

# GINs, a novel multiprotein complex required for chromosomal DNA replication in budding yeast

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Eukaryotic chromosomal DNA replication requires a two-step assembly of replication proteins on origins; formation of the prereplicative complex (pre-RC) in late M and G1 phases of the cell cycle, and assembly of other replication proteins in S phase to load DNA polymerases to initiate DNA synthesis. In budding yeast, assembly of Dpb11 and the Sld3–Cdc45 complex on the pre-RC at origins is required for loading DNA polymerases. Here we describe a novel replication complex, GINs (Go, Ichi, Nii, and San; five, one, two, and three in Japanese), in budding yeast, consisting of Sld5, Psf1 (partner of Sld five 1), Psf2, and Psf3 proteins, all of which are highly conserved in eukaryotic cells. Since the conditional mutations of Sld5 and Psf1 confer defect of DNA replication under nonpermissive conditions, GINs is suggested to function for chromosomal DNA replication. Consistently, in S phase, GINs associates first with replication origins and then with neighboring sequences. Without GINs, neither Dpb11 nor Cdc45 associates properly with chromatin DNA. Conversely, without Dpb11 or Sld3, GINs does not associate with origins. Moreover, genetic and two-hybrid interactions suggest that GINs interacts with Sld3 and Dpb11. Therefore, Dpb11, Sld3, Cdc45, and GINs assemble in a mutually dependent manner on replication origins to initiate DNA synthesis.

[*Keywords:* DNA replication; replication protein; Dpb11; Sld3; Sld5]

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The integrity of chromosomal DNA requires complete and precise DNA replication. To achieve this, eukaryotic DNA replication is highly governed by an ordered series of steps in which multiple macromolecular protein complexes are assembled and disassembled at replication origins. In *Saccharomyces cerevisiae*, chromosomal DNA replication is initiated in a restricted region, the autonomously replicating sequence (ARS; for review, see Campbell and Newlon 1991). The six-subunit origin recognition complex (Orc) binds to origins throughout the cell cycle (Aparicio et al. 1997; Tanaka et al. 1997). Inactivation of cyclin-dependent kinases (Cdks) from late M phase to G1 phase stimulates the recruitment of the minichromosome maintenance (Mcm) complex (Mcm2-7) onto origins by Cdc6 and Cdt1 to form the prereplicative complex (pre-RC). The Sld3–Cdc45 complex also as-

sociates with origins, and its association depends on the pre-RC (Aparicio et al. 1999; Zou and Stillman 2000; Kamimura et al. 2001). At the onset of S phase, when Cdk and Cdc7 protein kinases are activated, replication origins are unwound and the three DNA polymerases, Pol  $\alpha$ ,  $\delta$ , and  $\epsilon$ , essential for chromosomal DNA replication, are recruited to origins to initiate DNA synthesis.

For DNA polymerases to associate with origins, the Dpb11 protein is required. Dpb11 has four copies of the BRCT (Brca1 C-terminal) domain (Araki et al. 1995), which is important for protein–protein interactions (Bork et al. 1997; Callebaut and Moron 1997; Zhang et al. 1998), and forms a complex with Pol  $\epsilon$  and Sld2. The Dpb11–Pol  $\epsilon$  complex is detected predominantly in S phase using cross-linking reagent, and its formation depends on Dpb2, the second largest subunit of Pol  $\epsilon$ . Because the associations between origins and Dpb11 and Pol  $\epsilon$  are mutually dependent, the latter seems to associate with origins as a complex. Furthermore, the association of Dpb11 with origins is required for the loading of Pol  $\alpha$  (Masumoto et al. 2000). The Sld2–Dpb11 complex is essential for DNA replication (Kamimura et al.

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Takayama et al.

1998). In contrast to the Dpb11–Pol  $\epsilon$  complex, the Sld2–Dpb11 complex is detected without cross-linking. We recently showed that Sld2 is phosphorylated by S-Cdk and that this phosphorylation is necessary for the formation of the Sld2–Dpb11 complex, and we suggested that the association of polymerase with replication origins is regulated positively by S-Cdk activity via the complex formed between Sld2 and Dpb11 (Masumoto et al. 2002).

In higher eukaryotes, the same scenario seems to take place at replication origins, with some exceptions. Orc1 disappears in G2 and M phases, and counterparts of the three proteins, Dpb11, Sld2, and Sld3, have not been identified on the basis of amino acid sequence homologies, whereas homologs have been identified in fission yeast. Nonetheless, *Drosophila* Mus101 (Yamamoto et al. 2000) and human TopBP1 (Mäkinen et al. 2001), which have seven and eight BRCT domains, respectively, and play roles in DNA replication and repair, are thought to be functional homologs of Dpb11. Therefore, we expect that functional homologs of Sld2 and Sld3 will be found in higher eukaryotes in the near future.

We have isolated *sld1-6* (synthetic lethality with *dpb11-1*) mutations (Kamimura et al. 1998) to clarify the function of Dpb11. Sld1 is identical to Dpb3, the third largest subunit of Pol  $\epsilon$  (Araki et al. 1991). Sld4 is identical to Cdc45, which is required for the initiation of and elongation of chromosomal DNA replication (Hopwood and Dalton 1996; Zou et al. 1997; Tercero et al. 2000). Sld6 is identical to Rad53, which is required for cell-cycle checkpoints (Stern et al. 1991). The Sld2 and Sld3 proteins function for chromosomal DNA replication, and their roles in replication have been described above. In the present study, we analyzed one of the *SLD* genes isolated by screening, *SLD5*, and we demonstrate that the Sld5 protein is a component of GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese), a novel protein complex. GINS consists of Psf1 (partner of Sld five 1), Psf2, Psf3, and Sld5, all of which are well conserved in eukaryotic cells, and is essential for chromosomal DNA replication. We also show that GINS associates with replication origins and then with neighboring fragments during the S phase. Finally, from genetic and two-hybrid interactions between the genes, we infer that GINS mediates between the Sld3–Cdc45 and Dpb11–Pol  $\epsilon$  complexes to facilitate proper association between origins and DNA polymerases at the initiation of DNA replication.

## Results

### Isolation of a novel protein complex

We previously described cloning the *SLD5* gene (Kamimura et al. 1998). The *SLD5* gene corresponds to the YDR489w open reading frame (ORF; *Saccharomyces* Genome Database) and encodes a 34-kD protein essential for cell growth (Winzeler et al. 1999; M. Okawa, Y. Kamimura, A. Sugino, and H. Araki, unpubl.). Because we detected no obvious phenotype in the original *sld5-1* mu-

tation itself (Kamimura et al. 1998), four thermosensitive mutations (*sld5-2*, *sld5-8*, *sld5-12*, and *sld5-13*) were isolated using the plasmid-shuffling method (see Materials and Methods), and their mutation sites were determined (Fig. 1A).

To identify factors interacting with Sld5, we screened for a multicopy suppressor of the *sld5-12* mutation and isolated *PSF1* (Fig. 2A). The *PSF1* gene corresponds to the YDR013w ORF, which encodes a 24-kD protein and is essential for cell growth (Dardalhon et al. 2000; M. Okawa, Y. Kamimura, A. Sugino, and H. Araki, unpubl.). A *psf1-1* thermosensitive mutant was isolated using the plasmid-shuffling method (see Materials and Methods). We then screened for a multicopy suppressor of the *psf1-1* mutation and found a new gene, *PSF3* (Fig. 2A). *PSF3* corresponds to the YOL146w ORF and is also essential for cell growth. Although we did not isolate it during screening, high-copy *SLD5* weakly suppressed the *psf1-1* mutation. Furthermore, we identified Psf2 by co-immunoprecipitation with Psf1 (see below), and the *PSF2* gene on a high-copy plasmid also suppressed the *sld5-12* mutation (Fig. 2A). These genetic interactions between *SLD5*, *PSF1*, *PSF2*, and *PSF3* suggest that their products form a complex in the cell.

Therefore, we attempted to purify the complex containing Sld5 and Psf proteins. To this end, we substituted 6FLAG–*PSF1* for the wild-type *PSF1* gene. The resultant strain grew as well as the wild-type cells, suggesting that the 6Flag tag does not interfere with the biological function of the Psf1 protein. An anti-Flag immunoprecipitation was performed on both wild-type and 6Flag–Psf1 cell extracts, and the eluates with 3 $\times$ Flag peptide from immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 3A, lanes 1,2). After the gel was stained with silver, we identified four discrete protein bands (p35, p30, p25, and p22) present specifically in the 6Flag–Psf1 immunoprecipitate (Fig. 3A, lane 2). We analyzed the same eluates by 2-D gel electrophoresis and identified four discrete spots corresponding to these bands (data not shown). These results suggest that anti-Flag antibody precipitated four distinct protein species. To ascertain whether these proteins occur in the same complex, we subjected the eluates to gel filtration column chromatography and demonstrated that the four proteins coeluted from the column in almost the same fraction as aldolase (158 kD; Fig. 3B). This result strongly suggests that the four proteins form a complex in the cell.

To prove that Sld5 and Psf3 occur in the complex, they were tagged in 6Flag–Psf1 cells with glutathione *S*-transferase (GST) at the N terminus (Sld5) or with 3-hemagglutinin (3HA) at the C terminus (Psf3), and cellular proteins were immunoprecipitated with 6Flag–Psf1. The Psf3 fused with the HA tag shifted from a 22-kD band (Fig. 3A, lane 3) to a 27-kD band, which was detected by Western blotting using anti-HA antibody (data not shown). Therefore, we concluded that p22 is encoded by *PSF3*. Cells in which GST–*SLD5* was substituted for *SLD5* grew slowly, and the amount of immunoprecipitate was reduced. Although the intensity of the discrete

**A SLD5**

s21P (*sld5-8*)  
\*

Sc: MDINIDDLIARLEKETTAVBSTKITQSSSTTHRDANTIVGSSLDLNDKTIYVSPQODF  
 Sp: MEWDADDLLIEP---TTEVEN-----DY  
 Ce: M-AVTDSATIFLDFDDDEYDEMT-----TPEEVL  
 Dm: MSDVSDVPEQQLBIIVSDGAGLEDE-----DDDDMEQITPAQKVL  
 Hs: MTEEVDFIQGD---SDGSE-----VVLTAAELI  
 S66P (*sld5-8*) W67R (*sld5-12*)

\*\*

Sc: SDLMKSWKNERCSEPELLFYPHQMKRLNRLSMQSQLTENISMGLDMQNASNANPMPNP  
 Sp: EDLCIQVNERMAPDLLFAEIVSRVLDRETAQRFTLQ---LAIGTSS-----D  
 Ce: RKMTATWQNELCAPCLLPTMELVLEILLDQLCGMEENIGKQT-----D  
 Dm: EIIETAWINEMCAPHILPSQTDMLMLVMSQVAHMEQMRDLQ-----D  
 Hs: ERLECAWMEKFAPELLSEKPIVECVMEQLEHMEENLRRAK-----D  
 K150E (*sld5-2*)

\*

Sc: ESKPLLCMETELERLKFVIRSVIRCRLSKIDKHSLSLRQLNEDENSLISTDLLSKDEI  
 Sp: ATSYRSLMQTEBLERVKFVLRSMRTRINKIDKYAQYQSHP-----NLLLYLSPER  
 Ce: KMQLRISVHRVBLQRIG-----CRLQKIESNPHDAIDQHK-KRKEGKSDLLSESEM  
 Dm: KNDFRAVVHSMELERVRVIMASVLRCLRQKLEITFQHILNQE--ESR--EPDDKRLSPEET  
 Hs: REDLKVSIHOMEMERIRYVLSYLRCRIMKIEKFPFVLEKE--KTRPGEPPSSISPEEL

Sc: KYHDTSLIWLKIVNDSL-----KYMPBELQ  
 Sp: QYLLRHQQIVHRRHYMDSFL-----REVPAKVN  
 Ce: KPAEYALAESNLEOKTVLGTADGFLVENFFSLKKNCKISLNATWQFLQFFQNFKKKKFF  
 Dm: KPAQEFASNDVEYEHKVA-----QYMP-NOQ  
 Hs: APAREFMANTESYLKNVAL-----KHMEPNLQ

Sc: AINDTEGSSVMNIDE-----DWNKFFVIHVNCEP---DCKWNEPDLLOENEFKQ  
 Sp: KLDDKVGNSMNVASE-----DMDTAVFCVNVESV---EENFR---VSENEY--  
 Ce: QNSQKKTFFPKIKSKKKKFPKNSQFSSEFMPAALKKMPVFERGDHDDVMYAKVTSDDVGVN  
 Dm: RGEAEQ---RIVTP-----NEMSHVFLKANVA---PAVIVG---VDDEE---  
 Hs: KVDLFR---AVPKK-----DLDSYVFLRVREKQ---ENILVE---PDTDEKRD  
 L293P (*sld5-13*)

\*

Sc: PCYTVTIIPDLKEEVELTIGSIYVMRYEVRDLRDLRDKVALI  
 Sp: -----ITLDRKGVLLRYSVISDYLRLLGVVSLI  
 Ce: AIPDWQDLNGEVLLEMEPECHLIPFESVHQLVEDGNIQLM  
 Dm: -----VDMAGSQHTIIPQLVADLIQNNQAOLI  
 Hs: YV-----IDLEKGSOHLRYKTIAPLVASGAVOLI

**B PSF1**

Sc: MYGDLG---NKLVLAKRTKQLYARSNOIVNLFMYHEDIIRNLIKVEVSNRKNTEYLKQC  
 Sp: MENGLGNRSNKLIRDSKRTQYLDVLPYQADTVNDVVNETRAADRESLGLLONVTHEASQ  
 Ce: --MSSSDQNRGGVADKALQVLEMKRNP-VLPPYNTVELRVQCYQKIDELFKNAAVVEK  
 Dm: --MS---RQTKMFEKAFDILLKELERSSQ-TIPAFDDGVRQVLEBEIKATFRENVAQNS  
 Hs: -----MCEKAMEIIRELHRAPEGQLPAFNEDGLRQVLEEMKALYEQNSQDVNE  
 R84G (*psf1-1*)

\*

Sc: QQLGMLDQK--VAKQYFVTLCLMERN-----KRCLLAYQRLRTDILDSMANN  
 Sp: P-FQPQDHP--SEAAALLMFHSSSIYN-----KRCMLAYHNLRLQRLQYQVWS  
 Ce: IIRAGLPHSTLLQPRLAAMCHIRVSRERGRFLKYCSGSIIFRCMMAYVNERKNRIRSPRWK  
 Dm: YNASGDRS---LWF--LNFRAAALQRN-----KRCLLAYLYECCRRIKALRWE  
 Hs: AKSGCRS---LTF-TIKFRHCSLLRN-----RRCITVAYLYDRLLRTRALRWE

Sc: NNGLDMSSTITFSQDTNNLSHOFQEYLKEYCDITDKSGLDVIDLISGSLVPSVDVFI  
 Sp: G-GKRMECLDTS-----LSTYERDYLTRYSELAAAYK-GAWSELDLIGSLVPPKPNLFI  
 Ce: Y-GGALPASVFNALCDABITQFFN--EYSSTLARFQSNLIG-EGGV--NLLHSAPPKSLFV  
 Dm: F-GPIIFGDTKQALCEPVTFFN--NYSKSLAAYMCSAGYNGOGLPIDLNNRPPKSLYI  
 Hs: Y-GSVLENALFEHMAAEEMWEN--NYKRSATYMRSLGGDEGL--DITQDKMPKPSLYI

Sc: DVRVLKADAGEIQTEYG-VFNLIKDSQFVRQSDVERLIQQGYLQKI--  
 Sp: DVRVLKDVGDIEITYG-TINLTKNSQLHVRATDVERLIAQGFIAKL--  
 Ce: QVRALEDYGEFESDGTQVQLSKDSLHSLPRQDCEMLIRQGVLELVH-  
 Dm: EVRCMEDYKFELEDGEVLIHLKKNSSHYLERAQVESLVRQGIHHTIA-  
 Hs: EVRCLKDYGEFVEDDGTSLVLLKKNSSHFLPQWKCQLIRQGVLEHLS

**C PSF2**

Sc: --MSLPAHLQQTFSPEEIQFTVENPPIKTFERRITTRQKIRGDDRTGNHTRWOLITTTDDK  
 Sp: --MALPRELEISFPEEMEFAGNRYNINVPSETMDLP-----LVGS---A  
 Ce: -----MNAERCEFLAGNSLEVIIPSIDDRPIHLIS-----G  
 Dm: MPPHIFCLPIAIMDPSIIEPIGKCMISILPNFSNE-PLHLIY-----G  
 Hs: -----MDAAVEFLAEKLEVTIIPNFSLD-KIYLIIG-----G

Sc: ALNNMVMRSTEVVLIALLKQCKKSIIVAPQWLTTKELDRKIQYKTHPDRFSSELP-W  
 Sp: TIPIMKPKKCRVPLWLALELKKQNLARIVPEWMEIGKLEN-TRDDELENETFSSELP-F  
 Ce: DIGPFEAGVPCRIVWTAIIMKRKHNNKIVAPQWMDVDELKK-LLTSEFESQGLAKLP-D  
 Dm: PVGPFRAFVFPVPLWATHLRKQKCRIVPEWMDMILEE-IKEBEKRSKFFTKMCE  
 Hs: DLGPFNFGLPEVPLWLAINALKQRQKCRLLPEWMDVEKLEK-MRDHERKEETFTMPSP

Sc: NWLVLARILFNKAKDDPHDPIHELKGLQDLREIRQIKVLKQ---LKYLNESHQLDNL  
 Sp: RWLETAHLLNFCADDIEE-VEDTRIRILLDIREAROSKARTG---LEAINEVOLTLDNL  
 Ce: HFFRISHMLVRDAREDDIEF-VBAVKSIVQDIDYDRDAKLRSAPFELRQNTCHAQLDNLV  
 Dm: HYMVVQVLMSTAPDQVPER-CBELRTVIKIDIFDIRESKLRTSIDAFIKG-EGTYAKLDNL  
 Hs: YYMERTKLLNHSADNIEK-ADERTIVIKMMDWTRIAKLRVSDSIVRQ-QEAAKLDNL

Sc: SLELEINLRPFIETIMDKLREHTASLTAGTENDEEFNI-----  
 Sp: GAMEINRIRPIFREVMRMRKIVQVS-----QEE-----  
 Ce: QHTRASSARATIEACRQMGAVVR-----NKHESTPL-----  
 Dm: HLLBIHSVREIIPYSLDHIAVORTATASQRTSMLSSASMGSSGPNNSLFSQ  
 Hs: HLMBINSGTFLTQALNHYKLR---INLQPLESTQSQDF-----

**D PSF3**

Sc: MIYPSLRVLRDRKCRVRVKIAMELMPTLFLVLVYFYNRRASIERMGIYDIDIVADGTEFP  
 Sp: -----MDYDIDISLISENQKRVPC  
 Hs: M-----SEAYFRVSEGALGPENFELSDDIIMSHKLELV

Sc: KFQYDIPGLGVL-----ENNPGRPIKNTKLSLPLWARIATVGGDEALVDEPVPFV  
 Sp: TSTVSLPGLG-----HEGRMVTGSKVLELPPWLAEVLAIN-----SFV  
 Hs: RTETAMPRLGAPFLERSAGAEADTNAVPEQSSKLELPLWLAAGLFDNKR-----RIL

Sc: ELLPDMFSTKVMNAIKTDVVALDLHSINSRHFSLAIKWMIFSEKELANVSELLLQRA  
 Sp: SIHMAPPFSSVNRNALKANPNSVIRSDITTHYHYFAEKMLHLISDDSLVQTSINLTRSRA  
 Hs: SVLEKRIYQGWRTVFSADPNVVDLHKMGPHIYGFQSGQLLHFDSP-ENADISQSLQTFI

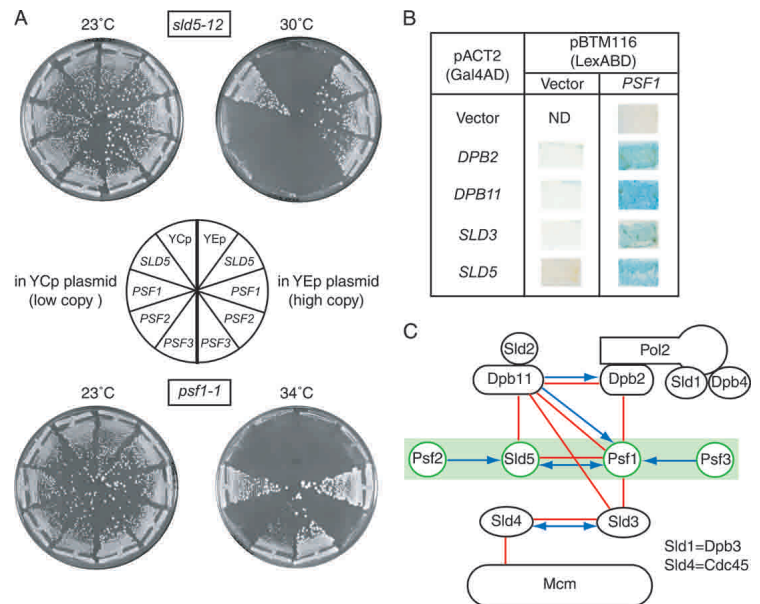
Sc: QELNHASSLSIDLNADSTGKNSANTNIATSTFLKLEEMEKETIYKSHESYKDTKRMMF  
 Sp: MLIADASLNPGALQONSQ-----FIEGLDDFEKHLIRVSHNAHRSLINWQ  
 Hs: GRFRIRIMDSSQNAYNEDTS-----ALVARIDEMERGLFQGTGKGLNDPQCWEK

Sc: KK-----  
 Sp: STS-----  
 Hs: GQASQITASNLVQNYKRRKFTMDM

**Figure 1.** Sld5, Psf1, Psf2, and Psf3 are conserved among eukaryotes. Amino acid sequence alignment of *S. cerevisiae* Sld5 (Sc; A), Psf1 (Sc; B), Psf2 (Sc; C), and Psf3 (Sc; D) and their homologs from *S. pombe* (Sp), *Caenorhabditis elegans* (Ce), *Drosophila melanogaster* (Dm), *Homo sapiens* (Hs). Amino acids conserved in all aligned sequences are shown with dark shadow, and those conserved in at least two sequences but not all are shown with light shadow. *sld5* mutation sites and *psf1-1* mutation sites are shown by asterisks with the amino acid changes in A and B. *sld5-2*, *sld5-12*, and *sld5-13* mutations were identified at positions 448 (A to G), 199 (T to C), and 878 (T to C) in the nucleotide sequence (nucleotide 1 is A of the first ATG of the ORF). These mutations change the amino acids of Sld5 from conserved lysine, tryptophan, and leucine to glutamic acid, arginine, and proline, respectively. The *sld5-8* allele has two alterations occurring at nucleotides 61 (T to C) and 196 (T to C), which both result in the replacement of serine with proline (A). However, we do not know whether two-site mutations are required for temperature sensitivity. The *psf1-1* mutation site was identified at position 250 (A to G) in the nucleotide sequence, which changes an arginine to glycine (B).

Takayama et al.

**Figure 2.** The genetic and two-hybrid interactions between Dpb2, Dpb11, Sld2, Sld3, Cdc45, Psf1, Psf2, and Psf3. (A) Suppression of thermosensitive growth of *sld5-12* and *psf1-1*. YYK38 (*sld5-12*) cells and YYT1 (*psf1-1*) cells carrying different plasmids were streaked onto YPDA plates and incubated at the indicated temperatures. The genes in the left half of the diagram were cloned into YCplac22 (the low-copy vector), and the genes in the right half were cloned into YEpl95 (the high-copy vector). (B) Two-hybrid interactions between Psf1 and Dpb2, Dpb11, Sld3, or Sld5. “Vector” and “Psf1” in pBTM116 denote plasmids that express LexABD and LexABD–Psf1, respectively. V, DPB2, DPB11, SLD3, and SLD5 in pACT2 denote plasmids that express Gal4AD and Gal4AD fusion with Dpb2, Dpb11, Sld3, and Sld5, respectively. Transformants of L40 each carrying a pair of plasmids were assayed for  $\beta$ -galactosidase activity by colony color with X-Gal. (C) Summary of genetic and two-hybrid interactions among Dpb2, Dpb11, Sld2, Sld3, Cdc45, Psf1, Psf2, and Psf3. The blue line shows multicopy suppression; the red line shows a positive signal in the two-hybrid assay.



bands was weak, we observed a novel 60-kD band in the immunoprecipitates (Fig. 3A, lane 7). Moreover, Western blots probed with anti-Sld5 antibodies reacted with p35 from cells expressing *SLD5* but not from cells expressing *GST-SLD5*, whereas both anti-Sld5 and anti-GST antibodies reacted with p60 (Fig. 3A, lanes 8–11). This result strongly suggests that p35 is encoded by *SLD5*. Because a weak signal was observed in the same position as p35 in wild-type cells (Fig. 3A, lane 1), the p35 signal remaining in cells expressing *GST-SLD5* might constitute nonspecific background.

The other two discrete protein bands were subjected to peptide mass fingerprinting analysis, and p30 was identified as Psf1. p25 was the product of a new essential gene, *PSF2*, encoded by the YJL072c ORF. Consistent with this finding, the p30 band was detected using not only anti-Flag but also anti-Psf1 antibodies (data not shown). Therefore, we concluded that p30 is 6Flag–Psf1. Replacement of Psf2 by Psf1 tagged with 13-Myc at its C terminus in 6Flag–Psf1 cells shifted the p25 band to a more slowly migrating band (Fig. 3A, lane 4). This shifted band was detected using anti-myc antibody (data not shown), indicating that *PSF2* encodes p25. Together with their genetic interactions, these results strongly suggest that Sld5, Psf1, Psf2, and Psf3 form a complex. We named this complex GINS. The abundance and composition of GINS were constantly maintained during the cell cycle (data not shown).

The amino acid sequences of Sld5, Psf1, Psf2, and Psf3 are well conserved among eukaryotic organisms, and some of their mutations occur at highly conserved amino acid residues (Fig. 1), suggesting that a similar complex exists in all eukaryotic cells. However, the N-terminal portion of the predicted amino acid sequence of Psf3 is absent in its homologs in other organisms. Moreover, the apparent molecular mass of Psf3 on SDS-PAGE is smaller than the predicted molecular mass (Fig. 3).

Therefore, we surmise that the fourth ATG in the predicted YOL146w ORF is used for translation.

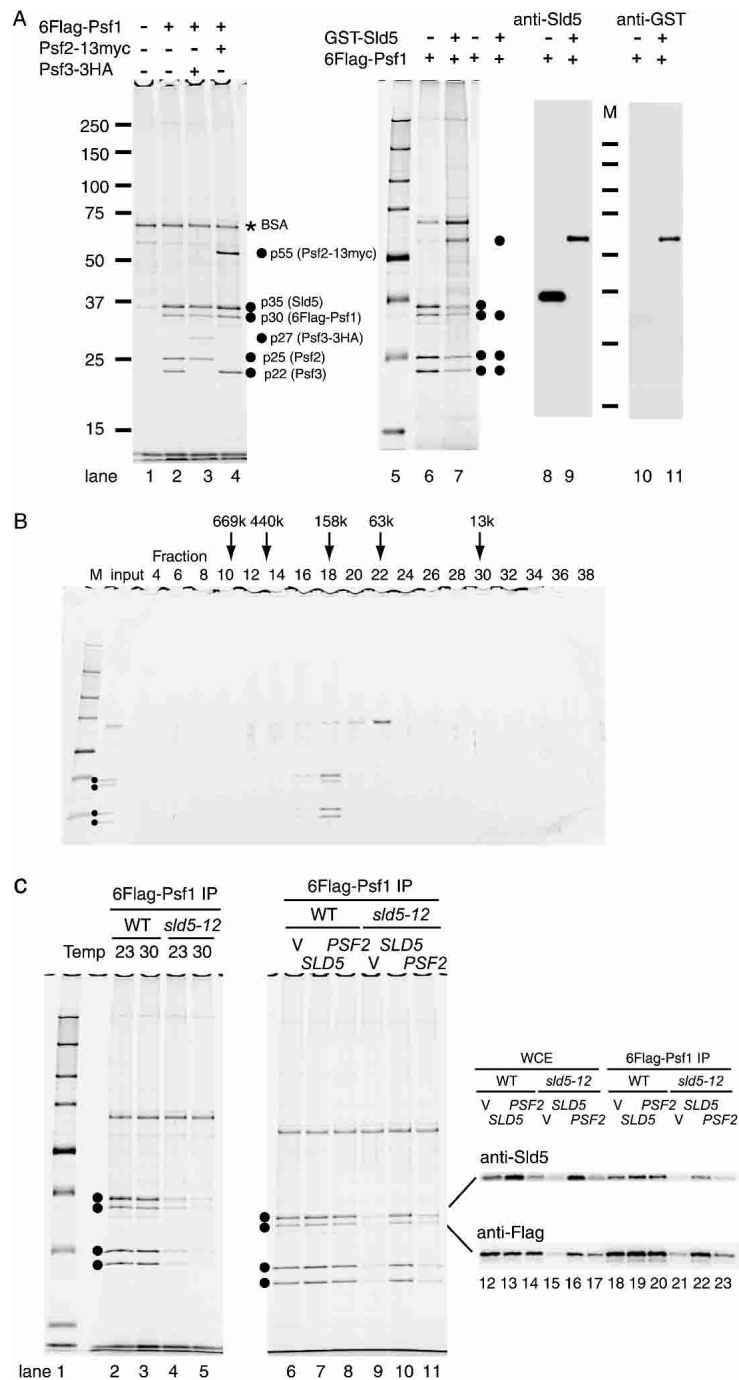
#### Genetic interactions between *SLD5*, *PSF1*, and *DPB11*

Because *sld5-1* was isolated as a synthetically lethal mutation with *dpb11-1* (Kamimura et al. 1998), we examined the synthetic lethality between *psf1-1*, *sld5-12*, and *dpb11-1*. We crossed all possible combinations of these mutants. The resultant diploids were allowed to sporulate and were then dissected. Of the 20 tetrads obtained from each diploid, no spore clones carrying two of these mutations were isolated. These results indicate that any pair of *psf1-1*, *sld5-12*, and *dpb11-1* is lethal, even at permissive temperatures, and suggest that both Psf1 and Sld5 interact with Dpb11. This is further supported by the finding that high-copy *DPB11* suppressed a *psf1-1* mutation (data not shown). We also found that *SLD5* on a high-copy plasmid weakly suppressed the growth defect of *dpb11* mutant cells at the restrictive temperature. Moreover, the growth defect of *dpb11* mutant cells was restored better by the simultaneous introduction of high-copy *SLD5*, *PSF1*, and *PSF3* than by *SLD5* alone (data not shown).

To confirm these genetic results, we examined whether Psf1 interacts with Dpb2, Dpb11, Sld3, and Sld5 in a two-hybrid assay. As shown in Figure 2B, Psf1 interacted with Dpb2, Dpb11, Sld3, and Sld5. These genetic and two-hybrid data are summarized in Figure 2C and will be discussed below.

#### *GINS* is required for essential function

Although the *SLD5*, *PSF1*, *PSF2*, and *PSF3* genes are all essential for cell growth, it was unclear whether the formation of GINS is essential for cell growth. If its forma-



**Figure 3.** Sld5, Psf1, Psf2, and Psf3 form a complex. (A) Protein extracts were prepared from cells of the indicated genotypes. +, an epitope-tagged gene; -, a wild-type allele. Anti-Flag immunoprecipitations were performed. The immunoprecipitated proteins were separated by 4%–20% SDS-PAGE and subsequently silver-stained (left and center panels). In the right panel, immunoprecipitates from 6Flag-Psf1 or 6Flag-Psf1 GST-Sld5 cell extracts were probed with anti-Sld5 or anti-GST antibodies. (B) A gel filtration chromatography of 6Flag-Psf1 immunoprecipitates using Superdex 200 was performed as described in Materials and Methods. The proteins in each column fraction were identified by 5%–20% SDS-PAGE followed by silver staining. The first lane contains protein molecular-weight standards (M), and the second lane contains the input sample. The Superdex 200 column was calibrated with tyroglobin (669 kD), ferritin (440 kD), aldolase (158 kD), BSA (63 kD), and RNase A (13 kD). (C) YYK46 (6Flag-Psf1, WT) and YYK50 (6Flag-Psf1 in *sld5-12*) cells were cultured at 23°C to  $1 \times 10^7$  cells/mL. Half of each culture was shifted to 30°C while the other half was maintained at 23°C, and incubations were continued until cells reached  $2 \times 10^7$  cells/mL (left panel). YEp195 (V), YEp195-SLD5 (SLD5), and YEp195-PSF2 (PSF2) plasmids were introduced into YYK46 (6Flag-Psf1, WT) and YYK50 (6Flag-Psf1 in *sld5-12*) cells. Cells carrying each plasmid were cultured at 23°C (center and right panels). Protein extracts were prepared from each culture, and anti-Flag immunoprecipitations were performed. The amount of GINS was estimated by staining with silver (left and center panels). In the right panel, whole cell extracts (WCE) and immunoprecipitates (IP) were immunoblotted with anti-Sld5 or anti-Flag antibodies.

tion is essential for cell growth, GINS may be impaired by some mutations in these components, and might be restored by the overproduction of other components. This seems to be true, because high-copy *SLD5* and *PSF* genes each suppressed the *sld5-12* and *psf1-1* mutations (Fig. 2A). Therefore, we examined the formation of GINS in *psf1-1* cells, because we have not yet succeeded in constructing a tagged version of Psf1-1. We found that the amount of GINS recovered from *sld5-12* cells was reduced to less than 1/5 that from wild-type

cells, even at the permissive temperature (23°C; Fig. 3C, lanes 4,5). As observed for the GST-Sld5 protein (Fig. 3A, lane 7), the amount of 6Flag-Psf1 in the precipitates was also reduced (to less than 1/5 that of wild-type cells; Fig. 3C, lanes 9,21). Moreover, Western blotting revealed that the amounts of Psf1 and Sld5-12 proteins in the crude extract prepared from *sld5-12* cells were also reduced (Fig. 3C, lane 15), as observed in the precipitates. These results suggest that the *sld5-12* mutation impairs the formation of GINS and that uncomplexed Psf1 is degraded. Furthermore, *sld5-12* cells carrying the high-

Takayama et al.

copy *PSF2* plasmid significantly restored the amount of GINS (Fig. 3C, lanes 11,23). As a control, the amount of GINS in wild-type cells carrying the vector, high-copy *SLD5*, and *PSF2*, was apparently constant (Fig. 3C, lanes 7,8,19,20). These results strongly suggest that GINS is required for cell growth and for chromosomal DNA replication (see below; Fig. 4).

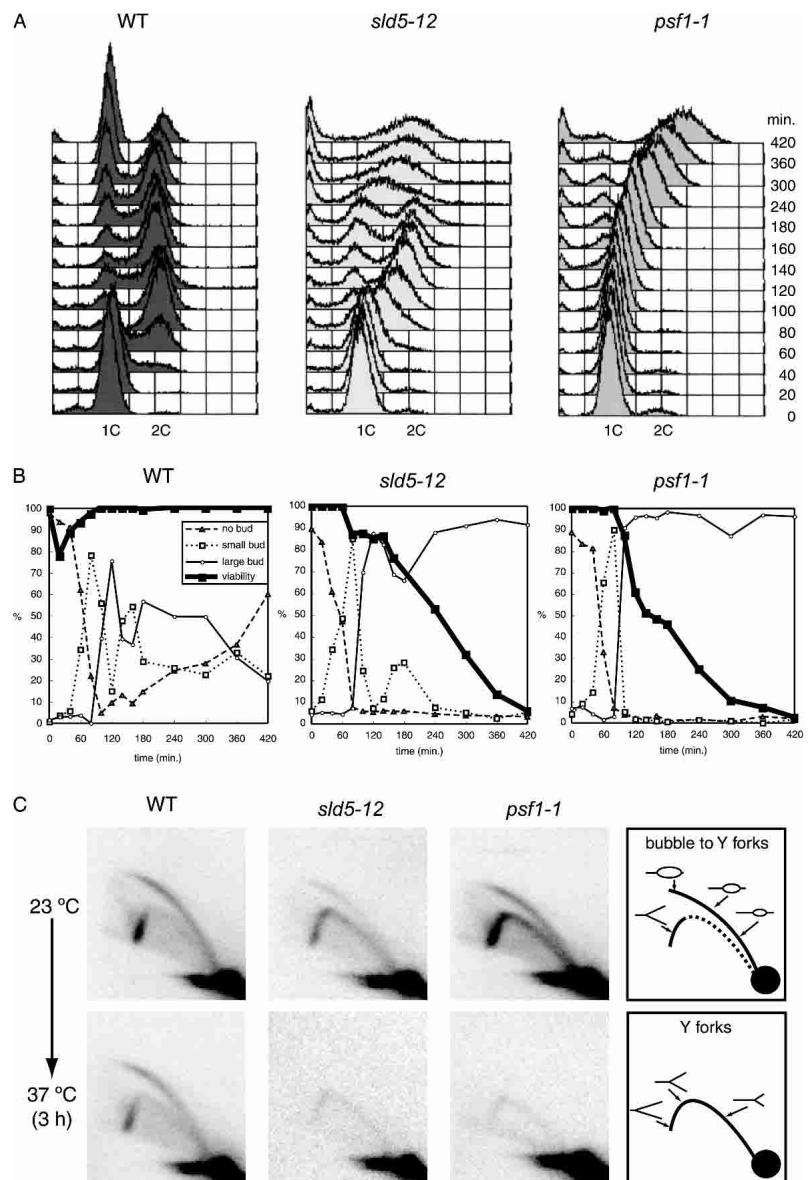
#### *Sld5 and Psf1 are required for DNA replication*

The results described above suggest that Sld5 and Psf1 proteins function through GINS. Therefore, we examined the phenotypes of the *sld5* and *psf1* thermo-sensitive mutants to clarify the biological function of GINS.

With a temperature shift up, the growth of all of the mutant *sld5* and *psf1-1* cells was arrested, and the cells

displayed the dumbbell shape and a single nucleus (data not shown) that is the typical terminal morphology for mutants defective in DNA replication. We measured DNA synthesis in mutant cells at the restrictive temperature by FACS analysis. When cells were arrested in G1 phase with  $\alpha$ -factor and released at 36°C, wild-type cells accumulated a 2C DNA content in 80 min, then entered a second cell cycle. Cells carrying the *sld5-12* mutation, which is the tightest allele of the *sld5* mutations described above, reached a 2C DNA content gradually, entered the second cell cycle, and again gradually reached a 2C DNA content (Fig. 4A). These kinetics suggest that *sld5-12* cells are partially defective in DNA replication. Defective DNA replication was more evident in the *psf1-1* cells; their DNA content did not increase for 120 min and then reached 2C gradually (Fig. 4A). These are the typical kinetics of bulk DNA

**Figure 4.** *sld5-12* and *psf1-1* cells are defective in DNA replication. (A) FACS analysis of cells released from G1-phase arrest. Wild-type (WT), *sld5-12*, and *psf1-1* cells were synchronized with  $\alpha$ -factor at 23°C and released from  $\alpha$ -factor at 36°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. (B) Viability and cell morphology of *sld5-12* and *psf1-1* mutant cells. Portions of the same samples incubated at 36°C and described in A were used to determine cell number and cell morphology. The cells were spread onto YPD plates to measure viability. ■, viable cells; △, cells without bud; □, cells with small bud; ○, cells with large bud. (C) N/N 2-D gel analysis of the chromosomal *ARS1* locus in wild-type (WT), *sld5-12*, and *psf1-1* cells. Cells were grown and harvested at 23°C or shifted to 37°C for 3 h prior to harvest. DNA was digested with *NcoI* and probed with *ARS1*-containing fragment. The diagrams besides photographs show the bubble to Y fork and Y fork arcs.



synthesis in mutants defective in the early step of DNA replication. On the other hand, when *psf1-1* cells were arrested in S phase by hydroxyurea (HU), and released at the restrictive temperature, they completed bulk DNA synthesis and entered the subsequent G1 phase, although the S-phase progression was substantially slowed relative to the wild-type control (data not shown).

Cells carrying *orc2-1* (Bell et al. 1993), *cdc45-1* (Zou et al. 1997), *dpb11-1* (Kamimura et al. 1998), *sld2-6* (Kamimura et al. 1998), and *sld3-5* (Kamimura et al. 2001) are defective in the initiation of DNA replication and begin to lose viability immediately after the cells start budding at the restrictive temperature. To determine the point at which the cells start losing viability, the viability and cell morphology of synchronized cells were examined. Both *sld5-12* and *psf1-1* mutants started losing viability when cells began to bud (Fig. 4B), like mutants defective in the initiation of DNA replication.

To examine the DNA replication defect in *sld5-12* and *psf1-1* cells in detail, an active origin, ARS1, was analyzed by neutral/neutral two-dimensional gel (N/N 2-D gel; Brewer and Fangman 1987). Wild-type, *sld5-12*, and *psf1-1* cells were grown to log phase at 23°C and shifted to 37°C for 3 h before harvest. At both 23°C and 37°C, the wild-type cells gave a clear transition signal from bubble to fork arcs, indicating that replication initiates in the ARS1 region. In contrast, a complete fork in addition to a bubble arc was observed in *sld5-12* and *psf1-1* cells at 23°C. Furthermore, the bubble arc signals were completely absent after temperature shift-up (Fig. 4C), suggesting the reduced frequency of initiation from ARS1.

The results described above, together with reduced associations between origins and the initiation proteins Cdc45 and Dpb11 in *psf1-1* cells (see below), suggest that GINS participates in the initiation step of chromosomal DNA replication, although its involvement in the elongation step is ambiguous.

Cells lacking DNA replication proteins are arrested with a dumbbell shape in the mitotic cell cycle, as well as after germination from spores. After germination from spores, deletion mutants for each of *SLD5*, *PSF1*, *PSF2*, and *PSF3* show the same morphologies, consistent with the formation of a complex by these gene products. However, the morphologies of those deletion mutants during germination are not typical for mutants defective in DNA replication; they appear as swelling spherical cells with small buds, split spheres, or lysed spheres, or dumbbell shapes, suggesting that these cells have a defect other than in DNA replication. In contrast to germinated cells, cells with a dumbbell shape that is typical for mutants defective in DNA replication accumulated in the mitotic cell cycle when the Psf1 plasmid was lost from YYK41 [ $\Delta psf1$  (YEpl95-*PSF1*)] cells during shuffling (data not shown). Moreover, both *psf1-1* and *sld5-12* cells are arrested in the cell cycle with a dumbbell shape. Therefore, GINS seems to participate in biological processes other than DNA replication in germination from spores.

### *Psf1 associates with origins in S phase*

Because it had been suggested that Psf1 is required for the early step of DNA replication, we examined whether the Psf1 protein associates with the ARS region in vivo using a chromatin immunoprecipitation (ChIP) assay (Strahl-Bolsinger et al. 1997). We used PCR primers to amplify ARS1, ARS305, ARS501, and their neighboring regions (Fig. 5A; Tanaka and Nasmyth 1998). ARS1 and ARS305 fire early in S phase, and ARS501 fires late in S phase (Ferguson et al. 1991).

When we used cells carrying 6FLAG-*PSF1* and anti-Flag antibody, the ARS1 fragment was specifically amplified (Fig. 5B). However, no significant amplification of the ARS1 fragment was observed when we used cells without 6Flag-Psf1 (Fig. 5B), suggesting that the ChIP assay is Flag-tag-dependent. Moreover, the ARS1 fragment in cells bearing a mutation in ARS1 was not amplified, whereas the ARS305 fragment was observed (Fig. 5C). This result indicates that Psf1 associates with functional origins.

Cells bearing 6FLAG-*PSF1* were arrested in G1 phase with  $\alpha$ -factor and released at 16°C to slow replication fork movement. The ChIP assay revealed that Psf1 associates with early origins, ARS1 and ARS305, from 60 min after cells are released from  $\alpha$ -factor and with late origin, ARS501, from 75 min after release (Fig. 5E). Later, Psf1-association with non-ARS regions near the origins was also observed. Therefore, it is suggested that GINS associates with replication origins in S phase.

### *Sld3 is required for the association of Psf1 with origins*

Sld3, which forms a complex with Cdc45, associates with early-firing origins from G1 phase (Kamimura et al. 2001). Therefore, we examined whether Sld3 is required for the association of Psf1 with origins. To slow the movement of replication fork and to prolong the period during which Psf1 might associate with origins,  $\alpha$ -factor-arrested cells were released in medium containing 0.15 M HU at 33°C. In wild-type cells, 6Flag-Psf1 associated with ARS1 and ARS305 30 min after release from  $\alpha$ -factor (Fig. 5F). In contrast, no significant association was detected in *sld3-5* cells at the restrictive temperature (Fig. 5F). These results suggest that Sld3 is required for the association of Psf1 with origins.

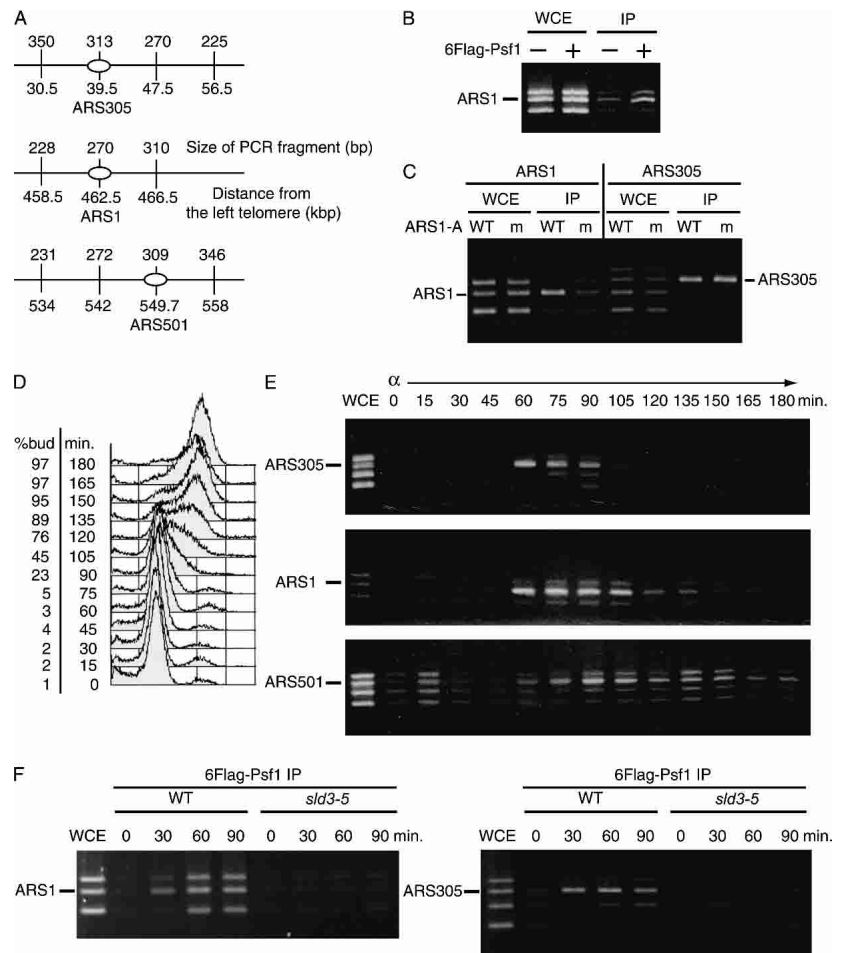
### *Cdc45 chromatin-binding step is impaired in psf1-1 cells*

The Cdc45 protein binds to chromatin in S phase. Because Psf1 associates with origins in S phase (Fig. 5), we examined whether the chromatin-binding of Cdc45 is affected by the *psf1-1* mutation.

*psf1-1* and wild-type cells were released from the G1 block and further incubated at 37°C to start the cell cycle. Budding occurred in these two strains with almost the same timing (date not shown). At 75 min, chromatin-bound Cdc45 in *psf1-1* cells was less abundant than in wild-type cells. Because it was reduced by DNase I treat-

Takayama et al.

**Figure 5.** Association of Psf1 with ARS regions. (A) Genomic intervals near or at ARSs amplified by PCR primers. (B) Flag-Psf1 specifically associated with ARS1 *in vivo*. YYK9 (tag-) or YYT2 (6Flag-Psf1) cells were grown in YPAR at 23°C. Immunoprecipitation (IP) was performed with anti-Flag antibody. PCR was performed on chromatin fragments isolated after IP or on those from the whole cell extracts (WCE). (C) Flag-Psf1 specifically associated with functional ARS1. YYT2 (6Flag-Psf1, ARS1-A; WT) or YYK51 (6Flag-Psf1, ARS1-A/860T → G; m) cells were arrested in G1 phase by  $\alpha$ -factor at 23°C for 3 h and released in YPAR containing 0.15 M HU at 23°C. Cells were withdrawn after 30 min. 6Flag-Psf1 was immunoprecipitated from each extract with an anti-Flag antibody. PCR was performed on immunoprecipitates derived from the same number of cells. (D) DNA content of synchronized cells used for the ChIP assay (E) was measured by FACS analysis. The percentage of budded cells is also shown. (E) Association of Flag-Psf1 with the ARS1, ARS305, or ARS501 regions. YYT2 cells were arrested in G1 phase with  $\alpha$ -factor and released in YPAD medium at 16°C. Cells were withdrawn from the culture every 15 min and fixed with formaldehyde. Cell lysates were sonicated and used for immunoprecipitation. PCR was performed either on immunoprecipitates (IP) derived from the same number of cells at each time point or on the 0-min chromatin fraction from whole cells extracts (WCE). (F) Association of Psf1 with the origin depends on Sld3. YYT2 (6Flag-Psf1; WT) and YYT3 (6Flag-Psf1; *sld3-5*) cells were arrested in G1 phase by  $\alpha$ -factor at 23°C for 3 h and released in YPAR containing 0.15 M HU at 33°C. Cells were withdrawn from the culture every 30 min. 6Flag-Psf1 was immunoprecipitated from each extract with an anti-Flag antibody. PCR was performed on immunoprecipitates derived from the same number of cells at each time point.



ment, Cdc45 in the pellet (P) fraction from the wild-type cells was associated with chromatin. In contrast, DNase I treatment did not reduce the amount of Cdc45 in the P fraction from *psf1-1* cells (Fig. 6). Furthermore, we did not detect chromatin-bound Cdc45 in *psf1-1* cells for 105 min after their release from  $\alpha$ -factor (data not shown). Therefore, Psf1 is required for the chromatin-binding of Cdc45. Because the association of Psf1 with origins depends on Sld3, which is essential for Cdc45 to function (Kamimura et al. 2001), the chromatin-binding of Cdc45 and the association of Psf1 with origins are mutually dependent.

#### Associations of Psf1 and Dpb11 with origins are mutually dependent

Dpb11, which is required for the association of DNA polymerases  $\alpha$  and  $\epsilon$  with origins, associates with replication origins at the same time as Psf1 (Fig. 5E; Masumoto et al. 2000). Therefore, we examined whether the association of Psf1 with replication origins depends on

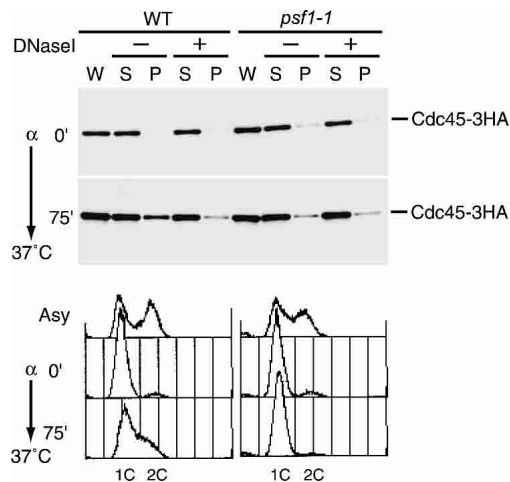
Dpb11. As shown in Figure 7A, the *dpb11-26* mutation, which is a tighter allele isolated recently, reduced the specific signals for the association of 6Flag-Psf1 with ARS1 and ARS305 in the ChIP assay, indicating that Dpb11 is required for the proper association of Psf1 with origins.

Next, we examined whether Psf1 is required for the association of Dpb11 with replication origins. As shown in Figure 7B, specific signals indicating the association of Dpb11-9myc with ARS1 and ARS305 in *psf1-1* cells were reduced significantly, implying that the association of Dpb11 with origins depends on Psf1. Therefore, we conclude that the associations of Psf1 and Dpb11 with origins are mutually dependent.

#### Discussion

In this study, we identified a novel protein complex, GINS, using genetic and biochemical methods. A similar complex has been identified in *Xenopus* egg extracts, in which anti-Sld5 antibodies depleted Psf1, Psf2, and Psf3,





**Figure 6.** Cdc45 association with chromatin is reduced in the *psf1-1* mutant cells. YYK20 (WT) and YYT4 (*psf1-1*) cells expressing Cdc45-3HA were synchronized in G1 phase by  $\alpha$ -factor and released at 37°C. The cells were collected at the  $\alpha$ -factor block or 75 min after release. Chromatin-binding assay was performed as described (Kamimura et al. 2001). The proteins present in the different fractions of the chromatin purification were examined by immunoblotting of SDS-PAGE: W, whole cell 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.

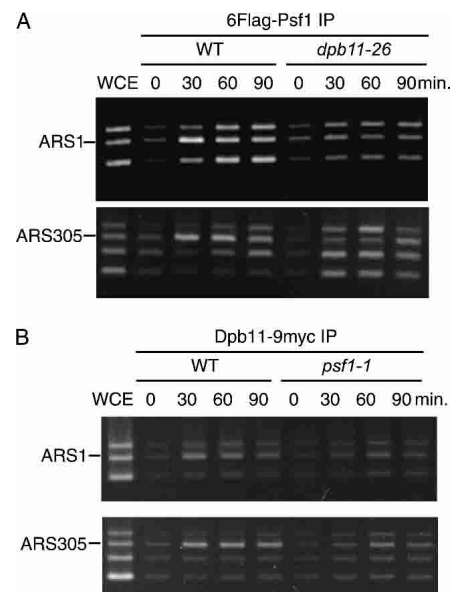
suggesting that these proteins exist as a complex (Kubota et al. 2003). This is also true for yeast GINS, because almost all of Sld5, Psf2, and Psf3 are depleted with 6Flag-Psf1 by anti-Flag antibody (Y. Kamimura and H. Araki, unpubl.). Further, we suggest that each protein functions through GINS, because high-copy *PSF2* restored the growth and complex formation in *sld5-12* cells (Figs. 2A, 3C). We therefore discuss the biological roles of GINS inferred from the results obtained using *sld5* and *psf1* mutants.

#### Assembly of replication proteins at replication origins

To initiate DNA replication, many replication proteins assemble on replication origins (for review, see Bell and Dutta 2002; Nishitani and Lygerou 2002). In *S. cerevisiae*, the pre-RC is formed on replication origins from late M phase to G1 phase. The Sld3-Cdc45 complex also associates with origins, and its association depends on the pre-RC. When S-Cdk and Cdc7 protein kinases are activated at the G1-S-phase boundary, DNA polymerases are recruited to origins to initiate DNA synthesis, and this step requires the function of Dpb11. Dpb11 forms a complex with both Sld2 and Pol  $\epsilon$ , and associates with origins, which is required for further associations between Pol  $\alpha$ -primase and origins (Masumoto et al. 2000). In the present study, we showed that Psf1 interacts with Dpb11, Dpb2, and Sld3, using a two-hybrid assay. It is therefore conceivable that GINS mediates the interaction between the Cdc45-Sld3 and Dpb11-Pol  $\epsilon$

complexes and thus promotes the association of the DNA polymerases with origins.

Interestingly, Dpb11 and Psf1 associate with origins interdependently (Fig. 7). This result does not support the idea that Psf1 and Dpb11 are recruited in a sequential manner, but rather suggests that they associate with origins concomitantly. Therefore, it is conceivable that once the macromolecular complex containing Dpb11, Sld2, Pol  $\epsilon$ , and GINS is formed at the G1-S-phase boundary, this complex associates with origins. Because the formation of the complex between Dpb11 and Sld2 is required for the initiation of DNA replication and is regulated positively by S-Cdk activity (Masumoto et al. 2002), the formation of the macromolecular complex may be regulated by S-Cdk through Sld2 phosphorylation. In *Xenopus* egg extracts, Cut5, the counterpart of Dpb11, associates with chromatin without GINS (Kubota et al. 2003). This apparent discrepancy is presumably attributable to the detection procedures for chromatin binding: the ChIP assay in yeast and chromatin fractionation in *Xenopus* extracts. A possible explanation that reconciles these data is that Dpb11/Cut5 associates with chromatin in the absence of GINS, whereas it requires GINS to associate with replication origins.



**Figure 7.** Associations of Psf1 and Dpb11 with origins are mutually dependent. (A) Association of Psf1 with origins requires Dpb11. YYT2 (6Flag-Psf1; WT) and YYK42 (6Flag-Psf1; *dpb11-26*) cells were arrested in G1 phase by  $\alpha$ -factor at 23°C for 3 h and released in YPAR containing 0.15 M HU at 33°C. Cells were withdrawn from the culture every 30 min. 6Flag-Psf1 was immunoprecipitated from each extract with an anti-Flag antibody. (B) Association of Dpb11 with origins requires Psf1. YHM011 (Dpb11-9myc; WT) and YYK43 (Dpb11-9myc; *psf1-1*) cells were arrested in G1 phase with  $\alpha$ -factor at 23°C for 3 h and released in YPAR containing 0.15 M HU at 34°C. Cells were withdrawn from the culture every 30 min. PCR was performed on immunoprecipitates derived from the same number of cells at each time point.

Takayama et al.

*Proteins associated with DNA replication forks*

ChIP assays have shown that Mcm, Cdc45, Sld3, and Pol  $\epsilon$  associate first with origins and then with their neighboring fragments (Aparicio et al. 1997; Masumoto et al. 2000; Kamimura et al. 2001). These observations suggest that these proteins move from origins to neighboring regions together with replication fork progress. They further suggest that these proteins participate in the elongation step of DNA replication. It is consistent that, under nonpermissive conditions after release from HU block that stalls replication forks, mutations in these proteins result in defective DNA replication. In the present study, we also observed that Psf1 associates with both the ARS fragment and with fragments surrounding the ARS [Figs. 5, 7A (WT)].

In contrast with the observation described above, *psf1-1* cells completed DNA synthesis and entered the subsequent phase of the cell cycle after release from HU block at the restrictive temperature, although S phase progressed very slowly. This phenomenon might be explained in two ways. Firstly, *psf1-1* cells are defective in late-origin firing, and the consequent reduced number of replication forks caused the slow progression of S phase. Secondly, the elongation step may be partially defective in *psf1-1* cells. We cannot yet discriminate between these two possibilities. In the case of Mcm and Cdc45, conditional degron mutants revealed their essentiality for the elongation step, whereas point mutations do not give a clear defect (Labib et al. 2000; Tercero et al. 2000). Sld5, Psf1, and Psf2 were recently found to be defective in DNA replication by genome-wide degron screening in which uncharacterized essential ORFs were fused to degrons. Moreover, the degron versions of Sld5 and Psf2 did not progress through the cell cycle at the restrictive temperature after release from HU block (the Psf1-degion is not tight enough to use in this experiment; M. Kanemaki, A. Sanchez-Diaz, and K. Labib, pers. comm.). Therefore, the *psf1-1* mutation may not be appropriate for the analysis of Psf1 function in the elongation step of chromosomal DNA replication, and it is likely that GINS also functions in the step(s) other than the initiation of DNA replication.

GINS requires Dpb11 for its association with origins. However, although it associates with origins, Dpb11 does not move with replication forks but dissociates from origins soon after its function. Therefore, the interaction between Dpb11 and GINS must be transient and limited to the assembly process. This is consistent with our failure to coimmunoprecipitate Dpb11 with Psf1 or Pol  $\epsilon$ . It should be noted that the Dpb11–Pol  $\epsilon$  complex is only detected using cross-linking reagent.

GINS purified from *Xenopus* egg extract showed a ring-like structure under electron microscope (Kubota et al. 2003). The ring-like shape of GINS may allow it to encircle the DNA as a clamp for the replication proteins during the elongation step of DNA replication, like a PCNA clamp. Considering the strong genetic interactions between Pol  $\epsilon$  and Dpb11, and between Dpb11, Sld5, and Psf1, GINS may work as the clamp for Pol  $\epsilon$ .

From the interactions between the genes encoding these components (Fig. 2C), we may predict the arrangement of these components in GINS. Because *sld5-12* is suppressed by high-copy *PSF1* and *PSF2* but not by *PSF3*, and because *psf1-1* is suppressed by *SLD5* and *PSF3* but not by *PSF2*, Sld5 seems to be sandwiched between Psf1 and Psf2, and Psf1 between Psf3 and Sld5. Therefore, we propose that they are arranged Psf2:Sld5:Psf1:Psf3. Consistent with this, a genome-wide two-hybrid analysis using Sld5 as bait also identified Psf1 and Psf2 but not Psf3 (Uetz et al. 2000; Ito et al. 2001). Furthermore, Psf1 coimmunoprecipitated with Sld5 and Psf2 but not with Psf3 under stringent conditions (Y. Kamimura and H. Araki, unpubl.).

Sld5 interacts weakly with Pol  $\delta$ , because the deletion of Pol 32, a nonessential subunit of Pol  $\delta$ , enhances the growth defect of *sld5-12* (Ohya et al. 2002). This raises the possibility that GINS also functions for Pol  $\delta$  as well as Pol  $\epsilon$ . In *Escherichia coli*, the  $\tau$  subunit of the replicative DNA polymerase, Pol III, connects two polymerases at the replication fork to coordinate leading- and lagging-strand synthesis (Kim et al. 1996). To date, no counterpart of the  $\tau$  subunit has been found in eukaryotic cells. Because Sld5 interacts with two replicative polymerases, GINS may coordinate leading- and lagging-strand synthesis, like the  $\tau$  subunit of Pol III.

*Conservation of replication proteins in eukaryotes*

Many replication proteins are conserved from yeast to human cells. This suggests that the replication mechanism of eukaryotes is well conserved. However, candidate homologs of Dpb11, Sld2, and Sld3 have not yet been identified in higher eukaryotes, whereas fission yeast has counterparts to all these proteins. Therefore, the amino acid sequences of these proteins may have diversified from yeast to higher eukaryotes. TopBP1 in human cells and Mus101 in *Drosophila* contain eight and seven BRCT repeats, respectively, and are thought to be functional homologs of Dpb11 because they are involved in DNA replication and repair (Yamamoto et al. 2000; Mäkinen et al. 2001). Therefore, we expect that the other two proteins, Sld2 and Sld3, have also diversified during the evolution from yeasts to higher eukaryotes and that their functional homologs will soon be found.

The diversity of these proteins probably reflects the regulatory mechanisms of DNA replication. For example, micro-organisms, including yeasts but not higher eukaryotes, are very sensitive to nutrient conditions in culture media, and regulate their cell cycle and consequently their DNA replication by responding to nutrient levels. In this context, these proteins act closely with the regulatory machinery of the cell cycle. In fact, the interaction between Dpb11 and Sld2 is regulated by the phosphorylation of Sld2 by Cdk (Masumoto et al. 2002). Moreover, *Schizosaccharomyces pombe* Sld3 (SpSld3) is also hyperphosphorylated in G2/M phase and hypophosphorylated in S phase, although the meaning of this phosphorylation has not been elucidated (Nakajima and

Masukata 2002). In contrast with these proteins, highly conserved proteins may work as the replication machinery itself. The proteins reported here are also highly conserved. We therefore anticipate that GINS works as one of the components of highly conserved replication machinery, for example, together with the DNA polymerases. Future biochemical studies will clarify this point.

## Materials and Methods

### Micro-organisms

Yeast strains used in this study are listed in Supplementary Table 1. *E. coli* DH5 $\alpha$  and BL21(DE3) were used for plasmid propagation and recombinant protein production, respectively.

### Plasmid construction

YCp22-*SLD5* and YEpl95-*SLD5* were constructed by subcloning the 1.8-kb *NheI*-*NdeI* *SLD5* DNA fragment into the *XbaI*-*SmaI* sites of YCplac22 and YEplac195 (Gietz and Sugino 1988), respectively. YCp22-*SLD5* was used as a template to engineer an *NdeI* site at the first methionine codon and an *XhoI* site after the stop codon by PCR. The PCR product was cloned into the *NdeI*-*XhoI* sites of pET15b. YIp211-GST-*SLD5* was constructed as follows: first, YCp22-*SLD5* was mutagenized by PCR, with the resultant plasmid containing an *EcoRI*-*BamHI* site before the first methionine of *Sld5*. The GST fragment was cloned into the *EcoRI*-*BamHI* sites, and the *KpnI*-*HindIII* fragment of YCp22-*SLD5* was cloned into *KpnI*-*HindIII* of the resultant plasmid. This plasmid was digested with *HpaI* before transformation into yeast. YCp22-*PSF1* and YEpl95-*PSF1* were constructed by subcloning the 1.6-kb *KpnI*-*EcoRI* *PSF1* DNA fragment into the *KpnI*-*EcoRI* sites of YCplac22 and YEplac195, respectively. pET15b-*PSF1* was constructed as follows: YCp22-*PSF1* was used as a template to engineer an *NdeI* site at the first methionine codon and an *XhoI* site after the stop codon by PCR. These PCR products were cloned into the *NdeI*-*XhoI* sites of pET15b. YIp211-6FLAG-*PSF1* was constructed by stepwise insertion of FLAG and 5FLAG fragments into the translation initiation site and truncation of the C-terminal portion of *PSF1* by *HpaII* cleavage. This plasmid was digested with *SnaBI* before transformation. To clone the *PSF2* gene, YJL072c was amplified by PCR using oligonucleotides 5'-ACGGTCCTAGCTTCAAA TGTGATATCTTTC-3' and 5'-TAACATGTTTCAGATATCCA GGAAGTGGC-3'. This PCR product was digested with *EcoRV* and cloned into the *EcoRV* site of pBlueScript KS<sup>+</sup> (pBS-*PSF2*). YCp22-*PSF2* and YEpl95-*PSF2* were constructed by subcloning the 1.0-kbp *PstI*-*SalI* *PSF2* fragment into the *PstI*-*SalI* sites of YCplac22 and YEplac195, respectively. All of the DNA inserts amplified by PCR were sequenced to confirm that there were no mutations in the PCR products.

### Disruption of the *SLD5*, *PSF1*, *PSF2*, and *PSF3* genes

The *NdeI*-*PvuII* *SLD5* fragment was cloned into the *XbaI*-*SmaI* sites of pBlueScript SK<sup>+</sup>. The *SalI*-*NdeI* fragment of *SLD5* was replaced with the *LEU2* fragment isolated from YDp-L (Berben et al. 1991). The resultant plasmid was cleaved with *PvuII* and used to transform W303-1A/1B cells to disrupt the *SLD5* gene.

The *StuI*-*EcoRI* *PSF1* fragment was subcloned into the *EcoRV*-*EcoRI* sites of pBlueScript SK<sup>+</sup>. The *BglIII* fragment of *PSF1* was replaced with the *LEU2* fragment isolated from YDp-L (Berben et al. 1991). The resultant plasmid was digested with *HindIII* and *PstI*, and introduced into W303-1A/1B cells.

*BamHI* sites were introduced just upstream and downstream from the *PSF2* ORF using PCR. The *BamHI* fragment of *PSF2* was replaced by the *LEU2* fragment isolated from YDp-L. The resultant plasmid was digested with *PstI* and *SalI* and introduced into W303-1A/1B cells.

To disrupt *PSF3*, the 600-bp upstream and 700-bp downstream fragments of *PSF3* amplified by PCR were cloned into pBlueScript SK<sup>+</sup>, and the *LEU2* fragment was inserted between them. The resultant plasmid was digested with *BglIII* and introduced into W303-1A/1B cells.

Southern blot analysis was performed on the Leu<sup>+</sup> transformants to confirm that one copy of the endogenous gene was successfully disrupted.

### Isolation of thermosensitive mutants

*SLD5* was amplified using PCR with *Taq* DNA polymerase, and used to transform YYK35 [*sld5Δ::LEU2* (YEpl95-*SLD5*)] cells with *PstI*- and *SalI*-cleaved YCplac22 (Muhrad et al. 1992). Approximately 7400 transformants grown at 23°C on Ura<sup>+</sup>Trp<sup>-</sup> plates were replica-plated onto two plates containing 0.1% 5-fluoroorotic acid (5-FOA). One plate was incubated at 23°C and the other at 37°C. Four clones showed temperature-sensitive growth. Plasmid DNA was recovered from each of them, and the DNA sequence of the *SLD5* portion was determined (Fig. 1A). The *EcoRI*-*PstI* fragment of the plasmid was cloned into Yiplac211 and was used to transform the YYK9 (W303-1Ab) strain after digestion with *HpaI*. Ura<sup>+</sup> transformants were grown at 23°C, spread onto 5-FOA plates, and a temperature-sensitive colony in which *URA3* is popped out was selected.

The procedures were the same for *PSF1* as those described for *SLD5*, with slight modification (below). YYK41 [*psf1Δ::LEU2* (YEpl95-*PSF1*)] was used. Of about 1500 transformants, three clones showed thermosensitive growth. One clone was found to have a single point mutation, and was designated *psf1-1*. The other two clones had two point mutations, one of which was the same as that occurring in *psf1-1*; the other was a silent mutation. The *KpnI*-*EcoRI* fragment encoding the *psf1-1* mutation was cloned into Yiplac211 and was used to transform strain YYK9 (W303-1Ab) after digestion with *Tth111I*. Ura<sup>+</sup> transformants were grown at 23°C, spread onto 5-FOA plates, and a temperature-sensitive colony was selected in which *URA3* is popped out.

### Multicopy suppressor screening

A YEpl24-based genomic library was transformed into *sld5-12* and *psf1-1* mutants. Cells were incubated at 23°C for a day on SD-Ura plates; incubation temperatures were shifted up to 30°C for *sld5-12* and 34°C for *psf1-1*. Of the *sld5-12* cells, 15 transformants grew at 30°C from about 20,000 Ura<sup>+</sup> transformants. Plasmid DNA was recovered from each of them, and the DNA sequences were determined. Twelve clones were found to carry *SLD5*, and the remaining three clones carried *PSF1*. Of the *psf1-1* cells, three transformants grew at 34°C from 7680 Ura<sup>+</sup> transformants. One clone was found to carry *PSF1*, and the other two clones carried *PSF3*.

### Immunoprecipitation

Cells (5 × 10<sup>9</sup>) were harvested, washed once with water, and resuspended in 0.8 mL lysis buffer [50 mM HEPES-KOH at pH 7.5, 300 mM KCl, 0.05% Tween-20, 0.005% NP-40, 10% glycerol, 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim), 1% protease inhibitor (Sigma), 2 mM  $\beta$ -glycerophosphate, 2 mM NaF, 0.4 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM Na-pyrophos-

Takayama et al.

phate] containing 5 mg/mL bovine serum albumin (BSA). Cells were disrupted with glass beads using a bead beater. Cell lysates were clarified by centrifugation for 20 min at 4°C. Protein extracts were adsorbed onto 0.5 mL (75% vol/vol slurry) of sepharose 4B Fast Flow (Pharmacia) for 30 min at 4°C. The beads were then pelleted, and the supernatant was recovered and mixed with 100  $\mu$ L anti-Flag (M2) beads (Sigma) for 5 h at 4°C. The beads were washed three times with lysis buffer containing 0.1 mg/mL BSA, then washed three times with 1 mL cold lysis buffer. The immunocomplex was eluted by incubating the beads with lysis buffer containing 100  $\mu$ g/mL 3 $\times$ Flag peptides (Sigma).

#### Preparation of antibodies

Sld5 and Psf1 proteins were expressed in *E. coli* BL21(DE3) cells and were recovered in the insoluble fraction after disruption of the cells. The proteins were either solubilized (Sld5) or separated on SDS-PAGE (Psf1), and then used to immunize rabbits.

#### Other methods

Gel filtration of 6Flag-Psf1 immunoprecipitates was performed using a Superdex 200 column (Amersham Pharmacia Biotech) equilibrated in lysis buffer (described above). The chromatin-binding assay was performed as described (Kamimura et al. 2001), except that spheroplasts were lysed in 0.2 M sorbitol buffer at the final step. Synchronization of yeast cells, N/N 2-D gel analysis, the two-hybrid assay, and ChIP assay were performed as described (Kamimura et al. 1998, 2001).

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## **GINs, a novel multiprotein complex required for chromosomal DNA replication in budding yeast**

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