld3 and Sld7 were applied separately to a glycerol gradient and centrifuged, they sedimented in the fractions corresponding to 65 kDa (Figure 2D). When the Sld3-Sld7 complex purified from yeast was applied, Sld3 and Sld7 cosedimented in the fraction corresponding to 180 kDa, indicating that they had formed a complex.

Using coprecipitation, we also examined the proportions of cellular Sld3 and Sld7 that exist as the Sld3-Sld7 complex. As shown in Figure 2E and F, most of the Sld3-9Myc and Sld7-3Flag-HA proteins coprecipitated when the tagged proteins were precipitated with anti-Flag antibody or anti-Myc antibody. Moreover, the Sld3 and Sld7 proteins coprecipitated throughout the cell cycle (Figure 2G). Therefore, most of the Sld3 and Sld7 proteins in the cells form a complex throughout the cell cycle.

(Supplementary Figure S4). These results are consistent with the observation that Sld7 and Cdc45 interact with the N-terminal and central portions of Sld3, respectively.

We also examined whether an increased dose of the *SLD3* gene restores the HU sensitivity of the *sld7Δ* mutant cells. As shown in Figure 4A, the *SLD3* gene on a high-copy plasmid restored the HU sensitivity of the *sld7Δ* mutant cells, whereas the *SLD3* gene on a low-copy plasmid did not. The level of the Sld3 protein in *sld7Δ* cells was reduced and was restored by

the introduction of *SLD3* on a low-copy plasmid. The introduction of *SLD3* on a high-copy plasmid more than doubled the Sld3 level (Figure 4B and C).

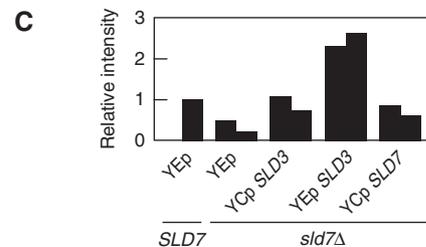
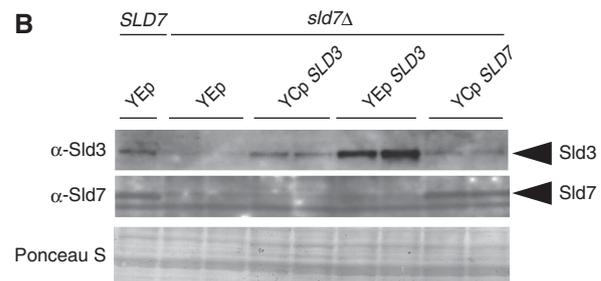
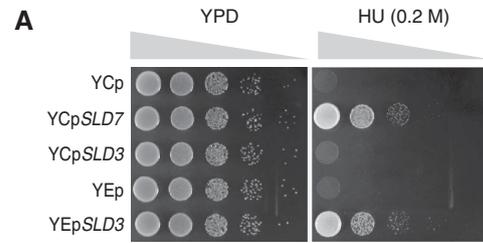
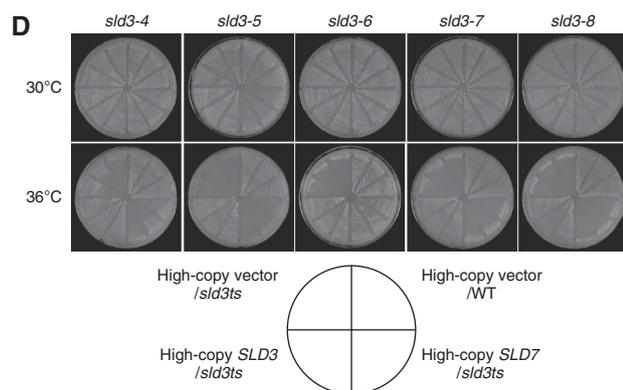
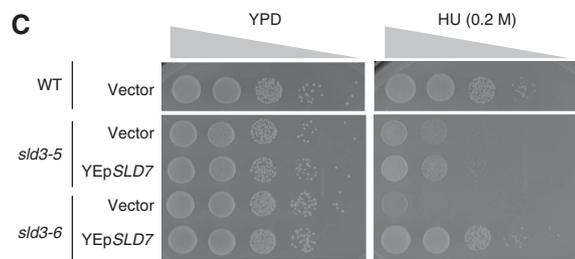
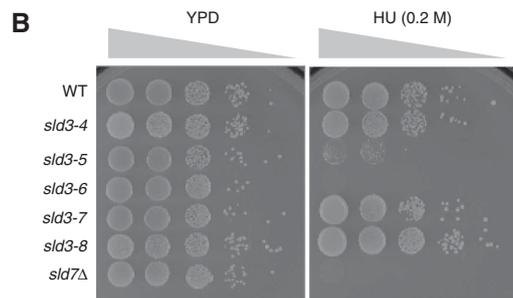
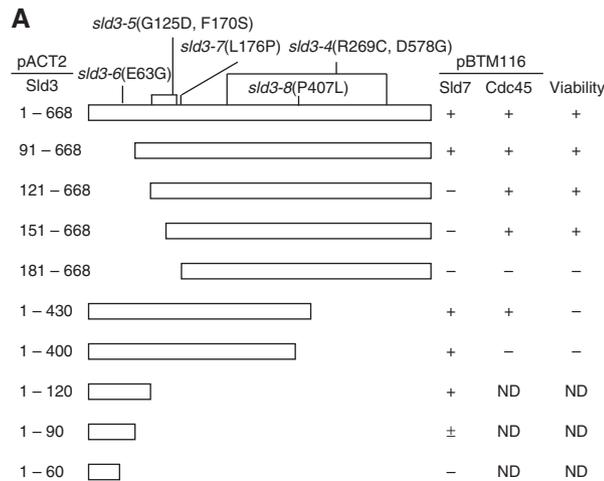
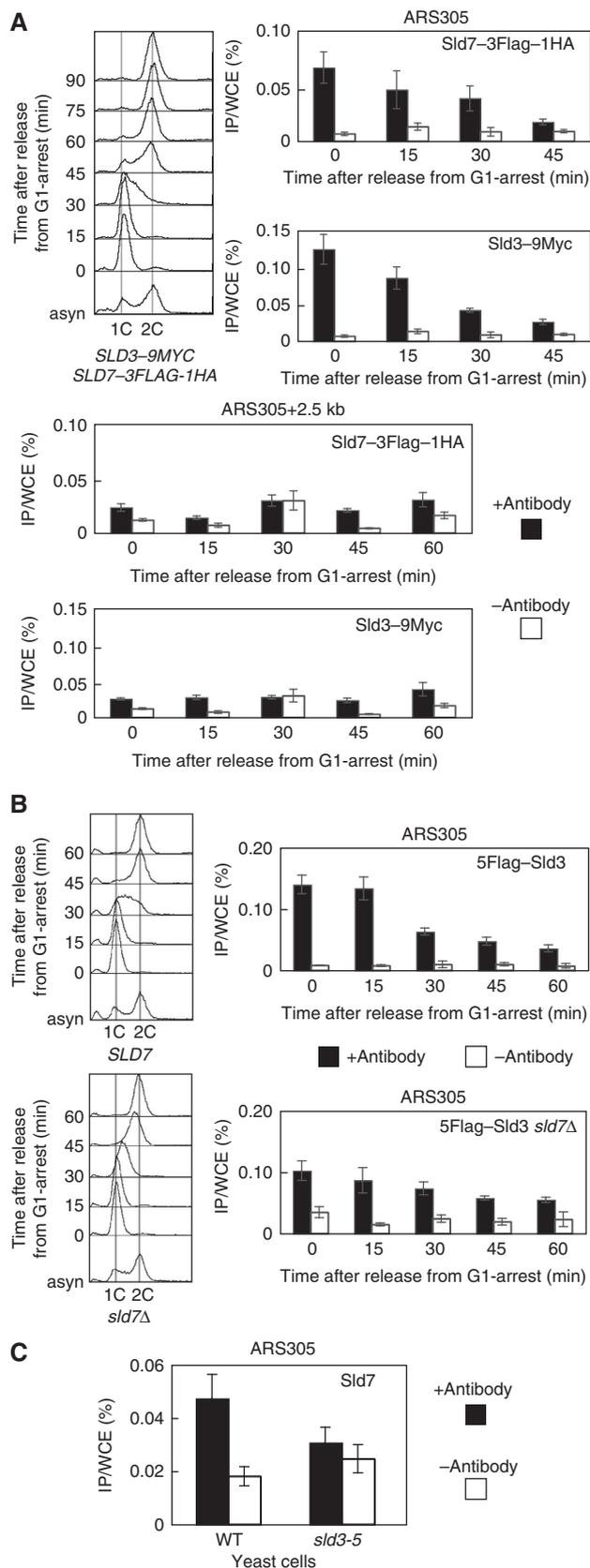


Figure 4 Reduced Sld3 protein levels in *sld7Δ* cells. (A) The *SLD3* gene on the high-copy plasmid YEp or on the low-copy plasmid YCp was introduced into YTT1 cells (*sld7Δ*). Tenfold dilutions of the cells were spotted onto YPD plates containing 0.2M HU and then incubated at 25°C for 4 days. (B, C) Restoration of the Sld3 protein levels in *sld7Δ* cells by the introduction of *SLD3* on a plasmid. The cells carrying the indicated plasmids were disrupted as described in 'Materials and methods' and subjected to SDS-polyacrylamide gel electrophoresis, followed by western blotting and probing anti-Sld3 and anti-Sld7 antibodies.

Figure 3 Interaction between Sld3 and Sld7. (A) The regions of Sld3 that interact with Sld7 and Cdc45 were determined. Various fragments derived from Sld3 were cloned into pACT2. The resultant plasmids and either pBTM116-Sld7 or pBTM116-Cdc45 were introduced into the yeast strain L40, and the interaction was detected by lacZ expression with colour. To determine whether the fragment is sufficient for cell growth, YCplac22 or pRS313 bearing various fragments of *SLD3* with its own promoter was introduced into YK13 cells (*sld3Δ::LEU2*[YEp195SLD3]) (Kamimura *et al*, 2001), and the resultant transformants were streaked onto FOA plates, which allow the growth of transformants expressing active Sld3 from YCplac22 or pRS313. (B) The HU sensitivity of the five temperature-sensitive mutants of *SLD3*: *sld3-4*, -5, -6, -7, and -8. Tenfold serial dilutions of the mutant cells were spotted onto YPD plates containing 0.2M HU. The cells were incubated at 25°C for 4 days. (C) High-copy plasmid (YEp) with or without the *SLD7* gene was introduced into the *sld3-5* and *sld3-6* cells. Then, 10-fold serial dilutions of the cells were spotted onto YPD plates containing 0.2M HU and incubated at 25°C for 4 days. (D) High-copy *SLD7* suppressed the temperature-sensitive growth of the *sld3-6* mutant, but not that of the *sld3-4*, -5, -7, or -8 mutant. The cells carrying YEplac195 (vector) or YEplac195SLD7 (YEpSLD7) were incubated at 30 or 36°C for 3 days.

Sld7 associates with replication origins in an Sld3-dependent manner

The Sld3 protein associates with early-firing origins in G₁ phase and with late-firing origins in S phase, and dissociates



from origins when DNA replication starts (Kamimura *et al*, 2001; Kanemaki and Labib, 2006). Because the Sld7 protein forms a complex with the Sld3 protein throughout the cell cycle, it was expected to associate with replication origins. Therefore, we confirmed the association between Sld7 and origins using a chromatin IP (ChIP) assay. Cells expressing Sld3-9Myc and Sld7-3Flag-HA were arrested in G₁ phase with α -factor and released at 25°C. Aliquots of the cells were sampled, and cross-linked DNA-protein complexes were immunoprecipitated using anti-Flag and anti-Myc antibodies. As shown previously, the Sld3-9Myc protein associated with ARS305, an early-firing origin, 0–30 min after release from the G₁ block (Figure 5A), which corresponds to the period from late G₁ to very early S phase on flow cytometry (Figure 5A). The origin-association signals of the Sld3-9Myc proteins decreased gradually. The ChIP assay of the Sld7-3Flag-HA protein showed exactly the same pattern as that of the Sld3-9Myc protein (Figure 5A). In the region neighbouring ARS305, the signals were at the background level. These results indicate that the Sld7 protein, as a component of the Sld3-Sld7 complex, associates with early-firing origins in G₁ phase, and then dissociates gradually from origins, and that this protein does not move with the replication fork.

Sld3 associated with ARS305 from G₁ phase, even in *sld7*Δ cells (Figure 5B). The reduced signals for the association between Sld3 and the origins might be attributable to the reduced level of Sld3 protein (Figure 4B and C). However, Sld3 in the *sld7*Δ cells dissociated from the origins slightly later than was observed in the wild-type cells. Therefore, the Sld3 protein associates with replication origins in the absence of the Sld7 protein, whereas the Sld7 protein is presumably required for a function of Sld3 other than its association with origins.

We then examined whether the association of Sld7 with origins is dependent on Sld3. Cells expressing temperature-sensitive alleles of *sld3* and Sld7-5Flag were inviable or very sick, probably because of the synthetic effect. Therefore, we used anti-Sld7 antibodies to perform a ChIP assay of the G₁-arrested cells at the nonpermissive temperature. As shown in Figure 5C, the association of the Sld7 protein with ARS305 was reduced in *sld3-5* cells at the nonpermissive temperature. We have previously shown that the Sld3-5 protein does not associate with ARS305 efficiently at the nonpermissive temperature (Kamimura *et al*, 2001). Therefore, this result indicates that Sld7 alone does not associate with the origins, but seems to associate with them only as a complex with Sld3.

Figure 5 Sld3-dependent association of Sld7 with chromatin. (A) Associations of Sld7-3Flag-HA and Sld3-9Myc proteins with chromatin. YTT5 cells expressing *SLD3-9MYC* and *SLD7-3FLAG-HA* were arrested in G₁ phase with α -factor and released at 25°C. Aliquots of the cells were sampled at 15 min intervals and subjected to a ChIP assay. (B) YTT6 (*5FLAG-SLD3*) and YTT7 (*5FLAG-SLD3 sld7*Δ) cells were synchronized and subjected to a ChIP assay, as described in (A). The cells expressing *5FLAG-SLD3* progressed to S phase faster than the parental cells. (C) YK19 (*sld3-5*) cells were grown at 25°C and α -factor was added to arrest the cells at G₁, after which the cells were incubated at 37°C for a further 2 h. The cells were collected and subjected to a ChIP assay. The levels of Sld7 protein in the *sld3-5* cells were almost the same as those observed in the wild-type cells (Supplementary Figure S5).

Sld7 affects the association of GINS with replication origins

When CDK is activated, the pre-LC, containing Dpb11, Sld2, Pol ϵ , and GINS, associates with replication origins through the interaction between CDK-phosphorylated Sld3 on origins and Dpb11, and Pol ϵ and GINS move with the replication fork when replication starts (Masumoto *et al*, 2000; Kanemaki and Labib, 2006; Muramatsu *et al*, 2010).

Therefore, we examined the chromatin association of Psf1, a subunit of the GINS complex, using a ChIP assay. In the wild-type cells, 6Flag-Psf1 associated with ARS305 in early S phase (its association level peaked at 30 min), then dissociated quickly from the origins. This was followed by its association with the neighbouring regions when replication started, indicating that Psf1 moves with the replication fork (Figure 6). In *sld7* Δ cells, the association of Psf1 with ARS305 occurred slightly later but reached the same level as that observed in the wild-type cells at 30 min. This high-level association continued to 45 min. As a consequence, the Psf1 displacement from the origin and its reassociation with the neighbouring sequences were substantially delayed (Figure 6B). This delay was more evident in its association with ARS501, a late-firing origin.

Sld7 reduces the association between Cdc45 and Sld3

Cdc45, a component of the CMG complex, forms a complex with Sld3 and associates with origins *in vivo* (Kamimura *et al*, 2001). However, once DNA replication has started, Cdc45 dissociates from origins, forms a replication fork as a component of the CMG complex, and moves along chromosomal DNA, whereas Sld3 dissociates from origins but does not move with the replication fork (Kanemaki and Labib, 2006). This suggests that the efficient association and dissociation of Cdc45 and Sld3 are important for the proper function of the CMG complex. Therefore, we compared the associations between Cdc45, Sld3, and the Sld3-Sld7 complex using purified proteins. Sld3 and Sld3-Sld7 bound specifically to Cdc45, whereas Cdc45 did not bind to GINS or Sld2 (Supplementary Figure S6). Moreover, Sld3 bound to Cdc45 more efficiently than did Sld3-Sld7 (Figure 7A). This result indicates that Sld7 reduces the association between Cdc45 and Sld3.

The genetic interactions were also consistent with the *in vitro* result. The overexpression of the *SLD7* gene from a galactose-inducible promoter suppressed the phenotype of the *sld3-6* mutant, as did the *SLD7* gene expressed from a multicopy plasmid (Figure 7B). However, the overexpression of *SLD7* inhibited the cell growth of other temperature-sensitive *sld3* mutants (*sld3-4*, -5, -7, and -8), which are suppressed by the multicopy *CDC45* gene (Kamimura *et al*, 2001) at the semipermissive temperatures of these mutants (Figure 7B). We surmise that the increased copies of Sld7 protein further reduced the weak association between Cdc45 and Sld3 in these mutant cells.

We also found that the *JET1* mutation partially suppressed the HU sensitivity of the *sld7* Δ cells (Figure 7C). *JET1* is a mutant allele of *CDC45* that bypasses the requirement for Sld3 phosphorylation by S-CDK (Tanaka *et al*, 2007). The phosphorylation of Sld3 is required for the formation of the complex between Sld3 and Dpb11, which is facilitated by Jet1 (Tanaka *et al*, 2007). We have previously shown that, in addition to *JET1*, high-copy *DPB11* also bypasses the require-

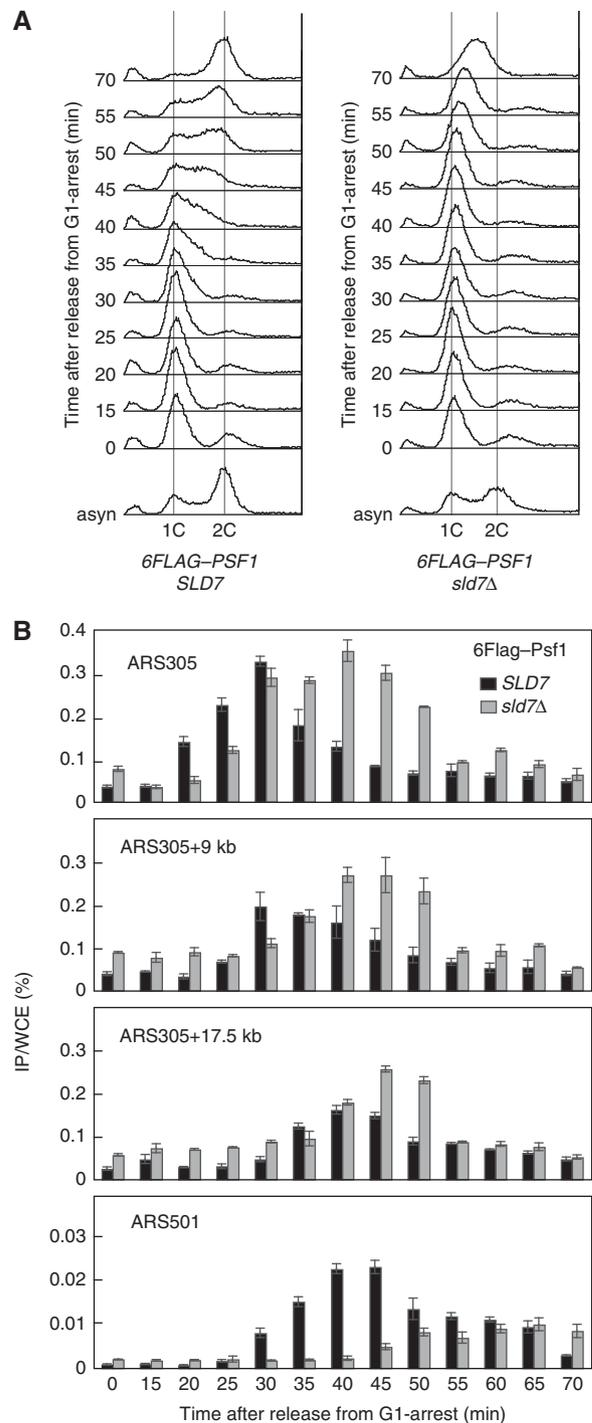


Figure 6 Chromatin association of GINS in the presence or absence of Sld7. Y2T2 (6FLAG-PSF1) or Y2T9 (6FLAG-PSF1 *sld7* Δ) cells were arrested in G₁ phase with α -factor and released at 25°C. Aliquots of the cells were sampled at 5 min intervals and subjected to flow cytometric analysis (A) and ChIP assay (B). The black and grey bars indicate the Y2T2 (*SLD7*) and Y2T9 (*sld7* Δ) cells, respectively.

ment for Sld3 phosphorylation by S-CDK, probably through the restoration of the Sld3-Dpb11 complex (Tanaka *et al*, 2007). This is also true for the suppression of the HU sensitivity of *sld7* Δ cells (Figure 7D). These observations are consistent with the increased association between Cdc45 and Sld3 in the absence of Sld7 (see Discussion).

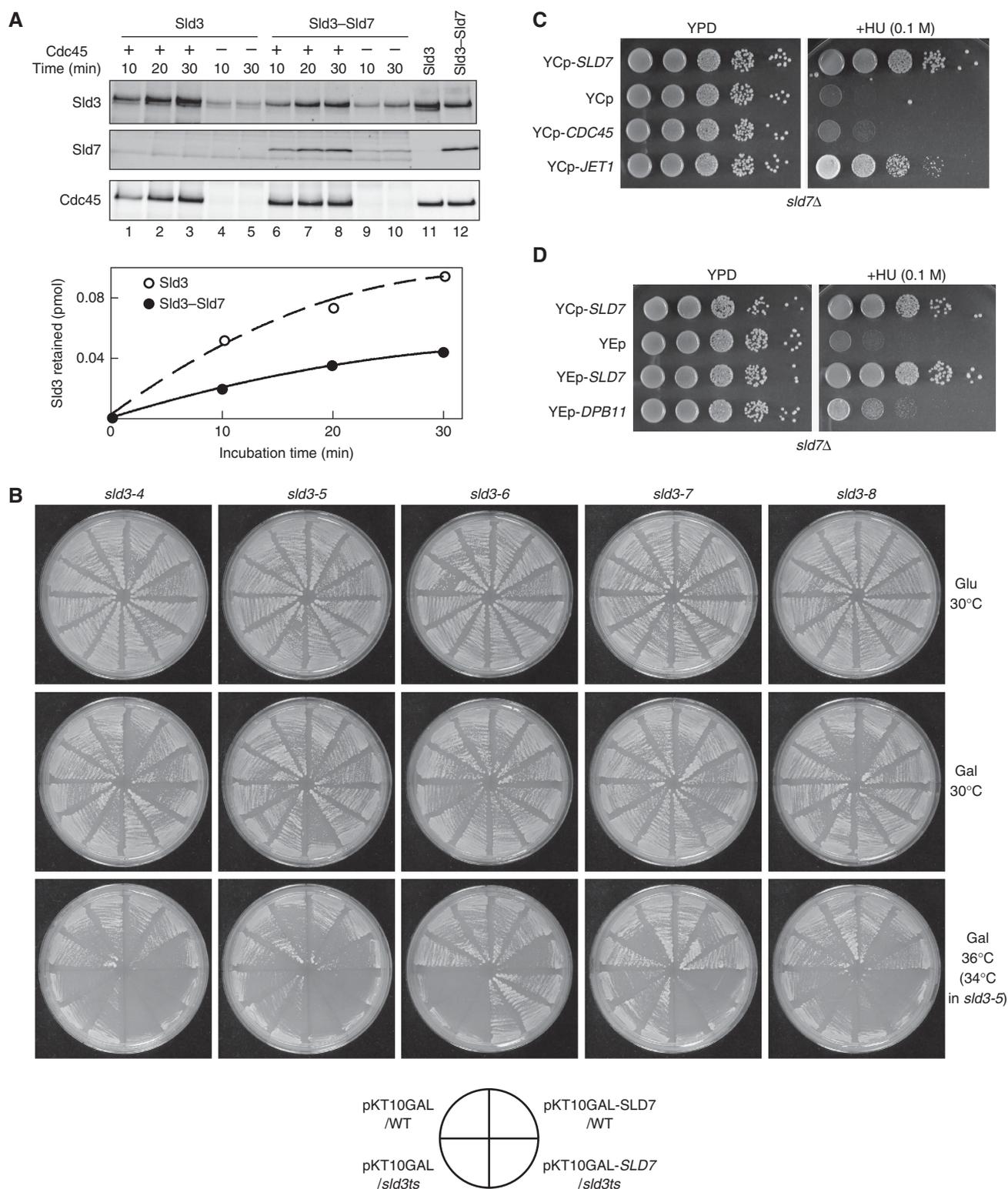


Figure 7 Reduced association between Cdc45 and Sld3 in the presence of Sld7. **(A)** The Sld3-Flag protein (1 pmol) and the Sld3-Flag-Sld7 complex (1 pmol) were incubated with Cdc45-Flag (0.4 pmol)-bound beads at 4°C for the indicated times. Half of the proteins bound to the beads were subjected to SDS-polyacrylamide gel electrophoresis, followed by western blotting, probed with anti-Sld3 and anti-Cdc45 antibodies. Lanes 11 and 12 contain samples of Sld3 (0.05 pmol), Sld3-Sld7 (0.05 pmol), and Cdc45 (0.2 pmol). **(B)** Plasmid encoding a Gal-inducible *SLD7* gene was introduced into the *sld3* temperature-sensitive mutants, *sld3-4*, -5, -6, -7, and -8. The cells were incubated on glucose or galactose plates lacking uracil at the indicated temperatures for 2 days. **(C, D)** Suppression of the HU sensitivity of the *sld7Δ* mutant by the introduction of *JET1*, an S-CDK bypass allele of the *CDC45* gene **(C)**, or *DPB11* on a high-copy plasmids **(D)**. YTT1 (*sld7Δ*) cells were transformed with the indicated plasmids. Tenfold dilutions of the cells were spotted onto plates and incubated at 25°C for 3 days.

Discussion

A recent genome-wide study of essential yeast genes strongly suggested that all the proteins involved in replication have been identified (Kanemaki *et al*, 2003). However, we have found a new replication protein, Sld7. This protein is not essential for DNA replication, although cells lacking it exhibit delayed chromosomal DNA replication. Presumably, its dispensability is the reason why Sld7 was not previously identified as a replication protein.

Role of Sld7 in DNA replication

In the absence of Sld7, the Sld3 level decreases (Figure 4B and C). Although we cannot exclude the possibility that Sld7 enhances the transcription and/or translation, it is most likely that the lack of Sld7 destabilizes Sld3. We speculate that Sld7 alters the conformation of Sld3 to increase its resistance to proteases and that the improper conformation of Sld3 in the absence of Sld7 makes it easily accessible to proteases. This Sld7-dependent conformational change may affect the efficiency of the initiation of DNA replication. Although the exact mechanisms underlying this inefficient initiation in *sld7Δ* cells are not known, we have shown that Sld7 reduces the association between Sld3 and Cdc45 (Figure 7A). Thus, it is conceivable that this reduced association contributes to the efficient displacement of Sld3 (Figure 5) and the CMG complex (Figure 6) from origins, allowing more efficient DNA replication.

Several phenomena observed in *sld7Δ* cells can be explained by the reduced levels of Sld3 and by the inefficient displacement of Sld3 from replication origins. A genome-wide study estimated the number of TAP-tagged Sld3 and Sld7 protein molecules to be 125 and 1690 per cell, respectively (Ghaemmaghami *et al*, 2003), and the number of replication origins to be about 247–400 per genome of *Saccharomyces cerevisiae*, depending on the methods used (Wyryck *et al*, 2001; Yabuki *et al*, 2002). In wild-type cells, the amount of Sld3 protein might be sufficient to be distributed evenly across the very early-firing origins during early S phase and to be redistributed to later-firing origins during mid or late S phase (Araki, 2010). However, because of the low levels of Sld3 protein, only a few origins in *sld7Δ* cells can associate with Sld3. Moreover, the redistribution of Sld3 in *sld7Δ* cells might be further delayed because the displacement of Sld3 from origins is delayed (Figure 5). Therefore, a very limited number of early-firing origins might fire at the same time in *sld7Δ* cells, and the redistribution of Sld3 may not be sufficient for the proper firing of origins. A computer simulation based on mathematical modelling predicted that the inefficient displacement of Sld3 from origins compromises the coherent firing of origins, thus causing their incomplete firing (Brümmer *et al*, 2010). This is consistent with the delay in S phase observed in *sld7Δ* cells (Figure 1B). In *sld7* cells expressing an increased dose of Sld3, higher than that in wild-type cells, Sld3 probably associates with more replication origins than in the wild-type cells, and the redistribution of Sld3 is enough for the proper firing of origins, regardless of the inefficient Sld3 displacement from origins. Consistent with this scenario, high-copy *SLD3* suppressed the HU sensitivity of *sld7Δ* cells (Figure 4A). The displacement of Sld3 from origins might require the establishment of the replication fork, and acceleration of the formation of the replication

fork might compensate for the redistribution of Sld3. This might happen through the enhanced interaction between Dpb11 and Sld3, which is caused by *JET1* and high-copy *DPB11* (Figure 7C and D).

Both the association and dissociation of the replication proteins from origins might be important. Many replication proteins associate with replication origins during the initiation step of DNA replication. Some of them form the replication machinery on origins and initiate DNA synthesis. In this step, the proteins associate first with origins and then dissociate from them to synthesize the DNA at a distance from origins, so the proteins must switch their affinity for replication origins from an association mode to a dissociation mode. The way in which they alter their affinities is not understood. However, our study of Sld7 provides a clue to this mechanism. Sld3 and Cdc45 form a complex that associates with origins, and dissociate later when replication starts. Because Cdc45 is a component of the CMG complex, which is an active DNA helicase at the replication fork, and Sld3 does not move with the replication fork, the lack of Sld7 seems to compromise the dissociation between Sld3 and Cdc45, which consequently delays the displacement of GINS from the origins. Although Sld3 binds to Sld7 throughout the cell cycle, the reduction in the affinity of Sld3 for Cdc45 caused by Sld7 suggests that Sld3 can alter its affinity for Cdc45 by interacting with other proteins and can alter the affinity modes (associations and dissociations) of protein–protein interactions. Because many proteins assemble and interact with each other at replication origins, the affinities of these complexes for the replication origins may change.

Subunit structure of the Sld3–Sld7 complex

The Sld7 and Sld3 proteins exist as a tight complex. However, why the Sld3 and Sld7 proteins exist as two distinct subunits is unknown. We propose three possible regulatory roles for this subunit structure. First, the subunit structure may maintain the concentration of the Sld3 protein at low levels, even if the level of either protein increases occasionally, because free Sld3 is unstable and is degraded. Although we observed no toxic effect of the simultaneous overproduction of Sld3 and Sld7 (our unpublished result), this regulation may prevent the precocious activation of replication origins. Second, the ratio of the two subunits, Sld3 and Sld7, may regulate the activity of Sld3. It is conceivable that the ratio of the Sld3 and Sld7 proteins varies under different conditions and that changes to the ratio in the complex have an important role in the regulation of Sld3 function. Third, the free subunits may also have a role. For example, the free Sld7 protein levels may be regulated by its interaction with Sld3. Therefore, if the amount of Sld7 that is associated with Sld3 decreases, free Sld7 may disperse inside or outside the nucleus, where it can interact with other factors to perform other functions.

Possible functions of Sld7, other than in DNA replication

The factors that act in DNA replication events also have roles in mitotic events (Shimada and Gasser, 2007; Hemerly *et al*, 2009). These factors may coordinate the distinct events of the cell cycle. We have demonstrated that Sld7 acts in DNA replication through its interaction with Sld3. At a glance, *sld7Δ* cells are unlikely to be defective in cell-cycle events except in S phase because the buds of synchronized *sld7Δ* cells appear at the same time as those of wild-type cells (Figure 1C). However, several lines of

evidence suggest that Sld7 has functions other than that in DNA replication. First, *sld7Δ* cells are sensitive to thiabendazole, an inhibitor of mitosis (our unpublished result). Second, a genome-wide study showed that the Sld7–GFP protein localized to the nucleus and spindle poles (Huh *et al*, 2003). Third, a mutation in the *SLD7* gene causes aberrant mitochondrial morphology (Altmann and Westermann, 2005). Fourth, the IP of Sld3 did not deplete the Sld7 protein completely from extracts (Figure 2F), and high-copy *SLD3* did not restore the sensitivity of these cells to thiabendazole to the level in the wild type (our unpublished result), unlike the restoration of the growth of *sld7Δ* cells. These results suggest that Sld7 interacts with a factor (or factors) other than Sld3 protein to function in situations other than replication. Further analysis is required to test this possibility.

Counterparts of Sld7 in higher eukaryotes

The N-terminal region of the Sld3 protein, which is less conserved between species than the central region (Supplementary Figure S2), binds to Sld7 (Figure 3), indicating that Sld7 protein has diverged more than Sld3. This appears to be the case because homologues of the Sld7 protein are only found in a limited range of yeast species closely related to *Saccharomyces*, whereas homologues of Sld3 protein have been found in yeast and fungi (Supplementary Figures S1 and S2). This *in silico* analysis suggests that homologues of Sld7 must exist in the fungi and yeast that have obvious homologues of Sld3, and leads us to expect that functional counterparts of Sld3 and Sld7 could even exist in higher eukaryotes.

Three TopBP1-interacting proteins, Treslin/Ticrr (Kumagai *et al*, 2010; Sansam *et al*, 2010), GEMC1 (Balestrini *et al*, 2010), and DUE-B (Chowdhury *et al*, 2010), have recently been reported as essential replication factors, required for the recruitment of Cdc45 to chromatin. TopBP1 is a possible counterpart of Dpb11. It has eight BRCT domains and its three N-terminal BRCT regions are essential for DNA replication in *Xenopus* egg extracts (Kumagai *et al*, 2010). Although a TopBP1–Treslin/Ticrr interaction has been observed in cells treated with roscovitine, an inhibitor of CDK (Sansam *et al*, 2010), TopBP1 associates with Treslin/Ticrr and GEMC1 in a CDK-dependent manner in *Xenopus* egg extracts (Balestrini *et al*, 2010; Kumagai *et al*, 2010). These characteristics are similar to those of Sld3. Moreover, a recent, sophisticated *in silico* analysis showed that Sld3 and Treslin/Ticrr have a central conserved region (Sanchez-Pulido *et al*, 2010), which corresponds to the region that binds to Cdc45 (Figure 3A). Furthermore, Treslin/Ticrr may have a subunit. Although the siRNA-mediated ablation of Treslin/Ticrr inhibited DNA replication in human cells and was rescued by an siRNA-resistant version of Treslin/Ticrr, DNA replication in Treslin/Ticrr-depleted egg extracts could not be restored by the addition of purified recombinant Treslin/Ticrr (Kumagai *et al*, 2010). The authors argued that either the recombinant Treslin/Ticrr was not active or Treslin/Ticrr forms a multi-protein complex with other unknown replication proteins.

Materials and methods

Microorganisms

The yeast strains used in this study are listed in Supplementary Table 1. To delete the *SLD7* gene, its whole open reading frame was replaced with *LEU2* using PCR.

Preparation of antibodies

Anti-Sld7 antibodies were raised against purified Sld7. The Sld7 protein was produced in *E. coli* and separated on sodium dodecyl sulphate (SDS)–polyacrylamide gels, from which the corresponding band was excised. The protein was eluted from the gel and used to immunize rabbits. The antibodies were purified from the sera of the immunized rabbits using Sld7-conjugated resin. The anti-Cdc45 monoclonal antibody was raised against the peptide TDAEVTDE-DEEDEDTISNKRGNSS, corresponding to amino acids 189–214 in the protein sequence of Cdc45.

Identification of Sld7-binding protein

YTT4 cells (7×10^7 cells/ml $\times 2.5$ l) expressing Sld7–3Flag–HA were harvested, washed once with distilled water, and disrupted with a mortar grinder (RM100, Retsch) under liquid nitrogen. The cell lysate was suspended in 5 ml of lysis buffer A (50 mM HEPES–KOH (pH 7.5), 300 mM KCl, 0.5% Tween 20, 0.05% NP40, 10% glycerol, $1 \times$ Complete (Roche), 1% protease inhibitor cocktail (Sigma), 50 mM NaF, 2 mM β -glycerophosphate, 0.4 mM Na_3VO_4 , 0.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, and 1 mM PMSF) and clarified by centrifugation at 12 000 r.p.m. at 4°C for 15 min. The protein extracts were adsorbed to 1 ml of Sepharose 4B (GE Healthcare) at 4°C for 30 min and incubated at 4°C for 2.5 h with 1 ml of anti-Flag M2 affinity gel (Sigma) that had been preincubated with lysis buffer A containing 5 mg/ml bovine serum albumin (BSA). The beads were washed three times with 3 ml of lysis buffer A containing 5 mg/ml BSA. The bound proteins were eluted twice by incubating the beads with 500 μ l of Flag elution buffer (lysis buffer A containing 150 μ g/ml of $3 \times$ Flag peptide) at 4°C for 1 h. The eluted solution was mixed with 120 μ l of anti-HA matrix (Roche) pretreated with anti-Flag M2 affinity gel and incubated at 4°C for 2.5 h. The beads were washed three times with 1 ml of buffer A containing 5 mg/ml BSA, once with 1 ml of buffer A containing 0.1 mg/ml BSA, and once with 1 ml of buffer A. The bound proteins were eluted by incubating the beads twice with 100 μ l of HA elution buffer (lysis buffer A containing 1.5 mg/ml $3 \times$ HA peptide [YPYDVPDYAYPYDVPDYAYPYDVPDYA]) at 37°C for 30 min. The proteins were precipitated by the addition of trichloroacetic acid and subsequent centrifugation. The precipitate was boiled for 3 min in 50 μ l of $1 \times$ SDS loading buffer. The proteins were separated in an SDS–polyacrylamide gel and stained by SilverQuest (Invitrogen). The protein band was excised from the gel, digested with trypsin, and analysed by mass spectrometry.

Purification of Sld3, Sld7, Sld3–Sld7, and Cdc45

Sld3–Flag was expressed in *E. coli* BL21–CodonPlus[®]–RIPL cells. The cells were disrupted by sonication, and the proteins were precipitated with 40% saturated ammonium sulphate. The proteins were incubated with M2 agarose beads at 4°C for 2 h and eluted with $3 \times$ Flag peptide (150 μ g/ml). The Sld3–Flag protein was purified further with successive HiTrap Heparin and HiTrap SP–XL column chromatography.

Sld7 was expressed as a glutathione S-transferase (GST)-fused protein connected with a PreScission Protease recognition site in *E. coli* BL21–CodonPlus–RIPL cells. The cells were disrupted by sonication, the nucleic acids were removed with Polymin P (final concentration, 0.3%), and the proteins were precipitated with 40% saturated ammonium sulphate. The proteins were purified with glutathione Sepharose and then HiTrap Heparin HP. The GST-fused protein was bound again to glutathione Sepharose and cleaved with GST–PreScission Protease. The cleaved Sld7 was purified with MiniS column chromatography.

The Sld3–Sld7 complex was purified from yeast or *E. coli*. In yeast, 5Flag–Sld3 and Sld7 were coexpressed in BJ2168 cells under the control of the galactose promoter from YEplac112 (Gietz and Sugino, 1988) and pKT10GAL (Kawamura *et al*, 1996), respectively. The cells were frozen in liquid nitrogen and ground with a mortar grinder. The 5Flag–Sld3–Sld7 complex was purified with anti-Flag M2 agarose affinity gel (Sigma) and then with HiTrap SP–XL. The 5Flag–Sld3–Sld7 complex was purified from *E. coli* as described for Sld3–Flag protein purified from BL21–CodonPlus–RIPL cells, producing Sld3–Flag and Sld7 simultaneously.

Cdc45–Flag was expressed in *E. coli* BL21–CodonPlus–RIPL cells. The cells were disrupted by sonication, and the proteins were incubated with M2 agarose beads at 4°C for 2 h and eluted with $3 \times$ Flag peptide (150 μ g/ml). The Cdc45–Flag protein was purified further using RESOURCE[™] Q column chromatography.

Complex formation between Sld3 and Sld7

E. coli BL21-CodonPlus-RIPL cells carrying the pETDuet-(6His)SLD3, pETDuet-SLD7, or pETDuet-(6His)SLD3-SLD7 plasmid were grown in 1 l of LB broth containing ampicillin (200 µg/ml) at 37°C. When the OD₆₀₀ reached 0.5, 0.1 mM IPTG was added to induce the expression of the proteins, and the cells were incubated at 20°C for 16 h. The cells were harvested and suspended in 5 ml of sonication buffer 300 (50 mM HEPES–KOH (pH 7.5), 300 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT, and 10% glycerol), and sonicated five times at 150 W for 30 s. The cell lysate was centrifuged at 15 000 r.p.m. at 4°C for 15 min. Most of the Sld3, Sld7, and Sld3–Sld7 proteins were precipitated. The precipitates were suspended in 1 ml of sonication buffer 500 (50 mM HEPES–KOH (pH 7.5), 500 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT, and 10% glycerol) and sonicated five times again at 150 W for 30 s. After incubation on ice for 1 h, the solution was clarified by centrifugation at 15 000 r.p.m. at 4°C for 15 min and the supernatant was then incubated with 50 µl of Ni–NTA agarose beads (Qiagen) at 4°C for 2 h. The beads were washed five times with 1 ml of washing buffer (50 mM HEPES–KOH (pH 7.5), 300 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT, 10% glycerol, and 20 mM imidazole), and the proteins were eluted with 200 µl of imidazole elution buffer (50 mM HEPES–KOH (pH 7.5), 300 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT, 10% glycerol, 250 mM imidazole, 0.1% Tween 20, and 0.01% Triton X-100). The eluates were boiled for 3 min in 1 × SDS loading buffer.

To mix the two purified proteins, 1.6 pmol of Sld3–Flag bound to M2 anti-Flag-antibody-conjugated Dynabeads M-270 was mixed with 12.5 pmol of Sld7 in 50 mM HEPES–KOH (pH 7.5) containing 0.2 M CH₃CO₂K, 0.2 M KCl, 2 mM Mg(CH₃CO₂)₂, 1 mg/ml BSA, 2.5 mg/ml casein, 0.1% Tween 20, 0.01% NP40, and 10% glycerol at 30°C for 10 min. After brief washing, the bound proteins were eluted with 20 µl of 200 µg/ml 3 × Flag at 4°C for 15 min.

Complex formation between Sld3 and Cdc45 in vitro

Cdc45–Flag was immobilized on anti-Cdc45-antibody-conjugated rProtein A Sepharose Fast Flow (GE Healthcare). The Cdc45–Sepharose beads (10 µl) containing 0.4 pmol of Cdc45 were incubated with 400 µl of reaction buffer (50 mM HEPES–KOH (pH 7.5) containing 0.2 M CH₃CO₂K, 0.2 M NaCl, 2 mM Mg(CH₃CO₂)₂, 2 mg/ml BSA, 2.5 mg/ml casein, 0.1% Tween 20, 0.01% NP40, and 10% glycerol) with 1 pmol of Sld3 or Sld3–Sld7 at 4°C for various times. After they were washed, the beads were incubated with

50 mM Tris–HCl (pH 6.8) containing 2% SDS and 10% glycerol at 65°C for 10 min to elute the proteins.

Glycerol gradient sedimentation

The samples were applied to a 15–30% glycerol gradient in 50 mM HEPES–KOH (pH 7.5), 0.15 M NaCl, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.01% Triton X-100 and centrifuged for 16 h at 42 000 r.p.m. at 4°C in an SW50.1 rotor.

Other methods

Proteins were extracted from yeast cells for electrophoretic analysis with the protocol described by Kushnirov (2000). The yeast two-hybrid assay was performed as described previously (Kamimura *et al*, 2001). The rapid *in vivo* cross-linking ChIP assay (Kohzaki and Murakami, 2007) was used for the ChIP assay. After western blotting, the proteins were visualized and quantified using fluorescence-conjugated secondary antibodies and the Odyssey Infrared Imaging System (LI-COR Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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