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Ordered assembly of Sld3, GINS and Cdc45 is distinctly regulated by DDK and CDK for activation of replication origins

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Initiation of chromosome DNA replication in eukaryotes is tightly regulated through assembly of replication factors at replication origins. Here, we investigated dependence of the assembly of the initiation complex on particular factors using temperature-sensitive fission yeast mutants. The *psf3-1* mutant, a GINS component mutant, arrested with unreplicated DNA at the restrictive temperature and the DNA content gradually increased, suggesting a defect in DNA replication. The mutation impaired GINS complex formation, as shown by pull-down experiments. Chromatin immunoprecipitation assays indicated that GINS integrity was required for origin loading of Psf2, Cut5 and Cdc45, but not Sld3. In contrast, loading of Psf2 onto origins depended on Sld3 and Cut5 but not on Cdc45. These results suggest that Sld3 functions furthest upstream in initiation complex assembly, followed by GINS and Cut5, then Cdc45. Consistent with this conclusion, Cdc7-Dbf4 kinase (DDK) but not cyclin-dependent kinase (CDK) was required for Sld3 loading, whereas recruitment of the other factors depended on both kinases. These results suggest that DDK and CDK regulate distinct steps in activation of replication origins in fission yeast.

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Introduction

Initiation of DNA replication is a dynamic reaction regulated during the cell cycle in eukaryotic cells. This is achieved through ordered assembly and disassembly of a number of replication factors onto specific chromosome loci, called replication origins (Bell and Dutta, 2002; Kearsey and Cotterill, 2003). The origin recognition complex (ORC) binds to replication origins throughout the cell cycle, at least in yeasts, although the mechanisms of recognition of origins may differ among organisms (Gilbert, 2001). When cells exit from mitosis, the mini-chromosome maintenance (MCM) complex binds to replication origins, dependent on ORC, Cdc6/Cdc18 and Cdt1, forming pre-replicative complexes (pre-RCs) (Diffley *et al*, 1994). Activation of pre-RCs requires further recruitment of several factors in S phase, including Cdc45 and Sld3, dependent on cyclin-dependent protein kinase (CDK) and Cdc7-Dbf4 protein kinase (Dbf4-dependent kinase, DDK) (Takeda and Dutta, 2005). This process is considered to activate the DNA helicase of MCM complex, resulting in origin DNA unwinding and assembly of RPA and DNA polymerases for initiation of DNA synthesis (Bell and Dutta, 2002).

Many of the replication factors involved in activation of replication in S phase have been identified through genetic screening in budding yeast. For example, *DPB11* has been isolated as a multicopy suppressor of a mutant of *DPB2* that encodes the second largest subunit of DNA polymerase ε (Araki *et al*, 1995). Three essential factors, Sld2, Sld3 and Sld5, have been identified by screening of synthetic lethal mutations with *dpb11-1* (Kamimura *et al*, 1998). Interaction of Sld2/Drc1 with Dpb11/Cut5/Rad4 is required for DNA replication in budding as well as fission yeasts (Masumoto *et al*, 2002; Noguchi *et al*, 2002). Sld3 has genetic and physical interactions with Cdc45 (Kamimura *et al*, 1998; Nakajima and Masukata, 2002). Sld5, Psf1 (partner of Sld five 1), Psf2 and Psf3 are components of a recently discovered Go, Ichi, Nii, and San; five, one, two, and three in Japanese (GINS) complex in budding yeast and *Xenopus* (Kanemaki *et al*, 2003; Kubota *et al*, 2003; Takayama *et al*, 2003). Although all these replication factors are essential for initiation of chromosome DNA replication, precise mechanisms of assembly of individual factors or functions in DNA replication have yet to be elucidated. In budding yeast, Sld3 and Cdc45 associate with early-firing replication origins in G1 phase in a mutually dependent manner (Kamimura *et al*, 2001), and the remaining factors bind to origins at the onset of S phase (Takayama *et al*, 2003). In contrast, fission yeast Sld3 is required for origin loading of Cdc45, but the reverse is not the case (Yamada *et al*, 2004). Relations of GINS with Sld3, Cut5 and Cdc45 in the process of origin binding remain unclear. In *Xenopus* extracts, chromatin binding of Cdc45, DNA Polε and DNA Polα depends on GINS, whereas chromatin association of Cut5 does not (Hashimoto and Takisawa, 2003; Kubota *et al*, 2003), suggesting that the precise processes of initiation complex formation differ among species. Thus, more information is required for understanding of the general scheme of initiation of replication.

Activation of replication origins at the onset of S phase requires two different serine/threonine kinases, CDK and Cdc7-Dbf4 kinase (DDK). S-phase CDK is required for tight association of Cdc45 with chromatin in budding yeast and *Xenopus* egg extracts (Mimura *et al*, 2000; Zou and Stillman,

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2000). Sld2 is the only protein, so far, whose phosphorylation by CDK is required for initiation of replication (Masumoto *et al*, 2002; Tak *et al*, 2006) and this requirement is conserved in fission yeast (Noguchi *et al*, 2002). Phosphorylation of Sld2/Drc1 is required for the interaction with Dpb11 (Masumoto *et al*, 2002). On the other hand, reactions regulated by DDK have not been identified, although it phosphorylates subunits of MCM and Cdc45 (Lei *et al*, 1997; Weinreich and Stillman, 1999; Nougarede *et al*, 2000; Takeda *et al*, 2001). The fact that the *bob1-1/mcm5* mutation bypasses the requirement for *CDC7* and *DBF4* in budding yeast replication (Hardy *et al*, 1997) suggests, however, that MCM complex phosphorylation by DDK does play an important role in initiation of replication.

In this study, we focused on dependence on replication factors in initiation complex formation, using a novel temperature-sensitive mutant for the GINS component. The results suggest that Sld3, GINS and Cdc45 act sequentially in initiation complex assembly. Consistent with this model, origin association of Sld3 depends on DDK but not on CDK, whereas recruitment of GINS and Cdc45 requires both DDK and CDK. Thus, DDK and CDK regulate different steps in initiation complex assembly.

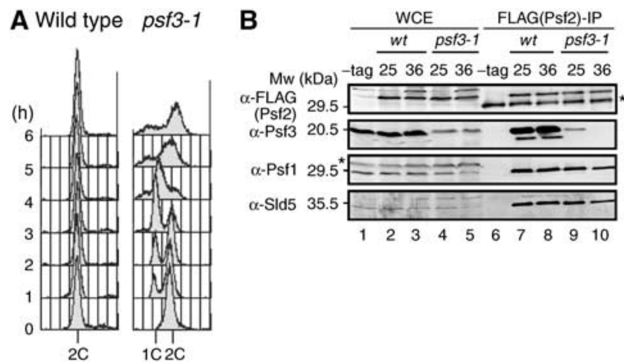

Figure 1 Defective chromosome replication owing to the absence of an intact GINS complex in the fission yeast *psf3* mutant. (**A**) Wild-type and *psf3-1* cells grown at 25°C in EMM medium were incubated at 36°C for the indicated time and DNA content was analyzed by flow cytometry. Positions of 1C and 2C DNA contents are indicated. (**B**) Untagged (−tag, lanes 1 and 6), *psf2-flag* (wt, lanes 2, 3 and 7, 8) and *psf2-flag psf3-1* (*psf3-1*, lanes 4, 5 and 9, 10) cells were grown at a permissive temperature (25°C) and portions were shifted to the restrictive temperature (36°C) for 2 h. Proteins in whole-cell extracts (WCE, lanes 1–5) and four times more amount of immunoprecipitates with anti-FLAG antibody (FL-IP, lanes 6–10) were separated by 15% polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-FLAG, Psf1, Psf3 and Sld5 antibodies. Positions of the expected molecular masses for Psf2-FLAG, Psf1, Psf3 and Sld5 along with those for molecular weight markers are indicated. The bands indicated by asterisks on anti-FLAG and anti-Psf1 staining are IgG light chain and a nonspecific protein, respectively.

Results

Requirement of GINS in early S phase

To elucidate the role of GINS in initiation complex formation upon onset of S phase, we isolated a temperature-sensitive (*ts*) mutant, *psf3-1*, by integration of randomly mutated *psf3* gene into the $psf3^{+}$ locus (see Materials and methods). To examine the possible defect of *psf3-1*, which alters the valine residue at position 56 into aspartic acid and the isoleucine at position 159 into leucine (V56D and I159L; Supplementary Figure S1), in DNA replication, wild-type and mutant cells grown at 25°C were shifted to 36°C and cellular DNA contents were analyzed by flow cytometry. The DNA contents of wild-type cells remained in 2C, owing to the long G2 phase in the fission yeast cell cycle (Figure 1A). In contrast, 1C DNA cells accumulated with *psf3-1* at 1–3 h and then the DNA content gradually increased at 4–5 h (Figure 1A), showing that DNA replication did not correctly initiate or proceed. A small fraction of cells with less than 1C DNA emerged at 4–6 h, probably resulting from cell division without completion of DNA replication. Cell viability of *psf3-1* was maintained for 2 h and then slowly decreased to about 60% by 6 h (Supplementary Figure S2). These results suggest that *psf3-1* cells arrest at a reversible step in DNA replication before they go into untimely mitosis.

GINS complex formation is impaired by psf3-1

For investigation of the effect of *psf3-1* mutation on DNA replication at the molecular level, we investigated interactions of FLAG-tagged Psf2 with other GINS components. FLAG-tagged derivatives of wild type (*psf2-flag*) and the mutant (*psf2-flag psf3-1*) were grown at 25°C or shifted to 36°C for 2 h and proteins in the cell extracts were analyzed by immunostaining with anti-FLAG antibody (Figure 1B, lanes 1–5). In case of wild type, a peptide migrating at around 30 kDa, slightly larger than the predicted molecular mass for Psf2-FLAG (23 kDa), was detected (Figure 1B, top panel, lanes 2 and 3), whereas the corresponding band was not present in the untagged strain (lane 1). By immunoprecipitation (IP) with FLAG-antibody, Psf2-FLAG was detected in *psf2-flag* but not untagged cells (Figure 1B, top panel, lanes 6–8). Immunostaining of IP products with anti-Psf3, Psf1 and Sld5 antibodies showed that Psf3 (around 20 kDa, Figure 1B, the second panel, lanes 7 and 8), Psf1 (around 29 kDa, the third panel) and Sld5 (around 35 kDa, the bottom panel) were co-precipitated with Psf2-FLAG. None of these components was detected in the IP fraction from the untagged cells (Figure 1B, lane 6). These results show that all GINS components form a complex.

In the *psf3-1* mutant, Psf3 detected by immunostaining was reduced both in the permissive and restrictive temperatures compared with those in wild type (Figure 1B, second panel, lanes 2–5). Notably, Psf3 was not co-immunoprecipitated with Psf2-FLAG at the restrictive temperature, although decreased but significant amount of Psf3 was detected in the IP fraction at the permissive temperature (Figure 1B, second panel, lanes 7–10). These results indicate that the intact GINS complex is not formed at the restrictive temperature, probably because the *psf3-1* mutant protein is unstable or defective in interaction with the other GINS components. It should be noted, however, that the amounts of Psf1 and Sld5 recovered in the Psf2-IP fraction from *psf3-1* were comparable to those in the wild type (lanes 7–10). These results suggest that Psf3 is not required for assembly of the remaining complex, although it is essential for the GINS function.

Integrity of the GINS complex is required for its localization at replication origins

To examine effects of the *psf3-1* mutation on association of GINS with replication origins, localization of Psf2-FLAG on chromosomes was analyzed by chromatin immunoprecipitation

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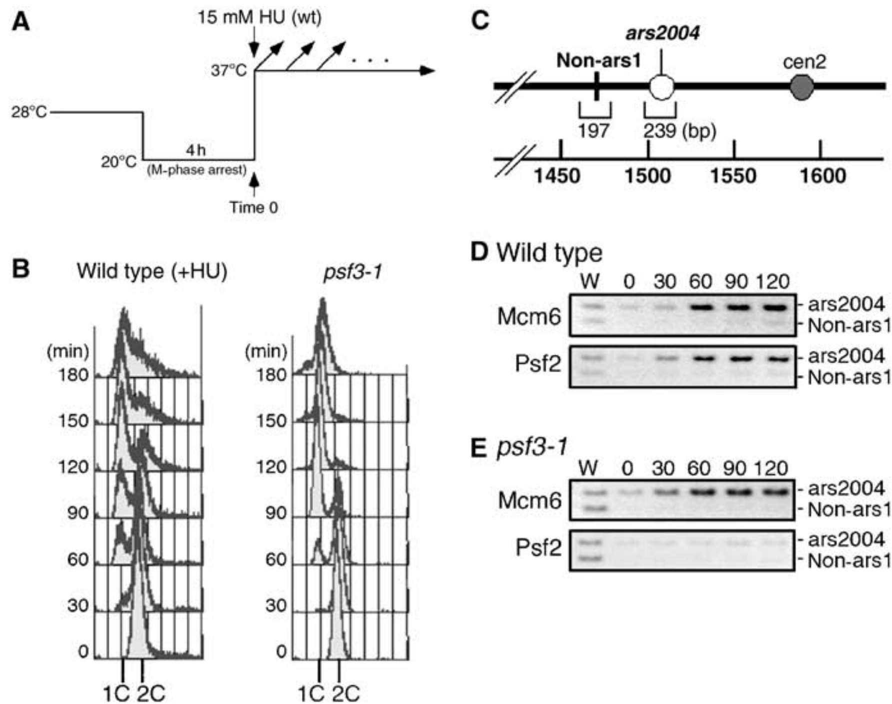


Figure 2 Origin association of GINS is impaired by *psf3* mutation. (**A**) The experimental scheme is shown. *psf2-flag nda3-KM311* (wild type) and *psf3-1 psf2-flag nda3-KM311* (*psf3-1*) cells were arrested at metaphase by incubation at 20°C for 4 h and then shifted to 37°C (time 0), with addition of hydroxyurea (HU, 15 mM) in the case of wild type. Aliquots of cultures taken at the indicated time points were analyzed by flow cytometry and ChIP assay. (**B**) DNA contents of wild type with HU and *psf3*-1 without HU were analyzed by flow cytometry. (**C**) Positions of *ars2004* and non-ars1 fragments, amplified by PCR in ChIP assay, are shown on the relevant portion of chromosome II. The distance (kb) from the left end of the chromosome and the length of the amplified fragment (bp) are indicated. (**D**) Immunoprecipitated DNA from wild-type extracts with anti-Mcm6 (upper panel) or with Psf2-FLAG (lower panel) at the indicated time points was used as template for PCR with *ars2004* (upper bands) and non-ars1 (lower bands) primers. PCR from the total cellular DNA prepared without IP yielded similar amplification of two fragments (lane W). (**E**) DNA immunoprecipitated from *psf3-1* cell extracts was analyzed as in (D).

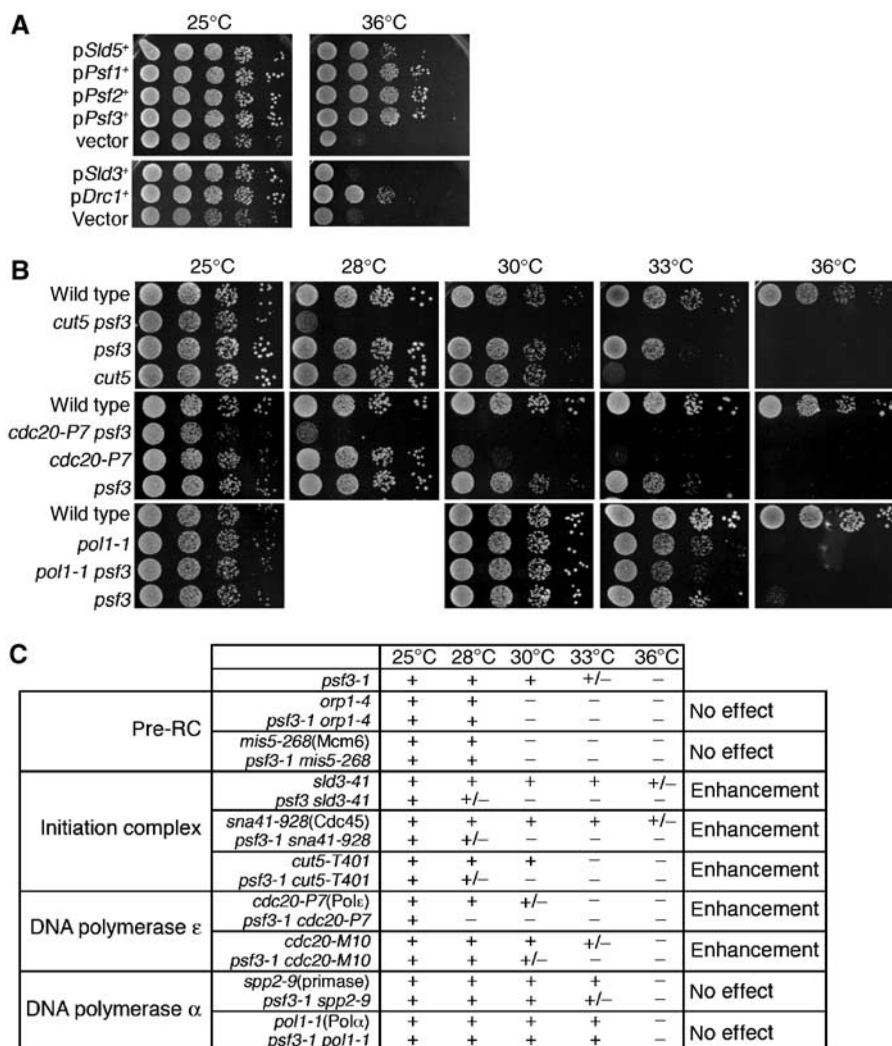
(ChIP) assay. For synchronization of the cell cycle, the cold-sensitive mutation *nda3-KM311*, which is defective for β-tubulin and causes metaphase arrest (Hiraoka *et al*, 1984), was employed to avoid premature inactivation of temperature-sensitive *psf3-1* mutation, and the arrested cells were released at the restrictive temperature for *psf3-1* (Figure 2A). Hydroxyurea, which depletes dNTP pools in cells, was added to wild-type cells at the release to detect transient localization of replication factors at replication origins more readily. In a later section, we have confirmed origin localization of factors in hydroxyurea-free wild-type cells (shown in Figure 6 and Supplementary Figure S3). Flow cytometry analysis showed that 1C DNA cells accumulated with both wild type (+HU) and *psf3* (−HU) (Figure 2B). The viability of *psf3-1* did not decrease for 120 min under these conditions, suggesting that cells were arrested at a reversible stage (Supplementary data and Supplementary Figure S2).

We first examined localization of Mcm6 and Psf2-FLAG in wild-type cells released from M phase and subsequently arrested with HU. Two sets of primers, amplifying the *ars2004* locus, an efficient replication origin, and non-ars1, 30 kb away from the origin (Figure 2C), generated similar amounts of PCR products from total cellular DNA without IP (Figure 2D, lane W as whole-cell extract). With Mcm6-IP, the *ars2004* fragment was preferentially enriched at 60–120 min period (Figure 2D, upper bands), whereas the non-ars1 fragment was not (Figure 2D, lower bands), consistent with G1–S-specific localization of MCM at replication origins (Ogawa *et al*, 1999). IP with Psf2-FLAG yielded amplification of the *ars2004* fragment in similar periods with Mcm6-IP (Figure 2D, lower panel). In a control experiment using immunoprecipitated DNA from untagged cells, neither fragment was significantly amplified (data not shown). IP of Psf2-FLAG from synchronized cells without HU yielded preferential recovery of the *ars2004* fragment (Supplementary Figure S3). These results show that Psf2 binds to the replication origin with a similar timing as Mcm6.

In the *psf3-1* mutant released at the restrictive temperature without HU, the *ars2004* fragment was enriched at 30–120 min with Mcm6-IP (Figure 2E, upper panel), showing that MCM was localized at the origin as in the HU-arrested wild type. In contrast, the *ars2004* fragment was not preferentially enriched by Psf2-IP at any time point (Figure 2E, lower panel), indicating that the origin association of Psf2 was abolished by the *psf3-1* mutation. Taken together with the results shown in Figure 1B, the integrity of GINS complex is required for association with replication origin. We confirmed that localization of Psf2 at another active replication origin *AT2024* (Segurado *et al*, 2003) was also impaired by the *psf3-1* mutation (Supplementary Figure S4).

Genetic interactions of $psf3^+$ with initiation factors

To identify replication factors that function in close relation with Psf3, we examined suppression of temperature sensitivity of *psf3-1* by increasing expression of the gene cloned on a multicopy plasmid. On incubation at the restrictive temperature after serial dilution, the temperature sensitivity was suppressed by $sld5^+$, $psf1^+$ and $psf2^+$, which encode



		25°C	28°C	30°C	33°C	36°C	
	psf3-1	+	+	+	+/–	–	
Pre-RC	*orp1-4*	+	+	–	–	–	No effect
	psf3-1 orp1-4	+	+	–	–	–	
	mis5-268(Mcm6)	+	+	–	–	–	No effect
	psf3-1 mis5-268	+	+	–	–	–	
Initiation complex	*sld3-41*	+	+	+	+	+/–	Enhancement
	psf3 sld3-41	+	+/–	–	–	–	
	sna41-928(Cdc45)	+	+	+	+	+/–	Enhancement
	psf3-1 sna41-928	+	+/–	–	–	–	
	cut5-T401	+	+	+	–	–	Enhancement
	psf3-1 cut5-T401	+	+/–	–	–	–	
DNA polymerase ε	*cdc20-P7*(Polε)	+	+	+/–	–	–	Enhancement
	psf3-1 cdc20-P7	+	–	–	–	–	
	cdc20-M10	+	+	+	+/–	–	Enhancement
	psf3-1 cdc20-M10	+	+	+/–	–	–	
DNA polymerase α	*spp2-9*(primase)	+	+	+	+	–	No effect
	psf3-1 spp2-9	+	+	+	+/–	–	
	pol1-1(Polα)	+	+	+	+	–	No effect
	psf3-1 pol1-1	+	+	+	+	–	

Figure 3 Genetic interactions of *psf3* with initiation complex components. (**A**) Gene-dosage suppression of the temperature sensitivity of *psf3-1*. Aliquots of *leu1-32 psf3-1* cells harboring the p940 vector alone, p940-*sld5*$^+$, p940-*psf1*$^+$, p940-*spf2*$^+$, p940-*psf3*$^+$, p940-*sld3*$^+$ and p940-*drc1*$^+$ were spotted on EMM plates after 10-fold serial dilution and incubated at permissive (25°C) and restrictive (36°C) temperatures. (**B**) Growth of double mutants. Ten-fold serial dilutions of indicated genotype cells were spotted onto YE plates followed by incubation at the indicated temperatures. The alleles used were *psf3-1*, *cut5-T401*, *cdc20-P7* (Polε) and *pol1-1* (Polα). (**C**) Summary of growth of double mutants. Growth of double mutants was examined as in (B). The plus sign shows growth as good as wild type and the minus and plus/minus signs indicate the absence of growth and reduced number of colonies, respectively.

components of GINS (Figure 3A), consistent with the finding that GINS complex formation is impaired by *psf3-1* mutation (Figure 1B). In addition, the temperature sensitivity of *psf3-1* was suppressed by *drc1*$^+$, a fission yeast counterpart of budding yeast *SLD2*, but not significantly changed by *sld3*$^+$ (Figure 3A), *cut5*$^+$ or *cdc45*$^+$ (data not shown). These results suggest that Drc1 acts in the same or closely related step with Psf3 in DNA replication.

It has been shown that combining two mutations of different genes results in synthetic lethality or enhanced temperature sensitivity when corresponding gene products function in the same process. To explore genetic relations of the other replication factors with Psf3, double mutants carrying *psf3-1* and a mutation of a component of the pre-RC, the initiation complex or DNA polymerases were constructed. As shown in Figure 3B, *psf3-1 cut5-T401* and *psf3-1 cdc20-P7* (Polε) double mutants exhibited more severe sensitivity than parental strains, whereas the sensitivity of *psf3-1 pol1-1* (Polα) did not significantly differ from those of single mutants. The results of similar experiments are summarized in Figure 3C. The temperature sensitivity of *psf3-1* was enhanced by *sld3-41*, *sna41-928/cdc45* and *cdc20-M10* (Polε). In contrast, the sensitivity was not affected by *orp1-4* and *mis5-268/mcm6*, which are components of pre-RC, or by *spp2-9* (primase) (Figure 3C). These results suggest that Psf3 functions in a process together with Sld3, Cdc45, Cut5, which are components of the initiation complex, and DNA Polε, which may have a role in initiation complex formation rather than DNA synthesis (Feng and D'Urso, 2001).

GINS is required for Cdc45 loading but not for Sld3 loading

We have shown that Sld3 and Cdc45 exhibit different dependence with regard to origin loading (Yamada *et al*, 2004). To examine whether GINS is required for origin loading of Sld3 and Cdc45, ChIP assays were carried out with wild type and *psf3-1* mutant expressing FLAG-tagged Sld3 or Cdc45. In wild-type cells released from M phase in the presence of

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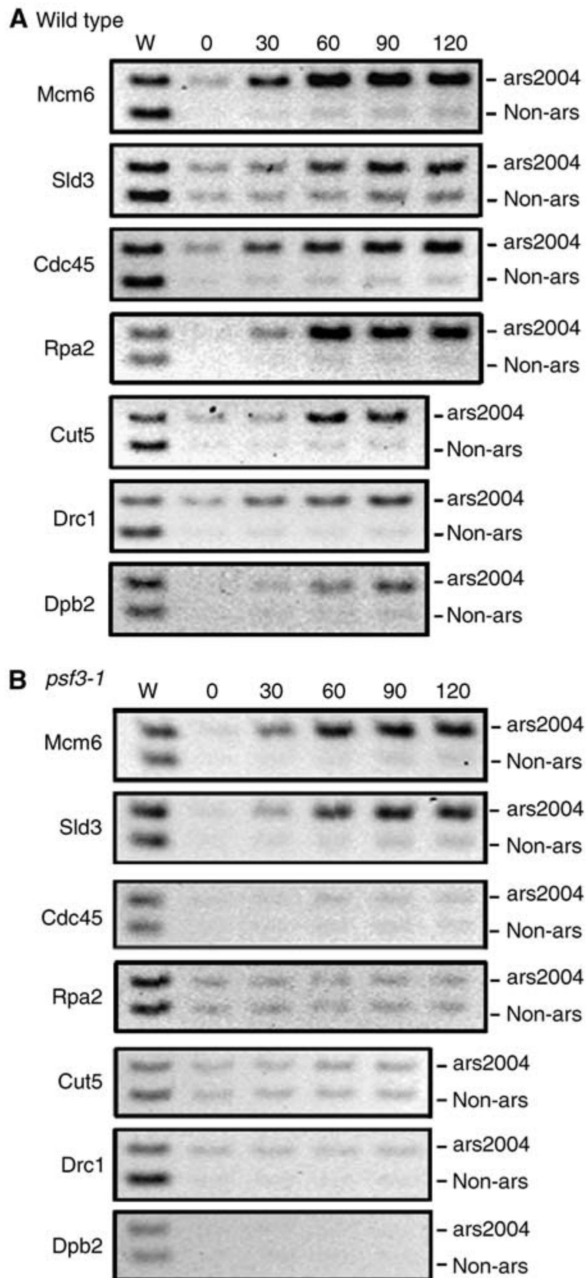

Figure 4 Localization of Sld3 but not Cdc45, Cut5, Drc1 or Dpb2 at replication origin in *psf3-1* mutant. Aliquots of *nda3-KM311* (**A**, wild type) and *psf3-1 nda3-KM311* (**B**, *psf3-1*) derivatives carrying *sld3-flag*, *cdc45-flag*, *cut5-flag*, *drc1-flag* or *dpb2-flag* were arrested at metaphase by culturing at 20°C for 4 h and then incubated at 37°C. Hydroxyurea (15 mM) was added to wild-type cells upon the shift to 37°C. Immunoprecipitated DNA with anti-Mcm6, anti-FLAG or anti-Rpa2 antibody was analyzed by PCR with the *ars2004* (upper bands) and non-ars1 (lower bands) primers. PCR products from total DNA without IP are shown in lane W.

HU, Mcm6, Sld3 and Cdc45 associated with the *ars2004* origin but not non-origin region at 30–120 min (Figure 4A). In contrast, in *psf3-1* mutant released without HU, localization of Cdc45 at the origin was greatly reduced, whereas Mcm6 and Sld3 were localized as in wild type (Figure 4B). Absence of Cdc45 at the origin was not due to an absence of HU, because it bound to the origin in wild type incubated without HU (Yamada *et al*, 2004). Specific reduction of Cdc45 loading onto another efficient replication origin, *AT2024*, was confirmed (Supplementary Figure S4). These results show that GINS is required for recruitment of Cdc45, but not for Sld3 and Mcm6 loading. As it has been suggested that Cdc45 is required for DNA helicase activity of MCM complex (Masuda *et al*, 2003), we examined binding of Rpa2, the second largest subunit of RPA complex that binds to single-stranded DNA. As expected, Rpa2 bound to *ars2004* origin in HU-arrested wild type but not in *psf3-1* (Figure 4A and B).

To examine whether GINS is required for recruitment of Cut5, Drc1 or DNA Polε, origin localization of FLAG-tagged Cut5, Drc1 or Dpb2, which is the second largest subunit of DNA Polε, was analyzed by ChIP assay. In wild-type cells released from M phase in the presence of HU, Cut5, Drc1 and Dpb2 were localized at the replication origin *ars2004* at 60–90 min (Figure 4A). Origin-specific localization of these factors was confirmed in the absence of HU (Supplementary Figure S3; data not shown). In contrast, localization of these factors at the origin was greatly decreased in *psf3-1* cells at the restrictive temperature in the absence of HU (Figure 4B), indicating that GINS is required for recruitment of Cut5, Drc1 and DNA Polε.

Sld3 and Cut5 but not Cdc45 are required for GINS recruitment

As GINS was required for loading of Cut5 and Cdc45 but not for Sld3, we investigated whether loading of GINS, in turn, depended on these factors. *sld3*, *cut5* and *cdc45* mutants expressing FLAG-tagged Psf2 were released from M phase at the restrictive temperature. Results of flow cytometry analysis showed that 1C DNA cells accumulated, although <1C DNA cells appeared on longer incubation in *sld3* and *cut5* (Figure 5A). ChIP assays showed that localization of Psf2-FLAG to the *ars2004* origin was severely reduced in *sld3* compared with the wild type, whereas Mcm6 was efficiently localized at the origins (Figure 5B, compare with Figure 2). With the *cut5* mutant, origin association of Psf2-FLAG was greatly reduced, similar to *sld3* mutant (Figure 5C). In contrast, in *cdc45* mutant, Psf2-FLAG was localized at the origin at 30–120 min after release (Figure 5D). Considering these results and our previous observation that the mutant Cdc45 protein does not bind to replication origins (Yamada *et al*, 2004), GINS appear to bind to the origin independent of association with Cdc45. Under these conditions, origin DNA did not appear to be unwound, because Rpa2 was not detected in cdc45 mutant (Figure 5D, compare with Figure 4A).

Considering Cdc45-independent loading of GINS together with mutually dependent loading of GINS and Cut5 (Figures 4B, 5C and D), it can be assumed that Cut5 loading is not dependent on Cdc45. In fact, Cut5-FLAG was localized at the replication origin in *cdc45* mutant released from M phase (Figure 5E). These results are consistent with the idea that GINS and Cut5 are recruited to replication origin independent of Cdc45.

DDK-dependent and CDK-independent origin loading of Sld3

Although both DDK and CDK are required for activation of replication origins at the onset of S phase, the specific reactions regulated by these kinases have yet to be elucidated. Dependence of Sld3, GINS and Cdc45 recruitment on DDK and CDK was examined using temperature-sensitive DDK and CDK mutants, in which kinase activities are

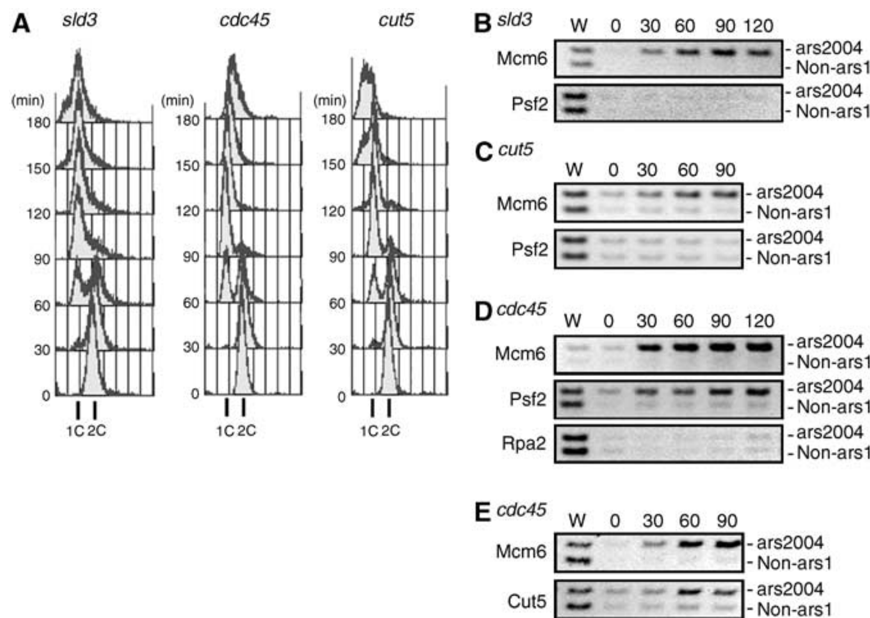

Figure 5 Origin loading of GINS depends on Sld3 and Cut5 but not Cdc45. (**A**) DNA contents of *sld3-10 psf2-flag nda3-KM311* (*sld3*), *sna41-928/cdc45 psf2-flag nda3-KM311* (*cdc45*) and *cut5-T401 psf2-flag nda3-KM311* (*cut5*) cells released from metaphase block were analyzed by flow cytometry. ChIP assays were carried out with anti-Mcm6 and anti-FLAG antibodies from *sld3* (**B**) and *cut5* (**C**), with anti-Mcm6, anti-FLAG and anti-Rpa2 antibodies from *cdc45* (**D**), as described in Figure 2, using *ars2004* (upper bands) and non-ars1 (lower bands) primers. PCR products from total cellular DNA without IP are shown in lane W. (**E**) Association of Mcm6 and Cut5-FLAG with origin in *sna41-928/cdc45 cut5-flag nda3-KM311* cells after release from metaphase block was analyzed by ChIP assay.

decreased (Nurse and Thuriaux, 1980; Booher *et al*, 1989; Takeda *et al*, 2001).

For this purpose, *nda3 hsk1-89* (DDK^{ts}) and *nda3* (wild type) cells expressing Sld3-FLAG and Cdc45-Myc or Psf2-FLAG were released from M-phase block at the restrictive temperature in the absence of HU. Flow cytometry analysis showed that *hsk1* cells were arrested in early S phase, whereas wild-type cells released without HU proceeded into S phase, as shown by the appearance of >2C DNA cells (Figure 6A). In wild-type cells, Sld3, Psf2 and Cdc45 were all preferentially localized at the *ars2004* at 50–80 min, slightly later than Mcm6 (30–70 min) (Figure 6B). In *hsk1* mutant, however, localization of Sld3, Psf2 or Cdc45 to the origin was severely impaired, whereas Mcm6 binding was not affected (Figure 6C). Thus, DDK is required for origin binding of Sld3, GINS and Cdc45.

However, ChIP assays with *nda3 cdc2-33* (CDK^{ts}) revealed that Sld3 was localized to the *ars2004* origin with a similar timing as in wild type, whereas localization of Psf2 or Cdc45 was greatly reduced (Figure 6D). Very similar results were obtained with two different alleles, *cdc2-M26 and cdc2-L7* (Supplementary Figure S5), suggesting that Sld3 loading does not depend on CDK. Taken together, DDK but not CDK is required for Sld3 loading, whereas loading of GINS and Cdc45 depends on both kinases. This is consistent with the ordered requirement of Sld3, GINS and Cdc45 in initiation complex formation as described above.

Discussion

Distinctly ordered assembly of initiation factors at onset of S phase

Individual replication origins are activated at onset of S phase through assembly of replication factors, including Sld3, GINS, Cut5, Drc1, Cdc45, RPA and the DNA polymerase α/primase complex. This process is tightly regulated by CDK and DDK. However, in fission yeast, it has not been elucidated whether the factors assemble onto replication origins in a specific order. Our present results indicate that specific subsets of replication factors bind to replication origins when a certain factor is not functional. These observations allow us to propose specific dependence in origin loading of replication factors.

Because initiation factors are assumed to execute their functions at replication origins, loading onto origins is the essential process. We have shown origin loading of GINS and Cut5 to depend on Sld3, whereas Sld3 binds to origins independently. Furthermore, recruitment of Cdc45 requires Sld3 and GINS (Figure 4), but not vice versa. These results are consistent with our previous observation that Sld3 binds to replication origins independent of Cdc45 in *sna41/cdc45* and *nda4/mcm5* mutants (Yamada *et al*, 2004), and indicate that loading of Sld3 is the most upstream reaction required for assembly of initiation complex at the beginning of S phase (Figure 7A, Step 1). GINS and Cut5 are then recruited onto origins depending on Sld3 in a mutually dependent manner (Step 2). Depending on all these factors, Cdc45 is recruited (Step 3). Replication origins may be unwound only after all these factors have assembled, consistent with the previous observation that Cdc45 is required for DNA helicase activity of MCM complex (Masuda *et al*, 2003).

One may argue that apparent independence in origin loading using conditionally lethal mutants may result from some remaining function of the mutant protein. However, Sld3 was loaded onto origins in *psf3-1* mutant, in which neither GINS complex formation nor loading of Psf2 was detected at the restrictive conditions (Figures 2 and 4). This demonstrates that Sld3 can bind to origins independently

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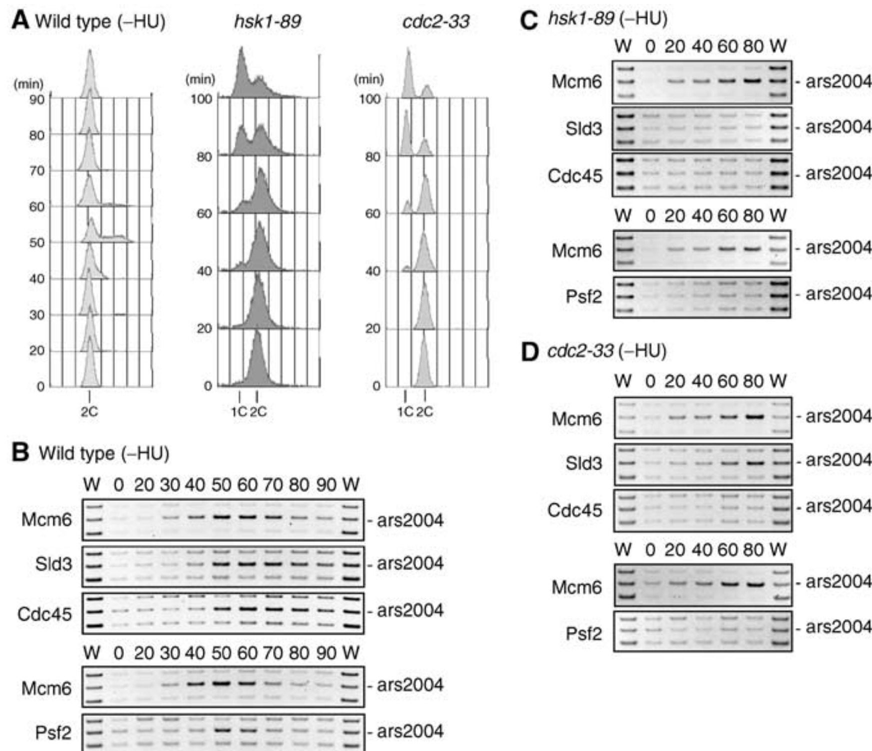

Figure 6 Origin association of Sld3 depends on DDK but not on CDK. Aliquots of *nda3-KM311 sld3-flag cdc45-myc* and *nda3-KM311 psf2-flag* cells (wild type) were released from metaphase block at 36°C, as described in Figure 2. DNA contents of *nda3-KM311 sld3-flag cdc45-myc* (wild type), *hsk1-89 nda3-KM311 sld3-flag cdc45-myc* (*hsk1*) and *cdc2-33 nda3-KM311 sld3-flag cdc45-myc* (*cdc2*) after release at 36°C from metaphase block, as described in Figure 2, were analyzed by flow cytometry (**A**). Localization of Mcm6, Sld3-FLAG, Cdc45-Myc and Psf2-FLAG at *ars2004* in wild type (**B**), *hsk1* (**C**) and (C) *cdc2* (**D**) derivatives was analyzed by ChIP assays. Three primer sets, non-arsA (upper bands), *ars2004* (middle bands) and non-arsB (lower bands), were used for PCR (Ogawa *et al*, 1999). PCR products without IP are shown in lane W.

from association of GINS, although a possibility that GINS assists Sld3 loading without tight binding to origins cannot be excluded. Consistently, Sld3 was localized at replication origin in the *cdc2* mutants, in which GINS did not associate with origins (Figure 6).

In budding yeast arrested in G1 phase in the presence of α-factor, both Cdc45 and Sld3 associate with the early firing replication origins in a mutually dependent manner, whereas GINS does not bind to origins (Figure 7B, Step 1) (Kamimura *et al*, 2001). The primary association of Cdc45, called the 'bound' state, is independent of CDK activity (Aparicio *et al*, 1999), whereas the association of Cdc45 becomes a tightly 'engaged' state depending on GINS, Dpb11, Sld2 and CDK (Takayama *et al*, 2003) (Figure 7B, Step 2). These observations suggest that budding yeast Cdc45 have two distinct association states before and after GINS loading. It has recently been shown that Sld3 does not associate or travel with replication fork components once DNA synthesis initiates in budding yeast, whereas both GINS and Cdc45 remain associated with MCM (Gambus *et al*, 2006; Kanemaki and Labib, 2006). These results are consistent with the idea that Sld3, GINS and Cdc45 have distinct roles in initiation of replication. Possible roles of Sld3 in recruitment of GINS and Cut5 might be conserved between budding yeast and fission yeast, although fission yeast Sld3 may not depend on Cdc45 in the interactions with pre-RC. The apparent requirement of Sld3 for Cdc45 loading could be a consequence of sequential assembly as described above. However, it may also be possible that Sld3 may participate in the loading of Cdc45 in fission yeast as shown in budding yeast, because tight genetic interactions were observed between *sld3*$^{+}$ and *cdc45*$^{+}$ (Nakajima and Masukata, 2002). The conserved role of Sld3 may be a kind of molecular chaperon that mediates stepwise formation of MCM–GINS–Cdc45 complex for active DNA helicase.

Regulation of initiation complex assembly by DDK and CDK

Although the requirement of CDK and DDK for initiation of replication is conserved in eukaryotes, the order of execution points is somewhat controversial and may differ among species. CDK function has been shown to be required before DDK in budding yeast (Nougarede *et al*, 2000), whereas DDK must execute its function before CDK for replication in *Xenopus* egg extracts (Walter, 2000). In this study, we found that Sld3 was not loaded onto origins in the *hsk1-89* mutant but bound to origins in three different *cdc2* mutants, where the CDK activity is below the level required for loading of Psf2, Cut5 and Cdc45 (Figure 6 and Supplementary Figure S5). Therefore, loading of Sld3, which is the most upstream reaction in initiation factor assembly, is regulated by DDK but not by CDK in fission yeast, although this conclusion does not exclude the possibility that pre-assembled CDK-dependent complex may be recruited to origins depending on DDK. This may occur for late firing origins. In fission yeast, a large number of replication origins may fire rather stochastically in a domain at the beginning of S phase (Dai *et al*, 2005; Patel *et al*, 2006), although a small number of late-firing

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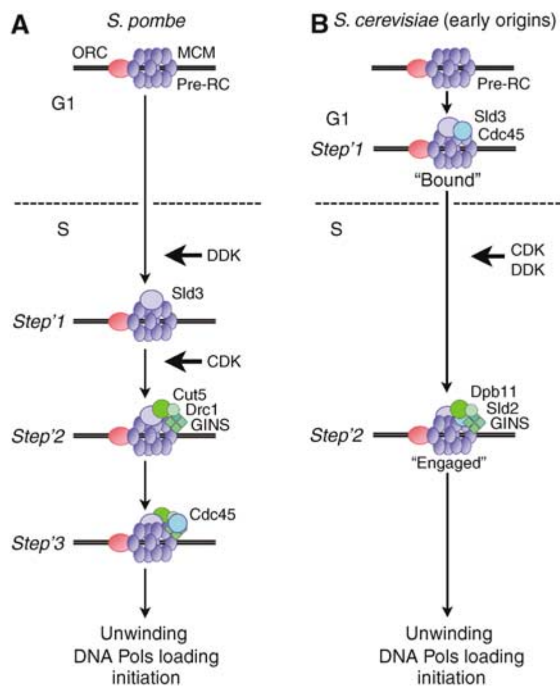

Figure 7 A model for ordered formation of initiation complex in fission yeast. (**A**) Based on dependence among Sld3, GINS, Cut5 and Cdc45 deduced from this study, a model for ordered assembly of replication factors at replication origins in fission yeast is presented. At the onset of S phase, Sld3 binds to replication origins independent of association of GINS, Cut5 or Cdc45 (Step 1). This step requires DDK function. Depending on Sld3 and CDK, GINS and Cut5 then bind to origins in a mutually dependent manner (Step 2). Finally, Cdc45 is recruited depending on all of Sld3, GINS, DDK and CDK (Step 3). At late-firing origins, association of Sld3 with Cut5 and Cdc45 may occur before their origin loading. (**B**) A model for assembly of replication factors on the early replication origins in budding yeast is presented. In G1 phase arrested by α-factor, Sld3 and Cdc45 associate with the early origins (Step 1). Upon activation of CDK and DDK at the beginning of S phase, Dpb11, Sld2 and GINS are recruited to early origins, in a mutually dependent manner, and Cdc45 becomes stably associated with the origins (Step 2).

origins have been identified (Kim and Huberman, 2001; Yompakdee and Huberman, 2004). In contrast, the timing of firing of individual origin appears to be distinctly determined in budding yeast (Friedman *et al*, 1997; Yamashita *et al*, 1997). Requirement of DDK before CDK in the initiation complex assembly in fission yeast and *Xenopus* egg extracts may be relevant to activation of a large number of origins at the beginning of S phase.

For Sld3 loading, DDK may phosphorylate pre-RC components or Sld3 itself. MCM subunits are the efficient substrates for DDK phosphorylation *in vitro* and *in vivo* (Weinreich and Stillman, 1999; Masai and Arai, 2002). In addition, requirements of Cdc7 and Dbf4 are bypassed by the *bob1/mcm5* mutation in budding yeast (Hardy *et al*, 1997), suggesting the possibility that certain conformational change in the MCM complex may be caused by DDK phosphorylation. Although the molecular mechanisms of interaction between pre-RC and Sld3 are not yet completely understood, interactions of Sld3 with Mcm2, Mcm4 and Mcm6, which are likely substrates for DDK phosphorylation, might play important roles in origin loading of Sld3 (S Azuma and H Masukata, unpublished results).

In contrast to Sld3 recruitment, loading of GINS, Cut5 and Cdc45 requires both DDK and CDK (Figure 6). At present, Sld2 is the only known substrate whose phosphorylation by CDK is required for initiation of replication. Interactions between Dpb11 and Sld2, and their origin loading require phosphorylation of Sld2 (Masumoto *et al*, 2002; Tak *et al*, 2006). The essential role of phosphorylation of Drc1/Sld2 appears to be conserved in fission yeast (Noguchi *et al*, 2002). GINS binds to replication origins in a mutually dependent manner with Cut5. Thus, dependence of GINS loading on CDK could be due to its dependence on Cut5 (Figure 5).

In budding yeast, activation of early or late origins is regulated by S-CDK, Dbf4/Cdc7 (DDK) and Rad53, an effector kinase in checkpoint regulation (Tanaka and Nasmyth, 1998). Chromatin association of Dbf4/Cdc7 during S phase and its kinase activity are downregulated by Rad53 and Cdc45 loading is implicated to be a target step of this regulation (Weinreich and Stillman, 1999). In fission yeast, because DDK function is required for Sld3 loading (Figure 6), possible reduction of DDK activity under checkpoint activation may prevent Sld3 loading. Therefore, Sld3 loading seems to be a key step for regulation of replication in fission yeast. Although a homolog of Sld3 is yet to be discovered in higher eukaryotes, a certain factor should play a role in recruitment of GINS, Cut5 and Cdc45 in initiation complex formation. Amino-acid sequences of Sld3 in higher eukaryotes may be diverse from those in budding and fission yeasts, which have only 24% similarity (Nakajima and Masukata, 2002).

Roles of GINS in DNA replication and genome stability

The results of co-precipitation of GINS components revealed that the *psf3-1* mutation impaired GINS complex formation (Figure 1B), possibly owing to reduction of the interaction between Psf3 and the other GINS components. This possibility is consistent with the finding that temperature sensitivity of the *psf3-1* was suppressed by copy number increase of *sld5*$^+$, *psf1*$^+$ and *psf2*$^+$ (Figure 3A). The mutations in *psf3-1* alter two amino-acid residues (V56D and I159L; Supplementary Figure S1). As the I159L is a change into a similar amino acid, the V56D might be responsible for the defect. Interestingly, other *psf3* mutants with similar phenotypes with the *psf3-1* carry amino-acid changes in the second exon near the Val^{56} (Supplementary Figure S1). Thus, the region around the second exon appears to be involved in interactions with other GINS components.

A complex containing Psf1, Psf2 and Sld5 but lacking Psf3 appears to be formed in the *psf3-1* mutant (Figure 1B). Similar complexes lacking Psf3 were detected in budding yeast and *Xenopus* egg extracts, although such partial complexes are not functional in DNA replication (Kanemaki *et al*, 2003; Kubota *et al*, 2003; Takayama *et al*, 2003). Here, we showed that Psf3 is required for recruitment of GINS to replication origins (Figure 2). A possible interpretation of this requirement is that the interaction of Psf3 with pre-RC components or Sld3 is required for GINS loading. We have detected the interactions of Psf3 with MCM subunits in yeast two-hybrid assay (S Azuma and H Masukata, unpublished results) and such interactions may facilitate association of GINS with replication origins.

Loading of GINS depends on Sld3 and Cut5 (Figure 5), suggesting tight relation between these factors. This is consistent with genetic interactions between *psf3*$^+$ with *sld3*$^+$

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and *cut5*$^+$ (Figure 3). GINS might interact directly or indirectly with Sld3 and Cut5. We have detected the interactions of GINS components with Sld3 and Cut5 in yeast two-hybrid assays (S Azuma and H Masukata, unpublished results). However, co-precipitation of GINS components with Sld3 or Cut5 in synchronized cells with or without crosslinking has not been detected under our experimental conditions (data not shown). Possible interactions of GINS with Sld3 or Cut5 may be transient or fragile during the process of initiation complex formation. In budding yeast, GINS components interact with Sld3 and Dpb11/Cut5 in two-hybrid assays (Takayama *et al*, 2003). However, Sld3 or Dpb11 is not found in the complex containing GINS, MCM and Cdc45 during S phase (Gambus *et al*, 2006; Kanemaki and Labib, 2006). These results suggest that Sld3 and Cut5/Dpb11 may act specifically for initiation step, probably for recruitment of GINS and Cdc45 to replication origins. In contrast, GINS, which interacts with DNA polymerase ε (Takayama *et al*, 2003; Seki *et al*, 2006), may act as a mediator between MCM helicase and DNA polymerases. Alternatively, GINS may function as accessory components for MCM helicase, as GINS is required for maintaining interaction between MCM and Cdc45 after initiation of replication (Gambus *et al*, 2006).

Some fraction of *psf3-1* cells enter mitosis without completion of DNA replication, as shown by the appearance of $<$1C DNA cells after accumulation of 1C DNA cells in the present study (Figure 1). A similar phenotype has been observed for *sld3-10*, *cut5-T401* and *hsk1-89* (Saka *et al*, 1994; Takeda *et al*, 2001; Nakajima and Masukata, 2002). On the other hand, neither *cdc45* allele causes abnormal mitosis when arrested at a restrictive temperature (Miyake and Yamashita, 1998; Uchiyama *et al*, 2001). These lines of evidence suggest that assembly of Sld3, GINS and Cut5 onto origins in Step 2 (Figure 7A) may play a role in preventing unscheduled cell division. Loading of Cut5 to replication origins may be important for its role in DNA damage checkpoint or for regulation of cytokinesis (Norden *et al*, 2006). Alternatively, GINS may be involved in mitosis or cell division, because *psf2*$^+$ on a multicopy plasmid suppresses the defect of the *bir1/cut17* mutation, which causes defects in chromosome segregation and spindle attachment (Huang *et al*, 2005).

Assembly of initiation machinery and its regulation are complex and elaborate mechanisms in eukaryotes. As components involved in various chromosome functions such as chromosome cohesion, segregation and cell division are likely to be recruited to appropriate locations on chromosomes before or during S phase, the mechanisms of initiation of replication may have been established in conjunction with these other functions. Significance of stepwise assembly of the initiation complex might be establishment of molecular links between replication machinery and other chromosome

Table I *S. pombe* strains used in this study

Strain	Genotype	Source
HM69	*h*$^-$ *cdc20-P7*	G D'Urso
HM140	*h*$^-$ *nda3-KM311*	M Yanagida
HM263	*h*$^-$ *mis5-268*	M Yanagida
HM374	*h*$^+$ *nda3-KM311 cdc45*$^+$ *::flag-cdc45 (ura4*$^+$*)*	Our Stock
HM508	*h*$^-$ *cut5-T401*	M Yanagida
HM509	*h*$^-$ *sld3-41*	Our stock
HM681	*h*$^-$ *nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*)*	Our stock
HM756	*h*$^-$ *psf3-1 (ura4*$^+$*)*	This work
HM915	*h*$^-$ *leu1-32 pol1-1*	P Nurse
HM948	*h*$^-$ *cut5*$^+$ *::cut5-3flag (kan-MX)*	K Tanaka
HM1175	*h*$^-$ *orp1-4/cdc30-2H4*	P Nurse
HYF40	*h*$^-$ *leu1-32 psf3-1 (ura4*$^+$*)*	This work
HYF57	*h*$^-$ *psf3-1 nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*)*	This work
HYF58	*h*$^-$ *psf3-1 nda3-KM311 cdc45*$^+$ *::flag-cdc45 (ura4*$^+$*)*	This work
HYF59	*h*$^-$ *psf3-1 nda3-KM311 dpb2*$^+$ *::4flag-dpb2*	This work
HYF67	*h*$^-$ *nda3-KM311 dpb2*$^+$ *::4flag-dpb2*	This work
HYF108	*h*$^-$ *psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
HYF113	*h*$^-$ *nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
HYF116	*h*$^-$ *psf3-1 nda3-KM311 drc1*$^+$ *:: drc1-3flag (kan-MX)*	This work
HYF118	*h*$^-$ *sld3-10 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
HYF124	*h*$^-$ *cut5-ts4 nda3-KM311 psf2*$^+$ *:: psf2-3flag (kan-MX)*	This work
HYF125	*h*$^-$ *nda3-KM311 drc1*$^+$ *::drc1-3flag (kan-MX)*	This work
HYF127	*h*$^-$ *sna41-928 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
HYF129	*h*$^-$ *psf3-1 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
HYF164	*h*$^-$ *sna41-928 nda3-KM311 cut5*$^+$ *::cut5-3flag (kan-MX)*	This work
HYF166	*h*$^-$ *psf3-1 nda3-KM311 cut5*$^+$ *::cut5-3flag (kan-MX)*	This work
YYY63	*h*$^-$ *cdc45/sna41-928*	Our stock
YYY242	*h*$^+$ *cdc2-33 nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*) cdc45*$^+$ *::cdc45-9myc*	This work
YYY243	*h*$^-$ *cdc2-M26 nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*)cdc45*$^+$ *::cdc45-9myc*	This work
YYY245	*h*$^-$ *nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*) cdc45*$^+$ *::cdc45-9myc*	This work
YYY253	*h*$^-$ *cdc2-L7 nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*) cdc45*$^+$ *::cdc45-9myc*	This work
YYY260	*h*$^+$ *cdc2-L7 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
YYY266	*h*$^-$ *cdc2-33 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
YYY271	*h*$^-$ *cdc2-M26 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work
YYY296	*h*$^-$ *cdc2-M26 nda3-KM311 cut5*$^+$ *::cut5-3flag (kan-MX)*	This work
YYY301	*h*$^-$ *hsk1-89 nda3-KM311 sld3*$^+$ *::5flag-sld3 (ura4*$^+$*) cdc45*$^+$ *::cdc45-9myc*	This work
YYY302	*h*$^-$ *hsk1-89 nda3-KM311 psf2*$^+$ *::psf2-3flag (kan-MX)*	This work

functions before actual DNA synthesis. Further investigation of molecular interactions between replication factors and those involved in other chromosome functions may be required for better understanding of genome integrity.

Materials and methods

Strains and media

Yeast strains used in this study are listed in Table I. Fission yeast strains were cultured in complete YE medium (0.5% yeast extract and 3% glucose) and minimal EMM minimum (Moreno *et al*, 1991), all solid media containing 2% agar.

Construction of plasmids

A 1.0 kb genomic fragment containing the *psf3*$^+$ gene was PCR-amplified from *Schizosaccharomyces pombe* HM17 (h^-, wild type) genome DNA, using primers 5′-AAAGGATCCCCTGTTGATGA AACTTGGCG-3′ and 5′-AAAGGATCCGCGTCTGATTATAGACAACC-3′ and the *Bam*HI fragment was cloned into pBluescript KS+ to obtain pBS-Psf3. A multicopy plasmid pXB940-Psf3 carrying *psf3*$^+$ was made by insertion of the *Bam*HI fragment into pXB940, an ARS plasmid containing the fission yeast origin *ars2004*. pXB940-Sld5, pXB940-Psf1 and pXB940-Psf2 were constructed by a similar procedure as described for *psf3*$^+$, using the following primers: 5′-AAAGGATCCGCTTAAACGACGCCAGTCTC-3′ and 5′-AAAGGATCCG TCTTGACAGAAGTCCC-3′ for pXB940-Sld5; 5′-AAAGGATCCCCC TGTCCGATGAAGCAAGC-3′ and 5′-AAAGGATCCGGATGCCGAA TAAACAGTCC-3′ for pXB940-Psf1; and 5′-AAAGGATCCCGA TATTGGCTGAGCGAAGG-3′ and 5′-AAAGGATCCCTGCCACACCGT GGAGAACG-3′ for pXB940-Psf2. For all constructions, the nucleotide sequences were confirmed after PCR cloning.

Epitope tagging of Psf2

The *psf2*$^+$ gene was tagged with DNA sequences encoding six histidine residues and 3xFLAG epitopes using PCR amplification, essentially as described previously (De Antoni and Gallwitz, 2000). The integration cassette was amplified from pU6H3VSV with primers containing 5′-TGATGGACAGAATGCGCAAAATTGTTCAAGT TTCCCAAGAAGAACACCACCATCATCATCACGG-3′, and 5′-AGGATT CATTATCATTATTTTTAAAGTACATCATCCACATGATAACTATAGGGA GACCGGCAGAT-3′, subsequently transformed to HM17 (h^-, wild type). Transformants grown on YE-geneticin (100 μg/ml) plates were analyzed by PCR and Western blotting to confirm *psf2-3flag*. The resultant strain, HFY108, grew as wild type, suggesting the FLAG-tagged Psf2 to be functional.

Isolation of psf3 mutants

For construction of the mutating plasmid, the *Bam*HI fragment of *psf3*$^+$ was cloned into pUCTL1 (Obuse *et al*, 1996), and the *Xba*I fragment of 1.7 kb *ura4*$^+$ was inserted at the *Xba*I site downstream of *psf3*-ORF, resulting in pTL-*ura4*-Psf3. Mutating PCR was carried out with ampli-Taq Gold (Perkin-Elmer) in a 100 μl reaction mixture containing 1.5 mM $MgCl_2$ (Perkin-Elmer) and 0.5 mM $MnCl_2$ using 5′-ATGCGTCTGATTATAGACAACC-3′ and 5′-AACCTGTTGATGAAA CTTGGCG-3′ as primers. PCR products purified by phenol/chloroform extraction followed by ethanol precipitation were used for transformation of HM 83 (h^+ *ura4-D18*). Among ura$^+$ transformants grown on MM plates at 25°C, temperature-sensitive mutants at 36°C were collected, and integration of the *psf3-ura4*$^+$ fragment at the *psf3*$^+$ locus was confirmed by PCR.

Cell cycle synchronization

To synchronize the cell cycle, derivatives carrying *nda3-KM311* were incubated at 20°C for 4 h to arrest at M phase and then released at 36°C, the restrictive temperature for the *psf3-1* and other mutants, except for *cdc45/sna41-928* (37.5°C).

Polyclonal antibodies against Psf1, Psf3, Sld5 and Rpa2

To express His6-tagged Psf3 peptides in *Escherichia coli*, the open reading frame of *psf3*$^+$ lacking introns was PCR-amplified from a cDNA library (donated by H Nojima) using 5′-AAAGGATCCCA TATGCATCATCATCATCATCATGATTACTATGATATTGAT-3′ and 5′-AA AGGATCCCGTCTCGCAGCATGCAATC-3′ as primers. The PCR products digested with *Bam*HI were cloned into pBluescript KS+ and the sequence was confirmed. The *psf3*$^+$ (without introns) fragment was cloned into pET21a, to obtain pET21a-His6-Psf3. pET21a-His6-Psf1 and pET21a-His6-Sld5 were constructed similarly except that the *psf1*$^+$ and *sld5*$^+$ genes were amplified from the genomic DNA using respective pairs of primers: 5′-AAAGGATCCTCATAACTTAGC CAAAAAACCTTGGG-3′ and 5′-AAAGGATCCCATATGGAGAATGGTT TGGGGAATCG-3′; and 5′-AAAGGATCCATATGGAGTGGGATGCTGAT-3′ and 5′-AAACGATCCTATATTAGTGAGACAACACC-3′. pET21a-His6-Rpa2, containing the *ssb2*$^+$, which encodes the second largest subunit of fission yeast RPA, was constructed from pET19b-rSpSSB (Ishiai *et al*, 1996). The His6-tagged N-terminus of *ssb2*$^+$ was amplified by using 5′-AAAGGATCCCATATGCATCATCATCATCAT CATGCTTATGATGCTTTTGGC-3′ and 5′-AAAGGATCCTTCAACGC CATCTATCTT-3′ as primers, and the *Nde*I–*Eco*RI fragment together with *Eco*RI–*Hin*dIII fragment from pET19b-rSpSSB was inserted into pET21a.

His6-tagged Psf1, Psf3 or Sld5 polypeptides were expressed in *E. coli* BL21 (DE3), purified as recommended by the manufacturer (Qiagen) and used to immunize rabbits (Hokudo Inc.). Anti-Psf3 polyclonal antibodies were purified as recommended by the manufacturer using a Hi-Trap NHS-activated column (Pharmacia).

Immunoprecipitation and immunostaining

For preparation of yeast cell extracts, a method of Moreno *et al* (1991) was used. *S. pombe* haploid cells ($0.5–1 \times 10^9$ cells) were suspended in 0.4 ml of HB buffer (25 mM MOPS (pH 7.2), 15 mM $MgCl_2$, 15 mM EGTA, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, 20 μg of aprotinin per ml) and disrupted with glass beads using a bead beater (BioSpec Products). The supernatant (0.4 ml) after centrifugation of the extracts at 12 000 *g* for 15 min was incubated with 25 μl of magnetic beads (Dynal) associated with mouse anti-FLAG antibody at 4°C for 2 h, and the beads were washed with HB buffer four times and then proteins were eluted with 50 μl of SDS buffer (120 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol) at 95°C for 5 min. Immunoblotting and immunostaining were carried out as described (Takahashi and Masukata, 2001). Antibodies were used at 1/3000 dilution for anti-FLAG (mouse IgG1) and 1/200–1/500 dilution for anti-Psf1, Psf3 and Sld5 antibodies (rabbit IgG).

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously (Yamada *et al*, 2004). PCR amplification with ampli-Taq Gold (Perkin-Elmer) was performed in 20 μl of PCR buffer containing 1.5 mM $MgCl_2$ (Perkin-Elmer) and 0.5–1 μl of immunoprecipitated DNA or total DNA as the template with two or three sets of primers: 5′-CTTTTGGGTAGTTTTCGGATCC-3′ and 5′-ATGAGTACTTGTCAC GAATTC-3′ for *ars2004*; and 5′-TCGAAGATCCTACCGCTTTC-3′ and 5′-CTTGCGCTGAAGCTTTAGTAAAAG-3′ for the non-origin region, as described previously (Yamada *et al*, 2004). PCR products were separated in 2.5% agarose gels and visualized with 0.5 μg/ml ethidium bromide.

Supplementary data

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

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References

Aparicio OM, Stout AM, Bell SP (1999) Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc Natl Acad Sci USA* **96:** 9130–9135

Araki H, Leem SH, Phongdara A, Sugino A (1995) Dpb11, which interacts with DNA polymerase II(ε) in *Saccharomyces cerevisiae*, has a dual role in S-phase progression and at a cell cycle checkpoint. *Proc Natl Acad Sci USA* **92:** 11791–11795

Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. *Annu Rev Biochem* **71:** 333–374

Booher RN, Alfa CE, Hyams JS, Beach DH (1989) The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. *Cell* **58:** 485–497

Dai J, Chuang RY, Kelly TJ (2005) DNA replication origins in the *Schizosaccharomyces pombe* genome. *Proc Natl Acad Sci USA* **102:** 337–342

De Antoni A, Gallwitz D (2000) A novel multi-purpose cassette for repeated integrative epitope tagging of genes in *Saccharomyces cerevisiae*. *Gene* **246:** 179–185

Diffley JF, Cocker JH, Dowell SJ, Rowley A (1994) Two steps in the assembly of complexes at yeast replication origins *in vivo*. *Cell* **78:** 303–316

Feng W, D'Urso G (2001) *Schizosaccharomyces pombe* cells lacking the amino-terminal catalytic domains of DNA polymerase epsilon are viable but require the DNA damage checkpoint control. *Mol Cell Biol* **21:** 4495–4504

Friedman KL, Brewer BJ, Fangman WL (1997) Replication profile of *Saccharomyces cerevisiae* chromosome VI. *Genes Cells* **2:** 667–678

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat Cell Biol* **8:** 358–366

Gilbert DM (2001) Making sense of eukaryotic DNA replication origins. *Science* **294:** 96–100

Hardy CF, Dryga O, Seematter S, Pahl PM, Sclafani RA (1997) *mcm5/cdc46-bob1* bypasses the requirement for the S phase activator Cdc7p. *Proc Natl Acad Sci USA* **94:** 3151–3155

Hashimoto Y, Takisawa H (2003) *Xenopus* Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. *EMBO J* **22:** 2526–2535

Hiraoka Y, Toda T, Yanagida M (1984) The *NDA3* gene of fission yeast encodes β-tubulin: a cold-sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39:** 349–358

Huang HK, Bailis JM, Leverson JD, Gomez EB, Forsburg SL, Hunter T (2005) Suppressors of Bir1p (Survivin) identify roles for the chromosomal passenger protein Pic1p (INCENP) and the replication initiation factor Psf2p in chromosome segregation. *Mol Cell Biol* **25:** 9000–9015

Ishiai M, Sanchez JP, Amin AA, Murakami Y, Hurwitz J (1996) Purification, gene cloning, and reconstitution of the heterotrimeric single-stranded DNA-binding protein from *Schizosaccharomyces pombe*. *J Biol Chem* **271:** 20868–20878

Kamimura Y, Masumoto H, Sugino A, Araki H (1998) Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication. *Mol Cell Biol* **18:** 6102–6109

Kamimura Y, Tak YS, Sugino A, Araki H (2001) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *EMBO J* **20:** 2097–2107

Kanemaki M, Labib K (2006) Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. *EMBO J* **25:** 1753–1763

Kanemaki M, Sanchez-Diaz A, Gambus A, Labib K (2003) Functional proteomic identification of DNA replication proteins by induced proteolysis *in vivo*. *Nature* **423:** 720–724

Kearsey SE, Cotterill S (2003) Enigmatic variations: divergent modes of regulating eukaryotic DNA replication. *Mol Cell* **12:** 1067–1075

Kim SM, Huberman JA (2001) Regulation of replication timing in fission yeast. *EMBO J* **20:** 6115–6126

Kubota Y, Takase Y, Komori Y, Hashimoto Y, Arata T, Kamimura Y, Araki H, Takisawa H (2003) A novel ring-like complex of *Xenopus* proteins essential for the initiation of DNA replication. *Genes Dev* **17:** 1141–1152

Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK (1997) Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev* **11:** 3365–3374

Masai H, Arai K (2002) Cdc7 kinase complex: a key regulator in the initiation of DNA replication. *J Cell Physiol* **190:** 287–296

Masuda T, Mimura S, Takisawa H (2003) CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in *Xenopus* egg extracts: possible activation of MCM helicase by association with Cdc45. *Genes Cells* **8:** 145–161

Masumoto H, Muramatsu S, Kamimura Y, Araki H (2002) S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. *Nature* **415:** 651–655

Mimura S, Masuda T, Matsui T, Takisawa H (2000) Central role for Cdc45 in establishing an initiation complex of DNA replication in *Xenopus* egg extracts. *Genes Cells* **5:** 439–452

Miyake S, Yamashita S (1998) Identification of *sna41* gene, which is the suppressor of *nda4* mutation and is involved in DNA replication in *Schizosaccharomyces pombe*. *Genes Cells* **3:** 157–166

Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* **194:** 795–823

Nakajima R, Masukata H (2002) SpSld3 is required for loading and maintenance of SpCdc45 on chromatin in DNA replication in fission yeast. *Mol Biol Cell* **13:** 1462–1472

Noguchi E, Shanahan P, Noguchi C, Russell P (2002) CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. *Curr Biol* **12:** 599–605

Norden C, Mendoza M, Dobbelaere J, Kotwaliwale CV, Biggins S, Barral Y (2006) The NoCut pathway links completion of cytokinesis to spindle midzone function to prevent chromosome breakage. *Cell* **125:** 85–98

Nougarede R, Della Seta F, Zarzov P, Schwob E (2000) Hierarchy of S-phase-promoting factors: yeast Dbf4-Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. *Mol Cell Biol* **20:** 3795–3806

Nurse P, Thuriaux P (1980) Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics* **96:** 627–637

Obuse C, Okuno Y, Okazaki T, Masukata H (1996) A replication-enhancing element with transcriptional silencer activity in autonomously replicating human chromosomal fragments. *Mol Biol Cell* **7:** 43–55

Ogawa Y, Takahashi T, Masukata H (1999) Association of fission yeast Orp1 and Mcm6 proteins with chromosomal replication origins. *Mol Cell Biol* **19:** 7228–7236

Patel PK, Arcangioli B, Baker SP, Bensimon A, Rhind N (2006) DNA replication origins fire stochastically in fission yeast. *Mol Biol Cell* **17:** 308–316

Saka Y, Fantes P, Sutani T, McInerny C, Creanor J, Yanagida M (1994) Fission yeast *cut5* links nuclear chromatin and M phase regulator in the replication checkpoint control. *EMBO J* **13:** 5319–5329

Segurado M, de Luis A, Antequera F (2003) Genome-wide distribution of DNA replication origins at A+T-rich islands in *Schizosaccharomyces pombe*. *EMBO Rep* **4:** 1048–1053

Seki T, Akita M, Kamimura Y, Muramatsu S, Araki H, Sugino A (2006) GINS is a DNA polymerase ε accessory factor during chromosomal DNA replication in budding yeast. *J Biol Chem* **281:** 21422–21432

Tak YS, Tanaka Y, Endo S, Kamimura Y, Araki H (2006) A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2–Dpb11. *EMBO J* **25:** 1987–1996

Takahashi T, Masukata H (2001) Interaction of fission yeast ORC with essential adenine/thymine stretches in replication origins. *Genes Cells* **6:** 837–849

Takayama Y, Kamimura Y, Okawa M, Muramatsu S, Sugino A, Araki H (2003) GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev* **17:** 1153–1165

Takeda DY, Dutta A (2005) DNA replication and progression through S phase. *Oncogene* **24:** 2827–2843

Takeda T, Ogino K, Tatebayashi K, Ikeda H, Arai K, Masai H (2001) Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. *Mol Biol Cell* **12:** 1257–1274

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Tanaka T, Nasmyth K (1998) Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J* **17:** 5182–5191

Uchiyama M, Arai K, Masai H (2001) *sna41^{goa1}*, a novel mutation causing G1/S arrest in fission yeast, is defective in a *CDC45* homolog and interacts genetically with *polα*. *Mol Genet Genomics* **265:** 1039–1049

Walter JC (2000) Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in *Xenopus* egg extracts. *J Biol Chem* **275:** 39773–39778

Weinreich M, Stillman B (1999) Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the *RAD53* checkpoint pathway. *EMBO J* **18:** 5334–5346

Yamada Y, Nakagawa T, Masukata H (2004) A novel intermediate in initiation complex assembly for fission yeast DNA replication. *Mol Biol Cell* **15:** 3740–3750

Yamashita M, Hori Y, Shinomiya T, Obuse C, Tsurimoto T, Yoshikawa H, Shirahige K (1997) The efficiency and timing of initiation of replication of multiple replicons of *Saccharomyces cerevisiae* chromosome VI. *Genes Cells* **2:** 655–665

Yompakdee C, Huberman JA (2004) Enforcement of late replication origin firing by clusters of short G-rich DNA sequences. *J Biol Chem* **279:** 42337–42344

Zou L, Stillman B (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol Cell Biol* **20:** 3086–3096

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