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GINS Inactivation Phenotypes Reveal Two Pathways for Chromatin Association of Replicative α and ε DNA Polymerases in Fission Yeast

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The tetrameric GINS complex, consisting of Sld5-Psf1-Psf2-Psf3, plays an essential role in the initiation and elongation steps of eukaryotic DNA replication, although its biochemical function is unclear. Here we investigate the function of GINS in fission yeast, using fusion of Psf1 and Psf2 subunits to a steroid hormone-binding domain (HBD) to make GINS function conditional on the presence of β-estradiol. We show that inactivation of Psf1-HBD causes a tight but rapidly reversible DNA replication arrest phenotype. Inactivation of Psf2-HBD similarly blocks premeiotic DNA replication and leads to loss of nuclear localization of another GINS subunit, Psf3. Inactivation of GINS has distinct effects on the replication origin association and chromatin binding of two of the replicative DNA polymerases. Inactivation of Psf1 leads to loss of chromatin binding of DNA polymerase ε, and Cdc45 is similarly affected. In contrast, chromatin association of the catalytic subunit of DNA polymerase α is not affected by defective GINS function. We suggest that GINS functions in a pathway that involves Cdc45 and is necessary for DNA polymerase ε chromatin binding, but that a separate pathway sets up the chromatin association of DNA polymerase α.

INTRODUCTION

The initiation of eukaryotic DNA replication requires a stepwise assembly of replication proteins at sites on the chromosome called replication origins (reviewed in (Bell and Dutta, 2002; Cotterill and Kearsey, 2008). In an initial step termed licensing or prereplicative complex (pre-RC) formation, Mcm2-7 complexes are loaded onto origins in a step dependent on the origin recognition complex (ORC), and the Cdc6 and Cdt1 proteins. Initiation of DNA synthesis requires subsequent activation of the pre-RC by protein phosphorylation events mediated by the S phase cyclin-dependent kinase (S-CDK) and the Dbf4-dependent kinase (DDK). In budding yeast, a key role of S-CDK phosphorylation is to promote the interaction of Sld2 and Sld3 with Dpb11, although the precise function of this complex is unknown (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007).

Other replication factors that associate with origins around the time of initiation include GINS and Cdc45 (Kubota *et al.*, 2003; Takayama *et al.*, 2003). The Mcm2-7 complex is thought to function as the replicative helicase (Bochman and Schwacha, 2008), unwinding double-stranded DNA ahead of the replication fork. Mcm2-7, GINS, and Cdc45 are each required for initiation but also for the elongation stage of replication. Consistent with this, these proteins are loaded onto origin DNA before initiation and move with the fork during elongation (Kanemaki *et al.*, 2003; Gambus *et al.*, 2006; Pacek *et al.*, 2006; Yabuuchi *et al.*, 2006).

One possible role for factors involved in initiation or elongation is to recruit and retain DNA polymerases on DNA. A number of studies suggest that both DNA polymerases α and ε (hereafter Pol α and ε) are recruited to replication origins as an early event in replication initiation. In *Xenopus*, recruitment of both polymerases requires GINS and Cdc45, and the binding of these factors is dependent on the Dpb11 orthologue Cut5, S-CDK activity, and prior origin licensing (Kubota *et al.*, 2003). However, chromatin binding of Pol ε can occur in the absence of Pol α, replication protein A (RPA), and proliferating cell nuclear antigen (PCNA), suggesting that the two polymerases do not share a common pathway for incorporation into replication complexes (Mimura *et al.*, 2000). Also, depletion of *Xenopus* RecQ4 (orthologous to yeast Sld2) specifically blocks Pol α binding, possibly via an effect on RPA, but this has no effect on GINS, Cdc45, or Pol ε binding (Matsuno *et al.*, 2006). The Pol α recruitment pathway appears to involve Mcm10 and associated proteins. In vertebrates, the Mcm10-associated protein And-1 (orthologous to yeast Ctf4/Mcl1) interacts with Pol α and is required for recruitment of this polymerase to chromatin (Zhu *et al.*, 2007); Mcm10 itself appears to have a

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Table 1. Yeast strains used

P11	*h⁻//h⁻*	972 diploid
P274	*cdc6–23 h⁻*	
P275	*cdc20-M10 ade6–704 h⁻*	
P412	*pol1-1 leu1–32 h⁻*	
P1411	*psf2⁺-YFP::kanMX6 ade6-M210 leu1-32 ura4-D18 h⁻*	Yang *et al.* (2005)
P1469	*pat1-114 ade6-M216 h⁻*	
P1470	*pat1-114 ade6-M210 h⁻*	
P1519	*psf1-HBD::kanMX6 h⁻*	This study
P1520	*psf2-HBD::kanMX6 h⁺*	Bøe *et al.* (2008)
P1524/Sp435	*psf2⁺-TAP::kanMX6 h⁻*	This study
P1555	*pol1⁺-YFP::kanMX6 ade6-M216 leu1-32 ura4-D18 h⁺*	This study
P1601	*pat1-114 ade6-M216 psf2-HBD::kanMX6 h⁻*	This study
P1602	*pat1-114 psf2-HBD::kanMX6 h⁻*	This study
P1613	*pat1-114 ade6-M210 psf2-HBD::kanMX6 h⁻*	This study
P1635	*cdc45⁺-YFP::ura4⁺ psf1-HBD::kanMX6 h?*	Derived from P1083 (Gregan *et al.*, 2003)
P1638	*psf2⁺-YFP::kanMX6 h⁺*	Derived from P1411 (Yang *et al.*, 2005)
P1644/Sp438	*psf3⁺-13myc::natMX6 h⁺*	This study
P1675	*psf1-HBD::kanMX6 pol1⁺-YFP::kanMX6*	This study
P1708	*psf3⁺-GFP::kanMX6 ade6-M210 leu1-32 ura4-D18 h⁻*	This study
P1739	*psf3⁺-GFP::kanMX6 h⁺*	This study
P1740	*psf3⁺-GFP::kanMX6 h⁻*	This study
P1742	*pol1⁺-YFP::kanMX6 h⁻*	This study
P1758	*dpb4⁺-GFP h⁺*	Spiga and D'Urso (2004)
P1760	*psf1-HBD::kanMX6 dpb4⁺-GFP h?*	Derived from Spiga and D'Urso (2004)
P1777	*pat1-114 ade6-M210 psf2-HBD::kanMX6 h⁻//pat1-114 ade6-M216 psf2-HBD::kanMX6 h⁻*	This study
P1778	*pol1-1 psf1-HBD::kanMX6 h?*	d'Urso *et al.* (1995)
P1780	*cdc6-23 (pol δ) psf1-HBD::kanMX6 h?*	Nurse *et al.* (1976)
P1781	*cdc20-M10 psf1-HBD::kanMX6 h?*	Nurse *et al.* (1976)
P1788	*pol1⁺-CFP::kanMX6 ade6-M210 leu1-32 ura4-D18 h⁻*	This study
P1880	*psf3⁺-YFP::ura4⁺ ade6-M210 leu1-32 ura4-D18 h⁻*	This study
P1833	*pol1⁺-CFP::kanMX6 nmt1(41X)-cdc13::LEU2 cig1Δ::ura4⁺ cig2Δ::ura4⁺ cdc13Δ::ura4⁺ ura4-D18 leu1-32 ade6-M210 h⁻*	Derived from Fisher and Nurse (1995)
P1839	*cdc10-V50 pol1⁺-CFP::kanMX6*	This study
P1907	*psf2-HBD-YFP::kanMX6 h⁻*	This study
P1922	*sna41ᵗˢ/goal-53 (cdc45) pol1⁺-YFP::kanMX6*	Uchiyama *et al.* (2001a)
P1968	*psf2-HBD::kanMX6 psf3⁺-YFP::ura4⁺ ura4-D18 h?*	This study
P2032	*psf1-HBD::kanMX6 dpb2⁺-4FLAG*	dpb2⁺-4FLAG from H. Masukata
P2034	*psf1-YFP::ura4⁺*	This study
P2053	*pol1⁺-GFP::kanMX6 psf1-HBD::kanMX6*	This study

similar role in *Saccharomyces cerevisiae* (Ricke and Bielinsky, 2004).

The relevance of factors involved in the elongation step of DNA replication has also been analyzed by looking at protein complexes at the fork and protein interactions in vitro. A large complex including Mcm2-7, Cdc45, GINS, and other factors involved in replisome progression does not seem to interact strongly with replicative polymerases (Gambus *et al.*, 2006; Pacek *et al.*, 2006). In contrast, in vitro studies suggest that GINS is an accessory factor for Pol α (De Falco *et al.*, 2007).

In this article we investigate the function of GINS in the fission yeast, *Schizosaccharomyces pombe*. GINS is a stable heterotetramer composed of four paralogous subunits Sld5-Psf1-Psf2-Psf3 (reviewed in Labib and Gambus, 2007). Some structural analyses suggest that GINS has a central cavity that could accommodate single-stranded DNA, consistent with a role as a DNA clamp (Boskovic *et al.*, 2007; Chang *et al.*, 2007), although this is disputed (Choi *et al.*, 2007; Kamada *et al.*, 2007). Previous work in fission yeast has established that Psf2 is required for DNA replication, and *psf2* mutants also have meiotic and mitotic chromosome segregation defects (Gomez *et al.*, 2005; Huang *et al.*, 2005). Psf3 also is required for DNA replication, and the *psf3-1* mutant is defective in the chromatin association of a number of replication factors, including other GINS subunits and Cdc45 (Yabuuchi *et al.*, 2006).

We show here that inactivation of the GINS Psf1 or Psf2 subunits using an β-estradiol hormone-binding domain (HBD) causes a tight arrest of mitotic and meiotic DNA replication. Psf1 is required for maintenance, as well as establishment, of Cdc45 binding and chromatin association of the Dpb4 subunit of Pol ε. In contrast, inactivation of Psf1 has no effect on Pol α origin association and chromatin binding in S phase, suggesting that this may occur via a separate pathway. Finally we show that although levels and nuclear localization of GINS subunits Psf2 and Psf3 are constant during the vegetative cell cycle, nuclear localization is lost on G1 arrest, which could be relevant to the regulation of GINS when cells are not actively cycling.

MATERIALS AND METHODS

Fission Yeast Methods

Strains used in this study are listed in Table 1. Diploid *pat1* strains were made by protoplast fusion. β-Estradiol at 125–200 nM in YES was used for growth of HBD mutants. With the Psf2-HBD-YFP strain we found that better inactivation of Psf2 (as judged by flow cytometry and viability) was obtained if cells were grown at 36°C before and after β-estradiol withdrawal. With Psf1-HBD, rapid inactivation of Psf1 was seen at all temperatures tested. Thiamine at 5 μg/ml was used to repress the *nmt1* promoter and hydroxyurea (HU) was

used at 12 mM. Nitrogen starvation was carried out using Edinburgh minimal medium (EMM) lacking NH_4Cl. Standard genetic methods and flow cytometry were as described previously (Gregan *et al.*, 2003; Yang *et al.*, 2005). Immunofluorescence was carried out using FITC-conjugated anti-rabbit secondary antibody (Sigma, St. Louis, MO; F0382, diluted 1:50) as previously described (Maiorano *et al.*, 1996).

Tagging Fission Yeast Proteins

Pol1 was C-terminally tagged with CFP or YFP (cyan and yellow fluorescent protein, respectively) by amplifying a *pol1*$^+$ fragment with oligos 5′ApaI-pol1C (5′-tttgggcccgagttttgtactttagctaaggagg-3′) and 3′XhoI-pol1C (5′-tttctcgagcgatgaaaatatcagtcccatatctac-3′) and inserting the fragment into ApaI and XhoI-cut pSMRY2+ or pSMRC2+ (Gregan *et al.*, 2003) to give pSMRY2+Pol1 or pSMRC2+Pol1. This resulting plasmid was linearized with BlpI to tag the endogenous *pol1*$^+$ gene with YFP or CFP. Pol1 was tagged with green fluorescent protein (GFP) by one-step gene tagging (Bahler *et al.*, 1998) using the product from a PCR reaction generated with primers 5′-pol1 (5′-atgccatcaacaaa aatatctctcgaataatgaacaaaaatgcgcgtgaatttgtagatatgggactgatattttcatcgcggatccccgggttaattaa-3′) and 3′-pol1 (5′- aacatttcttgtactgcatgagcaaatatctgttcgaggtgtcaagtagattacttaaacgggtagaatgaattaaagctgaattcgagctcgtttaaac-3′), using pFA6a-GFP-kanMX6 as template. Yeast transformants were selected using G418.

The *psf1*$^+$ gene was tagged with the HBD by one-step PCR-mediated gene targeting. Plasmid pFA6a-HBD-kanMX6 was used as template (Bøe *et al.*, 2008) with the following PCR primers: 5′accttactaaaaattcacaattgcatgtgcgtgctacagacgttgaacgactcattgcccaaggtttttggctaagttacggatcccgggttaattaa-3′ and 5′-atggtaggaatgaatggcttatggatgaaatttttagggtcagttcaaaaagcagtgaaactcttatttgggaaacagaggaattcgagctcgtttaaac-3′. Psf1 was tagged with YFP by amplifying a C-terminal region of the *psf1*$^+$ gene with the primers: 5′ ApaI-Psf1, 5′-TTTgggcccGTTTGGGGAATCGATCAAATAAATTAATTC-3′ and 3′ XhoI-Psf1, 5′-TTTctcgagTAACTTAGCCAAAAAACCTTGGGCAATGAG-3′. This PCR product was inserted into ApaI and XhoI cleaved pSMUY2+ and the resulting plasmid was cleaved with EcoRI for integration into the yeast genome.

To construct the *psf2*$^+$*-HBD-YFP* strain, a HBD fragment was amplified from pFA6a-HBD-kanMX6 using oligos 5′*Xho*I-HBD (5′-tttctcgagtctgctggagacatgagagctgcc-3′) and 3′SmaI-HBD (5′-ttttttcccgggcgactgtggcagggaaaccctctgc-3′), and the resulting fragment was subcloned into XhoI and SmaI-cut pSMRY2+Psf2 (Yang *et al.*, 2005) to give pSMRY2+Psf2-HBD. pSMRY2+Psf2-HBD was cleaved with EcoRV to direct integration into the *psf2*$^+$ locus. Psf2 was tagged with the TAP (tandem affinity purification) tag using the product of a PCR reaction derived from primers psf2-F (5′-tggaaattaacgaaatacgtcctatatttcgagaggtgatggacagaatgcgcaaaattgttcaagtttcccaagaagaacggatccccgggttaattaa-3′) and psf2-R (5′-atttcactactacaaagttggtattcataaacacttcgtaggattcattatcattatttttaaagtacatcatccacacggaattcgagctcgtttaaac-3′); pFA6a-CTAP2-kanMX6 (Tasto *et al.*, 2001) was used as template.

Psf3 was tagged with myc using the product of a PCR reaction derived from primers psf3-F (5′-caaaagctcgtgctgccacagtattgagagtatcacataatgcccatcgatccctaataaactggcaaaattccacttcgcggatccccgggttaattaa-3′) and psf3-R (5′-cgtaatattgaacaagtatatgttaaggattctttttcttttttctttgaacagaagggatttacgatcaacactcatttagaattcgagctcgtttaaac-3′); pFA6a-13myc-natMX6 was used as template (I. Charapitsa and S.A.M., unpublished data). To tag the *psf3*$^+$ gene with GFP or YFP, one-step PCR-mediated gene targeting was used with plasmid pSMRG2+ or pSMUY2+ as template. DNA for transformation was amplified using the primers 5′- caaaagctcgtgctgccacagtattgagagtatcacataatgcccatcgatccctaataaactggcaaaattccacttcgctcgagggtagatctggtgccc-3′ and 5′- cttttctttttttctttgaacagaagggatttacgatcaacactcatttagtgtttcacatatagattagcgtgctattctgagcccccgatttagagcttgac-3′.

Transformants were screened by colony PCR to confirm that the gene was successfully tagged (primers used are available on request).

Chromatin-binding Assay

Chromatin-binding assays and image analysis was carried out as previously described (Kearsey *et al.*, 2000; Gregan *et al.*, 2003; Kearsey *et al.*, 2005), although for some proteins the buffer conditions were modified. For analysis of Pol1 and Dpb4, a low-salt extraction buffer was used with the following composition: 20 mM Pipes-KOH, pH 6.8, 0.4 M sorbitol, 10 mM KAc, 0.5 mM spermidine, 0.15 mM spermine, and 1 mM EDTA. Chromatin-binding assays were performed at least twice and error bars show the statistical range. At least 100 cells were counted for each data point.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out basically as previously (Strahl-Bolsinger *et al.*, 1997). Briefly, cells were fixed for 15 min in 1% formaldehyde, and the reaction was stopped by adding glycine to 125 mM. After cell breakage, the extracts were sonicated in an MSE Soniprep 250, using six 15-s pulses. Immunoprecipitations were carried out using protein G Dynabeads (Invitrogen, Carlsbad, CA), preadsorbed with anti-GFP (monoclonal 3E1) or anti-FLAG (M2, Sigma F3165). Immunoprecipitated DNA was analyzed by 25–28 cycles of PCR, using *ars2004* primers 730 (5′-cttttgggtagtttcggatcc-3′) and 731 (5′-atgagtacttgtcacgaattc-3′) and non-ars primers 732 (5′-tcgaagatcctaccgctttc-3′) and 733 (5′-cttgcgctgaagctttagtaaaag-3′; Yabuuchi *et al.*, 2006). Primer concentrations used for PCR were as follows: 0.3 μM for 730 and 731 and 1 μM for 732 and 733.

Protein Analysis

Protein extracts were made by TCA extraction and analyzed by Western blotting as described previously (Ralph *et al.*, 2006). TAP-tagged proteins were detected with peroxidase–anti-peroxidase–soluble complex (P1291, Sigma). Psf3-Myc was detected using antibody M5546 (Sigma), and α-tubulin was detected with antibody T5168 (Sigma).

RESULTS

Conditional Inactivation of Psf1 and Psf2 Using the β-Estradiol Hormone-binding Domain

Previous work in several organisms has shown that proteins tagged with the HBD can be regulated with β-estradiol (reviewed in Picard, 2000). Hsp90 binds to the HBD in the absence of β-estradiol and can inactivate the tagged protein, usually by steric hindrance, but binding of β-estradiol to the HBD causes Hsp90 displacement and thus can reactivate the fusion protein. The C-terminal region of Psf1 is exposed on the surface of the complex and has been suggested to be important in mediating protein–protein interactions (Chang *et al.*, 2007; Kamada *et al.*, 2007). Therefore, we first determined whether Psf1 would tolerate the 30-kDa HBD as a C-terminal extension. Plate assays show that the *psf1-HBD* strain grows normally on plates containing β-estradiol, but is inviable in the absence of the hormone (Figure 1A). To examine the effect of Psf1 inactivation on DNA replication, the *psf1-HBD* strain was arrested in G1 using nitrogen starvation and released from the block in the presence or absence of β-estradiol. Flow cytometry shows an arrest of DNA replication in the absence of β-estradiol (Figure 1B), and cells became elongated (not shown). Cell viability remains high during the arrest and cells do not enter an untimely mitosis (data not shown), suggesting that the DNA replication checkpoint is activated. Inactivation of GINS function is readily reversible, because on readdition of β-estradiol DNA replication is activated within 1h (Figure 1B), and viability of the released cells is high (data not shown). Similar results were obtained with a strain where Psf2 is tagged with HBD (Bøe *et al.*, 2008).

In addition to examining the role of GINS in the vegetative cell cycle, we examined premeiotic S phase in cells containing Psf2-HBD. In a *pat1* haploid meiosis, inactivation of Psf2-HBD blocked premeiotic S phase (Supplementary Figure S1), and similar results were obtained in a diploid (not shown).

We investigated whether HBD regulation of GINS subunits affects their cellular localization by tagging HBD-tagged proteins with YFP. We were unable to derive a haploid *psf1-HBD-YFP* strain, but a *psf2-HBD-YFP* strain is viable in the presence of β-estradiol. This strain showed arrest of DNA replication in the absence of β-estradiol as with the *psf1-HBD* mutant (Figure 1C), and β-estradiol did not alter the Psf2-HBD-YFP level as assessed by Western blotting (Figure 1E). In the presence of β-estradiol, Psf2-HBD-YFP is constitutively nuclear during the vegetative cell cycle, as seen with Psf2-YFP, but after withdrawal of β-estradiol the protein became delocalized over the cell within 1 h (Figure 1D). Even when cells were first arrested in S phase using HU, when GINS is chromatin associated, withdrawal of β-estradiol caused nuclear localization of Psf2 to be lost (data not shown).

We also examined the effect of Psf2-HBD inactivation on the cellular localization of another GINS subunit, Psf3. Psf3-GFP is nuclear throughout the vegetative cell cycle (Figure 2A, −T). A chromatin-binding assay based on detergent extraction of permeabilized cells shows that this protein is retained on chromatin in binucleate cells, but not in uninu-

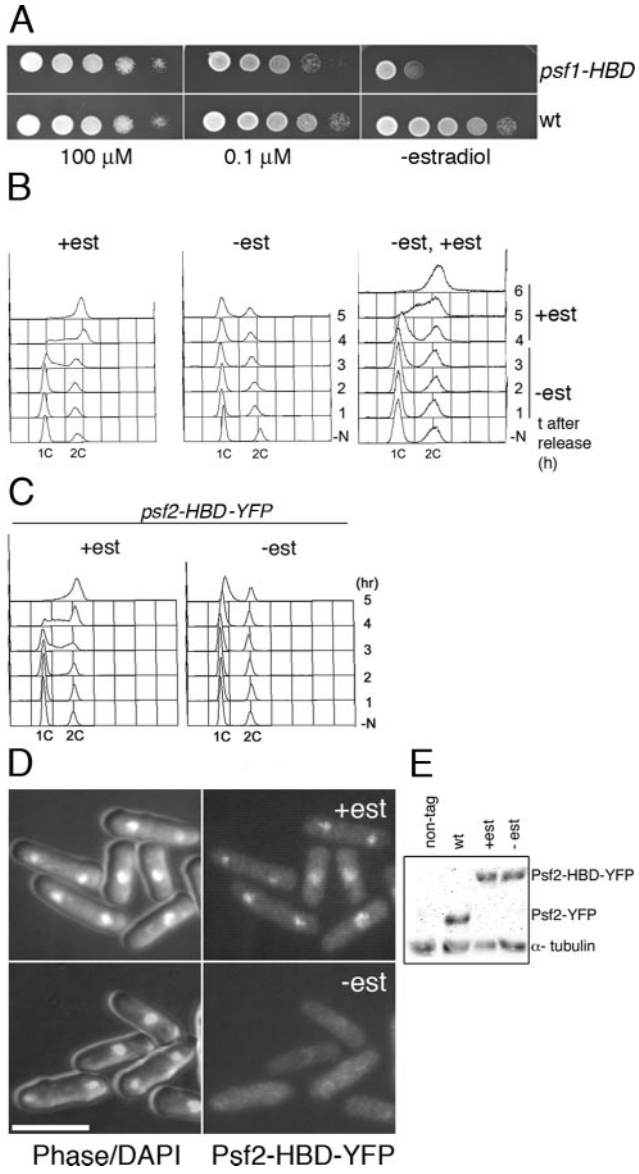

Figure 1. The β-estradiol HBD can be used to confer conditional function on GINS subunits Psf1 and Psf2. (A) Serial dilutions of a *psf1-HBD* strain (P1519) were spotted onto YES medium containing different concentrations of β-estradiol as indicated. (B) Flow cytometric analysis of a *psf1-HBD* strain after Psf1 inactivation. A *psf1-HBD* strain (P1519) was arrested in G1 by growing in EMM minus nitrogen medium for 16 h at 25°C and then was resuspended in EMM plus nitrogen medium either with or without 125 nM β-estradiol as indicated. The data in the −est+est panel relate to cells that were initially released back into the cell cycle in the absence of β-estradiol, and β-estradiol was added at 4 h. (C) Flow cytometric analysis of a *psf2-HBD-YFP* strain (P1907) after Psf2 inactivation. Cells were treated as in B except a release temperature of 36°C was used. (D) Cellular localization of Psf2-HBD-YFP before and after Psf2 inactivation. Cells (P1907) show nuclear localization of Psf2-HBD-YFP when grown in medium containing β-estradiol, but after 1-h growth in medium lacking β-estradiol, nuclear localization of the Psf2 fusion protein is lost. Bar, 10 μm. (E) Western blotting analysis of Psf2-HBD-YFP levels from cells grown in medium containing (+est) or lacking (−est) β-estradiol; α-tubulin is shown as a loading control.

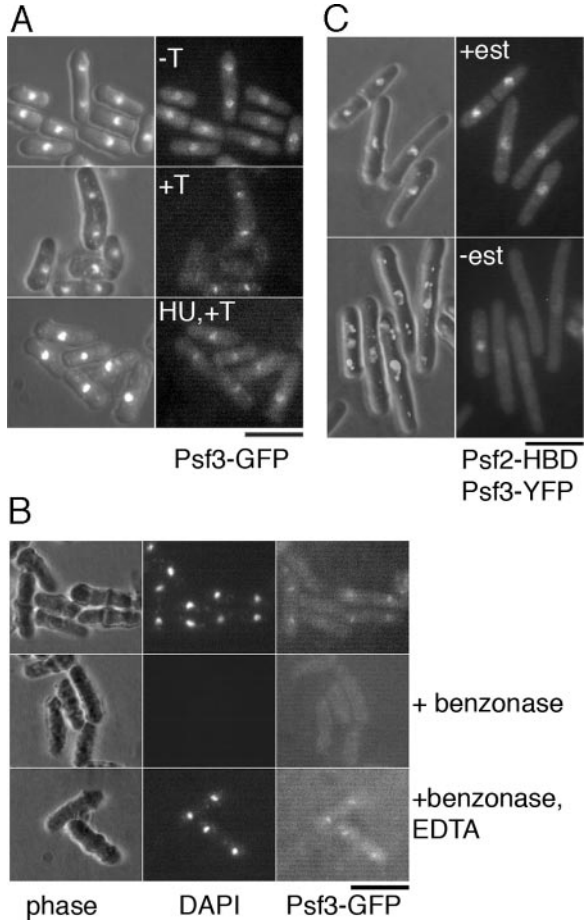

Figure 2. Cell cycle and Psf2-dependent chromatin binding of Psf3. (A) Psf3 binds to chromatin periodically during the cell cycle. Cells expressing Psf3-GFP (P1739) were either fixed directly (−T) or after extraction with detergent (+T) and examined by fluorescence microscopy. For the +HU,+T panel, cells were grown in medium containing HU for 2 h before detergent extraction and fixation. Retention of Psf3 in binucleate cells and after HU arrest is consistent with GINS chromatin binding in S phase. (B) Cells expressing Psf3-GFP (P1739) were detergent extracted in the presence or absence of benzonase and benzonase inhibitor as shown, before fixation and examination by fluorescence microscopy. Loss of nuclear Psf3 after digestion of DNA suggests that Psf3 is retained in detergent extracted cells by DNA binding. (C) Inactivation of Psf2 leads to loss of nuclear localization of Psf3-YFP. A *psf2-HBD* strain expressing Psf3-YFP (P1968) was grown in β-estradiol–containing medium (+est) and then transferred to medium lacking β-estradiol for 5 h (−est). Cells were fixed directly. Bar, 10 μm.

cleate (G2) cells (Figure 2A, +T), and this retention is dependent on DNA integrity (Figure 2B). After arrest in S phase by HU, Psf3 is retained in most cells after detergent extraction (Figure 2A, HU, +T), consistent with chromatin association of Psf3 in S phase, as suggested by ChIP analysis (Yabuuchi *et al.*, 2006). Inactivation of Psf2-HBD results in loss of nuclear localization of Psf3-YFP (Figure 2C, −est), implying that chromatin binding of GINS is lost and the entire complex is delocalized under these conditions.

One reason why GINS nuclear localization is lost in the HBD strains could be that GINS normally shuttles between nucleus and cytoplasm, and on β-estradiol withdrawal, GINS is trapped in the cytoplasm by Hsp90, which is predominantly cytoplasmic in *S. pombe* (Mishra *et al.*, 2005). We therefore examined whether there are any normal conditions when non-HBD–tagged GINS subunits show loss of nuclear localization. Although Psf2 and Psf3 are constitutively nuclear during the vegetative cell cycle (e.g., Figure 3,

A and B, log), we observed that the nuclear localization of these subunits is lost on G1 arrest by nitrogen starvation (Figure 3, A and B, −N, and E). When G1-arrested cells are released from the cell cycle block, clear nuclear localization of Psf2 and Psf3 is reestablished within about 4 h, around the time of S phase. These changes in cellular localization occur in the absence of any effect on protein levels (Figure 3, C and D). We observed a similar change in the nuclear localization of Psf1 on G1 arrest by indirect immunofluorescence, and also using a Psf1-YFP strain (Supplementary Figure S2).

Effect of GINS Inactivation on Cdc45 Chromatin Binding

Previous studies have emphasized the importance of GINS function in establishing Cdc45 association with chromatin in *Xenopus* (Kubota *et al.*, 2003). In *S. pombe*, ChIP analysis also shows that Psf3 function is also required for Cdc45 association with origins (Yabuuchi *et al.*, 2006). In contrast, *S. cerevisiae* Cdc45 associates with origins in the absence of GINS, although its stable incorporation into replication complexes that move away from the origin requires GINS function (Kanemaki and Labib, 2006). To clarify these different results, we examined the effect of Psf1-HBD inactivation on Cdc45, using a chromatin-binding assay for this protein (Gregan *et al.*, 2003) to compare with previous ChIP data. Psf1-HBD cells were released from a G1 block either in the presence or absence of β-estradiol, and chromatin binding of Cdc45-YFP was assessed by detergent extraction. Cells released in the presence of β-estradiol show Cdc45 chromatin binding coinciding with the onset of S phase as expected (Figure 4, A–C). However, Cdc45 chromatin binding is not seen in the absence of β-estradiol, showing that GINS is required to establish its chromatin association. If Psf1-HBD cells are arrested early S phase by Psf1 inactivation (i.e., β-estradiol withdrawal), subsequent reactivation of Psf1 leads to establishment of Cdc45 chromatin binding (Supplementary Figure S3) consistent with the reversibility of the Psf1 block shown in Figure 1B. We also showed that if *psf1-HBD* cells are released from G1 into an S phase (HU mediated) block in the presence of β-estradiol to allow chromatin association of Cdc45, subsequent inactivation of Psf1-HBD in the absence of HU leads to loss of Cdc45 chromatin binding (compare Psf1 on and Psf1 off panels, Figure 4E). This suggests that as well as being required to establish Cdc45 in replication complexes, continued functioning of GINS is necessary to maintain Cdc45 chromatin binding during the elongation stage of DNA replication, consistent with results from budding yeast (Kanemaki and Labib, 2006).

GINS Function and Chromatin Binding of Replicative Pol ε and Pol α

We carried out experiments to clarify the role of Psf1 function with respect to the chromatin binding of the replicative DNA polymerases. Pol ε is a four subunit complex, comprising of a large catalytic subunit Pol2/Cdc20, and three smaller subunits: Dpb2, Dpb3, and Dpb4. Previously, the Dpb2 subunit of Pol ε has been shown to bind to origins early in S phase fission yeast (Feng *et al.*, 2003) in a manner that is Psf3 dependent (Yabuuchi *et al.*, 2006). We examined the behavior of the Dpb4 subunit; this also shows cell cycle changes in chromatin binding consistent with S phase–specific DNA association (Figure 5A). Although cells arrested in S phase with HU show chromatin retention of Dpb4, this is abolished if Psf1 is inactivated (Figure 5B), suggesting that GINS promotes Pol ε chromatin binding. Consistent with these data, Psf1-HBD also shows a synthetic interaction with Pol ε (Cdc20); when a *psf1-HBD cdc20ts* strain is grown under conditions semipermissive for both alleles, the double mutant grows more poorly than either single mutant, suggesting that the two proteins function on the same pathway

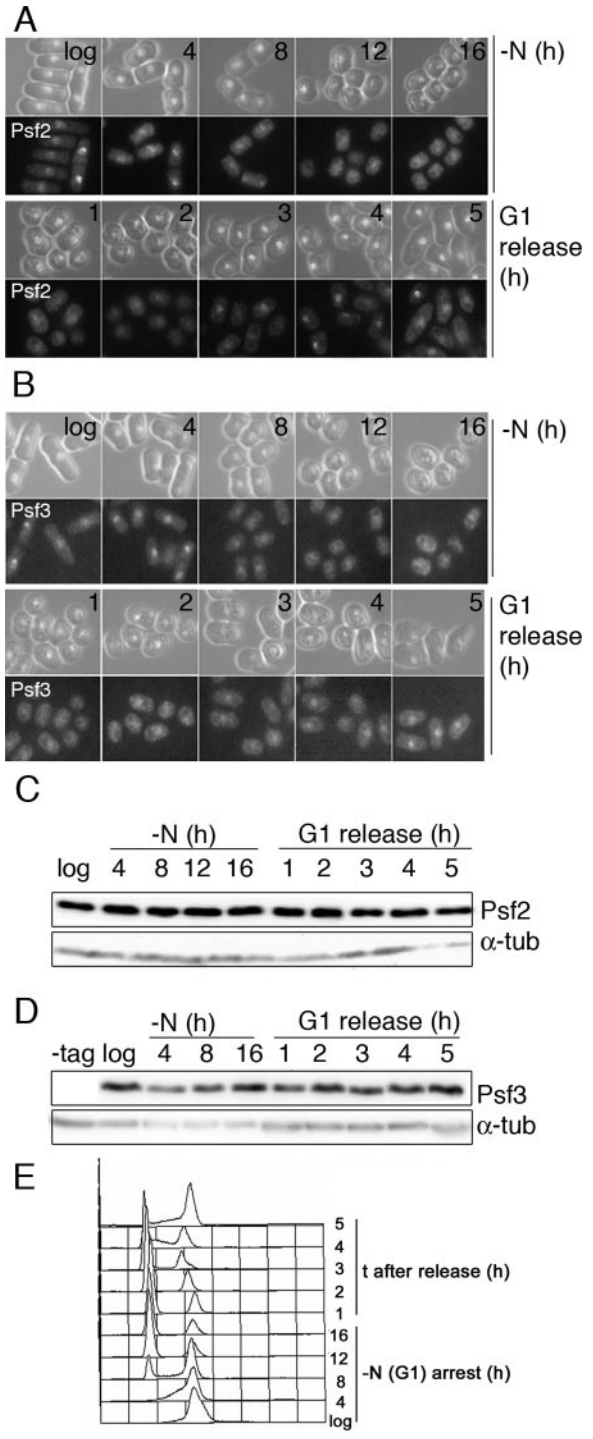

Figure 3. Nuclear localization of GINS subunits Psf2 and Psf3 is lost on nitrogen starvation in the absence of any effects on protein levels. (A and B) *psf2-YFP* (A, P1638) and *psf3-GFP* (B, P1739) strains were grown to log phase, transferred to minus nitrogen medium to arrest cells in G1, and finally released from the G1 arrest by transferring to nitrogen-containing medium. Samples of cells were analyzed after fixation by fluorescence microscopy (A and B) and Western blotting (C and D). (E) Flow cytometric analysis of experiment shown in B and D; DNA replication kinetics for the Psf2 strain were similar (not shown).

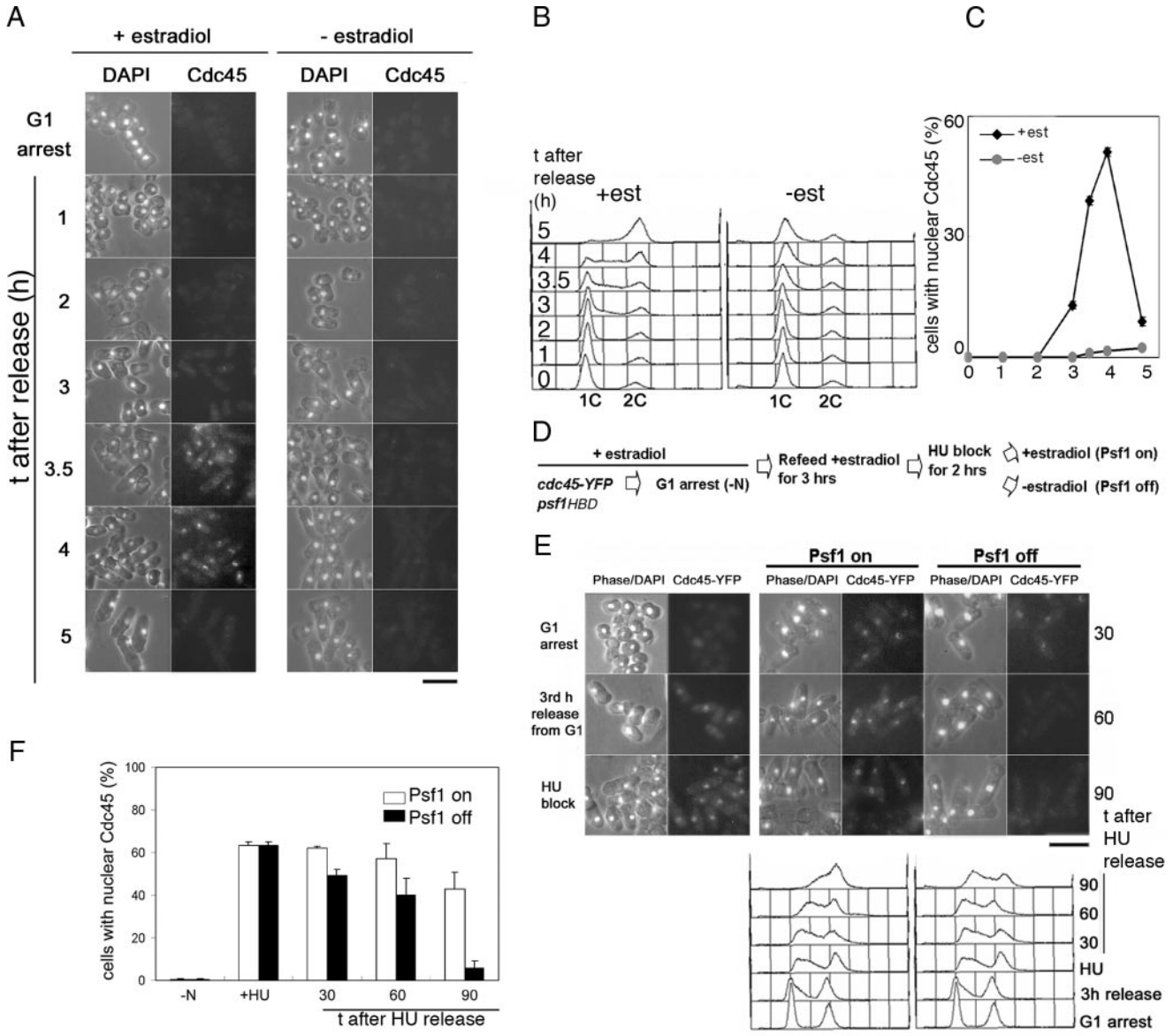

Figure 4. Psf1 function is required for establishment and maintenance of Cdc45 chromatin binding. (A and B) A *cdc45-YFP psf1-HBD* strain (P1635) was arrested in G1 by nitrogen starvation and released from the block in medium either containing or lacking β-estradiol. Samples of cells at time points indicated were detergent extracted, fixed, and analyzed by fluorescence microscopy to reveal whether Cdc45 is bound to chromatin (A). DNA contents were also analyzed by flow cytometry (B). Inactivation of Psf1 results in failure of Cdc45 to bind chromatin, which normally coincides with DNA replication (compare −est, +est, 3.5–4 h). (C) Quantitative analysis of cells shown in A. (D–F) Psf1 function is required to maintain Cdc45 chromatin binding. Experimental design is shown in D. A *cdc45-YFP psf1-HBD* strain (P1635) was arrested in G1 and released from the cell cycle block in medium containing β-estradiol; after 3 h, HU was added to arrest cells in S phase with chromatin-associated Cdc45 (E, left-hand bottom panels). Subsequently, the HU was washed out, and cells were transferred to medium either containing or lacking β-estradiol, and cells were analyzed for Cdc45 chromatin binding (E, top panel) and flow cytometry (E, bottom panel). Where Psf1 function is maintained, Cdc45 chromatin binding is seen and DNA replication resumes (E, center panels). Where Psf1 is inactivated, Cdc45 chromatin binding is lost, and DNA replication does not resume (E, right-hand panels). (F) Quantitative analysis of cells shown in E. Bar, (A and E) 10 μm.

(Figure 5C). We also see a similar synthetic interaction with the catalytic subunit of Pol δ (Figure 5C), agreeing with a previously reported Sld5-Pol δ interaction (Ohya *et al.*, 2002).

To investigate the role of GINS in Pol α function, we first examined whether the catalytic subunit of Pol α (Pol1) shows chromatin association during S phase. Pol1-CFP is retained in binucleate cells after detergent extraction, and this retention is blocked by CDK inactivation (Supplementary Figure S4, A and B), consistent with chromatin binding of this polymerase in S phase. Chromatin binding of Pol1 is also seen if cells are arrested in S phase with HU, but if Cdc10 is inactivated to block cells in G1, chromatin binding is not seen (Supplementary Figure S4, C–E). Inactivation of Cdc10 prevents pre-RC formation and DNA replication via a block to expression of Cdt1 and Cdc18; thus this result implies that chromatin binding of Pol1 requires pre-RC formation. However, unlike the findings with Pol ε, inactivation of Psf1 did not prevent chromatin binding of Pol α as assayed by detergent extraction (Figure 6). Psf1-HBD cells expressing Pol1-YFP were arrested in G1 and then released into the cell cycle in the presence and absence of β-estradiol. Under conditions where Psf1 is active, Pol α associated with

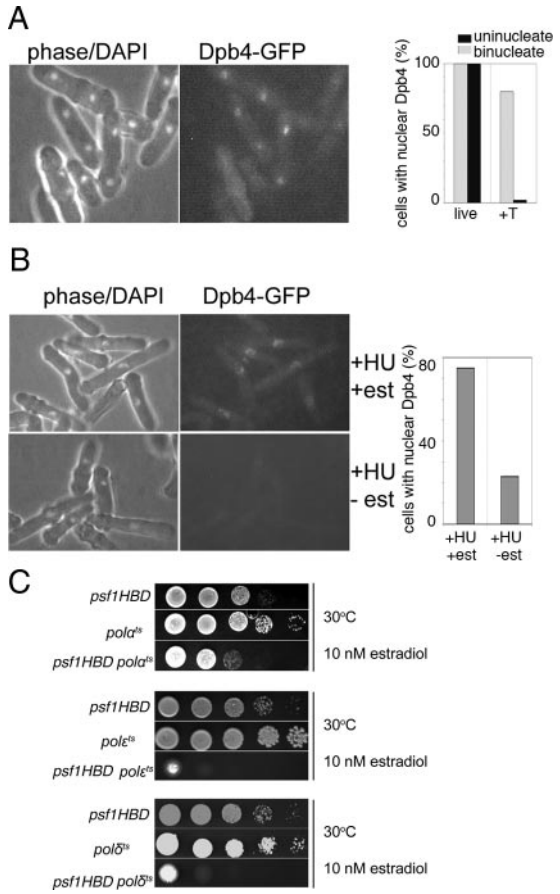

Figure 5. Inactivation of Psf1 prevents S phase chromatin binding of Pol ε subunit Dpb4-GFP. (A) Dpb4 associates with chromatin periodically in the cell cycle. Cells expressing Dpb4-GFP (P1758) were detergent extracted, fixed, and analyzed by fluorescence microscopy. Chromatin association is predominantly seen in binucleate (i.e., G1/S) cells after extraction (+T). (B) Effect of Psf1 inactivation of Dpb4 chromatin binding. *psf1-HBD dpb4-GFP* cells (P1760) were initially grown in β-estradiol–containing medium. In the top panel, cells were transferred to medium containing HU and β-estradiol, to block cells in S phase under conditions where Psf1 is active, and grown for 5 h; cells were then detergent-extracted and fixed. Under these conditions, Dpb4 is chromatin associated. In the bottom panel, cells were transferred to medium containing HU but lacking β-estradiol, before detergent extraction. Under these conditions, when Psf1 is inactivated, Dpb4 chromatin association is not observed. (C) Synthetic interactions between *psf1-HBD* and temperature-sensitive alleles of catalytic subunits of replicative DNA polymerases α, ε, and δ. Strains were grown on limiting β-estradiol to partially inactivate Psf1-HBD and at a semipermissive temperature to partially inactivate the DNA polymerase. Strains used were (top to bottom) P1519, P412, P1778, P1519, P275, P1781, P1519, P274, and P1780.

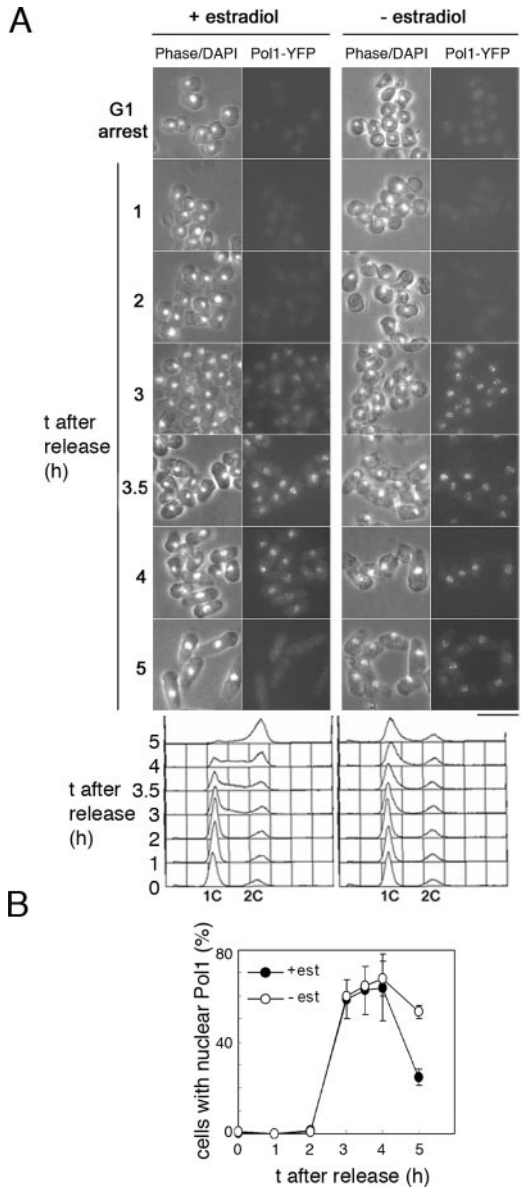

Figure 6. Inactivation of Psf1 does not affect the S phase chromatin binding of catalytic subunit of Pol α. (A) A *psf1-HBD pol1-YFP* strain (P1675) was arrested in G1 by nitrogen starvation and then released from the block in the presence or absence of β-estradiol. Cells were analyzed for Pol1-YFP chromatin binding by detergent extraction (top panel) and by flow cytometry (bottom panel). Releasing cells from the G1 block when Psf1 is inactive does not prevent Pol1-YFP chromatin binding. Bar, 10 μm. (B) Quantitative analysis of cells shown in A.

chromatin ca. 3 h after release and was released with the completion of S phase (Figure 6, A and B, + estradiol). When Psf1 was inactivated, Pol α bound with similar kinetics, but displacement did not occur (Figure 6, A and B, − estradiol). We interpret this to indicate binding of Pol α to chromatin in S phase, which is occurring in a GINS-independent manner, but in the absence of GINS function S phase cannot be completed, and Pol α remains chromatin associated, analogous to what is seen when cells are arrested in S phase with an HU arrest.

We carried out a similar experiment using ChIP to determine if GINS inactivation affected the interaction between Pol1 and the *ars2004* replication origin. A Psf1-HBD Pol1-GFP strain (P2053) was arrested in G1 and released from the block in the presence or absence of β-estradiol; in this experiment, HU was added to both cultures to slow the movement of replication forks. DNA precipitated with anti-GFP antibody was analyzed by PCR using primers for *ars2004* and a nonorigin sequence ~30 kb away from *ars2004*. Association of Pol1 with *ars2004* but not with the nonorigin sequence is seen 2 h after release when Psf1 is active (Figure 7A, +est+HU), somewhat in advance of bulk DNA replication seen in the absence of HU (Figure 7B, +est). This selective association with *ars2004* is not seen at later time points, presumably reflecting movement of replication forks

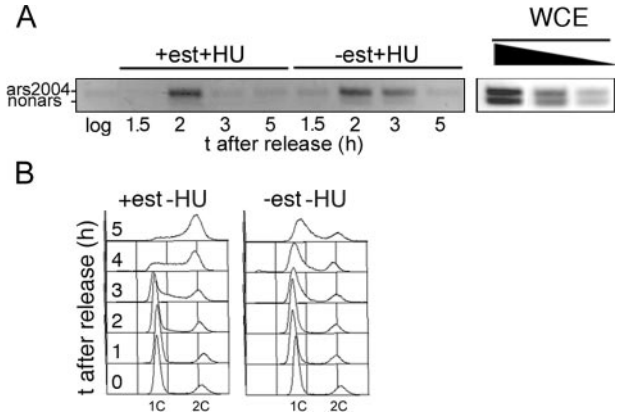

Figure 7. Association of the catalytic subunit of Pol α with replication origin *ars2004* is not affected by inactivation of GINS. (A) A Pol1-GFP Psf1-HBD strain (P2053) was arrested in G1 by nitrogen starvation and then released into the cell cycle in the presence of HU, to slow replication forks, and in the presence (Psf1 active) or absence (Psf1 inactive) of β-estradiol. At the time points shown, cells were processed for ChIP and used as template for PCR using primers for *ars2004* (top bands) or non-ars (bottom bands). WCE shows PCR result using dilutions of whole cell extract as template. A similar experiment showed a block to Pol ε association, with the origin in the absence of GINS function (Supplementary Figure S5). (B) Pol1-GFP Psf1-HBD cells were arrested as in G1 and released from the block as in A, except cells were released in the absence of HU and analyzed by flow cytometry. This shows the timing of S phase when Psf1 is active (+est) and confirms the block to S phase in the absence of β-estradiol.

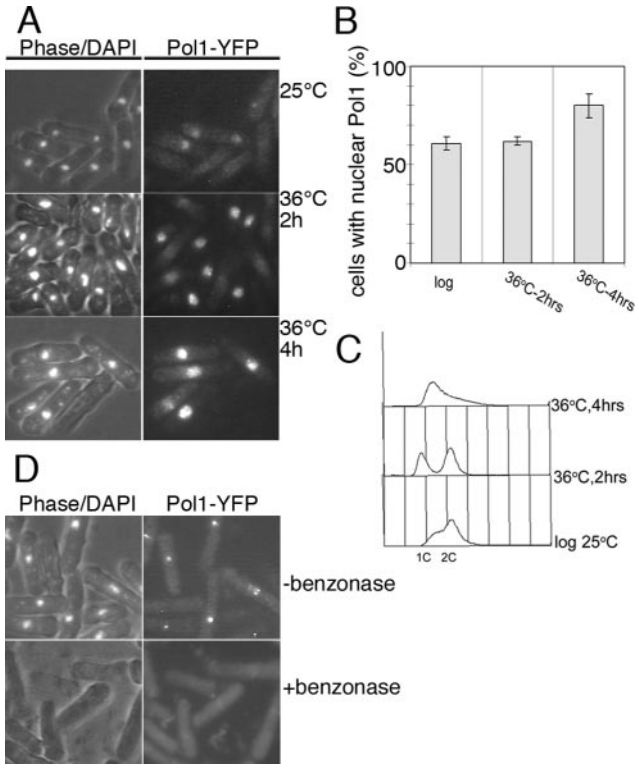

Figure 8. Inactivation of Cdc45 does not affect chromatin binding of Pol1-YFP. A strain temperature sensitive for Cdc45 function expressing Pol1-YFP (P1922) was grown to log phase at the permissive temperature and then transferred to 36°C for the times shown. Cells were analyzed by fluorescence microscopy after detergent extraction (A and B) and by flow cytometry (C). (D) Cells from the 4-h time point in A were digested with benzonase during detergent extraction. Loss of Pol1-YFP after nuclease digestion suggests that Pol1-YFP requires DNA integrity for retention.

even in the presence of HU. Consistent with the results seen with detergent extraction, association of Pol1 with *ars2004* was also seen after Psf1 inactivation, 2–3 h after release (Figure 7A, −est+HU). However, in a similar ChIP experiment, association of the Dpb2 subunit of Pol ε with *ars2004* was blocked by Psf1-HBD inactivation (Supplementary Figure S5A), consistent with results using a temperature-sensitive allele of *psf3* (Yabuuchi *et al.*, 2006). These results suggest that GINS function is not required for the association of Pol α with replication origins at the start of S phase, in contrast to the situation with Pol ε. Consistent with the notion that GINS and Pol α do not function on the same pathway, we failed to observe synthetic interactions between the catalytic subunit of Pol α and Psf1-HBD (Figure 5C).

These results imply that the pathway of assembly of replication factors involving GINS may not be required for Pol α chromatin association. Because GINS is required for Cdc45 chromatin binding, we examined whether Cdc45 inactivation has any effect on Pol α chromatin association, because this protein has been previously implicated in this process. After Cdc45 inactivation using a temperature-sensitive allele (Uchiyama *et al.*, 2001a), Pol1-YFP becomes resistant to detergent extraction in S phase–arrested cells, and in fact the level of chromatin-bound Cdc45 appears to be enhanced compared a normal S phase (Figure 8, A–C). The retained Pol1 is released on nuclease digestion, suggesting it is DNA bound (Figure 8D). This suggests that Cdc45 is not required for the chromatin association of Pol α, consistent with the analysis using Psf1-HBD. This result partly agrees with a previous study (Uchiyama *et al.*, 2001b), although this earlier article suggested that Pol α associates with chromatin in a Cdc10-independent manner, which we do not observe. Taken together, these results indicate that CDK activation leads to chromatin binding of Pol α in a manner that is not GINS or Cdc45 dependent and that separate pathways establish chromatin association of the replicative polymerases α and ε in fission yeast.

DISCUSSION

In this article we have shown that fusing the GINS subunits Psf1 and Psf2 subunits to the β-estradiol HBD is an effective strategy for inactivating the GINS replication factor in fission yeast. Inactivation of HBD fusion proteins appears to occur normally by steric hindrance after interaction with Hsp90 (reviewed in Picard, 2000), and the amenability of GINS to this approach could reflect its suggested role in mediating interactions between replication factors. With Psf2-HBD, however, we unexpectedly find that absence of β-estradiol rapidly leads to loss of nuclear localization of Psf2-HBD and at least one other GINS subunit. Although we were unable to derive a haploid Psf1-HBD-YFP strain, we also found that lack of β-estradiol causes loss of Psf1-HBD-YFP nuclear localization in a diploid strain containing *psf1*$^+$ (data not shown), suggesting that this behavior is seen with other HBD-tagged GINS subunits. This relocalization may simply be a reflection of the predominantly cytoplasmic localization of Hsp90/Swo1 in *S. pombe* (Mishra *et al.*, 2005). However, we also observed loss of nuclear localization of non-HBD tagged GINS subunits on G1 arrest by nitrogen starvation, suggesting that the nuclear localization of GINS can be subject to physiological regulation. Further work will be required to

determine whether this is of significance for replication control. A limited survey of fission yeast replication factors (Mcm2-7, Cdc45, Mcm10, Pol1, and Dpb3) shows that these proteins remain localized in the nucleus on G1 arrest (Gregan *et al.*, 2003; Namdar and Kearsey, 2006; and data not shown) unlike the situation with the Psf2 and Psf3 subunits of GINS.

As expected, inactivation of Psf1 prevents Cdc45 chromatin binding, although this arrest is reversible, suggesting that GINS function does not have to be provided at a critical step in replication activation. Inactivation of Psf1 function after replication complexes have been formed also leads to loss of Cdc45 chromatin binding, consistent with the notion that a complex of GINS and Cdc45 is active at the replication fork and that the function of GINS may be as a scaffold complex to retain other proteins in the replisome.

We also show that Psf1 is required for the chromatin binding and origin association of the replicative Pol ε, but not Pol α, indicating that there may be two pathways for establishing the chromatin association of these factors. Pol ε is likely to be the polymerase functional on the leading strand (Pursell *et al.*, 2007) and thus must presumably be loaded in an early step in DNA synthesis to take over from the priming Pol α. The mechanism of recruitment may involve Dpb11/Cut5, because Pol ε interacts with this factor. In fission yeast, Cut5 association with origins is Psf3 dependent (Yabuuchi *et al.*, 2006), thus providing a possible explanation for the Pol ε dependence on GINS function. Also an interaction between Psf1 and the Dpb2 subunit of Pol ε has been detected by two hybrid analysis (Takayama *et al.*, 2003). How Pol ε is retained at the fork is less clear, but coupling of the leading strand polymerase with the helicase would be expected to prevent the generation of excessive single-stranded DNA. Pol ε does not seem to be tightly associated with the GINS-containing replisome progression complex in vivo (Gambus *et al.*, 2006). Cut5/Dpb11 is probably not relevant to Pol ε tethering, as it is not required for elongation (Hashimoto and Takisawa, 2003). PCNA could have a Pol ε tethering role, although this protein stimulates Pol ε poorly in vitro (Chilkova *et al.*, 2007), and mutations in the putative PCNA-interaction motif of Pol ε are not lethal (Dua *et al.*, 2002). The mechanism to retain the leading strain polymerase must presumably be compatible with both Pol δ and ε, given that the catalytic activity of Pol ε is not essential (Kesti *et al.*, 1999; Feng and D'Urso, 2001).

In this study we show that the binding of the Pol α catalytic subunit to chromatin is independent of GINS and Cdc45, but still requires S-CDK and Cdc10 function. Two models for Pol α recruitment can be envisaged, one requiring activation of DNA helicase activity to generate ssDNA, which can then recruit RPA and Pol α (Tanaka and Nasmyth, 1998; Walter and Newport, 2000). In this scheme, Pol α recruitment would be indirectly dependent on initiation processes such as pre-RC formation, which helicase activation requires. A second model involves recruitment by interaction with chromatin-associated proteins. This could occur without the requirement for DNA unwinding, as suggested by some studies indicating Pol α chromatin association in G1 in advance of pre-RC formation (Desdouets *et al.*, 1998; Uchiyama *et al.*, 2001b). Replication factors capable of interacting with Pol α include ORC, which interacts with the B-subunit throughout the cell cycle, and recruits the Spp2 primase subunit around S phase (Uchiyama and Wang, 2004). In addition, Mcm10 and its interacting factor And-1 (Ctf4/Mcl1) interact with Pol α (Fien *et al.*, 2004; Ricke and Bielinsky, 2004; Zhu *et al.*, 2007). Mcm10 is recruited to pre-RCs in G1 (Ricke and Bielinsky, 2004), whereas And-1 recruitment to pre-RCs requires both Mcm10 and CDK activation (Zhu *et al.*, 2007). We have previously shown that Mcm10 inactivation in *S. pombe* also affects chromatin binding of a primase subunit of Pol α, with no effect on GINS binding (Yang *et al.*, 2005). The *S. pombe* orthologue of And-1 (Mcl1) also interacts with Pol α (Williams and McIntosh, 2005), and it would be interesting to determine whether it is required for Pol α chromatin association.

Although our results imply that GINS is not required for the chromatin recruitment of Pol α, they do not rule out the possibility of interactions between GINS and Pol α. Pol α may be retained near the fork by interaction with the helicase complex so that it is in a position for repeated Okazaki fragment priming on the lagging strand, as has been suggested for Archaeal GINS and primase (Marinsek *et al.*, 2006). In eukaryotes, this could occur via a Pol α–interacting protein such as Ctf4, which interacts with GINS in *S. cerevisiae* (Gambus *et al.*, 2006).

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