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Sister Chromatid Cohesion Role for *CDC28*-CDK in *Saccharomyces cerevisiae*

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ABSTRACT

High-fidelity chromosome segregation requires that the sister chromatids produced during S phase also become paired during S phase. Ctf7p (Eco1p) is required to establish sister chromatid pairing specifically during DNA replication. However, Ctf7p also becomes active during G_2/M in response to DNA damage. Ctf7p is a phosphoprotein and an *in vitro* target of Cdc28p cyclin-dependent kinase (CDK), suggesting one possible mechanism for regulating the essential function of Ctf7p. Here, we report a novel synthetic lethal interaction between *ctf7* and *cdc28*. However, neither elevated *CDC28* levels nor *CDC28* Cak1p-bypass alleles rescue *ctf7* cell phenotypes. Moreover, cells expressing Ctf7p mutated at all full- and partial-consensus CDK-phosphorylation sites exhibit robust cell growth. These and other results reveal that Ctf7p regulation is more complicated than previously envisioned and suggest that CDK acts in sister chromatid cohesion parallel to Ctf7p reactions.

CELL division is essential for embryonic growth, repair of damaged tissue, and the replacement of senescent cells. During S phase and long before a cell divides, chromosomes are replicated to produce identical sister chromatids. During mitosis, each sister chromatid orients to spindle pole microtubules opposite that of its sister chromatid, ensuring proper segregation of chromosomes into the newly forming daughter cells. In most vertebrate cells, chromosome replication and sister chromatid segregation are separated by extended periods of time and checkpoint functions. Thus, identifying the products of chromosome replication as sister chromatids during S phase and then maintaining that identity until anaphase are both fundamental facets of cell division and essential for progeny viability.

Early studies in yeast identified cohesin complexes (Mcd1p/Scc1p, Irr1p/Scc3p, Smc1p, and Smc3p) as the molecular glue that maintains sister chromatid pairing from S phase until anaphase onset (Strunnikov *et al.* 1993; Kurlandzka *et al.* 1995; Guacci *et al.* 1997; Michaelis *et al.* 1997; Toth *et al.* 1999). Biochemical and EM-based analyses revealed that a subset of cohesin subunits (Mcd1p, Smc1p, and Smc3p) form a huge triangular ring that likely encircles one or both chromatid fibers (Melby *et al.* 1998; Anderson *et al.* 2002; Haering *et al.* 2002; Gruber *et al.* 2003; Ivanov and Nasmyth 2005). Cohesin complexes are deposited along the chromosome length by a heterodimeric complex composed of Scc2p and Scc4p (Blat and Kleckner 1999; Megee *et al.* 1999; Tanaka *et al.* 1999; Ciosk *et al.* 2000; Laloraya *et al.* 2000; Glynn *et al.* 2004). Importantly, cohesins and their deposition onto sister chromatids are not sufficient for sister chromatid pairing. A third activity, termed establishment, is required to pair together cohesin-decorated sister chromatids. This function is provided by the essential and highly conserved Ctf7p (Ctf7p/Eco1p, EFO1/ESCO1, EFO2/ESCO2, DECO, ESO1) family of proteins (Skibbens *et al.* 1999; Toth *et al.* 1999; Tanaka *et al.* 2000; Bellows *et al.* 2003; Williams *et al.* 2003; Hou and Zou 2005; Vega *et al.* 2005). While the mechanism of cohesion establishment remains highly controversial, likely models are that Ctf7p catalyzes the pairing of cohesins associated with each sister chromatid or mediates cohesin dynamics during DNA replication (Skibbens *et al.* 2007).

In unperturbed cell cycles, Ctf7p functions exclusively during DNA replication after which Ctf7p becomes inactive (Skibbens *et al.* 1999; Toth *et al.* 1999). In response to DNA double-strand breaks during G_2/M, however, Ctf7p-dependent sister chromatid pairing becomes reactivated. Thus, S phase pairing and G_2/M re-pairing Ctf7p activities are tightly regulated (Strom *et al.* 2004, 2007; Unal *et al.* 2004, 2007). Prior studies revealed that Ctf7p is a phosphoprotein and an *in vitro* target of CDK activity (Ubersax *et al.* 2003), suggesting a mechanism for regulating Ctf7p. Here, we report on results from a synthetic lethal screen regarding novel interactions between *CTF7*, *CAK1*, and *CDC28* and site-directed mutagenesis of Ctf7p phosphorylation consensus sites. These findings test the model that Ctf7p function is regulated through CDK activity.

MATERIALS AND METHODS

Media and cell growth: Yeast and bacterial media, growth, sporulation, and transformation procedures were performed

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as described with minor modifications (Ito *et al.* 1983; Schiestl and Gietz 1989; Rose *et al.* 1990). Strains used in this study are listed in Table 1. Sectoring analyses to identify plasmid-dependent functions were based on previous genetic analyses and performed as previously described with minor modification (Koshland *et al.* 1985; Krantz and Holm 1990; Brands and Skibbens 2005).

Molecular methods and site-directed mutagenesis: YAB1004 was generated by crossing YBS514 four times into the W303 background strain YCH128 (Hardy and Pautz 1996). YAB1028 was generated by transformation with pAB1009. pAB1009 was generated by inserting a 5.5-kb *ADE3* locus-containing fragment into the *Sac*I site of pRS316-*CTF7* (Skibbens *et al.* 1999). All site-directed mutations were performed in pLC3 (*CEN TRP1 CTF7* using high-fidelity *Pfu* DNA polymerase and the entire resulting *CTF7* open reading frame sequenced in-house (ABI 310 DNA sequencer). Site-directed mutagenesis was performed using PCR and designed oligo pairs as previously described and following manufacturer instructions. DNA oligomers used in this study are available upon request.

CEN URA3 plasmids harboring various *cak1* alleles (generous gifts from Ed Winter, Thomas Jefferson University), were digested with *Pvu*I and the *cak1*-containing fragments ligated with *CEN TRP1* fragments harvested from *Pvu*I-digested pRS314.

Cell and colony morphological analyses and cohesion assays: Yeast cells harvested for phenotypic analyses were fixed in 3.7% formaldeyde for 30 min, washed, and the cell wall removed using zymolyase prior to adhering the cells to slides pretreated with poly-L-lysine. Cells were then permeabilized and flattened by immersion for 4 min in −20° methanol. Mounting mix containing DAPI to allow for DNA visualization was used to adhere coverslips to the glass slides. All images were acquired using a Nikon Eclipse 800 equipped with either a 100× Plan Apo 1.4 objective for cells or 10× Plan 0.25 objective for colonies and captured using a CoolSnap *fx* (Photometrics) cooled CCD camera operated by IPLab software (version 3.5.2).

Images of sectored colonies were acquired using a Nikon SMZ 1500 microscope equipped with a HR Plan Apo 1× WD 54 lens. Optivar settings of ~1.5 were used to achieve a 0.55 cm field of view applied to all sector colony assay images. Colony images were captured using a Nikon DXM 1200 CCD camera and ACT1 image software.

Cohesion defects were assessed by crossing *cak1-277* allele into our telomere proximal cohesion assay strain YLA1119 (*LacI-GFP:HIS3*, *LacO:KAN*, and *PDS1-13MYC:TRP*) previously described (Antoniacci and Skibbens 2006). The resulting diploids were sporulated, dissected, and independent isolates of progeny containing the appropriate markers identified. In parallel, *cak1-277* was incorporated into a centromere-proximal cohesion assay strain YBS1042 (*TetO:URA3*, *TetR-GFP:LEU2*, and *PDS1-13MYC:TRP1*) using a similar strategy (Kenna and Skibbens 2003). Cohesion assay diploid strains homozygous for either wild-type *CDC28* (AK1429), or *cdc28-B119* (AK1439), or *cdc28-B28* (AK1445) alleles were obtained as a generous gift from Ana Kitazono and Steven Kron (Kitazono *et al.* 2003). For *cdc28* mutant strains, the lac operator is integrated on chromosome III at the *LEU3* locus (Kitazono *et al.* 2003). Cohesion assays were performed as described previously with the following modifications (Kenna and Skibbens 2003; Antoniacci and Skibbens 2006). Cells synchronized in late G_1 or early S phase were preshifted to 37° during the final 30–45 min of the incubation. For *cak1* mutant cells, preanaphase cells were obtained by supplementing fresh medium with nocodazole. In contrast, *cdc28-B119* and *cdc28-B28* cells are deficient in spindle checkpoint function but they will arrest prior to anaphase onset in response to DNA damage (Kitazono *et al.* 2003 and this study). Thus, synchronized *cdc28* mutant cells were released into fresh medium supplemented with zeocin (100 μg/ml) to induce double-strand breaks and produce a G_2/M arrest. Pds1 is not epitope tagged in these cells, requiring analyses to be based on the 2C DNA bias resulting from zeocin exposure and in comparison to wild-type cells. All results represent data tallied from at least two separate experiments in which 100 cells were counted for each strain in each experiment.

TABLE 1

Strains and mutations used in this study

YAB1005	*MAT*α *ade2 ade3 lys2 ura3 his3 leu2 ctf7::HIS3 ctf7-203:LEU2*
YAB1010	*MAT***a** *ade2 ade3 trp1 ura3 his3 leu2 ctf7::HIS3* pAB1009 (*CEN ADE3 URA3 CTF7*)
YBS514	*MAT***a** *ura3 lys2 ade2 his3 trp1 leu2 ctf7::HIS3 ctf7-203:LEU2* (*D168V, R199K, I231F, G259R*) (Skibbens *et al.* 1999)
YLA1119	*MAT***a** *HIS3:LacI-GFP CLONAT: KAN:LacO:telomere IV PDS1-12MYC:TRP1* (Antoniacci and Skibbens 2006)
YBS1294	YLA1119 containing *cak1-277*
	Mutations in full/partial consensus CDK/Cdc28p-phosphorylation Ctf7p residues
YAB1200	= YAB 1010 pAB1067 (*CEN TRP1 ctf7-500* = *S67A*)
YAB1201	= YAB 1010 pAB1068 (*CEN TRP1 ctf7-502* = *T94A*)
YAB1202	= YAB 1010 pAB1066 (*CEN TRP1 ctf7-501* = *S99A*)
YAB1203	= YAB 1010 pAB1069 (*CEN TRP1 ctf7-503* = *S105A*)
YAB1204	= YAB 1010 pAB1074 (*CEN TRP1 ctf7-504* = *S67A, T94A, S99A, S105A*)

RESULTS

***ctf7-203* synthetic lethality screen identifies a novel *cak1* allele:** To identify novel cohesion establishment factors, we performed a synthetic lethal screen based on the conditional *ctf7-203* mutant strain. An *ade2, ade3* temperature-sensitive *ctf7-203* strain, which gives rise to white colonies, was transformed with a *CTF7 URA3 ADE3* plasmid. At temperatures permissive for ctf7-203p function, transformants remain viable despite plasmid loss and give rise to red/white sectored colonies. Transformed cells were mutagenized to a 20% survival rate using ethyl methylsulfonate (EMS). Cells harboring mutated genes synthetically lethal with *ctf7-203* are obligated to maintain the *CTF7 URA3 ADE3* plasmid and readily detected as giving rise to nonsectored red colonies (Figure 1). From a population of ~60,000 randomly mutagenized cells, we identified five nonsectoring strains that depended on plasmid-borne *CTF7* for viability. One of the nonsectoring strains was

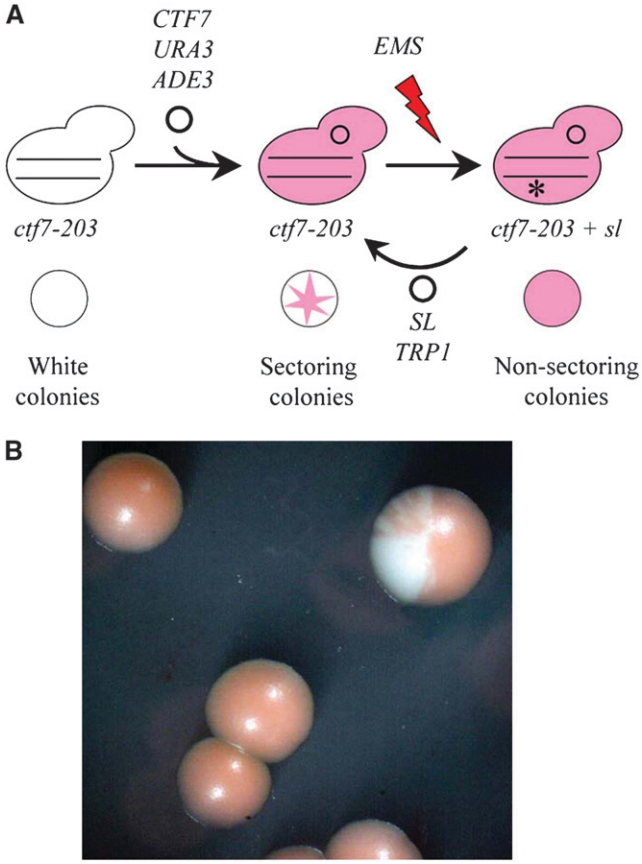

FIGURE 1.—*ctf7-203*-based synthetic lethal screen identifies a novel *cak1* allele. (A) Schematic of synthetic lethal screen: *ade2 ade3 ctf7-203* cells (top) produce white colonies (bottom). When transformed with *CTF7 URA3 ADE3*, cells give rise to red/white sectored colonies on nonselective 23° medium. Random EMS mutation of genes (asterisk) that are synthetically lethal (*sl*) with *ctf7-203* oblige cells to retain the *CTF7 URA3 ADE3* plasmid, producing nonsectored red colonies. Transformation of a genomic library (*TRP1 SL* rescuing plasmid) identified *CAK1* as allowing for loss of the *CTF7 URA3 ADE3* plasmid. (B) Micrograph shows transformation of synthetic lethal strain with *CAK1* genomic clone results in a sectored red and white colony.

temperature sensitive, suggesting that the new mutation occurred within an essential gene. *CEN TRP1* genomic library-based complementation of both temperature sensitivity at 37° and nonsectoring at 23° identified four genomic inserts that contained *CTF7* and three inserts that contained *CAK1* (Figure 1). Deletion and subcloning analyses confirmed that wild-type *CAK1* fully rescues the synthetic lethal interaction, documenting a genetic interaction between *CTF7* and *CAK1*. Sequence analyses identified a single G277D mutation within the *cak1* amino acid sequence of the synthetic lethal strain. From here on, we refer to this allele as *cak1-277*.

***cak1-277* cells exhibit bud growth, polarity, and nuclear division defects:** We first considered the possibility that we had identified a novel sister chromatid pairing role for Cak1p. However, an alignment between numerous kinases (including Cak1p, Cdc28p, Csk1p, and Fus1p) revealed that glycine 277 in Cak1p is invariably conserved through evolution (CHUN and GOEBL 1997) and thus not likely to uncover a unique Cak1p function. Indeed, phenotypic analyses of *cak1-277* mutant cells identified bud polarity defects, multiple and elongated bud structures, and chromosome segregation phenotypes previously documented in other *cak1* mutant cells (supplemental Figure 1) (KALDIS *et al.* 1996; THURET *et al.* 1996; CHUN and GOEBL 1997; SUTTON and FREIMAN 1997).

We next tested whether *ctf7-203* synthetic lethality could be recapitulated using other *cak1* alleles (WAGNER *et al.* 1997). The results show that *ctf7 cak1* cells that contain *cak1-17* plasmid required the *CEN URA3 CTF7* plasmid for viability and thus gave rise to red nonsectored colonies. *ctf7 cak1* cells transformed with either *cak1-22* or *cak1-K31R* provided only for infrequent *CEN URA3 CTF7* loss, consistent with prior studies that *cak1-K31R* and *cak1-17* mutations exhibit increased levels of activity relative to *cak1-22* (WAGNER *et al.* 1997). These observations reveal that *ctf7-203* interactions with *cak1-277* are not unique to this allele.

***CDC28*-dependent bypass of *cak1* phenotypes but not *ctf7* phenotypes:** Cak1p phosphorylates many substrates including Kin28p, Bur1p, and Ctk1p, but the only essential role for Cak1p kinase is to activate Cdc28p (ESPINOZA *et al.* 1996, 1998; CROSS and LEVINE 1998; YAO and PRELICH 2002; OSTAPENKO and SOLOMON 2005; GANEM *et al.* 2006). If the synthetic lethality that we observed between *ctf7* and *cak1* was in fact due to diminished CDK activation, we reasoned that Cak1p-bypass *CDC28* alleles (CROSS and LEVINE 1998) should rescue both *cak1-277* temperature sensitivity and *ctf7 cak1* synthetic lethality. To test the first of these predictions, wild-type *CAK1*-, *CDC28*-bypass alleles (4324 and 43244) and vector alone were transformed into *cak1-277* cells. Independent isolates for each strain were then plated on rich medium at permissive and restrictive temperatures. As expected, *CAK1* fully rescued *cak1-277* mutant cell temperature sensitivity while vector alone did not (Figure 2A). *CDC28*-bypass alleles fully rescued the conditional growth of the *cak1-277* strain, documenting that cak1-277p is conditionally defective in CDK activation (Figure 2A).

To confirm this interpretation, we tested whether *ctf7 cak1* synthetic lethality is based on reduced Cdc28p activation. *cak1 ctf7* double-mutant cells harboring *CEN CTF7 URA3* plasmid were transformed with *CEN TRP1* plasmids containing either *CTF7*, *CAK1*, *CDC28*, or *CDC28 CAK1*-bypass alleles (4324 and 43244). Independent isolates of the transformants were placed on medium supplemented with 5′ fluoro-orotic acid (5-FOA) to counterselect cells harboring the *CEN CTF7 URA3* plasmid (BOEKE *et al.* 1987). Both *CAK1* and *CTF7*, but not vector alone, supported cell growth at 23° (Figure 2B). *CTF7* provided for more robust cell growth, relative to *CAK1*, because the *ctf7-203* allele is compromised for

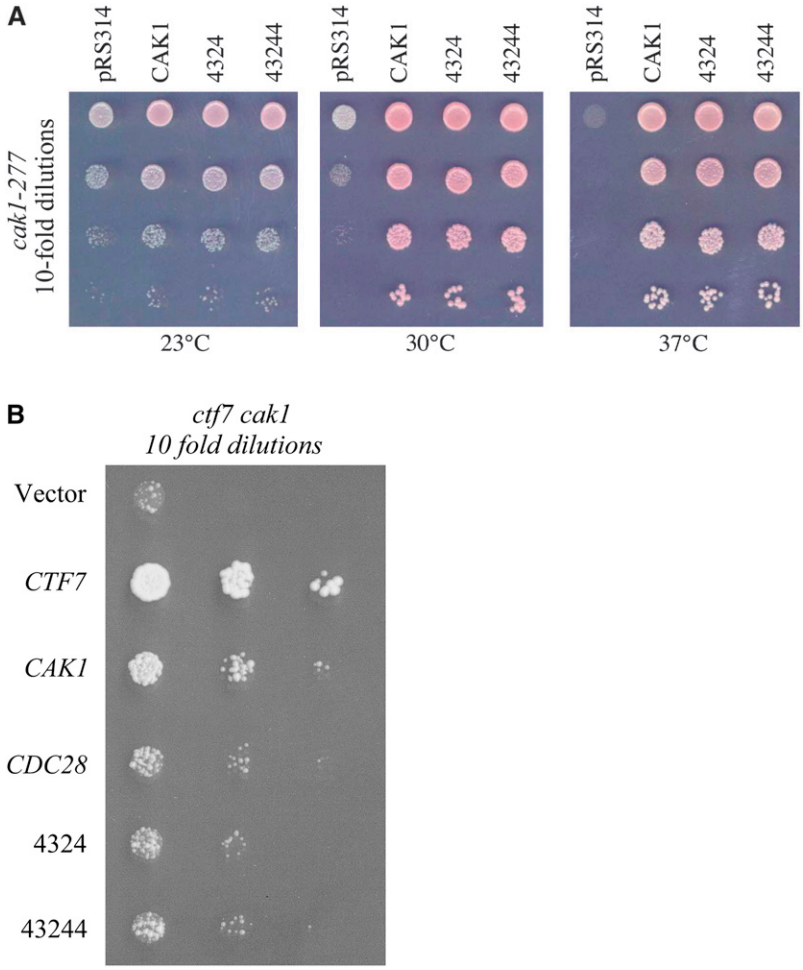

Figure 2.—(A) *CDC28*-bypass alleles suppress *cak1-277* temperature sensitivity. Tenfold serial dilutions of *cak1-277* mutant cells containing empty vector (pRS314), a *CAK1* genomic clone, or *CDC28*-bypass alleles (4324 and 43244) were spotted on selective medium plates and incubated at 23°, 30°, or 37°. (B) *CDC28*-bypass alleles partly suppress double-mutant *ctf7-203 cak1-277* synthetic lethality. Tenfold serial dilutions of *ctf7-203 cak1-277* double mutants harboring *CEN CTF7 URA3* and transformed with either *CEN TRP1* vector alone or vector containing *CTF7*-, *CAK1*-, *CDC28*-, or *CDC28*-bypass alleles (4324 or 43244) grown on medium containing 5-FOA at 23°.

growth and chromosome transmission fidelity even at 23° (Skibbens *et al.* 1999). *CDC28*-bypass alleles (4324 and 43244) also supported *cak1 ctf7* double-mutant cell viability, although not to the extent of either *CTF7* or *CAK1* (Figure 2B). Even a *CEN*-based increase of wild-type *CDC28* provided for partial rescue of *cak1 ctf7* double-mutant cell growth. While these findings do not exclude a minor role for Cak1p in Ctf7p function, the data reveal that *ctf7 cak1* synthetic lethality is largely due to diminished Cdc28p function.

***cdc28 ctf7* double-mutant cells are inviable:** To directly test the model that *ctf7* mutant cells are inviable in the presence of diminished CDK function, a reporter strain (*CTF7::HIS3*, *ctf7-203-LEU2, ade2, ade3* harboring a *CEN CTF7 URA3* plasmid) was mated to two different *cdc28* mutant strains (*cdc28-cte2-HIS3* or *cdc28-cte8-HIS3*). These *cdc28* alleles were of particular interest because they exhibit defects in genetic stability, consistent with a model that Cdc28p functions in chromosome segregation (Kitazono and Kron 2002). The resulting diploids were sporulated and eight His+ Leu+ Ura+ strains that also exhibited temperature sensitivity at 37° (attributable to *cdc28*) selected for further analyses (Figure 3). Tenfold serial dilutions of the parental *ctf7-203* strain and all eight spores were plated on uracil-deficient medium (to maintain the *CEN CTF7 URA3* plasmid) and then replica plated two times onto medium plates supplemented with 5-FOA to counterselect cells harboring the *CEN URA3 CTF7* plasmid. If *ctf7* and *cdc28* alleles are indeed synthetically lethal, approximately half of these strains should remain 5-FOA resistant while the other half should be 5-FOA sensitive and thus inviable. Indeed, four of the eight strains exhibited robust growth on 5-FOA plates identical to the *ctf7-203* parent strain (Figure 3). The remaining four strains were unable to survive exposure to medium containing 5-FOA. These findings reveal that these four strains contain both *cdc28* and *ctf7* alleles and that this combination is lethal (Figure 3).

Mutational analyses of candidate Ctf7p phosphorylation sites: ATP-analog-specific Cdc28p kinase reactions identified Ctf7p as an *in vitro* substrate for Cdc28p phosphorylation (Ubersax *et al.* 2003). Ctf7p is a small protein of 281 amino acids and contains only one full-CDK-consensus phosphorylation site (S99) and three partial-consensus sites (S67, T94, and S105). If Cdc28p phosphorylates Ctf7p to regulate cohesion establishment, then these candidate phosphorylation residues should be critical for cell viability. To test this model, we used PCR-based site-directed mutagenesis to replace

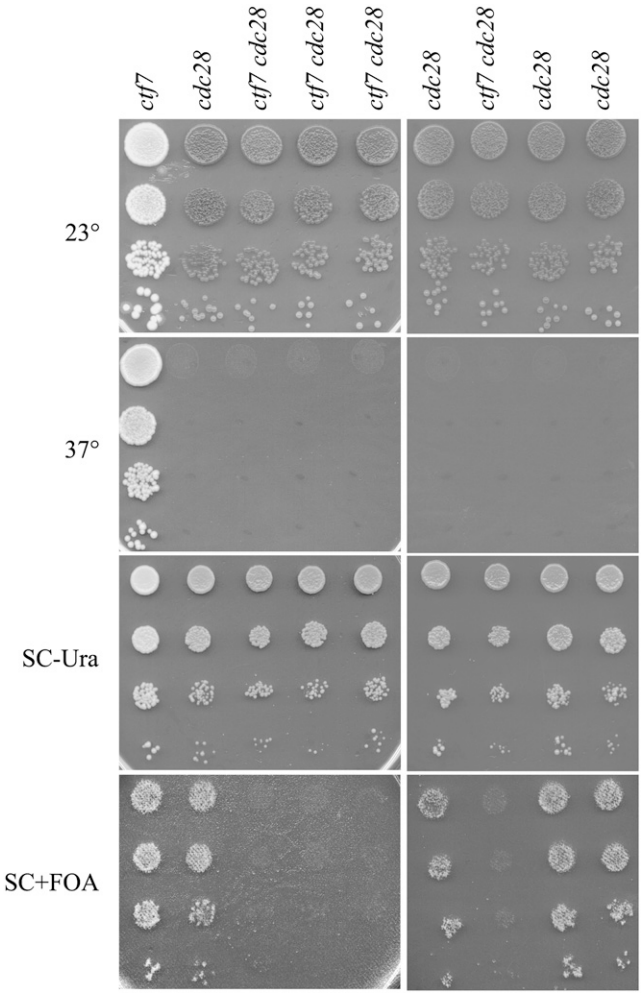

FIGURE 3.—*ctf7 cdc28* double-mutant strains are inviable. Tenfold dilutions of *ctf7* parental cells and eight spores obtained from *ctf7 cdc28* heterozygous diploids. All spores harbor *CEN CTF7 URA3* plasmid (growth on SC −Ura medium) but are inviable on rich medium placed at 37°, indicating that each spore contains the temperature-sensitive *cdc28* allele. Replica plating onto medium supplemented with 5-FOA counterselects for cells containing the *URA3*-based plasmid. See text for details.

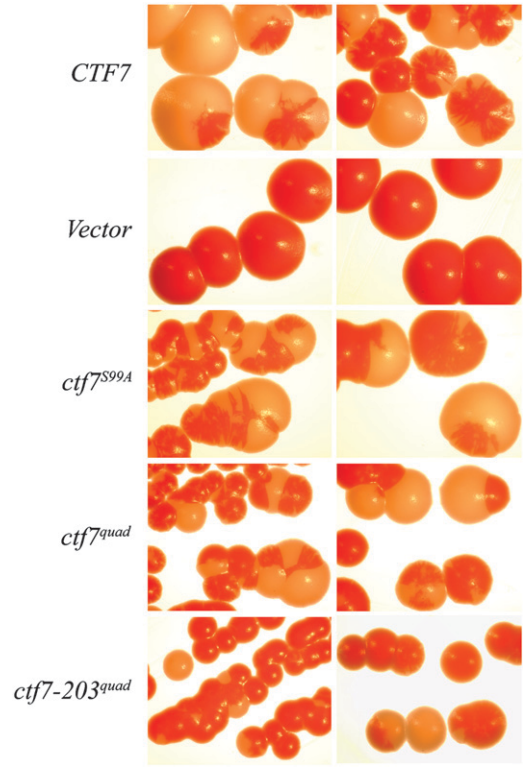

FIGURE 4.—*CTF7* mutated at candidate CDK phosphorylation sites maintain cell viability. *ctf7* null *ade2 ade3* strains harboring *CEN ADE3 URA3 CTF7* and *CEN TRP1* plasmids: the latter of which contains wild-type *CTF7, CTF7* mutated at either the full-consensus CDK phosphorylation site (*ctf7*^{S99A}), all candidate CDK phosphorylation sites (ctf7$^{S99A\ S67A\ T94A\ S105A}$ or quad mutant), the *ctf7-203* allele in which the quad mutations were also introduced (*ctf7-203*^{quad}), or vector alone (Vector). Ability of various *ctf7* constructs to support cell growth is observed as white and red sectored colonies on nonselective rich medium held at 23°. Two independent isolates for each strain are shown. Colony size differences reflect only cell crowding and local nutrient conditions, not growth defects associated with *ctf7* alleles. Each image in this and subsequent colony sectoring figures corresponds to ~0.55 cm.

each of these residues with alanine (herein referred to as *ctf7*$^{phos-}$ constructs). The mutated regions were cloned into pRS314 (*CEN TRP1* plasmid) and resequenced to ensure that no other mutations were generated. The resulting plasmids were then transformed into a *ctf7* null *ade2 ade3* strain in which viability was maintained by a *CEN ADE3 URA3 CTF7* plasmid. As controls, we included in our analyses *CEN TRP1* vector alone and vector harboring wild-type *CTF7*. Independent isolates of the resulting transformants were placed on rich nonselective medium and assayed for plasmid loss. As expected, *ctf7* null *ade2 ade3* cells harboring *CEN URA3 ADE3 CTF7* and transformed with a *CEN TRP* vector gave rise to solid red colonies while cells transformed with *CEN TRP CTF7* produced highly sectored red/white colonies (Figure 4). Importantly, cells transformed with any of the four single candidate *ctf7*$^{phos-}$ alleles also produced highly sectored red/white colonies (Figure 4). We then generated a quadruple *ctf7*$^{phos-}$ mutant (S99A, S67A, T94A, and S105A herein termed a "quad" mutant). When transformed into the reporter assay, this construct also gave rise to highly sectored red/white colonies (Figure 4). Thus, Ctf7p mutated at any or all consensus CDK phosphorylation residues is competent to provide for Ctf7p function at 23°.

We next decided to test whether conserved serine/threonine residues were crucial for Ctf7p function. From sequence analyses, we identified five additional serine and threonine residues (T76, S213, T228, S252, and T255) in budding yeast that are highly conserved in fission yeast, Drosophila, mouse, and human cells (BELLOWS *et al.* 2003). We mutated each of these five residues to alanine using PCR-based site-directed mutagenesis and confirmed each construct (*ctf7*$^{conserved\ S/T-}$) by sequencing.

Upon transformation into our reporter strain, all five *ctf7*$^{conserved\ S/T-}$ constructs supported robust cell growth at 23° (supplemental Figure S2).

Recent studies revealed that Ctf7p activity is required during G_2/M in response to DNA damage (Strom *et al.* 2007; Unal *et al.* 2007). Thus, we decided to test if any of the five *ctf7*$^{phos-}$ or five *ctf7*$^{conserved\ S/T-}$ constructs could support cell viability in the presence of various challenges such as elevated temperatures or exposure to genotoxic agents. To obtain cells that expressed *ctf7*$^{phos-}$ or *ctf7*$^{conserved\ S/T-}$ as the sole source of Ctf7p function, we isolated Trp+ Ura− cells that gave rise to solid white colonies (Figure 4 and supplemental Figure 2). Tenfold serial dilutions of the four single *ctf7*$^{phos-}$ strains, the quad *ctf7*$^{phos-}$ mutant, and the five different *ctf7*$^{conserved\ S/T-}$ mutant strains were then plated onto rich medium and maintained at 23°, 30°, or 37°. In parallel, duplicate samples were exposed to 75 μJ of UV irradiation or challenged with DNA double-strand breaks by incubating the cultures for 60 min in medium containing 100 μg/ml zeocin. The results show that each of the single and quad *ctf7*$^{phos-}$ alleles provided for robust growth identical to wild-type *CTF7* regardless of the type of challenge (Figure 5). Only *ctf7-203* mutant cells exhibited temperature and zeocin sensitivity, providing an internal control that further supported the finding that *ctf7*$^{phos-}$ strains are not genotoxic sensitive. In parallel, each of the 5 *ctf7*$^{conserved\ S/T-}$ constructs provided for robust growth, regardless of temperature and genotoxic challenges (supplemental Figure S3).

Analyses of *ctf7* alleles in combination with *cak1-277*: The synthetic lethality between *ctf7* and *cdc28* (and *cak1*) suggested that we might uncover phenotypes associated with either our *ctf7*$^{phos-}$ or *ctf7*$^{conserved\ S/T-}$ alleles if placed within the context of the original *cak1* allele. To test this possibility, each construct was transformed into the original synthetic lethal strain (*ade2 ade3 ctf7-203 cak1-277* harboring *CEN URA3 ADE3 CTF7* plasmid). Independent isolates of each were then plated to rich medium at 23° to allow for plasmid loss. As expected, *cak1 ctf7* double-mutant cells transformed with *CEN TRP1* vector alone were unable to lose the reporter plasmid and gave rise only to solid red colonies. In contrast, cells transformed with *CEN TRP1 CTF7* gave rise to highly sectored red and white colonies (Figure 6). Each of the *ctf7* mutant constructs tested (S99A, the quad allele, and all five conserved serine/threonine point mutation constructs) provided for robust cell viability despite the presence of *cak1-277* as the sole source of Cak1p function (Figure 6 and supplemental Figure S4). We included in our analyses a *CEN TRP ctf7-203* plasmid further engineered to contain all four quad mutations. Despite the additional copy of the *ctf7-203*^{quad} allele, these cells required the *CEN CTF7 URA3 ADE3* reporter plasmid for viability (Figure 6).

Testing *cak1* and *cdc28* mutant cells for cohesion defects: Synthetic lethality between *ctf7* and both *cak1* and *cdc28* raised the possibility that cells harboring mutations in either *CAK1* or *CDC28* might exhibit sister chromatid-pairing defects. To test the first of these possibilities, *cak1-277* mutant cells were crossed into a telomere cohesion assay strain (Antoniacci and Skibbens 2006) in which *LacO* arrays are integrated proximal to the telomere of chromosome IV and detected via GFP-tagged *LacI-GFP* expression. We inactivated cak1-277p starting both before and after START by first synchronizing log phase *wild-type* and *cak1-277* mutant strains in G_1 (α-factor) or early S phase (hydroxyurea) in 23° media. All cultures were shifted to 37° during the final 30–45 min of synchronization. The resulting cultures were washed and placed in 37° fresh media supplemented with nocodazole to arrest cells prior to anaphase

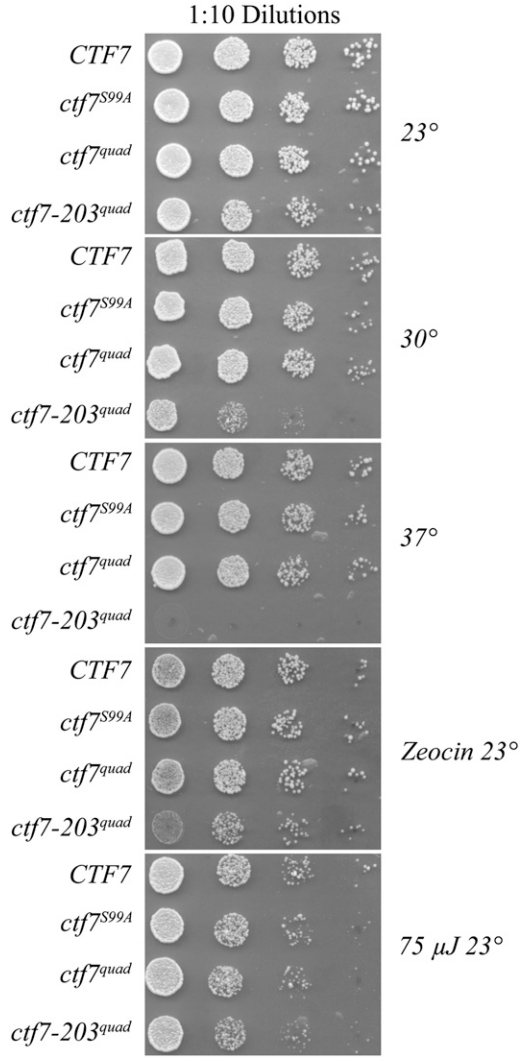

Figure 5.—Cells harboring mutations in candidate Ctf7p phosphorylation residues as the sole source of Ctf7p function exhibit robust growth not only at 23° but also despite exposure to increased temperatures (30° and 37°) and to genotoxic agents (zeocin and 75 μJ of ultraviolet irradiation). Tenfold serial dilutions of each strain were plated onto rich medium and treated as indicated. See text for details.

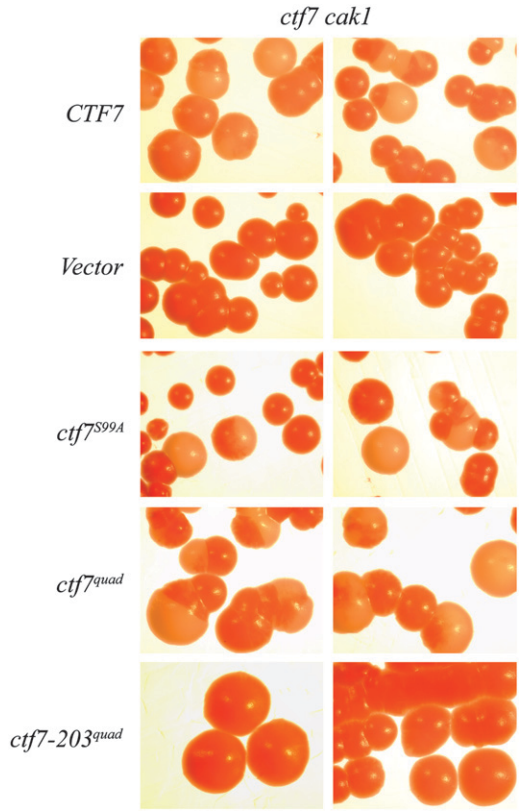

FIGURE 6.—*CTF7* mutated at all candidate CDK phosphorylation residues are competent to support cell viability in the presence of *cak1* mutations. See text for details.

onset. Parallel cell samples were then assessed for DNA content, cell morphology, Pds1p content (an inhibitor of anaphase onset) (COHEN-FIX *et al.* 1996), and disposition of sister chromatid loci via GFP. Both wild-type and *cak1* mutant cells exposed to nocodazole arrested as large budded cells with Pds1p coincident with DAPI staining (supplemental Figure 5). Results from numerous assays reveal that preanaphase *cak1* mutant cells retained a single GFP focus at levels indistinguishable from wild-type cells, regardless of whether the mutant cells were released from G_1 or early S phase synchronization (supplemental Figure 5).

Our telomere-based cohesion assay cannot rule out a model in which *cak1* mutant cells contain sister chromatids that are separated except at their telomeres. To address this issue, we repeated our assay but this time placed the *cak1-277* allele into a strain in which the *TetO* arrays are integrated within 40 kb of the centromere of chromosome V and visualized by TetR-GFP expression. As before, log phase wild-type and *cak1* mutant cells were synchronized in G_1 using α-factor at the permissive temperature, then washed and released into fresh medium supplemented with nocodazole, and maintained at the restrictive temperature. Three independent *cak1* isolates were tested for sister chromatid pairing defects in cells that retained Pds1p staining (supplemental Figure 5). Each of the *cak1* isolates exhibited precocious sister chromatid dissociation levels identical to that of wild-type cells.

One limitation of the above analyses is that the assay procedure includes a cell cycle arrest that may allow for Cdc28p to accumulate and thus obscure a cohesion defect. To directly test for Cdc28p function in cohesion, we obtained cohesion assay diploid strains homozygous for either wild-type *CDC28*, or *cdc28-B119*, or *cdc28-B28* alleles but heterozygous for the GFP-tagged loci (KITAZONO *et al.* 2003). *cdc28-B119* and *cdc28-B28* cells are deficient in spindle checkpoint function but arrest prior to anaphase onset in response to DNA damage (KITAZONO *et al.* 2003 and this study). Log phase wild-type and both *cdc28* mutant strains were synchronized in early S phase using hydroxyurea and then released into rich medium at 37°. Twenty to 30 min postrelease, the media was supplemented with zeocin (100 μg/ml final concentration) to induce postreplicative double-strand breaks. In response to continued exposure to zeocin, wild-type cells arrested tightly in G_2/M phase with very low levels (4%) of sister chromatid separation, indicative of a preanaphase arrest (Figure 7). In contrast, both *cdc28* mutant strains exhibited precocious sister chromatid separation: the weaker of the two alleles (*cdc28-B119*) exhibited a small but significant level (9%) of separation whereas the stronger of the two alleles (*cdc28-B28*) exhibited a more robust cohesion defect (15%)—roughly four times the level observed in wild-type cells. This latter defect level is similar to that reported for many DNA replication cohesion mutant strains (KENNA and SKIBBENS 2003; WARREN *et al.* 2004; MOLDOVAN *et al.* 2006).

DISCUSSION

How cells ensure the pairing of only sister chromatids while precluding the catastrophic pairing of nonsister chromatids remains one of the least understood and controversial mechanisms in the field of chromosome segregation (SKIBBENS 2008). Regardless of these models, the data clearly show that Ctf7p sister chromatid pairing reactions are tightly regulated in unperturbed cell cycles and inducible in response to specific challenges such as DNA double-strand breaks (SKIBBENS *et al.* 1999; MILUTINOVICH *et al.* 2007; STROM *et al.* 2007; ÜNAL *et al.* 2007).

One plausible mechanism of regulating cohesion establishment is predicated on the identification of Ctf7p as a phosphoprotein and potential Cdc28p substrate *in vitro* (UBERSAX *et al.* 2003). Here, we uncovered a synthetic lethal interaction between *ctf7-203* and a novel *CAK1* allele from a nonbiased genetic screen. The essential function of Cak1p is to activate Cdc28p-CDK (CROSS and LEVINE 1998). On the basis of this, we tested and subsequently found that *ctf7* is synthetically lethal when combined with *cdc28* alleles. That the interaction of interest occurs between *CTF7* and *CDC28* (and not

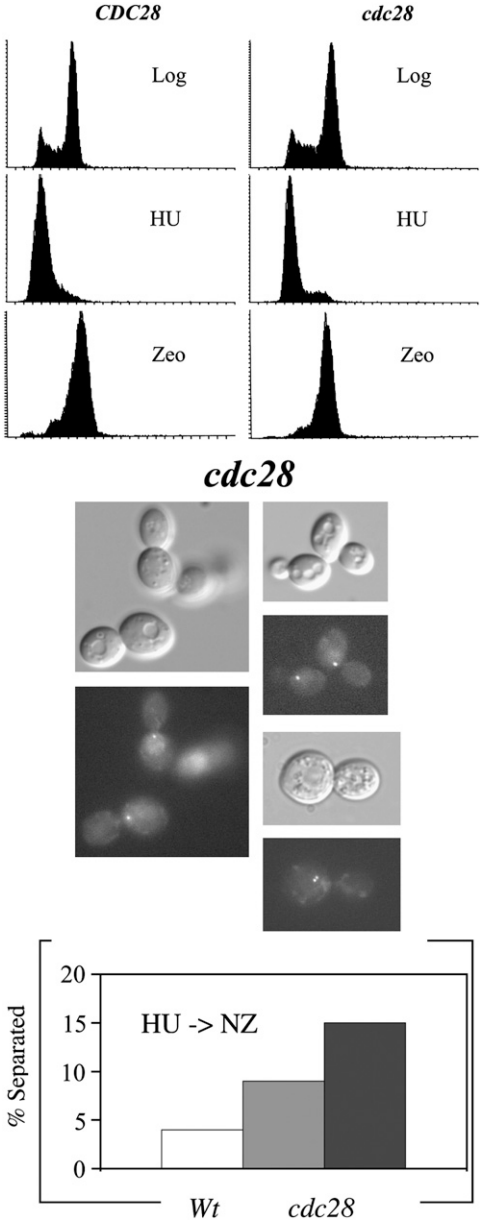

FIGURE 7.—*cdc28* mutant cells exhibit modest cohesion defects. (Top) Flow cytometer profiles reveal DNA contents of *wild-type* and *cdc28* cells during log growth (log), synchronized in early S phase (HU), and then released to the restrictive temperature in the presence of zeocin (Zeo) to obtain preanaphase cells. (Middle) Micrograph pairs of *cdc28* mutant cells show cell morphology above and sister chromatid loci immediately below. (Bottom) Quantification of cohesion defects in zeocin-treated wild-type cells and *cdc28* mutant cells. Analyses for wild type (open bars) and both *cdc28-B119* (shaded bar) and *cdc28-B28* (solid bar) are shown.

between *CTF7* and *CAK1*) is made clear by the findings that (1) *cak1-277* conditional lethality is completely rescued by *CDC28-CAK1*-bypass hypermorphs, (2) both *CDC28* hypermorphs and increased dosage of wild-type *CDC28* partially rescue *cak1 ctf7* synthetic lethality, and (3) *cdc28* mutant cells exhibit cohesion defects. In contrast, no cohesion defect was observed in *cak1* mutant cells. These results rule out an essential cohesion role for Cak1p. However, we cannot exclude a model in which Cak1p supplies some indirect role in cohesion, especially given the observation that *CAK1* suppresses *ctf7 cak1* lethality more efficiently than *CDC28*-Cak1p-bypass alleles (Figure 2).

A priori, these interactions raise numerous possibilities: (1) Cdc28p directly regulates Ctf7p, (2) Ctf7p and Cak1p coregulate Cdc28p, and (3) Cdc28p promotes cohesion in parallel to but distinct from Ctf7p reactions. The results presented here fail to support the first two of these models. First, *CDC28*-bypass alleles rescues *cak1-277* cell temperature sensitivity but do not bypass Ctf7p nor suppress *ctf7* cell phenotypes. Second, cells that express $ctf7^{phos-}$ mutant constructs exhibit robust growth at all temperatures tested and despite genotoxic challenges. Third, cells expressing either $ctf7^{phos-}$ or $ctf7^{conserved\ S/T-}$ mutant constructs failed to recapitulate the synthetic lethal interaction when combined with *cak1*. We also note that *ctf7-203*, the allele that genetically interacts with both *cak1* and *cdc28*, is wild type at all candidate phosphorylation residues. There is also a paucity of evidence to support a model that Ctf7p affects Cdc28p-dependent cell cycle regulation. *ctf7* phenotypes minimally overlap with the numerous and pleiotropic effects observed in either *cak1* or *cdc28* mutant cells (KALDIS *et al.* 1996; THURET *et al.* 1996; CHUN and GOEBL 1997; SUTTON and FREIMAN 1997; SKIBBENS *et al.* 1999; TOTH *et al.* 1999).

A more likely scenario is that Cdc28p affects sister chromatid pairing independent of Ctf7p. While speculative, this model is not without basis. Of the ~40 CDK *in vitro* substrates identified by Morgan and colleagues (UBERSAX *et al.* 2003), several promote efficient sister chromatid pairing or regulate cohesion dynamics. In addition to Ctf7p, these include ORCs, Kar3p, Slk19, Cdc5p, and Pds1p (CIOSK *et al.* 1998; MAYER *et al.* 2004; YU and KOSHLAND 2005; ZHANG *et al.* 2006; SHIMADA and GASSER 2007). How might diminished CDK function alter cohesion to the point of lethality when combined with mutations in *CTF7*? Using one of the CDK substrates listed above as an example, it is well established that Cdc28p phosphorylates origin of recognition complex (ORC) components and Cdc6p (DUNCKER *et al.* 1999; SANCHEZ *et al.* 1999; NGUYEN *et al.* 2001; WEINREICH *et al.* 2001; COOK *et al.* 2002; UBERSAX *et al.* 2003). There is now strong evidence that ORCs participate in cohesin-independent sister chromatid cohesion (DILLIN and RINE 1998; SUTER *et al.* 2004; SHIMADA and GASSER 2007). That ORCs act in parallel to but separate from Ctf7p/cohesin is supported by findings that both *orc2 smc1* and *orc2 ctf7* double-mutant cells produce additive cohesion defects, compared to single-mutant cells (SHIMADA and GASSER 2007). Additive cohesion defects are consistent with the notion that separate sister chromatid pairing reactions function in a loci-specific manner (SULLIVAN *et al.* 2004; CHANG *et al.* 2005;

Antoniacci and Skibbens 2006; Shimada and Gasser 2007). In combination, these results indicate that ORC's cohesion function is likely to be regulated by Cdc28p and act in parallel to Ctf7p/cohesin-based activities. These studies provide for a unique starting point from which to study CDK-dependent modes of cohesion. Our study further suggests that the field must pursue alternate models regarding Ctf7p regulation.

At present it remains unclear why *cak1* mutant cells exhibit negligible cohesion defects. One possibility is that the cohesion assay includes a cell cycle delay that allows for an accumulation of CDK. If true, this suggests that noncohesin-based mechanisms of cohesion maintenance (possibly via ORCs) require active CDK through G_2/M. We further note that *cak1-277* is not synthetically lethal when combined with a cohesin mutant: sporulation of a heterozygous *cak1-277 mcd1-1* diploid strain generated the expected frequency of viable double-mutant haploid cells (A. Brands and R. V. Skibbens, unpublished data).

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