Proper initiation of chromosomal DNA replication requires the Sld3-Sld7 complex in budding yeast

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ABSTRACT

Eukaryotic chromosomal DNA replication initiates from replication origins only once per cell cycle. Many proteins assemble on origins, form replication machinery, and then dissociate from origins to start DNA synthesis. However, the mechanism governing these processes has not been well elucidated. Here, I show that the Sld7 protein participates in the dissociation process between replication machinery and origins. *SLD7* (*S*ynthetic *L*ethality with *Dpb11-24 7*), a newly identified gene in a synthetic lethal screening, is not essential for cell growth but required for several cellular processes, such as an efficient chromosomal DNA replication. The Sld7 protein always forms a complex with the Sld3 protein, which functions for the initiation of chromosomal DNA replication, to enhance the Sld3 function. Thus, high-copy *SLD3* gene compensates for the lack of Sld7, and high-copy *SLD7* gene suppresses the *sld3-6* mutation that reduces the complex formation between Sld3 and Sld7 proteins. This Sld3-Sld7 complex associates with replication origins in G1-phase and dissociates gradually from them when replication initiates. Sld3 protein associates with and then dissociates from origins in the absence of Sld7. The Mcm7 protein, a component of replication machinery, associates with origins and then with neighboring regions of origins as it moves with replication forks. In the absence of Sld7 protein, although Mcm7 associates with origins as efficient as in WT cells, its association with neighboring region is significantly reduced. Therefore, the Sld7 protein seems to help the efficient displacement of replication machinery from origins.

ABBREVIATIONS

E. coli; Escherichia coli
S. cerevisiae; Saccharomyces cerevisiae
S. pombe; Schizosaccharomyces pombe
ARS; autonomously replicating sequence
bp; base pair
BRCT; Brca1 C-terminal
BSA; bovine serum albmin
C-terminal; carboxyl-terminal
ChIP; chromatin immunoprecipitaion
Da; dalton
DAPI; 4', 6-diamino-2-phenylindole
DTT; dithiothreitol
EDTA; ethylenediaminetetraacetic acid
5FOA; 5-fluoroorotic acid
HEPES; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
His; histidine
HU; hydroxyurea
IP; immunoprecipitaion
IPTG; isopropyl- β -D-thiogalactopyranoside
kb; kilo base pair
Leu; leucine
MCM; mini-chromosome maintenance
MMS; methylmethane sulfonate
2-ME; β -mercaptoethanol
N-terminal; amino-terminal
PAGE; polyacrylamide gel electrophoresis
PBS; phosphate buffered saline
PCR; polymerase chain reaction
PMSF; phenylmethylsulfonyl fluoride
Pol ε ; DNA polymerase ε
pre-RC; pre-replicative complex
SC; synthetic complete medium
SDS; sodium dodecyl sulfate

Trp; tryptophan

Ura; uracil

X-gal; 5-bromo-4-chloro-3-indolyl- β -galactoside

INTRODUCTION

S-phase, the DNA synthetic phase of the cell cycle

For cell reproduction, the components of the cells are duplicated and distributed into two daughter cells through the highly regulated sequence of events, called the cell cycle. The eukaryotic cell has an elaborate regulatory system for the correct order of the cell-cycle events. Eukaryotic chromosome is a huge molecule containing a long, thin DNA strand and DNA-binding and packaging proteins. This immense mass has to be duplicated once and only once per cycle for maintaining the genetic information from one generation to the next. This event occurs in a DNA-synthetic phase of the cell cycle, called S-phase. DNA replication initiates from the replication origins, which are scattered in large numbers along the chromosomes. In budding yeast, replication origins are defined by specific DNA sequence motifs, which are called autonomously replicating sequence (ARS), and protein complexes (Raghuraman *et al.*, 2001). In most eukaryotes, however, origins do not contain well-defined DNA sequences, and origin function is thought to depend on local chromatin structure (Gilbert, 2001). In eukaryotic organisms, to ensure the accurate timing of the chromosome duplication and prevent re-replication of an origin in a cycle, the initiation of DNA replication is strictly regulated, and many proteins are involved in this process (Kearsey and Cotterill, 2003).

Only once replication per cell cycle

For initiating DNA replication, a large complex of the initiator proteins, called pre-replicative complex (pre-RC) assembles at replication origins to prepare for their firing (Diffley *et al.*, 1994). The stepwise assembly of the initiator proteins provides multiple

points of control to ensure efficiency and fidelity of DNA replication. However, the mechanisms of protein assembly and origin activation remain elusive. At the onset of S-phase, the pre-RCs are activated through the action of two different kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent Cdc7 kinase (DDK) (Takeda and Dutta, 2005). Once an origin has been activated, the pre-RC disassembles, and its assembly is prevented until the next cell cycle (Arias and Walter, 2007). These processes ensure the only-once-replication system per cell cycle.

Cdk, the central component of the cell-cycle control system, governs the timing of the initiation of cell-cycle events. Cdks are activated through binding to cyclins, the regulatory proteins of Cdks. The different types of cyclins are produced in the specific phase of cell cycle and form a complex with the Cdk (CDK) (Bloom and Cross, 2007). This results in the oscillation of Cdk activity and phosphorylation of the specific substrates in appropriate time. CDK activity is important for the only-once replication (Nguyen *et al.*, 2001). S-phase cyclins and Cdk forms a complex (S-CDK) in S-phase to initiate DNA replication.

DDK activity is also regulated by the periodic stabilization of Dbf4 protein during the cell cycle. At the G1/S transition, and throughout S-phase, DDK is recruited to individual origins to facilitate concerted phosphorylation on the N-terminal side of multiple MCM subunits (described later) (Sheu and Stillman, 2006; Masai *et al.*, 2006), and this process is also required for the initiation of DNA replication.

DNA polymerases

DNA polymerases, the enzymes that copy the template DNA strands into new complementary DNA strands are the central factors to replicate DNA molecules. The

recruitment of DNA polymerases onto replication origins is a crucial step to assemble replication machinery and to begin DNA synthesis. There are three essential polymerases, Pol α, Pol δ, and Pol ε, which are conserved among eukaryotes (Rytkönen. *et al.*, 2006). Pol α is the only enzyme that can start DNA replication. Primase associated with Pol α synthesizes RNA primers, and Pol α synthesizes DNA using RNA primers (Ricke and Bielinsky, 2004), while Pol δ and Pol ε elongate the Pol α-initiated DNA strands (Garg and Burgers, 2005). Pol δ and Pol ε are believed to function as replicative polymerases in eukaryotes. However, the mechanisms how DNA polymerases are loaded on replication origins are still mystery.

MCM complex

For synthesizing DNA strand, DNA double helix has to be unwound. The enzyme, which has an activity to unwind the helices at replication forks, is called replicative helicase. It has been believed that the hexamelic protein complex, MCM is a component of replicative helicase because the purified complex consisting of Mcm4, Mcm6 and Mcm7 isolated from *HeLa* and yeasts extracts exhibited helicase activity (Ishimi *et al.*, 1997; Lee and Hurwitz, 2000; Kaplan *et al.*, 2003). Moreover, ChIP experiments indicated that Mcm4 and Mcm7 traveled with the DNA replication fork (Aparicio *et al.*, 1997; Labib *et al.*, 2000), and quantitative depletion of Mcm proteins from *Xenopus laevis* egg extract resulted in the defect in DNA unwinding (Shechter *et al.*, 2004). Purified MCM2-7, however, is devoid of DNA helicase activity (Takahashi *et al.*, 2005). This paradox has been an obstacle in the study of DNA replication for a long time.

Cdc45 protein and GINS complex

Recently, the studies about GINS and Cdc45 have grown into the clue for solving the problems in the study of DNA replication. Cdc45 is highly conserved in higher eukaryotes. Cdc45 activity is required for Pol α and Pol ε associations with origins in budding yeast, fission yeast, and *Xenopus* (Kearsey and Cotterill, 2003). Human Cdc45 interacts with MCM, Pol α, Pol δ, Pol ε and GINS complex (Kukimoto *et al.*, 1999; Bauerschmidt *et al.*, 2007). Moreover, density transfer analysis indicated that Cdc45 is involved in not only the initiation step, but also the elongation step of chromosomal DNA replication (Tercero *et al.*, 2000).

GINS complex, composed of four subunits, Sld5, Psf1, Psf2, and Psf3, was originally identified in budding yeast (see below; Takayama *et al.*, 2003) and its *Xenopus* homologue has been characterized (Kubota *et al.*, 2003). This complex is conserved in archaea (Marinsek *et al.*, 2006) and throughout eukaryotes and essential both for the initiation and progression of DNA replication. The recombinant human GINS physically binds to Pol α-primase and stimulates its polymerase activity (De Falco *et al.*, 2007). The structural analysis of human GINS revealed that it has unstructured regions on its surface, which are probably involved in its interaction with other proteins (Kamada *et al.*, 2007; Chang *et al.*, 2007; Boskovic *et al.*, 2007). The exact role of GINS is still to be elucidated.

In budding yeast, GINS is recruited to origins, and as a result, Cdc45 and GINS become stably engaged, and they move away from origins with replication forks (Takayama *et al.*, 2003; Kanemaki *et al.*, 2003; Calzada *et al.*, 2005; Kanemaki and Labib, 2006). It was reported that GINS in budding yeast (Takayama *et al.*, 2003), fission yeast (Yabuuchi *et al.*, 2006), and *Xenopus* (Kubota *et al.*, 2003) are required for the stable recruitment of Cdc45 to chromatin during S-phase.

RPC in *Saccharomyces cerevisiae* and CMG complex in *Drosophila melanogaster*

Recently, a huge protein complex 'replication progression complexes (RPCs)' including MCM, Cdc45, and GINS was purified from yeast extract (Gambus *et al.*, 2006). This complex is formed only during S-phase and dependent upon the prior loading of MCM at origins. In the absence of Psf2, Mcm2-7 and Cdc45 cannot associate during S-phase (Gambus *et al.*, 2006). These results indicated that GINS is required for the formation of a stable MCM-Cdc45 complex. Moreover, in the absence of GINS, MCM-Cdc45 does not move away from origins, and genomic footprinting indicates that the origin remains in the pre-replicative state (Kanemaki and Labib, 2006).

Since Cdc45 is required for unwinding of template DNA in *Xenopus* egg extract (Walter and Newport, 2000), it has been suggested that Cdc45 has a role as a processing factor for the MCM helicase (Masuda *et al.*, 2003; Pacek and Walter 2004). GINS has been proposed to act as an activator of MCM helicase activity in association with Cdc45 (Kubota *et al.*, 2003; Takahashi *et al.*, 2005; Pacek *et al.*, 2006). Remarkably, a complex, named 'CMG' including Cdc45, MCM, and GINS was purified from extracts of *Drosophira* early embryos. This complex is derived from S-phase chromatin and it has ATP-dependent helicase activity *in vitro* (Moyer *et al.*, 2006). Purified human GINS showed preferential binding for DNA structures containing single strand DNA using electrophoretic mobility shift assays, speculating about the possible role of GINS after its association with the other components of CMG complex (Boskovic *et al.*, 2007). GINS was also detected at the site of a paused DNA replication fork during replication of a plasmid in *Xenopus* egg extracts as a component of 'unwindsome' (Pacek *et al.*, 2006).

Dpb11 and the isolation of *SLD1-6*

A line of the studies about Dpb11 and Sld proteins in budding yeast also provides the key to unravel a tangle in replication field. Dpb11 in budding yeast is an essential protein for the initiation of DNA replication. It was originally identified as a DNA polymerase-interacting factor. An elevated dosage of *DPB11* suppresses the mutation in a Dpb2 subunit of Pol ε , and the mutation in the carboxyl terminal domain of the Pol2 of Pol ε , which is essential for DNA replication (Araki *et al.*, 1995; Dua *et al.*, 1999; Kesti *et al.*, 1999). Thus, Dpb11-Pol ε complex may be required for the DNA replication and the S-phase checkpoint. Actually, the Dpb11 controls the recruitment of Pol α and Pol ε onto origins (Masumoto *et al.*, 2000), and *DPB11* genetically and physically interacts with *DDC1*, which is a checkpoint factor (Wang and Elledge, 2002).

Dpb11 consists of four tandem BRCT repeats (Bork *et al.*, 1997), which have the ability to bind phosphorylated protein (Manke *et al.*, 2003, Yu *et al.*, 2003). Some of the proteins that have BRCT repeats, such as TopBP1, Cut5, and Mus101 are thought to be the functional homologues of Dpb11 in higher eukaryotes, and they have roles in many aspects of biological processes as well as DNA replication (Garcia *et al.*, 2005). A series of the genes that named *SLD* were isolated as genes whose mutations are synthetically lethal with *dpb11-1*, the temperature sensitive mutant of *DPB11* (Kamimura *et al.*, 1998). *SLD1-6* genes encode Sld1-6 proteins, respectively. Sld1 (Dpb3) is one of the subunits of DNA Pol ε . Sld4 is identical to Cdc45, essential for the initiation and progression of DNA replication. Sld6, also known as Rad53, is required for replication checkpoint. Sld2, Sld3, and Sld5 (described above) were unknown factors. Although these factors are essential for the initiation of DNA replication, precise functions of individual factors in DNA replication had not been elucidated. Recent studies have revealed some of the aspects of these proteins.

The roles of Sld2 and Sld3 proteins

Recent study revealed that Sld2 and Sld3 are the minimal set of the S-CDK targets required for DNA replication. (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). During S-phase, the Sld2 protein is phosphorylated by S-CDK, and Sld2 binds to the C-terminal BRCT repeats of Dpb11 in a phosphorylation dependent manner (Masumoto *et al.*, 2002; Tak *et al.*, 2006). The Sld2 phosphorylation triggers the formation of pre-Loading complex (pre-LC), included Dpb11, Sld2, Pol ε , and GINS, and the pre-LC is formed independently of pre-RC formation in S-phase (S. Sakamoto and H. Araki, unpublished result; described in Walter and Araki, 2006).

Drc1, a homologue of Sld2 in fission yeast is also phosphorylated by S-CDK and interacts with Cut5, a homologue of Dpb11 (Noguchi *et al.*, 2002). In higher eukaryotes, RecQ4 is a candidate of Sld2 homologue because N-terminal region of RecQ4 has some sequence similarity with Sld2 (Sangrithi *et al.*, 2005). RecQ4 interacts with Cut5 (Matsuno *et al.*, 2006), and in humans, mutations in RecQ4 are responsible for some cases of Rothmund-Thomson syndrome (Kitano *et al.*, 1999), suggesting that RecQ4 is important for preventing genome instability and that mutations in this protein lead to cancer.

S-CDK also phosphorylates Sld3 during S-phase, and phosphorylated Sld3 binds to N-terminal BRCT repeats (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). The Sld3 forms a complex with Cdc45 throughout the cell cycle (Kamimura *et al.*, 2001). Since these proteins are loaded onto replication origins mutually dependent manner in the absence of the CDK activity (Kamimura *et al.*, 2001), we suggest that the Sld3 acts as a docking site for pre-LC through the interaction between Sld3 and Dpb11. Therefore, the Sld2-Dpb11-Sld3 complex formation is required for the loading of the pre-LC, eventually

DNA polymerases onto the replication origins. As I mentioned above, it was shown that Cdc45 is required for elongation step of DNA replication and travels with replication forks (Aparicio *et al.*, 1997). On the other hand, Sld3 is required for initiation of DNA replication but not for the progression of replication forks (Kanemaki and Labib, 2006).

Fission yeast Sld3 (SpSld3), a counter part of budding yeast Sld3, interacts with SpCdc45 in early S-phase. This interaction is required for the loading and maintenance of the SpCdc45 on chromatin and the formation of SpCdc45-MCM complex (Nakajima and Masukata, 2002). However, unlike the budding yeast Sld3, the SpSld3 associates with origins independently of the SpCdc45 (Yamada *et al.*, 2004). The SpSld3 also interacts with Mcm proteins, and SpSld3-MCM interaction occurs before SpSld3-SpCdc45 interaction. Chromatin immunoprecipitation indicated that the SpSld3 associates with the replication origins in late G1 phases, and this association depends on DDK activity (Yamada *et al.*, 2004). Unlike Dpb11, Sld2, Cdc45, and GINS, Sld3 has been found only in budding and fission yeasts. However, since the N-terminal BRCT domain of Dpb11 is broadly conserved through the eukaryotes, Sld3 might exist in higher eukaryotes.

Since Dpb11 binds to Sld3 in a phosphorylation dependent manner (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007), and all the components of the CMG complex also bind to the Sld3 (Kamimura *et al.*, 2001; Takayama *et al.*, 2003), it is proposed that Sld3 acts as a link between the elongating DNA polymerases and the replicative DNA helicase. However, its accurate function remains unknown.

Sld7 protein

This study showed that novel Sld7 protein forms a complex with Sld3 protein through the direct interaction. This complex exists throughout cell cycle, and the

Sld3-Sld7 interaction is required for the function of the Sld3 protein. Additionally, this complex is loaded onto replication origins at G1-phase and displaced from origins with cell-cycle progression. The loading and displacement of Sld3-Sld7 complex may allow stable and active engagement of Cdc45 and GINS to the MCM complex on the origins and efficient progression of the active replicative helicase.

RESULTS

Isolation of the *SLD7* gene

Another *sld* screening using *dpb11-24* mutation identified the mutations in several genes (M. Kanemaki, Y. Kamimura, and H. Araki, unpublished result). In this screening, six mutations, which are synthetically lethal with *dpb11-24*, were isolated from 32000 colonies. Five of the isolated mutations occurred in known genes (two in *SLD3*, one each in *DPB2*, *MCM3*, and *MCM10*), and the remaining one was the mutation in unknown gene (*YOR060C*, Saccharomyces Genome Database, Valens *et al.*, 1997). This gene was located on chromosome XV and named *SLD7* (Synthetic Lethality with *Dpb11-24 7*). It encodes 29 KDa protein, which is consisted of 257 amino acids. This protein shares 25 % identity and 43 % similarity with *orf19.3531* in *Candida albicans*. However, this protein does not share any homology with other proteins in higher eukaryotes even in fission yeast. In addition, no functional motifs in this protein have been found so far. The isolated mutation (*sld7-1*) had a replacement of arginine for the 42nd glycine (G42R) of the Sld7 protein.

The *SLD7* gene is not essential but important for cell growth

First, I examined whether the *SLD7* gene is essential for cell growth. For this purpose, one of the *SLD7* gene copies in a *leu2/leu2* diploid cell was replaced with the *sld7 Δ ::LEU2*. Sporulation and tetrad dissection of the resultant *SLD7/sld7 Δ ::LEU2* heterodisruptant cells yielded two large and two small colonies (Fig. 1). All large colonies were Leu^- while all small colonies were Leu^+, indicating that small colonies were derived from a *sld7 Δ ::LEU2* allele. The two spores of 36 Leu^- spores (WT) and 15 of 36 Leu^+

spores (*sld7 Δ ::LEU2*) did not form colony. Microscopic observation revealed that some of these lethal spores carrying *sld7 Δ ::LEU2* allele died without germination, and some of them died after several division. Then, I cultured these colonies in YPD media at 30 ℃ and measured doubling time of these cells. The doubling time of WT cells was 77.5 minutes, while that of the cells carrying *sld7 Δ* was 86.2 minutes. Thus, the *SLD7* gene is not essential but important for cell growth.

It was reported that the null mutant of *SLD7*, constructed in a systematic high-throughput deletion study (Winzeler *et al.*, 1999), is inviable. However, even the *SLD7*-deleted yeast strain that was used in the systematic study (Winzeler *et al.*, 1999) and obtained from EUROSCARF was also viable as well as in W303 strain used in this study.

The Sld7 protein is essential for the growth of cells defective in specific replication protein

To know whether the genetic interaction between *SLD7* and *DPB11* gene is specific between *sld7-1* and *dpb11-24* mutations, I examined the synthetic lethality between *sld7 Δ* and *dpb11-24* and *dpb11-1* mutations, which is used for the screening of *SLD1-6* genes (Kamimura *et al.*, 1998). Sporulation and tetrad dissection of the *sld7 Δ ::LEU2/dpb11-24* and *sld7 Δ ::LEU2/dpb11-1* diploid did not yield *sld7 Δ dpb11-24* nor *sld7 Δ dpb11-1* spore clones (Fig. 2a and b), indicating that the *sld7 Δ* mutation is synthetically lethal with both *dpb11-24* and *dpb11-1* mutations. Microscopic observation revealed that the spores carrying *sld7 Δ ::LEU2* and *dpb11-24* germinate and arrest with dumbbell-like shape, a typical terminal-phenotype of the cells defective in replication genes (Fig. 2e). To confirm that disruption of the *SLD7* gene caused lethal spores, the *dpb11-24/sld7 Δ ::LEU2* or *dpb11-1/sld7 Δ ::LEU2* diploids transformed with

the plasmid carrying the *SLD7* gene (YEp-*SLD7*) were sporulated. Tetrad dissection yielded four large colonies (Fig. 2c and d), indicating that the disruption of the *SLD7* gene in *dpb11-24* or *dpb11-1* background causes the lethality of the cells. Therefore, the genetic interaction between *SLD7* and *DPB11* is not specific between *sld7-1* and *dpb11-24*, but rather general between *sld7* and *dpb11*.

Since the most *SLD* genes are required for DNA replication, the *SLD7* gene may play a role in DNA replication. Thus, I tested the synthetic lethality between the *sld7 Δ* mutation and mutations occurring in the replication genes, because two mutations occurring in related genes often cannot be combined even at the conditions permissive for a single mutation. The *sld7 Δ* mutation was synthetically lethal with the mutations in other *SLD* genes (*drc1-1*, *sld5-12*, *sld3-5*, *cdc45-27*) or the component of pre-LC (*pol2-11*, *psf1-1*), but not in the component of pre-RC (*mcm2-1*, *mcm3-1*, *orc2-1*, *orc5-1*) or the checkpoint gene (*ddc1 Δ*) (Table 3). These results suggest that *SLD7* plays a role in DNA replication, especially in the process working with the other *SLD*s and the pre-LC components.

Inefficient chromosomal DNA replication in the *sld7 Δ* mutant cells

Since *SLD7* was suggested to play a role in DNA replication, I examined DNA replication of *sld7 Δ* cells using flow cytometry. The *sld7 Δ* mutant cells and wild-type (WT) cells were synchronized in G1-phase with α-factor and released from a G1-arrest at 25 ℃. Aliquots of cells were sampled at 5 minutes intervals and DNA contents were measured by flow cytometry. DNA content of WT cells increased from 30 minutes and reached 2C 45 minutes after release (Fig. 3a), while DNA content of *sld7 Δ* mutant cells increased from 35 minutes and reached 2C 70 minutes after release, taking about 35

minutes to complete DNA replication. The buds appeared at same timing in both WT cells and *sld7 Δ* mutant cells (Fig. 3b), suggesting that the cell-cycle events except DNA replication are likely normal. This indicates that *SLD7* is required for efficient chromosomal DNA replication.

Many mutants defective in DNA replication show sensitivity to inhibitors of DNA replication and DNA damaging agents (Koç *et al.*, 2004, Wyatt and Pittman, 2006). Therefore, I examined whether the *sld7 Δ* mutant cells are sensitive to hydroxyurea (HU; replication stress) and methylmethane sulfonate (MMS; DNA-damaging agent). The *sld7 Δ* mutant cells were sensitive to 0.2 M HU and 0.01 % MMS (Fig. 4). On the other hand, the transient treatment of HU did not reduce the viability of the *sld7 Δ* mutant, suggesting that this mutant is not defective in checkpoint process. Taken together, these results are consistent with the idea that *SLD7* have a role in chromosomal DNA replication.

The Sld7 protein forms a complex with Sld3

To know the role of Sld7 protein in DNA replication, I have tried to identify proteins binding to the Sld7 protein. For this purpose, I employed immunoprecipitation (IP) using asynchronous cells harboring *3FLAG-1HA* tandem-tagged *SLD7* (Sld7-3Flag-1HA). First, the Sld7-3Flag-1HA protein was immunoprecipitated by anti-Flag M2 affinity gel (Sigma) and released from the gel using 3×Flag peptides. The released Sld7-3Flag-1HA protein was precipitated by anti-HA matrix (Roche) and released from the matrix using 3×HA peptides. Comparing with immunoprecipitates from the extracts of WT cells, 80 kDa protein was co-precipitated specifically with Sld7-3Flag-1HA (Fig. 5a). The band containing this protein was excised from the gel, and the sample was subjected to

analysis of mass spectrometry after digestion with trypsin. According to the peptide-sequence determined by mass spectrometry, the protein was identified as Sld3 protein. Western blotting using anti-Sld3 antibodies further confirmed that Sld3 protein co-precipitated with Sld7-3Flag-1HA protein (Fig. 5b). Conversely, Sld7-3Flag-1HA protein co-precipitated with 9Myc-tagged Sld3 protein (Sld3-9Myc) when the proteins were precipitated with anti-Myc antibodies (Fig. 6b). Thus, the Sld7 protein and the Sld3 protein form a complex.

To examine whether the interaction between Sld7 and Sld3 is direct or not, 6His-Sld3 protein and Sld7 protein were co-expressed in *E. coli* cells, and 6His-Sld3 protein was precipitated with Ni-NTA agarose (Qiagen) (Fig. 7a). In this assay, Sld7 protein was co-precipitated and eluted with 6His-Sld3 protein. To rule out the possibility that the protein(s) of *E. coli* connect Sld3 protein with Sld7 protein, I further purified these proteins expressed independently in *E. coli* cells, and the purified proteins were mixed. The purified Sld7 protein was also co-precipitated and eluted with 6His-Sld3 protein (Fig. 7b). Therefore, Sld7 protein binds to Sld3 protein directly.

The Sld3 protein exists as a complex with the Sld7 protein throughout the cell cycle

During co-precipitation assay, I found that depletion of Sld7-3Flag-1HA protein by anti-Flag antibody also depletes Sld3-9Myc protein from extracts prepared from asynchronous cultured cells (Fig. 6a), while the Sld7-3Flag-1HA protein remains in the extracts in which Sld3-9Myc protein is depleted (Fig. 6b). These results indicate that Sld3-free Sld7 proteins exist, while almost all the Sld3 proteins form a complex with the Sld7 proteins in cells. Consistent with this, the co-immunoprecipitation assay showed the Sld3-Sld7 complex in cells treated with α-factor, HU, and nocodazole (NOC), which

arrest cells in the specific phase of the cell cycle, G1, S, and G2/M, respectively (Fig. 8). Thus, Sld3-Sld7 complex exists throughout the cell cycle.

The Sld7 protein binds to the N-terminal portion of Sld3 protein

Next, to determine the Sld7-binding domain of Sld3 protein, the interaction between Sld7 protein and truncated Sld3 proteins was examined by yeast two-hybrid assay (Fig. 9). Sld3 is a 77 KDa protein, which is consisted of 668 amino acids (Saccharomyces Genome Database). The interaction was observed for Sld3 (61-668), Sld3 (91-668), and Sld3 (1-400), but not for Sld3 (121-668) (Fig. 9). This result suggests that the Sld7 protein binds to the N-terminal portion of Sld3 protein. This is also consistent with the suppression of *sld3-6* mutation occurring in the N-terminal portion by multi-copy *SLD7* gene (see below: Fig. 11). Inconsistent with these observations, the interaction between Sld7 and Sld3 (31-668) was not detected. I speculate that Sld3 (31-668) bears unusual structure for interacting with Sld7 protein.

The cells truncated 150 amino acids from N-terminal of Sld3 protein, Sld7-binding domain, were viable (T. Umemori and H. Araki, unpublished result). This result indicates that the binding of Sld7 protein to Sld3 protein is not essential for cell growth, consistent with the observation that *SLD7* is non-essential gene. On the other hand, Cdc45 protein binds to the central portion of Sld3 protein (Tanaka *et al.*, 2007). The deletion more than 180 amino acids from N-terminus of Sld3 protein resulted in lethal phenotype of the cells (T. Umemori and H. Araki, unpublished result). This is likely due to the loss of the Cdc45-binding domain.

Increased copy of *SLD3* suppresses the *sld7 Δ* mutation

The Sld7 protein is non-essential for cell growth and forms a complex with essential Sld3 protein throughout the cell cycle. Thus, it is conceivable that although the Sld3 protein alone can function with reduced activity, it enhances the activity of Sld3 protein. Since the increased copy of proteins with reduced activity is expected to restore their defects in the cells, I examined whether increased dosage of *SLD3* gene suppresses the phenotypes of *sld7 Δ* mutant cells. As shown in Fig. 10, the *SLD3* gene on multi-copy plasmid suppressed the phenotypes of *sld7 Δ* mutant cells, inefficient DNA replication (Fig. 10a) and the sensitivity to HU and MMS (Fig. 10b), while the *SLD3* gene on low-copy plasmid did not suppress the sensitivity to HU and partially suppressed the sensitivity to MMS. Then, to examine whether the suppression of *sld7 Δ* mutant is specific for *SLD3* gene, I also tested that HU sensitivity of *sld7 Δ* mutant in the presence of multi-copy *DPB11*, *SLD2*, *CDC45*, or *GINS* (*SLD5*, *PSF1*, *PSF2*, and *PSF3*). Any tested genes except *SLD3* did not suppress the HU sensitivity of *sld7 Δ* mutant (Fig. 10c).

Increased copy of *SLD7* suppresses the *sld3-6* mutation

It is expected that the *sld3* mutant, in which Sld3 protein does not bind to Sld7 protein, shows the same phenotypes as *sld7 Δ* mutant. So far, it has been known five temperature-sensitive (ts) mutants of *SLD3*, *sld3-4*, *-5*, *-6*, *-7*, and *-8* (Fig. 11a). Like *sld7 Δ* cells, *sld3-6* mutant cells were sensitive to HU, and *sld3-5* mutant cells were partially sensitive to HU, but not *sld3-4*, *-7*, and *-8* (Fig. 11b). Additionally, multi-copy *SLD7* gene suppressed the HU sensitivity of *sld3-6* mutant cells, but only partially suppressed the sensitivity of *sld3-5* cells (Fig. 11c). Furthermore, multi-copy *SLD7* gene suppressed the ts phenotype of *sld3-6* mutant, but not that of *sld3-4*, *-5*, *-7*, and *-8* mutants (Fig. 11d). These results suggest that *sld3-6* mutant have a defect in the binding of Sld3 protein to

Sld7 protein. Indeed, *sld3-6* protein interacted with Sld7 protein weakly in comparison with wild-type Sld3 protein in yeast two-hybrid assay (T. Umemori and H. Araki, unpublished result). Previous study showed that the increased dosage of *CDC45* gene suppressed the ts-phenotypes of *sld3-4*, *-5*, *-7*, and *-8* mutants, but not that of *sld3-6* mutant (Kamimura *et al.*, 2001). Taken together, these results suggest that *sld3-6* mutant is defective in the binding of Sld3 protein to the Sld7 protein, and the Sld7 protein enhances Sld3 protein activity.

Regulation of the Sld3 protein by the Sld7 protein

There are two possibilities for regulation of the Sld3 protein by the Sld7 protein. One is that Sld7 protein regulates the function of Sld3 protein qualitatively (qualitative regulation), and the other is that the Sld7 protein regulates abundance of Sld3 protein (quantitative regulation). Actually, the amount of Sld3 protein both in the *sld7 Δ* and *sld3-6* cells was smaller than in WT cells when it was detected by western blotting using anti-Sld3 antibodies (Fig. 12a and b). However, the quantitative regulation alone cannot account for the regulation of Sld3 protein by Sld7. This is because *SLD3* gene on low-copy plasmid did not suppress the HU sensitivity of *sld7 Δ* mutant cells and only partially suppressed the MMS sensitivity of this mutant (Fig. 10b) although the amount of Sld3 protein was the same level as WT cells (Fig. 13a). These results indicate that the Sld7 protein enhances the activity of Sld3 protein at least qualitatively, probably through their complex formation. The amount of Sld7 protein in *sld3-6* cells was also smaller than in WT cells (Fig. 12b). This is probably due to the instabilities of the Sld7 and Sld3 proteins by failure in the formation of the Sld3-Sld7 complex.

The enhancement of the Sld3 function by Sld7 was further supported by the

following observation. When the *SLD3* gene was expressed from galactose-induced promoter, the cells grew slowly in comparison with vector-introduced cells. On the other hand, over-expression of *SLD7* genes did not cause any defect in cell growth. Simultaneous over-expression of *SLD3* and *SLD7* genes led to severe growth defect of the cells on galactose plate than the over-expression of *SLD3* gene alone (Fig. 14).

Over-expression of the *SLD7* gene alone, however, aggravates the cell growth of temperature sensitive *sld3* mutant cells. Over-expression of *SLD7* gene from galactose-induced promoter suppressed the phenotype of *sld3-6* mutant as well as *SLD7* gene on multi-copy plasmid (Fig. 15). On the other hand, over-expression of *SLD7* gene aggravated the cell growth of other *sld3*ts mutants (*sld3-4*, *-5*, *-7*, and *-8*), which are suppressed by multi-copy *CDC45* gene (Kamimura *et al.*, 2001), even at semi-permissive temperature of these mutants (Fig. 15). These aggravations seem to be caused by the association mode among Cdc45, Sld7, and Sld3 proteins as follows. Binding domains for Cdc45 and Sld7 proteins in Sld3 protein overlap (see Fig. 9 and text), suggesting that Sld7 and Cdc45 proteins compete for binding to the Sld3 protein. Actually, in the presence of Sld7 protein, Sld3 binding to Cdc45 is inhibited in *E. coli* cells (S. Tanaka and H. Araki, unpublished result) and purified Sld3 protein forms a stable complex with Cdc45 protein, but not purified Sld3-Sld7 complex (Y. Li and H. Araki, unpublished result). In the mutant cells whose Sld3 protein has reduced affinity to the Cdc45 protein, the Sld7 protein might easily displace the Cdc45 protein from the Sld3 protein, and in consequence the growth inhibition by high-copy *SLD7* genes might be evident.

The over-expression of *SLD7* gene also aggravated the growth of *dpb11-24* mutant cells, but not that of *dpb11-1* mutant cells. The *dpb11-24* mutant, used in the screening of *SLD7*, has a mutation in N-terminal region of Dpb11 protein, while the

dpb11-1 mutant, used in the screening of *SLD1-6*, has a mutation in C-terminal region of the protein. The N-terminal region of the Dpb11 protein interacts with CDK-phosphorylated Sld3 protein and the *dpb11-24* mutation reduces this interaction (Tanaka *et al.*, 2007). Interestingly, this interaction requires the association between the Cdc45 and Sld3 proteins (Tanaka *et al.*, 2007). Over-expression of *SLD7* genes is expected to prevent the association between the Cdc45 and Sld3 proteins (see above) and then reduces the interaction between the Dpb11 and Sld3 proteins. This reduction may be more pronounced in *dpb11-24* cells and thus may aggravate the cell growth.

Sld7 protein associates with replication origins

The Sld3 protein associates with early-firing origins at G1-phase (Kamimura *et al.*, 2001; Kanemaki and Labib, 2006). Since the Sld7 proteins forms a complex with Sld3 protein throughout the cell cycle, it was expected to associate with replication origins. To examine whether Sld7 protein associates with replication origins, chromatin immunoprecipitation (ChIP) assay was performed.

The cells harboring *SLD3-9MYC* and *SLD7-3FLAG-1HA* were arrested at G1-phase with α-factor and released at 25 ℃. Aliquots of cells were sampled at 15 minutes intervals, and cross-linked DNA protein complex was immunoprecipitated using anti-Flag and anti-Myc antibodies. DNA fragments from immunoprecipitates of each sample were amplified by PCR primers for early-firing origin, ARS305, and their neighboring regions (Fig. 16a). As previously shown, the Sld3-9Myc protein associated with ARS305 from 0 to 30 minutes after release from G1 arrest (Fig. 16d), which corresponds to the period from late G1 to very early S-phase in flow cytometry (Fig. 16b), and origin-association signals of Sld3-9Myc proteins gradually decreased. The ChIP assay

of Sld7-3Flag-1HA protein showed the same pattern as Sld3-9Myc protein (Fig. 16c). In the assay using primers for the neighboring region of ARS305, the amplification signals were very low as background level (Fig. 16e and f). These results suggest that the Sld7 protein, like the Sld3 protein, associates with replication origins at G1-phase, then gradually dissociates from origins, and this protein does not move with replication forks.

The Sld3 protein associates with origins in the absence of Sld7

Next, I examined whether Sld3-loading to replication origins is dependent on Sld7 protein by ChIP analyses of 5Flag-Sld3 protein in WT and *sld7 Δ* cells. The 5Flag-Sld3 protein in the *sld7 Δ* cells was not reduced severely as observed for non-tagged Sld3 protein (Fig. 12) probably because the tag stabilizes Sld3 protein. The 5Flag-Sld3 protein associated with ARS305 at G1-phase, and gradually dissociated from the origins (Fig. 17b) similarly as Sld3-9Myc protein (Fig. 16d). Moreover, although S-phase was delayed in flow cytometry (Fig. 17a), the 5Flag-Sld3 protein still associated with ARS305 in *sld7 Δ* cells at slightly reduced efficiency, with similar pattern to that in WT cells (Fig. 17c). In the assays using primers for the neighboring region of ARS305 (Fig. 17d and e), no differences were detected in the chromatin-association of 5Flag-Sld3 protein between in the presence/absence of Sld7 protein. Thus, it is supposed that the Sld3 protein associates with replication origins mostly independent from the Sld7 protein, and the Sld7 protein regulates the function of Sld3 protein other than Sld3-binding to replication origins.

It remains unknown whether Sld7-association with replication origins is dependent on Sld3 protein. Since the Sld3 protein associates with the origins even in the absence of Sld7 protein, association of the Sld7 protein is supposed to be dependent on

Sld3 protein. To examine this possibility, I constructed the yeast strain harboring *SLD7-3FLAG-1HA* in *sld3-6* mutant background because the *sld3-6* mutation generates the defect in the interaction between the Sld3 and Sld7 proteins. The cells harboring *SLD7-3FLAG-1HA* and *sld3-6* were synchronized in G1-phase by α -factor treatment, and released at 25 ℃ and 37 ℃. However, although the S-phase progression of the *sld3-6* cells was slower than that of WT cells, the cells were not arrested in the first cell cycle even at 37 ℃ for 2 hours. Thus, the transient exposure of the *sld3-6* cells to high temperature is not enough to inactivate the function of Sld3 protein, and this mutant cells are not further analyzed.

Chromatin association of MCM is altered in the absence of Sld7

Since the Sld3 protein binds to all the components of the CMG complex (see Introduction), and Sld7 protein enhances the function of the Sld3 protein, it is conceivable that loss of the Sld7 protein affects the chromatin association of Cdc45 protein and other components of the CMG complex. So far, the yeast strain harboring tagged-*CDC45* and *sld7* Δ has not been obtained probably because tagged-Cdc45 protein reduces its activity and is intolerable for lack of Sld7 protein. Thus, chromatin-association of Mcm7, a subunit of MCM complex, which is a component of CMG complex, was examined by ChIP analyses of Mcm7-3Flag-1HA in WT and *sld7* Δ cells. With ARS305 in WT cells, Mcm7-3Flag-1HA protein associated at G1-phase and immediately after release from G1-arrest, dissociated at 45 minutes (Fig. 18b), and then associated again from 90 minutes (late M-phase). Mcm7-3Flag-1HA protein also associated with neighboring region of the origins (Fig. 18d), indicating its traveling with replication forks. In the absence of Sld7 protein, Mcm7-3Flag-1HA protein associated with ARS305 at G1-phase and early S-phase

and re-associated 105 minutes form release, later than WT cells (Fig. 18c). The association signals with ARS305 were slightly reduced in comparison with WT cells. Mcm7 also associated with neighboring region of the origins, while its association signal was significantly reduced in comparison with the signals in the presence of Sld7 protein (Fig. 18e). These results suggest that the Sld7 protein helps the efficient moving of Mcm7 protein, a subunit of MCM complex, with replication forks, but not its association with the origins.

Other functions of Sld7 protein

Interestingly, *sld7 Δ* mutant cells showed elongated, tadpole-like cell morphology in the presence of HU (Fig. 19a). Thus, it is supposed that Sld7 protein has a role in other process of the cell cycle rather than DNA replication. Indeed, *sld7 Δ* mutant cells were sensitive to nocodazole (NOC) and thiabendazole (TBZ) (Fig. 19c), inhibitors for polymerization of microtubules. Although multi-copy *SLD3* gene restored the abnormal cell morphology (Fig. 19b), they did not restore the sensitivities to NOC and TBZ to wild-type level (Fig. 19c). These results imply that the loss of Sld7 protein leads to the defect in some other processes rather than DNA replication, such as cell division, and further suggest that Sld7 protein does not work with Sld3 protein at all times for these functions. This is consistent with observation of Sld3-free Sld7 protein (Fig. 6).

DISCUSSION

Function of Sld7 protein

Many proteins including replication enzymes assemble at replication origins. Then, some of the assembled proteins form replication machinery on the origins and start DNA synthesis. In this step, the proteins of replication machinery, associate first with the origins and then dissociate from them to synthesize DNA distant from origins. That is, the proteins must switch its affinity to replication origins from the association mode to the dissociation mode. Besides the proteins of replication machinery, the initiation proteins seem to change their affinity to replication origins. For example, Sld3 associates with replication origins and just falls out from chromatin DNA when replication initiates (Kamimura *et al.*, 2001; Kanemaki and Labib, 2006; this study). Thus, these initiation proteins may also work to displace replication machinery from origins. However, how they alter their affinity has been far from understanding.

This study gives a clue to the above problem. Sld3 and Sld7 proteins always form a complex (Fig. 6 and 8), associate with replication origins and then dissociate from them when replication initiates (Fig. 16). Even in the absence of Sld7, Sld3 associates with replication origins at almost same efficiency as observed in WT cells (Fig. 17). However, one of the replication proteins, Mcm7, associates with origins but not moves with replication forks efficiently in the absence of Sld7 (Fig. 18). Mcm7, as a subunit of MCM complex, associates with replication origins from late M to G1 phase before CDK activation in a Sld3-independent manner (Kamimura *et al.*, 2001) and then moves with replication forks. Thus, Sld7 seems to ease the dissociation between replication origins and MCM complex.

MCM forms a huge CMG complex with two other replication factors, Cdc45 and GINS, which associate with origins after CDK activation. Because the purified CMG complex shows a helicase activity at least in *Drosophila* (Moyer *et al.*, 2006), this complex is thought to be a replicative helicase associating with replication forks. Interestingly, Sld3 interacts with all the components of CMG complex (Kamimura *et al.*, 2001; Takayama *et al.*, 2003; Tanaka, 2007 *et al.*) and is suggested to work for the formation of CMG complex because Sld3 connects Cdc45, GINS, and MCM *in vitro* assay (Y. Li and H. Araki, unpublished result). This study suggested that Sld7 and Cdc45, a component of CMG complex, compete for binding to Sld3 (Fig. 9 and 15; see results for detail explanation). Thus, the absence of Sld7 protein confers Sld3 tighter association with Cdc45 protein. The tight Sld3-Cdc45 association may affect MCM dissociation from origins in three ways (Fig. 20). First, Cdc45 in the CMG complex does not dissociate from Sld3 on the origin efficiently. Second, the CMG complex is formed inefficiently. Once the CMG complex is formed, its components reduced the affinity to replication origins, so that it dissociates easily from origins. Third, although the CMG complex is formed as efficient as WT cells, the complex is fragile. The fragile complex may dissociate from replication forks frequently. Although these possibilities are not mutually exclusive, at least the last possibility explains the observations that in the cells lacking Sld7, the origin-association signals of Mcm7 gradually decreased after release from G1-arrest as in WT cells (Fig. 18) in spite of the longer S-phase than in WT cells (Fig. 3). Moreover, the HU sensitivity of *sld7 Δ* cells (Fig. 4) may intimate the break of replication forks consisted of fragile CMG complex by HU treatment.

The quantitative regulation of Sld3 by Sld7

The qualitative regulation of Sld3 by Sld7 protein is important for the initiation of DNA replication as described in results. However, I cannot rule out the possibility that the quantitative regulation also takes place. In genome-wide study, the number of TAP-tagged Sld3 and Sld7 proteins are estimated about 125 and 1690 molecules per cell, respectively (Ghaemmaghami *et al.*, 2003), and the number of replication origin is estimated about 400 per a genome of *Saccharomyces cerevisiae* (Nieduszynski *et al.*, 2006). In WT cells, the amount of the Sld3 proteins is enough to distribute evenly on early-firing origins during early S-phase, and on late-replicated origins during mid or late S-phase. On the other hand, because of smaller amount of Sld3 protein in *sld7 Δ* and *sld3-6* cells than WT cells, one Sld3 molecule probably has to take charge of larger number of the origins. Thus, it is possible that the Sld3 protein cannot fire the origins evenly on time, and it causes the delay in S-phase. It remains unclear whether the activation of late-firing origins in *sld7 Δ* and *sld3-6* cells is delayed in comparison with in WT cells. If the ratio of the number of origins and Sld3 protein affects the on-time replication, the association of the Sld3 protein with late-firing origins may be delayed in the *sld7 Δ* and *sld3-6* cells.

The role of N-terminal portion in Sld3 protein

The Sld7 protein binds to the N-terminal portion of Sld3 protein (Fig. 9 and 11), and the *sld3-6* mutation occurring in this portion seems to reduce the interaction with Sld7 protein. Both *sld7 Δ* and *sld3-6* mutations cause the reduction of Sld3 protein level (Fig. 12). However, the *sld7 Δ* mutant cells are viable even at 37 ℃ (data not shown), while the *sld3-6* mutant cells cannot grow at 36 ℃ (Fig. 11d). What is the difference between these mutants caused by? Sld3 (61-668) and Sld3 (91-668) proteins interact with Sld7 protein, but not Sld3 (31-668) protein (Fig. 9). Moreover, *sld3 (61-668)* mutant cells

grew slower than WT cells (T. Umemori and H. Araki, unpublished result). I speculate that the mutations in the N-terminal region of Sld3 protein including the *sld3-6* mutation affect the interactions with the Sld3-binding proteins, such as Dpb11, Cdc45, and GINS as well as with Sld7 protein. As a consequence, it probably causes more severe defect than the *sld7 Δ* mutation. Another formal explanation of the difference between these mutations is that the Sld7 protein is functionally redundant, and some other proteins that bind to the N-terminal portion of Sld3 protein compensate for the lack of the Sld7 protein. However, since *sld3 (151-668)* mutant lacking the interaction between Sld3 and Sld7 proteins is still viable (T. Umemori and H. Araki, unpublished result), this hypothesis is unlikely.

Other functions of Sld7

It has been reported that the factors, which act in DNA replication event, also play a role in mitotic events (Prasanth *et al.*, 2002; Huang *et al.*, 2005; Shimada and Gasser, 2007). These factors may coordinate the distinct events in the cell cycle. I demonstrated that Sld7 protein acts in DNA replication through the interaction with Sld3 protein. At a glance, the *sld7 Δ* cells are not likely defective in the cell-cycle events except S-phase because the buds of synchronized *sld7 Δ* cells appeared at the same time as those of WT cells (Fig. 3b). However, as shown in Fig. 19, it is suggested that other functions of Sld7 protein in some other process than DNA replication. This idea is consistent with the observations that immunoprecipitation of Sld3 protein did not completely deplete Sld7 protein from extracts (Fig. 6b), and the sensitivity of *sld7 Δ* mutant to TBZ was only suppressed partially by multi-copy *SLD3* gene (Fig. 19c). These results suggest that Sld7 interacts with the factor(s) other than Sld3 protein. Further analysis is required for verifying this possibility. Interestingly, genome-wide study showed that the Sld7-GFP protein

localizes to nuclei and spindle poles (Huh *et al.*, 2003), and mutation in *SLD7* gene resulted in aberrant mitochondria morphology (Altmann and Westermann, 2005).

The counterparts of Sld3 and Sld7

The Sld3 and Sld7 proteins form a complex throughout the cell cycle. It remains unknown the significance of the existence of these proteins as two distinct proteins. The ratio of the Sld3 and Sld7 proteins in a complex is estimated from 1:2 to 1:4 (T. Umemori and H. Araki, unpublished result). It is possible that changing their ratio in a complex is important to regulate the initiation of DNA replication. It is also possible that the Sld7 protein disperses outside of the nucleus and interacts with other factors to connect the Sld3 protein with other proteins.

So far, the counterpart of Sld3 protein has been found only in yeast and fungi. On the other hand, the counterparts of Sld7 protein have not been found in other species even in fission yeast. In budding and fission yeasts, the N-terminal regions of the Sld3 protein and SpSld3 protein (Sld3 homologue in fission yeast) are divergent. Thus, it is suggested that because of the binding of Sld7 protein to the N-terminal region of the Sld3 protein, the Sld7 protein itself differentiates even in these species, further in higher eukaryotes.

MATERIALS AND METHODS

Microorganisms

Yeast strains used in this study are listed in Table 1. *Escherichia coli* XL10 Gold was used for plasmid propagation.

Plasmid construction

YCplac111 (*LEU2*), YCplac33 (*URA3*), YCplac22 (*TRP1*), YEplac181 (*LEU2*), YEplac195 (*URA3*), and YEplac112 (*TRP1*) were used for cloning (Gietz and Sugino, 1988). YCp-*SLD7* and YEp-*SLD7* were constructed by subcloning the 1.6 kb *Spe*I-*Spe*I *SLD7* fragment into the *Xba*I site of YCplac and YEplac plasmids, respectively. YCp-*SLD7* was used as a template to amplify the *Nde*I-*Xho*I *SLD7* fragment by using *SLD7-Bam*HI/*Nde*I-F and *SLD7-Xho*I-R primers. The *Nde*I-*Xho*I *SLD7* fragment was cloned into *Nde*I-*Xho*I site of pET20b(+) (Novagen) or pETDuet-1 (Novagen) plasmids (pET20b(+)-*SLD7*, pETDuet-*SLD7*). The *Bgl*II-*Pst*I *SLD3* fragment was cloned into the *Bam*HI-*Pst*I site of the pETDuet-*SLD7* plasmid (pETDuet-*SLD3-SLD7*). pBTM116, pACTII, and pBTM116-*SLD3* were used for a two-hybrid assay (Bartel and Fields, 1995; Bai and Elledge, 1997; Kamimura *et al.*, 2001). The pBTM116-*SLD3* plasmid was used as a template to amplify the N-terminal truncated *SLD3* fragments amplyfied by *sld3* (73-102) *Nde*I-F, *sld3* (73-102) *Nde*I-F, *sld*3 (167-196) *Nde*I-F, *sld3* (257-286) *Nde*I-F, *sld3* (346-375) *Nde*I-F, *sld3* (566-668) *Nde*I-R, respectively. The PCR products were digested by *Nde*I and filled up by Klenow Fragment (TaKaRa). The resultant fragments were cloned into *Nde*I-*BamH*I site of pBTM116 plasmid (pBTM-*sld3* (31-668), pBTM-*sld3* (61-668), pBTM-*sld3* (91-668), pBTM-*sld3* (121-668)). The C-terminal truncated *SLD3* fragments

were amplified by PCR using *sld3* (1-400) *Bam*HII-F and *sld3 Bam*HI-R primers, digested by *Bam*HI, and cloned into *Bam*HI site of pBTM116 plasmid (pBTM-*sld3* (1-400)). *Eco*RI-*Bam*HI *SLD7* fragment was amplified by *SLD7-Eco*RI-F and *SLD7-Bcl*I-R primers and cloned into *Eco*RI-*Bam*HI site of pBTM116 plasmid (pBTM-*SLD7*). *Eco*RI-*Xho*I *SLD7* fragment was amplified by *SLD7-Eco*RI2-F and *SLD7-Xho*I-R primers and cloned into *Eco*RI-*Xho*I site of pACTII plasmid (pACT-*SLD7*). The *Eco*RI-*Sal*I fragment from pBTM-SLD7 was cloned into the *Eco*RI-*Sal*I site of pKT10-GAL plasmid (Tanaka *et al.*, 1990) (pKT10-*SLD7*).

Oligos and peptides

Oligo nucleotides and peptides used in this study are listed in Table 2.

Yeast media

YPD medium (2 % bacto peptone, 1 % yeast extract, 2 % glucose) and SD (0.67 % bacto-yeast nitrogen base (without amino acids), 2 % glucose) medium were used to cultivate yeast cells. Minimal sporulation medium (1 % potassium acetate) was used to for sporulation of yeast cells.

Preparation of anti-Sld7 antibodies

E. coli BL21 (DE3) cells harboring pET20b(+)-*SLD7* plasmids were grown in 1L LB broth (1 % bacto tripton, 0.5 % yeast extract, 0.5 % NaCl) containing ampicillin (200 μ g/ml) and chloramphenicol (34 μ g/ml) at 30 ℃. When OD_{600} reached to 0.2, IPTG (0.4 mM) was added to culture, and the culture was incubated at 30 ℃ for 3 hours. The cells were harvested and suspended in the sonication buffer (50 mM HEPES-KOH (pH 7.5),

300 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, 10 % glycerol). The suspension was sonicated 3 times at 40 W for 30 seconds (OHTAKE WORKS). Cell lysates were clarified by centrifugation at 4 ℃ for 15 minutes and the pellet fraction was separated in 4-20 % SDS-polyacrylamide gel. Protein band containing the Sld7 protein were excised from the gel, and the protein was eluted from the gel. The eluted protein was injected into rabbit.

Disruption of *SLD7* gene

The DNA fragment amplified by *SLD7*-480*Spe*-F and *SLD7*-20*Bam*-R primers was cloned into the *Spe*I-*Bam*HI site of pBluescript SK+ (pBS-*SLD7*-upper), and the DNA fragment amplified by *SLD7*-780*Xho*/*Bam*-F and *SLD7*-1148*Spe*/*Xho*-R primers was cloned into the *Xho*I-*Bam*HI site of pBS-*SLD7*-upper. The *LEU2* fragment was inserted into the *Bam*HI site of the resultant plasmid (pBS-*sld7 Δ::LEU2*). The plasmid including *sld7 Δ::LEU2* fragment was cleaved with *Spe*I and used for transformation of W303-1A/1B to disrupt the *SLD7* gene. Southern blot analysis was performed on the Leu^+ transformants to confirm that one copy of the endogenous *SLD7* was successfully disrupted (T. Umemori and H. Araki, unpublished data).

Epitope Tagging of *SLD7* gene

DNA fragment was amplified by pFA-*SLD7*-F2 and pFA-*SLD7*-R1 primers. pFA6a-kanMX6 plasmid (Longtine *et al.*, 1998) was used as a template DNA. The fragments were used for transformation of the cells to add an epitope-tag to the *SLD7* gene.

Synchronization of yeast cells

Cells were grown to 5×10^6 cells/ml, and then arrested with 30 ng of α-factor (α-factor mating pheromone, Peptide institute) per ml at 25 ℃ for 2.5 hours. α-factor was removed by centrifugation , and the cells were suspended in fresh YPD medium (2 % bacto peptone, 1 % yeast extract, 2 % glucose) containing 100 μg of actinase E (Kakenseiyaku) per ml.

Flow cytometry

Yeast cells were fixed in 70 % Ethanol at -20 ℃ overnight. The fixed cells were washed with 50 mM Na-citrate (pH 7.5). The 4×10^6 cells were suspended in 500 μl of 50 mM Na-citrate (pH 7.5) containing 250 μg of RNase A per ml at 50 ℃ for 1 hour. Then, 1 mg/ml proteinase K (Merck) was added and incubated at 50 ℃ for 1 hour. The cells were stained by 500 μl of 50 mM Na-citrate (pH 7.5) containing 8 μg of propidium iodide (Nacalai tesque) per ml and sonicated. Stained cells were analyzed by FACScan (Becton-Dickinson).

Protein extraction from yeast cells

The 5×10^7 cells were collected by centrifugation and washed by distilled water. These cells were resuspended in 100 μl of distilled water, added 100 μl of 0.2 M NaOH, and incubated for 5 minutes at room temperature. Then, cells were pelleted, resuspended in 50 μl of 1×SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2 % SDS, 0.1 % Bromophenol blue, 10 % Glycerol), and boiled for 3 minutes.

Immunoprecipitation

Immunoprecipitation in Figure 5

Cells were harvested, washed with distilled water and grind down by homogenizer (RM100, Retsch) with liquid nitrogen. The cell lysate was resuspended in 5 ml lysis buffer A (50 mM HEPES-KOH (pH 7.5), 300 mM KCl, 0.5 % Tween20, 0.05 % NP40, 10 % Glycerol, 1×complete (Roche), 1 % protease inhibitor cocktail (Sigma), 50 mM NaF, 2 mM β-glycerophosphate, 0.4 mM Na_2VO_4, 0.5 mM Na-pyrophosphate, 1 mM PMSF) or lysis buffer B (50 mM HEPES-KOH (pH 7.5), 100 mM KCl, 0.5 % Tween20, 0.05 % NP40, 10 % Glycerol, 1×complete (Roche), 1 % protease inhibitor cocktail (Sigma), 50 mM NaF, 2 mM β-glycerophosphate, 0.4 mM Na_2VO_4, 0.5 mM Na-pyrophosphate, 1 mM PMSF), and clarified by centrifugation of 12000 rpm at 4 ℃ for 15 minutes. The soluble protein was quantitated by a Bradford assay (Bio-Rad). The extracts were adsorbed against Sepharose 4B (Parmacia Biotech) at 4 ℃ for 30 minutes. Then, beads were pelleted and the supernatant was mixed with anti-Flag M2 affinity gel (Sigma), which was primarily washed by lysis buffer A or B including 5 mg/ml BSA, at 4 ℃ for 2 hours. Then, the supernatant was removed and the beads were washed by lysis buffer A or B. The precipitates were resuspended in 500 μl of Flag elution buffer (lysis buffer A or B including 150 μg/ml of 3×Flag peptide). The eluted solution was mixed with anti-HA matrix (Roche) and incubated at 4 ℃ for 2 hours. Then, supernatant was removed and the beads were washed by lysis buffer A or B. The precipitates were resuspended in 100 μl of HA elution buffer (lysis buffer A or B including 1 mg/ml of 3×HA peptide) and incubated at 37 ℃ for 30 minutes. 5.8 % trichloroacetic acid was added to the eluted solution. The mixture was incubated at 4 ℃ for 10 minutes and pelleted by centrifugation. Supernatant was removed and the precipitate was boiled for 3 minutes in 50 μl of 1×SDS loading buffer. The proteins were separated by SDS-PAGE and stained by SilverQuest (Invitrogen). The band containing the protein was excised from

the gel and analyzed by mass spectrometry after digestion with trypsin.

Immunoprecipitation in Figure 6

Cell extract was prepared as described for Figure 5. The extracts were adsorbed against Sepharose 4B (Parmacia Biotech) or Dynabeads Protein A (Invitrogen) at 4 ℃ for 30 minutes. Then, beads were pelleted and the supernatant was mixed with anti-Flag (anti-Flag M2 antibody, Sigma) or anti-Myc (c-myc Ab-1 9E11, NeoMarkers) antibodies at 4 ℃ for 2 hours. Then, supernatant was removed and the beads were washed by lysis buffer A. The precipitates were boiled for 3 minutes in 1×SDS loading buffer.

Yeast two-hybrid analysis

Plasmids were introduced into TAT7 cells, and the transformants were spotted onto SD-Leu-Trp plate. When transformants grew, cells were replicated to filter paper (Whatmann 50). The filter paper was frozen in liquid nitrogen and soaked in Z buffer (10 mM KCl, 1 mM $MgSO_4$, NaPO4 (pH 7.0)) containing X-gal and β-mercaptoethanol.

Protein production in *E. coli* cells

E. coli BL21 (DE3) cells harboring pETDuet-*SLD3*, pETDuet-*SLD7* or pETDuet-*SLD3*-*SLD7* plasmids were grown in 1L of LB broth containing ampicillin (200 μg/ml) at 37 ℃. When OD_{600} reached to 0.5, 0.1 mM IPTG was added to induce the proteins, and incubated at 20 ℃ for 16 hours. 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 $MgCl_2$, 0.1 mM DTT, 10 % glycerol), and sonicated 5 times at 150 W for 30 seconds (OHTAKE WORKS). Cell lysate was centrifuged 15000 rpm at 4 ℃ for 15 minutes, and

supernatant was removed. Precipitants were washed by lysis buffer 300 and resuspended in 1 ml of sonication buffer 500 (50 mM HEPES-KOH (pH 7.5), 500 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, 10 % glycerol), and sonicated 5 times at 150 W for 30 seconds again. The cell lysate was clarified by centrifugation of 15000 rpm at 4 ℃ for 15 minutes.

in vitro binding assay

The supernatant including the proteins was adsorbed against 100 μl of Sepharose 4B (Parmacia Biotech) at 4 ℃ for 30 minutes. Then, beads were pelleted and the supernatant was mixed with Ni-NTA agarose (Qiagen) at 4 ℃ for 2 hours. The supernatant was removed, and the beads were washed by washing buffer (50 mM HEPES-KOH (pH 7.5), 300 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, 10 % glycerol, 20 mM imidazole). 100 μl of imidazole elution buffer (50 mM HEPES-KOH (pH 7.5), 300 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, 10 % glycerol, 250 mM imidazole, 0.1 % Tween 20, 0.01 % Triton X-100) was added to the washed beads. The supernatants were boiled for 3 minutes in 1×SDS loading buffer.

Chromatin immunoprecipitation assay

The rapid *in vivo* cross-linking chromatin immunoprecipitation assay (Kohzaki and Murakami, 2007) was referred for this assay.

Cells were treated with 1 % formaldehyde for 20 minutes at 25 ℃ to promote cross-linking. After addition of 125 mM glycine and incubated at 25 ℃ for 5 minutes, cells were harvested and washed with cold TBS (20 mM Tris-HCl (pH 8.0), 150 mM NaCl). The cells were resuspended in 500 μl of lysis buffer C (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Na-deoxycholate, 1×complete (Roche),

1 % protease inhibitor cocktail (Sigma)) and broken up with glass beads. The cell extracts were sonicated to fragment the DNA by ultrasonic homogenizer (channel 4, 220 W on a Bioruptor UCW-201, COSMO BIO). The cell lysates were clarified by centrifugation of 12000 rpm at 4 ℃ for 5 minutes. The extracts from cells were mixed with Dynabeads Protein A (Invitrogen) at 4 ℃ for 30 minutes. 2 μl of the extracts were diluted in 98 μl of distilled water and the diluted solutions were used as whole cell extracts (WCE). The remnant of the extracts from cells were immunoprecipitated by anti-Flag (anti-Flag M2 antibody, Sigma) or anti-Myc (c-myc Ab-1 9E11, NeoMarkers) antibodies with Dynabeads Protein A at 4 ℃ for 2 hours. The beads were washed by lysis buffer C, lysis buffer C containing 360 mM NaCl (final concentration 0.5 M), washing buffer (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 0.5 % NP 40, 0.5 % Na-deoxycholate, 1 mM EDTA), and TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The beads were resuspended in 40 μl of TE buffer. The presence of ARS-containing fragments in WCE and immunoprecipitates was determined by real-time PCR amplification using SYBR Premix Ex Taq II system (TaKaRa).

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TABLES AND FIGURES

Table 1. Yeast strains used in this study

Strain name	Genotype	Reference
W303-1A	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
BY4743 (YSC1050)	*MATa/MATα his3Δ0/his3Δ0 leu2Δ0/leu2Δ0 lys2Δ0/WT met15Δ0/WT ura3Δ0/ura3Δ0*	open biosystems
BY4743 (YSC1021)	*MATa/MATα his3Δ0/his3Δ0 leu2Δ0/leu2Δ0 lys2Δ0/WT met15Δ0/WT ura3Δ0/ura3Δ0 yor060cΔ::kanMX/WT*	open biosystems
W303-1Ab	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ*	Y. Kamimura
YTT1	W303-1Ab, *sld7Δ::LEU2*	This work
W303-6D his4	*MATα ade2-1 can1-100 his4 leu2-3,112 trp1-1 ura3-1*	Y. Kamimura
YTT2	W303-6D his4, *sld7Δ::LEU2*	This work
dpb11-24	*dpb11-24*	Y. Kamimura
dpb11-1	W303-1A, *dpb11-1*	Y. Kamimura
sld2-6	W303-1A, *sld2-6*	Y. Kamimura
cdc45-27	W303-1Ab, *cdc45-27*	Y. Kamimura
sld5-12	W303-6D, *sld5-12*	Y. Kamimura
psf1-1	W303-1Ab, *psf1-1*	Y. Kamimura
8534-M2	*MATα mcm2-1 his4-Δ 34 leu2-3 112 ura3-52*	Y. Kawasaki and A. Sugino
R61-3C	*MATα mcm-3-1 his4Δ 34 leu2-3 112 ura3-52*	Y. Kawasaki and A. Sugino
JRY4125	*MATα ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100 orc2-1 uba1-o1 orc2-1*	lab stock
JRY4249	*MATα ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100 orc2-1 uba1-o1 orc5-1*	lab stock
YTT3	W303-1Ab *ddc1Δ::TRP1*	This work
sld3-5N	W303-1A, *sld3-4*	Y. Kamimura
YYK19 (sld3-9N)	W303-1A, *sld3-5*	Y. Kamimura
YYK16 (sld3-11N)	W303-1A, *sld3-6*	Y. Kamimura
sld3-14N	W303-1A, *sld3-7*	Y. Kamimura
sld3-21N	W303-1A, *sld3-8*	Y. Kamimura
TAT7	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1::LexA-LacZ Lys2::LexA-HIS3*	lab stock
YTT4	W303-1Ab, *SLD7-3FLAG-1HA*	This work
YTT5	W303-1Ab, *SLD7-3FLAG-1HA SLD3-9MYC*	This work
YTT6	W303-1Ab, *5FLAG-SLD3*	This work
YTT7	W303-1Ab, *5FLAG-SLD3 sld7Δ::LEU2*	This work
YNIG198-2	W303-1Ab, *MCM7-3FLAG-1HA::kanMX*	S. Sakamoto
YTT8	W303-1Ab, *MCM7-3FLAG-1HA::kanMX sld7Δ::LEU2*	This work

Table 2. Primers and peptides used in this work

Primer name	Sequence (5'-3')
pFA-*SLD7*-F1	ACTAAGAAATGATAGACTGCCTTGAAAGGGGACAGCATTCACCAATTCGCCTGACTTTTGCGGATCCCCGGGTTAATTAA
pFA-*SLD7*-F2	GTTTCGTTTGAATCATTGCAGGAAACTGTGGAAACTCTTCTGAAGCTCTTTACCAAATCACGGATCCCCGGGTTAATTAA
pFA-*SLD7*-R1	ATGGTTGATCACACGAAGTTTTCTACCGATTATTGCAGTAAAGAGTTGCCACCCTTTCTTGAATTCGAGCTCGTTTAAAC
pFA-*DDC1*-F2	TTCCCAGAATGACACAAGTAATCACAAGAAACAGGACAATAAAGAGATGGAAGATGGGCTGGGTCTAACACAAGTAGAAA-AGCCAAGGGGTATATTTGACCGGATCCCCGGGTTAATTAA
pFA-*DDC1*-R1	TTAGTTTGTTCCCCGACCGTGATGGACCATCAGCCGCACGCTCACCAAATTGAGACAGAAAAAAAAAAAAAAAACAGCGAT-CGATATTATCATGATAATTGGAATTCGAGCTCGTTTAAAC
*SLD7-Bam*HI/*Nde*I-F	CAATTCGCCTGACTTTTGATGTCACGG
*SLD7-Xho*I-R	CTGAAAATCCTCGAGGATCATGATTTGGT
SLD7-480*Spe*I-F	TCGTAGTACTAGTATTCTTGTGGTCTCTCC
SLD7-20*Bam*HI-R	TTCCGTGGATCCAAAAGTCAGGCGAATTGG
SLD7-780*Xho*I/*Bam*HI-F	CATGATCTCGAGGGATCCTCAGTATATAAG
SLD7-1148*Spe*I/*Xho*I-R	CAATATCCTCGAGCACTAGTTTAAATTCGG
pBS-*SLD3-Bam*HI-F	TAGGAACGATCTGACGGATCCTATG
pBS-*SLD3-Pst*I-R	GGTGGTCTCGCTGCAGCTTATTCTATGTGGATTCT
pBS-*Bam*HI-R	CGGCCGCTCTAGAACTAGTGGATCC
BTM-*SLD3-Bam*HI-F	ATTCCCGGGGATCCATGGAAACATGGG
BTM-*SLD3-Pst*I-R	TGGCTGCAGTCTATGTGGATTCTGGAG
sld3 (73-102) *Nde*I-F	CTTATTATTAAACATATGGACGTGCCCAGT
sld3 (167-196) *Nde*I-F	GAAAAGAGCATATGCTACTAGAGGAATATG
sld3 (257-286) *Nde*I-F	GGCGTACGCATATGGTAACGACAGTTGAAA
sld3 (346-375) *Nde*I-F	CTTCATTTACATATGGAAGAACCCAAGGGC
sld3 (1-400) *Bam*HII-F	TGGTACCTTTTTGGGATCCTATGGAATAAG
*sld3 Bam*HI-R	CGATCTGAGGGATCCTAATGGAAACATGG
sld3 (566-668) *Nde*I-R	ACCTCTCATATGGAAACATGG
*SLD7-Eco*RI-F	AGCATTCACGAATTCGCCTGACTTTTGATG
*SLD7-Bcl*I-R	ATCTTATATACTGAAAATCCCCGTTGATCA
*SLD7-Eco*RI2-F	ACCAATTCGCCTGAATTCTGATGTCACGGA
*SLD7-Xho*I-R	CTGAAAATCCTCGAGGATCATGATTTGGT
ARS305 realtime LEFT1	AACGTTCCGAAACAGGACAC
ARS305 realtime RIGHT1	GCGGCCTGAAATACTGTCAT
305+2.5kb realtime LEFT1	GATTGGCGAAGCCGTAGTTAA
305+2.5kb realtime RIGHT1	CACCGGCAGCGATTGC

Table 2. Primers and peptides used in this work

Peptide name	Sequence (N-C)
3×Flag	DYKDDDDKDYKDDDDKDYKDDDDK
3×HA	YPYDVPDYAYPYDVPDYAYPYDVPDYA

Table 3. Synthetic lethality between the *sld7Δ* mutation and mutations occurring in the replication genes.*

Gene	alelle	phenotype	complex	function
DPB11	*dpb11-24*	lethal	Dpb11-Sld2	Replication initiation (pre-LC formation)
	dpb11-1	lethal		
SLD2	*drc1-1*	lethal		
POL2	*pol2-11*	lethal	Polε	
SLD5	*sld5-12*	lethal	GINS	
PSF1	*psf1-1*	lethal		
SLD3	*sld3-5*	lethal	Sld3-Cdc45	Replication initiation
CDC45	*cdc45-27*	lethal		
MCM2	*mcm2-1*	viable	MCM	Replication initiation (pre-RC formation)
MCM3	*mcm3-1*	viable		
ORC2	*orc2-1*	viable	ORC	
ORC5	*orc5-1*	viable		
DDC1	*ddc1Δ*	viable	9-1-1	Checkpoint

*Synthetic lethality between *sld7Δ* mutation and mutations occurring in the replication genes was tested by tetrad dissection as described in Fig. 1 and 2. Spores were incubated at 25 °C for 6 days.

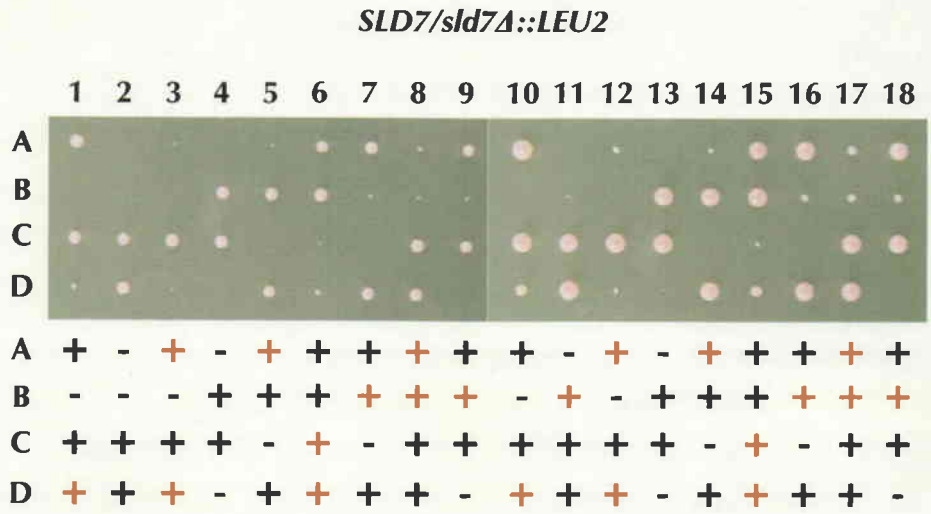


Figure 1. Tetrad dissections of the *SLD7/sld7Δ::LEU2* diploid. *SLD7/sld7Δ::LEU2* heterodisruptant cells were sporulated on minimal sporulation medium for 5 days, and the spores were dissected. The dissected spores were incubated on YPD plate at 30 **℃** for 3 days (1-9) or 4 days (10-18). All large colonies were Leu$^-$. Viable spore clones are shown +, non-viable spore clones are shown -. Black letters indicate the spores carrying *SLD7* allele. Red letters indicate the spores carrying *sld7Δ::LEU2* allele.

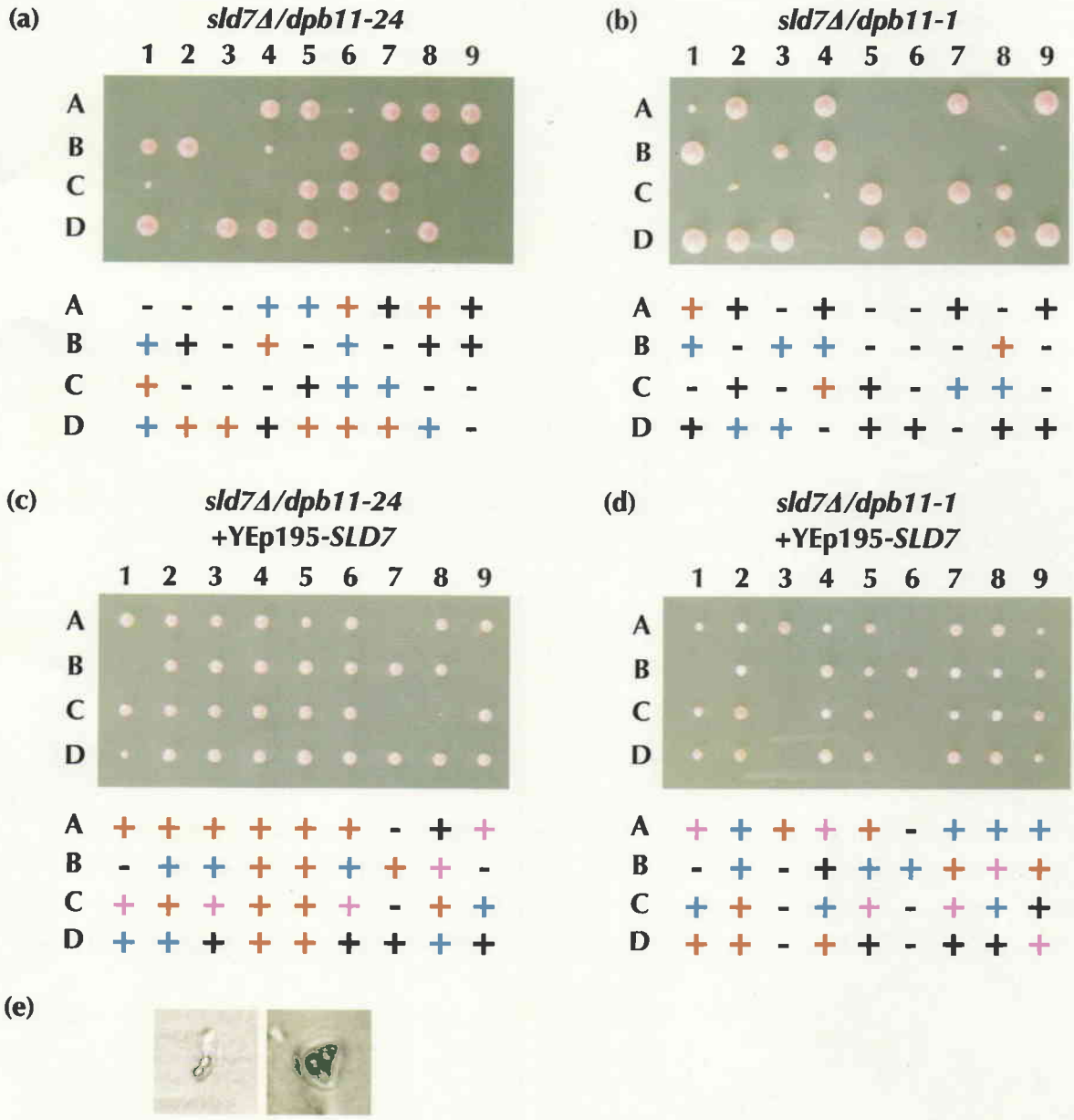


Figure 2. Tetrad dissections of the *sld7Δ::LEU2/dpb11-24* or *sld7Δ::LEU2/dpb11-1* diploids.
(a) (b) The *sld7Δ::LEU2/dpb11-24* or the *sld7Δ::LEU2/dpb11-1* diploid cells were sporulated on minimal sporulation medium for 5 days, and the spores were dissected. The dissected spores were incubated on YPD plate at 30 ℃ for 4 days. (c) (d) YEp195-*SLD7* plasmid was introduced into the *sld7Δ::LEU2/dpb11-24* and the *sld7Δ::LEU2/dpb11-1* diploids, and the cells were sporulated. Spores were dissected and incubated at 30 ℃ for 4 days. Viable spore clones are shown +, non-viable spore clones are shown -. Black letters indicate the spores carrying *SLD7* allele. Red letters indicate the spores carrying *sld7Δ::LEU2* allele. Blue letters indicate the spores carrying *dpb11-24* or *dpb11-1* allele. Pink letters indicate the spores carrying *sld7Δ::LEU2 dpb11-24* or *sld7Δ::LEU2 dpb11-1* alleles. (e) Microscopic observation of the lethal cells carrying *sld7Δ::LEU2 dpb11-24*.

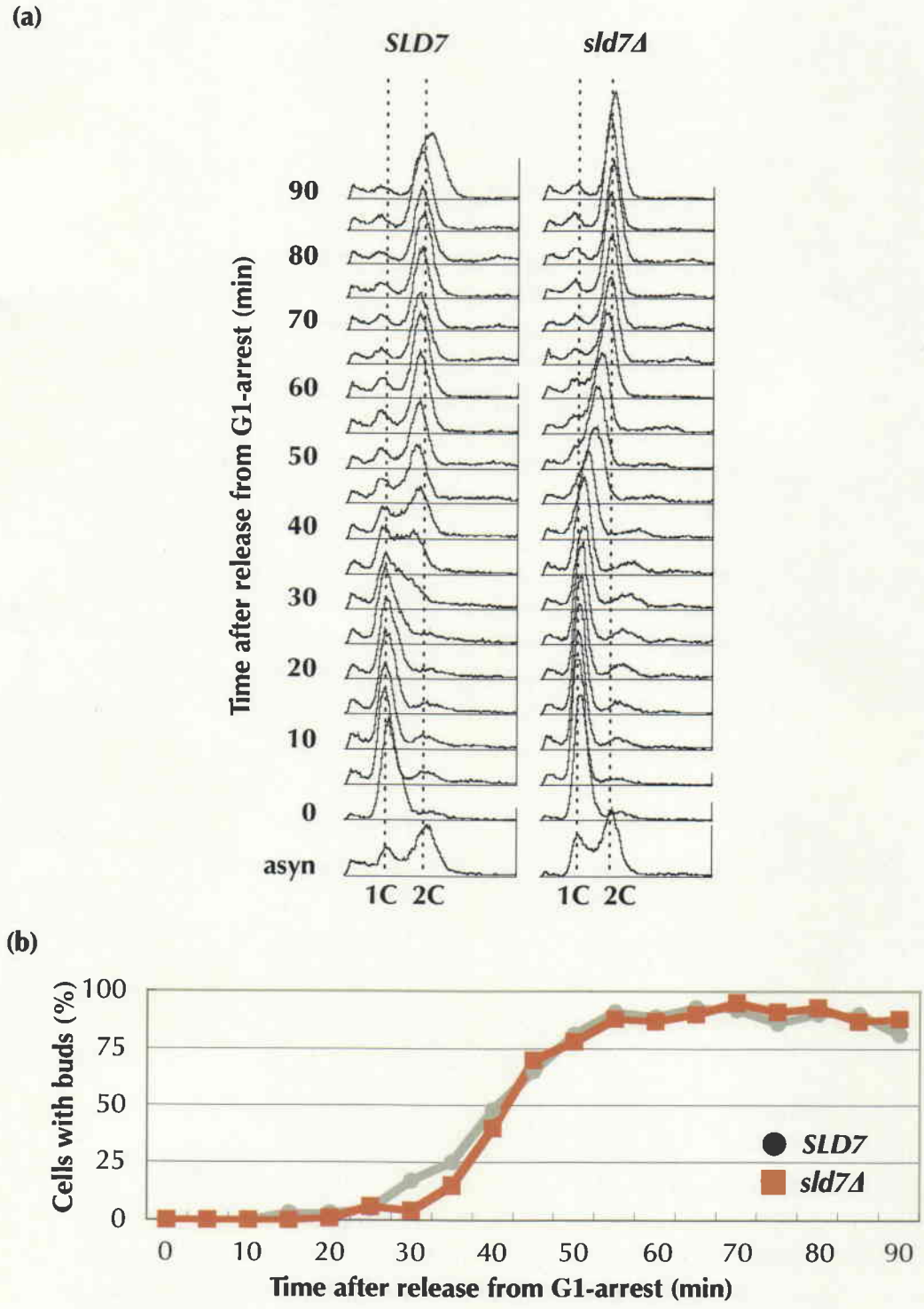


Figure 3. DNA replication in *sld7Δ* cells.
(a) The *sld7Δ* mutant cells and wild-type (WT) cells were synchronized in G1-phase with α-factor and released from a G1-arrest at 25 ℃. Aliquots of cells were sampled at 5 minutes intervals and DNA contents were measured by flow cytometry. (b) The buds appeared at the same timing in both WT cells and *sld7Δ* mutant cells.

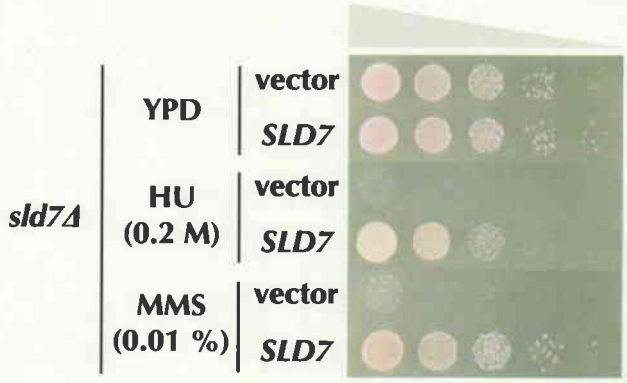


Figure 4. Sensitivity of *sld7Δ* cells to HU and MMS.
The plasmids with or without *SLD7* gene were introduced into *sld7Δ* cells. Then 10-fold serial dilutions of the cells were spotted onto YPD plates containing 0.2 M hydroxyurea (HU), or 0.01 % methylmethane sulfonate (MMS). The cells were incubated at 25 **℃** for 4 days.

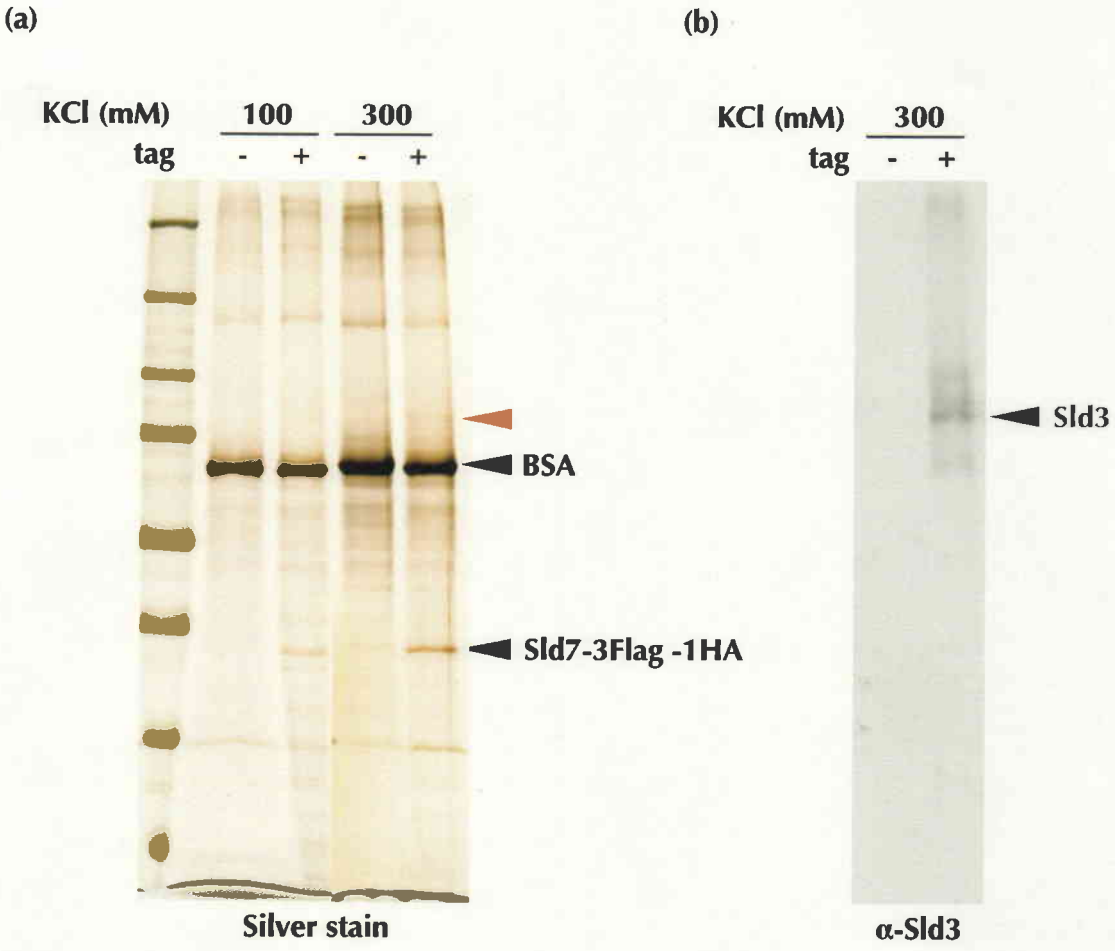


Figure 5. Immunoprecipitation of Sld7-3Flag-1HA protein.
(a) The Sld7-3Flag-1HA protein was immunoprecipitated by anti-Flag M2 affinity gel (Sigma) and released from the gel using 3×Flag peptides. The released Sld7-3Flag-1HA protein was precipitated by anti-HA matrix (Roche) and released from the matrix using 3×HA peptides. Comparing with immunoprecipitates from the extracts of WT cells, 80 kDa protein shown by red arrowhead was co-precipitated specifically with Sld7-3Flag-1HA. According to peptide-sequence analysis by mass spectrometry, this protein was identified as Sld3. (b) Western blotting using anti-Sld3 antibodies further confirmed that Sld3 protein co-precipitated with Sld7-3Flag-1HA protein.

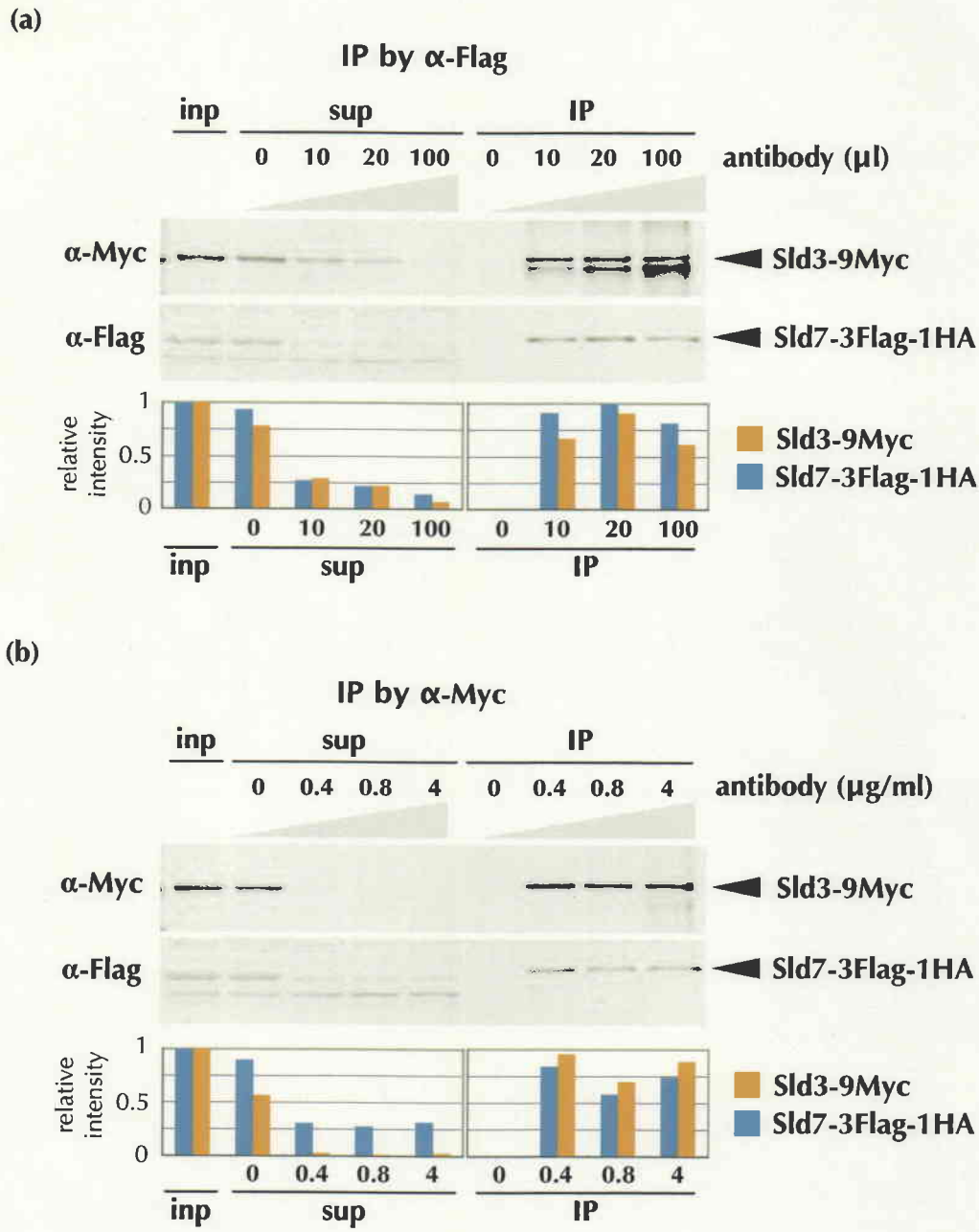


Figure 6. Immunoprecipitation of Sld7-3Flag-1HA and Sld3-9Myc proteins. (a) Immunoprecipitation was performed as described in materials and methods. Depletion of Sld7-3Flag-1HA protein by anti-Flag antibody (anti-Flag M2 affinity gel, Sigma) also depletes Sld3-9Myc protein from extracts prepared from asynchronous cultured cells. (b) The Sld7-3Flag-1HA protein remains in the extracts in which Sld3-9Myc protein is depleted by anti-Myc antibody (c-myc Ab-1 9E11, NeoMarkers).

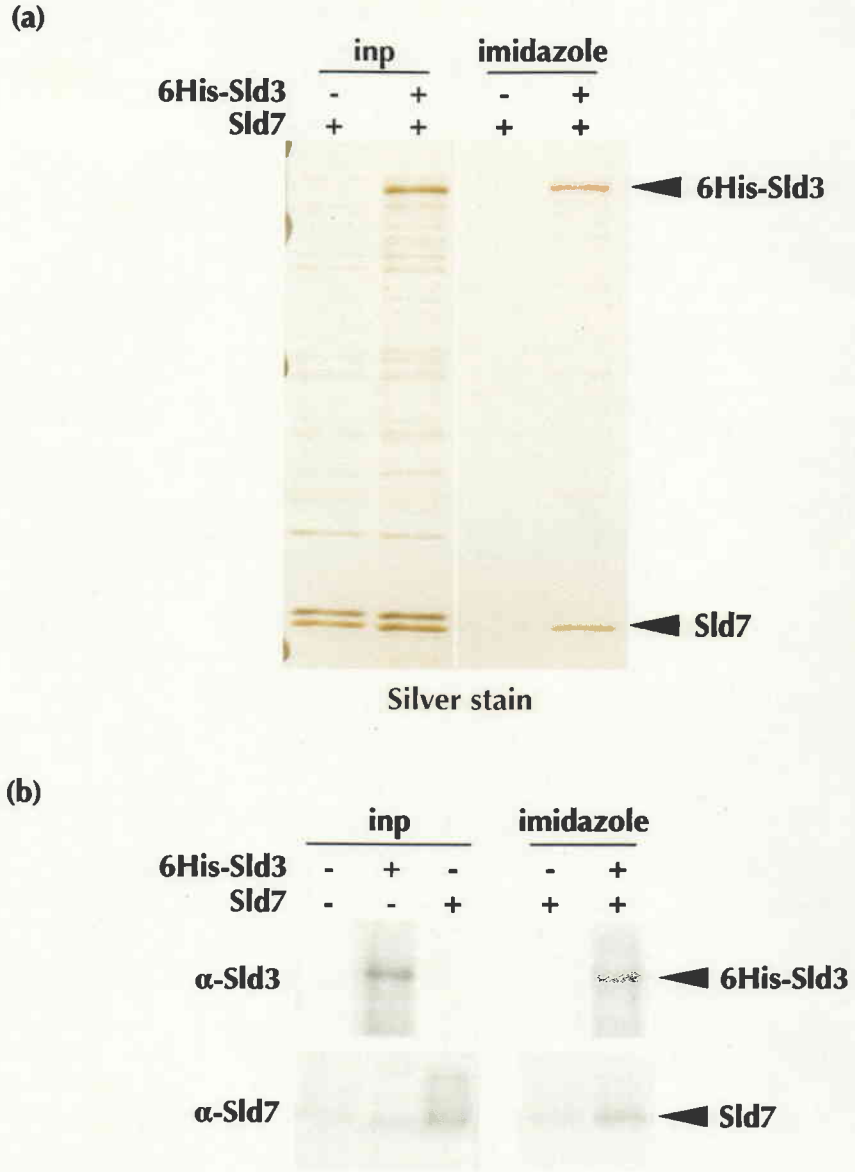


Figure 7. Purification of 6His-Sld3 and Sld7 proteins
(a) 6His-Sld3 protein and Sld7 protein were co-expressed in *E. coli* cells, and prepared as described in materials and methods. 6His-Sld3 protein was precipitated with Ni-NTA agarose (Qiagen) in the sonication buffer 500 at 4 ℃ for 2 hours. (b) 6His-Sld3 and Sld7 proteins were expressed independently in *E. coli* cells, and prepared as described in materials and methods. The 100 μl of the extracts including the 6His-Sld3 or Sld7 proteins prepared independently were mixed and precipitated with Ni-NTA agarose as described above.

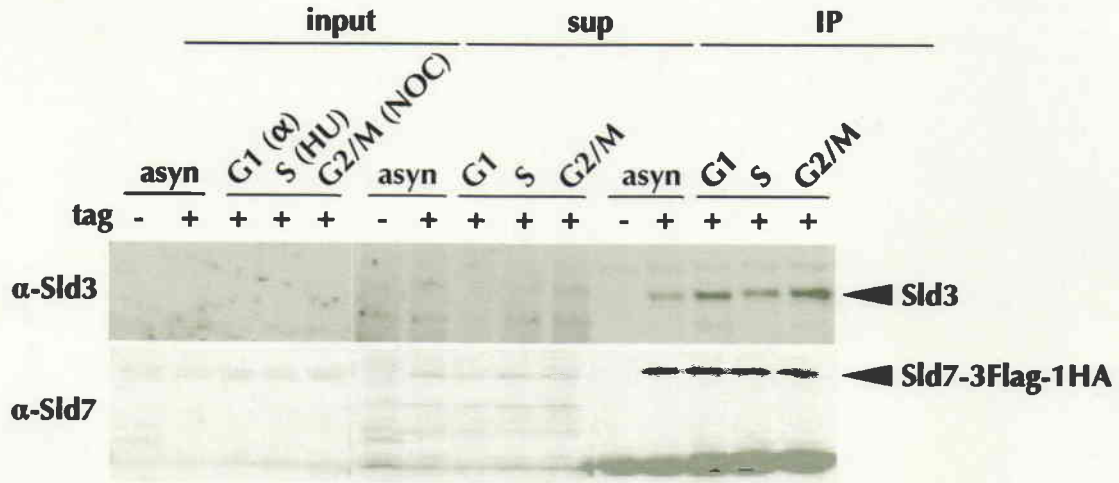


Figure 8. Immunoprecipitation of Sld7-3Flag-1HA in the cells treated with α-factor, HU, and NOC.
The cells harboring *SLD7-3FLAG-1HA* were treated with α-factor, hydroxyurea (HU), and nocodazole (NOC), which arrest cells in the specific phase of the cell cycle, G1, S, and G2/M, respectively. Extracts from the cells were immunoprecipitated by anti-Flag antibody (anti-Flag M2 affinity gel, Sigma).

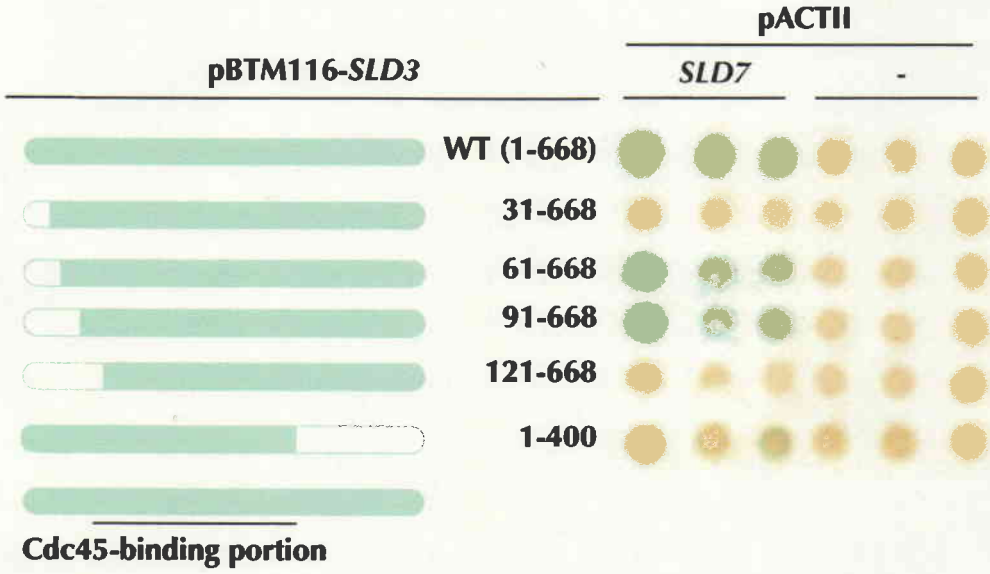
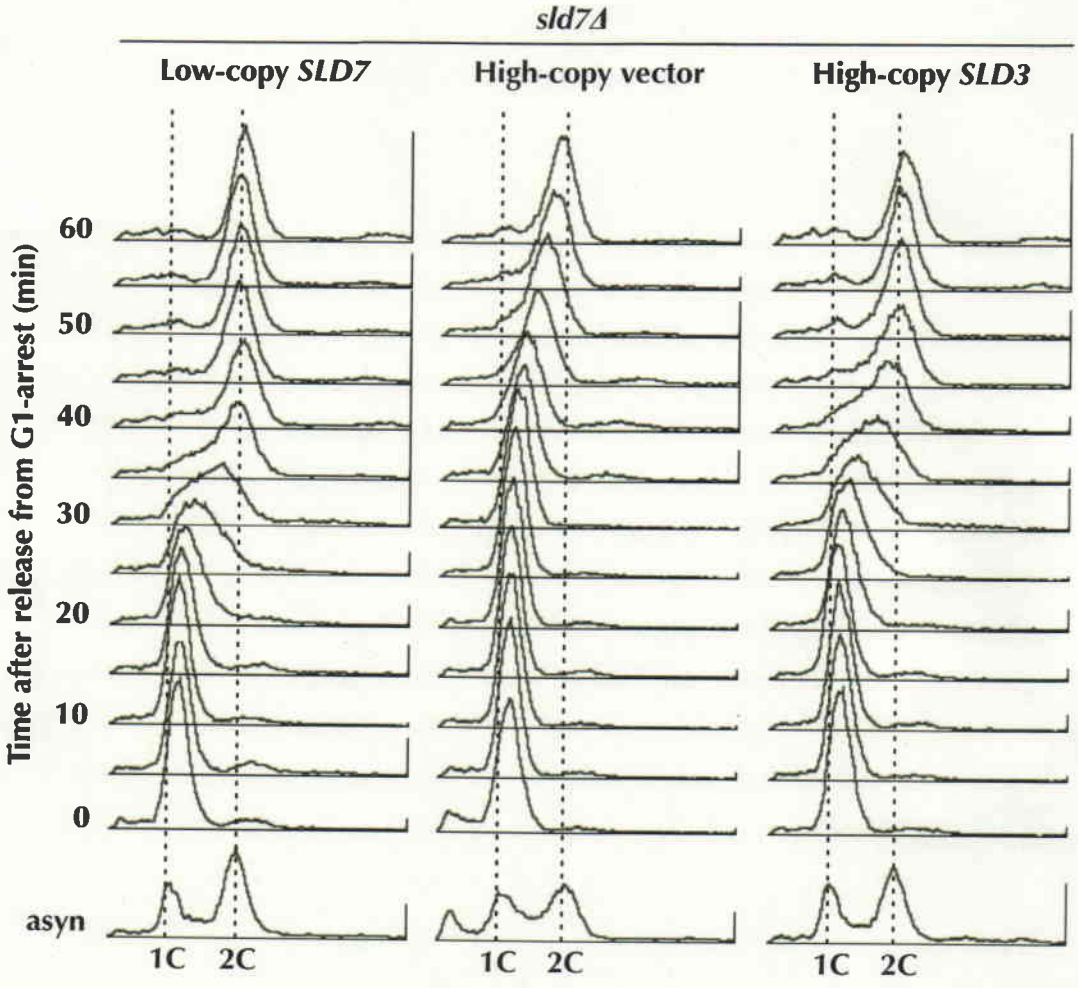
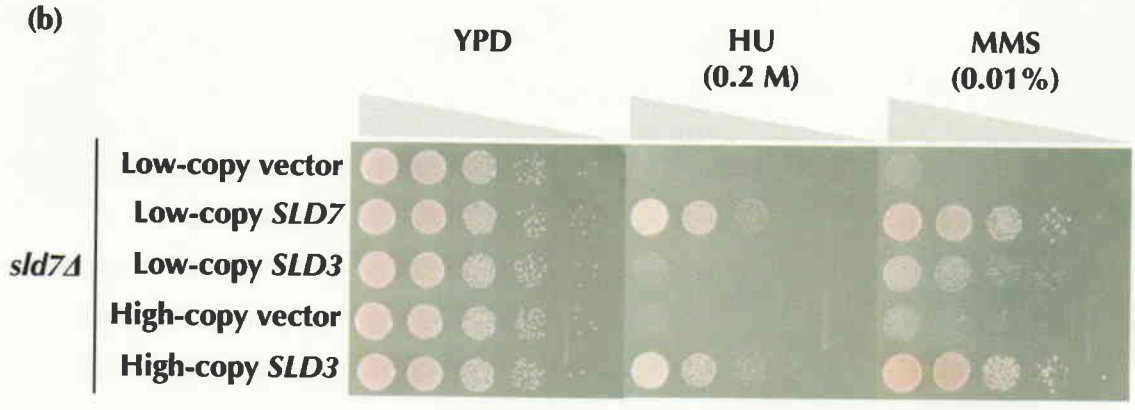


Figure 9. Yeast two-hybrid assay for Sld7 and truncated Sld3 proteins.
Plasmids were introduced into TAT7 cells, and the transformants were spotted onto SD-Leu-Trp plate. When transformants grew, cells were replicated to filter paper (Whatmann 50). The filter paper was frozen in liquid nitrogen, soaked in Z buffer containing X-gal and β-mercaptoethanol, and incubated at 30 ℃ for 1 hour.

(a)


sld7Δ
Low-copy SLD7
High-copy vector
High-copy SLD3
Time after release from G1-arrest (min)
60
50
40
30
20
10
0
asyn
1C
2C
1C
2C
1C
2C

(b)


YPD
HU
(0.2 M)
MMS
(0.01%)
sld7Δ
Low-copy vector
Low-copy SLD7
Low-copy SLD3
High-copy vector
High-copy SLD3

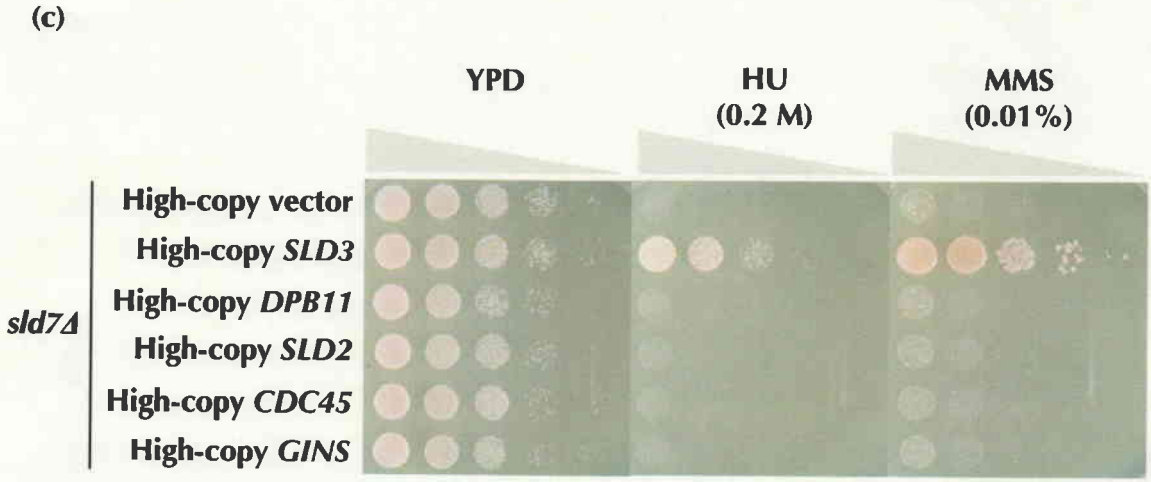
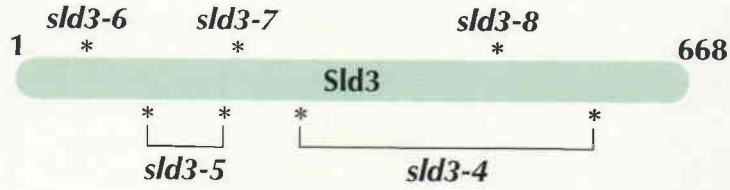


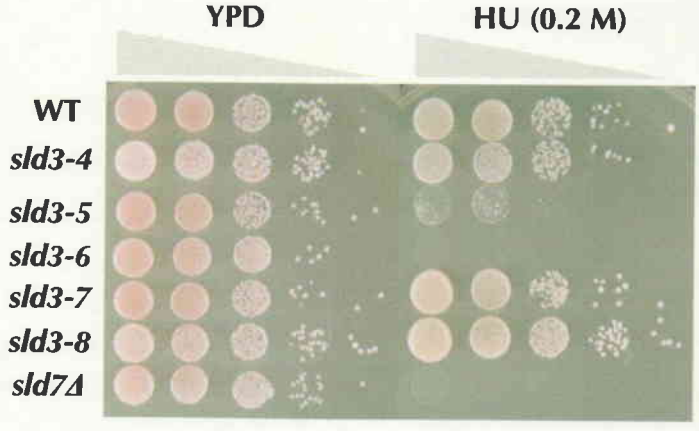
Figure 10. Suppression of the phenotypes of *sld7Δ* mutant by high-copy *SLD3* gene. (a) *SLD3* genes on high-copy plasmid suppressed inefficient DNA replication of *sld7Δ* mutant cells. (b) Low- and high-copy plasmids with *SLD3* or *SLD7* gene were introduced into *sld7Δ* cells. Then 10-fold serial dilutions of the cells were spotted onto YPD plates containing 0.2 M hydroxyurea (HU), or 0.01 % methylmethane sulfonate (MMS), respectively. The cells were incubated at 25 ℃ for 4 days. (c) High-copy plasmids with *SLD3, DPB11, SLD2, CDC45,* or *GINS* (*SLD5, PSF1, PSF2,* and *PSF3*) gene were introduced into *sld7Δ* cells. The sensitivities of these cells were tested as described above.

(a)

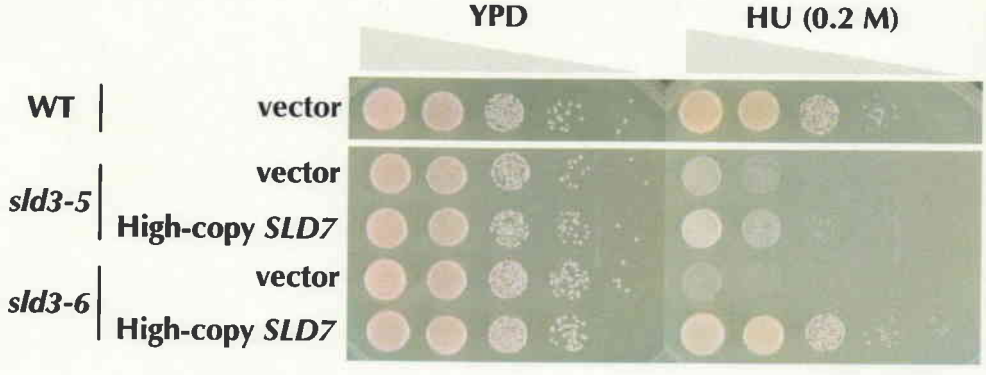
	Mutant name	Mutation(s)
sld3ts mutants	*sld3-4*	R269C, D578G
	sld3-5	G125D, F170S
	sld3-6	E63G
	sld3-7	L176P
	sld3-8	P407L



(b)



(c)



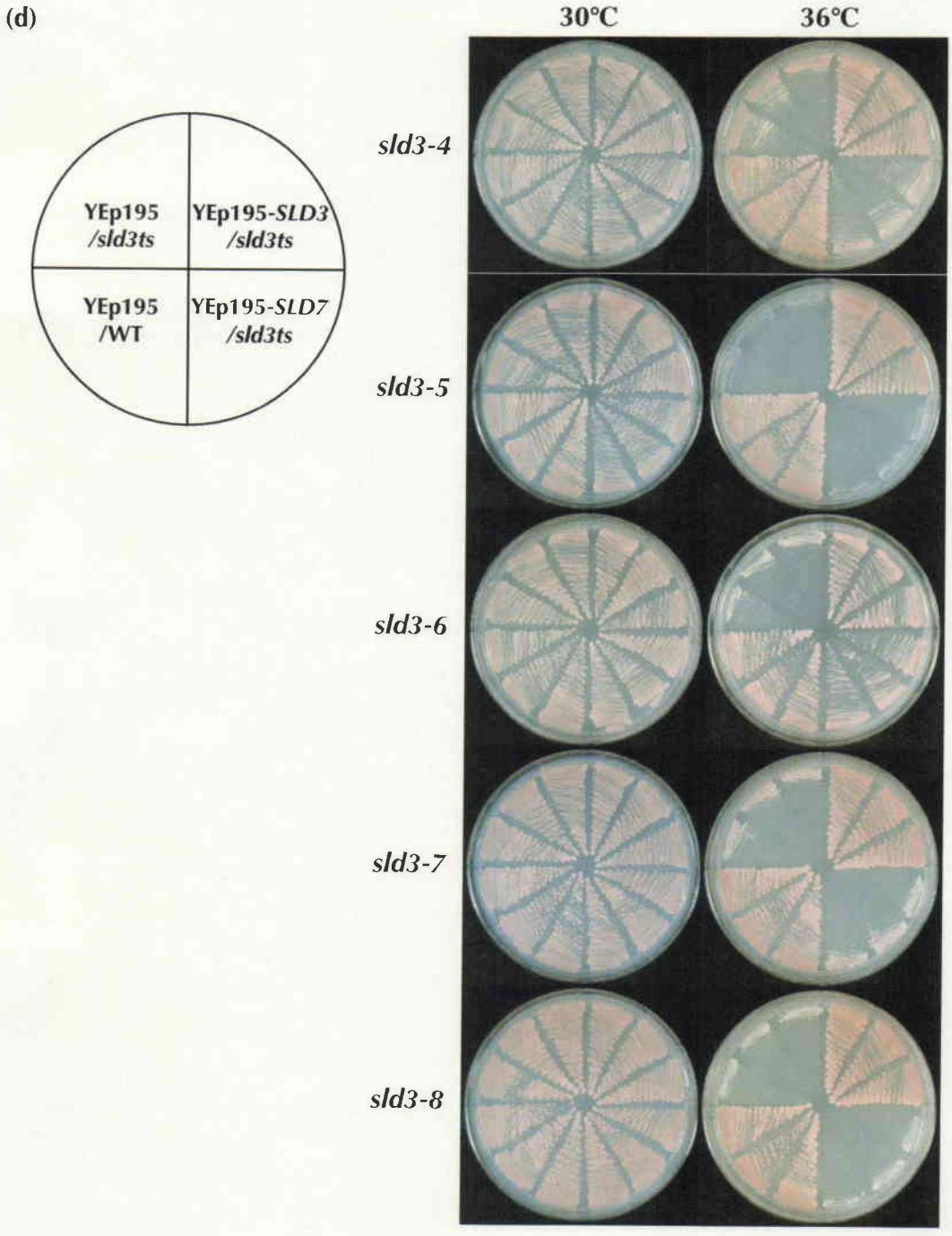


Figure 11. Suppression of the phenotypes of *sld3-6* mutant by high-copy *SLD7* genes. (a) Five temperature-sensitive (ts) mutants of *SLD3*, *sld3-4*, *-5*, *-6*, *-7*, and *-8*. (b) 10-fold serial dilutions of the mutant cells were spotted onto YPD plates containing 0.2 M hydroxyurea (HU). The cells were incubated at 25 **°C** for 4 days. (c) High-copy plasmids with or without *SLD7* gene were introduced into the *sld3-5* and *sld3-6* cells. Then 10-fold serial dilutions of the cells were spotted onto YPD plates containing 0.2 M HU. The cells were incubated at 25 **°C** for 4 days. (d) High-copy *SLD7* genes suppressed the ts phenotype of *sld3-6* mutant, but not that of *sld3-4*, *-5*, *-7*, and *-8* mutants.

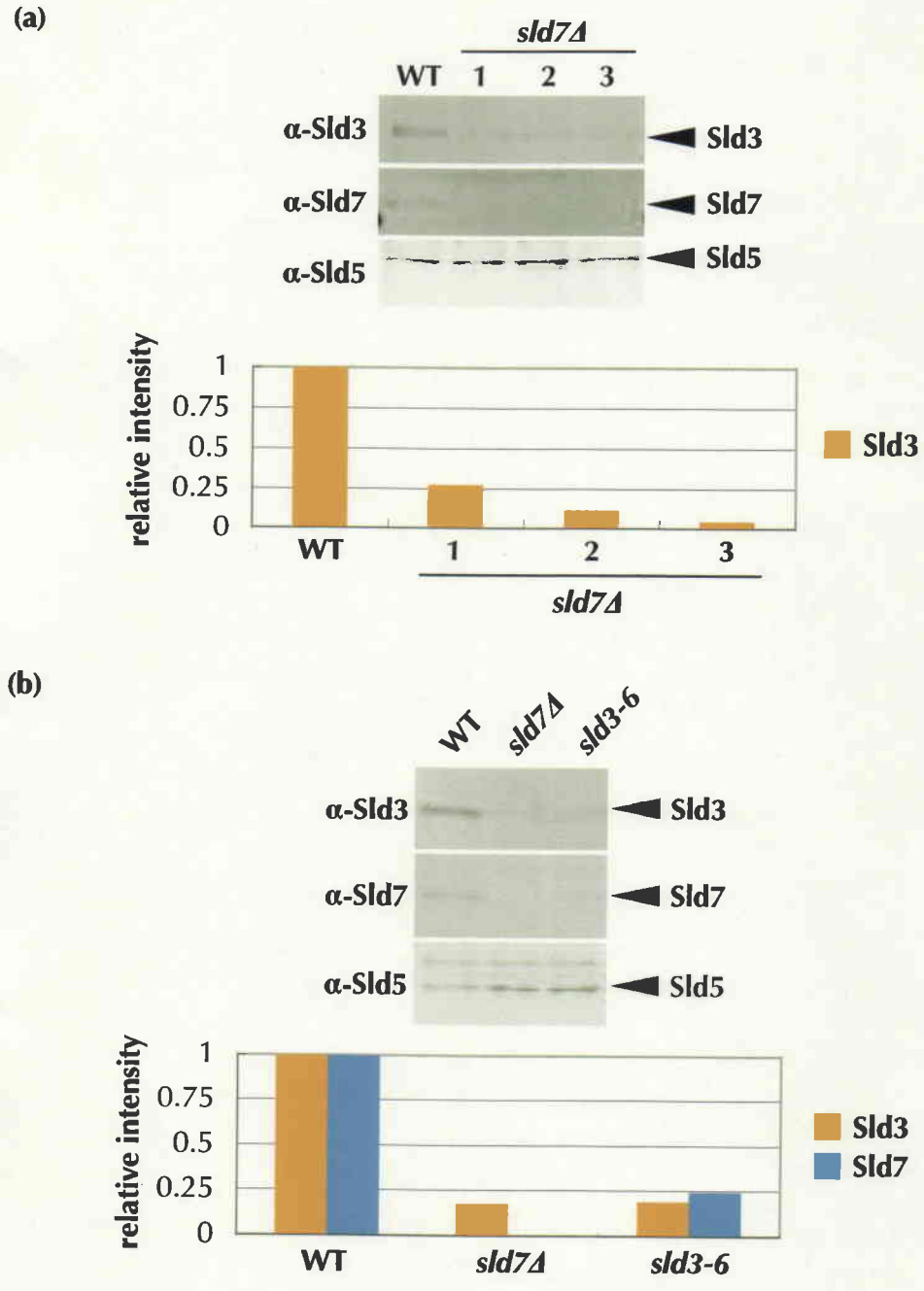
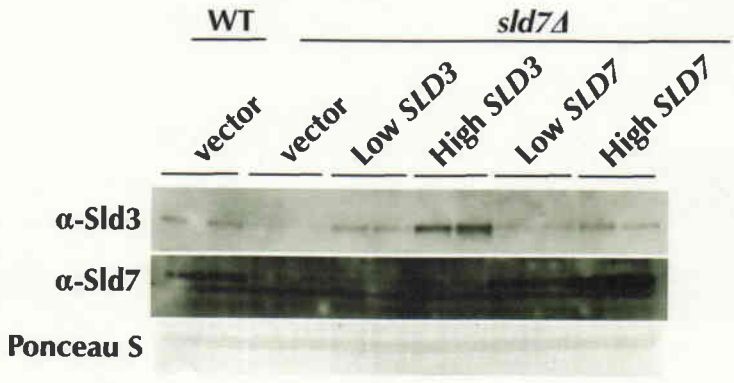
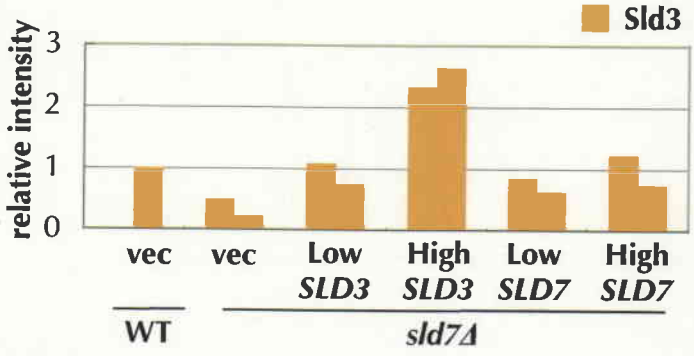
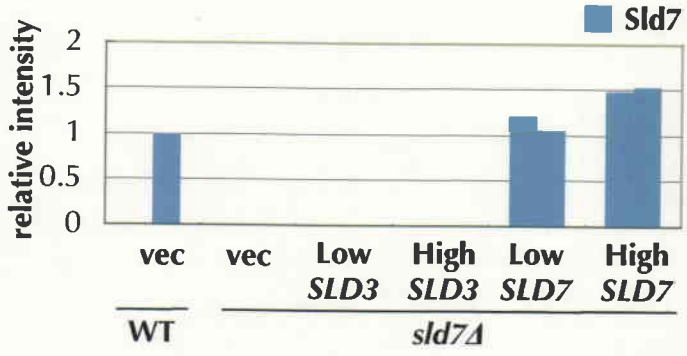


Figure 12. Abundance of Sld3 protein in *sld7Δ* and *sld3-6* cells.
Proteins were extracted from WT, *sld7Δ*, or *sld3-6* cells as described in materials and methods. (a) The Sld3 protein in three independent *sld7Δ* cells (1, 2, 3) was detected by western blotting using anti-Sld3 antibodies. (b) The amount of the Sld3 and Sld7 proteins in *sld3-6* cells. The Sld3 and Sld7 proteins were detected by anti-Sld3 and anti-Sld7 antibodies. Sld5 was also detected by anti-Sld5 antibodies and used as an internal control.

(a)


WT
sld7Δ
vector
vector
Low SLD3
High SLD3
Low SLD7
High SLD7
α-Sld3
α-Sld7
Ponceau S


Sld3
relative intensity
3
2
1
0
vec
vec
Low SLD3
High SLD3
Low SLD7
High SLD7
WT
sld7Δ


Sld7
relative intensity
2
1.5
1
0.5
0
vec
vec
Low SLD3
High SLD3
Low SLD7
High SLD7
WT
sld7Δ

(b)

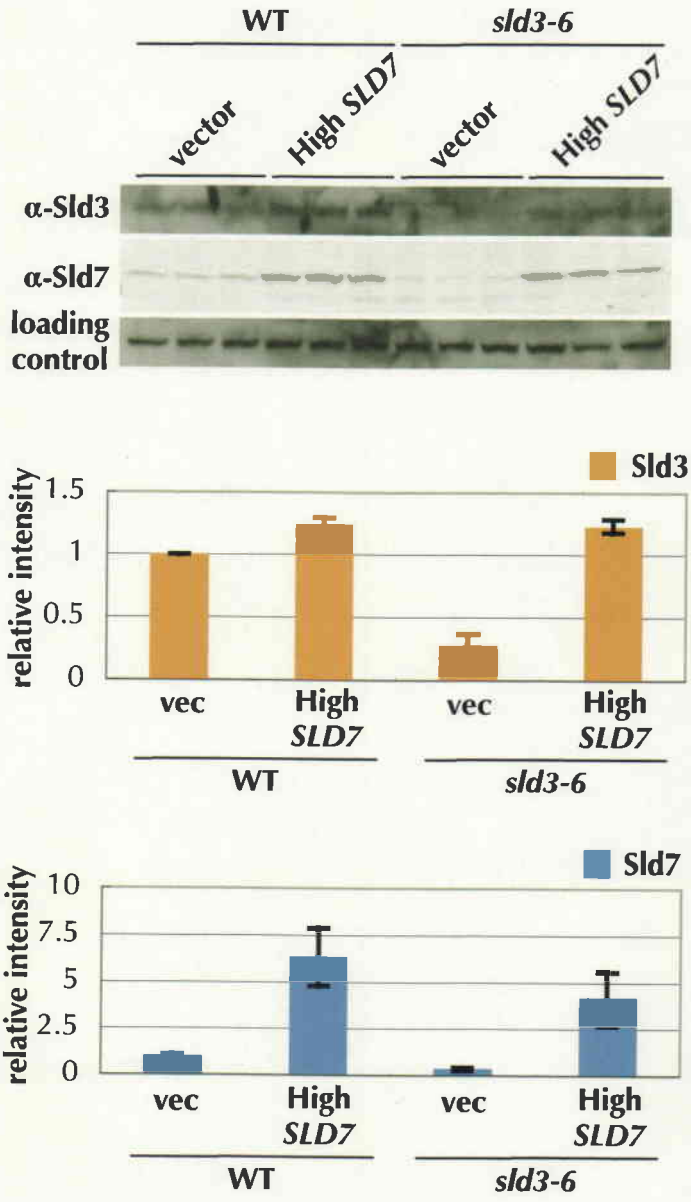


Figure 13. Restoration of the Sld3 protein level in *sld7Δ* and *sld3-6* cells. Low- and high-copy plasmid with *SLD3* or *SLD7* gene were introduced into WT, *sld7Δ*, *sld3-6* cells. Proteins were extracted from the cells as described in Materials and methods. (a) The *SLD3* genes on low-copy plasmid restored the abundance of Sld3 protein in the *sld7Δ* cells to the same level as WT cells, and the *sld7Δ* cells harboring *SLD7* genes on low- and high-copy plasmids. The protein level in each lane was quantified and shown independently. (b) The *SLD7* genes on high-copy plasmid restored the abundance of the Sld3 protein in *sld3-6* cells to the same level as WT cells. The non-specific protein detected by anti-Sld3 antibodies was shown as a loading control. The protein level was quantified in each lane and shown as an average with the standard deviation.

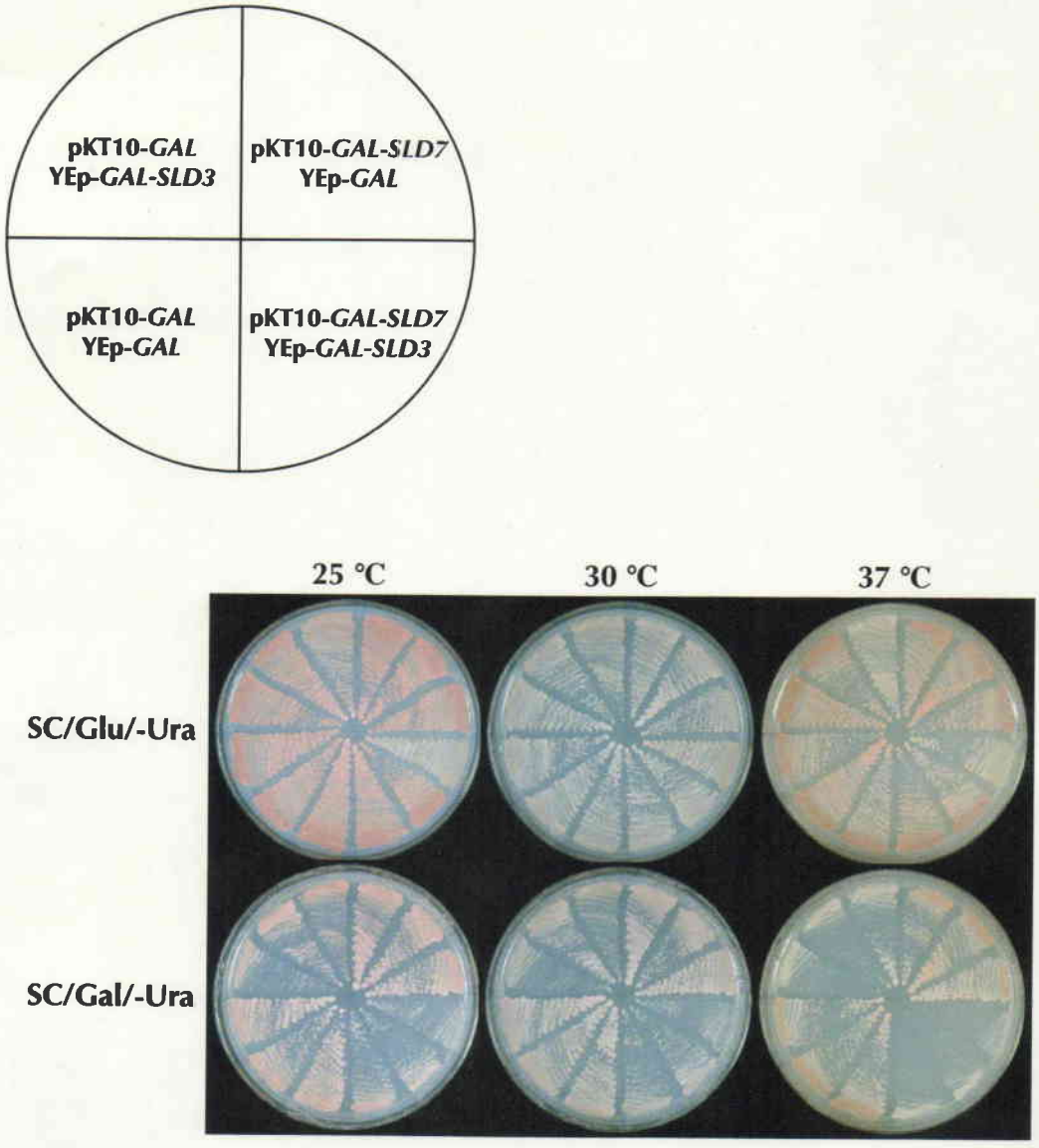


Figure 14. Simultaneous over-expression of *SLD3* and *SLD7* genes.
The *SLD3* and *SLD7* genes under control of galactose promoter on plasmids were introduced and over-expressed in W303 cells, and the cells were incubated on Glucose or Galactose plate for 2 days.

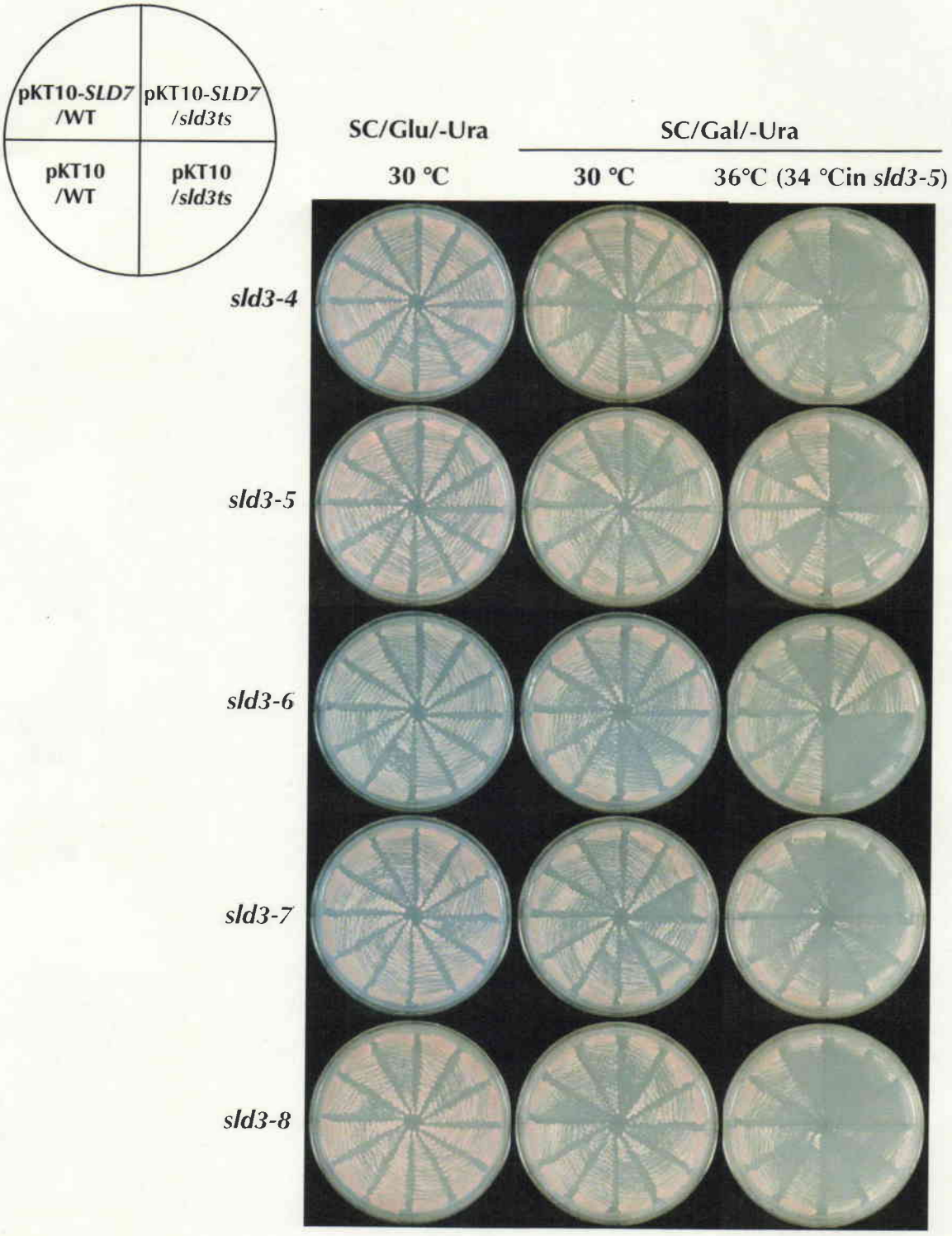
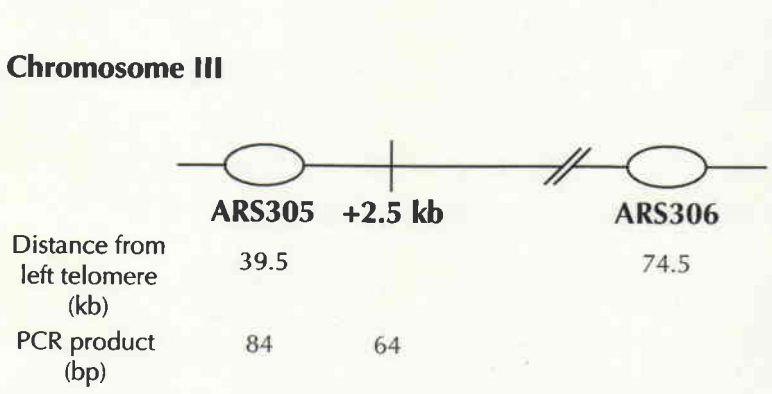
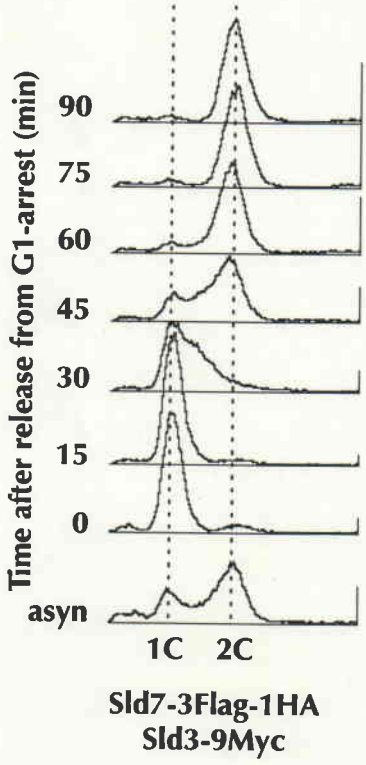


Figure 15. Over-expression of *SLD7* in *sld3ts* mutant cells.
The plasmids with Gal-inducible *SLD7* gene were introduced into *sld3ts* mutant cells. The cells were incubated on Glucose or Galactose plate for 2 days.

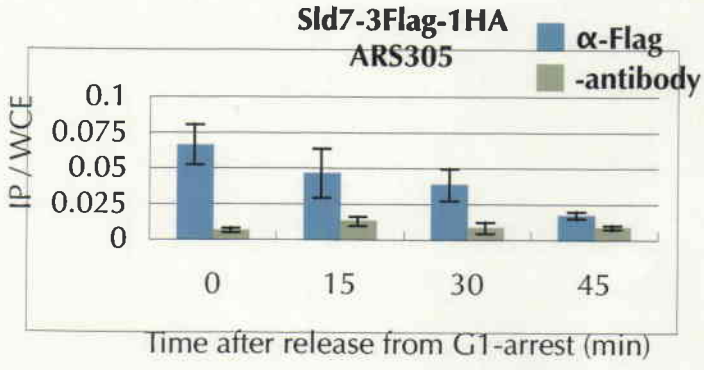
(a)


Chromosome III
ARS305
+2.5 kb
ARS306
Distance from left telomere (kb)
39.5
74.5
PCR product (bp)
84
64

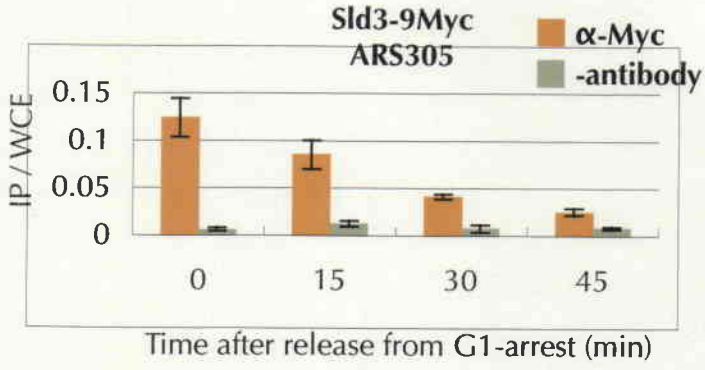
(b)


Time after release from G1-arrest (min)
90
75
60
45
30
15
0
asyn
1C
2C
Sld7-3Flag-1HA
Sld3-9Myc

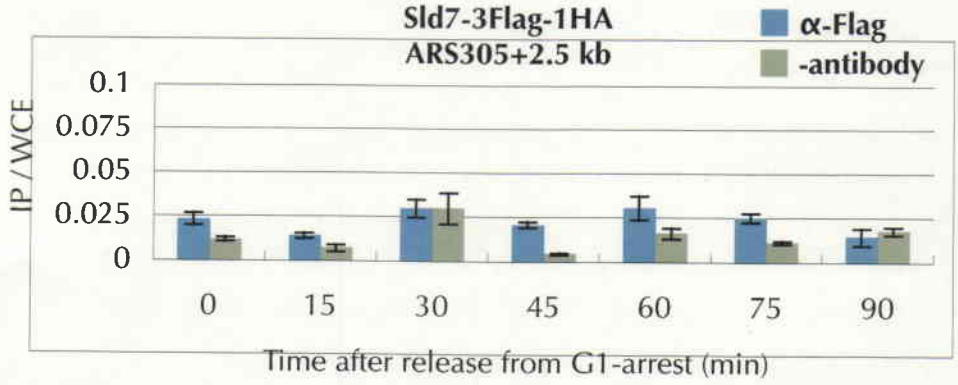
(c)


Sld7-3Flag-1HA
ARS305
α-Flag
-antibody
IP / WCE
0.1
0.075
0.05
0.025
0
0
15
30
45
Time after release from G1-arrest (min)

(d)


Sld3-9Myc
ARS305
α-Myc
-antibody
IP / WCE
0.15
0.1
0.05
0
0
15
30
45
Time after release from G1-arrest (min)

(e)



(f)

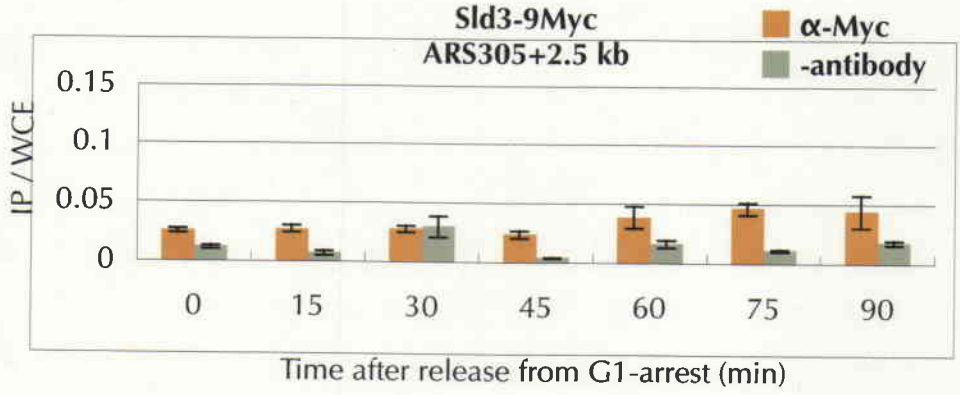
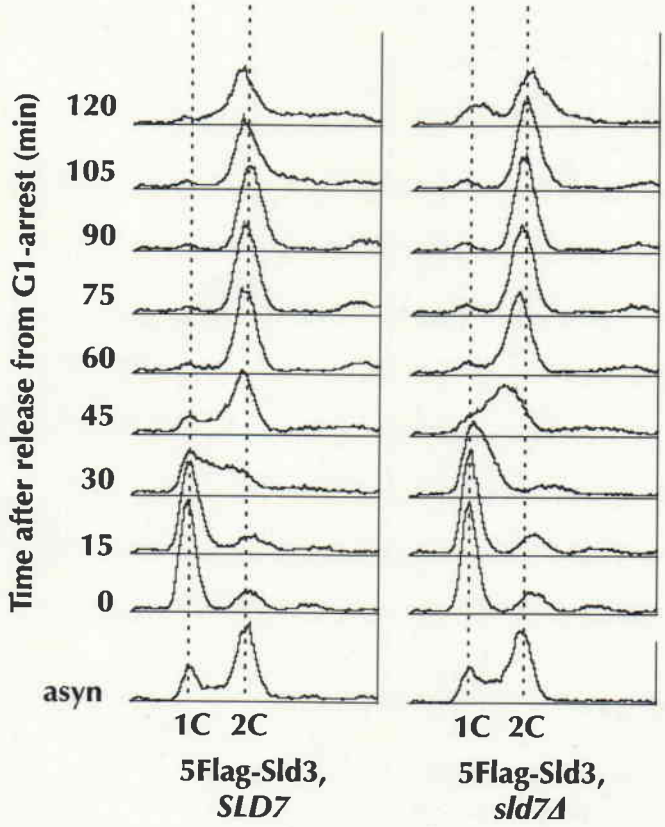
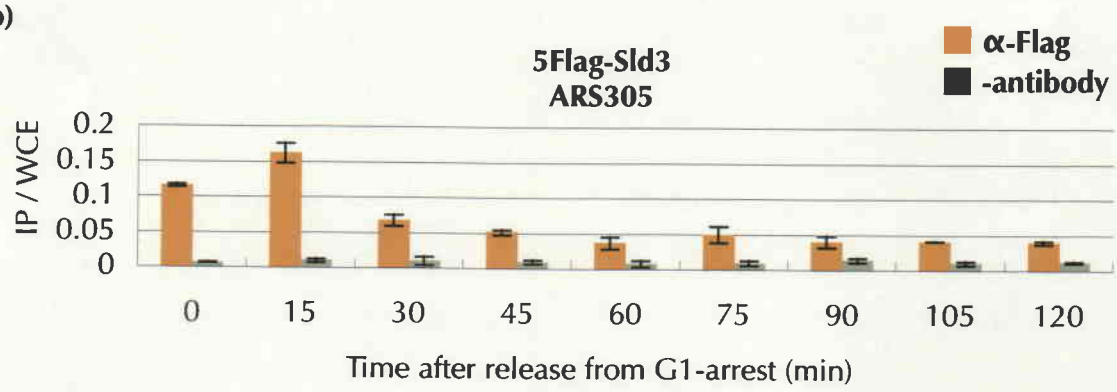


Figure 16. Chromatin immunoprecipitation (ChIP) of Sld7-3Flag-1HA and Sld3-9Myc proteins.
(a) ARS305 and its neighboring regions on chromosome III. (b) The cells harboring *SLD3-9MYC* and *SLD7-3FLAG-1HA* were arrested at G1-phase with α-factor and released at 25 ℃. Aliquots of cells were sampled at 15 minutes intervals. (c) DNA fragments from immunoprecipitates of each sample were amplified by PCR primers for ARS305 (ARS305 realtime LEFT1 and ARS305 realtime RIGHT1). (d) ChIP of Sld3-9Myc protein by anti-Myc antibody (c-myc Ab-1 9E11, NeoMarkers). (e), (f) The assays using primers for the neighboring region of ARS305 (305+2.5kb realtime LEFT1 and 305+2.5kb realtime RIGHT1).

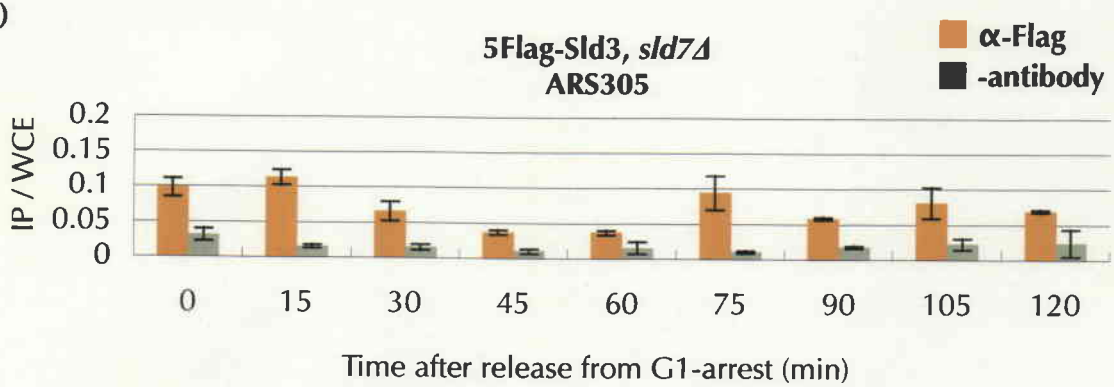
(a)


Time after release from G1-arrest (min)
120
105
90
75
60
45
30
15
0
asyn
1C 2C
1C 2C
5Flag-Sld3, SLD7
5Flag-Sld3, sld7Δ

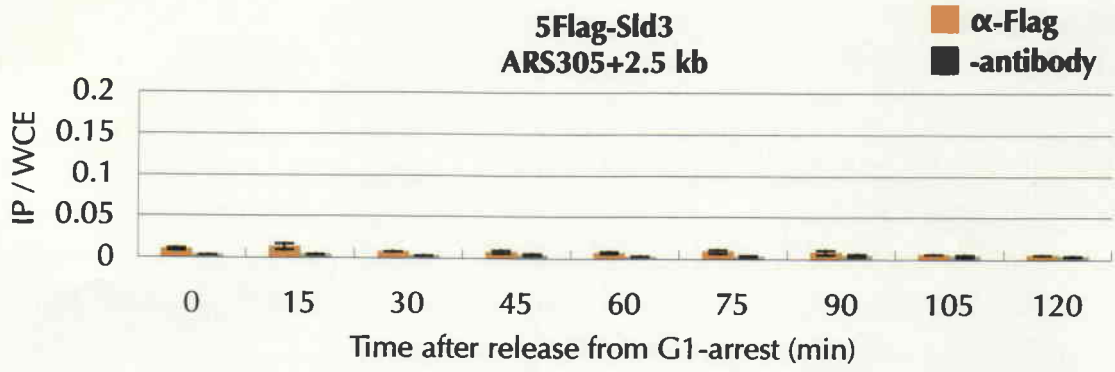
(b)


5Flag-Sld3
ARS305
α-Flag
-antibody
IP / WCE
0.2
0.15
0.1
0.05
0
0 15 30 45 60 75 90 105 120
Time after release from G1-arrest (min)

(c)


5Flag-Sld3, sld7Δ
ARS305
α-Flag
-antibody
IP / WCE
0.2
0.15
0.1
0.05
0
0 15 30 45 60 75 90 105 120
Time after release from G1-arrest (min)

(d)



(e)

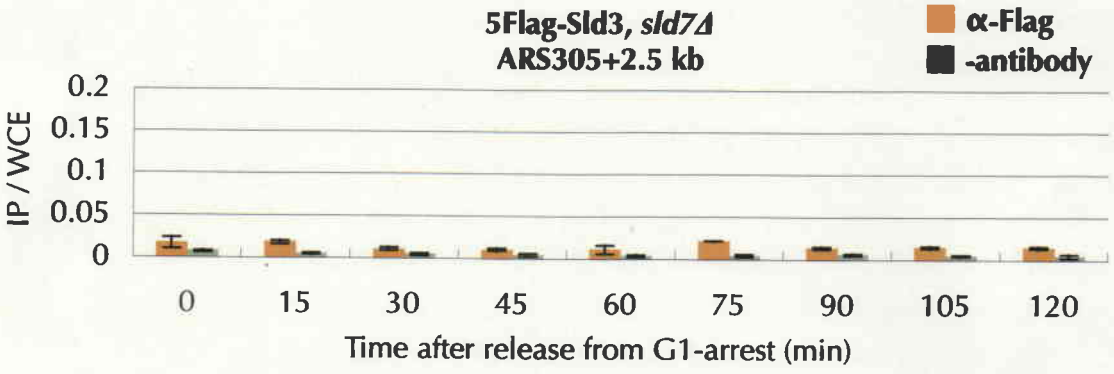
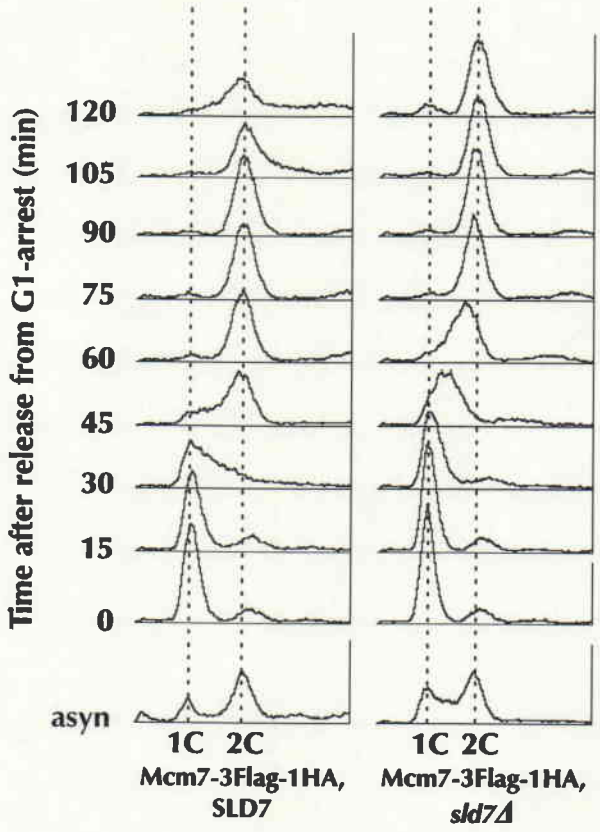
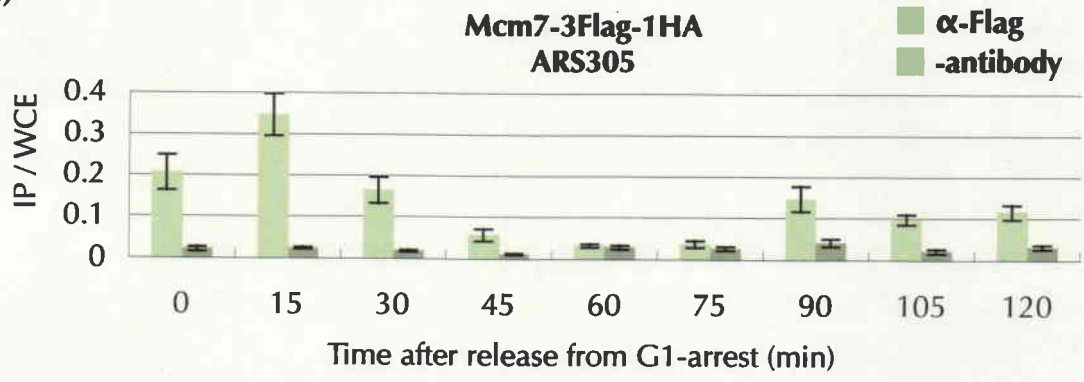


Figure 17. Chromatin immunoprecipitation (ChIP) of 5Flag-Sld3 protein in the presence or absence of Sld7 protein.
(a) The cells harboring *5FLAG-SLD3* or *5FLAG-SLD3, sld7Δ* were arrested at G1-phase with α-factor and released at 25 ℃. Aliquots of cells were sampled at 15 minutes intervals, and DNA content was measured by flow cytometry. (b) DNA fragments from immunoprecipitates of each sample were amplified by PCR primers for ARS305 (ARS305 realtime LEFT1 and ARS305 realtime RIGHT1). (c) The association signals of the 5Flag-Sld3 protein in *sld7Δ* cells. (d) 5Flag-Sld3 association with the neighboring region of ARS305 (305+2.5kb realtime LEFT1 and 305+2.5kb realtime RIGHT1).

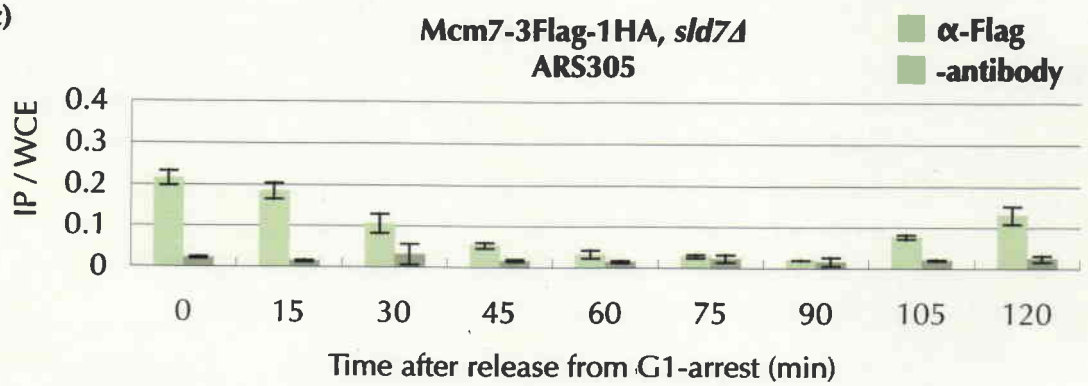
(a)


Time after release from G1-arrest (min)
120
105
90
75
60
45
30
15
0
asyn
1C 2C
Mcm7-3Flag-1HA, SLD7
1C 2C
Mcm7-3Flag-1HA, sld7Δ

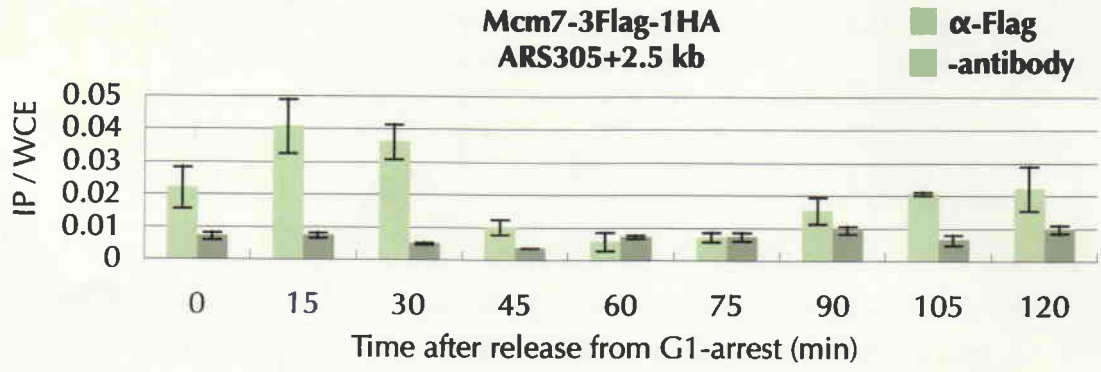
(b)


Mcm7-3Flag-1HA
ARS305
α-Flag
-antibody
IP / WCE
0.4
0.3
0.2
0.1
0
0 15 30 45 60 75 90 105 120
Time after release from G1-arrest (min)

(c)


Mcm7-3Flag-1HA, sld7Δ
ARS305
α-Flag
-antibody
IP / WCE
0.4
0.3
0.2
0.1
0
0 15 30 45 60 75 90 105 120
Time after release from G1-arrest (min)

(d)



(e)

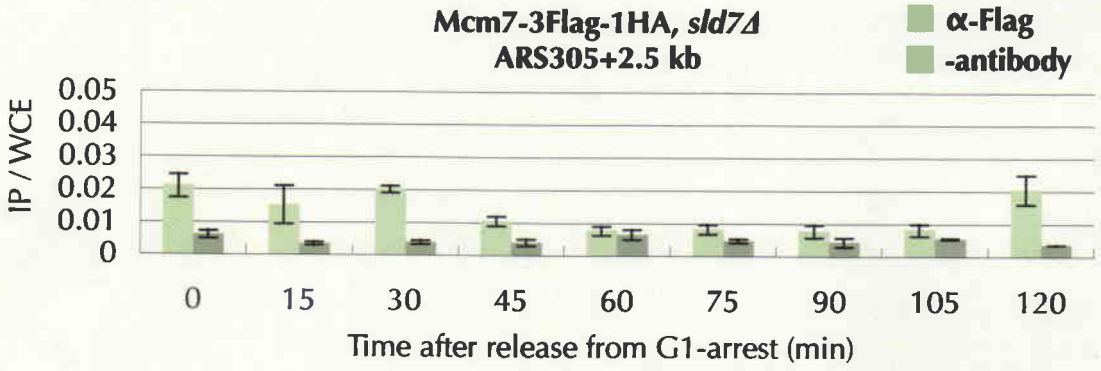
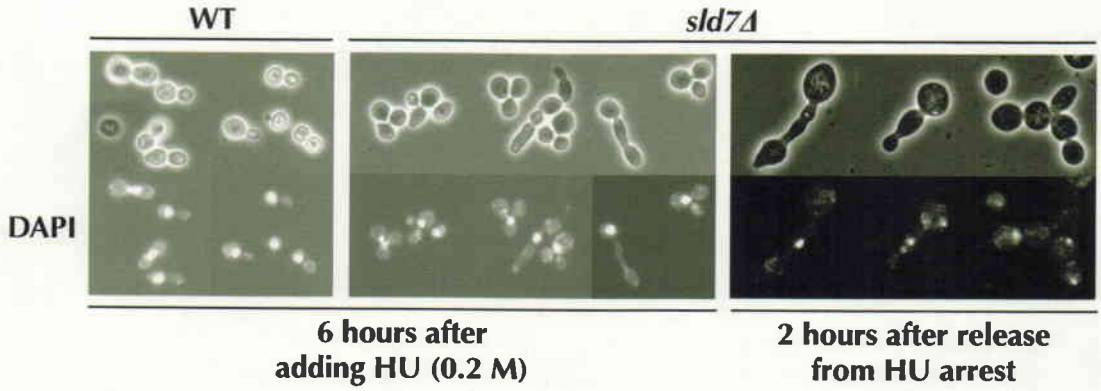
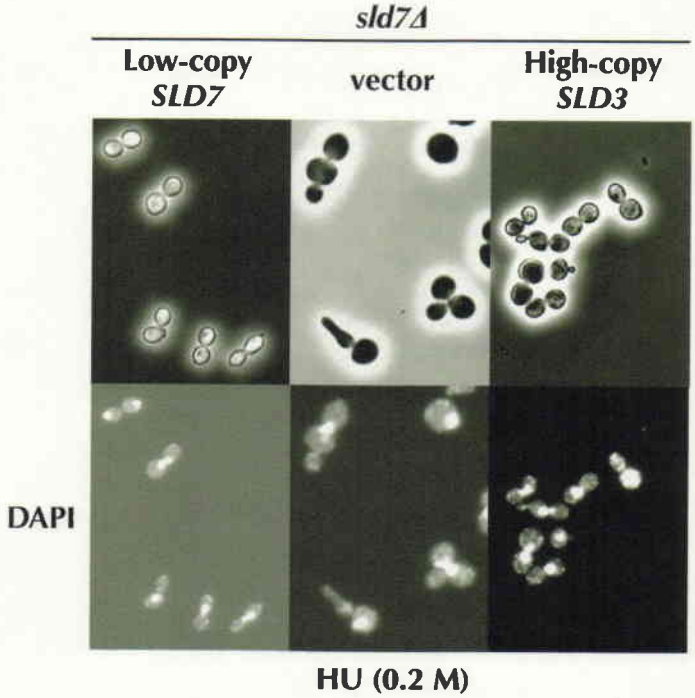


Figure 18. Chromatin immunoprecipitation (ChIP) of Mcm7-3Flag-1HA protein in the presence or absence of Sld7 protein.
(a) The cells harboring *MCM7-3FLAG-1HA* or *MCM7-3FLAG-1HA, sld7Δ* were arrested at G1-phase with α-factor and released at 25 ℃. Aliquots of cells were sampled at 15 minutes intervals, and DNA content was measured by flow cytometry. (b), (c) DNA fragments from immunoprecipitates of each sample were amplified by PCR primers for ARS305 (ARS305 realtime LEFT1 and ARS305 realtime RIGHT1). (d), (e) Mcm7-3Flag-1HA association with neighboring region of the origins (305 +2.5kb realtime LEFT1 and 305+2.5kb realtime RIGHT1).

(a)


WT
sld7Δ
DAPI
6 hours after
adding HU (0.2 M)
2 hours after release
from HU arrest

(b)


sld7Δ
Low-copy
SLD7
vector
High-copy
SLD3
DAPI
HU (0.2 M)

(c)

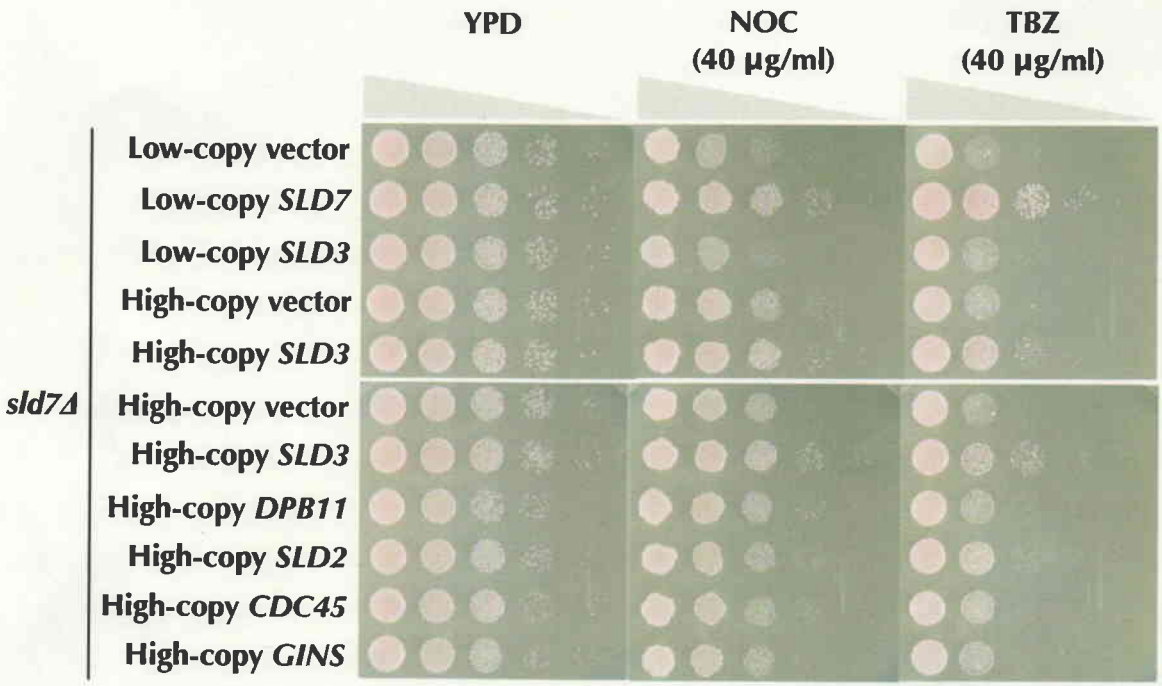


Figure 19. Functions of Sld7 protein other than DNA replication.
(a) Cell morphology treaded with hydroxyurea (HU). *sld7Δ* and WT cells were incubated in YPD medium with 0.2 M HU at 30 ℃ for 6 hours. Then, the cells were transferred to fresh YPD medium and incubated at 30 ℃ for 2 more hours. (b) The *SLD3* gene on high-copy plasmid suppressed the abnormal morphology of the *sld7Δ* cells.The *SLD7* gene on low-copy plasmid or *SLD3* gene on high-copy plasmid was introduced into the *sld7Δ* cells. The cells were incubated in YPD medium with 0.2 M HU at 30 ℃ for 6 hours. (c) Sensitivity of *sld7Δ* cells harboring low- or high-copy plasmids with the *SLD3* and *SLD7* genes to inhibitors of microtubule polymerization. 10-fold serial dilutions of the cells were spotted onto YPD plates containing 40 μg/ml nocodazole (NOC), or 40 μg/ml thiabendazole (TBZ). The cells were incubated at 25 ℃ for 4 days.

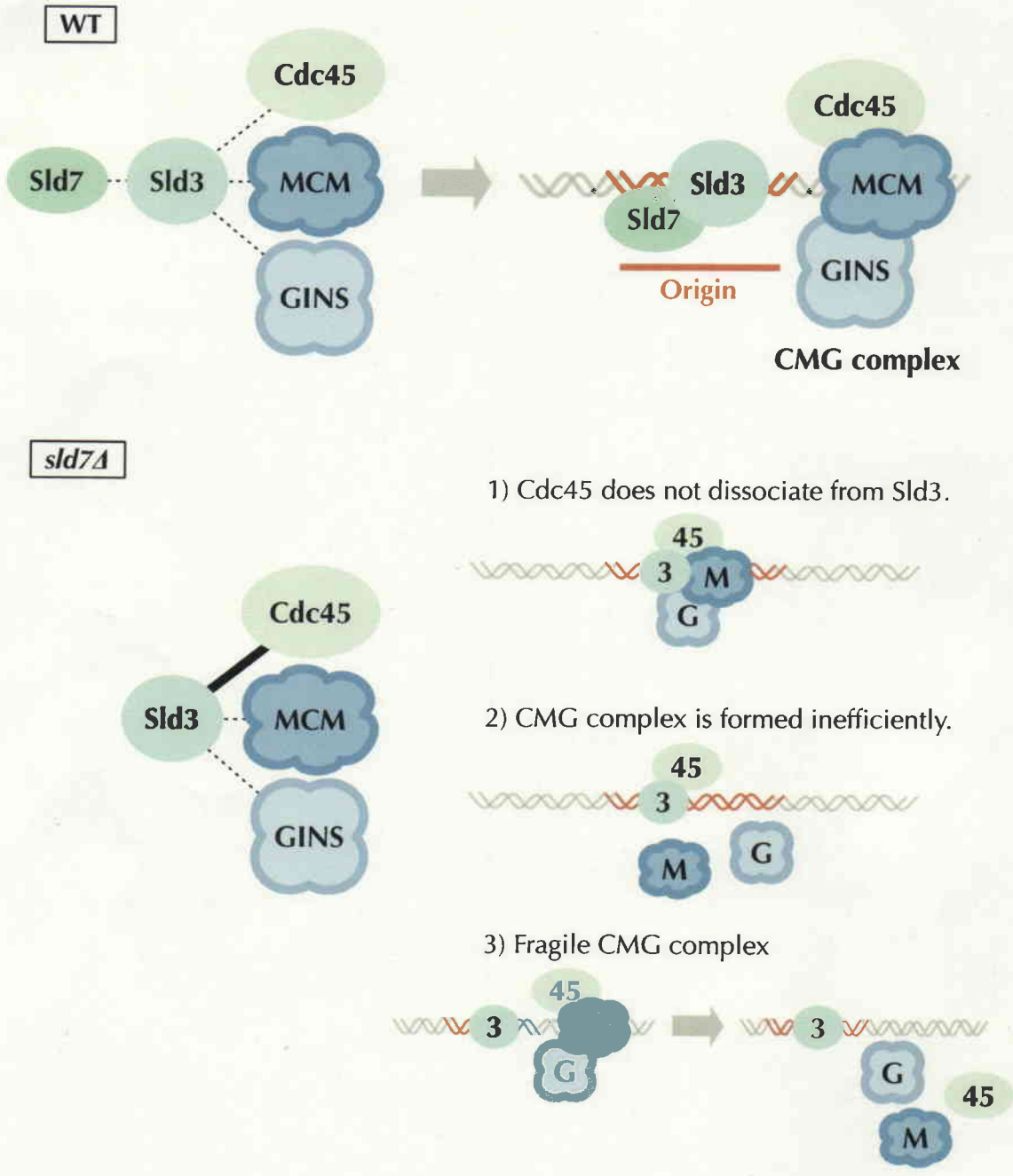


Figure 20. Model for the initiation step of DNA replication in *S. cerevisiae*. This study suggested that Sld7 and Cdc45, a component of CMG complex, compete for binding to Sld3. Thus, the absence of Sld7 protein confers Sld3 tighter association with Cdc45 protein. The tight Sld3-Cdc45 association may affect MCM dissociation from origins in three ways. 1) Cdc45 in the CMG complex does not dissociate from Sld3 on the origin efficiently. 2) The CMG complex is formed inefficiently. Once the CMG complex is formed, its components reduced the affinity to replication origins, so that it dissociates easily from origins. 3) Although the CMG complex is formed as efficient as WT cells, the complex is fragile. The fragile complex may dissociate from replication forks frequently.