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Sgt1p and Skp1p Modulate the Assembly and Turnover of CBF3 Complexes Required for Proper Kinetochore Function

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Kinetochores are composed of a large number of protein complexes that must be properly assembled on DNA to attach chromosomes to the mitotic spindle and to coordinate their segregation with the advance of the cell cycle. CBF3 is an inner kinetochore complex in the budding yeast *Saccharomyces cerevisiae* that nucleates the recruitment of all other kinetochore proteins to centromeric DNA. Skp1p and Sgt1p act through the core CBF3 subunit, Ctf13p, and are required for CBF3 to associate with centromeric DNA. To investigate the contribution of Skp1p and Sgt1p to CBF3 function, we have used a combination of in vitro binding assays and a unique protocol for synchronizing the assembly of kinetochores in cells. We have found that the interaction between Skp1p and Sgt1p is critical for the assembly of CBF3 complexes. CBF3 assembly is not restricted during the cell cycle and occurs in discrete steps; Skp1p and Sgt1p contribute to a final, rate-limiting step in assembly, the binding of the core CBF3 subunit Ctf13p to Ndc10p. The assembly of CBF3 is opposed by its turnover and disruption of this balance compromises kinetochore function without affecting kinetochore formation on centromeric DNA.

INTRODUCTION

Kinetochores are specialized DNA–protein complexes that are essential for the proper segregation of chromosomes. Kinetochores consist of multiple, discrete protein complexes that are recruited to centromeric (*CEN*) DNA in what is believed to be a hierarchical manner; specialized nucleosomes and specific DNA binding complexes nucleate the recruitment of other kinetochore proteins required for the attachment and movement of chromosomes along the mitotic spindle. The kinetochore is also critical for the spindle checkpoint, a surveillance system that ensures proper kinetochore-microtubule attachments are formed before the onset of anaphase. The recent identification of a large number of kinetochore proteins suggests that the processes of microtubule attachment, chromosome movement, and coordination with the cell cycle are complex and require the organization of a large number of proteins at the centromere (Cheeseman *et al*., 2002; McAinsh *et al*., 2003).

Remarkably, in the budding yeast *Saccharomyces cerevisiae*, this large array of proteins is organized on a compact centromere of 125 base pairs that is divided into three well conserved regions: *CDEI*, *CDEII*, and *CDEIII* (Clarke and Carbon, 1985). Although each region plays an important role in kinetochore function, mutational analyses suggest that only the *CDEIII* region is essential for kinetochore function (Hegemann *et al*., 1988). Biochemical purification of proteins that bind CDEIII and subsequent genetic analyses identified the CBF3 complex, which is comprised of three proteins: Ctf13p (p58), Cep3p (p64), and Ndc10p (p110) (Lechner and Carbon, 1991; Doheny *et al*., 1993; Jiang *et al*., 1993; Lechner, 1994; Strunnikov *et al*., 1995). Additional genes critical for the proper function of CBF3 were identified in dosage suppression studies; *SKP1* was found as a dosage suppressor of a mutation in *CTF13* (*ctf13-30*); similarly, *SGT1* was found as a dosage suppressor of a mutation in *SKP1* (*skp1-4*) (Connelly and Hieter, 1996; Kitagawa *et al*., 1999). Mutations in *SKP1* and *SGT1* abolish CBF3 and in vitro studies suggest that Skp1p and Sgt1p are required for the biochemical activity of Ctf13p (Kaplan *et al*., 1997; Kitagawa *et al*., 1999). Although CBF3 is required for kinetochore function, it does not directly contact microtubules. Instead, it acts to nucleate and organize a large number of outer kinetochore complexes (Basu *et al*., 1999; Ortiz *et al*., 1999; He *et al*., 2001; Jones *et al*., 2001; Janke *et al*., 2002; Li *et al*., 2002; Measday *et al*., 2002). Although its interaction with microtubules is indirect, it has been recently shown that Skp1p directly interacts with the spindle checkpoint protein Bub1p, suggesting that CBF3 may be intimately connected to the regulatory machinery that ensures proper kinetochore–microtubule attachment has occurred before the onset of anaphase (Kitagawa *et al*., 2003).

Studies of CBF3 organization suggest that multiple DNA contacts made by all three subunits are required for CBF3 to stably bind *CEN* DNA and that Ctf13p is the core subunit of the complex, making contacts with both Cep3p and Ndc10p (Espelin *et al*., 1997; Russell *et al*., 1999). The importance of Ctf13p is underscored by its central position in the complex and by the fact that mutations in the *SKP1* and *SGT1* genes inactivate Ctf13p, leaving it unable to form a functional CBF3 complex (Kaplan *et al*., 1997; Kitagawa *et al*., 1999). Skp1p was shown to interact directly with a conserved amino terminal F-box motif in Ctf13p and mutations in the F-box disrupt Skp1p association with Ctf13p, inactivating Ctf13p and abolishing CBF3 function (Russell *et al*., 1999).

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Table 1. Strains used in this study

S. cerevisiae strain	Genotype	Reference
YPH500	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ1his3-Δ200 leu2-Δ1*	Sikorski and Hieter, 1989
YPH1015	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3.L.YPH983) HIS3 SUP11*	Connelly and Hieter, 1999
YPH1161	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-4::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP11*	Connelly and Hieter, 1999
YPH1172	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-3::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP1*	Connelly and Hieter, 1999
JK418	*Mata ura3 leu2-3, 112 trp1 ndc10-1*	Goh and Kilmartin, 1993
KSC433	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13*	This study
KSC1028	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-TRP1::CTF13*	This study
KSC820	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 CEP3-GFP::TRP1*	This study
KSC563	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-4::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP11 GAL1-3XHA-kan::CTF13*	This study
KSC564	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-3::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP11 GAL1-3XHA-kan::CTF13*	This study
KSC951	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 13MYC-TRP::SPC24*	This study
KSC953	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 13MYC-TRP::OKP1*	This study
KSC1118	*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-TRP1::CTF13 13MYC-HIS::DAM1*	This study
KSC1087	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 CFP-kan::SPC42 GFP-TRP::DAM1*	This study
KSC1008	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 GFP-TRP::OKP1*	This study
KSC816	*Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-HIS3::CTF13 GFP-TRP::SPC24*	This study
KSC1122	*Mata ura3 leu2-3, 112 trp1 ndc10-1 GFP-HIS::DAM1*	This study

Similarly, a mutation in *SGT1* that blocks its interaction with Skp1p inhibits the formation of CBF3 (Kitagawa *et al*., 1999). Biochemical and genetic studies suggest that Skp1p and Sgt1p modify Ctf13p, possibly through protein phosphorylation or the action of HSP90 type chaperones (Kaplan *et al*., 1997; Stemmann *et al*., 2002). However, the precise biochemical changes required for Ctf13p activation and how activation contributes to CBF3 function remain unclear. Given the central role of CBF3 in nucleating kinetochore formation and its involvement in the spindle checkpoint, it is critical to precisely understand how Skp1p and Sgt1p contribute to CBF3 function.

Here, we examine how Skp1p and Sgt1p contribute to the function of the CBF3 complex and how this process relates to the normal function of the kinetochore. We report that Skp1p and Sgt1p are required for the assembly of CBF3, which occurs in a stepwise manner. Mutations in *SKP1* suggest that Skp1p plays a dual role contributing to complex turnover as well as to complex assembly. CBF3 assembly is not restricted during the cell cycle and inhibition of CBF3 assembly compromises chromosome segregation independent of kinetochore formation. We propose that Skp1p and Sgt1p may have a general role balancing protein complex assembly and turnover, a property that is important for the proper function of kinetochores.

MATERIALS AND METHODS

Plasmids, Yeast Strain Construction and Antibodies

The coding sequence of *SGT1* was polymerase chain reaction (PCR)-amplified from yeast genomic DNA. Primers (sequences provided upon request) were designed with appropriate restriction sites to insert SGT1 DNA into the pFastBac (Invitrogen, Carlsbad, CA) and pFBNhis10HA baculovirus vectors. The sequence was confirmed, recombinant viruses were generated, and High-Five cells were used to produce recombinant proteins as described previously (Kaplan *et al*., 1997; Russell *et al*., 1999). The *CTF13* gene used to generate recombinant protein was synthetically constructed using short oligonucleotides to optimize codon usage (kindly provided by X. He and P. Sorger, Massachusetts Institute of Technology, Cambridge, MA). Yeast strains were constructed in the indicated backgrounds (Table 1) by homologous recombination by using standard PCR-based targeting techniques (Longtine *et al*., 1998). The integrity of both 5′ and 3′ recombination sites was confirmed using PCR, and the fusion verified by immunoblotting.

Viral infections of insect cells were carried out and lysates were prepared as described previously (Russell *et al*., 1999). Recombinant MRGS-10 × his-HA-Sgt1p was purified using standard metal chelate chromatography conditions. Polyclonal antibodies against Sgt1p were produced in rabbits after injection of purified recombinant MRGS-10 × his-HA-Sgt1p by using standard procedures (Harlow, 1988). Antiserum against Ctf13p, Cep3p, and Skp1p were made as described previously (Kaplan *et al*., 1997; Russell *et al*., 1999). The monoclonal antibody to the hemagglutinin tag (12CA5) was purchased from Roche Diagnostics (Indianapolis, IN), and the rat tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Yeast Growth and Extract Preparation

Yeast strains were grown at 30°C unless otherwise indicated in standard YEP media supplemented with 2% dextrose or 2% raffinose and 2% galactose as indicated previously (Guthrie and Fink, 1991). For chromosome loss assays, yeast were grown to log phase in minimal media lacking histidine and containing 2% galactose and raffinose. Cells were transferred to YEP containing the indicated carbon source for 1 h. Cells were plated on YEP-agar plates containing 2% galactose and raffinose. The number of red colonies (more than half red) and sectored colonies (more than two sectors but less than one-half red) were determined, corrected for the number of cell divisions and the fold increase of each colony type calculated by comparing cells with a wild-type CTF13 promoter.

Cells were arrested in G1 with 15 μg/ml α-factor or in G2/M with 10 μg/ml nocodazole (American Peptide Co., Inc., Sunnyvale, CA). Additional α-factor was added every 90 min to maintain arrest. Cells were immediately chilled on ice and harvested by centrifugation for 15 min at 4°C, washed once in ice cold water, once in extract buffer (50 mM bis-Tris-propane, pH 7.2, 200

mM KCl, 5 mM EGTA, 5 mM EDTA, 100 mM β-glycerol-phosphate, 10% glycerol, 14 mM 2-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride, 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone) and then resuspended in a volume of extract buffer supplemented with a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone and 10 μg/ml leupeptin, pepstatin, and chymostatin) equal to the size of the cell pellet. The resulting cell slurry was immediately frozen in liquid N_2 and processed as described previously (Kaplan and Sorger, 1997).

Chromatin Immunoprecipitation

The protocol used for chromatin immunoprecipitation analysis was performed as described previously (De Wulf *et al.*, 2003). Cells containing temperature-sensitive alleles were grown to log phase at 25°C, shifted to 37°C for 5 h, and fixed in 1% formaldehyde for 15 min. For Ctf13p depletion, cells containing *GAL1-3HA-CTF13* were grown to log phase at 30°C in YEP media containing 2% galactose and 2% raffinose, and either washed with YPD media (2% dextrose) and resuspended in fresh YPD, or washed with YEP with 2% raffinose and resuspended in fresh YEP containing 2% raffinose, and grown for 5 h before harvesting. After immunoprecipitations, samples were analyzed by PCR by using primers for CEN VI (5′-GACGTTACACTACGAACTTAAGGCC-3′ and 5′-CGGTCGTCCAATATCATCG-3′) and URA3 (5′-GAGGGCACAGTTAAGCCGC-3′ and 5′-CCACCCATGTCTCTTTGAGC-3′).

Protein Purification and Analyses

Equal amounts of GST-Ctf13p were purified from nuclear fractions prepared from infected insect cells, diluted in 250 μl of glutathione (GST) binding buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton, 50 mM NaF, 50 mM β-glycerolphosphate, 10% glycerol, and 1 mM dithiothreitol [DTT], and a cocktail of protease inhibitors) and incubated with glutathione Sepharose (Amersham Biosciences, Piscataway, NJ). Resin was isolated by centrifugation (5000 × *g* for 15 s) and washed twice in GST binding buffer and once in binding buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, and 1 mM DTT) for 20 min at 4°C. Bound proteins were eluted as indicated in text.

The tandem affinity purification (TAP)-Ctf13p fusion was purified from 10 mg of yeast extract as described previously (Riedel *et al.*, 1986; Rigaut *et al.*, 1999). Equivalent amounts of starting extract, flow-through, and 5 μl of each fraction were resolved by SDS-PAGE and visualized by immunoblot or standard silver staining techniques (Morrissey, 1981).

Proteins were immunoprecipitated from 2 mg of yeast extract. The indicated extracts were diluted in immunoprecipitation buffer (50 mM HEPES, pH 8.0, 50 mM β-glycerolphosphate, 50 mM NaF, 150 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, and a cocktail of protease inhibitors) and incubated at 4°C with 1 to 2 μl of antibody solution for 1 h followed by incubation with 25 μl of protein A-Sepharose (Amersham Biosciences) for 30 min. Beads were harvested by centrifugation at 10,000 × *g* for 20 s, washed three times in immunoprecipitation buffer, and bound proteins were eluted by boiling in Laemmeli buffer for 4 min. Proteins were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked for 1 h at room temperature in Tris-buffered saline/Tween 20–2% dried milk (25 mM Tris, 140 mM NaCl, and 0.2% Tween 20), and probed with the indicated primary antibodies diluted at 1/5000 in Tris-buffered saline/Tween 20–2% bovine serum albumin as described previously (Russell *et al.*, 1999). Bound antibodies were detected using standard luminescent chemistry (Amersham Biosciences) and used to expose film. Films were scanned and analyzed using Image Quant 5.0 software (Amersham Biosciences) or NIH Image.

CBF3 Bandshift Assay

The indicated amounts of recombinant proteins or yeast extracts (40–80 μg) were incubated with a radiolabeled 88 base pair *CDEIII* probe as described previously (Espelin *et al.*, 1997; Kaplan *et al.*, 1997; Kaplan and Sorger, 1997; Russell *et al.*, 1999). Yeast extracts containing TAP-Ctf13p were cleaved with TEV protease to remove the TAP tag before bandshift analysis. Greater than 95% of the TAP-fusion was cleaved after digestion as determined by immunoblotting.

Fluorescence Microscopy

Cells were grown as indicated, incubated in 3.7% formaldehyde for 1 h at the culture temperature, and processed for 4,6-diamidino-2-phenylindole and tubulin staining (tubulin antibodies diluted 1/400 in phosphate-buffered saline, 0.2% gelatin, and 0.02% NaN3) as described previously (Guthrie and Fink, 1991). Cells containing green fluorescent protein (GFP)/cyan fluorescent protein (CFP) gene fusions were collected at indicated time points and placed on agarose pads containing the identical carbon source as the culture media as described previously (Hoepfner *et al.*, 2000). Fluorescent images were collected using a Nikon E600 epifluorescence microscope equipped with a Nikon 60× oil immersion lens, numerical aperture 1.4, and recorded with a Hamamatsu Orca ER charge-coupled device camera controlled by Simple PCI software (www.cimaging.net). Color was added to images using Adobe Photoshop version 6.0.

RESULTS

Ctf13p, Skp1p, and Sgt1p Form a Single Complex Involved in the Activation of Ctf13p

Skp1p and Sgt1p are required to activate Ctf13p, a critical event for the formation of CBF3 and kinetochores in yeast. Active Ctf13p is defined by its ability to mediate the formation of CBF3 complexes that can bind stably to centromeric (*CEN*) DNA. To understand how Skp1p and Sgt1p contribute to CBF3 formation, we analyzed the interactions of recombinant CBF3 proteins by using wild-type or mutant versions of *SKP1*. To validate the relevance of this in vitro system, we first characterized the behavior Sgt1p when coexpressed with recombinant CBF3 subunits in insect cells. Although Sgt1p is not required to activate Ctf13p in insect cells (Kaplan *et al.*, 1997), presumably because the insect Sgt1p can substitute, we observed a 13-fold increase in the specific activity of Ctf13p when coexpressed with yeast Sgt1p (Figure 1A). Furthermore, the increase in specific activity of Ctf13p remained dependent on Skp1p, because Ctf13p coexpressed with Sgt1p failed to be activated in the absence of Skp1p or in the presence of Skp1-4p (Figure 1B). We note that the small amount of active Ctf13p observed in Skp1-4p lysates may reflect the partial suppression of the *skp1-4* mutation due to overexpression of Sgt1p, consistent with previously reported genetic interactions (Kitagawa *et al.*, 1999). The contribution of Sgt1p to Ctf13p activation and its dependence on Skp1p allowed us to further characterize the role of Sgt1p and Skp1p in CBF3 formation.

Mutations in *SGT1* that block the interaction between Sgt1p and Skp1p prevent the formation of CBF3 complexes. To test whether this is the case for *SKP1* mutations, we coexpressed Sgt1p with either wild-type Skp1p or Skp1-4p in insect cells, Sgt1p complexes were isolated using a polyclonal serum against Sgt1p and, as expected, Skp1p specifically coprecipitated with Sgt1p, consistent with their demonstrated interaction in yeast extract (Kitagawa *et al.*, 1999). In contrast, Skp1-4p failed to coprecipitate with Sgt1p and the complementary immunoprecipitation confirmed the reduction in affinity between Skp1-4p and Sgt1p (Figure 1, C and D). The small amount of Sgt1p that coprecipitates with Skp1-4p is consistent with the partial suppression we observed in the bandshift assay (Figure 1B). Thus, a SKP1 mutation that blocks Ctf13p activation also prevents Skp1p and Sgt1p interaction.

Although previous studies suggest that the interaction between Skp1p and Sgt1p is critical for CBF3 function, it is not clear to what context Sgt1p contributes to the formation of CBF3 complexes (Kitagawa *et al.*, 1999). To address whether Skp1p, Sgt1p, and Ctf13p form a single complex, we coexpressed a GST-Ctf13p fusion with Sgt1p and either Skp1p or Skp1-4p. After the isolation of GST-Ctf13p, we observed the copurification of Skp1p and low but specific levels of Sgt1p copurifying (Figure 2A). The higher affinity of Sgt1p for Ctf13p in the absence of Skp1p or in the presence of Skp1-4p argues that Sgt1p binds directly to Ctf13p and that this interaction is destabilized by Skp1p association (Figure 2A, compare lane 2 with lanes 3 and 4). To assess whether Skp1p, Sgt1p, and Ctf13p can form a single complex, we analyzed the migration of Skp1p on glycerol gradients after fractionating extracts containing Skp1p and Sgt1p, Skp1p and Ctf13p, or all three proteins. Significantly, a subfraction of Skp1p (16%) migrates in more dense fractions when coexpressed with Sgt1p and Ctf13p, consistent with the formation of a single complex containing all three proteins (Figure 2B, fractions 9–12). The low percentage of triple complex is consistent with the relatively weak inter-

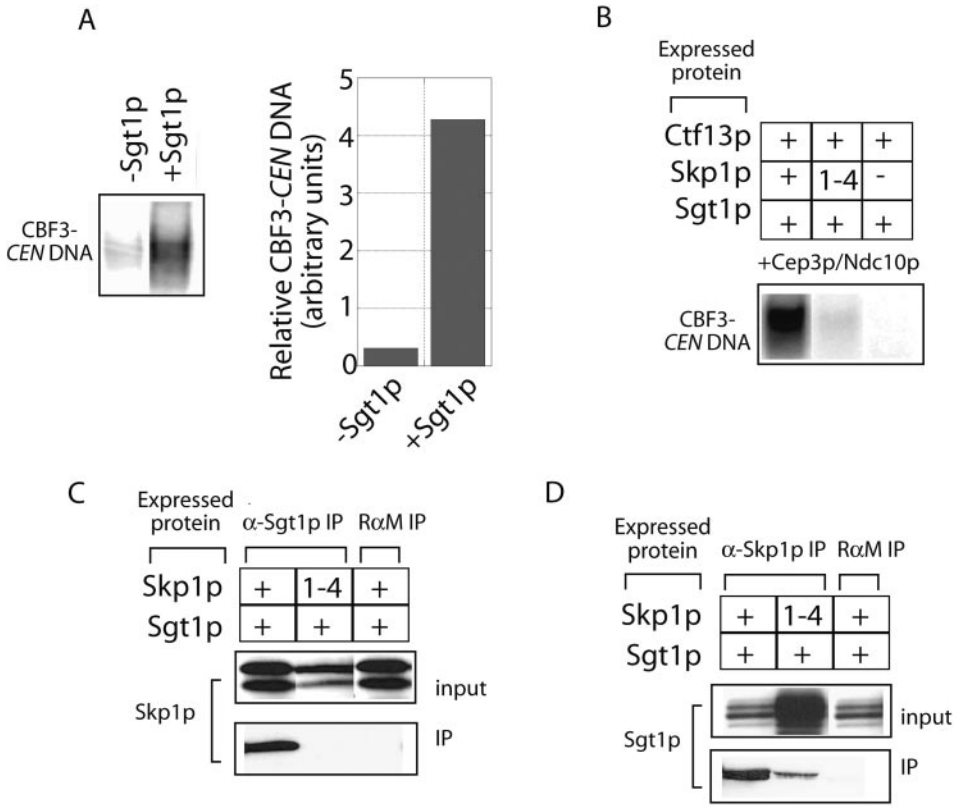

Figure 1. Ctf13p, Skp1p, and Sgt1p form a complex required for Ctf13p activation. (A) Insect cells were infected with recombinant viruses to express Ctf13p and Skp1p, in the presence or absence of Sgt1p. Extracts containing equal amounts of Ctf13p were combined with recombinant Cep3p, Ndc10p, and an 88-base pair radiolabeled *CEN* DNA probe and analyzed by bandshift assay. The relative levels of CBF3-*CEN* DNA complexes were quantified using a Storm PhosphorImager system. (B) Insect cell lysates containing equal amounts of Ctf13p and the indicated proteins were analyzed as described above. (C) Insect cell lysates containing equal amounts of Sgt1p and the indicated recombinant proteins were immunoprecipitated with antibodies to Sgt1p. Immune complexes were eluted and analyzed by immunoblot with Skp1p polyclonal antibodies. (D) The same extracts were equalized for Skp1p, immunoprecipitated with antibodies to Skp1p, and analyzed by immunoblot with Sgt1p polyclonal antibodies. The specificity of immunoprecipitates was confirmed using rabbit antimouse antibodies (labeled RαM). Input lanes represent 10% of the protein immunoprecipitated.

action observed between Sgt1p and the Ctf13p-Skp1p heterodimer in the pull down assays (Lingelbach and Kaplan; unpublished data). Our results indicate that both Skp1p and Sgt1p contact Ctf13p, raising the possibility that interactions between all three proteins contribute to the activation of Ctf13p.

Activation of Ctf13p Is Required for Its Association with Ndc10p

Skp1p and Sgt1p may be important for assembly of CBF3 complexes, or they may facilitate the binding of the already assembled complex to *CEN* DNA. To distinguish between these possibilities, we asked whether there are Skp1p-dependent interactions between Ctf13p and the other CBF3 subunits. We expressed GST-Ctf13p alone or in the presence of Skp1p or Skp1-4p. We confirmed that Skp1p is required for GST-Ctf13p activity by demonstrating that it can be complemented with separately expressed Cep3p and Ndc10p in a bandshift reaction (Figure 3A). As observed in our binding experiments, Sgt1p partially suppressed the Skp1-4p extracts, resulting in a low level of active GST-Ctf13p. We took advantage of this material to evaluate the interaction of Cep3p and Ndc10p with active, partially active or inactive Ctf13p. Active GST-Ctf13p produced in the presence of wild-type Skp1p bound specifically to both Ndc10p and Cep3p (Figure 3B). In contrast, partially active GST-Ctf13p, produced in the presence of Skp1-4p, bound similar levels of Cep3p but more than twofold less Ndc10p. Inactive GST-Ctf13p produced in the absence of Skp1p bound similar levels of Cep3p, but bound only background levels of Ndc10p (Figure 3, B and C). Although unable to bind Ndc10p, inactive GST-Ctf13p does not seem misfolded because it could bind both Cep3p and Skp1p or Skp1-4p. We conclude that the Skp1p-Sgt1p–mediated activation of Ctf13p is required for the assembly of the CBF3 complex and specifically for the binding of Ndc10p to Ctf13p.

The Binding of Ndc10p to Ctf13p Is a Rate-limiting Step for CBF3 Assembly In Vivo

The short half-life of Ctf13p in yeast and the stability of both Cep3p and Ndc10p suggest that production of active Ctf13p may limit the assembly of CBF3 complexes (Kaplan *et al.*, 1997). To characterize the steps that lead to CBF3 formation, we synchronized the assembly of CBF3 complexes in vivo by placing the chromosomal copy of *CTF13* under control of the *GAL1* promoter and fusing *CTF13* to a TAP tag. This strain grows with wild-type characteristics when cultured in the presence of galactose with no obvious cell cycle delays or loss of viability (our unpublished data). When switched to media containing dextrose, the levels of TAP-Ctf13p quickly decrease in agreement with previous measurements of Ctf13p half-life (Figure 4A; Kaplan *et al.*, 1997). The rapid loss of Ctf13p in dextrose-containing media and the stability of other CBF3 components (our unpublished data) make this a unique system for asking questions about how Ctf13p activation is related to CBF3 assembly. When cells are shifted to media containing galactose, significant levels of TAP-Ctf13p were induced after only 10 min and a peak of CBF3 was observed after 40 min (Figure 4A). Quantification of TAP-Ctf13p and CBF3 levels during induction demonstrates that the appearance of Ctf13p precedes CBF3 formation by 15–20 min, suggesting the presence of a rate-limiting step in CBF3 assembly (Figure 4B).

To identify the rate-limiting step in CBF3 assembly, we harvested yeast in 10-min intervals after the induction of TAP-Ctf13p and purified these complexes using a two-step affinity chromatography protocol (Rigaut *et al.*, 1999). Isolated complexes were analyzed by immunoblot or silver

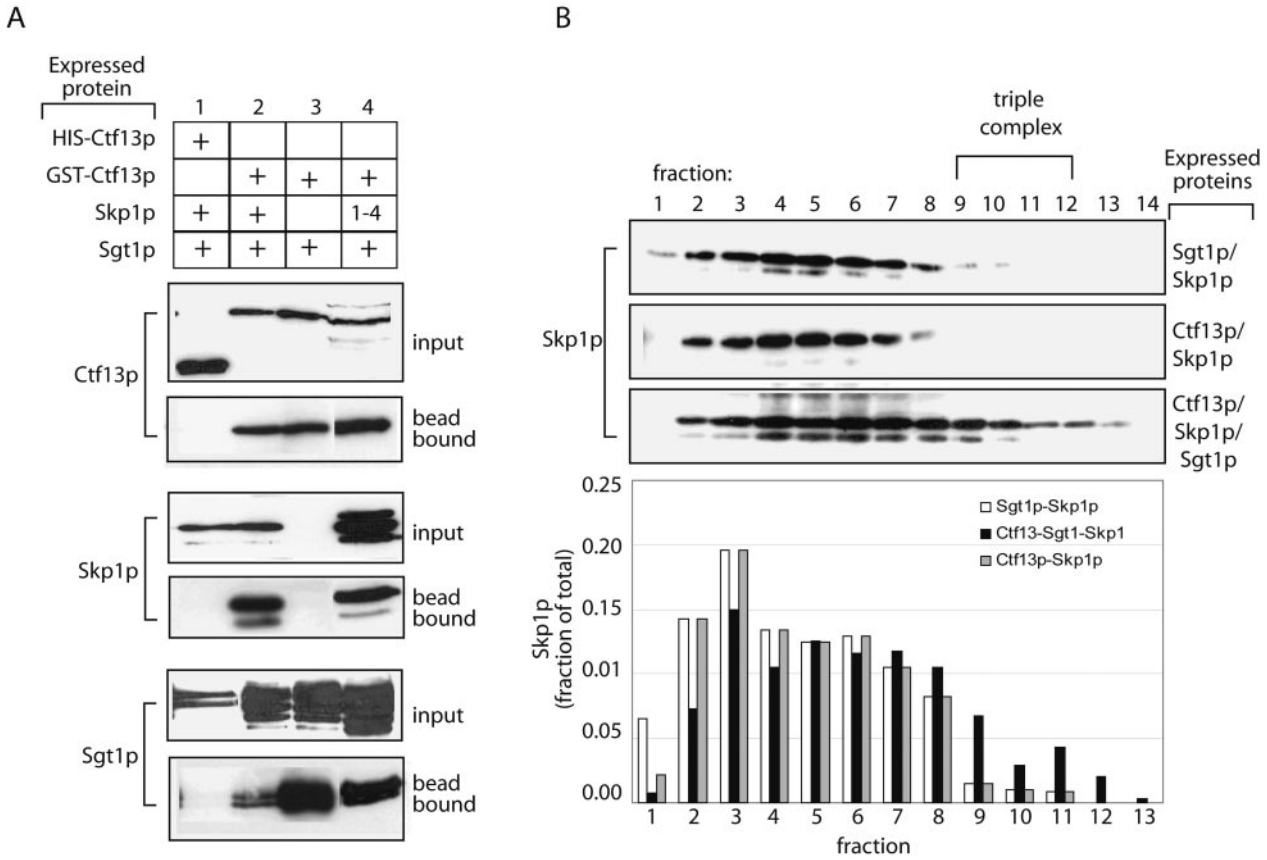

Figure 2. Sgt1p contributes transiently to the activation of Ctf13p. (A) Insect cell lysates containing equal levels of GST-Ctf13p and the indicated recombinant proteins were bound to glutathione Sepharose. Associated proteins were detected by immunoblot using antibodies against the indicated proteins. Lysates containing a 10 × His-Ctf13p instead of GST-Ctf13p were used to determine the level of background binding to glutathione beads. Input lanes represent the levels of protein before purification. (B) Insect cell lysates containing the indicated recombinant proteins were equalized for the levels of Ctf13p and Skp1p and separated by glycerol gradient sedimentation. Fractions were collected from the top of the gradient to the bottom (fractions 1–14), and the position of Skp1p was determined by immunoblotting with polyclonal antibodies against Skp1p. The fractions containing the triple complex of Ctf13p, Skp1p, and Sgt1p are indicated with a bracket. (C) Quantification of Skp1p levels are plotted for each glycerol gradient as a fraction of the total protein detected on the gradient.

staining of gels after SDS-PAGE. A small amount of Ctf13p was detected in cells grown in dextrose, suggesting that not all of the TAP-Ctf13p was degraded or that there is leaky repression of the *GAL1* promoter under these conditions (Figure 4C, arrow 3). A 64-kDa band copurified specifically with TAP-Ctf13p at all times after induction and was confirmed by immunoblot to be Cep3p (Figure 4C, arrow 2; our unpublished data). Skp1p was also observed to copurify with TAP-Ctf13p at all time points by immunoblot and was particularly enriched in later time points (Figure 4C, arrow 4; our unpublished data). Although not detectable by silver staining, Sgt1p was observed by immunoblot to be weakly associated with these complexes (our unpublished data). Notably, the band corresponding to Ndc10p (110 kDa) occurred only at later time points, beginning to peak at 40 min (Figure 4C, arrow 1). Quantification of protein levels confirms that Ctf13p and Cep3p association precedes CBF3 formation and that the kinetics of Ndc10p binding exactly match those of CBF3 formation (Figure 4D). These data argue that the association of Ctf13p with Ndc10p represents the rate-limiting step in CBF3 assembly.

What prevents the interaction between Ctf13p and Ndc10p at early stages of the induction time course? We entertained two possibilities: Ndc10p in extracts may be inactive or, as our in vitro binding studies would suggest, Ctf13p may only become active at later time points (i.e., >20 min). To distinguish between these possibilities, we added recombinant, active Ctf13p or Ndc10p to extracts derived from cells starved for Ctf13p, and from cells after Ctf13p induction for 15 and 45 min; in this experiment, a strain containing a 3 × HA-fusion to Ctf13p under control of the *GAL1* promoter was used to facilitate analyses. Similar levels of 3 × HA-Ctf13p were observed at 15 and 45 min after induction (Figure 4E), although CBF3 complexes were not observed until 45 min (Figure 4F, buffer controls). Addition of recombinant Ndc10p resulted in only a minor increase in CBF3 formed in extracts, whereas the addition of Ctf13p completely complemented extracts to wild-type levels (Figure 4F), arguing that both Ndc10p and Cep3p are functional in these extracts. These results indicate that the lag in CBF3 assembly is due to a lack of active Ctf13p. Thus, activation of Ctf13p is required for Ndc10p binding and is rate limiting in the assembly of CBF3.

To address the assembly of CBF3 complexes in living cells, we analyzed the behavior of a Cep3-GFP fusion during the synchronized assembly of CBF3. Because the binding of Cep3p to *CEN* DNA depends on intact CBF3, we reasoned that Cep3p would be a good marker to monitor CBF3 assembly in live cells. Consistent with the distribution of other kinetochore proteins in yeast, Cep3-GFP was observed to

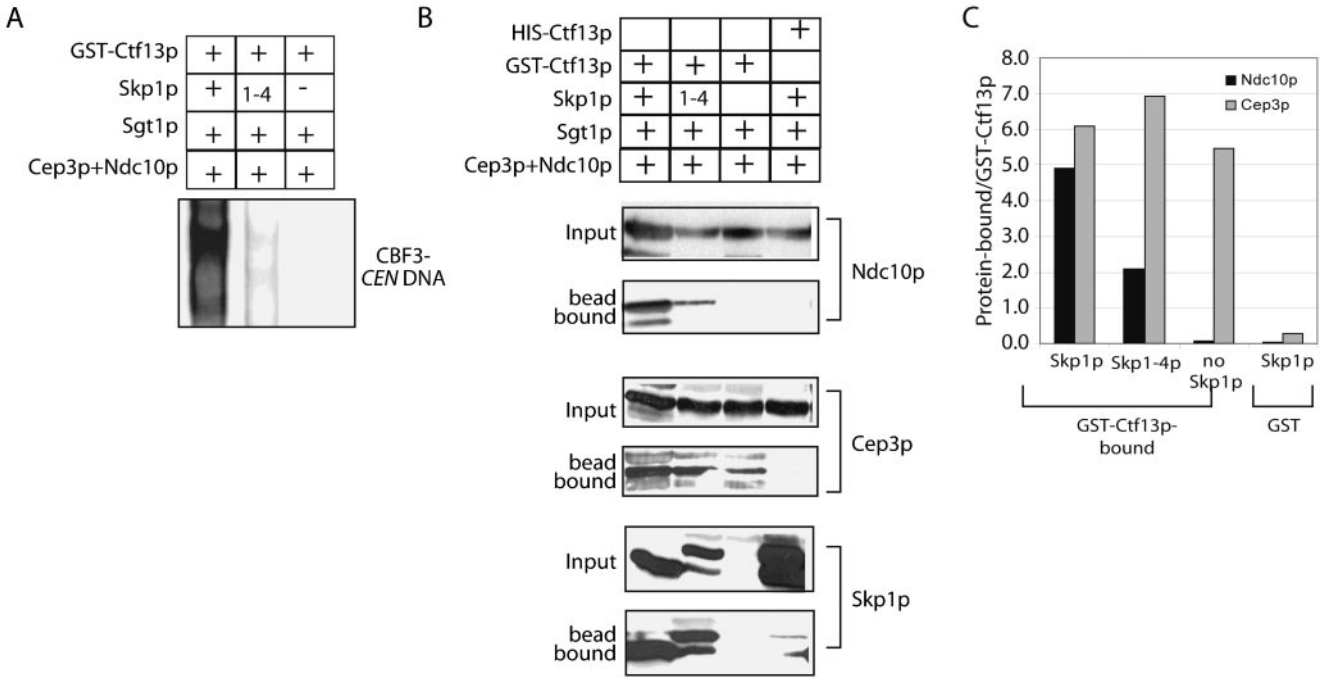

Figure 3. Ctf13p activation is required to interact with Ndc10p. (A) Cell lysates with the indicated recombinant proteins were prepared as described above (Figure 1), combined with recombinant Cep3p and Ndc10p and analyzed in a bandshift assay. (B) Glutathione Sepharose was incubated with the lysates containing the indicated recombinant proteins. Bound proteins were eluted and immunoblotted. Input lanes represent the levels of protein before isolation. The slower migration of Skp1-4p is due to the addition of a MRGS-His10-HA amino-terminal epitope tag. (C) Levels of GST-Ctf13p or GST bound Cep3p or Ndc10p were equalized to the levels of GST fusion in the binding assay and plotted.

cluster in cells grown in the presence of galactose (Figure 4G, arrow, gal/raf). After cells were grown in dextrose for 300 min, Cep3p-GFP was dispersed throughout cells (Figure 4G, Dex, 300′). After the return of cells to galactose containing media for 15 min, Cep3-GFP was observed in puncta (Figure 4G, arrows in gal/raf 15′; our unpublished data) and by 45 min, Cep3-GFP returned to its clustered position in the majority of cells, consistent with its binding to centromeres (Figure 4G, arrow in 45′). In separate experiments, we observed Cep3p localization by indirect immunofluorescence to reflect the behavior of the GFP fusion and, after the assembly of CBF3, to cluster near spindle poles overlapping with chromosomes (Figure 1, S-). We note that this pattern is distinct from the localization of outer kinetochore proteins under these same conditions (Figure 7), suggesting that Cep3-GFP uniquely transits through multiple cellular compartments during CBF3 assembly. However, our main conclusion that CBF3 assembly as measured in extracts correlates with kinetochore formation in vivo is supported by these data.

The Assembly and Turnover of CBF3 Complexes Are Affected by Mutations in SKP1

To examine how *SKP1* mutations affect CBF3 complexes in vivo, we compared the assembly of CBF3 in wild-type, *skp1-4*, and *skp1-3* strains containing a 3 × HA-fusion to Ctf13p under control of the *GAL1* promoter. Whereas the *skp1-4* mutation has been shown to block Ctf13p activation, the *skp1-3* mutation has been reported to affect the SCF ubiquitin ligase and arrests cells in G1 with wild-type CBF3 levels (Connelly and Hieter, 1996; Kaplan *et al.*, 1997). Cells were grown in the presence of raffinose to inhibit 3 × HA-Ctf13p expression and induced with galactose at 37°C for 90 min, sufficient time for CBF3 to assemble in wild-type strains. When grown in the presence of raffinose, 3 × HA-Ctf13p decreased to similar levels in all strains, regardless of the temperature cultured (Figure 5A). CBF3 assembly was monitored after the induction of 3 × HA-Ctf13p in each of the strains. After 90 min of induction, CBF3 was observed at similar levels in wild-type and *skp1-3* cells at both permissive and nonpermissive temperatures (Figure 5B). As expected, CBF3 levels were reduced in *skp1-4* cells at permissive temperature and entirely absent in cells grown at nonpermissive temperature (Figure 5B). We confirmed that *skp1-4* blocked 3 × HA-Ctf13p activation by showing biochemical complementation of CBF3 with activated recombinant Ctf13p but not with Ndc10p or Cep3p (Figure 5C, 37°C).

A prediction based on our in vitro binding studies is that the *skp1-4* mutation blocks Ctf13p activation by preventing the interaction between Skp1p and Sgt1p. To examine this interaction during the synchronized assembly of CBF3, we immunoprecipitated Sgt1p from extracts and examined the levels of coprecipitated Skp1p. In wild-type cells, barely detectable levels of Skp1p were observed to coprecipitate with Sgt1p before the induction of 3 × HA-Ctf13p even though both proteins were present at wild-type levels (Figure 5D, Skp1p visible on longer film exposures; our unpublished data). Interestingly, significantly higher levels of Skp1-3p coprecipitated with Sgt1p under all conditions. These results suggest that the Skp1-3p has a higher affinity for Sgt1p. In contrast, Skp1-4p failed to coprecipitate with Sgt1p under any conditions, consistent with our in vitro

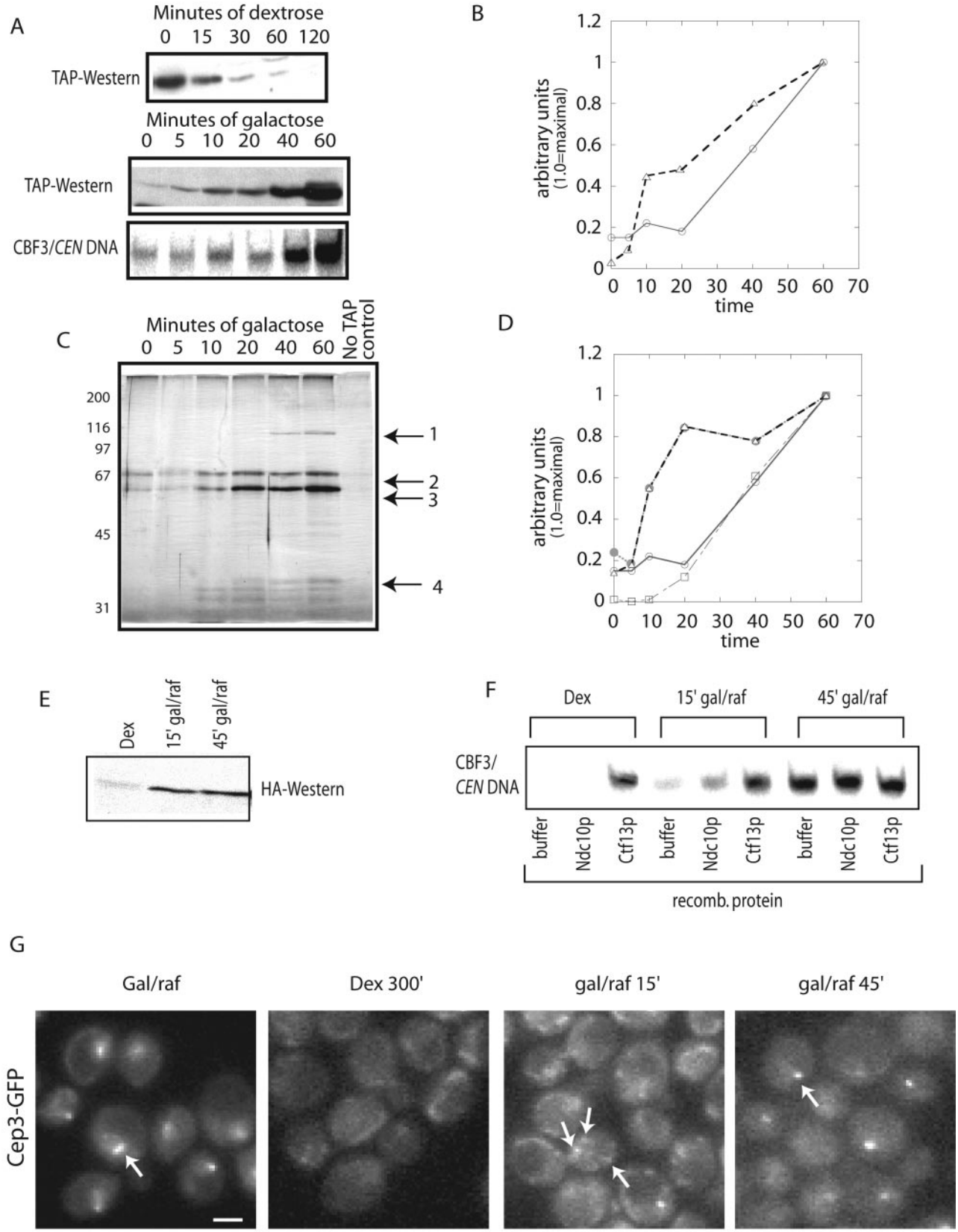

Figure 4. Ndc10p binding to TAP-Ctf13p is the final step in the synchronous assembly of CBF3 in yeast. (A) Cells containing the *GAL1* promoter upstream of *TAP-CTF13* were cultured in galactose (0 min, top) and then transferred to media containing dextrose for 15, 30, 60, and 120 min; extracts were prepared and immunoblotted to detect the TAP-fusion. After 5 h of growth in dextrose (0 min, middle), cells were transferred to media containing galactose for 5, 10, 20, 40, and 60 min, and extracts were prepared and immunoblotted as described above

observations that the *skp1-4* mutation disrupts the interaction with Sgt1p (Figure 5D).

To test the prediction that inactive Ctf13p results in the partial assembly of CBF3, we performed immunoprecipitations of 3 × HA-Ctf13p followed by immunoblot detection of Cep3p. We observed similar levels of Cep3p coprecipitate with 3 × HA-Ctf13p from wild-type and mutant strains, grown at permissive or nonpermissive temperature (Figure 5D). The small variations in the levels of Cep3p that coprecipitate with 3 × HA-Ctf13p at 90 min reflects differences in the amount of 3 × HA-Ctf13p induced after addition of galactose (Figure 5A). Together, these results argue that CBF3 assembles in at least two steps; the first step involves the interaction between Ctf13p, Cep3p, Skp1p, and Sgt1p; the second step only occurs when Skp1p and Sgt1p interact directly to activate Ctf13p, allowing Ndc10p binding and the formation of CBF3 complexes.

In addition to demonstrating that Skp1p and Sgt1p are required for the assembly of CBF3, these experiments also highlight the transient nature of the CBF3 complex. Depletion of 3 × HA-Ctf13p causes the rapid disappearance of CBF3 from extracts with a half-life of 40 min (our unpublished data). Interestingly, CBF3 levels are not reduced in strains carrying the *skp1-3* mutation when grown in the presence of raffinose (Figure 5B, 0-min time point). Because 3 × HA-Ctf13p levels are equally reduced in all strains cultured in raffinose, this stabilization is not due to a general failure to degrade 3 × HA-Ctf13p (Figure 5A, compare 0-min time points). Therefore, we speculate that *skp1-3* may prevent the turnover of CBF3 complexes and argues that Skp1p is involved in both the assembly and the turnover of CBF3 complexes.

The Machinery Required to Assemble CBF3 Is Active throughout the Cell Cycle

The assembly of CBF3 and its binding to *CEN* DNA is required for the association of all other kinetochore complexes with *CEN* DNA, and thus it can be considered the first step in de novo kinetochore formation. De novo formation of kinetochores may be restricted to discrete points in the cell cycle. For example, assembly of CBF3 may be limited to G1 or S when newly replicated centromeres are assembling a kinetochore. To address whether the pathways that control the assembly of CBF3 are influenced by cell cycle progression, we monitored CBF3 assembly in cells containing *GAL1-3 × HA-CTF13* at specific stages of the cell cycle. In the presence of raffinose and α-factor cells became arrested in G1 with depleted 3 × HA-Ctf13p. The addition of galactose induced the production of 3 × HA-Ctf13p and examination of bud morphology, fluorescence-activated cell sorting profiles, and tubulin staining suggest that cells remain largely arrested in G1 (Figure 6, A–C; our unpublished data). Normal kinetics and levels of CBF3 were observed under these conditions (compare Figure 6A with Figure 5). In separate experiments, we also observed the movement of Cep3-GFP to kinetochores in G1 cells with similar kinetics (Figure 4G; our unpublished data). These results argue that CBF3 can assemble during the G1 stage of the cell cycle, possibly to enable de novo kinetochore formation at newly replicated centromeres.

To examine whether CBF3 assembly is restricted to G1, we analyzed assembly in cells that had been arrested in G2/M with the microtubule poison nocodazole. Cells arrested with large-bud morphology as expected; Clb2 levels accumulated and remained high throughout the experiment, consistent with cells being arrested in mitosis. Surprisingly, CBF3 assembly occurred with normal kinetics, whereas cells were arrested in mitosis (Figure 6, D–F). To rule out that microtubule depolymerization artificially induced CBF3 assembly, we analyzed cells enriched in G2 after release from α-factor arrest. Cells were arrested with α-factor, depleted for 3 × HA-Ctf13p, and released from the arrest in raffinose-containing media. After the appearance of medium budded cells, galactose was added to the media and samples were collected at the indicated time points (Figure 6G). The appearance of 2N DNA levels and the increase in Clb2p levels suggest that these cells have progressed into G2 (Figure 6, G–I). Induction of 3 × HA-Ctf13p resulted in the formation of CBF3 complexes with similar kinetics as observed in cells arrested with nocodazole (Figure 6D). Together, these results suggest that the machinery required to assemble CBF3 is active throughout the cell cycle and this could allow for the continual assembly of kinetochores.

The Balance of CBF3 Assembly and Turnover Is Required for Proper Kinetochore Function

To understand how CBF3 assembly contributes to kinetochore function, we tested the effect of altering CBF3 levels on chromosome segregation. To this end, we first examined the segregation of a chromosome III fragment carrying *SUP11*, a suppressor of the *ade2-101* mutation, in a strain where *CTF13* is under control of the *GAL1* promoter. Cells with compromised kinetochore function lose the chromosome fragment at high rates, resulting in the failure to suppress the *ade2-101* mutation and the accumulation of red or red-sectored colonies (Spencer *et al*., 1990). When cells were grown in the presence of galactose to induce *3 × HACTF13*, we observed a significant increase of red sectored-colonies (colonies with more than 2 sectors) compared with the background chromosome loss observed in the wild-type strain with *CTF13* under normal transcriptional control (10.8-fold), although only a small increase in red colonies (1.7-fold), suggesting that elevated levels of CBF3 cause a modest negative defect in chromosome segregation (Figure 7A). In contrast, we observed a significant increase in the percentage of red colonies when cells were grown for short periods (as little as 1 h) in the presence of raffinose or dextrose to inhibit CBF3 assembly before plating on media containing galactose (4.8- and 8.9-fold, respectively), suggesting that limiting CBF3 assembly also interferes with normal chromosome segregation (Figure 7A). We conclude that maintaining the proper balance of CBF3 complexes is critical for kinetochore function and the most dramatic effect on chromosome segregation is observed when CBF3 assembly is inhibited.

To address in more detail how inhibition of CBF3 assembly compromises kinetochore function, we examined the ability of kinetochores to form on *CEN* DNA. After the

Figure 4 (facing page) or analyzed by bandshift gel. (B) Levels of TAP-Ctf13p (△) and CBF3 (○) shown in A were quantified using a Storm PhosphorImager system (maximum value 1.0). (C) TAP-Ctf13p fusions were purified from extracts prepared in A, resolved on SDS-PAGE, and proteins were visualized by silver staining. (D) Levels of Ctf13p (△), Cep3p (●), Ndc10p (□), and CBF3 (○) shown in C were quantified as described above; Ctf13p and Cep3p lines overlap for much of the time course. (E) Cells containing the *GAL1* promoter upstream of *3 × HA-CTF13* were cultured for 5 h in dextrose and then transferred to galactose-containing media for 15 and 45 min. Extracts were immunoblotted with antibodies against HA (E) and analyzed by bandshift gel in the presence of the indicated recombinant CBF3 subunits (F). (G) Images of cells expressing Cep3-GFP were digitally captured at the indicted times during the assembly of CBF3. Bar, 1 μm.

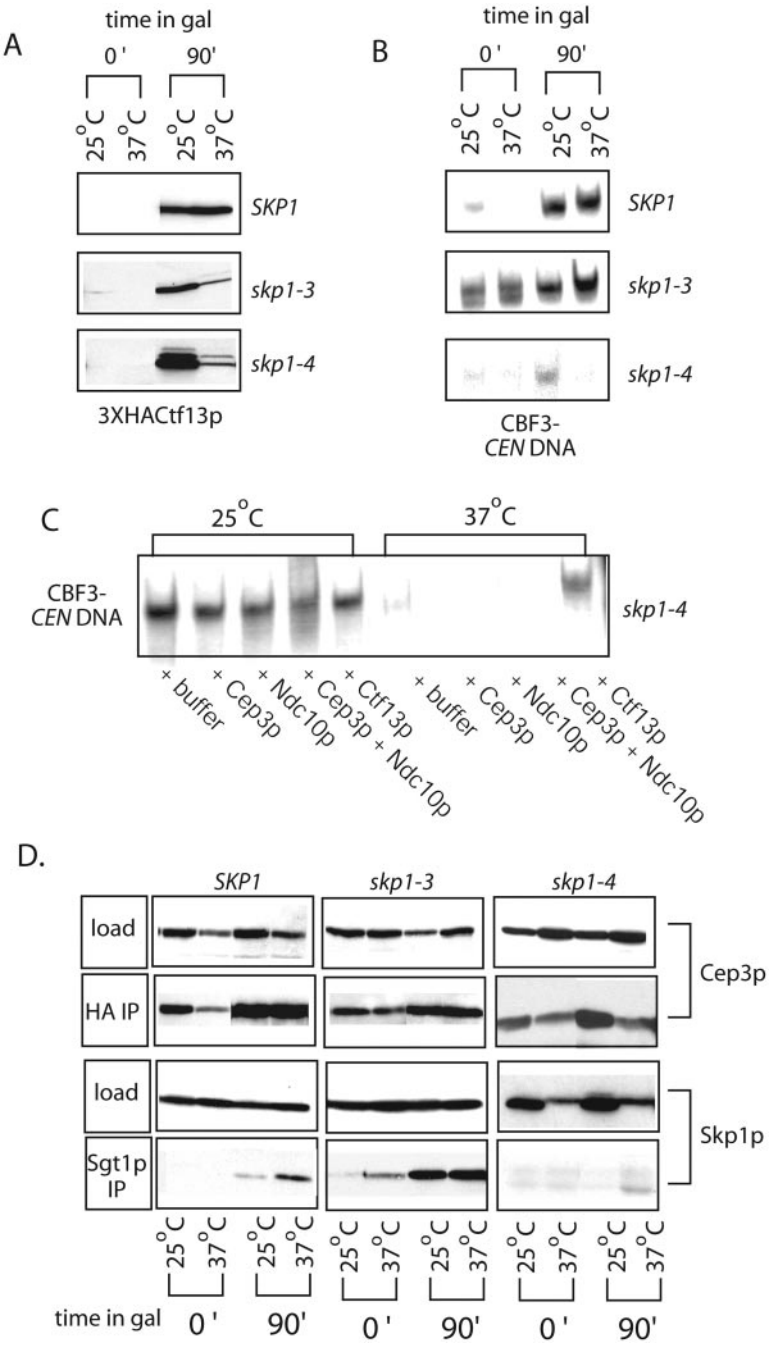

Figure 5. SKP1 is required for the stepwise assembly of CBF3 in yeast. *SKP1*, *skp1-3*, and *skp1-4* cells containing the *GAL1* promoter upstream of 3 × *HA-CTF13* were cultured at 25 or 37°C in media containing dextrose (0′) and then transferred to media containing galactose for 90 min (90′). Extracts were immunoblotted with antibodies to HA (A) and analyzed by bandshift assay (B). (C) Extracts from *skp1-4* cells were supplemented with the indicated recombinant CBF3 subunits and analyzed as described in B. (D) Levels of Cep3p and Skp1p in extracts were detected by immunoblot with appropriate antibodies (load) and then immunoprecipitated and immunoblotted with indicated antibodies.

inhibition of CBF3 assembly, we were unable to detect CBF3 complexes in extracts by bandshift analysis (Figures 4 and 5); however, chromatin immunoprecipitation revealed levels of *CEN* DNA coprecipitating with Cep3p antibodies comparable with wild-type cells (Figure 7B). We also examined the association of CBF3 with *CEN* DNA in a *skp1-4* strain and observed that similar levels of *CEN* DNA precipitated with antibodies to Cep3p at both permissive and nonpermissive temperatures (Figure 7C). In contrast, the *ndc10-1* allele dramatically reduced the ability of CBF3 to associate with *CEN* DNA, consistent with published reports that this allele prevents kinetochore nucleation (Figure 7D). To determine whether inhibiting CBF3 assembly affected the recruitment of other kinetochore proteins to *CEN* DNA, we performed chromatin immunoprecipitation on strains with Spc24-myc, Okp1-myc, and Dam1-myc. No significant changes in the levels of coprecipitating *CEN* DNA were observed when CBF3 assembly was inhibited (Figure 7E). We conclude that mutations that block CBF3 assembly (i.e., *skp1-4* and *sgt1-3*; our unpublished data) do not release CBF3 or outer kinetochore proteins from *CEN* DNA; however, mutations in the core DNA binding subunits (i.e., *ndc10-1* and *ctf13-30* (our unpublished data) cause CBF3 to dissociate from DNA.

Although kinetochores may form on *CEN* DNA, it is possible that they are not properly organized. One characteristic of properly organized kinetochore proteins in yeast is their juxtaposition to spindle poles throughout the majority of the cell cycle (Jin *et al.*, 2000). To examine

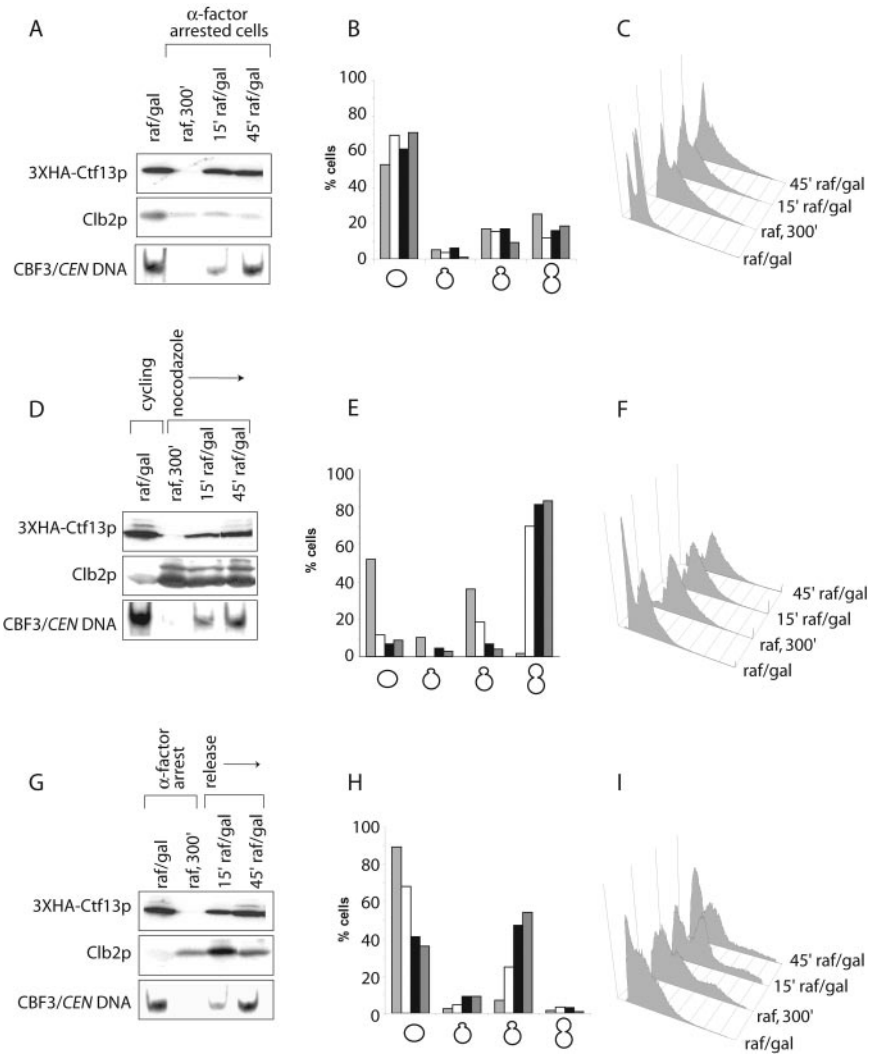

Figure 6. CBF3 can be assembled in G1 and G2/M. Cells containing the *GAL1* promoter upstream of 3 × *HA-CTF13* were grown in the presence of raffinose and α-factor, galactose was added, and samples were collected at the indicated times (A–C). Cells grown in galactose were washed into raffinose and treated with 10 μg/ml nocodazole; galactose was added to the culture and samples collected at the indicated times (D–F). Cells were arrested using α-factor first in galactose then in raffinose-containing media; washout of α-factor was followed by the addition of galactose and samples were collected at the indicated times (G, H, and I). Extracts were immunoblotted with antibodies to HA and Clb2p and analyzed by bandshift assay (A, D, and G). The bud index (relative bud size indicated by cartoon below each set of columns) was determined for cells grown under the following conditions: galactose (light gray column), raffinose for 300 min (□), return to galactose for 15 (■) minutes, and for 45 (dark gray column) minutes (B, E, and H). Cells from the indicated time points were processed for DNA staining and flow cytometry to determine DNA content (C, F, and I).

the affect of inhibiting CBF3 assembly on the positioning of kinetochore proteins near spindle poles, we monitored the localization of the outer kinetochore protein Dam1p after fusion to GFP in a strain also containing Spc42-CFP to mark spindle poles. When cells were grown in galactose, Dam1-GFP clustered adjacent to spindle poles in G1 or late mitotic cells or between replicated spindle poles in early mitotic cells as expected (Figure 7F, arrows indicate early mitotic cells and arrowheads indicate late mitotic cells). Inhibition of CBF3 assembly after growth of cells in dextrose had no detectable effect on Dam1-GFP localization (Figure 7F). Similarly, GFP fusions to Spc24p and Okp1p also maintained their normal localization after inhibition of CBF3 assembly (our unpublished data). In contrast, the mutation *ndc10-1* at nonpermissive temperature caused a dramatic loss of Dam1-GFP from their juxtapolar position, presumably because the CBF3 complex itself was released from *CEN* DNA (Figure 7H). We conclude from these data that interfering with normal CBF3 assembly by overexpressing Ctf13p, or more dramatically, by inhibiting CBF3 assembly compromises chromosome segregation, independent of kinetochore formation on *CEN* DNA. We cannot rule out that depleting CBF3 results in a subtle perturbation of kinetochores that we cannot detect by these assays. However, it is interesting to speculate that CBF3 may have a noncentromeric role required for proper chromosome segregation.

DISCUSSION

The large number of kinetochore proteins and their organization into multiple discrete complexes highlights the importance of understanding their assembly into a functional kinetochore. Our studies of Skp1p and Sgt1p implicate these well conserved proteins in the assembly of CBF3, raising the possibility that they may perform a general role in the assembly of protein complexes. Specifically, we have shown that Skp1p and Sgt1p are required for the final and rate-limiting step in CBF3 assembly, a step that is essential for its ability to associate with *CEN* DNA. Finally, our work suggests that the de novo assembly of CBF3 occurs throughout the cell cycle and is required for proper kinetochore function, independent of its role in recruiting proteins to *CEN* DNA. We propose that a balance between CBF3 assembly and turnover may help to modulate kinetochore function.

Mechanism of Skp1p-Sgt1p Action

Skp1p and Sgt1p are well conserved and implicated in the function of a number of protein complexes important for the

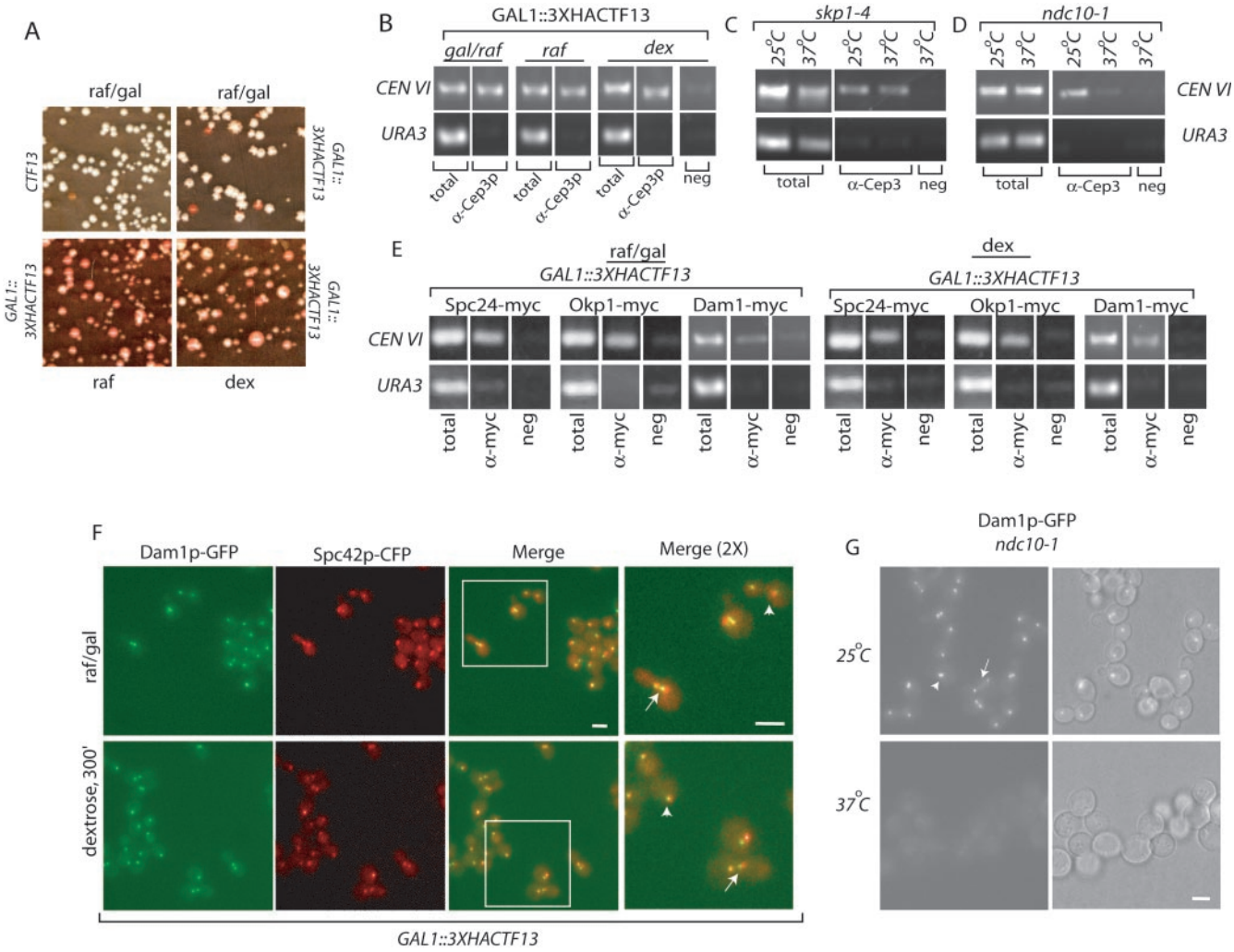

Figure 7. Inhibition of CBF3 assembly compromises chromosome segregation but not kinetochore formation. (A) Strains were grown in media containing the indicated carbon source for 4 h and plated on media containing galactose and raffinose. (B) Cells containing the *GAL1* promoter upstream of *3 × HA-CTF13*, (C) *skp1-4*, or (D) *ndc10-1* were grown in media containing the indicated carbon source at either permissive (25°C) or nonpermissive (37°C) temperatures. After cross-linking, lysates were immunoprecipitated with antibodies to Cep3p or a nonspecific control (neg). Total chromatin or precipitated DNA was subject to PCR analysis by using primers to identify *CEN IV* and *URA3*. (E) Cells containing the *GAL1* promoter upstream of *3 × HA-CTF13* and the indicated myc-tagged kinetochore proteins were analyzed as described in B. (F–G) Cells containing the *GAL1* promoter upstream of *3 × HA-CTF13*, or the *ndc10-1* mutation, and the indicated fusions to GFP (green) or CFP (red) were grown in the appropriate carbon source and living cells were visualized on agarose pads. Images represent a projection of six to seven 0.2-μm optical sections. Arrows indicate metaphase-like localization of Dam1-GFP between the poles and arrowheads indicate the examples of late mitotic cells where Dam1-GFP and Spc42-CFP overlap. White square outline indicates region that was chosen to magnify twofold. The white box indicates the region that is magnified. Bars, 5 μm.

regulation of cell growth, cell cycle advance, and chromosome segregation (Kitagawa *et al.*, 1999; Dubacq *et al.*, 2002). Although a biochemical function for these proteins has not yet been determined, CBF3 offers a useful model complex to dissect their actions. Our results indicate that Skp1p and Sgt1p form important contacts with each other and with the F-box protein Ctf13p that allow Ctf13p to associate with Ndc10p and ultimately bind *CEN* DNA. Two models have been proposed to explain the "activation" of Ctf13p: 1) posttranslational modification in the form of phosphorylation (Kaplan *et al.*, 1997), and 2) the recruitment of HSP90 chaperones (Stemmann *et al.*, 2002). Our efforts to distinguish between these two possibilities suggest that Ctf13p activation may be controlled by multiple pathways. We have observed a mobility shift for Ctf13p, Skp1p, and Sgt1p during the synchronous assembly of CBF3 that is reversed by phosphatase treatment, but mutation of modified sites in Ctf13p and Sgt1p has not revealed an essential function (our unpublished data). Also, our results suggest that the role of HSP90 chaperones in Ctf13p activation is not straightforward; inhibition of HSP90 function in insect cells expressing recombinant Ctf13p reduces the levels of protein produced but not its specific activity (our unpublished data). Given the complicated relationship between CBF3 assembly and turnover, it is not unreasonable to speculate that multiple pathways work in parallel to maintain proper CBF3 levels. Although this work does not resolve the mechanism by which Skp1p and Sgt1p activate Ctf13p, it provides important details concerning the role of these proteins in

maintaining CBF3 complexes and thus provides a critical framework from which to more carefully dissect the molecular mechanisms underlying Skp1p-Sgt1p function.

Skp1p and Sgt1p also have been reported to play critical roles in the function of the SCF ubiquitin ligase. In this light, it is interesting to note certain similarities between CBF3 and the SCF. Both complexes are composed of an F-box protein, Skp1p, and Sgt1p. The F-box protein in both complexes provides a protein interaction function; as a scaffold in CBF3 to bind other subunits and as a ligand receptor in the context of SCF. Perhaps a more intriguing similarity is that both complexes exhibit a transient quality. In both cases, F-box proteins are degraded after ubiquitin modification, a process proposed to be important for the formation of new SCF complexes (Zhou and Howley, 1998). The turnover of Ctf13p may also contribute to the ability to create new CBF3 complexes, a process that may be linked to maintenance of the spindle checkpoint (Kitagawa *et al*., 2003). Interestingly, it has been reported that Skp1p is required for the turnover of Rcy1p, a protein involved in the recycling of SNARE complexes, independent of its role in SCF (Galan *et al*., 2001); this result and our observations concerning the stability of CBF3 in *skp1-3* cells raises the possibility that Skp1p contributes to the turnover of proteins independent of ubiquitin-mediated degradation. Whether degradation of Ctf13p itself serves as the lynchpin for CBF3 turnover, or some other process involving chaperones is involved, will require a more detailed examination of how CBF3, and perhaps SCF, complexes are turned over.

Assembly of CBF3 Is Important for Chromosome Segregation but Not for Kinetochore Formation

The relatively constant levels of *CTF13* message (Cho *et al*., 1998) and our results that CBF3 can assemble throughout the cell cycle raise the possibility that the continual assembly of CBF3 is important for proper kinetochore function. Indeed, both overexpression and inhibition of CBF3 assembly dramatically increases chromosome loss even though kinetochore complexes are properly recruited to *CEN* DNA. There are several possible explanations for this observation: 1) inhibition of CBF3 results in a small number of defectively formed kinetochores, leading to a low rate of chromosome loss without detectable dissociation of kinetochore proteins from *CEN* DNA; 2) inhibition of CBF3 assembly preferentially blocks the recruitment of factors to *CEN* DNA required to maintain the fidelity of chromosome segregation; and 3) CBF3 plays some extracentromeric role that is required for accurate chromosome segregation. Although it is possible that only a few kinetochores are defective in every cell, the high rate of chromosome loss and the drop in cell viability observed after inhibition of CBF3 assembly suggests a massive failure of kinetochore function. More comprehensive analyses of the composition of kinetochores and the movement of chromosomes after inhibition of CBF3 assembly will help to distinguish between these possibilities.

In summary, our results document the biochemical details that underlie the formation of kinetochores in the budding yeast *S. cerevisiae*. The job of assembling such a large protein structure is likely to be carefully orchestrated. The data we present suggest that maintaining careful control over CBF3 assembly and turnover is important for proper chromosome segregation.

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