Regulatory mechanism of the initiation of DNA replication by cyclin-dependent kinase

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Contents

Abstract

In *Sacchromyces cerevisiae*, the initiation of chromosomal DNA replication requires the origin recognition complex (ORC) that binds to DNA and provides a site on the chromosome where additional replication factors can bind. Associations of Cdc6 and Cdt1 with ORC allow the complex to load the presumptive replicative helicase (Mcm proteins) onto chromatin. This process, called licensing or prereplicative complex (pre-RC) formation, confers competence on the origin to fire a single time in S phase. Onset of DNA synthesis requires the action of the Cdk and Cdc7 kinases, which trigger the binding of additional factors such as Cdc45, Sld3, GINS, Dpb11 and Sld2 at pre-RCs. Cdc45 binds to origins tightly when Cdk and Cdc7 activated, and this association is dependent on pre-RC formation. Since this event appears to be linked to origin unwinding, I first isolated series of thermosensitive alleles for *CDC45* to elucidate the initiation mechanism. In this screening, eight thermosensitive *cdc45* mutants are isolated, and are found to be defective in the initiation of chromosomal DNA replication. The chromatin binding of Cdc45 significantly reduces even after S-Cdk activation in thermosensitive alleles at *SLD2* and *DPB11*, respectively. Moreover, early origin association of both Sld2 and Dpb11 depends on Cdc45 by ChIP assay, implying that the associations of Cdc45, Sld2 and Dpb11 with chromatin appear to be mutually dependent.

Second, I have analyzed the interaction between Sld2 and Dpb11, which is regulated by Cdk activity. Sld2 has a cluster of eleven Cdk-dependent phosphorylation sites

(S/T-P), six of which are preferred Cdk phosphorylation sites. Pohsphorylation of Sld2 enhances its interaction with Dpb11, and this appears to be necessary for onset of DNA replication. In this study, I show that Sld2 forms a complex with Dpb11 independently of Cdc45 or Cdc7 kinase. Moreover, using various truncated forms of the proteins in yeast two-hybrid assay, I mapped the interaction region of Sld2 and Dpb11. Dpb11 has four copies of the BRCT domain, which is important for protein-protein interaction. C-terminal pair of BRCT domains of Dpb11 binds to specific region of Sld2. I also delimitated a binding domain of Sld2 for Dpb11 into a 39-amino acids stretch, partially overlapping with a cluster of the phosphorylation sites. While a 39-aa stretch binds to Dpb11 in phosphorylation-independent manner, the binding of Dpb11 with Sld2 depends on phosphorylation when this 39-aa stretch is connected to a cluster of the phosphorylation sites of Sld2. *In vitro* binding assay, developed by this study using the purified proteins, demonstrates that the Sld2 directly interacts with Dpb11 and Cdk-dependent phosphorylation of Sld2 changes its affinity to Dpb11. Therefore, I propose that a cluster of phosphorylation sites in Sld2 regulates the affinity of 39-aa binding domain to Dpb11.

Abbreviations

E.coli	*Escherichia coli*
5-FOA	5-fluoroorotic acid
Ala	alanine
BRCT	Brca1 C-terminal
C - terminal	carboxyl - terminal
CBB	coomassie brilliant blue
Cdk	cyclin-dependent kinase
DAPI	4',6-diamino-2-phenylindole
dNTP	deoxynucleotide triphosphophates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulfonicacid
HU	hydroxyurea
IP	immunoprecipitation
IPTG	isopropyl-□-D-thiogalactopyranoside
N - terminal	amino-terminal
NAD	nicotinamide adenine dinucleotide
Na_3VO_4	sodium orthovanadate
NaF	sodium fluoride
NaPPi	sodium pyrophosphate
ORF	open reading frame
PCR	polymerase chain reaction
PIPES	piperazine-1,4-bis(2-ethansulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
Thr	threonine
X-gal	5-bromo-4-chloro-3-indolyl-□-galacosidase

Introduction

A definite feature of eukaryotic organism is a DNA replication mechanism able to cope with the duplication of large genomes. The replication of a large genome of eukaryotic cells is carried out by the use of multiple replication origins per chromosome. Multiple origins must, however, be carefully regulated in order that all chromosomal sequences are to be replicated once and only once during each cell cycle division. Some origins fire early during S phase whereas others fire late, but none fire more than once, except in rare cases where cells undergo endoduplication (Diffley, 1996;Stillman, 1996). In *Sacchromyces cerevisiae*, it is well known that bidirectional replication initiates at sequence specific locus on chromosome, called the autonomously replicating sequences (ARSs) (Campbell and Newlon, 1991). Till now, many factors and regulators involved in replication have been identified and their function and binding order onto the origins for the activation have been characterized (Bell and Dutta, 2002; Mendez and Stillman, 2003). The roles of replication proteins and mechanisms in chromosome replication seem to be well conserved through eukaryotes (Kearsey and Cotterill, 2003).

The initiation of DNA replication requires the origin recognition complex (ORC), which contains six subunits related to each other and binds directly to the origins of replication. Sequentially, the ORC complex recruits two binding factors critical for the activation

(Kelly and Brown, 2000), Cdc6 and Cdt1, which allow assembly of the mini-chromosome maintenance (MCM) 2-7 complex onto the origins. Association of Mcm proteins which probably constitute the replicative helicase (Labib and Diffley, 2001), completes assembly of the prereplicative complex (pre-RC), in both yeast and *Xenopus* (Zou and Stillman, 2000; Jares and Blow, 2000;Walter and Newport, 2000). At the G1/S transition, the pre-RC allows replication initiation by action of two protein kinases, which are activated around the same times as the firing of early origins. These kinases include S-phase cyclin-dependent kinase (S-Cdk; Cdc28 in budding yeast) whose activity depends on cyclin regulatory subunits (Clb5 and Clb6 in budding yeast, cyclin E and A in animal cells) and Cdc7 whose activity depends on a regulatory subunit, Dbf4. Thereafter Cdc45-Sld3, Dpb11-Sld2 and GINS complex associate with origins, depend ently on the pre-RC (Takayama et al., 2003; Kubota et al., 2003), and DNA polymerases (, and) are recruited to origins to initiate DNA synthesis. In the next step called elongation, replication forks travel through replication machinery until they meet other forks to complete duplication of the genome during S-phase (Fig. 1). Thus if cells have a problem with S-phase progression, this could be caused by a defect either in origin firing or in elongation.

An essential protein for DNA replication, Cdc45, is known to be involved in the both processes: initiation at origins and elongation of replication forks. A two-dimensional gel electrophoresis experiment has shown that a mutant allele for *CDC45*, *cdc45-1*, have a defect in the initiation step (Zou et al., 1997). However, this mutation allele does not

show any defect in elongation probably due to leakiness of the mutant as *cdc45-1* cells complete chromosome replication at the restrictive temperature when the protein is inactivated after early origin activation. Analysis of another mutant, heat-inducible-degron *cdc45-td* cells, in which the degron specifically targets the Cdc45 for degradation by a particular ubiquitin-dependent pathway at 37°C, showed that Cdc45 plays an essential role in elongation as they cannot finish chromosome replication at the restrictive condition after early origin activation (Tercero et al., 2000). These observations suggest that Cdc45 could be a good candidate to be studied for elucidating how origin activation occurs and how elongation would be accomplished during DNA synthesis.

In *S.cerevisiae*, Dpb11 has been shown to play a pivotal role in loading DNA polymerases onto replication origins. Dpb11 was originally identified as a multicopy suppressor of the *pol2-12* and *dpb2-1* mutations, which are defective in the catalytic and the second largest subunits of DNA polymerase ε, respectively. *DPB11* is an essential gene required for DNA replication (Araki et al., 1995). The inability of *dpb11* mutants to restrain mitosis in the presence of incomplete replication suggests that Dpb11 is also needed for the replication checkpoint (Araki et al., 1995; Kamimura et al., 1998; Wang and Elledge, 1999). Genetic interactions between Dpb11, Cdc45 and the replication protein Sld2, further link Dpb11 to the replication machinery. Dpb11 has four copies of the BRCT (*Br*ca1 *C-t*erminal) domain (Bork et al., 1997; Callebaut and Mornon, 1997; Zhang et al., 1998), and forms a complex with Pol εand Sld2 (Masumoto et al., 2000;

Kamimura et al., 1998; Wang and Elledge, 1999). BRCT domains are autonomously folding modules consisting of about 100 amino acids that were first recognized as a repeat in the C-terminus of the Breast Cancer Susceptibility gene 1 (BRCA1). Recently, it has been shown that BRCT domains are capable of binding proteins that have been phosphorylated on either serine or threonine residues (Manke et al., 2003; Yu et al., 2003). Therefore, to account for a number of important complexes that form at the action involving in DNA replication/repair processes, elucidation of the molecular mechanism in the interaction between BRCT domain and its physiological targets might be helpful to elucidate the cell cycle control including S phase regulation.

The Sld2-Dpb11 complex is formed *in vivo* and is essential for chromosomal DNA replication (Kamimura et al., 1998). Sld2 has six closed matches to the preferred Cdk motif, S/T-P-X-K/R, S/T-P-K/R and K/R-S/T-P (X= any amino acid), and additional five S/T-P sites, which are clustered in 200-aa stretch (Fig. 7A; Pearson and Kemp, 1991). Our previous study showed that Sld2 is phosphorylated by S-Cdk and that this phosphorylation is necessary for the formaion of the Sld2-Dpb11 complex. Remarkably, the mutant allele of *SLD2* that replaces all the serine or threonine residues in the preferred Cdk phosphorylation motif by alanine residues is inviable and is also defective in formation of Sld2-Dpb11 complex (Masumoto et al., 2002). In *Schizosaccharomyces pombe*, Drc1 has a homology to Sld2, and mutant Drc1 that lacks CDK phosphorylation sites is nonfunctional and fails to interact with Cut5, a homolog of Dpb11 (Noguchi et al., 2002). Although these phosphorylations promote interaction with Dpb11/Cut5 *in vivo*, it

is not well understood how the complex is formed between Sld2 and Dpb11.

To understand the initiation of replication regulated by Cdks, I have studied two events in early S phase: stable association of Cdc45 with chromatin and association of Dpb11-Sld2 complex with origins. Although the association of Cdc45 with the pre-RC has been observed in even G1 phase by chromatin immuno-precipitation (ChIP) assay, its stable association to chromatin occurs only after activation of S-Cdks in G1/S transition (Aparicio et al., 1999; Zou and Stillman, 2000; Kamimura et al., 2001). In *Xenopus* egg extract loading of Cdc45 onto chromatin in S phase is the last known step before origin unwinding and the commencement of DNA synthesis (Mimura et al., 2000). These results suggest that Cdc45 is limiting factor for the initiation of replication. While Dpb11 does not associate with origin without Cdk activity*, Xenopus* Cut5/Mus101 binds to chromatin in advance of Cdc45, rather than in a mutually dependent manner, but is needed for Cdc45 and polymerase □ association steps (Hashimoto and Takisawa, 2003; Van Hatten et al., 2002).

In this study, I isolated series of thermosensitive allele for *CDC45* defective in the initiation of DNA replication. In thermosensitive alleles for *SLD2* and *DPB11*, *drc1-1* and *dpb11-26,* respectively, the chromatin binding assay revealed that the chromatin-bound Cdc45 is significantly reduced even after Cdk activation. A mutant allele, *cdc45-26*, isolated in this study, abolishes association of Sld2-Dpb11 with the early-origins, but not affect the Sld2-Dpb11 complex formation. These results suggest that S-Cdk dependent stable association of Cdc45 and association of Dpb11-Sld2 complex with the

origins are mutually dependent.

Moreover, I defined a Dpb11 binding region in Sld2 within a 39-amino acids stretch, partly overlapping with a cluster of the phosphorylation sites. The 39-aa peptide binds to C-terminal pair of BRCT domain in Dpb11 irrespective of phosphorylation. When I fused 39-aa to a cluster of phosphorylation sites the interaction between the fragment of Sld2 and Dpb11 was phosphorylation dependent. Therefore, Sld2 seems to have the binding and regulatory domains: The regulatory domain controls the binding activity by Cdk-dependent phosphorylation. This interaction mode will be discussed further.

Results

Novel thermosensitive alleles for *CDC45*

We usually employ thermosensitive alleles to analyze the function of respective genes. However, no thermosensitive alleles of *CDC45* have been available. I therefore isolated a series of thermosensitive mutants for *CDC45* for further understanding of Cdc45. To isolate high temperature sensitive mutations for *CDC45*, eight alleles that formed colonies at 25°C but not at 37°C were isolated from 3200 clones containing a plasmid with mutagenized *CDC45* by the plasmid shuffling method (see Materials and Methods), and their mutation sites in *CDC45* were determined (Table 1 and Fig. 2A). Then the endogenous *CDC45* was replaced with one of most stringent thermosensitive alleles, *cdc45-26* (Table 1). At the restrictive temperature, *cdc45-26* cells arrested with a dumbbell-shape with a single nucleus, which is a typical terminal morphology for mutants defective in chromosomal DNA replication (Fig. 2F).

To investigate whether the chromosomes were duplicated at the restrictive temperature, *cdc45-26* cells were arrested in G1 phase with the mating-type pheromone (□-factor) and then temperature was raised to 37°C to inactivate the protein. After release from G1 arrest, the cells were fixed at every 20 min and DNA contents of the cells were analyzed with a fluorescence-activated cell sorter (FACS). As shown in Figure 2B, the wild-type cells reached at 2C DNA content by 60 min while DNA content of

cdc45-26 cells did not increase to 2C DNA content even at 180 min. The delay of S phase observed in *cdc45-26* cells could be due to a defect in either initiation or elongation of DNA replication. Time course shows that nuclear division without DNA replication occurs in most *cdc45-26* cells 2-3 hr after shift to 37˚C (Fig. 2D and E).

To distinguish between the initiation and elongation steps, the cells were arrested with hydroxyurea (HU), which inhibits ribonucleotide reductase and so reduces cellular dNTP pools. Under such condition, initiation occurs at early origins but DNA replication fork then stall within a few kilobase (Santocanale and Diffley, 1998; Yabuki et al., 2002). First, cells were arrested at G1 phase with □-factor and then released into a medium containing 0.2 M HU at 25˚C. It was followed by temperature shift to 37˚C, and subsequently release from the HU block. FACS analysis showed that both the wild-type and *cdc45-26* cells rapidly finished chromosome replication after release from HU block (Fig. 2C). This means that S phase delay observed in the first G1-arrest-and-release experiment was largely rescued by allowing the cells to initiate origin firing at the permissive temperature. Other alleles also slowed a defect in the initiation but not elongation (summarized in Table 1).

Cdc45 is required for the association of Sld2 and Dpb11 with origins of replication

In order to activate origins of replication, active S-phase Cdks (S-Cdks) are thought to drive S-phase entry by regulating activity of one or more replication proteins (Kelly and Brown, 2000). One of such candidates is Sld2 that forms a complex with another DNA replication protein, Dpb11 (Kamimura et al., 1998; Masumoto et al., 2002). Dpb11 has been shown to play an essential role for the loading of DNA polymerases onto replication origins (Masumoto et al., 2000). The ChIP assay has demonstrated that Cdc45 associates with origins during G1- and early S phase that is before origin activation, and Cdc45 is also required for both Pol□ and Pol□ to associate with origins (Aparicio et al., 1999).

I therefore examined whether Cdc45 is required for association of Sld2 and Dpb11 with origins of replication. The *cdc45-26* and wild-type cells, both of which contain either Sld2-10flag or Dpb11-9myc, were arrested at G1 phase by □-factor under permissive condition and then temperature was raised to nonpermissive temperature (34˚C) to inactivate the mutant protein. The cells were released from G1 arrest into a medium containing 0.15 M HU in order to slow the movement of replication forks around origins (Fig. 3D). Cells were withdrawn from cultures every 30 minutes and fixed with formaldehyde to crosslink protein-DNA. The proteins from cell lysates were immunoprecipitated using anti-flag or anti-myc antibody. DNA was extracted from the

immunoprecipitates and analyzed by polymerase chain reaction (PCR) to determine the relative abundance of specific sequences bound to the immunocipitated flag- or myc-tagged proteins. I used PCR to detect two origins, ARS1 and ARS305, and their neighboring regions as controls (Fig 3A; Tanaka and Nasmyth, 1998). Both origins fire early during S phase (Campbell and Newlon, 1991). In wild-type cells, Sld2-10flag and Dpb11-9myc associated with ARS1 and ARS305 at 30 minutes after release from G1 arrest (Fig 3B and C; WT). However, the associations of both Sld2 and Dpb11 in *cdc45-26* mutant cells at the restrictive temperature (34˚C) were greatly reduced, and little or no bulk DNA replication was observed (Fig 3B; Sld2-10flag, 3C; Dpb11-9myc and 3D; FACS analysis). These results suggest that the Cdc45 is required for both Sld2 and Dpb11 to associate with early origins.

Binding of Cdc45 onto chromatin requires the functional Sld2 and Dpb11

It has been shown that Cdc45 binding to chromatin is detected in chromatin binding assay when S-Cdk is activated (Zou and Stillman, 2000) although Cdc45 is loaded on origins even at G1 phase in the absence of S-Cdk activity in ChIP assay (Aparicio et al., 1999). This is probably due to a difference of its binding affinity between G1 and S phase. The chromatin binding of Cdc45 has been thought as a landmark for S-Cdk

activation.

I therefore examined whether chromatin binding of Cdc45 depends on Sld2 and Dpb11 (Fig. 4A). For this purpose, I studied its chromatin binding in mutant alleles for *SLD2* and *DPB11*, *drc1-1* and *dpb11-26,* respectively. The cells were synchronized at G1 with α-factor under a permissive condition. Then temperature was raised to nonpermissive temperature at 36˚C to inactivate either Sld2 or Dpb11, and the cells were released from G1 arrest. Budding occurred in these strains almost at the same timing (Fig. 4C; see the budding index) as wild-type cells. At 60 min, the amount of Cdc45 in pellet fraction in both *drc1-1* and *dpb11-26* alleles was less than that in the wild-type cells. In the wild-type cells, Cdc45 in pellet fraction was dissociated by DNaseI treatment (Fig. 4B, P2), meaning that the protein indeed binds to chromatin DNA (Fig. 4B, upper panel). On the contrary, DNase I treatment did not reduce the amount of Cdc45 in the pellet fraction in both *drc1-1* and *dpb11-26* alleles, indicating that Cdc45 in the pellet fraction in the mutants did not associate with chromatin DNA (Fig. 4B, middle and lower panels, compare P1 with P2). Therefore, both Sld2 and Dpb11 are required for chromatin binding of Cdc45. Because the association of both Sld2 and Dpb11 with origins depends on Cdc45 (Fig 3B and C), origin binding of Cdc45, Sld2 and Dpb11 is likely to be mutually dependent.

Sld2 forms a complex with Dpb11 in the absence of functional Cdc45, Mcm5 or Cdc7/Dbf4

The Sld2-Dpb11 complex formation and chromatin association of Cdc45 require S-Cdk activity (Masumoto et al., 2002; Zou and Stillman, 2000; Aparicio et al., 1999). Therefore, I asked whether Cdc45 is required for the complex formation between Sld2 and Dpb11. To investigate it, a co-immunoprecipitation experiment was performed with the cells having both Sld2-10flag and Dpb11-9myc in the wild-type or *cdc45-26* background. The cells were arrested at G1 phase by □-factor, the temperature was raised to 34˚C, and then cells were released from G1 arrest. The wild-type cells started DNA synthesis in 30 min (S phase) and the most of them reached to a 2C DNA content (G2/M phase) at 60 min after release from G1 arrest (Fig. 5B, the left panel). The *cdc45-26* cells hardly started DNA synthesis even at 60 min (Fig. 5B, the right panel). In wild-type cells, immunoprecipitation of Sld2-10flag at each time points showed that Dpb11-9myc co-precipitated with Sld2 at 30 min (S phase) and the association was greatly reduced at 60 min (M phase), which is consistent with previous results (Fig. 5A, upper panel, lanes 8 and 9; Masumoto et al., 2002). On the other hand, in *cdc45-26* cells the Sld2-Dpb11 complex formation was observed at 30 min after G1 release and lasted even at 60 min probably because these cells did not complete DNA replication (Fig. 5A, lanes 11 and 12). These results suggest that physical interaction between Sld2 and Dpb11 is independent of functional Cdc45.

To investigate whether the interaction between Sld2 and Dpb11 is regulated by other replication factors, I examined the Sld2-Dpb11 complex formation in *mcm5-1* and *dbf4-1* cells. The Mcm2-7 complex is essential for the initiation of chromosomal DNA replication (Tye, 1999). A thermosinsitive mutant for one of these *MCM 2-7*, *mcm5-1*, exhibits growth arrest at the G1/S boundary. Cdc7 is another protein kinase that is required for G1/S transition, and for the binding of unstable regulatory subunit, Dbf4 is essential for activation (Sclafani, 2000).

A co-immunoprecipitation experiment was performed with cells having both Sld2-10flag and Dpb11-9myc in *mcm5-1* or *dbf4-1* background. The cells were arrested at G1 phase by □-factor and were released from G1 arrest at 37˚C. The phosphorylation of Sld2 was detected not only in the wild-type cells but also in the both mutants since the band corresponding Sld2 decreased mobility when these cells were released from G1 arrest (Fig. 5C, lower panel, lanes 2, 4 and 6). As shown in Figure 5C, the Sld2-Dpb11 complex was observed in *mcm5-1* and *dbf4-1* mutants, indicating that the complex formation does not depend on Mcm5 and Dbf4. These results suggest that formation of Sld2-Dpb11 complex is not affected by the single-out replication factor important for DNA replication, Cdc45, Mcm5 and Cdc7/Dbf4 kinase. It is likely that the Sld2-Dpb11 complex formation is regulated only through phosphorylation in Sld2 mediated by S-CDKs.

Mapping the interaction domains between Dpb11 and Sld2

A previous study suggested that the C-terminal portion of Dpb11 plays an important role for physical interaction with Sld2 (Kamimura et al., 1998), because thermosensitive Dpb11-1, has a nonsense mutation in C-terminal portion (Fig. 6) and a defect of the interaction at the restrictive temperature.

To determine the region of Dpb11 responsible for the interaction with Sld2, two-hybrid analysis was employed. I constructed the plasmids by fusing the various truncated Dpb11 proteins to the C terminus of the Gal4 activating-domain (Gal4AD) of pACT2 and the ORF of *SLD2* was fused to the LexA binding domain (LexABD) of pBTM116. They were introduced into L40 cells, and expression of the *lacZ* gene reporter was examined in □-galactosidase filter assay (Bartel and Fields, 1995). As a positive control, cells harboring both of Dpb11 and Sld2 proteins fused with GAL4AD and LexABD were turned blue (Fig. 6 or 7B). As shown in Figure 6, the region in Dpb11 responsible for Sld2 interaction is located in the C-terminal pair of BRCT domain, whereas the N-terminal pair of BRCT repeats [1-309 aa] did not interact with Sld2. Therefore the C terminal pair of BRCT repeats appeared to have the region critical for Sld2 binding.

To determine the region of Sld2 required for its association with Dpb11, a yeast two-hybrid assay was also employed using various truncated forms of Sld2 fused to LexABD. Figure 7B shows that N-terminal 39 amino acid of Sld2 [79-117 aa, S9], which encompassed a part of the putative phosphorylation consensus region was required for

the interaction with Dpb11. S9 [79-117 aa] showed a stronger signal than the wild-type Sld2 whereas S9 itself gave a weak signal (Fig. 7B). Thus, it is difficult to compare the affinity of S9 with that of the wild-type Sld2.

Previous study has shown that the phosphorylation of Sld2 enhanced its interaction with Dpb11 (Masumoto et al., 2002). I thus investigated whether the two-phosphorylation sites (Thr84 and Ser100) in S9 affect its interaction with Dpb11. To examine this, I have constructed alanine substituted mutations, LexA-S9-1A, in which Ser^{100} was replaced by Ala, LexA-S9-2A, which both of Thr^{84} and Ser^{100} were replaced by Ala. As shown in Figure 7C, both of these mutant proteins interact with Dpb11. Thus the phosphorylation of this region is not essential for binding with Dpb11 (Fig. 7C, S9-1A and –2A).

The stretch from residue 79 to 263 of Sld2 also interacted with Dpb11 (Fig. 7B, S8). This region contains 11 phosphorylation sites of Sld2. I considered the possibility that the mutation of Cdk sites, which causes little co-immunoprecipitation with Dpb11 (Masumoto et al., 2002), might affect interaction with Dpb11. To examine this, I constructed phosphorylation-defective mutants, LexA-S4-6A, which have Ala substitution at Ser^{100} (S100A), Ser^{128} (S128A), Thr^{167} (T167A), Ser^{172} (S172A), Ser^{208} (S208A) and Thr^{241} (T241A). The mutation of six-phosphorylation sites, LexA-S4-6A, dramatically reduced Dpb11 interaction (see S4-6A in Fig. 7C). This result suggests that binding of S4 with Dpb11 is dependent on its phosphorylation of Sld2.

As the fragment became shorter than S8, the residues from 1 to 140 and the residues

from 1 to 228 (S2 and S3), the signal for Dpb11 binding diminished. Since the expression levels of all the truncated Sld2 proteins were alternative same (data not shown), the entire structure of S8, containing multiple phosphorylation sites, may also be required for the interaction with Dpb11. As expected, no interaction was observed with N-terminal truncated form of Sld2, residues 142 to 453 (S6) and residues 198 to 453 (S7), without N-terminal 39 amino acids.

Taken together, these results indicate that the N-terminal 39 amino acid of Sld2 (S9) and the stretch from 79 to 263 (S8) interacts with Dpb11. Since the non-phosphorylated form of S9 fragment was capable of interacting with Dpb11, the 39-amino acid region seems to constitute a binding domain of Sld2. Moreover, S8 fragment containing phosphorylation consensus region interacted with Dpb11 in a phosphorylation-dependent manner. Therefore, a cluster of phosphorylation sites seems to regulate the binding domain for its affinity to Sld2 as a regulatory domain.

Sld2 and Dpb11 directly bind each other *in vitro*

In vivo experiments showed that Dpb11-C terminal half interacts with Sld2 in a phosphorylation-dependent manner. However, these experiments cannot exclude the possibility that other factors regulated the complex formation between Dpb11 and Sld2.

To confirm the interaction detected in two-hybrid system, an *in vitro* binding assay

was performed. Considering practical difficulties of purifying the full-length recombinant Sld2 and Dpb11, truncated Sld2 and Dpb11 based on the results from yeast two-hybrid assay were purified and used for the *in vitro* assay. I used the histidine-tagged C-terminal Dpb11 [291-636 aa] and Sld2-S8 [79-263 aa] or Sld2-S9 [79-117 aa] fused to GST, respectively. These proteins were purified from *Escherichia coli* using affinity resins (see Materials and Methods) and the amount of purified protein was estimated on SDS-PAGE with CBB staining (Fig. 8B). In the *in vitro* binding assay, GST-Sld2-S8 or GST-Sld2-S9 was first bound to glutathione sepharose beads and then approximately the same amount of His-Dpb11 was added. After washing the beads, proteins bound the beads were dissociated by boiling with SDS-loading buffer. As shown in Figure 8A, His-Dpb11 bound to both GST-Sld2-S8 and GST-Sld2-S9 but not GST, indicating that Sld2 directly and specifically binds to Dpb11 *in vitro* (lanes 1, 2 and 4, upper panel).

Next I examined whether phosphorylation of Sld2 affects the interaction between the two proteins. Since different specificity of phosphorylation sites of Cdk1 and 2 with various cyclins has not been reported so far, I employed human Cdc2 (Cdk1)-cyclin B. Cdc2-cyclin B kinase was added to GST-Sld2-S8 and GST-Sld2-S9 prior to the binding assay (see Materials and Methods). Phosphorylation of GST-Sld2-S9 was detected by anti-phospho-Ser^{100} (Fig. 8A lower panel, lane 3) and phosphorylation of GST-Sld2-S8 was detected from mobility shift on SDS-10% polyacryamide gel (Fig. 8A lower panel, lane 5). These phosphorylated Sld2 (GST-S9, 0.52 □M; GST-S8, 0.68 □M) proteins were immobilized on glutathione sepharose beads and again those were incubated with

His6-Dpb11 (0.4 □M) protein and then the bound protein fractions were analyzed by western blotting using anti-His antibody. His-Dpb11 fragment bound both of phosphorylated GST-Sld2-S9 and unphosphorylated GST-Sld2-S9 (1% of input His-Dpb11 fragment; Fig. 8A.,upper left panel, compare lanes 2 and 3). On the other hand, His-Dpb11 efficiently bound to phosphorylated GST-Sld2-S8 fragment (8% of input His-Dpb11 fragment), in contrast, 1.3 % of input His-Dpb11 recovered in intact GST-Sld2-S8 fragment (Fig. 8A, compare lanes 4 and 5). This result indicates that Sld2 can bind directly to Dpb11 and phosphorylation enhances the efficiency of the complex formation between Sld2 and Dpb11. Each experiment was repeated at least three times and the same results were obtained.

Our previous *in vivo* study showed that Sld2 only binds to Dpb11 when Sld2 is phospohrylated by S-Cdk (Fig. 5A, WT; Masumoto et al., 2002). However, *in vitro* binding assay showed that S8 fragment directly binds to Dpb11 although the efficiency of complex formation is low. These observations are compromised if I assume that cellular concentration of Sld2 and Dpb11 protein is not high enough to form a complex *in vivo* without phosphorylation. This assumption predicts that Sld2 and Dpb11 form a complex *in vivo* without phosphorylation when these proteins are overproduced. I therefore examined whether the overexpressed sld2 form a complex with overexpressed Dpb11 in the absence of S-Cdk activity. Cells having plasmids with one of wild-type, non (6A)- or phosphomimetic (6E) Sld2-10flag under control of the GAL1/10 promoter were grown to mid-log phase in a raffinose-containing medium and then transferred to a

fresh medium containing galactose and □-factor. Under this condition both Sld2 and Dpb11 were overexpressed and cells were blocked in G1 phase with low Cdk activity. Co-immunoprecipitation experiment showed that overexpressed Sld2-10flag binds to Dpb11-9myc in G1 cells (Fig. 8C, lane 8) as expected. Moreover, this interaction was also observed with 6A-Sld2-10flag (Fig 8C, lane 10) as well as phosphomimetic 6E-Sld2-10flag (Fig 8C, lane 9). Therefore, phosphorylation seems to regulate the affinity between Sld2 and Dpb11 even *in vivo*.

Next, I further examined whether Sld2-Dpb11 complex induces DNA synthesis in the absence of S-Cdk activity. To inhibit Cdc28 activity, non-degradable form of Sic1 (*SIC1□NT*) was used because Cdc28 activity is inhibited by new degradation of Sic1 (Noton and Diffley, 2000). Cells were arrested in G1 in galactose-containing medium for 1 hr to induced *GAL*-Sic1□NT and then transferred to fresh galactose-containing medium to repress Cdc28 activity (Fig. 8D, see the experimental scheme). FACS analysis showed that the cells released from G1 block were not competent for the bulk DNA synthesis of chromosomal DNA replication (Fig. 8D).

Discussion

Interaction sites between Sld2 and Dpb11

(1) A tandem BRCT repeat of Dpb11 interacts with a specific region of Sld2

The BRCT domain has been shown to mediate protein-protein interactions (Zhang et al. 1998; Aravind 1999). For example, the interaction between BRCT domains are reported for human proteins XRCC (X-ray repair cross-complementing) 4 and DNA ligase IV (Critchlow et al., 1997; Grawunder et al., 1997), XRCC1and DNA ligase III (Nash et al., 1997), and XRCC1 and poly (ADP-ribose)-polymerase (PARP) (Masson et al., 1998). BRCT containing proteins also interact specifically with other proteins lacking this domain. For example, the 53BP1, the C-terminal region of which contains two tandem BRCT domains, binds specifically with not only TopBP1, which contains eight BRCT domains and interacts with DNA topoisomerase II (Yamane et al., 1997), but also p53 (Joo et al., 2002). Binding of Dpb11 to Sld2 is similar to that of 53BP1 to p53 since C-terminal pair of BRCT domains binds to Sld2. This is also true for Cut5 except the N-terminal tandem repeat of BRCT has been reported to be sufficient for interaction with Drc1/Sld2 while C-terminal pair of BRCT was not examined for its interaction with Drc1 (Noguchi et al., 2002).

The suppression of *dpb11* mutations by a high copy number of *SLD2* is also consistent with the interaction between the C-terminal BRCT repeats and Sld2. The *dpb11-1*

mutation occurred at C-terminus reduces the affinity of Dpb11 to Sld2 (Kamimura et al., 1998), predicting that the increased copy of either Dpb11-1 or Sld2 might restore the complex formation between Dpb11 and Sld2. Actually, high-copy *SLD2* suppresses the *dpb11-1* mutation, whereas high copy *SLD2* fails to suppress the *dpb11-26* mutation occurring at N-terminal half (Muramatu and Araki, personal communication).

(2) Domain of Sld2 for binding to Dpb11

The binding domain of Sld2 with Dpb11 is delimited to the 39-amino acids (S9 fragment). This region is highly conserved in not only *Saccharomyces* species but also other yeast species (Fig. 9; Kellis et al., 2003; Cliften et al., 2003), suggesting importance of this region. Interestingly, several mutations occurring in the 39-aa stretch were isolated during the screening for *sld* mutations (Fig. 7A). These mutations probably reduce the efficiency of Sld2-Dpb11 complex formation, supporting the 39-aa stretch for binding domain.

The S9 fragment contains two putative Cdk-dependent phosphorylation sites. Since non-phosphorylated form of S9 showed the interaction with Dpb11, the phosphorylation of S9 seems not to be essential for binding to Dpb11 (Fig. 7C). This is also strengthened by the *sld2* mutations. The *sld2-3*, *-4* and *-6* mutations occurred in Cdk-phosphorylation motifs of S9, PTP^{85}Q (P85S in *sld2-6*) and S^{100}P^{101}IK (S100L in *sld2-4* and P101L in *sld2-3*). Although these mutations are synthetically lethal with *dpb11-1* and seems to reduce the affinity of Sld2 to Dpb11, these mutations in wild-type genetic background do not cause lethal defect at permissive conditions, indicating that these

mutant Sld2 proteins keep the enough affinity to Dpb11. This evidence again suggests that phosphorylation of S9 is not essential for binding to Dpb11 as already mentioned above.

Recently, Yu *et al*. and Manke *et al*. reported that the BRCT domains are phospho-peptide binding motifs that are able to distinguish between the phosphorylated and non-phosphorylated peptides (Yu et al., 2003; Manke et al., 2003). This is not the case for Sld2-Dpb11 binding, because the binding domain (the 39-aa stretch, S9) of Sld2 does not require its phosphorylation to bind Dpb11. Manke *et al*. discovered that some BRCT domain (53BP1, Rad9, Mdc1) fails to recognize the ATR/ATM specific phosphopeptide library. This observation demonstrates that not all known interactions mediated by BRCT domains are regulated by phosphorylation as observed in Sld2 binding to Dpb11. In the case of Sld2, however, phosphorylation of the portion other than binding domain regulates its binding to Dpb11, which may be caused by the novel mechanism described below.

Complex formation between Sld2 and Dpb11

Ghaemmaghami et al. estimated copy numbers of yeast proteins per cell in a log-phase random culture using tagged version of each protein. According to this estimation, the levels of of Dpb11 and Sld2 are 5.40×10^2 ($\pm 5.54 \times 10^1$) and 6.56×10^2 protein molecules/cell, respectively. These proteins are localized in the nucleus that is a

round-lobate organelle, some 1.5 μm in a diameter. Thus, the estimated concentration of Sld2 and Dpb11 in a nucleus is approximately 0.7 μM. Since I used 0.4 μM of His-Dpb11 and 0.68 μM of GST-S8 for *in vitro* binding assay, the protein concentrations *in vitro* mimic the *in vivo* situation very well, suggesting that we can discuss the *in vivo* reaction mechanism from the analysis of the *in vitro* reaction.

The Sld2-Dpb11 complex was recovered poorly *in vitro* (8% in phosphorylated S8 and 1% in unphosphorylated S8 and S9) as well as *in vivo* (ca 10%). This is probably because complex formation between Sld2 and Dpb11 is rapidly dissociated. The preliminary results obtained from real-time kinetic analysis using Biacore system showed that K_D for unphosphorylated S8 to Dpb11 is 5.69×10^{-5} M (I have not obtained reliable value for phosphorylated S8 due to its non-specific binding to a sensor chip). This K_D well explains 1% level of *in vitro* Sld2-Dpb11 complex formation in sub-micromolar concentration.

In contrast with *in vitro* study, *in vivo study* do not show the Sld2-Dpb11 complex formation in G1 phase (low Cdk activity). The Sld2 and Dpb11 protein-levels do not fluctuate drastically throughout the cell cycle when they are tagged with myc-epitope (Masumoto et al., 2002). Although I do not know the exact reason for this discrepancy, it is easy to speculate that Dpb11 in G1 phase form a complex with other partner(s) or it is dimerized.

In the wild-type cells without any epitope tag, the protein level of intact Dpb11 is apparently constant whereas that of Sld2 fluctuates and peaks at S phase immediate

after the transcript level of *SLD2* peaks. Consequently, the protein level of Sld2 in G1 phase is 1/6 of that in S phase (Muramatsu and Araki, personal communication). Therefore, the complex formation is regulated *in vivo* by not only phosphorylation but also protein concentration.

The phosphorylation of S8 enhances the complex formation *in vitro*. Since the levels of complex formation with a unphosphorylated S8 and S9 are similar, phosphorylation of a cluster of Cdk sites may stabilize the complex. However, the recovery of the complex is still low (8%). This recovery can be estimated from one order lower value (sub-ten micromolar level) of K_D obtained from unphosphorylated form. In the case of the complex formation between BRCA1 BRCT domains and phospho-BACH1, K_D is 68 nM (Yu et al., 2003), 100-fold lower than phospho-Sld2. Thus, mode of the Sld2-Dpb11 complex formation differs from the complex formation between phosphopeptides and BRCT domains. This is because that the BRCT domains of Dpb11 do not bind directly to the phosphopeptide, and the binding domain (39-aa stretch, S9) and regulatory domain (a cluster of phosphorylation sites) are separable.

I therefore propose a following model for the complex formation between Sld2 and Dpb11. The binding domain (S9) interacts with Dpb11 in a phosphrylation-independent manner (Fig. 10A). When the regulatory domain is phosphorylated, the complex is stabilized probably by the conformation change of Sld2 (Fig. 10B).

Initiation of DNA replication regulated by Sld2

It has been shown that many replication proteins are phosphorylated (Ubersax et al., 2003) by Cdk although it is still unclear how phosphorylation is involved in this process. After activation of S-Cdks, phosphorylated Sld2 forms a complex with Dpb11. Once the Sld2-Dpb11 complex is formed at boundary of G1-S phase, a large replication complex, containing Sld2-Dpb11, Cdc45-Sld3, GINS and Pol ε, may be formed at the origins. How the Sld2-Dpb11 complex formation controls the assembly of other proteins on replication origins is intriguing. In addition to Sld2, Dpb11 interacts with Dpb2, Sld3, and a Psf1 subunit of GINS complex in the two-hybrid assay (Takayama, Y., Kamimura, Y. and Araki, H., personal communication). Thus, Dpb11 seems to function as a scaffold for those proteins. That is, Sld2 may change the conformation of Dpb11 to associate with other replication proteins, such as Sld3, Dpb2 and GINS.

Materials and Methods

Strains

Yeast strains used in this study are listed in Table2. *E.coli* DH5□ was used for cloning and *E.coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene) was used for the expression of his-tagged Dpb11 and truncated Sld2-GST fusion proteins.

Isolation of thermosensitive *cdc45* alleles

The diploid strain containing the disrupted *CDC45* gene (*cdc45□::LEU2*) was transformed with YEp195*CDC45*, and the resultant Ura^+ transformants were sporulated and dissected. One Ura^+ Leu^+ segregant, YS4, was used for further study. The *CDC45* was amplified using PCR with *Taq* polymerase, and was used for transformation of YS4 with *Xba*I- and *Hin*dIII- cleaved YCplac22. Approximately 3200 transformants grown at 25˚C on Ura^- Trp^- plates were replica-plated to 5-FOA plate and grown at 25˚C. Subsequently, these 5-FOA plates were replica-plated to one set of YPD plates and incubated at 25 and 37˚C, respectively. Eight clones showed temperature-sensitive growth. From these thermosensitive clones, plasmid DNAs were recovered and then DNA sequence of *CDC45* was determined. A 2.2 kb *Bam*HI-*Hin*dIII fragment of the plasmids were transferred to YIplac211, which was used for transformation of W303-1A after digestion with *Bgl*II. Ura^+ transformants were grown at 25˚C, spread onto 5FOA plates and temperature-sensitive colonies were selected (Table 1).

Measurement of DNA content

The DNA concentration was measured as described previously (Kamumura et al., 1998). Yeast cells were fixed in 70% ethanol and then washed with 50 mM Na-citrate (pH 7.5). The $4X10^6$ cells were suspended in 0.5 ml buffer containing 250 □g RNase A at 50˚C for 1 hr. Proteinase K was added to 1 mg/ml and incubated at 50˚C for 1 hr. They were added 0.5 ml of 50 mM Na-citrate containing 8 □g of propidium iodide per ml and sonicated. Stained cells were analyzed with FACscan (Becton-Dickinson).

Chromatin immunoprecipitation assay (ChIP assay)

The ChIP assay used in this study was performed as described (Kamimura et al., 2001), except that lysis buffer contains 50 mM HEPES-KOH [pH7.5], 140 mM NaCl, 1 mM EDTA, 1% triton-X-100, 0.1% Na-deoxychloate, 1 mM PMSF, 20 mM □-glycerophosphate, 50 mM NaF, 0.1 mM Na_3VO_4 and 0.5 mM NaPPi. This chromatin-containing suspension was sonicated to yield an average DNA size of 500 bp (range, 100 to 1000), clearified by centrifugation, and subjected to immunoprecipitation with anti-flag (M2) or anti-myc (9E10) conjugated to Dynabeads proteinA (DYNAL). PCR was carried out in 50 □l containing 1/5 of the anti-flag or anti-myc antibody immunoprecipitates, respectively, or 1/500 of the cross-linked DNA samples derived from the whole cell extracts. *Taq* polymerase and the corresponding buffer system were used. The PCR products were separated in a 2.3% agarose gel and stained with ethidium bromide. Seqences of PCR primers used are summarized in Table 3.

Chromatin-binding assay

Chromatin binding assay was performed as described by Takayama et al. (2003). Cell extracts were incubated for 15 min on ice with or without 0.46 U/μl of DNaseI (Worthington Biochemical Coporation). Lysates were underlayered with 50% volumn of 30% sucrose (volumn refers to the volumn of spheroplast suspension), and spun at 14000 rpm for 15 min at 4˚C.

Immunoprecipitation assay

Cells (3×10^8) were harvested by centrifugation and resuspended in 5 ml of 0.1 M PIPES-KOH [pH9.4] containing 10 mM DTT. After incubation for 10 minutes at 25˚C, pellets were resuspended with 2.5 ml of YPD medium containing 0.6 M sorbitol, 10 mM DTT and 25 μl of Lyticase (Sigma) and incubated for 15 minutes at 25˚C. Harvested cells were washed with lysis buffer [20 mM PIPES-KOH buffer at pH 6.8, 400 mM sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM □-glycerophosphate, 50 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM NaPPi, 1 mM PMSF, 1× Complete (Roche Diagnostics) and 1% protease inhibitor cocktail (Sigma)] for three times and resuspended with 0.6 ml lysis buffer containing 1 % triton-X-100. Dyna beads protein A pre-coated with 1 μg of anti-Flag M2 antibody (Sigma) were mixed with cell lysate and rotated for 1 hr. The immune complex was recovered after the beads were washed for 30 min three times with 1 ml of cold lysis buffer. The precipitated samples were boiled for 5 min in the presence of sodium dodecyl sulfate (SDS) and DTT

and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to Immobilon P filters (Millipore) by electroblotting. The filters were blocked in 5% skim milk in TBS (10 mM Tris-HCl [pH8.0], 50 mM NaCl, 0.05% Tween 20) for 1 hr, probed with primary antibody for 1 hr, and then incubated with alkaline phosphatase-conjugated secondary antibodies for 1hr. Filters were washed with TBS after both the primary and secondary antibody incubations. Detection was done with an ECL western blotting detection reagent (Amersham pharmacia).

Two-hybrid assay

Yeast two-hybrid analysis was performed as described by S. Field (Bartel and Field, 1995). Basically, truncated Sld2 were cloned into pBTM116, a LexA binding domain vector (LexA-BD). PCR-amplified DNA from the DPB11 or truncated DPB11 was cloned into pACT2, a Gal4 activation domain (Gal4-AD). Primers used for cloning are summarized in Table3.

Plasmids were introduced into yeast strain L40, and the transformants were selected on medium lacking tryptophan and leucine. Colonies thus isolated were spot onto medium lacking the same amino acids. When grown, cells were replicated to filter paper (Whatman no. 50). The filter paper was frozen in liquid nitrogen and color was developed with Z buffer (10 mM KCl, 1 mM $MgSO_4$, Na-PO_4 pH7.0) containing X-gal and □-mercaptoehanol.

Plasmid construction and mutagensis

Site-directed mutagenesis was performed as described (Sawano et al., 2000). The anti-sense primers for a various substitution of Sld2 are summarized Table 3. They were phosphorylated at their 5' end with T4 polynucleotide kinase. PCR was carried out in a 50 □l mixture using 50 ng template plasmid DNA [pBlueScript*SLD2*], 14 pmol of each primer, 10 nmol of dNTPs, 2.5 U of cloned *Pfu* DNA polymerase (Stratagene) in 0.5 X *Pfu* polymerase reaction buffer and 20 U of *Taq* DNA ligase (New England Biolabs) in 0.5 X *Taq* DNA ligase buffer containing 50 nmol of NAD. The terminal cycler was programmed as follows: pre-incubation at 65˚C for 5 min allowing the ligase to repair any nick in the template; initial denaturation at 95˚C for 2 min; 18 cycles at 95˚C for 30 s, 55˚C for 30 s, 65˚C for 7 min; post-incubation at 75˚C for 7 min. The time at 65˚C was relatively long, so that the extended primers could be fully ligated. One microliter (20 U) of *Dpn*I was added to the sample (50 □l), and incubated at 37˚C for 1 hr. Then the sample (51 □l) was subjected to denaturation at 95˚C for 30 s, followed by 2 cycles at 95˚C for 30 s, 55˚C for 1 min and 70˚C for 7 min. Five microliters of the final sample was used to transform competent *E.coli* cells [DH5□].

Construction, Expression and Purification of fusion proteins

A DNA fragment encoding the residues 79 to 117 or 79 to 263 of *SLD2* were amplified by PCR and cloned into the pGEX6P-1 vector via *Eco*RI and *Sal*I restriction sites. The PCR amplified sequence of this construction was verified by DNA sequence analysis.

The His-tagged fusion protein containing the C-terminus BRCT domain (residues 291 to 636) of DPB11 was cloned into pET28C vector via *Bam*HI and *Xho*I restriction sites. The GST- and His- fusion proteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL by induction with 100 nM isopropyl-□-D-thiogalactopyranoside (IPTG) at 25˚C for 2 hr. The cells were harvested and suspended in lysis buffer (50 mM Tris-Cl (pH7.5), 100 mM NaCl and 1 mM PMSF) and then frozen with liquid Nitrogen. After thawing, cell pellet was resuspended in lysis buffer containing 5 mM DTT, 0.1% triton-X-100, 1 mM PMSF and 0.1 mg/ml lysozyme and incubated at 30˚C for 15 min. The lysed cells were sonicated on ice and centrifuged at 15000 X g for 30 min. Each supernatant was loaded onto ~1 ml to Glutathione S-transferase (GST)-sepharose (Amersham Pharmacia) and washed with buffer150 (lysis buffer containing 150 mM NaCl). The fusion proteins were eluted with 20 mM reduced glutathionine in buffer150. His-tagged recombinant protein was purified by Ni-NTA (Quiagen) resin and eluted with 250 mM immidazole. Eluted proteins dialyzed against a solution containing 50 mM Tris-Cl (pH7.5), 100 mM NaCl, 0.1% triton-X-100 and 20% Glycerol and were stored at –80˚C.

In vitro phosphorylation and *in vitro* binding assay

(i) *In vitro* phosphorylation: A Cdc2 (NewEngland Biolabs) was used for the phosphorylation of recombinant GST-Sld2 proteins. Various amount of GST-Sld2 by measuring CBB staining were incubated with 1 □l (20 units) of Cdc2 kinase for 1 hr at 30˚C. Phosphorylation of GST fusion proteins was analyzed by SDS-PAGE.

Phosphorylation of GST-Sld2-S9 was detected using rabbit anti-Phospho-Ser100 polyclonal antibody at 1:1000 dilution. Phosphorylation of GST-Sld2-S8 was observed band shift on 10% SDS-PAGE by CBB staining or immunoblotting by anti-GST monoclonal antibody.

(ii) *In vitro* binding assay: The purified recombinant His-Dpb11 and GST-fusion proteins were used for an *in vitro* binding assay. Various amount of GST-S8 (0.68 □M) or GST-S9 (0.52 □M) in 0.5 ml reaction buffer (50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 5 mM DTT, 0.5% triton-X-100, 15% glycerol, 1 mM PMSF and phosphatase inhibitors) were bound to glutathione-agarose beads (20 □l bed volumes) for 1 hr at 4˚C. The beads were washed and then added to His-Dpb11 (0.4 □M) in 0.5 ml reaction buffer for 2 hr at 4˚C. The protein mixture of His-Dpb11 and GST-Sld2 proteins were washed three times by reaction buffer containing 150 mM NaCl, and then boiled with 20 □l of SDS-loading buffer. 1:20 dilution of bound complex was loaded on 10% SDS-PAGE and analyzed by immunoblotting using anti-His monoclonal antibody.

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Tables and Figures

Table 1. Mutation sites of the thermosensitive *cdc45* alleles

CDC45 alleles	Amino acid	from	to	Restrictive temperature (˚C)	Terminal phenotype
cdc45-21	19	Ser	Pro	35.5	dumbbell
	408	Gly	Arg		
cdc45-25	131	Leu	Pro	35.5	dumbbell
cdc45-26	481	Trp	Arg	33	dumbbell
cdc45-27	131	Leu	Pro	34	dumbbell
cdc45-28	124	Asp	Asn	35.5	dumbbell
cdc45-29	87	Gly	Glu	35.5	dumbbell
cdc45-30	127	Arg	Gly	35.5	dumbbell
cdc45-35	242	Leu	Pro	35.5	dumbbell

Table 2. *S.cerevisiae* strains used in this study

Strain	Genotype	Source
W303-1A	*MAT**a** ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1*	This lab.
W303-1Ab	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 Δbar1*	Y.Kamimura
L40	*MAT***a** *his3-Δ200 trp1-901 leu2-3112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ*	This lab.
YYK3	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 sld2Δ::Leu2[YCpSLD2]*	Y.Kamimura
HMS55	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 Δbar1 dpb11::DPB11-9myc::URA3 sld2::SLD2-10FLAG::LEU2*	H.Masumoto
YS132	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 Δbar1 cdc45-26 dpb11::DPB11-9myc::URA3 sld2::SLD2-10FLAG::LEU2*	This study
YS136	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-11,15 can1-100 Dbar1 dbf4-1 dpb11::DPB11-9myc::URA3 sld2::SLD2-10FLAG::LEU2*	This study
YS138	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-11,15 can1-100 Dbar1 mcm5-1 dpb11::DPB11-9myc::URA3 sld2::SLD2-10FLAG::LEU2*	This study
YS140	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 ura3::GAL1-SIC1ΔNT::URA3, Δbar1::G418, trp1::GAL10-SLD2-10flag::TRP1*	This study
YS23	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 Δbar1 cdc45-26*	This study
YS4	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1 Δbar1 Δcdc45::LEU2[YEp195CDC45]*	This study
YS93	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 ura3-1 Δbar1 trp1::CDC45-3HA::TRP1*	Y.Kamimura
YS94	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 ura3-1 Δbar1 drc1-1 trp1::CDC45-3HA::TRP1*	This study
YS95	*MAT***a** *ade2-1 can1-100 his3-1115 leu2-3112 ura3-1 Δbar1 dpb11-26 trp1::CDC45-3HA::TRP1*	This study

Table 3. Primers used

primer	Insertion plasmid	Sequence
SLD2-F	pBTM116	5'-CTAGTGAGGCGAATTCTGTAGT
SLD2-R	pBTM116	5'- ACTTAATAGGTGTCGACAAATTACA
SLD2-77F	pBTM116	5'-CCACGACGAATTCGTAGAGATA
SLD2-139F	pBTM116	5'-TCGCCTGAATTCCGTACGTTA
SLD2-195F	pBTM116	5'-CACTAGAATTCGGCAAACCATC
SLD2-268F	pBTM116	5'-AATCCCTGATAGAATTCGCCAG
SLD2-114R	pBTM116	5'-GTCTTGGAGTCGACATTGTTAAC
SLD2-227R	pBTM116	5'-TGAAGTCGACGTTTAGTGCTTG
SLD2-260R	pBTM116	5'-TATCAGGGATGTCGACAGTGGTCT
SLD2-340R	pBTM116	5'-GTCTCTTTCTTGTCGACGTATC
DPB11-288F	pACT2	5'- GATATGGATCCAGGCAATGTCTCTACACC
DPB11-390F	pACT2	5'-TTATAGATGATAATGATGGGATCCTTACAG
DPB11-R	pACT2	5'- CGTGTCGACTTATTGCGATCC
DPB11-636R	pACT2	5'- CGTATCTTTCCTCGAGTGCTCCGATAATG
DPB11-681R	pACT2	5'-GGCAGTGACTCGAGCTTGATCCTTTTCG
DPB11-R1	pACT2	5'-CTAGGGCAGTCGACATTATCG
SLD2-Kpn1R	pGEX6P-1	5'-GAGGCAGATCGGTACCCAGTCACGATG
SLD2-Mun1F	pGEX6P-1	5'-CACAGGAAACACAATTGATGTCCCCTATAC
SLD2-S100E	(mutagenesis)	5'- ACATGAATTTGGAGCCTATAAAGCC
SLD2-S128E		5'-CAATTTCTAATGAATCTGAGCCACG
SLD2-S172E		5'-ACGTGAACCGACTTCAGAACCATGC
SLD2-S208E		5'-CCTAATGAACCGCTAAAATTGGATG
SLD2-T168A&S172E		5'-ACGTGCTCCGACTTCAGAACCATGC
SLD2-T84A		5'-GTAGAGATAGGCCCTGCTCCCCAAGTTTAC
ARS1-458.5	(ChIP assay)	5'- AAGCGCCCCTGATTGACAAG
		5'- GCTGGAGGAATTCGAGAATG
ARS1-462.5		5'- TGGTGTTGATGTAAGCGGAG
		5'- AAAGTCAACCCCCTGCGATG

Table 3. (continue)		**Sequence**
ARS1-466.5	(ChIP assay)	5’- AGTGCCCTAGAAGGTGCTAC
		5’- GGAAACACCACCGGCAAACT
ARS305-30.5		5’-TGCAAACAGTATTCCGGCAC
		5’-ACACGATCCACGCTGTCCCA
ARS305-39.5		5’- TTTCAGAGCCTTCTTTGGAG
		5’-CAAACTCCGTTTTTAGCC CC
ARS305-47.5		5’-GAAGATGCTAAGAAATGCAG
		5’-AGTTGAGGCGCAGAATCCCA
ARS305-56.5		5’-TTCGCTAGAAAATGAGTAAG
		5’-CAGCTATGAAAGATGCGTAG

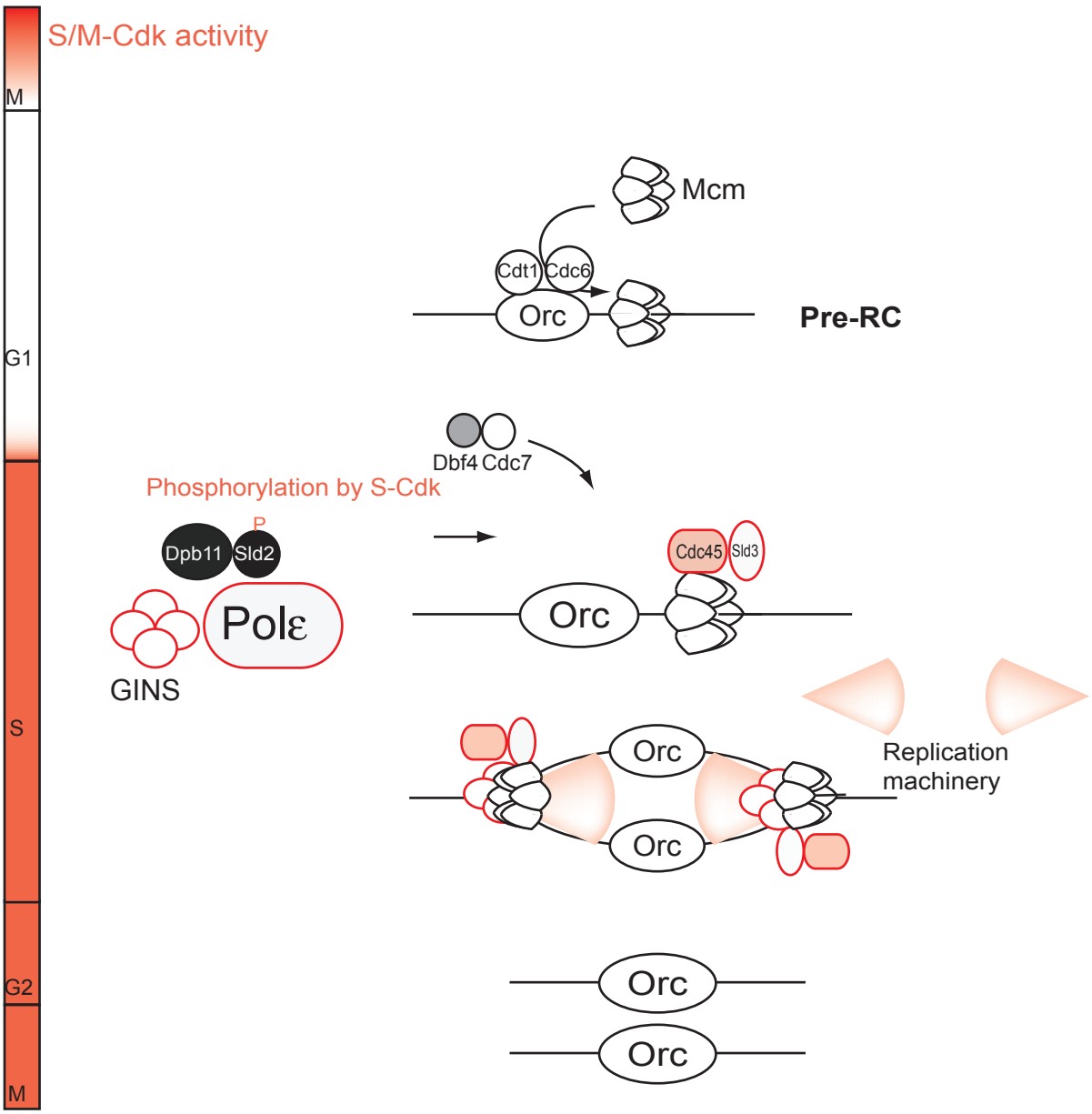
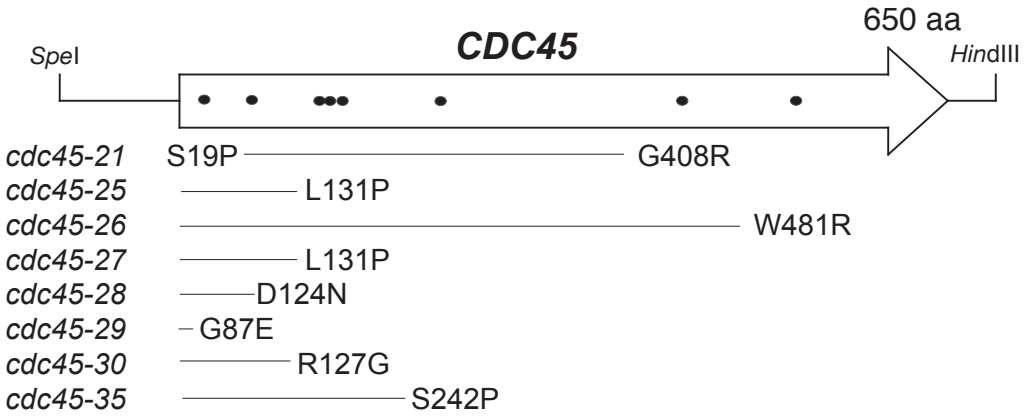


Figure 1. Chromosomal DNA replication in *S.cerevisiae*.
At the end of M phase and during G1 phase, ORC, Cdc6, Cdt1, and MCM proteins are sequentially assembled onto replication origins, leading to the formation of prereplicative complex (pre-RC). Through the action of two kinases, S-CDK and Cdc7/Dbf4, subsequent loading of the initiation factors, Cdc45-Sld3, Sld2-Dpb11, GINS complex and polymerases (replication machinery) occurs.

A.


650 aa
CDC45
SpeI
HindIII
cdc45-21 S19P G408R
cdc45-25 L131P
cdc45-26 W481R
cdc45-27 L131P
cdc45-28 D124N
cdc45-29 G87E
cdc45-30 R127G
cdc45-35 S242P

B.

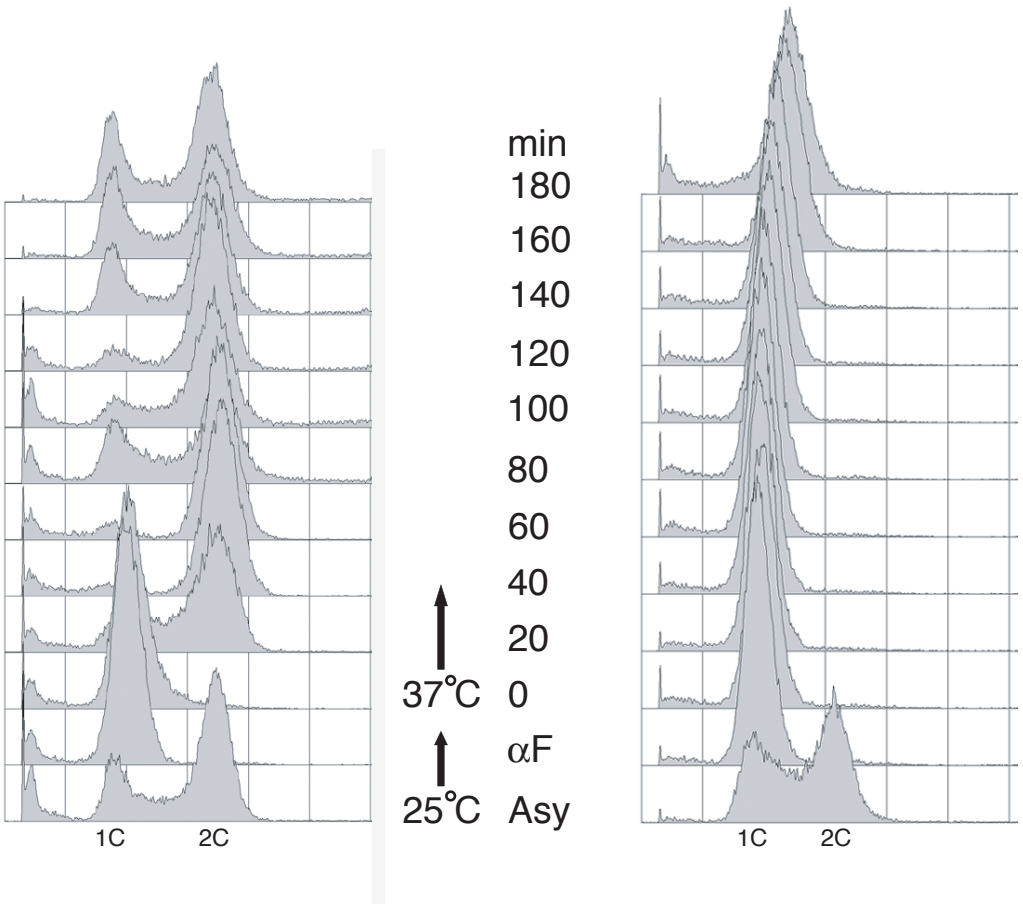

WT
cdc45-26
min
180
160
140
120
100
80
60
40
20
37℃ 0
αF
25℃ Asy
1C 2C
1C 2C

Fig. 2-1

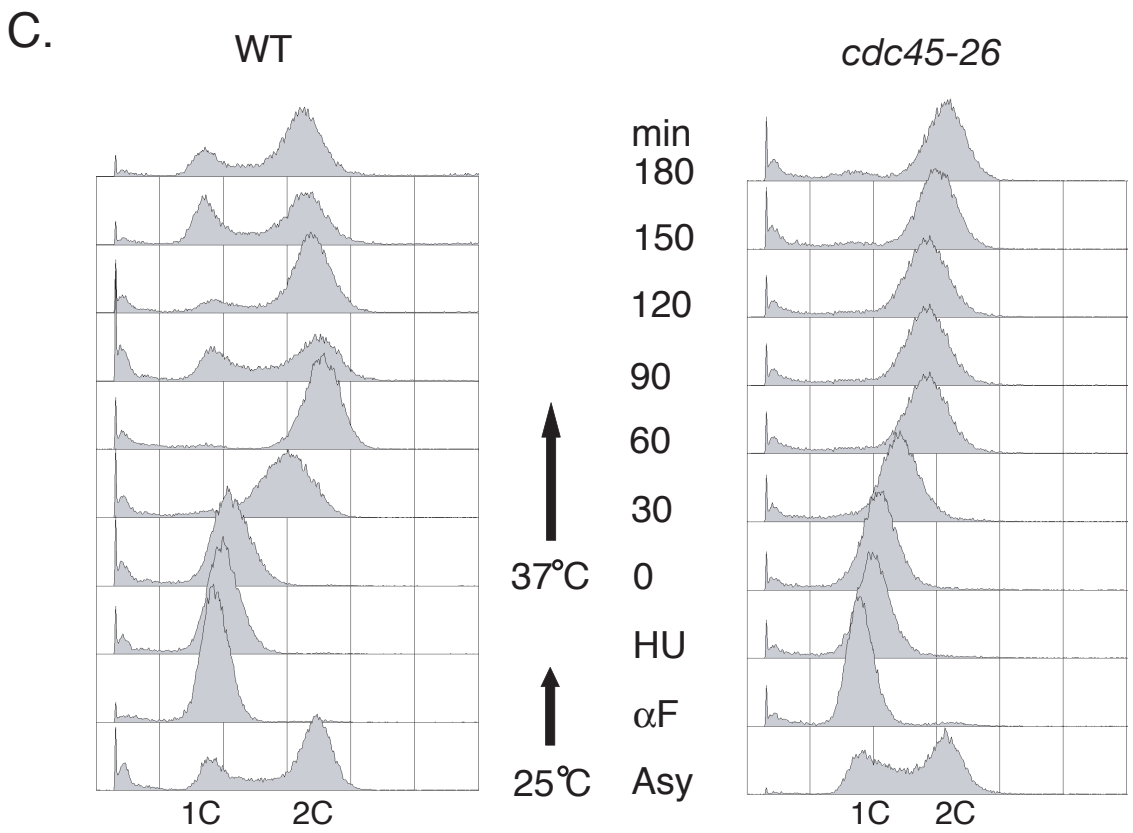
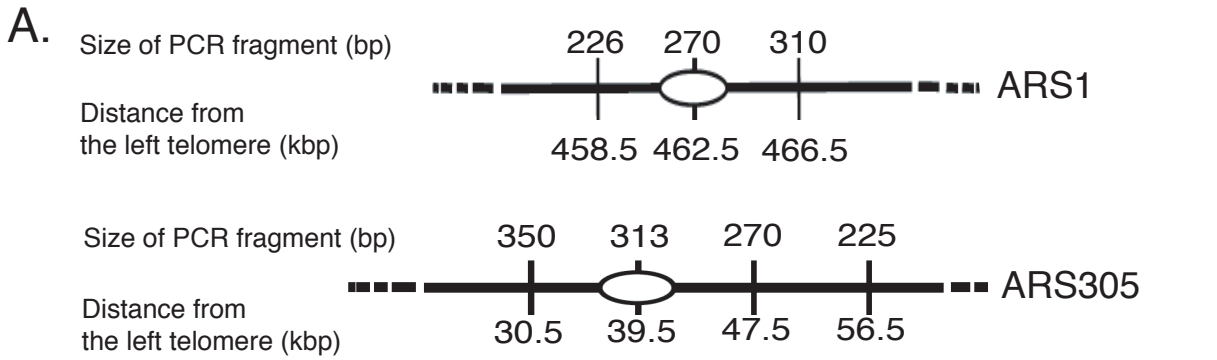
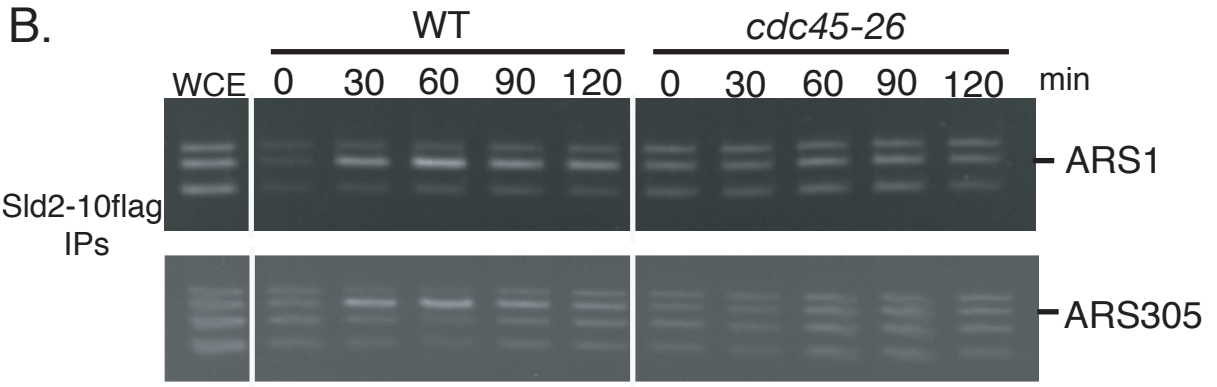

C.
WT
cdc45-26
min
180
150
120
90
60
30
0
HU
αF
Asy
37℃
25℃
1C
2C
1C
2C
D.
(αF)
G1
S
G2
M
E.
αF, 0 hr
3 hr
Nuclear division (%)
100
60
20
0
1
2
3
4
Time (hr)
WT
cdc45-26
F.
HU, 0 hr
3 hr

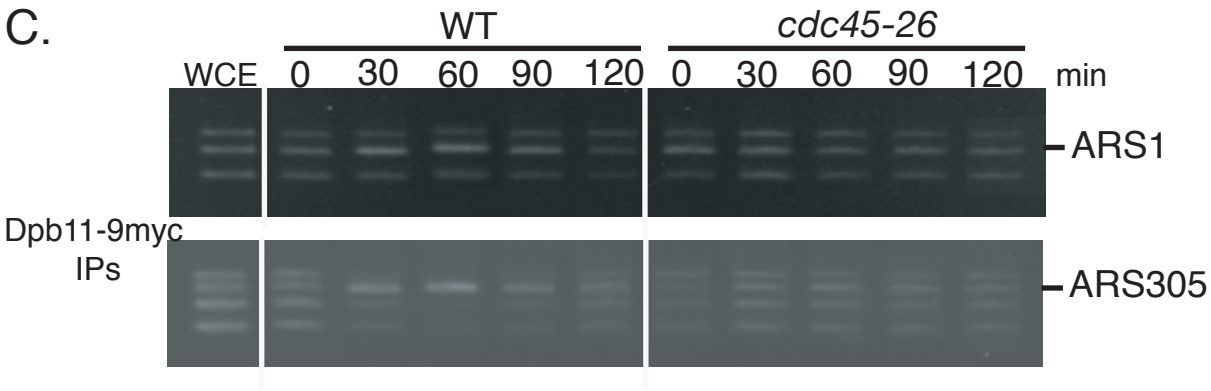
Fig. 2-2

Figure 2. *cdc45-26* cells are defective in DNA replication.

(A) Locations of mutation sites in *CDC45*. The amino acid substitution is shown for each mutant allele. Nucleotide substitution occurring in each mutation is as follows (nucleotide 1 is A in the first ATG of the ORF): C for T at nucleotide 55 and A for G at nucleotide 1222 in *cdc45-21*; C for T at nucleotide 392 in *cdc45-25* and *cdc45-27*; A for G at nucleotide 1441 in *cdc45-26*; A for G at nucleotide 370 in *cdc45-28*; G for A at nucleotide 378 in *cdc45-30*; C for T at nucleotide 724 in *cdc45-35*. (B) FACS analysis of cells released from G1-phase arrest. Wild-type (WT) and YS8 (*cdc45-26*) cells were arrested with α-factor at 25˚C and then released from α-factor at 37˚C. At the indicated times, aliquots were treated with propidium iodide and the DNA content was measured by FACScan. 1C and 2C indicate DNA contents of G1 and G2/M cells. (C) Wild-type (WT) and YS8 (*cdc45-26*) cells were arrested with α-factor at 25˚C for 3 hr and released to 0.2 M HU at 25˚C and further incubated for 2 hr, and then released from HU block at 37˚C. (D) Morphology of the different stage of cell cycle in *S.cerevisiae*. (E) Terminal morphology of the *cdc45-26* cells. *cdc45-26* cells were arrested with α-factor at 25˚C and then released at 37˚C. Cells from the 0 or 3 hr time point were stained with DAPI and nuclear morphology was examined by fluorescence microscopy. (F) Cells were synchronized with α-factor, released to YPD medium containing 0.2 M HU at 25˚C for 90 min and then transferred to 37˚C. After 30 min incubation, cells were washed and released from HU block at 37˚C.


A.
Size of PCR fragment (bp)
226 270 310
Distance from the left telomere (kbp)
458.5 462.5 466.5
ARS1
Size of PCR fragment (bp)
350 313 270 225
Distance from the left telomere (kbp)
30.5 39.5 47.5 56.5
ARS305


B.
WT
cdc45-26
WCE 0 30 60 90 120 0 30 60 90 120 min
Sld2-10flag IPs
ARS1
ARS305


C.
WT
cdc45-26
WCE 0 30 60 90 120 0 30 60 90 120 min
Dpb11-9myc IPs
ARS1
ARS305

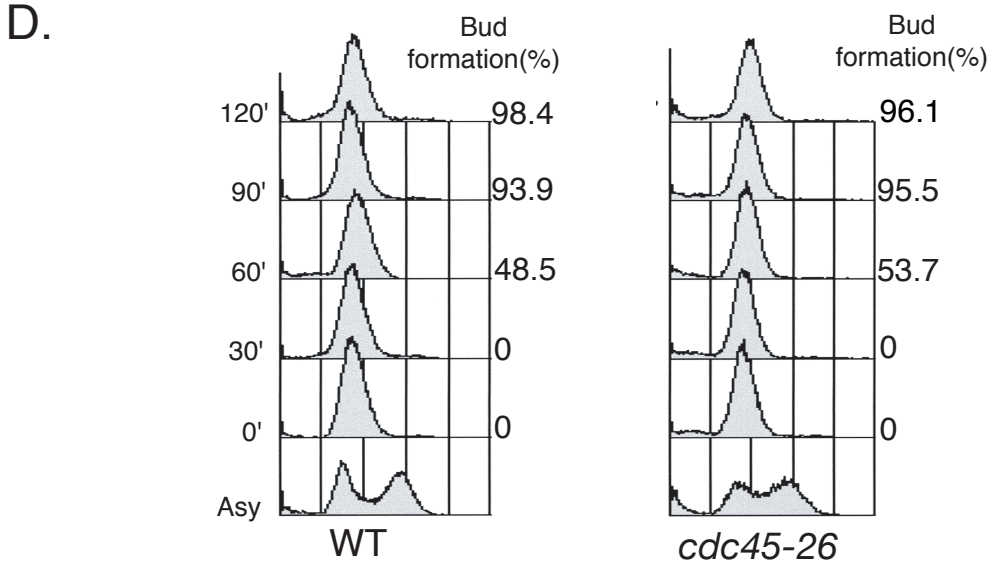

D.
Bud formation(%)
120' 98.4
90' 93.9
60' 48.5
30' 0
0' 0
Asy
WT
Bud formation(%)
96.1
95.5
53.7
0
0
cdc45-26

Fig. 3

Figure 3. The association of Sld2 or Dpb11 with ARS regions is dependent on Cdc45.

(A) Genomic intervals near or at ARSs amplified by PCR primers. (B) Cdc45 is required for the association of Sld2-10flag with ARS1 and ARS305. WT and YS103 (cdc45-26) cells were grown in YPAR at 25 ℃ containing 0.15 M HU and released at 35.5 ℃. Cells were withdrawn after 30 min and then immunoprecipitation (IP) was performed with anti-flag M2 antibody. PCR was carried out on chromatin fragment isolated after IP or on those from whole cell extract (WCE). (C) Cdc45 is required for the association of Dpb11-9myc with ARS1 and ARS305. Experiment was described above except using anti-myc (9E10) antibody for IP. (D) Cell cycle progression of HU-treated cells used for the ChIP assay.

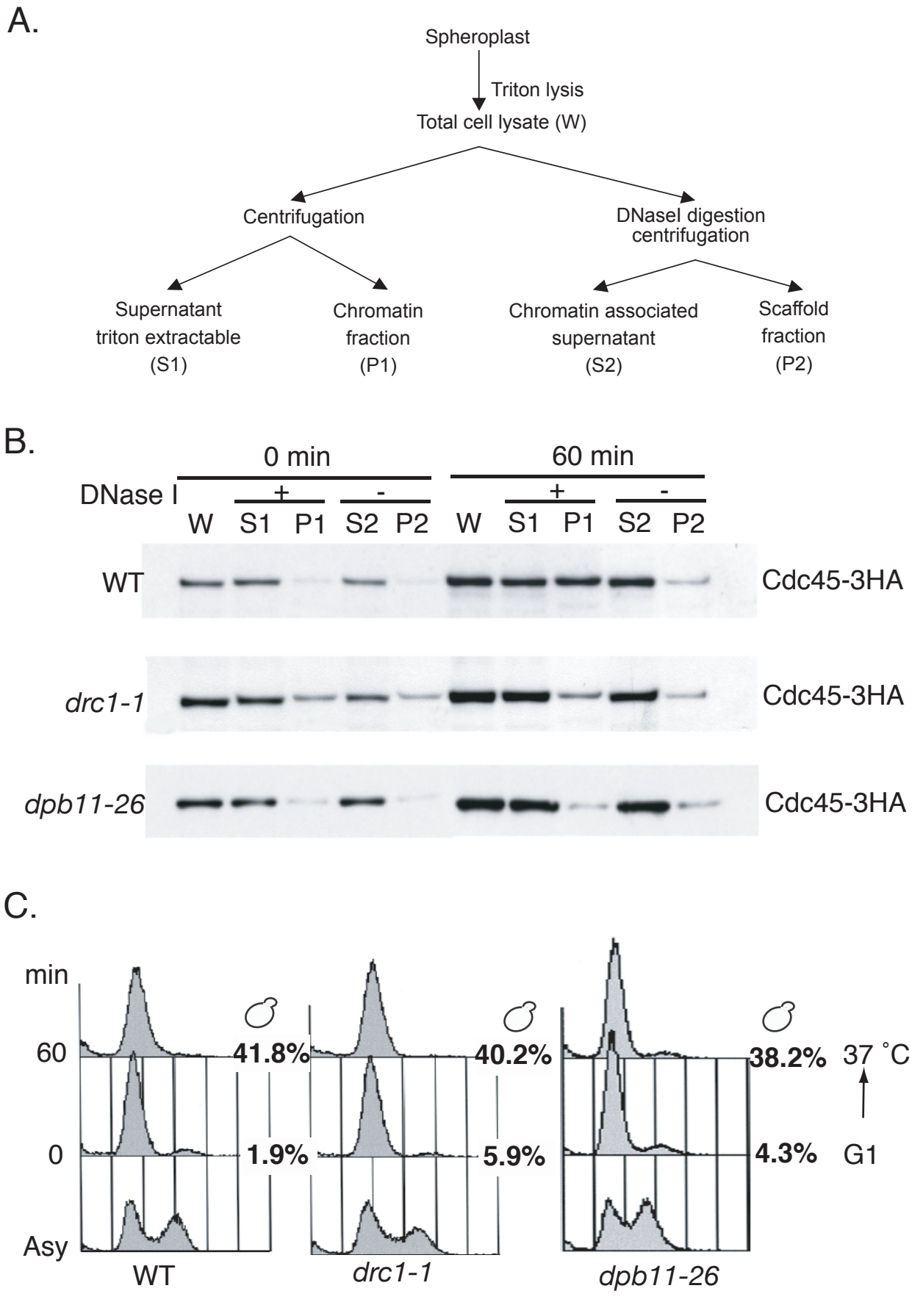
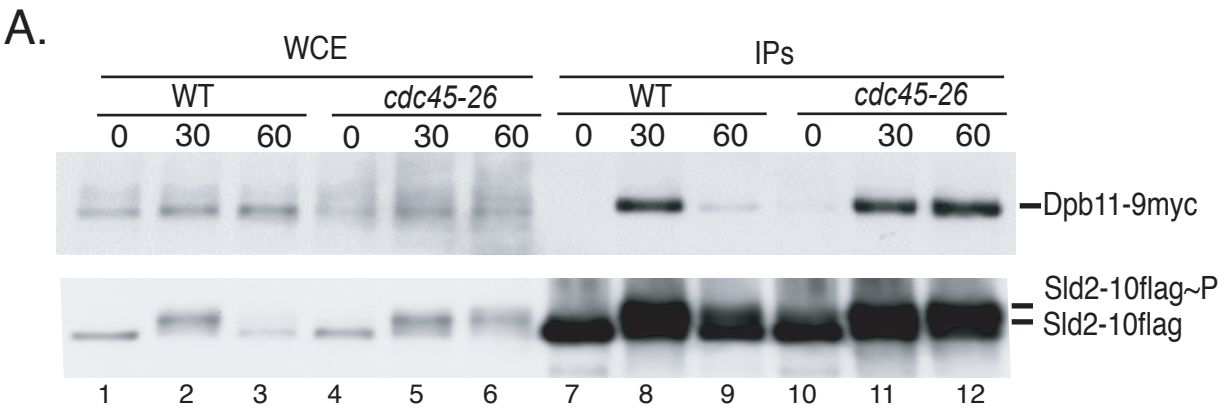
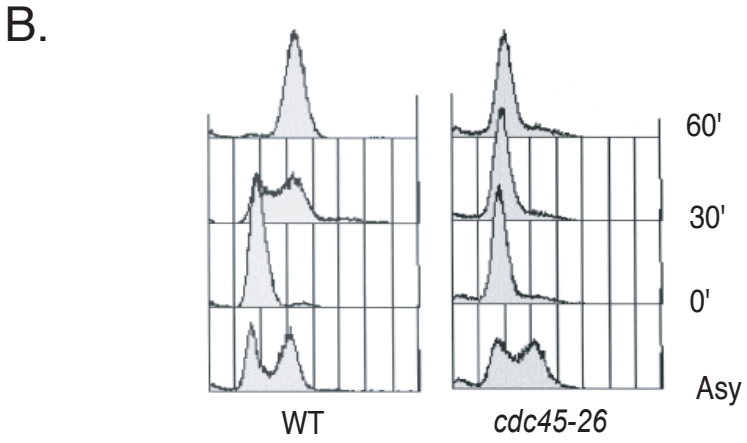
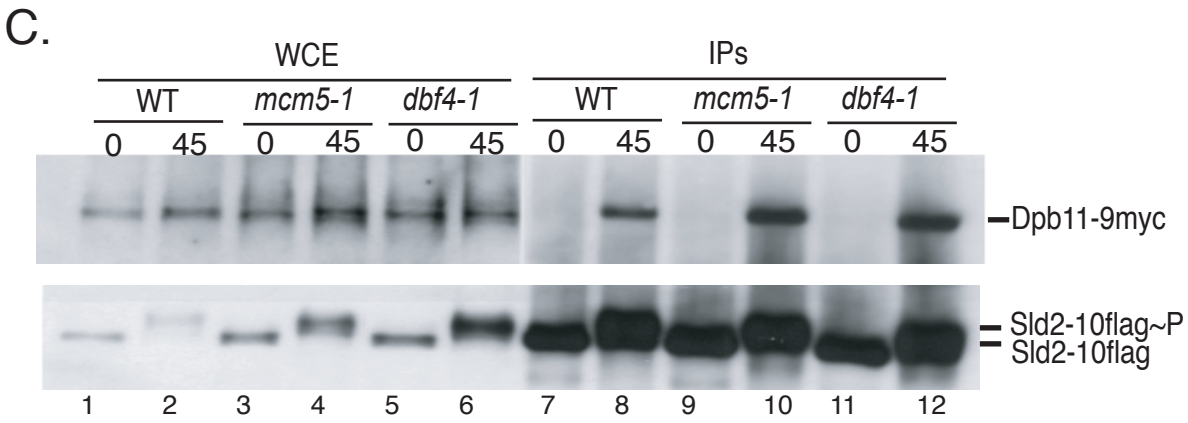

A.
Spheroplast
Triton lysis
Total cell lysate (W)
Centrifugation
DNaseI digestion centrifugation
Supernatant triton extractable (S1)
Chromatin fraction (P1)
Chromatin associated supernatant (S2)
Scaffold fraction (P2)
B.
0 min
60 min
DNase I
+
-
+
-
W S1 P1 S2 P2 W S1 P1 S2 P2
WT
Cdc45-3HA
drc1-1
Cdc45-3HA
dpb11-26
Cdc45-3HA
C.
min
60
0
Asy
41.8%
1.9%
40.2%
5.9%
38.2%
4.3%
37 ˚C
G1
WT
drc1-1
dpb11-26

Fig. 4

Figure 4. Binding of Cdc45 onto chromatin is reduced in the *drc1-1* and *dpb11-26* mutant cells. YS93 (wild-type), YS94 (*drc1-1*) and YS95 (*dpb11-26*) cells expressing Cdc45-3HA were synchronized in G1 phase by α-factor and released at 36 ˚C. The cells were collected at the α-factor block or 60 min after release from α-factor. Chromatin-binding assay was performed as described (see Materials and Methods). The proteins present in the different fractions of chromatin purification were examined by immunoblotting of SDS-PAGE: W, whole extract extract; S, supernatant; P. pellet fraction. Extracts were incubated on ice either without (-) or with (+) DNase I. The bottom panel shows the DNA content of the samples used in the top panel.


A.
WCE
IPs
WT
cdc45-26
WT
cdc45-26
0 30 60 0 30 60 0 30 60 0 30 60
Dpb11-9myc
Sld2-10flag~P
Sld2-10flag
1 2 3 4 5 6 7 8 9 10 11 12


B.
60'
30'
0'
Asy
WT
cdc45-26


C.
WCE
IPs
WT
mcm5-1
dbf4-1
WT
mcm5-1
dbf4-1
0 45 0 45 0 45 0 45 0 45 0 45
Dpb11-9myc
Sld2-10flag~P
Sld2-10flag
1 2 3 4 5 6 7 8 9 10 11 12

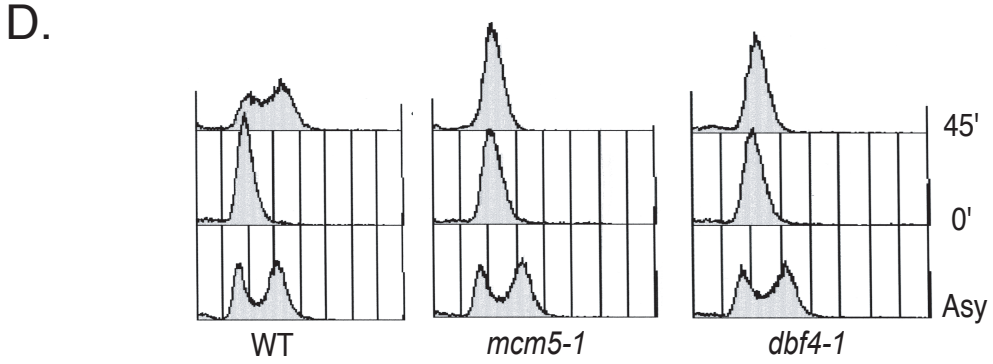

D.
45'
0'
Asy
WT
mcm5-1
dbf4-1

Fig. 5

Figure 5. Formation of Sld2-Dpb11 complex is not dependent on Mcm5, Dbf4 and Cdc45 (A) Co-immunoprecipitation of Sld2-10flag and Dpb11-9myc in *cdc45-26* mutant cells. YS132 (*SLD2-10flag DPB11-9myc cdc45-26*) cells were treated with α-factor (30 ng/ml) for 3 hr at 25°C. After wash with pre-warmed YEPR, the cells were resuspended in YEPR at 33.5°C. Co-immunoprecipitation of Sld2 and Dpb11 was preformed with anti-flag M2 antibody. (C) Co-immunoprecipitation of Sld2-10flag and Dpb11-9myc in *mcm5-1* or *dbf4-1* mutant cells. Lysates were prepared as described. (B) and (D) The DNA contents of the samples used in the (A) and (C).

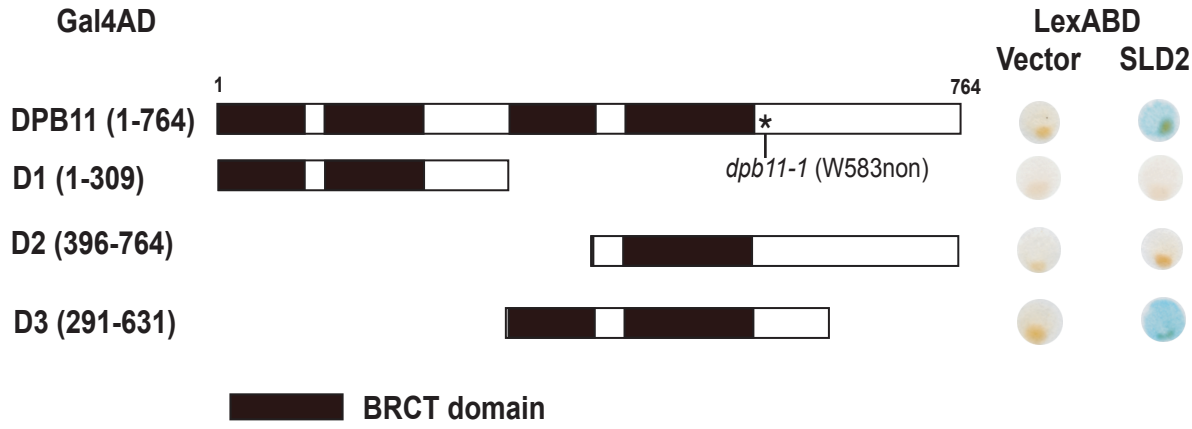
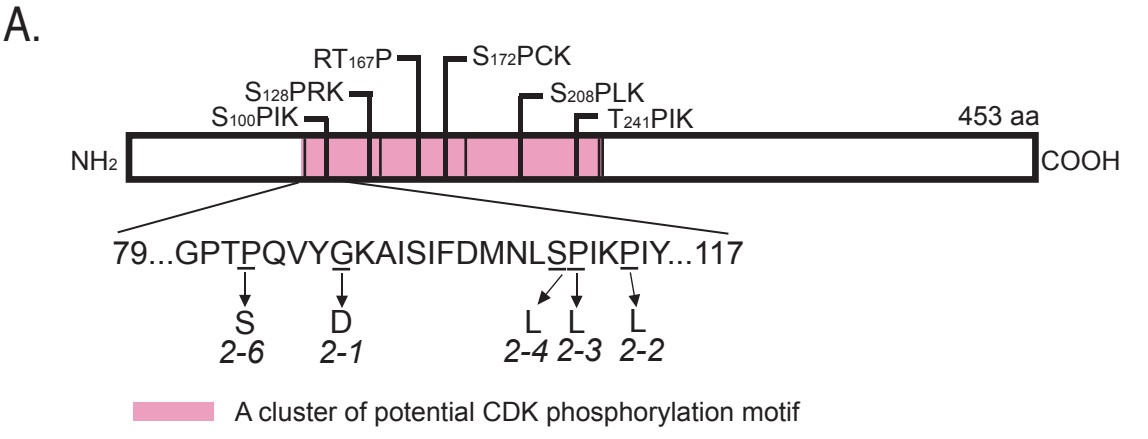
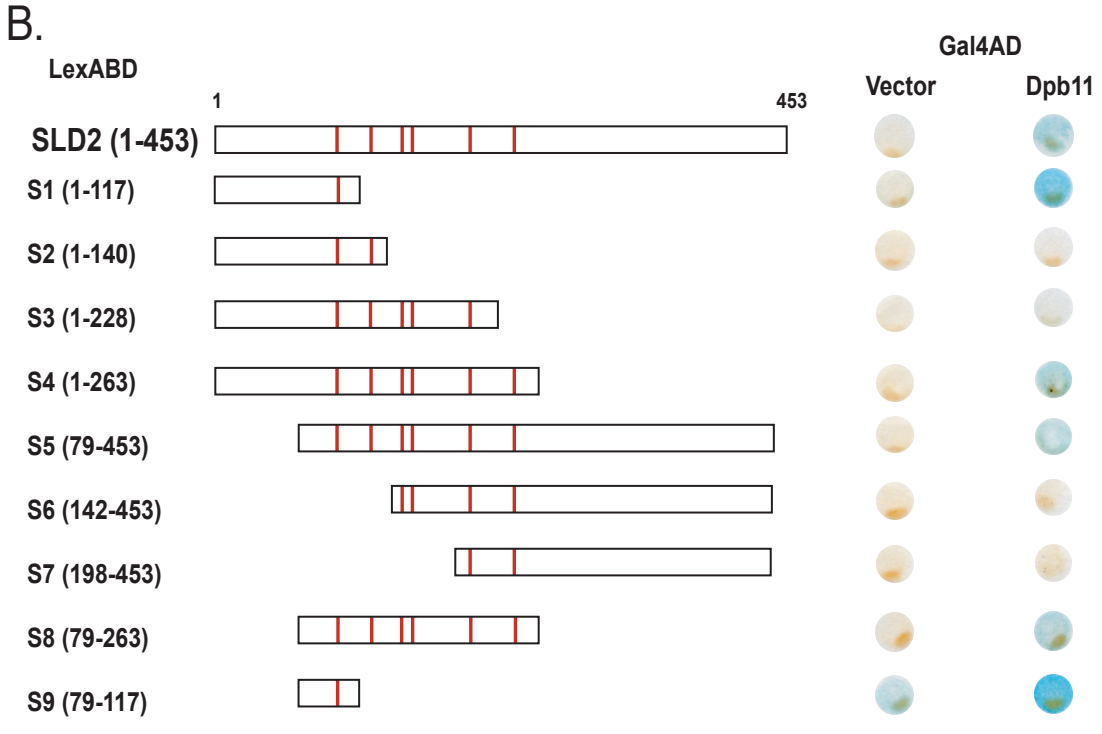


Figure 6. Mapping the interaction domain of Dpb11 with Sld2.

Interaction between full-length or truncated Dpb11 fused with GAL4AD and Sld2 fused with LexABD were analyzed. Black boxes in Dpb11 indicate the BRCT domain regions. In the *dpb11-1* allele, 583rd codon for tryptophan (W) is replaced by non-sense codon (indicated by asterisk).


A.
RT167P
S172PCK
S128PRK
S208PLK
S100PIK
T241PIK
453 aa
NH2
COOH
79...GPTPQVYGKAISIFDMNLSPIKPIY...117
S
2-6
D
2-1
L
2-4
L
2-3
L
2-2
A cluster of potential CDK phosphorylation motif


B.
LexABD
Gal4AD
Vector
Dpb11
1
453
SLD2 (1-453)
S1 (1-117)
S2 (1-140)
S3 (1-228)
S4 (1-263)
S5 (79-453)
S6 (142-453)
S7 (198-453)
S8 (79-263)
S9 (79-117)

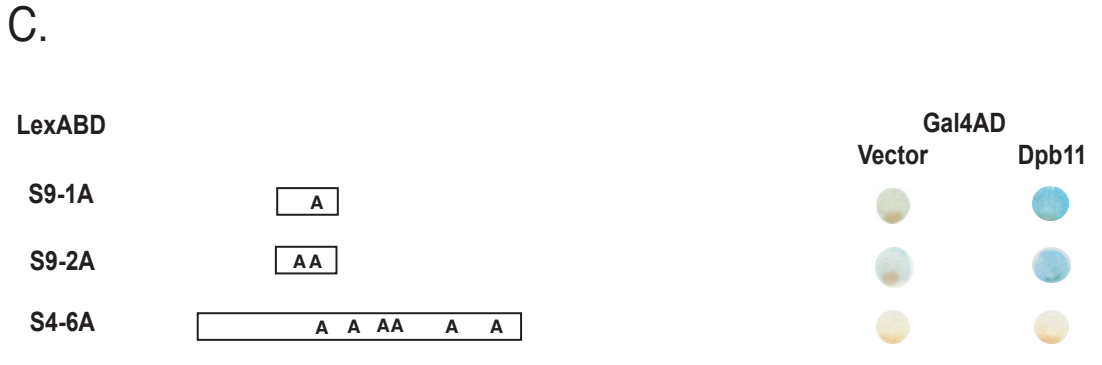

C.
LexABD
Gal4AD
Vector
Dpb11
S9-1A
A
S9-2A
AA
S4-6A
A A AA A A

Fig. 7

Figure 7. Mapping the interaction domain of Sld2 with Dpb11. (A) Schematic presentation of Cdk phosphorylation consensus sequences in Sld2. S/T-P sites are indicated by vertical lines and preferred Cdk sites are depicted with their amino-acid sequences. (B) Interaction between full-length of Dpb11 fused with GAL4AD and full-length or various truncated Sld2 fused with LexABD. Vertical lines indicate the phosphorylation sites. (C) Interaction between alanine substituted and truncated Sld2 fused with LexABD and full-length Dpb11 fused with GAL4AD. Amino acid residues are presented in *parentheses*. Transformants of L40 each carrying a pair of plasmids were assayed for β-galactosidase activity by colony color with X-Gal.

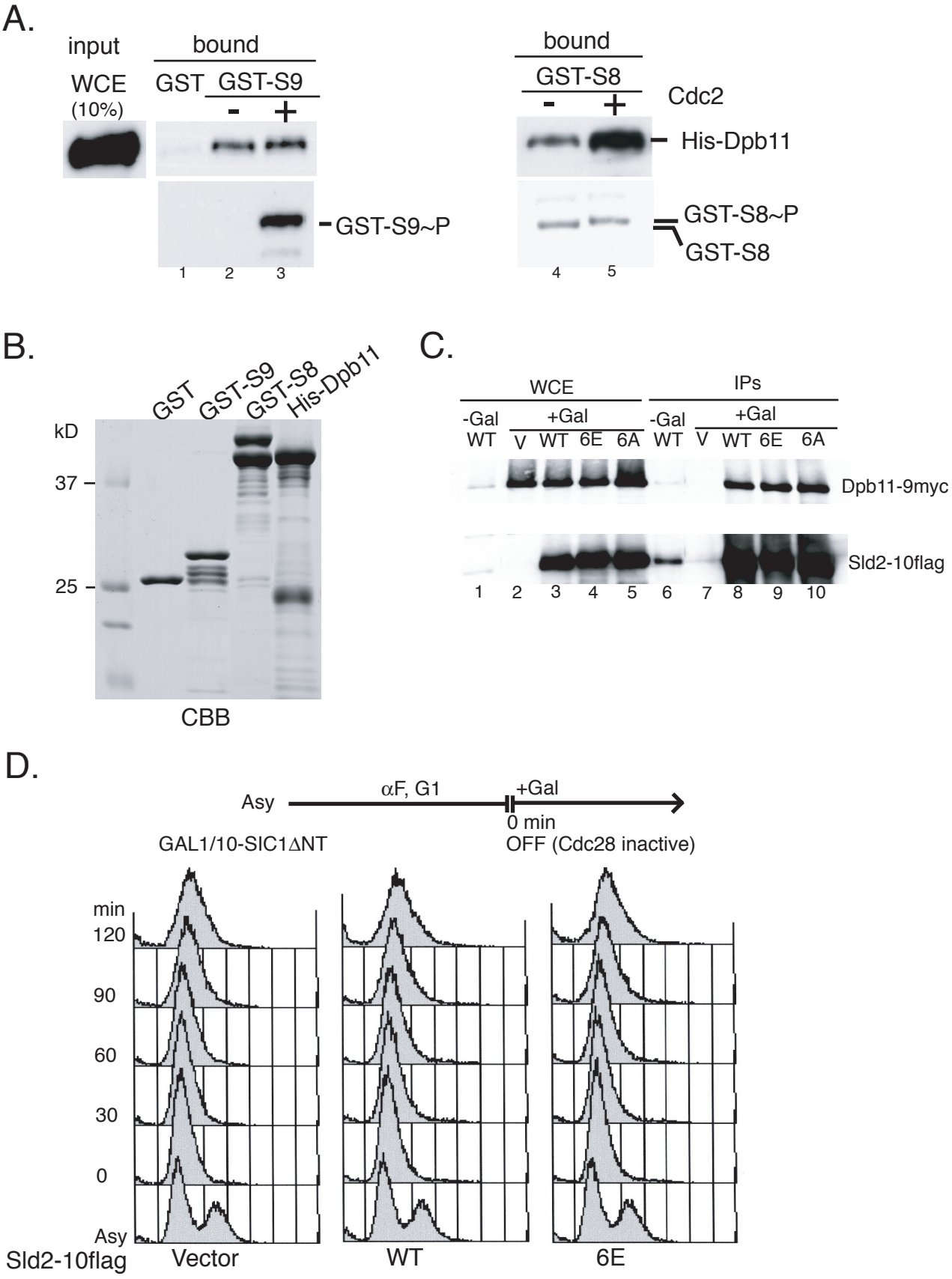

A.
input
WCE
(10%)
bound
GST
GST-S9
-
+
GST-S9~P
1
2
3
bound
GST-S8
-
+
Cdc2
His-Dpb11
GST-S8~P
GST-S8
4
5
B.
GST
GST-S9
GST-S8
His-Dpb11
kD
37
25
CBB
C.
WCE
IPs
-Gal
+Gal
WT
V
WT
6E
6A
Dpb11-9myc
Sld2-10flag
1
2
3
4
5
6
7
8
9
10
D.
Asy
αF, G1
+Gal
0 min
OFF (Cdc28 inactive)
GAL1/10-SIC1ΔNT
min
120
90
60
30
0
Asy
Sld2-10flag
Vector
WT
6E

Fig. 8

Figure 8. A physical interaction between Sld2 and Dpb11 *in vitro* and *in vivo*. (A-B) *In vitro* binding assay; GST fusion protein of Sld2-S8 or -S9 was phosphorylated by Cdc2 kinase for 1 hr at 30 °C. The GST-Sld2-S8 or –S9 bound to glutathione-agarose beads were used (GST; 0.6 μM, GST-S9; 0.52 μM and GST-S8; 0.68 μM),respectively and then incubated with equal amounts of purified recombinant Dpb11 domain with His-tagged (0.4 μM each) in 0.5 ml lysis buffer. Bound complexes were solubilized in SDS sample buffer, subjected to SDS-PAGE, and by immunoblotting using monoclonal antibody against His (upper panel). Phosphorylation of GST-S9 was detected using anti-phospho-Ser100 of Sld2 (α-(p)S100, lower, left panel), and the phosphorylation of GST-S8 was observed by band mobility on 10% SDS-PAGE (α-Sld2, lower, right panel). Protein concentration was estimated with CBB staining. M, molecular weight; lane6, GST; lane7, GST-S9; lane8, GST-S8; lane9, His-Dpb11. (C) *In vivo*, overproduced Sld2-10flag and Dpb11-9myc co-immunoprecipitate even in G1. HMS55 (*ura3::GAL-SIC1ΔNT::URA3*,pRS423*DPB11-9myc*) cells harboring [YEp112*GAL-SLD2-10flag*]; cells were incubated in YEP plus 2% raffinose to log phase and then added 2% galactose and α-factor (30 ng/ml). Cell lysates were analyzed by immunoprecipitation and immunoblotting as indicated. As a control, cell lysates without galactose were examined since expression of Sld2-10flag is controlled by GAL1/10 promotor. (D) Overproduced Sld2 and Dpb11 are insufficient for DNA synthesis. Strains were used above. Cells were grown at YEP+raffinose and subjected to following experiment depicted on the top. DNA contents were determined by FACS analysis. V,[YEp112]; WT, [YEp112*GAL-SLD2-10flag*]; 6A, [YEp112*GAL-SLD2 (6A)-10flag*]; 6E, [YEp112*GAL-SLD2 (6E)-10flag*].

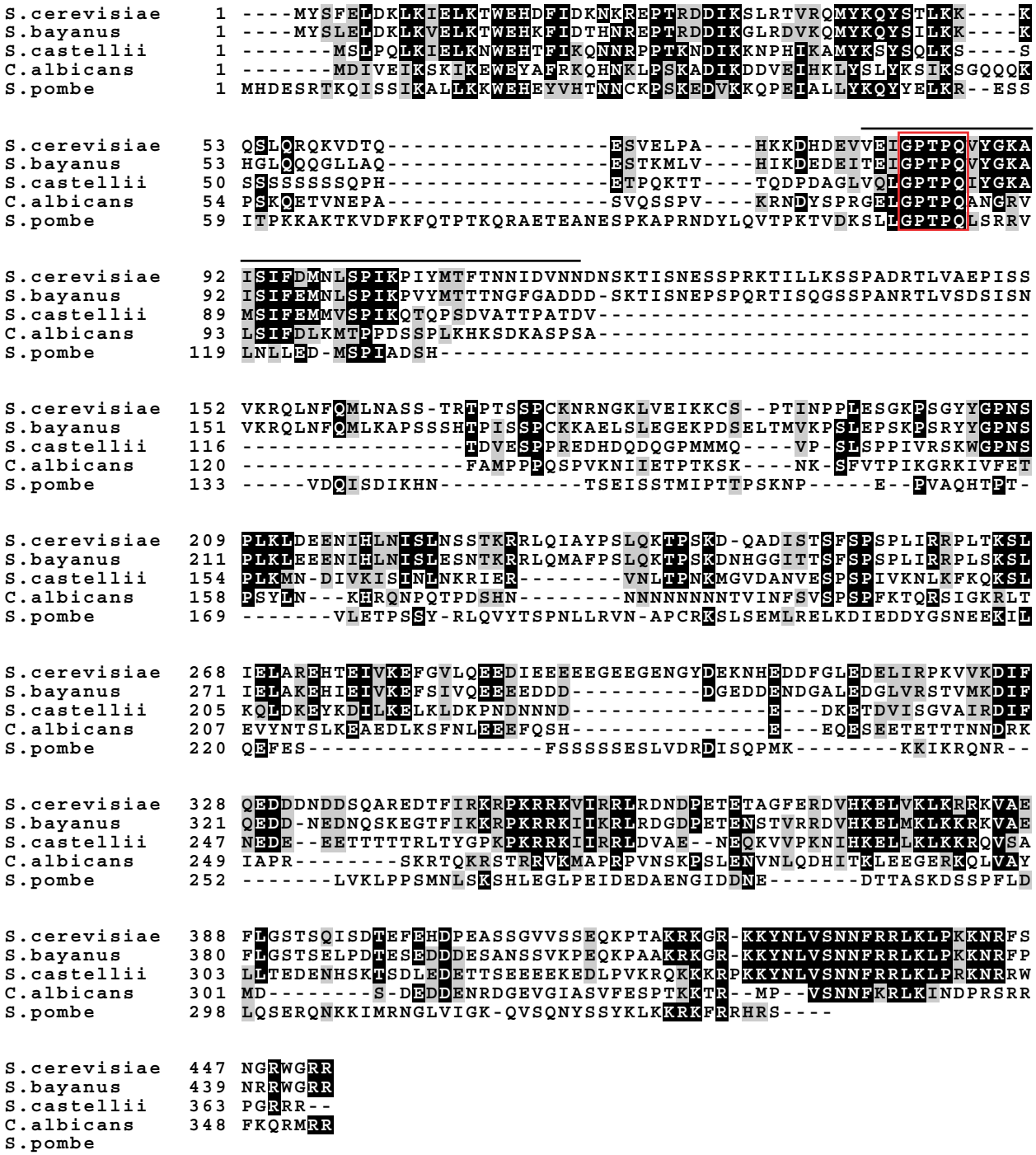


Figure 9. Sequence aligement of Sld2 from *Sacchromyces*, *Candida albicans* and *Schizosaccharomyces pombe*. The 39-aa stretch of *S.cerevisiae* is lined. Identical amino acids are boxes, and amino acids conserved in the 39-aa stretch in *S.cerevisiae* are shown with red box.

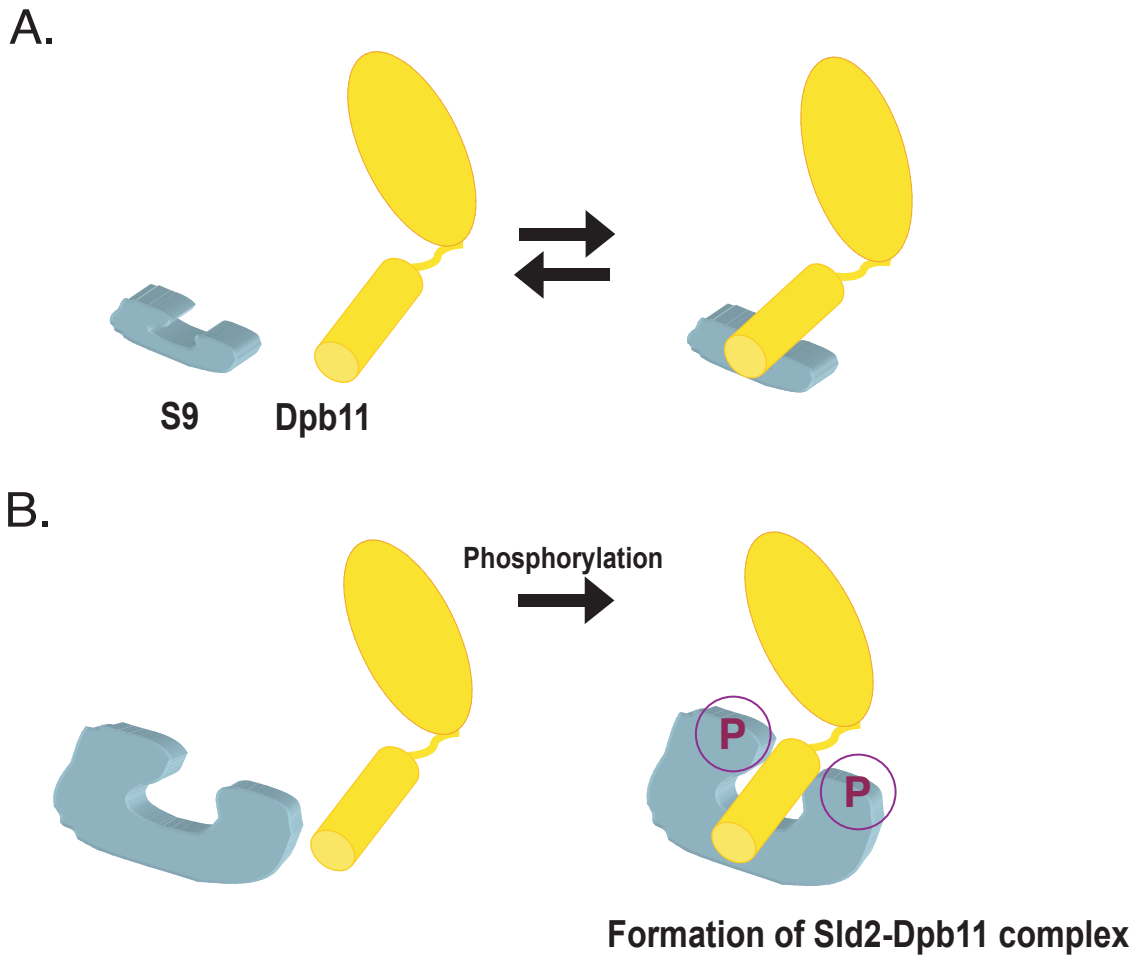


Figure 10. Possible mechanism for the interaction between Sld2 and Dpb11.

(A) The 39-aa stretch (S9) interacting with Dpb11. This interaction occurs in phosphorylation-independent manner. (B) Sld2 hardly interact with Dpb11. Here, the phosphorylation of Sld2 increases the stability of Sld2-Dpb11 complex.