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De Novo Kinetochore Assembly Requires the Centromeric Histone H3 Variant[D]

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Kinetochores mediate chromosome attachment to the mitotic spindle to ensure accurate chromosome segregation. Budding yeast is an excellent organism for kinetochore assembly studies because it has a simple defined centromere sequence responsible for the localization of >65 proteins. In addition, yeast is the only organism where a conditional centromere is available to allow studies of de novo kinetochore assembly. Using a conditional centromere, we found that yeast kinetochore assembly is not temporally restricted and can occur in both G_1 phase and prometaphase. We performed the first investigation of kinetochore assembly in the absence of the centromeric histone H3 variant Cse4 and found that all proteins tested depend on Cse4 to localize. Consistent with this observation, Cse4-depleted cells had severe chromosome segregation defects. We therefore propose that yeast kinetochore assembly requires both centromeric DNA specificity and centromeric chromatin.

INTRODUCTION

Accurate chromosome segregation in mitosis and meiosis is essential for the maintenance of genomic stability. Chromosomes attach to the mitotic spindle at the kinetochore, the protein complex that assembles onto centromeric DNA. Although kinetochore function is conserved, the underlying centromeric DNA is highly variable. Budding yeast contain a 125-base pair sequence-specific centromere that is sufficient for kinetochore formation (Fitzgerald-Hayes *et al.*, 1982). In contrast, centromeres in multicellular eukaryotes are composed of megabases of highly repetitive DNA that lack sequence specificity (for review, see Sullivan *et al.*, 2001). In these organisms, kinetochore assembly seems to be propagated by unidentified epigenetic component(s) (Karpen and Allshire, 1997; Sullivan *et al.*, 2001).

The best-characterized kinetochore is in budding yeast where >65 components have been identified that constitutively localize to the kinetochore (for reviews, see Biggins and Walczak, 2003; McAinsh *et al.*, 2003). Most of the yeast kinetochore proteins are found in biochemically distinct complexes known as the CBF3, CTF19/COMA, MTW1, NDC80, and DAM1 complexes that seem to assemble on a single centromeric nucleosome (Meluh *et al.*, 1998). Although the exact architecture of the kinetochore is not known, dependency relationships subdivide the kinetochore into inner, central, and outer domains. The inner kinetochore contains the CBF3 complex (Ndc10, Cep3, Skp1, and Ctf13) as well as the DNA binding proteins Mif2, Cbf1, and the yeast centromeric histone H3 variant (CenH3) Cse4. CBF3 binds directly to the centromeric DNA and is thought to nucleate kinetochore assembly because all kinetochore proteins require it for localization (Russell *et al.*, 1999; Goshima and Yanagida, 2000; He *et al.*, 2001; Janke *et al.*, 2001, 2002). The central kinetochore contains the MTW1 (Mtw1, Dsn1, Nnf1, and Nsl1) and CTF19/COMA (Ctf19, Mcm16, Mcm19, Mcm21, Mcm22, Ctf3, Chl4, Okp1, Ame1, Iml3, Nkp1, and Nkp2) complexes. CTF19/COMA can be further divided into two subcomplexes, with Ame1 and Okp1 in one subcomplex (C2.105) and Mcm21 and Ctf19 in another subcomplex (C2.100) (De Wulf *et al.*, 2003). Outer kinetochore complexes include the conserved NDC80 (Ndc80, Spc24, Spc25, and Nuf2) and DAM1 (Dam1, Ask1, Duo1, Dad1, Dad2, Dad3, Dad4, Spc19, Spc34, and Hsk3) complexes. DAM1 is considered to be the outermost complex because it requires microtubules and all other complexes for kinetochore localization (Enquist-Newman *et al.*, 2001; Janke *et al.*, 2002; Li *et al.*, 2002).

One hallmark of all kinetochores is the essential CenH3 (Palmer *et al.*, 1987; Stoler *et al.*, 1995; Buchwitz *et al.*, 1999; Henikoff *et al.*, 2000; Takahashi *et al.*, 2000; Sanyal and Carbon, 2002; Talbert *et al.*, 2002; Zhong *et al.*, 2002; Edwards and Murray, 2005). CenH3s contain a unique N terminus and a well-conserved C terminus that is highly homologous to histone H3 (Malik and Henikoff, 2003). Because all active centromeres contain CenH3, it may be the epigenetic factor that specifies the site of kinetochore formation. Consistent with this hypothesis, RNA interference (RNAi) studies in worm, fly, and human cells have demonstrated that CenH3

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Abbreviations used: αF, α factor; CenH3, centromeric histone H3 variant; ChIP, chromatin immunoprecipitation; *cCEN*, conditional centromere; dox, doxycycline; *eCEN*, endogenous centromere; FACS, fluorescence-activated cell sorter; IP, immunoprecipitate; SPB, spindle pole body.

is required for the localization of many kinetochore proteins (Blower and Karpen, 2001; Oegema *et al.*, 2001; Goshima *et al.*, 2003). However, several kinetochore proteins do not seem to require CenH3 for localization, suggesting that CenH3-independent assembly pathways also exist (Goshima *et al.*, 2003; Hayashi *et al.*, 2004; Regnier *et al.*, 2005). In addition, the overexpression of human CenH3 fails to nucleate complete kinetochore assembly, despite driving CenH3 localization to euchromatin (Van Hooser *et al.*, 2001). The precise function of CenH3 remains unclear for several reasons. Because RNAi-mediated CenH3 depletion occurs over many cell cycles, it is not known what phenotypes are direct consequences or secondary effects of the loss of CenH3. In addition, these studies cannot distinguish between maintenance and assembly of the kinetochore in the absence of CenH3. Finally, it is difficult to determine whether the CenH3 depletion is complete in multicellular eukaryotes that contain a large number of centromeric nucleosomes.

Little is known about kinetochore assembly in any organism. Budding yeast provide an excellent system to investigate kinetochore assembly due to the large number of identified kinetochore proteins and the defined centromeric DNA sequence. Here, we use a conditional centromere (*cCEN*), a system unique to budding yeast, to investigate kinetochore assembly in vivo (Hill and Bloom, 1987, 1989).

MATERIALS AND METHODS

Microbial Techniques

Media and microbial techniques were essentially as described previously (Sherman *et al.*, 1974; Rose *et al.*, 1990). All experiments in which cells were released from a G_1 arrest were carried out by αF arrest and release, by using αF at 1 μg/ml. Nocodazole was used at 10 μg/ml. Doxycycline (dox) was used at 25 μg/ml, and dox treatment was maintained throughout all *Degron-CSE4* experiments. Strains containing the *cCEN* were maintained in media containing 2% galactose. The *cCEN* was activated by growth in media containing 2% glucose. Yeast strains used in this study are listed in Table 1 and are derived from the W303 background. *cCEN* yeast strains were generated by integration of pR285#7 (*pGAL-CEN3-URA3*; Hill and Bloom, 1989) that was digested with *XhoI* to direct integration to the *HIS4* locus. Strains containing *pGAL-UBR1-myc* were made by transforming *PmeI*-digested pLK54#300 (Labib *et al.*, 2000) to direct integration to the *UBR1* locus. All strains made by transformation of pLK54#300 were screened by immunoblot to ensure equal expression of UBR1-myc protein. All kinetochore proteins were epitope tagged by the PCR integration technique and generated fusions that are functional at the permissive temperature (Longtine *et al.*, 1998). Primer sequences are available upon request.

Strains Used in Figures

The following strains were used in the figures. For Figure 1, Ctf13-myc13 (SBY1524) (B), Mif2-myc13 (SBY1525) (C), Ctf3-myc13 (SBY1526) (D), Okp1-myc13 (SBY3920) (E), Mtw1-myc13 (SBY2061) (F), and Nuf2-myc13 (SBY2770) (G). For Figure 2, Dam1-myc9 (SBY3665). For Figure 3, *Degron-CSE4*, *pGAL-UBR1-myc* (SBY4355). For Figure 4, Ndc10-myc13 (*CSE4*$^+$, SBY4713) and (*Degron-CSE4*, SBY4806) (A and E), Mif2-myc13 (*CSE4*$^+$, SBY4453) and (*Degron-CSE4*, SBY4452) (B), Mtw1-myc13 (*CSE4*$^+$, SBY4457) and (*Degron-CSE4*, SBY4456) (C and F), and Okp1-myc13 (*CSE4*$^+$, SBY4459) and (*Degron-CSE4*, SBY4458) (D). For Figure 5, Ctf19-myc13 (*CSE4*$^+$, SBY4711) and (*Degron-CSE4*, SBY4708) (A), Ndc80-myc13 (*CSE4*$^+$, SBY4461) and (*Degron-CSE4*, SBY4460) (B), Dam1-myc9 (*CSE4*$^+$, SBY1823) and (*Degron-CSE4*, SBY3456) (C), and Ask1-myc13 (*CSE4*$^+$, SBY3910) and (*Degron-CSE4*, SBY3912) (D). For Figure 6, *Degron-CSE4*, *pGAL-UBR1-myc*, *cse4*Δ, *mad2*Δ (SBY3933). For Figure 7, *Degron-CSE4*, *mad2*Δ, Spc42-GFP, *cse4*Δ, *pGAL-UBR1-myc* (SBY4391) and *mad2*Δ, Spc42-GFP, *pGAL-UBR1-myc* (SBY4547). For Supplemental Figure, *pGAL-UBR1-myc* (SBY4356) (A), Okp1-myc13 (*CSE4*$^+$, SBY4459) and (*Degron-CSE4*, SBY4458) (B), Mif2-myc13 (*CSE4*$^+$, SBY4453) and (*Degron-CSE4*, SBY4452) (C), and Stu2-myc13 (*CSE4*$^+$, SBY4712) and (*Degron-CSE4*, SBY4805) (D).

Protein and Immunological Techniques

Protein extracts were made and immunoblotted as described previously (Minshull *et al.*, 1996). 9E10 antibodies (Covance, Princeton, NJ) that recognize the Myc tag were used at a 1:10,000 dilution.

Chromatin Immunoprecipitation (ChIP) and Quantification

ChIP analysis was performed as described with the following modifications (Strahl-Bolsinger *et al.*, 1997). Samples were fixed for 15 min, except Dam1 and Ask1 epitope-tagged cells, which required a 2-h fixation. Samples were washed once in 25 ml of Tris-buffered saline. Cells were lysed in 500 μl of ice-cold lysis buffer with glass beads by beating in the cold room (mini bead-beater; Biospec Products, Bartlesville, OK) for 2 × 30 s (1-min rest). Chromatin was sheared by sonicating 5 × 10 s using a Sonifier cell disruptor (model W185; Misonix, Farmingdale, NY) on setting 3 (average fragment size 500 base pairs) and clarified for 10 min in microfuge. Rabbit polyclonal anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or Cse4 antibodies (Pinsky *et al.*, 2003) were used for immunoprecipitations with protein G-conjugated Dyna beads (Dynal Biotech, Lake Success, NY). Washes were 1 min each. Primers used to amplify the *eCEN3* locus (202 bp) were SB773 and SB774, the *cCEN* locus (389 bp) were SB712 and SB717, and *PGK1* (242bp) were SB775 and SB776. Specific sequences are available upon request. *Taq* polymerase was used for all PCR amplifications (New England Biolabs, Beverly, MA). For all nonquantitative analyses, input and immunoprecipitated (IP) template concentrations were titrated into the linear range. PCR volumes were 25 μl, and primers were added to each reaction at a final concentration of 1 μM. Fivefold serial dilutions of the crude lysates (input) and immunoprecipitated DNA (IP) are shown in all figures. All IPs were confirmed to be equal by immunoblotting.

For quantitative analyses, input and IP template concentrations were diluted to give linear PCR amplification with 24 cycles. Only samples that fell in the linear range were included in the analysis, and all experiments shown are averages of at least two independent experiments with error bars representing 1 SD. PCR reagent concentrations were the same as described above. PCR products were resolved on 6% nondenaturing polyacrylamide gels for SYBR Green analysis and on 1.4% agarose gels for Vistra Green analysis. SYBR Green and Vistra Green were used at 1:10,000 in 1× Tris-Borate-EDTA for 10 and 60 min, respectively. SYBR Green was used in Figures 1–3 and 5, and Vistra Green was used in Figure 4. Relative amplification of each PCR product was determined using the Typhoon PhosphorImager and ImageQuant software (GE Healthcare, Little Chalfont, England). The efficiency with which each PCR product was amplified in the input was used to normalize the corresponding IP sample PCR products.

Flow Cytometry

Flow cytometry was performed as described previously (Hutter and Eipel, 1979) with propidium iodide (Sigma-Aldrich, St. Louis, MO). A BD Biosciences FACScan flow cytometer was used and analyzed on CellQuest software (BD Biosciences, San Jose, CA).

Microscopy

Microscopy was performed as described previously (Biggins *et al.*, 1999) on a Nikon Eclipse E600 microscope with a 60× A/1.40 oil immersion lens. A CoolSNAPfx camera (Photometrics, Tucson, AZ) was used to acquire images. 4,6-Diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) was used at 1 μg/ml final concentration. At least 100 cells were analyzed for all reported experiments.

RESULTS

Yeast Kinetochores Can Assemble in G_1 Phase

It is not known what cell cycle stages are permissive for kinetochore assembly in budding yeast. One possibility is that kinetochore assembly is temporally restricted to occur after centromere replication. We therefore set out to address the cell cycle requirements for kinetochore assembly by using a *cCEN* (Figure 1A). The *cCEN* is integrated 49 kb to the left of endogenous centromere 3 (*eCEN3*) and is controlled by the galactose promoter (*pGAL*). When cells are grown in galactose media, transcription through the *cCEN* keeps it inactive. The addition of glucose represses transcription at the *cCEN*, allowing a functional kinetochore to form within 20 min (Hill and Bloom, 1989; Neff and Burke, 1992; Dewar *et al.*, 2004; Tanaka *et al.*, 2005).

To begin analyzing the cell cycle restrictions over kinetochore assembly, we first determined whether kinetochores could assemble in G_1 cells. Kinetochore assembly at the *cCEN* was analyzed using ChIP. *cCEN* cells containing an epitope-tagged kinetochore protein were arrested in G_1 by using the mating pheromone α factor (αF) and then shifted

Table 1. Yeast strains used in this study

Strain	Genotype
SBY1524	*Mata CTF13-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY1525	*Mata MIF2-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY1526	*Mata CTF3-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY1823	*Mata DAM1-myc9:TRP1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY2061	*Mata MTW1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY2770	*Mata NUF2-myc13:KanMX ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY3456	*Mata DAM1-myc9:TRP1 ura3-1 leu2-3112 his3-11 trp1-1 lys2Δ can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX HIS4:pGAL-CEN3-URA3*
SBY3910	*Mata ASK1-myc13:HIS3 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY3912	*Mata ASK1-myc13:HIS3 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX HIS4:pGAL-CEN3-URA3*
SBY3665	*Mata DAM1-myc9:TRP1 ura3-1 leu2-3112 his3-11 trp1-1 lys2Δ can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY3920	*Mata OKP1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 HIS4:pGAL-CEN3-URA3*
SBY3933	*Mata bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 mad2ΔURA3*
SBY4355	*Mata CTF3-myc13:KanMX ura3-1 leu2-3112 his3-11 trp1-1 lys2Δ can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4356	*Matα ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4391	*Mata bar1 ura3-1 leu2-3112 his3-11 trp1-1:SPC42-GFP:TRP1 lys2Δ can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 mad2ΔURA3*
SBY4452	*Mata MIF2-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 lys2Δ can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4453	*Mata MIF2-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4456	*Mata MTW1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4457	*Mata MTW1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4458	*Mata OKP1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4459	*Mata OKP1-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4 pGAL-CEN3-URA3*
SBY4460	*Mata NDC80-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4461	*Mata NDC80-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4547	*Mata bar1 ura3-1 leu2-3112 his3-11 trp1-1:SPC42-GFP:TRP1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 mad2ΔURA3*
SBY4708	*Mata CTF19-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4711	*Mata CTF19-myc13:KanMX bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4712	*Mata STU2-myc13:HIS3 bar1 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4713	*Mata NDC10-myc13:KanMX ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1 pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4805	*Mata STU2-myc13:HIS3 ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*
SBY4806	*Mata NDC10-myc13:KanMX ura3-1 leu2-3112 his3-11 trp1-1 can1-100 ade2-1:7tetop-UB-R-CSE4:ADE cse4ΔKanMX pGAL-UBR1-myc:HIS3 HIS4:pGAL-CEN3-URA3*

All strains were generated for this study and are isogenic with the W303 background.

for 30 min into either galactose to keep the *cCEN* inactive or glucose to activate the *cCEN*. ChIP was performed to compare the localization of each protein at the endogenous centromere (*eCEN*) relative to the *cCEN* and a control locus, *PGK1*. We tested a representative subset of the yeast kinetochore complexes, including the inner kinetochore proteins Mif2-myc13 and Ctf13-myc13 (CBF3 complex), the central kinetochore proteins Ctf3-myc13 (CTF19/COMA complex), Okp1-myc13 (CTF19/COMA complex), and Mtw1-myc13 (MTW1 complex), and the outer kinetochore protein Nuf2-myc13 (NDC80 complex). As expected, all of the proteins were enriched to similar levels at the *eCEN* relative to *PGK1* in both glucose and galactose, indicating that none of the proteins are limiting for assembly (our unpublished data). To determine the occupancy of proteins at the *cCEN*, the *eCEN* and *cCEN* localization were normalized to the control locus *PGK1*. We then set the *eCEN* enrichment to 1.0 and calculated the relative amount of protein at the *cCEN*. When the *cCEN* was inactive (GAL), the relative protein occupancy at the *cCEN* relative to the *eCEN* varied (Figure 1, B–G). Ctf13, Mif2, Okp1, and Nuf2 were at 10–30% occupancy relative to the *eCEN*, whereas Ctf3 and Mtw1 were not detected at the *cCEN* (Figure 1B). Although it is unclear whether partial occupancy reflects a population of cells with reduced protein binding or a small population of cells with fully occupied kinetochores, none of the proteins that we tested fully localized to the inactive *cCEN* during G_1 phase.

We next tested whether the proteins could achieve full occupancy at the *cCEN* when it was activated (GLU). All proteins tested assembled to levels comparable with the *eCEN* (Figure 1), suggesting that the G_1 phase of the cell cycle is permissive for kinetochore assembly. In addition, because the levels of protein assembled at the *cCEN* are comparable with the *eCEN*, the *cCEN* is a valid system for kinetochore assembly studies.

Although all of the kinetochore proteins tested assembled at the *cCEN* during a G_1 arrest, these experiments did not address whether the newly assembled kinetochore is functional to bind microtubules. Because kinetochore proteins

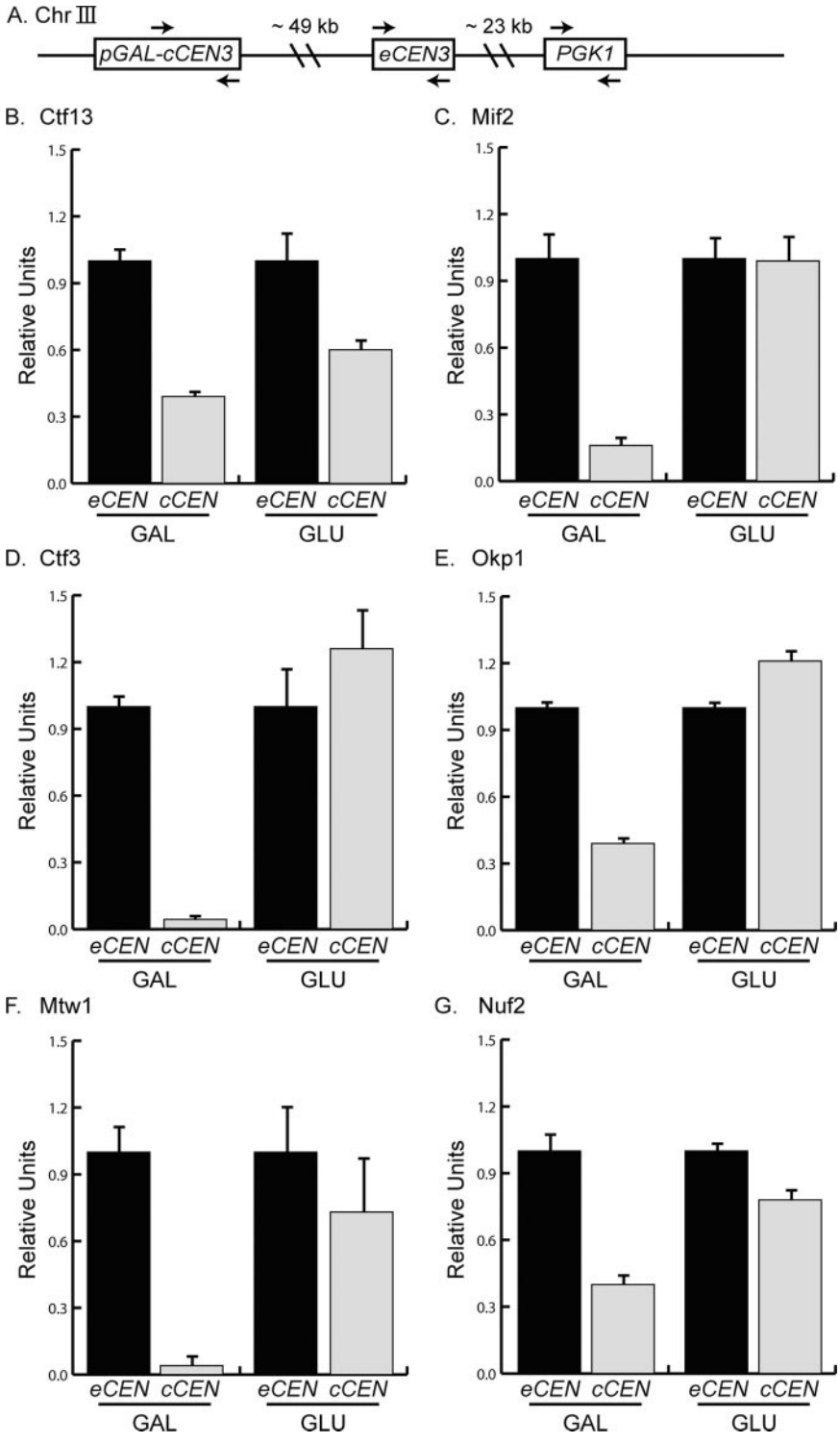

Figure 1. Kinetochore proteins from each subcomplex can assemble in G_1. (A). Schematic of chromosome III (Chr III) indicates the position of the *cCEN* (*pGAL-cCEN3*) and the negative control locus (*PGK1*) with respect to the *eCEN* (*eCEN3*). Arrows indicate the PCR primer sets used to detect these loci in ChIP experiments shown in the subsequent figures. (B–G). Strains expressing Ctf13-myc13, Mif2-myc13, Ctf3-myc13, Okp1-myc13, Mtw1-myc13, and Nuf2-myc13 were arrested in G_1 with αF and then transferred to media containing GAL or GLU. ChIP was performed with anti-myc antibody. Relative units indicate the *eCEN:PGK1* ratio or the *cCEN:PGK1* ratio and are normalized to an *eCEN:PGK1* value of 1.0 for each experiment. All kinetochore proteins tested localize to the active *cCEN* in G_1.

localized to the *cCEN* cannot be distinguished from the *eCEN* by microscopy, we could not perform a functional assay for microtubule binding to the *cCEN* (our unpublished data). We therefore analyzed the localization of the Dam1 protein to the *cCEN* as an indirect test for microtubule binding because Dam1 requires microtubules for its assembly at the kinetochore. Cells expressing Dam1-myc9 were arrested in G_1 and then transferred into either galactose (GAL) or glucose (GLU) media and harvested for ChIP analysis. Similar to the other kinetochore proteins we assayed, Dam1-myc9 localized to the active *cCEN* in G_1-arrested cells (Figure 2). To confirm that Dam1 localization to the *cCEN* is microtubule dependent, cells expressing Dam1-myc9 were arrested in G_1 and then treated with or without the microtubule-destabilizing drug nocodazole (NOC) for 60 min. The cultures were then split into GAL or GLU media. As expected, ChIP analysis confirmed the previously reported observation that Dam1 localization to the *eCEN* de-

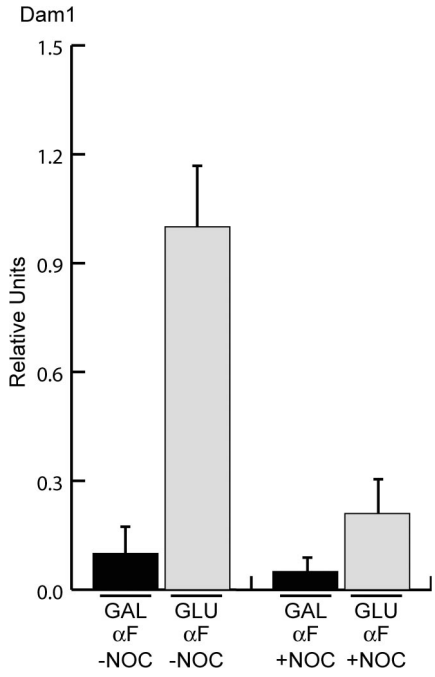

Figure 2. The G_1 *cCEN* requires microtubules for kinetochore assembly. Cells expressing Dam1-myc9 were arrested in G_1 with αF in galactose and then treated with (+NOC) or without (−NOC) the microtubule-depolymerizing drug nocodazole. Subsequently, the cultures were split into media containing GAL or GLU while maintaining the nocodazole treatment in αF. ChIP was performed with anti-myc antibody. Relative units indicate the *cCEN:PGK1* ratio and the amount of Dam1-myc9 in GLU-NOC is normalized to a value of 1.0. Dam1 localization to the *cCEN* in G_1 depends on microtubules.

pends on microtubules (our unpublished data) (Enquist-Newman *et al*., 2001; Li *et al*., 2002). To determine Dam1 occupancy at the *cCEN*, Dam1 localization to the active *cCEN* relative to *PGK1* in the absence of nocodazole was set to 1.0 and then compared with the *cCEN:PGK1* ratio in the other conditions. We found that Dam1 association with the *cCEN* also depends on microtubules (Figure 2). Thus, a kinetochore assembled on the *cCEN* in G_1 can bind microtubules, suggesting that a functional kinetochore can assemble in G_1 cells.

Cse4 Localizes to the Inactive cCEN *and Can Be Removed by Using a Degradable Cse4 Protein*

Because all of the kinetochore proteins tested localize to the *cCEN* in a glucose-dependent manner, the *cCEN* is an excellent system to further study kinetochore assembly. We were particularly interested in understanding the role of the budding yeast CenH3 in de novo kinetochore assembly. We first investigated whether Cse4 localizes to the inactive *cCEN* by performing ChIP with Cse4 antibodies in asynchronously growing cells, G_1-arrested cells and prometaphase-arrested cells. Unlike the other kinetochore proteins analyzed above, Cse4 localized to the inactive *cCEN* at levels comparable with the *eCEN* (Figure 3A; our unpublished data).

To analyze kinetochore assembly in the absence of Cse4, it was first critical to deplete Cse4 from the inactive *cCEN*. Cse4 was therefore fused to an N-degron sequence (*Degron-CSE4*) to conditionally target it for destruction (Collins *et al*., 2004). Transcription of *Degron-CSE4* is repressed by the addition of dox, and the protein is targeted for ubiquitin-mediated proteolysis by the N-end rule (Dohmen *et al*., 1994; Turner and Varshavsky, 2000). To enhance the kinetics of Cse4 depletion, the ubiquitin-protein ligase *UBR1* was overexpressed from the inducible galactose promoter (*pGAL*) (Labib *et al*., 2000). Strains expressing *Degron-CSE4* as the sole copy of Cse4 had similar occupancy at the inactive *cCEN* as the endogenous Cse4 protein (our unpublished data), consistent with our observations that Degron-Cse4 can complement a *cse4* deletion (Collins *et al*., 2004). To determine whether Cse4 could be depleted from the inactive *cCEN*, *pGAL-UBR1-myc cCEN Degron-CSE4* cells were grown in galactose and arrested in prometaphase with nocodazole to eliminate cell cycle variation and to prevent cells from attempting mitosis with dicentric chromosomes. Dox was

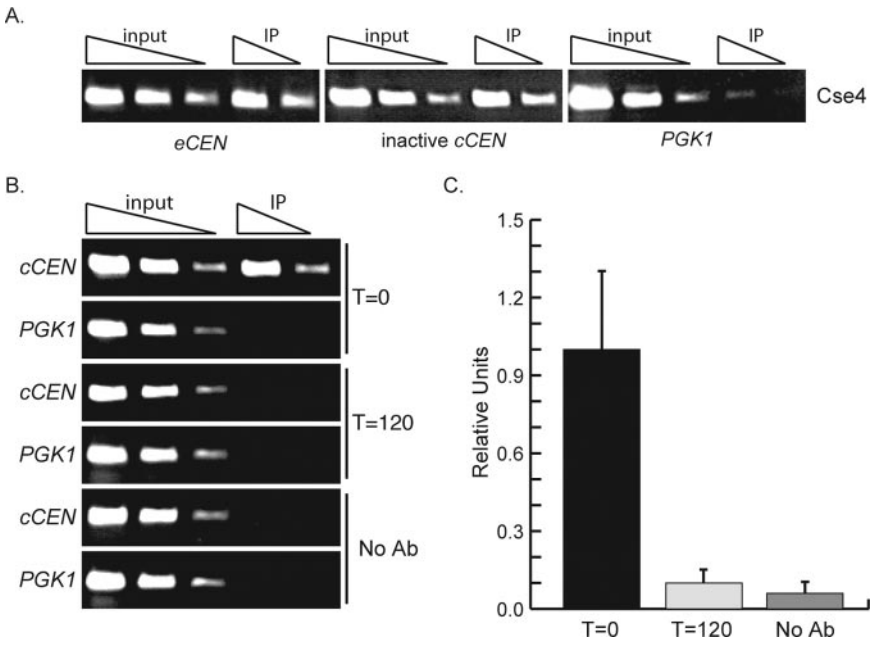

Figure 3. Cse4 localizes to the inactive *cCEN* and can be depleted using a Degron-Cse4 protein. (A) *Degron-CSE4 pGAL-UBR1-myc* cells were grown in galactose and arrested with nocodazole. ChIP was performed with anti-Cse4 antibody. Serial dilutions of lysates (input) and immunoprecipitated DNA (IP) are shown. Cse4 localizes to the inactive *cCEN* at levels similar to the *eCEN*. (B) Cells were grown as described above (A), and dox was added to repress transcription of *Degron-CSE4*. ChIP was performed using either anti-Cse4 antibody or no antibody at the indicated times after dox addition. Cse4 is depleted from the *cCEN* within 120 min. (C). Quantification of B. Relative units indicate the *cCEN:PGK1* ratio.

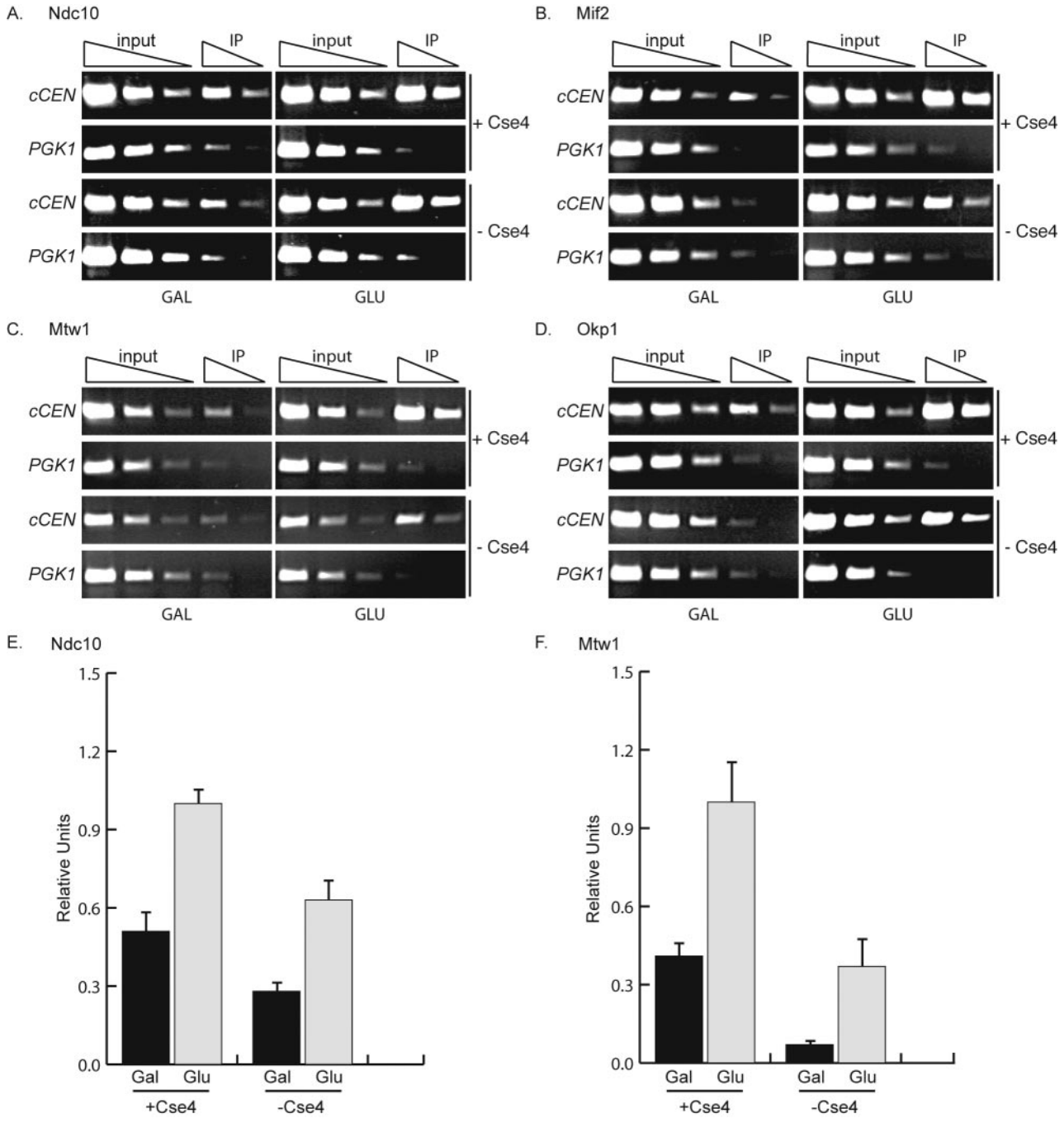

Figure 4. Cse4 is required for the full occupancy of inner and some central kinetochore components. (A–F). *pGAL-UBR1-myc cCEN* cells expressing the indicated myc-epitope tagged kinetochore protein and either wild-type *CSE4* (+Cse4) or *Degron-CSE4* (−Cse4) were grown in media containing galactose and arrested with nocodazole. Dox was added to repress transcription of *Degron-CSE4*, and cells were transferred into media containing GAL (inactive *cCEN*) or GLU (active *cCEN*) and analyzed by ChIP with anti-myc antibody. (E and F) Quantification of A and C. Relative units indicate the *cCEN:PGK1* ratio. The *cCEN:PGK1* value in GLU +Cse4 is normalized to a value of 1.0. Cse4 is required for the complete occupancy of Ndc10-myc13, Mif2-myc13, Mtw1-myc13, and Okp1-myc13 (also see Supplemental Figure 1, B and C).

added to repress transcription of *Degron-CSE4* and ChIP analysis revealed that the Degron-Cse4 protein is depleted from the inactive *cCEN* by120 min (Figure 3B). To confirm that Cse4 was completely depleted, we performed quantitative PCR and found that the amount of Degron-Cse4 that remained at the inactive *cCEN* after 120 min is comparable with the control ChIP where antibody was not added (Figure 3C). In contrast, a significant amount of Degron-Cse4 protein remained at the *eCEN* 120 min after dox addition (Collins *et al*., 2004). The difference in the amount of Cse4 that remains at the *eCEN* compared with the *cCEN* may be due to the kinetochore structure protecting Cse4 from destruction at the *eCEN* and/or transcription aiding depletion at the *cCEN*.

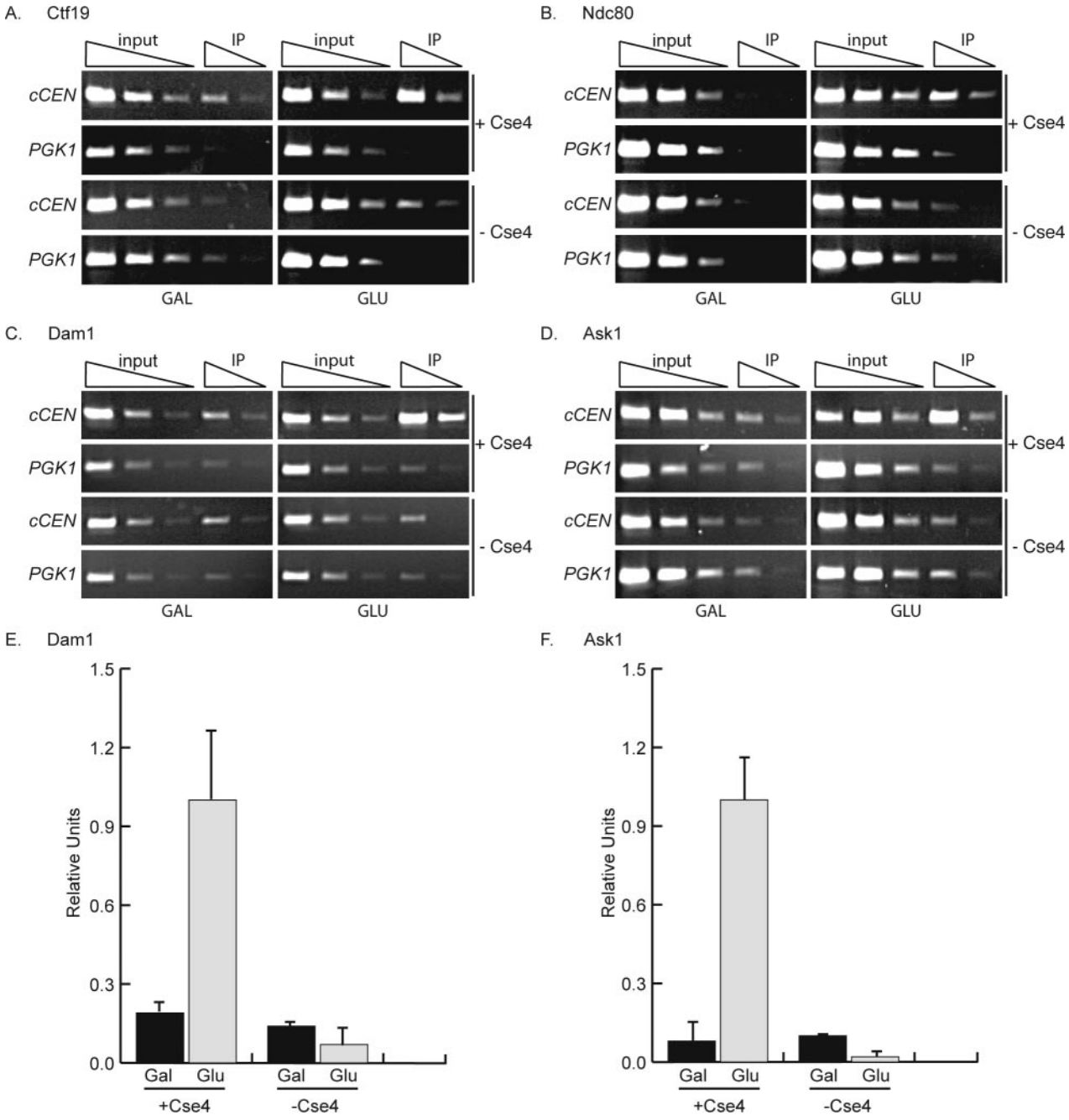

Figure 5. Cse4 is required to localize some central and outer kinetochore components. (A and B). *pGAL-UBR1-myc cCEN* cells and the indicated myc-epitope tagged kinetochore protein with either wild-type *CSE4* (+Cse4) or *Degron-CSE4* (−Cse4) were grown in media containing galactose (*cCEN* inactive) and arrested in prometaphase with nocodazole. Dox was added to repress transcription of *Degron-CSE4*, and cells were transferred into media containing GAL (inactive *cCEN*) or GLU, active *cCEN*) and analyzed by ChIP with anti-myc antibody. Cse4 is required to localize Ctf19-myc13 and Ndc80-myc13 to the *cCEN*. (C and D). Cse4 was depleted from the *cCEN* in cells expressing Dam1-myc9 or Ask1-myc13 by incubation with dox for 4 h. Cells were transferred to media containing GAL (inactive *cCEN*) or GLU (active *cCEN*) in the presence of dox and then harvested for ChIP with anti-myc antibody. Cse4 is required to localize Dam1-myc9 and Ask1-myc13. (E and F). Quantification of C and D. Relative units indicate the *cCEN*:*PGK1* ratio. The *cCEN*:*PGK1* value in GLU +Cse4 is normalized to a value of 1.0.

Cse4 Is Required for Kinetochore Assembly

Having established a way to deplete Degron-Cse4 from the inactive *cCEN*, we next investigated the assembly of representative members of the kinetochore complexes in the absence of Cse4. *pGAL-UBR1-myc cCEN* cells containing an epitope-tagged kinetochore protein and either *Degron-CSE4* or wild-type *CSE4* were grown in galactose and arrested in prometaphase with nocodazole (*cCEN* inactive). Dox was added to repress transcription of *Degron-CSE4*, and then cells were shifted into either galactose (GAL, inactive *cCEN*) or glucose (GLU, active *cCEN*) and harvested for ChIP analysis. Quantitative PCR confirmed that Cse4 remained de-

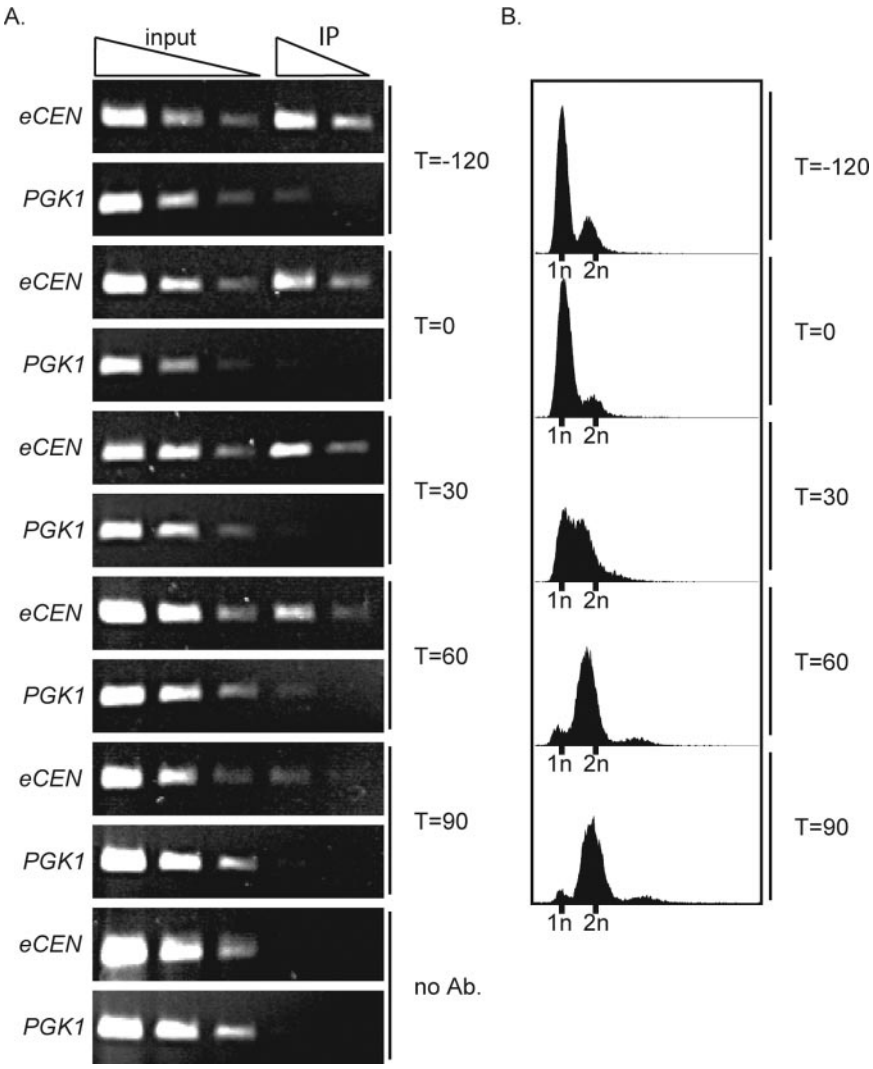

Figure 6. Cse4 can be depleted from the *eCEN*. Cells expressing *Degron-CSE4* (*Degron-CSE4 pGAL-UBR1-myc cse4*Δ *mad2*Δ) were arrested in αF in media containing galactose. Dox was added to repress transcription of *Degron-CSE4*, and then cells were released into the cell cycle in the absence of Cse4. Cells were harvested for Cse4 ChIP (A) and FACS (B) at −120 min (when dox was added) and 0, 30, 60, and 90 min after αF release. Cse4 is completely depleted from the *eCEN* within 90 min.

pleted when the cells were harvested (our unpublished data). In addition, a *cCEN* strain that expressed *pGAL-UBR1-myc* as the only myc-tagged protein was used in control ChIP experiments. As expected, Ubr1-myc does not localize to the *cCEN*, or the negative control locus, *PGK1* (Supplemental Figure 1A). We also confirmed that *pGAL-UBR1-myc* expression does not alter the levels of any of the kinetochore proteins tested in Figures 4 and 5 (our unpublished data).

We first assayed kinetochore assembly in the absence of Cse4 by analyzing the localization of the inner kinetochore proteins Ndc10-myc13 (CBF3 complex) and Mif2-myc13 as well as the central kinetochore proteins Mtw1-myc13 (MTW1 complex) and Okp1-myc13 (CTF19/COMA complex). All of these proteins were enriched at the *cCEN* when it was activated in either the presence or absence of Cse4 (Figure 4, A–D). However, because the proteins did not seem to be enriched to the same extent, we performed quantitative PCR to determine whether the occupancy of the proteins at the *cCEN* was the same in the presence and absence of Cse4. The amount of protein that localized to the active *cCEN* relative to *PGK1* in the presence of Cse4 was set to 1.0 and compared with the *cCEN:PGK1* ratio for the other conditions. Because Cse4 at the *eCEN* was partially reduced under these conditions (Collins *et al*., 2004), a comparison of the *cCEN* to the *eCEN* was not possible. Ndc10 and Mtw1 both partially localized to the inactive *cCEN* in the presence of Cse4 (Figure 4, E and F). The occupancy of these proteins at the inactive *cCEN* was further reduced in the absence of Cse4, suggesting that Cse4 may have a role in localizing these proteins. Consistent with this observation, Ndc10 and Mtw1 did not achieve the same level of occupancy at the active *cCEN* in the absence of Cse4 compared with the presence of Cse4. Similar results were obtained for Mif2 and Okp1 (Figure 4, B and D, and Supplemental Figure 1, B and C), indicating that Cse4 is required to fully localize these proteins to the *cCEN*.

We extended our analysis of kinetochore assembly in the absence of Cse4 to the central kinetochore component Ctf19 (CTF19/COMA complex) and the outer kinetochore components Ndc80 (NDC80 complex), Dam1, and Ask1 (DAM1 complex). Cells containing Ctf19-myc13 and Ndc80-myc13 were grown as described above for the inner kinetochore proteins and harvested for ChIP. Ctf19-myc13 (Figure 5A) and Ndc80-myc13 (Figure 5B) were not enriched at the inactive *cCEN* and showed little enrichment at the active *cCEN* in the absence of Cse4. Immunoblot analysis confirmed that equal amounts of Ctf19-myc13 and Ndc80-myc13 were immunoprecipitated (our unpublished data).

We also analyzed Dam1 and Ask1, members of the outer kinetochore DAM1 complex. However, because this com-

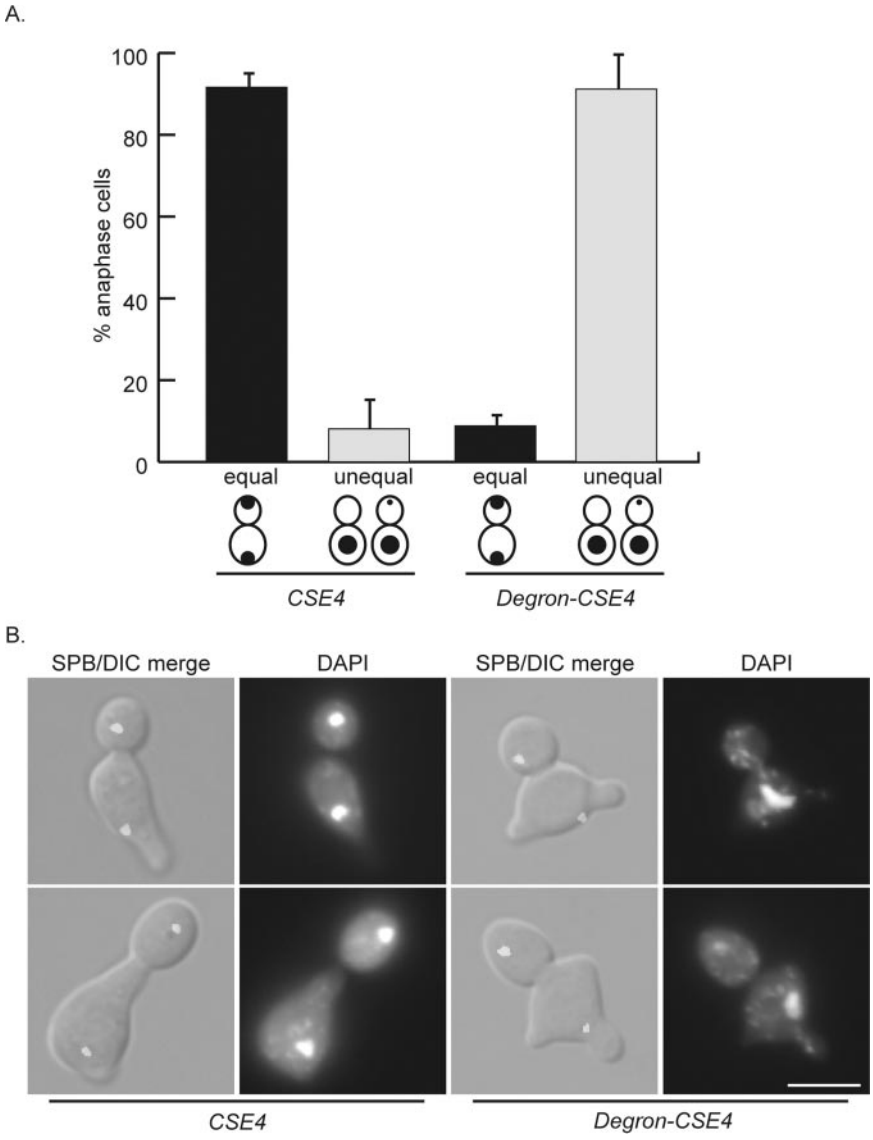

Figure 7. Cse4-depleted cells are defective in chromosome segregation. Cells expressing *Degron-CSE4* (*mad2*Δ Spc42-GFP *cse4*Δ *Degron-CSE4 pGAL-UBR1-myc*) or wild-type Cse4 (*mad2*Δ Spc42-GFP *pGAL-UBR1-myc*) were grown in galactose and arrested in αF. Dox was added, and cells were released into the cell cycle in the absence of Cse4. After 150 min, cells were fixed and stained with DAPI. (A). At 150 min, cells that had segregated spindle pole bodies (anaphase cells) were scored as having equal DAPI masses (equal) or unequal DAPI masses (unequal). (B). Representative cells at the 150-min time point. The top images of the *Degron-CSE4* show a cell with one, decondensed DAPI mass, and the bottom images represent a cell that has undergone unequal segregation where a small amount of DNA is in the bud. Bar, 5 μm.

plex requires microtubules for assembly, the experiment was performed using a four-hour asynchronous Cse4 depletion instead of arresting cells in prometaphase with nocodazole. After 4 h of Cse4 depletion, 87% of the cells were arrested in metaphase. Therefore, although nocodazole was not added, the cells were arrested at the same cell cycle stage as the other experiments. In addition, quantitative PCR analysis determined that Cse4 depletion at the *cCEN* was complete under these conditions (our unpublished data). We found that Cse4 is required for the localization of the outer kinetochore proteins Dam1-myc9 and Ask1-myc13 (Figure 5, C–F). Consistent with the mislocalization of the outer kinetochore components, the microtubule associated protein Stu2-myc13 also failed to localize to the active *cCEN* in the absence of Cse4 (Supplemental Figure 1D). Immunoblot analysis confirmed that equal amounts of Dam1-myc9, Ask1-myc13, and Stu2-myc13 were immunoprecipitated (our unpublished data). In conclusion, inner kinetochore proteins and some central components show a partial dependency on Cse4 for localization, whereas other central and all outer kinetochore components completely require Cse4 for kinetochore localization. Together, these data indicate that Cse4 is essential for kinetochore assembly.

Cse4 Can Be Depleted from the Endogenous Centromere

To determine how the defects in kinetochore assembly that occur in the absence of Cse4 alter chromosome segregation, we analyzed the phenotype of Cse4-depleted cells. To do these experiments, we first investigated whether cells could be depleted of Cse4 at the *eCEN*. We hypothesized that cells would need to pass through S phase in the absence of Cse4 to achieve depletion at the *eCEN* because fluorescence recovery after photobleaching experiments demonstrated that Cse4 is replaced at the centromere during S phase (Pearson *et al.*, 2004). To facilitate G_1 arrest and allow the analysis of cells at anaphase, *Degron-CSE4 pGAL-UBR1-myc* cells were also deleted for the *MAD2* spindle checkpoint gene, which halts the cell cycle when there is a defect in proper spindle assembly (for review, see Lew and Burke, 2003). Although *Degron-CSE4* cells do not respond to αF, *Degron-CSE4 mad2*Δ cells arrest in αF for reasons that are unclear (Biggins *et al.*, 2001). To analyze Cse4 depletion at the *eCEN*, *Degron-CSE4*

pGAL-UBR1-myc mad2Δ cells were arrested with αF, and dox was added to repress transcription of *Degron-CSE4*. Cells were then released into the cell cycle and monitored by ChIP and fluorescence-activated cell sorting (FACS) for Cse4 localization and DNA content, respectively. Consistent with our hypothesis, Cse4 was completely depleted from the *eCEN* after cells had completed replication at 90 min, although some Cse4 remained if cells did not pass synchronously through S phase (Figure 6; our unpublished data). Quantitative PCR was used to confirm that Cse4 was completely depleted at the *eCEN* (our unpublished data).

Cells Depleted of Cse4 Exhibit Severe Chromosome Segregation Defects

The depletion of Cse4 at the *eCEN* allowed us to characterize the phenotype of cells completely lacking Cse4. Although phenotypic analyses of temperature sensitive *cse4* alleles have been carried out (Stoler *et al*., 1995; Keith *et al*., 1999; Chen *et al*., 2000; Glowczewski *et al*., 2000; Keith and Fitzgerald-Hayes, 2000; Biggins *et al*., 2001), the consequences of depleting the centromeric nucleosome have not been analyzed previously. *Mad2Δ pGAL-UBR1-myc* cells containing *Degron-CSE4* or wild-type *CSE4* were arrested in G_1 with αF, treated with dox to repress transcription of *Degron-CSE4*, and then released into the cell cycle. Cells also contained a fluorescent spindle pole body (SPB) component, Spc42-GFP, to allow determination of the cell cycle stage. Cells were analyzed for chromosome segregation when the majority of cells were in anaphase, as defined by having fully separated SPBs. Chromosome segregation was scored as equal when similar amounts of DNA were at each pole (equal). Chromosome missegregation fell into two categories (unequal): one completely unsegregated DAPI mass, or unequal segregation where a small amount of DNA was in the daughter cell and the majority of the DNA was in the mother cell. Most control cells underwent normal DNA segregation (92%). However, the majority of Cse4-depleted cells (91%) underwent massive chromosome missegregation and had either completely unsegregated DNA (50%), or a very small amount of DNA in the daughter cell (41%) (Figure 7). It is important to note that even the cells with unequal segregation had very little DNA that had actually segregated to the daughter cell (Figure 7B). Thus, Cse4-depleted cells have severe defects in chromosome segregation, consistent with the defects in kinetochore assembly observed at the *cCEN* in the absence of Cse4.

DISCUSSION

Using a *cCEN*, we found that yeast cells do not restrict kinetochore assembly to a specific cell cycle phase. In addition, every kinetochore protein tested exhibited some dependence on Cse4 for assembly, consistent with our observation that Cse4-depleted cells cannot segregate chromosomes. We therefore propose that yeast use a combination of centromeric chromatin and centromeric DNA specificity to mediate kinetochore assembly.

A Conditional Centromere Provides an Assay to Study Yeast Kinetochore Assembly

We used a *cCEN* that is controlled by transcription to analyze kinetochore assembly. Because kinetochore proteins at the *cCEN* cannot be distinguished from the *eCEN* by microscopy (our unpublished data), we analyzed the assembly of representative proteins from each kinetochore complex using ChIP. There was variation in the occupancy of proteins at the inactive *cCEN* depending on the subcomplex analyzed. Cse4 was the only protein that fully localized to the inactive *cCEN*, suggesting that Cse4 may have sequence-specific centromere binding like other inner centromere proteins. Although our results contrast with a previous study that did not detect Cse4 at the inactive *cCEN* (Mythreye and Bloom, 2003), our results are consistent with the maintenance of the centromere-specific chromatin structure at the inactive *cCEN* (Hill and Bloom, 1987). Although no other protein fully occupied the inactive *cCEN*, several inner (Ctf13, Ndc10, and Mif2) and central kinetochore proteins (Okp1, Nuf2, and Mtw1) showed enrichment that is likely because of their transient association with the inactive *cCEN*. Because the inner proteins are required for outer kinetochore assembly, their transient association should be longer than the outer proteins and therefore easier to detect. Consistent with this idea, we did not detect outer kinetochore proteins at the inactive *cCEN*. Despite the variation at the inactive *cCEN*, all of the proteins tested localized to the active *cCEN* at levels comparable with the *eCEN*.

Although we detected full occupancy of Cse4 at the inactive *cCEN*, it is not clear why we detected only partial localization of Ndc10, because Ndc10 is required for the localization of Cse4 (Ortiz *et al*., 1999). One possibility is that Cse4 and Ndc10 transiently bind to the inactive *cCEN*, but Cse4 is easier to detect because it has a stronger binding affinity. Another possibility is that the partial localization of Ndc10 is sufficient for Cse4 localization. Further studies will be required to understand the dependency of Cse4 localization on Ndc10.

Yeast Kinetochore Assembly Is Not Cell Cycle Restricted

We found that representative proteins from all kinetochore complexes assembled at the *cCEN* during G_1 phase and prometaphase, suggesting that the entire kinetochore assembles. Due to the rapid assembly of kinetochore proteins (within 10 min), kinetic studies to test the order of assembly were not feasible (our unpublished data). Similar to the *eCEN*, Dam1 recruitment to the G_1 *cCEN* was microtubule dependent, strongly suggesting that a fully functional kinetochore can assemble in G_1. Together, these data suggest that budding yeast kinetochores can assemble and become functional throughout the cell cycle.

This is the first study to demonstrate that yeast cells maintain the ability to assemble kinetochores in multiple cell cycle stages. The lack of temporal restriction on kinetochore assembly may allow the repair of damaged kinetochores. Alternatively, yeast cells may not temporally restrict kinetochore assembly because a specific centromere sequence is required for assembly. The lack of cell cycle control indicates that all posttranslational modifications required for assembly are either maintained from the previous cell cycle or can occur during G_1. In addition, none of the kinetochore proteins seem to be limiting for assembly because changes in *eCEN* localization were not detected when the *cCEN* was activated.

Although yeast cells maintain the ability to assemble kinetochores at several cell-cycle stages, it remains unclear when kinetochores normally form. It is likely that assembly is coupled to centromere replication because yeast kinetochores quickly achieve bioriented attachments after centromere duplication and Cse4 deposition occurs at this time (Goshima and Yanagida, 2000; He *et al*., 2000; Pearson *et al*., 2004).

The Functions of CenH3

We analyzed kinetochore assembly at the *cCEN* in the absence of the CenH3. We were not able to analyze the main-

tenance of a previously assembled kinetochore because cells needed to pass through S phase to deplete Cse4 from the *eCEN*. Because kinetochores likely disassemble during S phase (Tanaka *et al*., 2005), studies at the *eCEN* would not distinguish between kinetochore assembly and maintenance. All proteins tested exhibited some dependence on Cse4 for localization to the *cCEN*. The inner and central kinetochore proteins Ndc10, Mif2, Mtw1, and Okp1 achieved ~50% occupancy at the active *cCEN* in the absence of Cse4, whereas the central protein Ctf19 and all outer proteins (Ndc80, Dam1, Ask1, and Stu2) completely failed to localize. Although residual Cse4 may remain, the partial occupancy of the inner kinetochore proteins may instead be due to their Cse4-independent sequence-specific binding (Lechner and Carbon, 1991; Meluh and Koshland, 1995; Stoler *et al*., 1995). It is likely that central kinetochore proteins achieve partial localization in the absence of Cse4 due to interactions with inner kinetochore proteins. The differences in the dependence of the CTF19/COMA complex proteins Ctf19 and Okp1 may reveal different requirements for Cse4 in localizing the C2.100 (Ctf19 and Mcm21) and C2.105 (Ame1 and Okp1) subcomplexes (De Wulf *et al*., 2003).

We extended our analyses to examine the phenotype of Cse4-depleted cells in a single cell cycle. In contrast to studies on *cse4* temperature-sensitive alleles that exhibited chromosome missegregation (Stoler *et al*., 1995; Keith *et al*., 1999; Biggins *et al*., 2001), the majority of DNA did not segregate in the Cse4-depleted cells. Strikingly, this phenotype most strongly resembles *ndc10-1* mutants that completely lack kinetochore assembly and microtubule attachment (Goh and Kilmartin, 1993), suggesting that microtubule–kinetochore attachments are not made in the absence of Cse4. This is consistent with our ChIP experiments that revealed severe defects in kinetochore assembly. Although *ndc10-1* cells do not engage the spindle checkpoint (Goh and Kilmartin, 1993), we were unable to determine whether Cse4-depleted cells are also defective in the checkpoint. *Degron-CSE4* cells do not respond to αF unless the *MAD2* spindle checkpoint gene is mutated, so we used cells defective in the checkpoint to facilitate the cell cycle arrest for the reported experiments.

Our studies of Cse4-depleted cells revealed two additional phenotypes that were not observed in *cse4* temperature-sensitive mutants. First, Cse4-depleted cells had a larger and more diffuse DAPI mass than wild-type cells, suggesting a role in condensation. This may be a conserved function because the *Caenorhabditis elegans* CenH3 is also required for condensation (Chan *et al*, 2004; Maddox, Portier, Desai, and Oegema, unpublished data) and a *Drosophila* condensin protein Cap-G interacts with CenH3 (Jager *et al*., 2005). We also found that Cse4-depleted cells were delayed in cytokinesis (our unpublished data), similar to the delay observed in human cells overexpressing a CenH3 mutant (Zeitlin *et al*., 2001). Future work will be aimed at determining the role of Cse4 in condensation and cytokinesis.

The ability to deplete Cse4 in a single cell cycle allowed us to perform the first study of de novo kinetochore assembly in the absence of CenH3. In agreement with other studies using RNAi to deplete CenH3 over multiple cell cycles, we found that Cse4 is required for the localization of many kinetochore proteins (Howman *et al*., 2000; Oegema *et al*., 2001; Regnier *et al*., 2005). However, in contrast to studies that suggested the existence of CenH3-independent assembly pathways based on immunofluorescence data (Oegema *et al*., 2001; Goshima *et al*., 2003; Hayashi *et al*., 2004; Regnier *et al*., 2005), we found that all proteins tested exhibited some dependence on Cse4 when the ChIP technique was used to quantify occupancy. Although the inner kinetochore complex CBF3 was previously proposed to nucleate yeast kinetochore assembly (Russell *et al*., 1999; Goshima and Yanagida, 2000; He *et al*., 2001; Janke *et al*., 2001, 2002), our work shows that the centromeric nucleosome also has an essential role in kinetochore assembly. We therefore favor a model where the centromeric nucleosome creates a chromatin structure that cooperates with the binding of the inner CBF3 complex to initiate yeast kinetochore assembly.

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