

Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery

Robert V. Skibbens,^{1,3,5} Laura B. Corson,² Doug Koshland,³ and Philip Hieter^{1,4}

¹Department of Molecular Biology and Genetics, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205 USA;

²Predoctoral Training Program in Human Genetics, Johns Hopkins University, Baltimore, Maryland 21210 USA; ³Carnegie Institution of Washington, Baltimore, Maryland 21210 USA

CTF7 (chromosome transmission fidelity) gene in budding yeast encodes an essential protein that is required for high-fidelity chromosome transmission and contains regions of identity conserved from yeast to man. *ctf7* mutant cells arrested prior to anaphase onset contain separated sister chromatids. Thus, Ctf7p is essential for cohesion. Cohesion is established during S phase and then maintained until mitosis. However, Ctf7p activity is required only during S phase, suggesting that Ctf7p functions in the establishment of cohesion. In addition, *ctf7* genetically interacts with DNA metabolism mutations *pol30* (PCNA) and *ctf18* (an RF-C like protein) and *ctf7* temperature sensitivity and chromosome loss are rescued by high levels of *POL30*. These findings provide the first evidence that links the establishment of sister chromatid cohesion to the DNA replication machinery and suggest that the assembly of cohesion (and possibly condensation) complexes are coupled to PCNA-dependent DNA replication. The analysis of Ctf7p also reveals an important connection between sister chromatid cohesion, spindle integrity and the spindle assembly checkpoint.

[Key Words: *CTF7*; chromosome loss; *BUB3*; *MAD2*; *POL30*; chromatid cohesion; condensation]

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Cohesion between replicated sister chromatids is fundamental to cell viability. Cohesion tethers replicated sister chromatids together along their entire lengths from S phase until anaphase. Chromatid cohesion also sterically constrains sister kinetochores so that they face away from each other, promoting proper chromosome attachment to microtubules nucleated from opposing spindle poles (Rieder and Salmon 1994; Hyman and Sorger 1995). On attachment of kinetochores to spindle microtubules, each sister chromatid experiences microtubule-dependent poleward forces. These poleward forces are resisted by cohesion so that sister chromatids do not separate but instead come under tension. Observations made in meiotic cells have led to the proposal that tension is monitored by the cell to ensure that each sister chromatid is properly associated with microtubules. Only after all of the chromosomes are placed under tension does the cell signal for the onset of anaphase (Nicklas 1997). Thus, chromatid cohesion may also be a critical link in the mechanism that regulates anaphase onset. At anaphase,

cohesion complexes are inactivated and the sister chromatids segregate from one another and move toward their spindle poles.

Despite the essential role of chromatid cohesion in mitosis and cell division, only recently has progress been made in elucidating the molecular basis of this form of chromosome metabolism. In budding yeast, genetic and biochemical analyses have identified a complex that contains two regulators of sister chromatid cohesion, Pds1p and Esp1p (Cohen-Fix et al. 1996; Yamamoto et al. 1996 a,b; Ciosk et al. 1998). Pds1p is an inhibitor of anaphase onset and is degraded at the metaphase-to-anaphase transition by a ubiquitin-dependent process involving the anaphase promoting complex (APC). Pds1p proteolysis is thought to free Esp1p, which in turn promotes the inactivation of chromatid cohesion. The Pds1p–Esp1p complex is functionally related to the fission yeast Cut1p–Cut2p complex and potential mammalian homologs of Esp1p have been identified (Funabiki et al. 1996). In addition to these regulatory proteins, several candidate cohesion proteins have been identified: Mcd1p/Scc1p, Scc2p, Smc1p, and Smc3p from budding yeast (Guacci et al. 1997; Michaelis et al. 1997) and Mis4p from fission yeast (Furuya et al. 1998). Smc1p and Smc3p are members of the recently discovered gene fam-

⁴Present address: Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, British Columbia V5Z 4H4, Canada

⁵Corresponding author.

E-MAIL skibbens@mail1.ciwemb.edu; FAX (410) 243-6311

ily required for the structural maintenance of chromosomes (SMC) and are related by protein motifs first identified in Smc1p (Strunnikov et al. 1993). Inactivation of Mcd1p, Scc2p, Smc1p, Smc3p, or Mis4p causes precocious sister separation in M phase. Consistent with Mcd1p's role in the maintenance of cohesion, Mcd1p associates with chromosomes from S phase through M phase. Mcd1p both exists in a complex with Smc1p and requires Smc1p to associate with chromatin (Guacci et al. 1997; Michaelis et al. 1997). *Xenopus* homologs of Mcd1p, Smc1p, and Smc3p, along with two other proteins, form a cohesin complex that is required in interphase to maintain cohesion in the subsequent mitosis (Losada et al. 1998). These similarities between yeast and vertebrate proteins suggest that the mechanisms of chromatid cohesion is likely to be conserved in all eukaryotes.

In budding yeast, the cohesion factor Mcd1p is also required for chromosome condensation (Guacci et al. 1997), revealing an important, but poorly understood, link between cohesion and condensation. Chromosome condensation ensures both disentanglement of sisters and compaction of the chromosome arms away from the cytokinetic furrow (Koshland and Strunnikov 1996). Interestingly, two other SMC family members, Smc2p and Smc4p, are required for condensation and appear conserved across evolution (Chuang et al. 1994; Hirano and Mitchison 1994; Saitoh et al. 1994; Saka et al. 1994; Strunnikov et al. 1995; Jessberger et al. 1996; Sutani and Yanagida 1997). In *Xenopus*, Smc2p (XCAP-E) and Smc4p (XCAP-C) homologs combine with three additional proteins to form a condensin complex that associates with chromosomes (Hirano et al. 1997). Biochemical analysis of *Xenopus* extracts have revealed that cohesin and condensin complexes function independent of one another, indicating that the processes of cohesion and condensation are uncoupled in this cell system (Losada et al. 1998).

Although recent advances have been made in the identification of proteins required for cohesion and condensation, little is known about how cohesion proteins are assembled onto chromatin and, once assembled, how those complexes are regulated. For instance, temporal analysis of cohesion in human cells and genetic analysis of cohesion in yeast have shown that cohesion is established during S phase (Selig et al. 1992; Guacci et al. 1997; Michaelis et al. 1997; Furuya et al. 1998; Uhlmann and Nasmyth 1998). Yet, specialized factors that assemble cohesion complexes or load those complexes onto chromatin remain to be discovered. Another fundamental issue pertains to the regulation of chromatid cohesion. In early mitotic vertebrate cells, sister chromatids are paired along their entire length. In metaphase-arrested cells, however, the chromosome arms are no longer paired, whereas the centromeres can remain paired for an extended period of time (Sumner 1991). A similar spatial and temporal regulation is observed in meiotic cells: Cohesion is lost from the chromosome arms during meiosis I to resolve recombinant intermediates, but cohesion is retained at the centromeres until meiosis II. In budding yeast, *mcd1* mutant cells are de-

fective in cohesion at both the centromere and arms (Guacci et al. 1997; Michaelis et al. 1997). These results suggest that although a common cohesion mechanism exists for the entire chromosome, cohesion along the chromosome can be differentially regulated. Finally, Losada et al. (1998) recently reported that the Mcd1-Smc1-Smc3 protein complex in *Xenopus* dissociates from chromatin early in mitosis, suggesting that the process of cohesion may be more mechanistically complicated than anticipated and that other factors involved in cohesion have yet to be identified.

Previously, a collection of 136 chromosome transmission fidelity (*ctf*) mutants was identified in *Saccharomyces cerevisiae* on the basis of the increased rate of loss of a nonessential chromosome fragment (Spencer et al. 1990). *ctf* mutants that allowed both transcription to proceed through a centromere and the stable propagation of a dicentric chromosome (in vivo assays for kinetochore integrity) proved to contain mutations in genes subsequently shown to encode kinetochore components that bind the yeast centromere (Lechner and Carbon 1991; Doheny et al. 1993; Goh and Kilmartin 1993; Jiang et al. 1993). However, the vast majority of *ctf* mutants do not appear to correspond to integral kinetochore components and therefore constitute a powerful resource to identify proteins involved in novel processes crucial for proper chromosome transmission. In this paper, we report that *CTF7* encodes an essential nuclear protein that is required for cohesion. The analysis of *CTF7* also establishes a novel connection between cohesion and DNA replication.

Results

Ctf7p is an essential nuclear protein and contains domains that are highly conserved

In this study, we characterized *ctf7* mutants, one complementation group of the *ctf* collection that exhibit elevated loss of a nonessential chromosome fragment (Spencer et al. 1990). The *CTF7* gene was cloned on the basis of complementation of the chromosome loss phenotype and confirmed by integrative mapping (Materials and Methods). *CTF7* was shown to encode an essential gene by both tetrad analyses of a heterozygous *CTF7/ctf7Δ* strain and plasmid shuffle methods (see Materials and Methods). *CTF7* is identical to the unannotated ORF YFR027W on chromosome VI (Murakami 1995) and encodes a 281 amino acid protein with a predicted molecular mass of 31.8 kD (Fig. 1A). A search of the expressed sequence tag database (dBEST) revealed that portions of Ctf7p contained strong homologies to several human and mouse EST sequences (Fig. 1B). Similar homology was found for an ORF of unknown function in fission yeast. These results show that Ctf7p, an essential yeast protein, contains a motif that is conserved across kingdoms and suggests that Ctf7p-related proteins may play a role important for chromosome transmission in all eukaryotic cells. Overexpressed HA-tagged Ctf7p localized to the nucleus (HA-tagged Ctf7p under the control of the

A

GAT AAA ATG AGA TTA TTT TTT TCT TTT ATG TAG AAA CAT CGA TTC GCT TCT GTG GGT TCC
 CAT ATC CTC GAG GAG AAC TTC TAA TAT ATT CTG TAT AAT TAA TAT IAT AGC ATT AAC AAC
 GGT ATA ATC TCA GTC ATT ATC CCC AAA TTC ACC TAT TTT CCC ATC ADA CCG ACC TTT GCG
 AAT ACT TGA GCA TGF AAA TCA GAG ATT CTA TTT ACC AGC TGC CCA GAA ACC AGA AAA CTT
 CTC TTA ACC ACT TCC TCT APT GPT GGC ATG TTG AAA CCT CTT GCC GGC TTA ATC ATC CTT CGA
 CTT AGG GAA GAA TCT TAA CAA ATG AAA TTT AAC GTC ACC GCG TTA TAG AAA AAA AGC CTT
 TCG CPT ACT GTA ATC AGT AAT AAT ACA TAA GAA GAT GCG AGC TAC AAC GGA CAT CAA CAT
 ATA TCT ATG TTT ACA TAT TAG GGT TCA ACA GAA TAT AAA TCG TTG CAC AAA ATG AAA GCT
 M K A
 AGG AAA TCG CAG AGA AAA GCG GGC AGT AAA CCA AAT CTT ATC CAG TCT AAA TTG CAA GPT
 R K S Q R K A G S K P N L I Q S K L Q V
 AAT AAT GGT TCG AAA TCG AAT AAA ATA GTC AAG TGT GAT AAA TGT GAG ATG TCA TAT TCC
 N N G S K S H K A I V K C D A A C E M S Y S
 TCG ACA TCA ATA GAA GAT GGC GCC ATC CAC GAG AAA TAC CAC ACT TTA CAG CTC CAT GGA
 S T S I E D R A I H E K Y H C T L Q L H G
 CGT AAA TGG TCG CCG AAT TGG GGT TCT ATOA GTA TAC ACA GAG CGA AAC CAT TCA AGG ACG
 R K W S P N G W G S I V Y T E R N H S R T
 GTG CAT CTA TCA AGA TCG ACA GGG ACA ADA ACG CCA TTG AAC TCC TCA CCT TTG AAA AAA
 V H L S R S T G T I T P L N S S P L K K
 APT AGT CCG TCT APT ACC CAT CAG GAG GAG AAG APT GTA TAT GTG AGA CCA GAT AAG TCG
 S S P S I T H Q E E K I V Y V R P D K S
 AAT GGT GAA GTC GCC ATG ACG GAG ATA ATG ACA CTA CTS AAT AAC GAG CTS AAT GCG
 N G E V R A H T E I M T L V N N E L N A
 CCA CAC GAT GAG AAT GTC ATT TGG AAC AGT ACC ACA GAA AAA GGC AAA GCS TTT GTA
 P H D E N V I W N S T T E R K G K A P V
 TAC ATA AGA AAT GAC AGG GGC GTC GGA ATA ATT ATA CAG AAC CTT TAT GCG GGC AAT
 Y I R N D R A V G I I I I E N L Y G G N
 GGT AAA ACA TCT AGT CGT GGA CGT TGG ATG GTT TAT GAT TCT AGA AGA TTG GTA CAG AAT
 G K T S S R G R W M V Y D S R R L V Q N
 GTG TAC CCC GAT TTT AAG ATT GGC ATA TCG AGA AAT TGG GTG TCC AGG ACA GCA AGG AAG
 V Y P D P F T K I G I S R I W V C R T A R K
 TTG GGT ATC CCA ACC AAA TTG AIT GAC GTT GCA AGA GAA AAT ATT GTT TAC GPT GAA GTT
 L G I A T K L I D V A R E N I V Y G E V
 ATT CTT AGG TAC CAG GTA CCA TWS TCG CAA CCC ACA GAC AGC GPT GGA AAA CTS GCT AGC
 I F K Y Q V A G S Q P T D S G G K L A S
 AAA TAC AAC GGC APT ATT GAT AAA TCA GGC AAG TTA CTA TTG CCG GTA TAC ATA TGA CTC
 K Y N H R S G K L L P V Y I *
 TTT CGA AAA GAC AGC GAG AAG GGA CAC TGG AAA AAT GGC ATA AAA CTT TTC AAG GTT ATT
 APT AGT AAG TTT TAT TAT AGT ACG ATA TAT AAT TTA AAA ATG AAA TAA APT APT TCT
 TGA TGG AGC CAC TTA TTT TTC TTA TAC CAC CCG CGC GCG TTC TAG TCC TTC TCC CTG ACG
 TTA CTC TCC TAT TCT TGG GCA ATA GTT CTC TTA GTA TCG TAT CAT CTS CGG CAT TAT TCA

B

Ctf7p 29 SNKIVKCDKCEMSYSSTSIEDRAIHEKYHPLQLHG 63
 +N V C +C M Y-SIS ED +H +++ L G
 S. pombe 649 ANSTVTCSECSMEYNSSTSEEDILLHSRFRSVLGG 683
 Ctf7p 211 GISRIWVCTARLGIATKLIDVARENIVVEVPIRYQVANSOFTDSGKLSKYNG 267
 GISRIWV + RK GIA+ L+D A + +YG VI +VA-SQP++SG
 S. pombe 799 GISRIWVSASRRKQGIASLLLDNALKFFIYGVISPAEVAFSPQSESG 846
 GISRIWV R+ IA+++I+ R N +YG + + ++A+S PT G A+++ G
 M. musculus 281 GISRIWVFSMMRRKLIASRMIECLRSNFIVGSLYSKEIAFSDPTFDGKLFATQYCG 337
 GISRIWV R R+ IA +L+D R ++G + ++A+S PT G A+KY
 H. sapiens 116 GISRIWVFLRRKRRLIARLVDLNRNCFMFCFLSTDEIAFSDPTFDGKLFATKY 170

Figure 1. (A) Nucleotide and amino acid sequences of *CTF7*. The *MluI* cell cycle box in the 5' untranslated region, which directs the expression of many genes required during S phase, is boxed (Johnston and Lowndes 1992; Toyn et al. 1995). (B) *Ctf7p* homology to fission yeast, mouse and human ESTs are shown. Identical residues are indicated by placement of the amino acid above the sequence, conserved residues are indicated with a plus. The carboxy-terminal region of *Ctf7p* (211–267 amino acids) is 49%, 40%, and 41% identical and 70%, 63%, and 60% similar to fission yeast, mouse, and human ESTs, respectively.

endogenous *CTF7* promoter was undetectable; Materials and Methods), consistent with *Ctf7p*'s essential role in chromosome transmission.

ctf7 mutants delay in mitosis with a 2C DNA content, separated DNA masses, and partially elongated spindles

To investigate the function of *Ctf7p* in vivo, three temperature-sensitive alleles of *CTF7* were obtained by a PCR-based mutagenesis strategy and integrated into the genome (see Materials and Methods). At the permissive temperature, all three temperature-sensitive alleles, *ctf7-201*, *ctf7-202*, and *ctf7-203*, exhibited increased chromosome fragment loss (Fig. 2A), recapitulating the original *Ctf* phenotype. At the restrictive temperature, *ctf7* mutant strains rapidly lost viability compared with wild type, indicating that all three alleles caused an irreversible defect (Fig. 2B).

To ascertain the terminal phenotype of the *ctf7* mutant strains, log phase cultures of each mutant were shifted from 25°C to 37°C and processed to assess DNA content and cell morphology. At 25°C, both wild-type and mutant strains exhibited similar DNA contents, as expected for cycling cells. However, after 3 hr growth at 37°C, a significant increase in the number of cells with a 2C DNA content was observed in *ctf7* mutant strains compared with *CTF7* control cells (Fig. 2C). Analyses of cell morphologies from this time point showed that ~51% of *ctf7* mutant cells were large budded and of these, 63% contained separating or bi-lobed DNA masses (Fig. 2D; Table 1A). The bilobed DNA masses often remained entirely within the mother cell and occasionally contained unequal amounts of DNA. Spindles

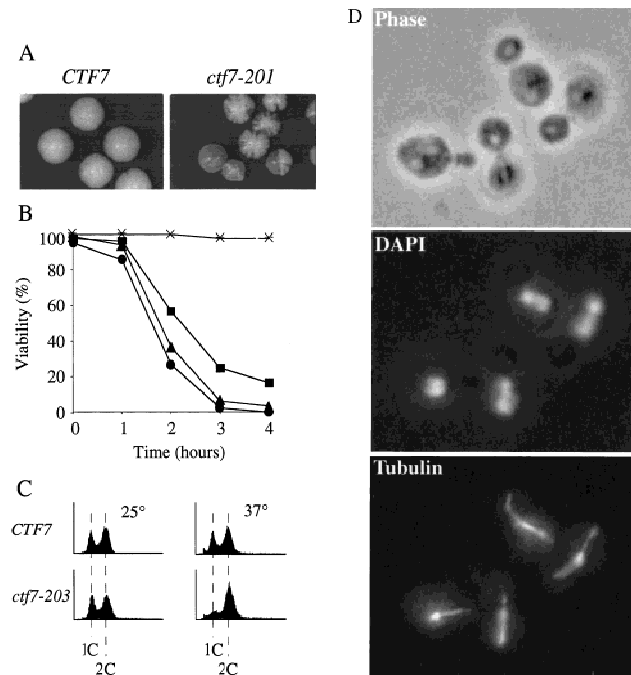



Figure 2. Phenotypes of *ctf7* conditional mutants. (A) *ctf7* cells (*ctf7-201* shown) exhibit elevated chromosome loss over wild type. Faithful maintenance of a reporter chromosome fragment yields white colonies. Loss of the reporter chromosome results in red sector colonies. (B) *ctf7* mutants are temperature-sensitive lethal. Log-phase cultures were shifted from 25°C to 37°C and assayed for cell viability. (●) *ctf7-201*; (■) *ctf7-202*; (▲) *ctf7-203*; (*) wild type. (C) *ctf7* mutants delay in mitosis. The DNA content of log phase cultures grown at 25°C or 37°C for 3 hr were assessed by flow cytometry (*ctf7-203* shown). (D) Micrographs of *ctf7* mutant cell (Phase), DNA (DAPI), and microtubule (Tubulin) morphologies after 3 hr growth at 37°C.

Table 1. *ctf7* morphologies at 37°C


	1	2	3	4	5	6	7
A							
CTF7	63.2	13.5	6.2	11.9	3.1	0	2.1
<i>ctf7-203</i>	25.1	9.1	9.7	8.6	31.9	5.7	9.9
B							
CTF7	69.4	12.2	5.2	6.1	4.5	0	2.6
<i>ctf7-203</i>	19.1	15.3	7.4	0	56.3	1.9	0
<i>ctf7-203</i> <i>mad2-1</i>	59.2	8.2	3.1	9.3	15	3.1	2.1

(A) *ctf7* mutant cells accumulate as large budded cells containing a bilobed/elongated nucleus. Cell and nuclear morphologies of log-phase *CTF7* and *ctf7-203* strains were assayed after 150 min growth at 37°C. (B) The bilobed nuclear morphology of *ctf7* mutants depends on the *MAD2* checkpoint. Log-phase cultures of *CTF7*, *ctf7-203*, and *ctf7-203/mad2-1* were synchronized in G₁ used α -factor, washed, and grown at 37°C for 3 hr before analysis.

from these cells were also atypical in that they were entirely contained within the mother cell (Fig. 2D) and appeared longer than preanaphase spindles. To quantify this last observation, log phase cultures of *CTF7*, *ctf7-203*, and *ctf13-30* (a kinetochore mutant that arrests prior to anaphase onset) were shifted to 35°C for 3 hr, fixed, and immunostained for tubulin to visualize the spindle. Measurements of spindle lengths revealed that *ctf7* mutant cells with bilobed DNA masses contained partially elongated spindles of 3.7 μ m (± 0.4 , $n = 7$) in length; ~ 2.5 times longer than the 1.4 μ m (± 0.3 , $n = 8$) preanaphase spindles in *ctf13-30* mutants but roughly half as long as the 6.9 μ m (± 0.9 , $n = 11$) elongated spindles in anaphase *CTF7* wild-type cells. The increased chromosome loss, large-budded cell morphology, 2C DNA content, abnormal spindle morphology, and unequal chromatid distribution observed in *ctf7* mutation strains indicate that loss of Ctf7p function leads to a defect in mitosis and a mitotic delay.

The mitotic delay in *ctf7* cells requires the kinetochore/spindle assembly checkpoint

Mitotic delays typically arise by activation of either the DNA damage or kinetochore/spindle assembly checkpoint pathways. To determine whether *ctf7* cells required either of these checkpoint mechanisms to delay in mitosis, we constructed strains containing *ctf7* in combination with either mutations in the DNA damage checkpoint pathway (*rad9 Δ* and *mec1-1*) (Weinert and Hartwell 1988, 1990; Weinert et al. 1994) or mutations in the kinetochore/spindle assembly checkpoint pathway (*mad2-1* and *bub3 Δ*) (Hoyt et al. 1991; Li and Murray 1991). Log phase cultures of the resulting strains were shifted to 37°C for 3 hr and their DNA content measured by flow cytometry. Neither *rad9 Δ* nor *mec1-1* mutations abrogated the *ctf7-203* mediated 2C DNA accumulation (Fig. 3, data not shown). In contrast, both *ctf7-203*

mad2-1 and *ctf7-203 bub3 Δ* strains exhibited a complete loss of the 2C DNA accumulation at the restrictive temperature (Fig. 3; data not shown). Consistent with this result, the *ctf7-203 mad2-1* double mutant strain also failed to accumulate partially elongated spindles or bilobed DNA masses, but instead proceeded through mitosis as evidenced by the reappearance of G₁-like (unbudded) cells (Table 1B). These results show that the mitotic delay in *ctf7* cells requires a functional kinetochore/spindle assembly checkpoint but not a functional DNA damage checkpoint.

ctf7 cells delay in metaphase prior to anaphase onset

Typically, an activated kinetochore/spindle assembly checkpoint causes cells to arrest prior to anaphase onset and those cells contain short spindles and a single DNA mass. However, the *MAD/BUB*-dependent mitotic delay in *ctf7* mutant cells lead to an accumulation of cells that contained partially elongated spindles and separated DNA masses. This unusual phenotype could represent an aberrant metaphase. An alternative possibility was that *ctf7* cells were delayed in midanaphase, suggesting a novel postmetaphase role for Mad/Bub checkpoint proteins. To determine whether the mitotic delay occurred prior to anaphase onset, we assessed the levels of Pds1p in wild-type and *ctf7* mutant cells. Pds1p is an inhibitor of anaphase onset and is degraded at the metaphase-to-anaphase transition (Cohen-Fix et al. 1996; Yamamoto et al. 1996a,b).

Wild-type and *ctf7* mutant cells were transformed with a plasmid directing the expression of HA-Pds1p (Cohen-Fix et al. 1996). The level of HA-Pds1p was analyzed after synchronized G₁ cells were released into rich medium and grown for 3 hr at 37°C. Western blot analysis revealed that wild-type cells had low levels of HA-Pds1p because most cells had already completed mitosis

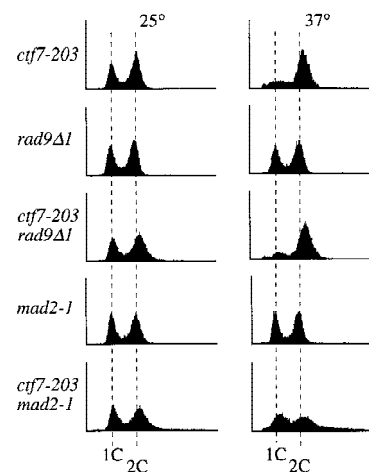


Figure 3. The mitotic delay in *ctf7* cells requires the kinetochore/spindle checkpoint pathway. Flow cytometry analysis of DNA content of log phase *ctf7*, checkpoint mutant, and double-mutant strains grown for 3 hr at 25°C (left) or 37°C (right).

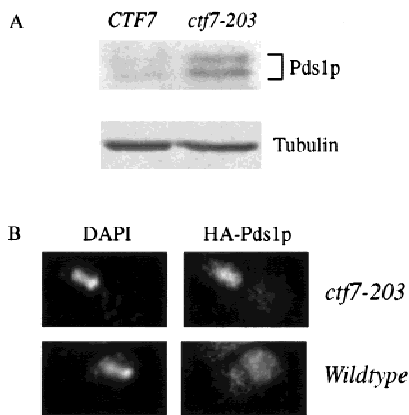


Figure 4. *ctf7* cells are delayed prior to anaphase onset. (A) The Western blot shows HA-Pds1p levels in wild-type and *ctf7* cells that were synchronized in G₁ and then released into medium and grown at 37°C for 3 hr. Tubulin levels verified that equal protein loads were present in each lane. (B) Micrographs show the localization of DNA (DAPI) and HA-Pds1p (HA-Pds1p) in *ctf7* mutant and wild-type cells treated as in A.

(Fig. 4A). In contrast, *ctf7* cells contained high HA-Pds1p levels, indicating that *ctf7* cells were delayed prior to anaphase onset. To confirm that *ctf7* cells were delayed in metaphase, HA-Pds1p was visualized by immunofluorescence in wild-type and *ctf7* cells treated as above. As expected, wild-type cells with separating DNA masses did not contain HA-Pds1p (Cohen-Fix et al. 1996). In contrast to wild-type cells, a significant population of *ctf7* cells with separating DNA masses contained high levels of HA-Pds1p (Fig. 4B). Thus, these *ctf7* cells were delayed prior to anaphase onset. It was not surprising that some *ctf7* cells with separating DNA masses did not contain detectable HA-Pds1p levels, because *ctf7* cells are only delayed in the cell cycle and eventually exit mitosis. These results reveal that partial spindle elongation and DNA separation occurs prior to anaphase onset in *ctf7* mutant cells.

Ctf7p is required for sister chromatid cohesion

How can cells delayed prior to anaphase onset have separated DNA masses? One possibility was that defects in sister chromosome cohesion allowed sister chromatids to separate prematurely, resulting in the movement of disjointed sisters toward the spindle poles. To test this model, wild-type and *ctf7-203* cells were synchronized in G₁ with α -factor, released into medium that contained nocodazole, and grown for 3 hr at 37°C. Nocodazole blocks both microtubule assembly and anaphase onset such that sister chromatids typically remain closely apposed for extended periods of time. FISH has been used previously to demonstrate the pairing of sister chromatids in M-phase yeast cells and can further assess the pairing at different loci such as the centromere and chromosome arms (Guacci et al. 1994). Therefore, we used

FISH to ask if sister chromatid cohesion was normal in *ctf7* mutant cells. In wild-type cells arrested in mitosis, only ~10% of the DNA masses contained two FISH signals when using a *CEN16* proximal DNA probe (Fig. 5A,B). In contrast, ~40% of the DNA masses from *ctf7* cells contained two FISH signals, often with sister centromeres residing at opposite sides of the nuclear masses (Fig. 5A,B). The *ctf7* defect affected other chromosomes, ~40% of DNA masses in *ctf7* cells contained two FISH signals with a *CEN1* proximal probe (Fig. 5B). Finally, a similar loss of cohesion was obtained by use of centromere-distal (arm) probes to chromosome XVI, indicating that Ctf7p was required for cohesion along the entire length of the chromosome (Fig. 5B). These findings reveal that Ctf7p is essential for sister chromosome cohesion and supports previous findings that cohesion is mediated by a mechanism common throughout the chromosome (Guacci et al. 1997).

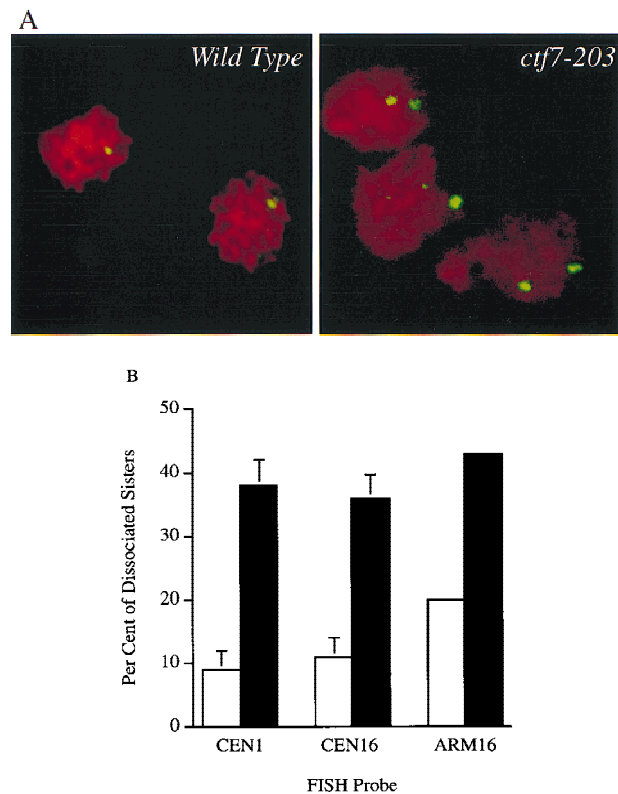


Figure 5. Ctf7p is required for sister chromatid cohesion. (A) Micrographs of wild-type and *ctf7* mutant cells grown for 3 hr at 37°C in medium containing nocodazole and processed for FISH. A single FISH signal represents paired sister chromatids, two FISH signals represents disjointed sister chromatids. (red) Propidium iodide staining DNA mass; (green) hybridization to *CEN16* probe. (B) Quantification of single (nondisjoined chromosomes) vs. double FISH signals (disjointed chromosomes) within propidium iodide staining DNA masses. Synchronized G₁ wild-type and *ctf7* cells contained an equal percentage of single FISH signals (data not shown), indicating that the increase in two FISH signals observed for *ctf7* mutant cells was not due to pre-existing aneuploid.

Ctf7p is required during S phase

Sister chromatid cohesion is apparently established during S phase and maintained until anaphase onset. To assess the role of Ctf7p in the establishment and maintenance of cohesion, we used reciprocal temperature-shift experiments in *ctf7* cells to map that portion of the cell cycle that requires Ctf7p function (Fig. 6). A shift-up experiment marks the earliest point in the cell cycle when Ctf7p can be inactivated without reducing cell viability (i.e., when Ctf7p function is no longer essential). *ctf7-203* cells were synchronized in G₁ with α -factor and then released into medium at 23°C to allow the cells to proceed through the cell cycle. Starting at the release from α -factor ($t = 0$), aliquots were shifted to 37°C at 20 min intervals. At the time of the shift, aliquots were also harvested for flow cytometry and immunofluorescence analyses to position cells within the cell cycle (see Materials and Methods). At the end of the experiment, all samples were plated at 23°C to assess viability [Materials and Methods]. Cultures exhibited low viability when shifted to 37°C any time during the first 60 min (after

α -factor release but prior to DNA replication) (Fig. 6A,B). Cultures exhibited high viability when shifted to 37°C after 80 min of growth at 23°C (Fig. 6A). The increase in viability correlated with progression of DNA replication: Maximum viability was realized only after the bulk of DNA replication was completed (80 min) (Fig. 6B). Furthermore, maximal cell viability preceded anaphase onset (spindle elongation) by >20 min (Fig. 6A). These results suggest that Ctf7p is required during S phase but not during mitosis.

A shift-down experiment marks the latest point in the cell cycle when Ctf7p must be active to produce viable cells. *ctf7-203* cultures were synchronized in G₁ with α -factor at 23°C, then released into 37°C rich medium in the absence of α -factor to allow cells to proceed through the cell cycle. Aliquots were then shifted down to 23°C at 20 min intervals and also harvested for flow cytometry and immunofluorescence analyses as described above. On release from α -factor ($t = 0$), cells retained high viability for 80 min of growth at 37°C prior to shifting down to 23°C (Fig. 6C). Decreased cell viability (starting at $t = 80$) correlated with entry into DNA replication (Fig. 6C,D)

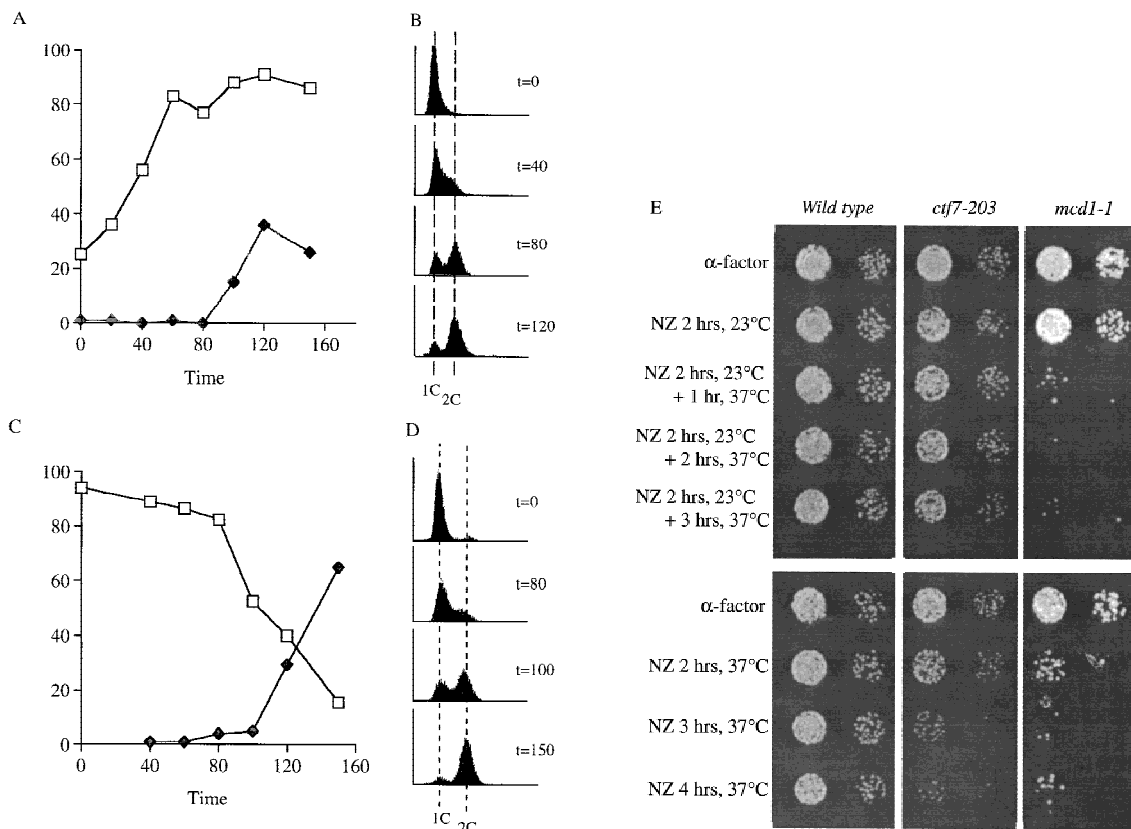


Figure 6. Ctf7p is required only during S phase. (A,B) Shift-up experiment: G₁ *ctf7-203* cells were released from α -factor and shifted from 23°C to 37°C at 20–30 min increments. (C,D) Shift-down experiment: G₁ *ctf7-203* cells were released from α -factor into 37°C medium and shifted down to 23°C at 20–30 min increments. Wild-type strains retain high viability throughout either procedures (data not shown). For each time point, an aliquot was assayed for spindle elongation/DNA mass separation (\blacklozenge), viability (\square), and DNA content (flow cytometry profile). (E, top) Viability of G₁ synchronized wild-type, *ctf7-203*, and *mcd1-1* cells released into medium containing nocodazole and permitted to arrest in mitosis at 23°C prior to shifting to 37°C. (E, bottom) Viability of G₁ synchronized wild-type, *ctf7-203*, and *mcd1-1* cells released into medium containing nocodazole at 37°C. A 10-fold dilution for each culture is shown.

and a further decline in cell viability coincided with progression through S phase. Thus, Ctf7p must be active as cells enter S phase to obtain viable cells. Activation later in the cell cycle resulted in cell death. A further finding from this experiment was that *ctf7* mutants progressed through S phase with kinetics similar to wild-type cells (data not shown). Thus, the lesion incurred during S phase in *ctf7* mutant cells is not manifested as a defect in DNA replication. The combination of results from the shift-up and shift-down experiments in *ctf7* cells suggest that Ctf7p function is essential during S phase when cohesion is established but not in mitosis when cohesion is maintained.

We decided to use a second method to test whether Ctf7p is required during M phase and S phase. *Mcd1p* appears to be required during both M phase and S phase and was included as an indicator for loss of cohesion (Guacci et al. 1997). To test whether Ctf7p was required during mitosis, wild-type, *ctf7-203*, and *mcd1-1* mutant cells synchronized in G₁ were released into medium containing nocodazole and allowed to proceed from G₁ into mitosis at the permissive temperature of 23°C. Aliquots of the cultures were then shifted to 37°C at 1 hr intervals. After incubation at 37°C, the cultures were plated and allowed to grow for 2 days at 23°C to assess viability (Fig. 6E). Wild-type cells were not adversely affected by this procedure and maintained high viability. As expected, *mcd1-1* cells exhibited a dramatic decrease in viability within the first hour of incubation at 37°C. In contrast, *ctf7-203* cells retained high viability even after 3 hr incubation at 37°C. Thus, Ctf7p is not required during mitosis when sister chromatid cohesion is maintained. However, Ctf7p is required during S phase when cohesion is established. *ctf7-203* and *mcd1-1* mutant cells both exhibited decreased viability when synchronized cells were allowed to proceed from G₁ into mitosis at 37°C. The combination of these results suggest that Ctf7p plays a role in cohesion distinct from *Mcd1p*.

The high viability of *ctf7-203* cells allowed to proceed into mitosis before shifting them to the restrictive temperature implied that Ctf7p was not required for the maintenance of cohesion. To demonstrate this unambiguously, we performed FISH analysis on wild-type and *ctf7-203* cells. As before, G₁ synchronized cells were released into medium containing nocodazole and allowed to proceed into mitosis at the permissive temperature. After 3 hr at 23°C, the cultures were shifted to 37°C and held at this restrictive temperature for 2 hr. Aliquots of the cultures were then processed for FISH analysis by use of both *CEN1* and *CEN16* proximal probes. Wild-type cells retained sister chromatid cohesion following this procedure, as only 22% (*CEN1*) and 23% (*CEN16*) of the DNA masses contained two FISH signals. *ctf7-203* cells also retained sister chromatid cohesion, 26% (*CEN1*) and 27% (*CEN16*) of the DNA masses contained two FISH signals (data not shown). The slight increase in the number of DNA masses that contained two FISH signals observed for both strains was most likely due to the prolonged mitotic arrest and exposure to nocodazole. Thus, by use of both criteria of viability and localization of

sister centromeres, Ctf7p is not required for the maintenance of sister chromatid cohesion.

Genetic interactions between CTF7 and the DNA replication machinery

Typically, high copy suppressors are proteins that either interact with the target protein or function in a parallel pathway. To gain further insight into the role of Ctf7p in cohesion, high-copy suppressors of the temperature sensitive *ctf7* phenotype were identified. Three plasmids were obtained that enabled *ctf7* mutant cells to grow at 34°C but not at 37°C. The three plasmids contained overlapping DNA sequences, suggesting suppression was due to a single gene. *ctf7* temperature sensitivity was suppressed by a minimal DNA sequence defined by *Bgl*III to *Stu*I restriction sites (Fig. 7A). Sequence analysis revealed that this 1.7-kb fragment contained *POL30*, which encodes the DNA replication processivity factor proliferating cell nuclear antigen (PCNA) (Bauer and Burgers 1990; Burgers 1991). A frame-shift mutation in the *POL30* ORF rendered the resulting plasmid incapable of rescuing *ctf7-203* temperature sensitivity, indicating that *POL30* is a high-copy suppressor of *ctf7-203*. Overexpression of *POL30* also rescued the temperature

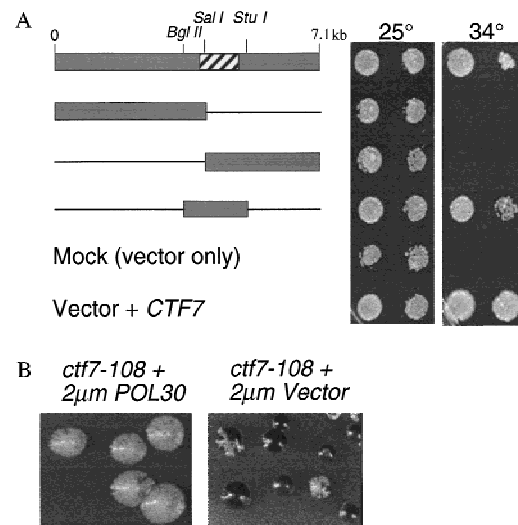


Figure 7. *POL30* is an overexpression suppressor of *ctf7* mutant phenotypes. (A) A 7.1-kb insert was identified that suppressed *ctf7* temperature sensitivity at 34°C. Neither vector alone (Mock) nor vector containing insert DNA sequences to either the left or right of the *Sal*I digestion site rescued the *ctf7* mutant at 34°C. A 1.7-kb fragment flanked by *Bgl*III and *Stu*I restriction sites rescued *ctf7* mutant temperature sensitivity but not to the same level of growth as CTF7 (Vector + CTF7). The hatched box in starting clone denotes *POL30* ORF. (B) *ctf7* mutations containing reporter chromosomes were transformed with 2 µm vector ± *POL30* and grown on medium that allowed for chromosome fragment loss but retention of the 2 µm plasmid [*ctf7-108* is a non-temperature-sensitive strain obtained from the original *ctf* collection].

sensitivity of *ctf7-201* but failed to rescue a *ctf7Δ1* deletion strain (not shown).

On the basis of the finding that *POL30* is a high-copy suppressor of *ctf7* temperature sensitivity, we asked whether *POL30* could also rescue *ctf7* chromosome loss. To test this possibility, *POL30* was overexpressed in strains containing either *ctf7* temperature-sensitive alleles (*ctf7-201* and *ctf7-203*) or a non-temperature-sensitive allele (*ctf7-108*) that was obtained in the original *ctf7* collection (Spencer et al. 1990). Identical results were obtained in each case: Overexpression of *POL30* decreased the chromosome loss associated with all *ctf7* mutations tested (Fig. 7B).

To both test whether the genetic interaction between *CTF7* and *POL30* reflected an important link between the establishment of sister chromatid cohesion and DNA replication machinery, and to further identify gene products that might require *CTF7* function, we assayed for synthetic lethal interactions. A strain harboring the *ctf7-203* allele was mated to strains containing either checkpoint, kinetochore, DNA metabolism, or other chromosome loss mutants. The inability to recover double-mutant spores was scored as a synthetic lethal interaction (Table 2). Synthetic lethal interactions were observed for *ctf7-203* in combination with either *ctf18Δ1* or *pol30-104*. *CTF18* encodes an RF-C-like protein and RF-C complexes are known to load PCNA onto double-stranded DNA (Burgers 1988; Kouprina et al. 1994; Ayayagari et al. 1995; Amin and Holm 1996). In contrast to these findings, no synthetic lethal interactions were observed when *ctf7-203* was combined with checkpoint mutants (*rad9Δ*, *mec1-1*, *mad2-1*, or *bub3Δ*), kinetochore mutants (*ctf13-30*, *ctf14-42*, *skp1-4*, or *skp1-3*), or other mutants involved in DNA replication (*ctf4-25*, an allele of the Pol α -binding protein, and *cdc6/ctf10-103*, an allele of the ORC interacting protein). Of 12 of the mutant strains that we tested (Table 3), synthetic lethality was only observed for *ctf7* in combination with *pol30* or *ctf18* mutations. The specificity of these genetic interactions is substantiated by the related functions of PCNA and Ctf18p and by the finding that *POI30* is a

high-copy suppressor of *ctf7* phenotypes. These genetic interactions link Ctf7p with proteins that are required during S phase, consistent with our mapping experiments, and together suggest that the establishment of sister chromatid cohesion is facilitated by components of the DNA replication machinery.

Discussion

Ctf7p, a novel link between sister chromatid cohesion, S phase, and the DNA replication machinery

In this study, we have identified Ctf7p as a protein required for sister chromatid cohesion. HA-Ctf7p is a nuclear protein and mutations in *CTF7* result in elevated chromosome loss, consistent with Ctf7p's role in chromosome segregation (this paper; Spencer et al. 1990). We discovered further that Ctf7p is required for cohesion at both centromeres and chromosome arms, as found for the cohesion factor Mcd1p (this paper; Guacci et al. 1997; Michaelis et al. 1997). Independent analysis has also shown that Ctf7p (called Eco1p) is required for cohesion (A. Toth, R. Ciosk, F. Uhlmann, M. Galova, A. Schleiffer, K. Nasmyth, pers. comm.). Therefore, although there is evidence from vertebrate cell chromosomes that cohesion is differentially regulated along the length of the chromosome (Sumner 1991), Ctf7p and Mcd1p appear to function in aspects of cohesion that are common to all regions of the chromosome.

Cell viability and sister chromatid cohesion are abolished when *ctf7* cells are placed at the restrictive temperature during S phase but not during mitosis. These findings demonstrate that Ctf7p, unlike Mcd1p, functions specifically in the establishment of cohesion (this paper; Guacci et al. 1997; Michaelis et al. 1997). An independent allele of *ctf7* (*eco1-1*) is also defective in the establishment but not the maintenance of cohesion (A. Toth, R. Ciosk, F. Uhlmann, M. Galova, A. Schleiffer, K. Nasmyth, pers. comm.), indicating that the inability to reveal a function for Ctf7p in the maintenance of cohesion is not an allele-specific effect. The model in which

Table 2. *ctf7-203* is synthetically lethal with *ctf18Δ1* and *pol30-104*

	Observed	Expected
<i>pol30-104^{mmms}-LEU2</i> × <i>ctf7Δ::HIS3 ctf7-203^{ts}:LEU2</i>	61% viable (68 of 112 spores)	
wild type	32 ⁺	28
<i>pol30</i>	28	28
<i>ctf7</i>	8	14
<i>pol30 ctf7</i>	0	14
<i>ctf18Δ::URA3</i> × <i>ctf7Δ::HIS3 ctf7-203^{ts}:LEU2</i>	55% viable (57 of 104 spores)	
wild type	30	26
<i>ctf18Δ</i>	13	26
<i>ctf7</i>	14	13
<i>ctf18Δ ctf7</i>	0	13

ctf7-203 was mated with *pol30-104* and *ctf18Δ1*, and the resulting diploid dissected to identify spores that contained both mutations. The expected and observed number of wild-type, single and double mutant spores are shown for each cross. Total spore viability is also shown.

Table 3. Strains used in this study

Strains	Genotypes	References
YBS2	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ/leu2-Δ1 ctf7Δ1::HIS3/CTF7 CFIII (CEN3.L) TRP1 SUP11</i>	this study
YBS3	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CTF7:HIS3</i>	
YBS5	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CTF7::HIS3 CFIII (CEN3.L) TRP1 SUP11 pRS316CTF7</i>	
YBS204	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 CTF7:LEU2 rad9Δ1::TRP1</i>	
YBS205	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 ctf7-203:LEU2 rad9Δ1::TRP1</i>	
YBS213	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1,63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 CTF7:LEU2 mad2-1</i>	
YBS214	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1,63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 ctf7-203:LEU2 mad2-1</i>	
YBS504	<i>MATa ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 CTF7:LEU2 bub3Δ1::LEU2</i>	
YBS568	<i>MATa ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 ctf7-203:LEU2 bub3Δ1::LEU2</i>	
YBS255	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 CTF7:LEU2</i>	
YBS514	<i>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ctf7Δ1::HIS3 ctf7-203:LEU2</i>	
YBS268	<i>MATα ura3 trp1 his3 leu2 ctf7Δ1::HIS3 ctf7-203:LEU2 mec1-1:HIS3</i>	
YBS271	<i>MATα ura3 trp1 his3 leu2 ctf7Δ1::HIS3 CTF7-LEU2 mec1-1:HIS3</i>	
YCTF108	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ1 ctf7-108 CFIII (CEN3.L) URA3 SUP11</i>	Spencer et al. (1990)
YCTF103	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ1 ctf10-103 CFIII (CEN3.L) URA3 SUP11</i>	
YCTF42	<i>MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf14-42 CFIII (CEN3.L) URA3 SUP11</i>	
YCTF30	<i>MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf13-30 CFIII (CEN3.L) URA3 SUP11</i>	
YCTF25	<i>MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 cf4-25 CFIII (CEN3.L) URA3 SUP11</i>	
YCTF10	<i>MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf7-10 CFIII (CEN3.L) URA3 SUP11</i>	
YPH1182	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1-4:LEU2</i>	Connelly and Heiter (1996)
YPH1188	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1-3:LEU2</i>	
YCH2165	<i>MATa leu2-3,112 ura3-52 POL30:LEU2</i>	Amin and Holm (1996)
YCH2161	<i>MATa leu2-3,112 ura3-52 pol30-104:LEU2</i>	
YJE106	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ctf18Δ1:LEU2</i>	Kouprina et al. (1994)
YJE188	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 ctf18Δ1::URA3</i>	
DLY285	<i>MATa ura3 trp1 his3 leu2 trp1 mec1-1-HIS3</i>	Paulovich (1995)
YCB624	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rad9Δ1::TRP1</i>	Brachmann (1995)
YFS451	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1,63 his3-Δ200 leu2-Δ1 mad2-1 CFIII (CEN3.L) URA3 SUP11</i>	Pangilinan (1996)
YFP74	<i>MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 bub3Δ1::LEU2</i>	
VG955-7D	<i>MATa mcd1-1 trp1 leu2 bar1 gal1</i>	Guacci et al. (1997)

See Materials and Methods for details.

Ctf7p functions in the establishment of cohesion during S phase is further supported by the genetic interactions observed between mutations in *CTF7* and the DNA metabolism genes *POL30* and *CTF18* (Kouprina et al. 1994; Amin et al. 1996). *POL30* was the only gene found in a genome-wide screen that, at high copy, suppressed *ctf7* temperature sensitivity and chromosome loss. In addition, *pol30-104* and *ctf18Δ1* are synthetically lethal with *ctf7-203*. The significance of these genetic interactions is augmented by their specificity; no synthetic lethal interactions were observed for *ctf7* in combination with mutations that affect either the initiation of DNA replication, kinetochore function, or DNA damage or spindle assembly checkpoint pathways. The genetic interactions

between *CTF7*, *POL30*, and *CTF18* do not appear to reflect a general response of *ctf7* mutant strains to alterations in S-phase progression. For instance, both *ctf18Δ1* and *pol30-104* are cold-sensitive mutations that, at elevated temperatures, exhibit normal S-phase kinetics (Kouprina et al. 1994; Amin and Holm 1996). In addition, *ctf7* mutant strain temperature sensitivity was not suppressed by levels of hydroxyurea that slow DNA elongation (R.V. Skibbens and D. Koshland, unpubl.). Taken together, these observations suggest that Ctf7p functions during S phase for the establishment of cohesion and reveals an important link between DNA replication and sister chromatid cohesion.

The connection between *CTF7* and *POL30* is particu-

larly intriguing. PCNA forms doughnut-like sliding clamps that are continuously loaded behind DNA polymerases at the replication fork (Krishna et al. 1994). Others have noted that PCNA both appears to be landing pads for a variety of proteins not directly related to DNA replication and may play a role in chromatin remodeling (Kelman 1997). In combination with our results, these data suggest that the loading of cohesin complexes onto chromatin is coupled with PCNA-dependent DNA replication. This model is attractive because it places cohesin complexes proximal to newly replicated sister chromatids to ensure the pairing of sister chromatids but preclude the pairing of nonsister chromatids. Loading of cohesin complexes onto chromatin can be uncoupled from replication, as has been observed in *Xenopus* extracts and budding yeast cells, but at least in yeast this association fails to generate functional sister chromatid cohesion (Losada et al. 1998; Uhlmann and Nasmyth 1998).

In budding yeast, Mcd1p is required for both cohesion and condensation along chromosome arms, providing the first link between these two processes (Guacci et al. 1997). Given this precedent, we found that Ctf7p was also required for condensation at the rDNA locus, although the defect in *ctf7* mutants was not as pronounced as that observed in *mcd1* mutants (R.V. Skibbens and D. Koshland, unpubl.). Concomitant with our study, Trf4 protein has also been shown to be required for both cohesion and condensation (Castano et al. 1996; I. Castano and M. Christman, pers. comm.). Thus, their study of Trf4p and our study of *ctf7*p further establishes that yeast cohesin and condensin complexes share either common components, regulators, or assembly factors. In contrast, Losada et al. (1998) reported that depleting cohesin components from *Xenopus* extracts did not adversely affect chromosome condensation. In addition, *Xenopus* 13S condensin and 14S cohesin complexes were found to associate with chromatin independent of one another, indicating a separation of function (Losada et al. 1998). It will be important to pursue whether the coupling/uncoupling of cohesion and condensation reflect differences in organisms, cell systems (somatic versus embryonic) or segregation cycles (mitotic versus meiotic). Such differences have been well documented and what once appeared as conflicting data has historically proven to be of tremendous scientific value.

Cohesion, checkpoints, and spindle integrity

Our results showed that *ctf7* mutant cells exhibited a mitotic delay that required the kinetochore/spindle assembly checkpoint. Consistent with the preanaphase delay typically imposed by this checkpoint, *ctf7* cells contained high levels of the anaphase inhibitor Pds1p. On the other hand, *ctf7* cells contained elongated spindles and separated DNA masses, uncharacteristic of preanaphase cells. This aberrant metaphase delay raises three issues that further reveal important functions of sister chromatid cohesion.

First, how does the *ctf7* mutation disrupt spindle

structure? In preanaphase budding yeast cells, spindle pole bodies are mechanically linked by a few interdigitating polar microtubules and by many kinetochore microtubules that are indirectly connected by virtue of their attachment to paired sister chromatids (O'Toole et al. 1997). We propose that the dissolution of sister chromatid cohesion complexes weakens this linkage and profoundly affects spindle integrity. Abnormal spindles have been reported for other mutants defective in cohesion such as *smc1* and *mcd1* (Strunnikov et al. 1993; Guacci et al. 1997; Michaelis et al. 1997). Thus, sister chromatid cohesion appears to function as part of the mechanical linkage that is required to maintain spindle integrity.

The second question raised by our observations is why do *ctf7* mutants activate the kinetochore/spindle assembly checkpoint and delay in mitosis? To date, the kinetochore/spindle damage checkpoint pathway has been implicated in monitoring defects in centromere, kinetochore, or spindle function and responds by delaying cells in mitosis (Skibbens and Hieter 1998). Currently, we cannot rule out a model whereby *ctf7* cells contain both defective kinetochores and sister chromatid cohesion complexes. However, it is not necessary to invoke a kinetochore defect to explain the checkpoint-mediated delay in *ctf7* cells. Interestingly, other cohesion mutants also exhibit a mitotic delay, although it is unknown whether this delay is dependent on a spindle assembly checkpoint (Strunnikov et al. 1993; Guacci et al. 1997; Michaelis et al. 1997). On the basis of our evidence, we envision cohesion defects to activate this checkpoint by altering the state of tension exerted on chromosomes. Chromosomes attached to microtubules experience poleward forces that place the chromosome under tension. Evidence from meiotic cell systems revealed that tension exerted on chromosomes is critical for signaling anaphase onset (Nicklas 1997): A decrease or loss of tension generates a mitotic delay. One predicted effect for the dissolution of chromatid cohesion is to decrease the level of tension exerted on chromosomes by spindle microtubules and activate the spindle assembly checkpoint (Wells and Murray 1996). This model suggests that sister chromatid cohesion is also a fundamental component of the mechanism that maintains tension on chromosomes.

Typically, checkpoint mechanisms maintain cell viability by delaying the cell cycle and allowing cells time to repair damage or correct mistakes. In mutations that induce such damage, the additional loss of checkpoint function often results in synthetic lethality. Therefore, a third question is why is *ctf7* not synthetically lethal with mutations in the kinetochore/spindle assembly checkpoint? The loss of the checkpoint has little impact on *ctf7* cells for two reasons. First, one component of the checkpoint mechanism is to block sister separation. However, *ctf7* cells are defective in sister chromatid cohesion and subvert this component of checkpoint function. Second, sister separation is an irreversible event. Thus, whether the cell delays or not in response to checkpoint function, cells with precociously separated

sisters are beyond rescue. Our results are analogous to those reported over a decade ago, double-strand breaks generated in G_1 haploid cells resulted in a *RAD9*-mediated cell cycle delay at G_2/M . Despite this delay, the cells were inviable (Weinert and Hartwell 1988).

Materials and methods

Yeast strains and medium

Medium used for growth and sporulation was described previously (Rose et al. 1990). Yeast transformations were performed as described (Ito et al. 1983; Schiestl and Gietz 1989). S288C-derived *Saccharomyces cerevisiae* yeast strains (YPH) and plasmids (pRS) were described previously (Sikorski and Hieter 1989; Doheny et al. 1993; Connelly and Hieter 1996). Other strains are listed in Table 3. To establish genetic interactions, two independent double heterozygous diploids were obtained, sporulated, and haploid spores containing both mutations identified. The nonessential reporter chromosome of ~150 kb contains the short arm and centromere of chromosome III for stable propagation and *SUP11* to suppress the formation of the red byproduct of the adenine biosynthetic pathway in *ade2* mutations (Spencer et al. 1990).

Cloning and molecular characterization of *CTF7*

A region of DNA that rescued the chromosome fragment-loss phenotype in a *ctf7* mutant strain (*ctf7-108*) from the original *ctf* collection (Spencer et al. 1990) was identified from a *CEN/LEU2* library and subcloned following procedures and reagents described previously (Gerring et al. 1990). This 2.1-kb region was shown to encode the chromosome transmission fidelity locus of *CTF7* in the following manner. The 2.1-kb fragment was cloned into pRS303, linearized within the *CTF7* ORF with *Xba*I and integrated into the genome of YPH250. His⁺ transformants were shown to contain *HIS3*-marked *CTF7* integrated at the proper locus by Southern analysis on the basis of the known restriction map of the region. The resulting strain (YBS3) was mated with a strain from the original collection that contains *ctf7-10* (Spencer et al. 1990) and sporulated. His⁺ spores (22 of 22) exhibited wild-type levels of chromosome loss, whereas all 14 His⁻ spores tested sectorized dramatically when grown on non-selective medium. Database searches of the yeast genome indicated that *CTF7* is contained on Chromosome VI. This chromosome assignment was confirmed by hybridization to yeast genomic clone contigs (L. Riles and M. Olson, pers. comm.).

The *CTF7* ORF was replaced with the selectable marker *HIS3* by a PCR strategy described previously (Baudin et al. 1993). The deletion DNA fragments were generated by PCR by the primers 5'-AGTAATAATACATAAGGAGATGCCAGCTACAACGGACATCAACATATATCTAGATTGTAAGTACTGAGAGTGCACC-3' and 5'-ACCTTGAAAAAGTTTTATGCCATTTTTCCAGTGTCCCTTCTCGCTGTCTTTCTGTGCGGTATTTTCACACCG-3'. The wild-type diploid strain YPH987 was transformed with PCR product and His³⁺ colonies identified to obtain a heterozygous *CTF7/ctf7Δ* deletion strain (YBS2). *CTF7* deletion was confirmed by Southern blot analysis. Tetrad analysis yielded 2+:2- spore death with no recovery of His⁺ spores. To confirm that *CTF7* encodes an essential protein, the diploid YBS2 (*ctf7Δ::HIS3/CTF7*) was transformed with pRS316-*CTF7* and sporulated. All His⁺ Ura⁺ spores tested were inviable on medium plates containing 5-FOA (Boeke et al. 1987).

Generation, integration and suppression of conditional *ctf7* alleles

PCR-based mutagenesis of pRS316-*CTF7* was performed as described (Kassenbrock et al. 1993) with minor modifications. YBS5 (*ctf7Δ* covered by pRS316-*CTF7*) was cotransformed with pooled PCR product and *Mlu*I-*Nde*I-gapped pRS315-*CTF7* (this digestion removes the entire *CTF7* ORF but leaves 5' and 3' ends homologous to the *CTF7* genomic locus). Of 12,500 Leu⁺ transformants screened, 11 scored positive as temperature-sensitive nonpetite strains by virtue of their inability to grow on 5-FOA plates at 37°C and growth on glycerol plates at 25°C. Plasmids containing putative mutated *CTF7* sequences were recovered from those strains. YBS5 was then transformed with the rescued plasmids to confirm plasmid-dependent temperature sensitivity. Three of the eleven plasmids that exhibited the lowest reversion rate and conferred the tightest temperature sensitivity were chosen for integration into the genome. The 2.1-kb insert was cloned into pRS304 and the resulting plasmid linearized with *Hpa*I to target integration to the *LEU2* locus in YBS5. After passage on 5-FOA, His⁺ Leu⁺ Ura⁻ transformants were temperature sensitive for growth. The temperature-sensitive phenotype was rescued on retransformation with pRS316-*CTF7*. Hence, these mutations are recessive.

For overexpression suppression, *ctf7-203* was transformed with a 2 μm of *URA3* library (Connelly et al. 1996). Of 24,900 transformants, 26 plasmids were obtained that, on retransformation, rescued growth at 34°C. Twenty-three of the twenty-six plasmids contained the *CTF7* ORF. The remaining three were found to contain overlapping sequences and one plasmid was subcloned to identify the suppressor ORF.

Morphological and growth analyses

Flow cytometry, Western blot analysis, and indirect immunofluorescence (IIF) were performed as described previously (Gerring et al. 1990; Cohen-Fix et al. 1996) with minor modifications. Viability was determined as described previously (Morrow et al. 1995) with minor modifications. Briefly, cells were plated after experimental procedures onto rich YPD medium plates and grown at the permissive temperature for 18 hr. The plates were then assessed microscopically for the formation of microcolonies. Typically, microcolonies were rescored after an additional 24 hr. For execution experiments, the incidence of anaphase onset was based on either spindle elongation or separation of DNA masses. In shift-down experiments, the percent of anaphase cells rose dramatically because of the cell cycle delay observed in *ctf7* mutants, consistent with the accumulation of a 2C DNA content. For localization of Ctf7p, the entire *CTF7* ORF was placed in-frame with the encoded HA epitope tag contained on pAD5 (Neiman et al. 1993). YBS5 was transformed with this plasmid and pRS316-*CTF7* shuffled out from the resulting strain by use of medium plates containing 5-Fluorotic acid, FOA (Boeke et al. 1987) prior to immunolocalization. Immunostaining was performed by use of the anti-HA antibody 12CA5 (BabCo) in combination with fluorescein- or Cy3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch) to visualize epitope-tagged Ctf7p and Pds1p (Cohen-Fix et al. 1996). Not all nuclei stained with 12CA5, indicating that protein levels varied considerably when constructs are carried on a 2 μm plasmid. YBS5 similarly transformed with the pAD5 vector only (containing no *CTF7* insert) exhibited no nuclear staining, nor was a nuclear signal observed with secondary antibody alone. YOL1/34 (Serotec) and fluorescein- or rhodamine-conjugated goat anti-rat antibodies (Cappel and Boehringer Mannheim) were used to visualize microtubules. DNA

masses were visualized with DAPI (Sigma). For Western blot analysis of HA-Pds1, cell densities were normalized by optical density. Equal loads of cell extracts were confirmed by Western blot analysis of tubulin levels.

Reagents used to perform FISH analysis and detailed procedures are described previously (Guacci et al. 1994, 1997).

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