

# In vivo dissection of the chromosome condensation machinery: reversibility of condensation distinguishes contributions of condensin and cohesin

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The machinery mediating chromosome condensation is poorly understood. To begin to dissect the in vivo function(s) of individual components, we monitored mitotic chromosome structure in mutants of condensin, cohesin, histone H3, and topoisomerase II (topo II). In budding yeast, both condensation establishment and maintenance require all of the condensin subunits, but not topo II activity or phospho-histone H3. Structural maintenance of chromosome (SMC) protein 2, as well as

each of the three non-SMC proteins (Ycg1p, Ycs4p, and Brn1p), was required for chromatin binding of the condensin complex in vivo. Using reversible condensin alleles, we show that chromosome condensation does not involve an irreversible modification of condensin or chromosomes. Finally, we provide the first evidence of a mechanistic link between condensin and cohesin function. A model discussing the functional interplay between cohesin and condensin is presented.

## Introduction

Mitotic chromosome condensation serves to shorten and organize chromosomes to facilitate their segregation. This higher order compaction is a complex process, and a number of different chromosomal components, conserved from yeast to man, have been identified as potential mediators of condensation. A key component of the molecular condensation machinery is the five subunit complex called condensin (Hirano et al., 1997; Sutani et al., 1999; Freeman et al., 2000; Kimura et al., 2001). All five components of yeast condensin are essential for cell viability (Saka et al., 1994; Strunnikov et al., 1995; Sutani et al., 1999; Freeman et al., 2000; Lavoie et al., 2000; Biggins et al., 2001). Two of its subunits, Smc2p (cut14/XCAP-E/MIX-1/dSMC2/hCAP-E) and Smc4p (cut3/XCAP-C/dSMC4(gluon)/hCAP-C), are members of the ubiquitous structural maintenance of chromosome (SMC)\* proteins, which possess characteristic coiled-

coil and ATPase domains (for reviews see Cobbe and Heck, 2000; Hirano, 2000). In addition to Smc2/4p, the condensin complex contains three other proteins, Brn1p/Ycs3p, the yeast homologue of *Drosophila Barren* (XCAP-H/cnd2/BRRN1), Ycg1/Ycs5p (XCAP-G/cnd3/hCAP-G), and Ycs4p (pEg7/XCAP-D2/cnd1/hCAP-D2). All five condensin proteins are required for condensation in vivo and in vitro (Hirano et al., 1997; Cubizolles et al., 1998; Sutani et al., 1999; Kimura and Hirano, 2000; Lavoie et al., 2000; Ouspenski et al., 2000; Kimura et al., 2001). In vitro biochemical analyses suggest that the SMC heterodimer can bind DNA, whereas the non-SMC subunits form a subcomplex that mediates chromatin binding of the condensin, and condensin can introduce positive supercoils into a circular template (Hirano, 2000). However, the in vivo relevance of these activities has not been tested.

In addition to condensin, other proteins have been implicated in mitotic chromosome condensation; in particular phosphohistone H3 (phospho-H3), topoisomerase II (topo II), and proteins involved in mediating sister chromatid cohesion (Hirano, 2000). Whether they are required for condensation in all eukaryotes and their exact roles in this process remain unclear. For example, the posttranslational modification of histone H3 by phosphorylation at serine 10 has long been correlated with the onset of mitosis, and mutants in this residue show condensation defects in *Tetrahymena* (Wei et al., 1999). In contrast, a recent report suggests a poor correlation

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\*Abbreviations used in this paper: FISH, fluorescence in situ hybridization; HU, hydroxyurea; IIF, indirect immunofluorescence; Nz, nocodazole; phospho-H3, phosphohistone H3; SMC, structural maintenance of chromosome; topo II, topoisomerase II; ts, temperature sensitive; WT, wild type.

Key words: sister chromatid cohesion; mitotic chromosome structure; SMC proteins; chromosome dynamics; MCD1/SCC1

between H3 phosphorylation and levels of condensation (Adams et al., 2001). Similarly, in both budding yeast and *Sordaria*, chromosome condensation requires the cohesion machinery, which mediates pairing between sister chromatids from their replication until their separation in anaphase (Hirano, 2000; Koshland and Guacci, 2000; Nasmyth et al., 2000; Skibbens, 2000). Mutations in cohesion factors such as *MCD1/SCC1*, *TRF4*, *PDS5/SPO76*, and *CTF18* not only perturb sister chromatid cohesion but also fail to establish and/or maintain chromosome condensation (Castano et al., 1996; Guacci et al., 1997; Hartman et al., 2000; Hanna et al., 2001). To account for these data, it has been proposed that cohesins bound to chromosomes could modulate the extent of chromosome compaction according to the density of cohesin binding sites (Guacci et al., 1997). Although the current structural data on cohesins correlate well with these models, several issues remain to be resolved. Direct interactions between the cohesins and condensins have not been described, and the two machineries neither colocalize on chromosomes nor show interdependent chromatin binding in any system (Losada et al., 1998; Toth et al., 1999). Furthermore, cohesin-depleted extracts support condensation in vitro (Losada et al., 1998; Sonoda et al., 2001). Thus the question arises, are the in vivo roles of cohesins and condensins in condensation mechanistically linked?

As a first step toward understanding the in vivo mechanism of mitotic chromosome folding, we have used a comparative approach to probe known and putative components of the condensation apparatus using conditional alleles that allow rapid protein inactivation and therefore minimize secondary consequences of chromosome transmission defects. We define essential parameters of in vivo condensation in

*Saccharomyces cerevisiae* and discuss the implications of our in vivo data in relation to in vitro models.

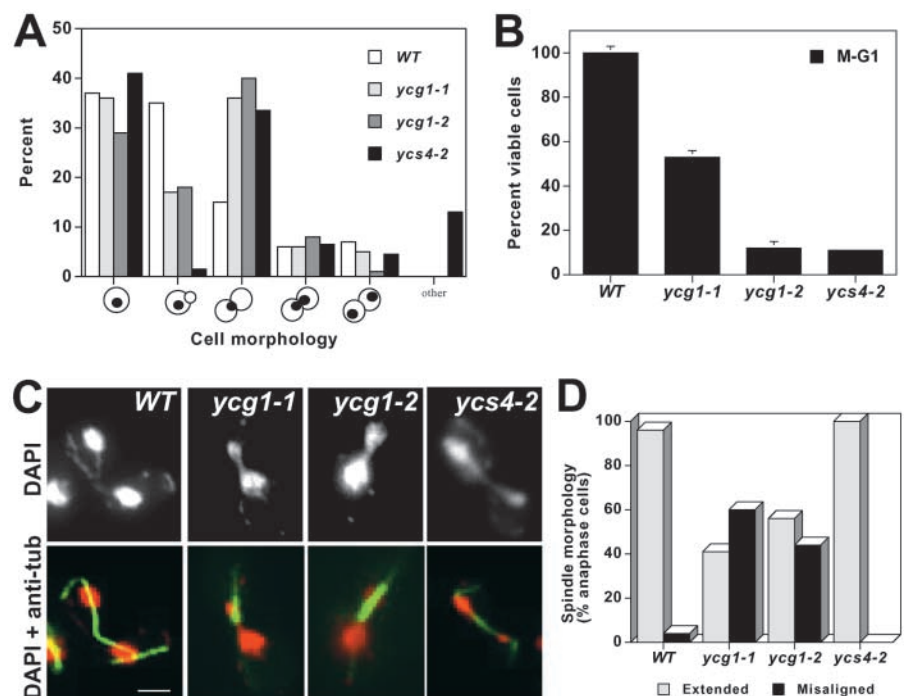
## Results

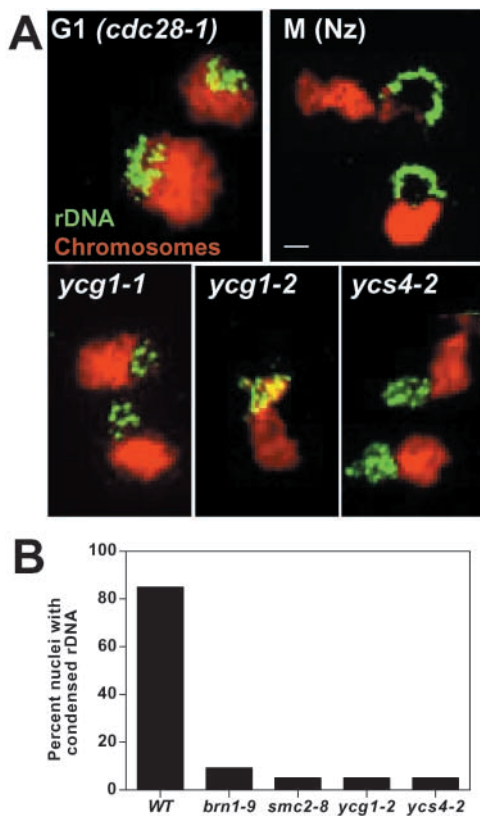
### Novel condensin mutants

In *S. cerevisiae*, gross defects in chromosome segregation and condensation have been described for mutants in three condensin subunits: Smc2p, Smc4p, and Brn1p (Strunnikov et al., 1995; Freeman et al., 2000; Lavoie et al., 2000; Ouspenski et al., 2000). In an attempt to better understand how the remaining two condensins, *YCG1* and *YCS4*, contribute to mitotic chromosome structure/function and to extend our armory with a way to distinguish between functions of individual subunits, temperature-sensitive (ts) alleles of *ycs4* and *ycg1* were generated. After shifting asynchronously growing *ycg1-1*, *ycg1-2*, and *ycs4-2* strains to the restrictive temperature for 3 h, cells were fixed and monitored microscopically for both cell and DNA morphology. Although none of the mutants arrested homogeneously, an enrichment in large budded cells with the nucleus at or near the bud neck suggested a mitotic defect (Fig. 1 A).

To address whether *ycg1* and *ycs4* perform an essential mitotic function, condensins were inactivated in a window of the cell cycle where chromosome segregation occurs. Cultures were synchronized in M phase using the microtubule-depolymerizing drug nocodazole (Nz), shifted to the restrictive temperature, released, and allowed to proceed to G1. Under these conditions, cell viability was greatly compromised, consistent with a mitotic role for these two proteins (Fig. 1 B). Indeed, DAPI and tubulin staining of mitotic cells revealed gross defects in chromosome segregation in all of the mutants (Fig. 1, C and

**Figure 1. Phenotypic analysis of condensin mutants.** (A) Cell morphology distribution. Asynchronously growing cultures of BLY03 (*WT*), BLY04 (*ycg1-1*), BLY05 (*ycg1-2*), and ZW206 (*ycs4-2*) were shifted from 23°C to 37°C for 3 h. Cells were fixed and scored by differential interference contrast and fluorescence (DAPI) microscopy. Greater than 100 cells/sample were counted. (B) Viability of condensin mutants after passage through mitosis. Cultures, as in A, were synchronized in M phase (Nz) at 23°C, shifted to 37°C for 1 h, released, and rearrested in G1 before counting and plating to monitor viability (see Materials and methods). (C) IIF of condensin mutants in anaphase. G1-arrested cells were released into 37°C media and allowed to proceed into mitosis. *WT* (60 min after release) and mutant (80 min) cultures were processed for IIF using antitubulin antibodies to detect spindles (shown in green with yellow overlap). Chromosomes were visualized by DAPI staining (false colored red). Bar, 2  $\mu$ m. (D) Quantitation of spindles shown in C. Greater than 50 anaphase cells/sample were scored. Spindles were scored as extended if they joined both dividing chromosomal masses and ranged from 7 to 15  $\mu$ m. Misaligned spindles refer to partially extended spindles, 4–5  $\mu$ m in length, that localize near one side of the dividing chromosomes.





**Figure 2. Establishment of rDNA condensation in condensin mutants.** (A) BLY03 (*WT*), BLY04 (*ycg1-1*), BLY05 (*ycg1-2*), and ZW206 (*ycs4-2*) strains were synchronized in G1 and shifted to 37°C before release and rearrest in M phase. Fixed cells were processed for rDNA FISH (see Materials and methods). For the G1 control, strain 4522-282 (*cdc28-1*) was shifted to 37°C for 3 h. Micrographs depict chromosomes in red and rDNA FISH signal in green. Bar, 2 μm. (B) Quantitation of rDNA condensation in A. Additional conditional mutant strains CH2524 (*brn1-9*) and 1aAS330 (*smc2-8*) were grown as in A. Chromosomes adopting a looped rDNA morphology were scored as condensed. Greater than 100 nuclei/sample were scored.

D). A characteristic “cut” phenotype was readily apparent, with nuclei failing to partition equally between mother and daughter cells, often leaving a trail of chromosomes spanning the plane of cytokinesis. In addition, whereas the *ycs4-2* mutant had normal spindle morphology, both *ycg1* alleles displayed spindle abnormalities. In particular, a partially elongated (4–5 micron) spindle associated with only one side of the dividing DNA mass was observed in up to 50% of anaphase cells. Despite these gross abnormalities, cell cycle progression from M to G1 was not blocked, because the appearance of anaphase cells occurred as in wild-type (*WT*) strains (~80 min after release from G1 arrest), and their disappearance showed only a modest delay (~20 min; unpublished data). Taken together, these data indicate that equal chromosome segregation, and thus successful passage through mitosis, requires both *YCG1* and *YCS4*. Moreover, the aberrant anaphases induced by either *ycg1* or *ycs4* did not markedly affect cell cycle progression, indicating that they elicit little or no checkpoint response.

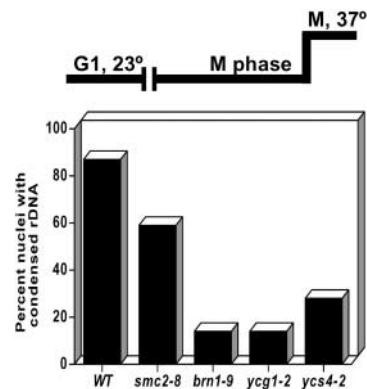
**Protein requirements for chromosome condensation**

To ascertain whether *YCG1* and *YCS4* are required for the establishment of chromosome condensation, fluorescence in

situ hybridization (FISH) was performed. Previous studies have shown that in G1 cells, euchromatic probes are maximally separated and the rDNA adopts a disordered, puffed morphology (Fig. 2 A, *cdc28-1*; Guacci et al., 1994). In contrast, in early M phase cells (Nz arrest), the distance between euchromatic probes is minimized, and the rDNA adopts a distinct looped or line-like structure (Fig. 2 A, Nz; Guacci et al., 1994). The rDNA serves as an excellent reporter for the condensation state of chromosomes because it reflects the action of condensin at both repetitive and unique sites (Guacci et al., 1994, 1997; Strunnikov et al., 1995; Freeman et al., 2000; Hartman et al., 2000; Lavoie et al., 2000). To inactivate *Ycg1p* and *Ycs4p* function over a window of the cell cycle when chromosome condensation is established and maintained, *ycg1-1*, *ycg1-2*, and *ycs4-2* strains were synchronized in G1, shifted to 37°C, released into Nz-containing medium to rearrest in mitosis, and then processed for FISH. Under these conditions, all three mutants exhibited G1-like rDNA rather than the looped structures characteristic of M phase (Fig. 2). Thus, *Ycs4p* and *Ycg1p*, like *Brn1p* and *Smc2p*, are required for the establishment of condensation.

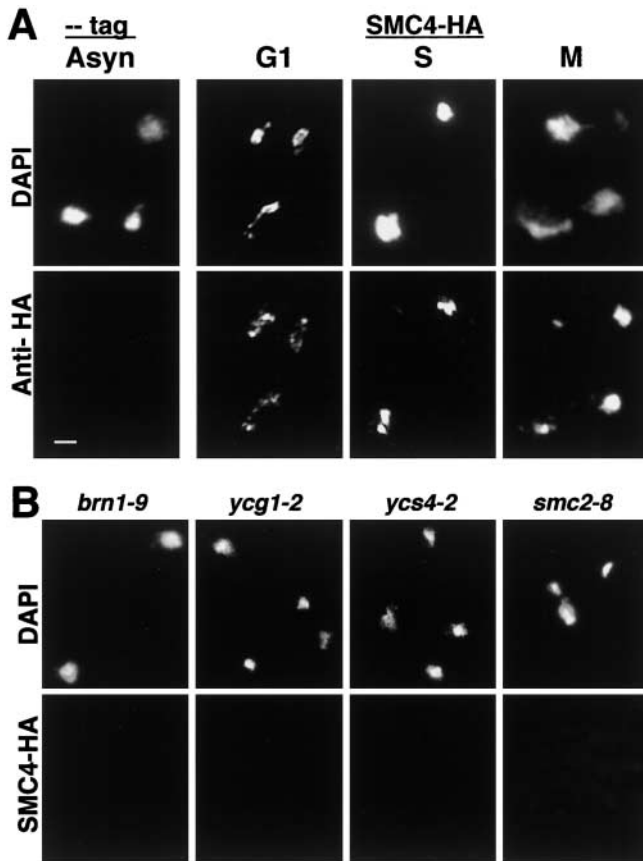
**Maintenance of condensation in M phase requires YCG1 and YCS4**

Although the inactivation of the non-SMC subunits from G1 to M phase abrogates chromosome condensation, these experiments do not address whether *Ycg1p* and *Ycs4p* function solely as accessory loading factors or play an ongoing role in maintaining the structure of folded chromosomes in vivo. To address this directly, mutant strains were arrested in M phase at the permissive temperature to allow condensation to occur. The cells were then shifted to 37°C for 30 min to inactivate the mutant proteins. Under these conditions, inactivation of any of the three non-SMCs efficiently disrupted chromosome condensation (Fig. 3), indicating that *Ycg1p* and *Ycs4p* are required to maintain the mitotic chromosome structure of the rDNA. Notably, temperature shifting the *smc2-8* strain had a much less dramatic effect on rDNA condensation, suggesting that partial function may



**Figure 3. Maintenance of chromosome condensation.** The experimental outline is shown. Strains BLY03 (*WT*), 1aAS330 (*smc2-8*), CH2524 (*brn1-9*), BLY05 (*ycg1-2*), and ZW206 (*ycs4-2*) were arrested in M phase at 23°C to allow condensation of the rDNA to occur (see Materials and methods). After a temperature shift for 1 h (37°C), the extent of rDNA condensation remaining was assessed by FISH. In all experiments, >100 nuclei/sample were scored.





**Figure 4. Chromatin spreads of Smc4-HA.** (A) Cell cycle analysis. WT strain BLY08 (*SMC4-HA3*) was synchronized in G1 phase ( $\alpha$ -factor), in S phase (HU), and in M phase (Nz). The unfixed cells were processed for spreads and Smc4-HA3p binding to chromatin was detected by IIF using anti-HA antibodies (see Materials and methods). The control untagged culture (YPH499) was grown asynchronously to mid-log phase. DNA staining was with DAPI. (B) Chromatin spreads of Smc4p in the absence of non-SMC subunits. BLY08 (*SMC4-HA3*), BLY10 (*brn1-9 SMC4-HA3*), BLY11 (*ycg1-2 SMC4-HA3*), BLY12 (*ycs4-2 SMC4-HA3*), and BLY26 (*smc2-8 SMC4-HA3*) were synchronized in G1, shifted to the restrictive temperature, and rearrested in M phase. Spreads are as in A. All images were generated using 1-s exposures with a CCD camera, and background was adjusted to the same range using Scanalytics software (see Materials and methods). Bar, 2  $\mu$ m.

remain under these conditions. Taken together, these data indicate that the non-SMCs are required not only to fold chromosomes but also to keep them folded.

### Chromatin binding of condensin requires all three non-SMC subunits

We next asked whether the SMCs could bind chromatin in the absence of the non-SMCs in vivo. For this purpose, a triple HA epitope-tagged Smc4 protein was generated as a reporter for the condensin complex. This allele fully complements a *smc4* knockout strain (unpublished data) and was used to replace the endogenous *SMC4* gene. The binding of Smc4-HA3p to chromatin was followed by chromatin spreads (Michaelis et al., 1997). In this method, cells are simultaneously lysed and fixed such that the chromatin remains bound to the slide but the nucleoplasm and cytoplasm are washed away.

As chromatin spreads have not been extensively used to assess condensin binding, we initially characterized Smc4-HA binding in WT cells. Smc4p staining was detected on chromosomes at all times during the cell cycle (Fig. 4 A), and in >95% of asynchronously growing cells (unpublished data). In addition, the Smc4p signal appeared concentrated over the rDNA locus, which stains poorly by DAPI (Fig. 4 A). This localization likely reflects that of the condensin complex, because a similar pattern is observed for both Ycs4-MYC12p and Ycg1-HA3p (unpublished data), and the complex is known to exist in the nucleus throughout the cell cycle (Freeman et al., 2000). In addition, recent in vivo studies have shown a nucleolar enrichment of GFP-tagged condensin (Freeman et al., 2000).

We next addressed whether all of the other components of condensin are required for the chromatin association of Smc4p (Fig. 4 B). *Brn1-9*, *ycg1-2*, *ycs4-2*, and *smc2-8* mutant strains containing Smc4-HA3p were synchronized in G1 (23°C), temperature shifted, and released to an M phase block (37°C). Consistent with the proposal that Smc2p and Smc4p form a heterodimer, Smc4p binding was disrupted after the inactivation of Smc2-8p. In addition, a dramatic reduction in chromosomal staining of Smc4-HA3p was apparent in the three non-SMC mutants, even though Smc4 protein levels were not greatly affected (unpublished data). Similarly, when any of the non-SMCs were inactivated in M phase, the Smc4-HA3p signal was also lost (unpublished data). From these data, we infer that the decondensed chromosomes observed in our mutants correspond to the null state for condensin function.

### Chromosome condensation is reversible in a single mitosis

The transient inactivation of the non-SMCs just before chromosome segregation leads to a loss of condensin chromatin binding and chromosome decondensation. However, when the cells are returned to the permissive temperature, cell viability remains high (Fig. 5 A). This apparent contradiction can be resolved if condensins rapidly regain activity upon return to the permissive temperature and recondense their chromosomes before chromosome segregation ensues. To test this possibility, *BRN1* and *brn1-9* cultures were synchronized in M phase at 23°C to allow condensation to occur. As shown in Fig. 5 B, both *BRN1* and *brn1-9* cells show similar levels of condensation at the permissive temperature. Upon a shift to 37°C, condensation was disrupted in the *brn1-9* mutant and, significantly, was efficiently regained within 1 h of returning to the permissive temperature. This recondensation must be biologically relevant, because the viability of cells after transient inactivation in M phase is high. Furthermore, the fact that condensin can promote a second round of condensation suggests that neither the chromosomes nor the condensins are irreversibly modified (or inactivated) during condensation. Thus, the activity of condensin is maintained at least until the metaphase checkpoint.

### Topo II and phospho-H3 are not required for condensation in budding yeast

Our study of the condensin subunits provided a basis to analyze the contribution of other potential condensation fac-

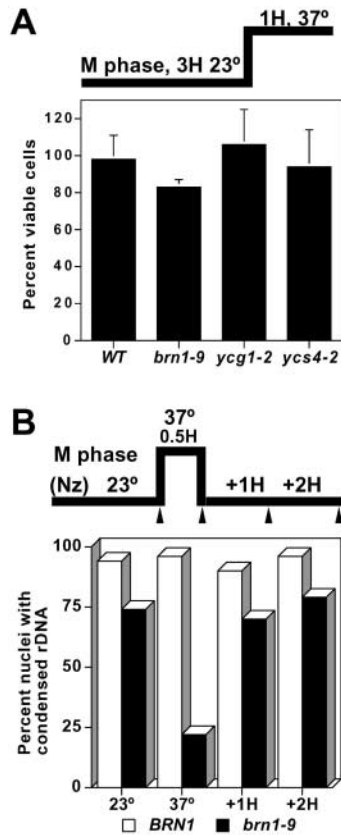


Figure 5. **Reversibility of rDNA condensation.** (A) Transient inactivation of condensins in mitotic cells. An experimental schematic is shown above. In brief, WT (BLY03), *brn1-9* (CH2524), *ycg1-2* (BLY05), and *ycs4-2* (ZW206) cultures were arrested in M phase (Nz) at 23°C, and then shifted to 37°C for 1 h. The cells were then released to the permissive temperature by plating. Viability was determined by counting the number of colony forming units versus the number of cells plated (see Materials and methods). (B) The experimental schematic is shown above. M phase-arrested CH2523 (*BRN1*) and CH2524 (*brn1-9*) strains were temperature shifted as indicated; arrows denote time points where samples were taken, fixed, and processed for rDNA FISH. rDNA loops were scored as condensed. Greater than 100 nuclei/sample were scored.

tors such as topo II and phospho-H3. In fact, in budding yeast neither of these had been directly tested for a role in condensation. To do this, a well-characterized mutant of topo II (*top2-4*) was used to inhibit the double strand passage activity of topo II from G1 phase to mitosis, yielding chromosomes that remain topologically interlinked (Koshland and Hartwell, 1987; Holm et al., 1989). Similarly, the phosphorylation of histone H3 was precluded through mutagenesis of serines 10 and 28 to alanines, because phosphorylation of both of these positions has been correlated with chromosome condensation in numerous organisms (Goto et al., 1999; Hirano, 2000). Neither of these mutants displayed an effect on chromosome condensation; both the morphology of chromosomes (unpublished data) and the levels of condensation were similar to WT cells and differed markedly from condensin mutants (Fig. 6). Thus, both topo II activity and phospho-H3 appeared dispensable for the establishment of mitotic chromosome structure in *S. cerevisiae*, and were not pursued further.

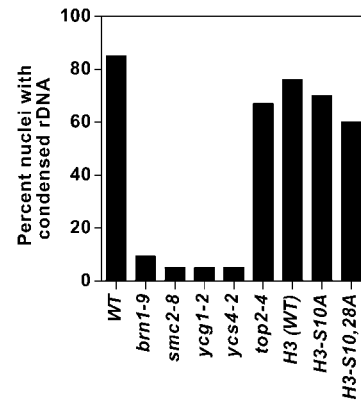
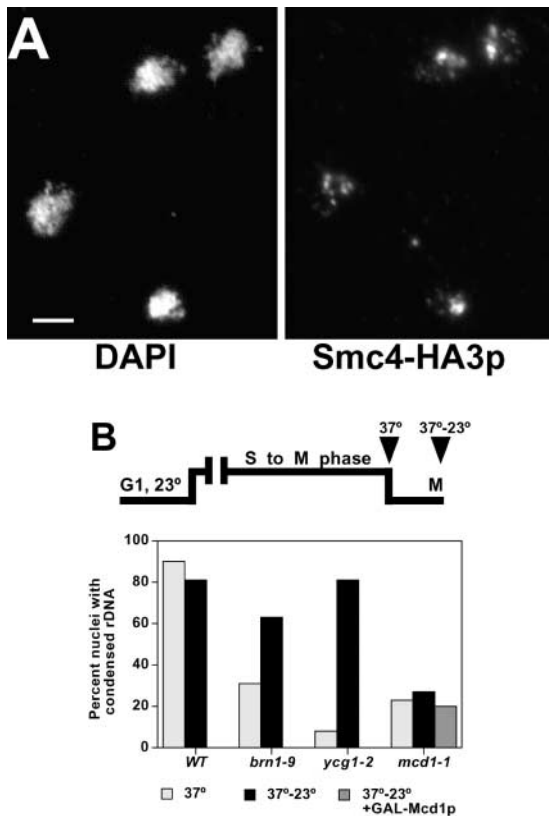


Figure 6. **Topo II and phospho-H3 are not essential for rDNA condensation.** CH325 (*top2-4*), JHY90 (WT), JHY91 (*H3-S10A*), and JHY93 (*H3-S10,28A*) cultures were synchronized in G1, and released into a Nz block either at 37°C (*top2-4*) or 23°C. After rearrest in M phase, cells were fixed and processed for rDNA FISH. rDNA loops were scored as condensed. Greater than 100 nuclei/sample were scored. Data for the condensins is from Fig. 2 and is shown for comparison.

### The interplay of condensins and cohesins

In contrast to histone H3 and topo II, mutations in components of the sister chromatid cohesion machinery do cause severe condensation defects; in fact, these appear indistinguishable from that seen in condensin mutants (Castano et al., 1996; Guacci et al., 1997; Hartman et al., 2000; Hanna et al., 2001). It is not known however how cohesins impact condensin function. One possibility was the cohesins might be important for condensin binding to chromatin. However, by chromatin spreads, Smc4–HA3p is readily detected on chromosomes in the cohesin mutant background (*mcd1-1*), and neither the levels of condensin binding nor its enrichment on the rDNA appear altered (Fig. 7 A). These data agree with previous reports that cohesins and condensins bind chromosomes independently (Losada et al., 1998; Toth et al., 1999); therefore, cohesins must affect condensation at a step after condensin binding.

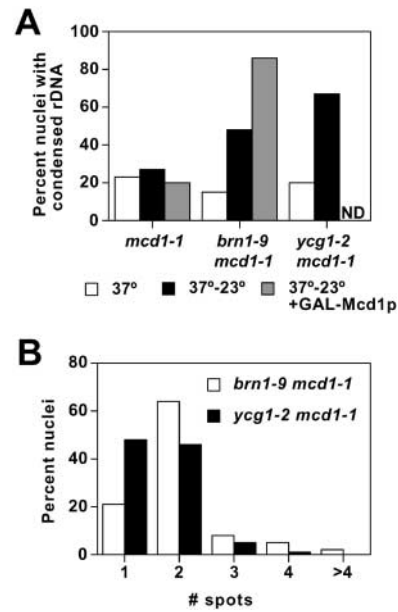
Two simple models can be envisaged: either cohesins are needed to activate condensin function or, alternatively, cohesins are required to ensure correct chromosome folding by condensins. These models can be distinguished by following the state of the mitotic chromosomes after a loss of cohesin activity. In the first scenario, the chromosomes remain in an interphase state, and thus would condense upon the readdition of cohesin and the subsequent “activation” of condensin. In contrast, the latter scenario predicts that misfolded chromosomes would result from the inappropriate action of condensin, and these would likely prove refractory to refolding. To test this, we asked whether chromosome condensation is reversible in the cohesin mutant *mcd1-1*. In contrast to both the *brn1-9* and *ycg1-2* condensin mutants, the condensation defect in the *mcd1-1* strain was not reversible (Fig. 7 B). One trivial explanation is that no new functional Mcd1-1p protein is made after the shift to the permissive temperature. Failure of the Mcd1-1p to refold, coupled with the fact that *MCD1* transcription is cell cycle regulated so that most expression occurs during S phase,



**Figure 7. Condensation is not reversible in the absence of cohesin.**

(A) Chromatin spreads of BLY13 (*mcd1-1 SMC4-HA3*). Strains were grown, spread, and stained for Smc4-HA3p as in Fig. 4. Bar, 2  $\mu$ m. (B) Reversibility of condensation. CH2523 (WT), CH2524 (*brn1-9*), BLY07 (*ycg1-2*), 985-7c (*mcd1-1*), and 985-7c-101 (*mcd1-1 GAL1-MCD1-HA6*) cultures were synchronized in G1, released, and rearrested in M phase (Nz, 37°C) as indicated in the schematic. Cells were taken for rDNA FISH either just before (37°C) or after a 1 h recovery at the permissive temperature (37°–23°C) (see Materials and methods). To ensure the presence of active Mcd1p in the *mcd1-1* mutant before the temperature downshift, tagged WT Mcd1p was integrated downstream of an inducible GAL1 promoter; 985-7c-101 cells were grown as above, except in YPRaff. After M phase arrest at 37°C, WT Mcd1p was induced 30 min before the shift to 23°C by the addition of 2% galactose to the culture. After a 1 h recovery at 23°C, the cells were fixed and rDNA condensation was assessed by FISH. Nuclei with rDNA loops were scored as condensed. In all experiments, >100 nuclei/sample were scored.

could result in a lack of functional protein (Guacci et al., 1997; Michaelis et al., 1997). To address this, a galactose-inducible *MCD1-HA6* allele was integrated into the *mcd1-1* strain. Mcd1-HA6p was then induced in M phase-arrested *mcd1-1* cells 30 min before temperature downshift, to allow functional protein to accumulate (as seen by Western blot; unpublished data). Although this newly expressed Mcd1p goes to cohesin sites appropriately (Megee, P., personal communication; unpublished data), it does not restore sister chromatid cohesion (Toth et al., 1999). Similarly, the production of functional Mcd1p in M phase did not rescue rDNA condensation despite the presence of condensin on chromosomes (Fig. 7). Thus, the decondensed chromosomes in cohesin and condensin mutants are neither structurally nor functionally equivalent.



**Figure 8. Reversal of condensation in condensin/cohesin double mutants.** (A) 985-7c (*mcd1-1*), 985-7c-101 (*mcd1-1 GAL1-MCD1-HA6*), BLY22 (*brn1-9 mcd1-1*), BLY22-101 (*brn1-9 mcd1-1 GAL1-MCD1-HA6*), and BLY25 (*ycg1-2 mcd1-1*) strains were grown as in Fig. 7. GAL1-inducible MCD1 strains were grown in the presence of raffinose before Mcd1p induction. After recovery at 23°C, the cells were fixed and rDNA FISH was performed. Nuclei with rDNA distinct from G1-type puffs were scored as condensed. In all experiments, >100 nuclei/sample were scored. (B) Separation of sister chromatids in the double mutants. *Brn1-9 mcd1-1* and *ycg1-2 mcd1-1* strains (as above) were grown as in A, but were analyzed by FISH using a CEN16 proximal probe to assess sister chromatid cohesion in Nz-arrested cells. Number of spots per nuclei (>100 per sample) were scored.

Although these data argue against an activator role for cohesin, they are consistent with a regulatory function. In this scenario, the inability of chromosomes to properly condense in cohesin mutants results from the malfunction of condensin. To demonstrate that the irreversible state observed in the *mcd1-1* strain was dependent on condensin activity, we generated double mutants. Both condensin and cohesin function were inactivated from G1 to M phase in *brn1-9 mcd1-1* and *ycg1-2 mcd1-1* strains. Upon return to the permissive temperature in M phase, the double mutants showed a dramatic restoration of condensation, indicating that chromosomes remain good substrates for folding when both condensin and cohesin are inactivated (Fig. 8 A). Furthermore, in contrast to that seen in the *mcd1-1* strain, the expression of functional Mcd1p before the temperature downshift stimulated the level of condensation in the *brn1-9 mcd1-1* strain, suggesting that cohesin action must precede that of condensin, as would normally occur in vivo. One explanation for the restoration of condensation in the double mutants versus the *mcd1-1* mutant is that condensin mutants suppress sister chromatid cohesion defects. To assess this directly, we performed FISH using a CEN16 proximal probe to monitor the extent of precocious separation of sisters in both *brn1-9 mcd1-1* and *ycg1-2 mcd1-1* cells, after the temperature downshift (Fig. 8 B). In both cases, two spots were

detected in >50% of the nuclei, indicating a precocious dissociation of sister chromatids that was not restored after the return to the permissive temperature. Thus, the failure of chromosome condensation in an *mcd1-1* mutant is unlikely to result from the irreversible loss of sister chromatid cohesion, because sister chromatid pairing per se does not appear essential for chromosome condensation.

From these results three important conclusions can be drawn. First, the inability to reverse the condensation defect of the *mcd1-1* single mutant cannot be attributed to the failure to produce functional Mcd1p, because similar levels of expression in the double mutant allow condensation. Second, the expression of Mcd1p in M phase, after sister separation has occurred, stimulates chromosome condensation in the double mutant, suggesting that cohesins may function in condensation independently of their role in cohesion. Third, the cause of the irreversibly misfolded state in the *mcd1-1* single mutant is apparently active condensin. Thus, cohesins are not needed to activate the enzymatic function of condensin; instead, they regulate condensin activity on chromosomes and promote correct folding.

## Discussion

### Requirements for chromosome condensation

The non-SMC subunits Ycg1p and Ycs4p are required for cell viability, chromosome segregation, and condensation. Our data corroborate earlier studies indicating that the condensin subunits are essential for condensation of all chromosomes (Saka et al., 1994; Strunnikov et al., 1995; Lieb et al., 1998; Sutani et al., 1999; Freeman et al., 2000; Lavoie et al., 2000; Ouspenski et al., 2000; Steffensen et al., 2001). Importantly, we have extended these data to show that in vivo, all three non-SMC subunits (including Brn1p) are required for condensin binding to chromosomes, which explains their requirement in both the establishment and maintenance of condensation. This finding has significant impact on models for the function of both SMC and non-SMC subunits in condensin. First, in the absence of the non-SMCs, the Smc2/4p heterodimer (called 8S complex and accounts for up to 50% of these subunits in *Xenopus* extracts) is not sufficient for chromatin binding in vivo. These data lend credence to in vitro findings where neither the 8S nor the 11S (containing the non-SMCs) were found to bind isolated chromatin, although the 8S did show weak DNA binding activity (Kimura and Hirano, 2000). Second, the fact that all three non-SMC condensins share a common in vivo function is consistent with their existence in the 11S regulatory complex (Kimura and Hirano, 2000). Indeed, in vitro, the phosphorylation of *Xenopus* HCAP-D2 (Ycs4p) promotes binding of condensin to mitotic chromosomes, thereby restricting condensin function to mitosis (Kimura et al., 1998). Interestingly, chromatin binding of budding yeast condensin also requires the non-SMCs, but this requirement does not appear to be cell cycle regulated (this study; Freeman et al., 2000) because the chromatin binding of condensin requires the non-SMCs in G1, when chromosomes are decondensed (unpublished data). Thus, the function of condensin is likely to involve additional regulatory mechanisms.

The characterization of multiple condensin subunits provided a basis to analyze the contribution of other potential condensation factors such as phospho-H3 and topo II. In fact, in budding yeast, neither of these proteins had been directly tested for a role in condensation, and we now show that in contrast to condensins, neither phospho-H3 nor topo II activity are required for condensation (this study). These results are consistent with the robust viability of histone H3 mutants in budding yeast (Hsu et al., 2000). In addition, studies in *Drosophila* found a poor correlation between H3 phosphorylation and chromosome condensation, suggesting that the link between phospho-H3 and condensation is not absolute (Adams et al., 2001). Similarly, we show that topo II activity is not required for condensation in budding yeast, however this contrasts with previous work in *S. pombe* (Uemura et al., 1987). One possible explanation for this difference is that condensation of the rDNA fundamentally differs from that at euchromatic sites. This seems unlikely because our work, and that of others, has shown that condensin function is closely mirrored between unique and repetitive DNA regions. In addition, the rDNA is a bona fide in vivo substrate of condensin, because both its morphology and transmission are condensin dependent (this study; Freeman et al., 2000; Lavoie et al., 2000; Ouspenski et al., 2000). Alternatively, the observed rDNA condensation in the topo II mutant could reflect the relatively small size of budding yeast chromosomes. Although a precise role for topo II in higher order chromosome folding remains controversial (Adachi et al., 1991; Hirano and Mitchinson, 1993), it is noteworthy that our results concur with recent in vivo studies of mammalian chromosome condensation in the presence of topo II inhibitors (Andreassen et al., 1997). Taken together, these data fail to provide compelling evidence that either phospho-H3 or topo II activity are ubiquitous components of the in vivo condensation machinery, and indicate that condensin remains the only functionally conserved component known to date.

### Reversibility of chromosome condensation in mitosis

Using a reversible condensin mutant, we demonstrate that condensation can occur twice in a single mitosis. Therefore, the process of condensation itself does not lead to an irreversible change in either chromosome structure or the condensin complex. This recondensation is not specific to the *brn1-9* allele, because the *yeg1-2* mutant also supports efficient chromosome refolding. Furthermore, the restoration of viability indicates that this recondensation is both morphologically and functionally correct. The condensation state of chromosomes can be modulated in mitosis through the activity of the non-SMC subunits, but, could the cell exploit this potential in a more physiological context? One possibility exists in vertebrate cells that initiate the decondensation of chromosomes and a reversal of the cell cycle after either irradiation in early prophase (Rieder and Cole, 1998) or a treatment with microtubule-depolymerizing drugs (Rieder and Cole, 2000). Our data suggest that the mechanism by which this decondensation and subsequent refolding occurs could involve changes in the activity of the non-SMCs. Consistent with this, *cdc2* kinase is down-regulated as early prophase nuclei revert into interphase nuclei (Rieder and Cole, 1998),



and this kinase controls the chromatin association of *Xenopus* condensin through phosphorylation of the non-SMC components (Kimura et al., 1998, 2001). It will therefore prove interesting to determine the phosphorylation state of condensins in irradiated prophase cells.

### Role of cohesins in condensation

In striking contrast to the reversibility of condensation in condensin mutants, an irreversible uncondensed rDNA state is produced in the absence of cohesin. This uncondensed rDNA can be distinguished both structurally and functionally from that produced in condensin mutants; in the absence of functional Mcd1p, condensin binds chromatin yet fails to promote condensation. We infer that condensin is active and misregulated because the irreversible, presumably misfolded, chromosome structure generated in the absence of cohesin is condensin dependent.

These data provide the first evidence of a mechanistic link between condensins and cohesins; therefore, these proteins are not acting in independent pathways. How then, do cohesins regulate chromosome condensation? As higher order chromosome structure is highly reproducible, it is likely to be orchestrated through cis determinants. These determinants could in principle be provided by site-specific binding of condensins. In fact, such sites have been suggested by a previous study (Freeman et al., 2000), however, we have been unable to reproduce these results using multiple tagged subunits (unpublished data). Alternatively, cohesin-defined domains could dictate condensin distribution along chromosomes (Guacci et al., 1997; Hartman et al., 2000). Cohesins are known to bind chromosomes regularly, roughly every 10 kb (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999; Laloraya et al., 2000), and this coincides with biochemical estimates of condensin (1 complex per 8–10 kb in *S. pombe*; Sutani and Yanagida, 1997). Cohesins could then act in a manner reminiscent of boundary elements, restricting condensin activity to defined domains and consequently imposing a regular array of loops. Indeed, the cohesin subunit Smc1 has been implicated in boundary element activity at the MAT mating type locus (Donze et al., 1999). In the absence of such “stops,” condensin function would not be confined to regions of chromatin, and this deregulation would preclude the normal higher order chromosome structure (Fig. 9).

One attractive feature of this model is that cohesins could act as cis determinants of condensation, independent of their function as molecular glue between sisters. As the cohesin/condensin double mutants effectively condense chromosomes in the absence of cohesion, it seems unlikely that cohesion itself would be an essential requirement for condensation. Indeed, condensation in *brn1-9 mcd1-1* cells is stimulated by the expression of functional Mcd1p in M phase, when cohesion can no longer be established (Toth et al., 1999). This is consistent with in vitro data where unreplicated chromosomes, which by definition lack cohesion, are substrates for condensin (Hirano et al., 1997). Ultimately, the distinction between the requirement for cohesin versus cohesion is biologically satisfying, because a role for cohesin proteins in condensa-

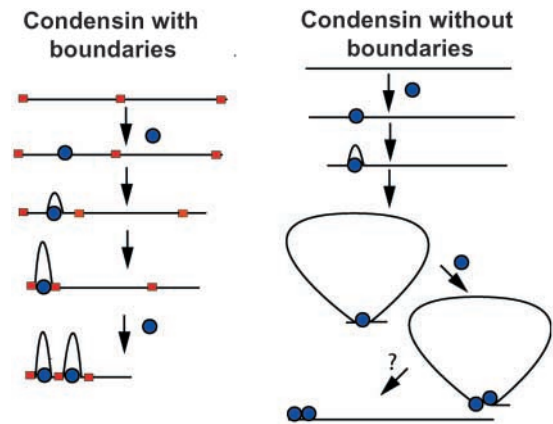


Figure 9. **Model for cohesin function in condensation.** Cohesin-bound sites (red squares) serve as “stops” to define cis boundaries of condensation. In the presence of cohesins, condensin (blue circles) functions between stops to generate an ordered chromosomal structure. When cohesins are disrupted, unregulated condensin activity no longer promotes the characteristic structure of mitotic chromosomes.

tion maintenance would explain how chromosomes traverse the metaphase to anaphase transition without inducing chromosome decondensation.

A second attractive feature of this model is the prediction that condensin function is regulated by cis determinants on chromosomes. Whether cohesins delimit domains of condensin action in all eukaryotes remains to be demonstrated. Current in vitro and in vivo studies suggest that in higher eukaryotes, the loss of cohesins does not abrogate chromosome condensation (Losada et al., 1998; Sonoda et al., 2001). Consistent with this, and in contrast to budding yeast, bulk cohesins are removed from chromosomes in prophase (Losada et al., 1998; 2000; Waizenegger et al., 2000), suggesting that low levels of cohesins are sufficient to promote condensation and/or additional regulatory factors are required in these systems. Indeed, the demarcation of condensation domains could be provided by other boundary-like elements such as AT-rich sequences, which have been proposed to act as cis determinants for higher order chromosome structure (Hart and Laemmli, 1998). Alternatively, condensin could modulate only a portion of the total mitotic chromosome compaction observed in higher eukaryotes, such that its loss or deregulation in the absence of cohesins would not eliminate condensation. This idea is consistent with in vivo data where a loss of condensin in flies causes partial defects in condensation (Bhat et al., 1996; Steffensen et al., 2001), and could explain why significant chromosome condensation was observed in Mcd1/Sccl-depleted TD40 cells (Sonoda et al., 2001). It does not, however, account for the dramatic loss of chromosome condensation in condensin-depleted extracts in vitro (Hirano et al., 1997), and further experiments will be required to resolve these issues. Indeed, because direct comparisons of condensin versus cohesin knockouts have as yet only been done in vivo in budding yeast, the extension of this approach to other organisms should prove helpful in determining the roles of cohesins and condensins in higher order chromosome dynamics.



Table I. *S. cerevisiae* strains used in this study

Strain	Mating type	Genotype	Background	Reference
YPH501	MATa/x	ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 lys2-801/lys2-801 ade2-101/ade2-101	S288C	Sikorski and Hieter, 1989
YPH499	MATa	ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101	S288C	Sikorski and Hieter, 1989
2627-1D	MATa	bar1 ura3 leu2 trp1 ade2 his3Δ200 gal1 can1	A364A	This study
BLY03	MATa	bar1 ura3 leu2 trp1 ade2 his3Δ200 gal1 can1 YCG1:KAN	A364A	This study
BLY04	MATa	bar1 ura3 leu2 trp1 ade2 his3Δ200 gal1 can1 ycg1-1-HA3:his5+	A364A	This study
BLY05	MATa	bar1 ura3 leu2 trp1 ade2 his3Δ200 gal1 can1 ycg1-2:KAN	A364A	This study
BLY06	MATa	ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101 YCG1:KAN	S288C	This study
BLY07	MATa	ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101 ycg1-2:KAN	S288C	This study
BLY08	MATa	ura3-52 lys2-801 ade2-101 trp1Δ63 leu2Δ1 his3Δ200 SMC4-HA3:his5+	S288C	This study
BLY10	MATa	ura3-52 trp1Δ63 leu2Δ1 HIS3? lys2-801 brn1-9:TRP1 SMC4-HA3:his5+	S288C	This study
BLY11	MATa	ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101 ycg1-2:KAN SMC4-HA3:his5+	S288C	This study
BLY12	MATa	ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 SMC4-HA3:his5+ HIS3:ycs4-2-MYC12	S288C	This study
BLY13	MATa	ura3-52 ade2-101 trp1Δ63 leu2Δ1 his3Δ200 SMC4-HA3:his5+ mcd1-1	S288C	This study
BLY22	MATa	trp1 ura3 leu2Δ1 brn1-9:TRP1 mcd1-1	other	This study
BLY22-101	MATa	mcd1-1 brn1-9:TRP1 ura3:URA3 GAL1-MCD1-HA6 trp1 leu2Δ1	other	This study
BLY25	MATa	ura3 trp1 leu2 lys2-801 ycg1-2:KAN mcd1-1 gal1	other	This study
BLY26	MATa	smc2-8 ura3-52 leu2Δ1 his3Δ200 ade2-101 lys2-801 SMC4-HA3:his5+	S288C	This study
CH2523	MATa	leu2Δ1 trp1Δ63 ura3-52 BRN1:TRP1	S288C	Lavoie et al., 2000
CH2524	MATa	brn1-9:TRP1 ura3-52 leu2Δ1 trp1Δ63	S288C	Lavoie et al., 2000
JHY90	MATa	ura3-52 ade2-101 his3Δ1 leu2-3,112 trp1-289 lys2-801 Δ(hht1-hhf1) Δ(hht2-hhf2)/CEN TRP1 HHT2 HHF2	S288C	This study
JHY91	MATa	ura3-52 ade2-101 his3Δ1 leu2-3,112 trp1-289 lys2-801 Δ(hht1-hhf1) Δ(hht2-hhf2)/CEN TRP1 hht2-3(H3-S10A) HHF2	S288C	This study
JHY93	MATa	ura3-52 ade2-101 his3Δ1 leu2-3,112 trp1-289 lys2-801 Δ(hht1-hhf1) Δ(hht2-hhf2)/CEN TRP1 hht2-5(H3-S10,28A) HHF2	S288C	This study
ZW206	MATa	bar1 ura3-52 leu2 his3Δ-200 trp1 ade2-101 HIS3:ycs4-2-MYC12	A364A	This study
CH325	MATa	ura3-52 his4-539amber lys2-801 SUC2+ top2-4	S288C	Holm et al., 1989
1aAS330	MATa	smc2-8 ura3 leu2 lys2 his3 ade2	S288C	Freeman et al., 2000
985-7c	MATa	mcd1-1 trp1 ura3 bar1 gal1	A364A	Guacci et al., 1997
985-7c-101	MATa	mcd1-1 trp1 ura3 bar1 gal1 ura3::URA3 GAL-MCD1-HA6	A364A	This study

## Materials and methods

### Media and reagents

Yeast cultures were grown in rich media (Guthrie and Fink, 1991) with 2% dextrose (YPD) or 2% raffinose for galactose inductions (YPRaff). Raffinose, α-factor, hydroxyurea (HU), and Nz were from Sigma-Aldrich. Restriction enzymes were from Boehringer. PCR cloning was performed using the TOPO-TA cloning kit (Invitrogen). rDNA and CEN16 proximal FISH probes were generated as previously described (Guacci et al., 1994). Antibodies used were as follows: mAb YOL1/34 (rat anti-tubulin; Serotec), mAb 12CA5 (anti-HA; BAbCo), mouse antidigoxigenin and pig anti-goat-FITC (Boehringer), goat anti-mouse-FITC (BAbCo), rabbit anti-GFP (CLONTECH Laboratories, Inc.), and goat anti-rat-FITC and CY3 anti-rabbit (Jackson ImmunoResearch Laboratories). G418 was from GIBCO BRL.

### Yeast strains and plasmids

Yeast strains are in Table I and were constructed using standard techniques (Guthrie and Fink, 1991). *YCG1* and *YCS4* genes were cloned in pRS316 (Sikorski and Hieter, 1989) or YCplac33 (Gietz and Sugino, 1988) by gap repair from YPH501-generating pBL235 and pZF1. Because of a sequence error in *Saccharomyces* Genome Database (missing G at position 1117105), the initiating methionine for Ycg1p is misidentified, predicting a protein that has a 16-amino acid NH<sub>2</sub>-terminal extension. The actual start codon is at 1117118. Both *ycg1::KAN* and *ycs4::KAN* knockouts were generated by single step gene replacement in YPH501, selecting for growth on 150 μg/ml G418. Diploids were sporulated and 20 tetrads were dissected. 2:2 segregation confirmed that both *YCG1* and *YCS4* are essential (no surviving spores were G418<sup>R</sup>).

Ts alleles were generated by cloning each gene into YCplac111, and random mutations were introduced using the XL1-RED mutator strain (Stratagene). Two ts alleles of *ycg1* and one of *ycs4* were isolated, integrated into YPH501 and 2627-1D using the *KAN* (G418<sup>R</sup>) marker, and

confirmed by colony PCR. All characterizations of ts alleles were performed in both A364A and S288C backgrounds and gave similar results. Strains containing ts alleles and tagged condensins were more readily isolated in S288C, thus chromatin spreads were done in this background.

To generate *SMC4-HA3*, single step gene replacement used the *S. pombe* his5<sup>+</sup> gene to complement *ura3* deficiency in YPH501. The 3xHA-his5<sup>+</sup> was amplified from p473 with 5' and 3' sequences corresponding to *SMC4*. Integration into the correct site was verified by colony PCR and Western blotting. After sporulation and dissection, 18/20 tetrads showed 4:0 viability and 2:2 segregation of the tagged allele. Galactose-inducible Mcd1p strains were generated by integration of NcoI-linearized pPCM87 (*URA3: GAL1-MCD1-HA6*; a gift from Paul C. Megee, University of Colorado, Denver, CO) into 985-7c and BLY22. The JHY90, JHY91, and JHY93 strains were gifts from J.Y.A. Hsu and M.M. Smith (University of Virginia, Charlottesville, VA).

### Cell growth and viability

Permissive and restrictive temperatures were 23°C and 37°C, respectively. For viability assays, strains were grown in YPD at 23°C, synchronized in M phase with 20 μg/ml Nz for 3 h, shifted to the restrictive temperature for 1 h, sonicated, counted, and plated. Percent viability was scored as the number of colony forming units divided by the number of cells plated (×100). For the M/G1 viability experiment, an aliquot from the M phase-arrested culture was taken and released from the Nz block by washing the cells three times in warmed YPD. The cells were then resuspended in their original volume of warmed YPD, containing α-factor (10<sup>-8</sup> M), and incubated for 1 h at 37°C until >85% of the cells had a schmoo morphology. The cells were then sonicated, counted, and plated.

### Cell cycle synchronization

Cultures were first synchronized in G1 (10<sup>-8</sup> M α-factor for *bar1* strains vs. 3 × 10<sup>-5</sup> M for *BART1*) for 2.5 h at 23°C (85–95% schmoos), shifted to 37°C

for 0.5 h, released by washing the cells three times with 37°C YPD containing 0.1 mg/ml pronase E (Sigma-Aldrich), and incubated for 2.5 h in 20 µg/ml Nz (37°C), as described in Lavoie et al. (2000). For indirect immunofluorescence (IIF) or FISH, cells were fixed with 0.36% formaldehyde for 1.5 h. Arrests were verified microscopically by cell and nuclear morphology and confirmed by FACS<sup>®</sup>. Rescues of condensation experiments were performed similarly except that cells were shifted back to 23°C for 1 h after only 2 h at 37°C, to maintain the arrest. Maintenance experiments were performed by synchronizing cells in G1, releasing into Nz at 23°C for 2 h, and then shifting to 37°C for 1 h. All mutants possessed >85% rDNA loops at 23°C. S phase arrests were performed by adding HU to cells (0.2 M final concentration) for 3 h at 23°C.

### IIF, chromatin spreads, and FISH

IIF of tubulin was performed using 1:500 Yo11/34 rat mAb-tubulin (Serotec) and 1:500 anti-rat-FITC (Sigma-Aldrich) as described in Lavoie et al. (2000). After G1 arrest, cultures were shifted to 37°C, released, and then followed microscopically until a maximal level of anaphase cells was reached (80–100 min after release). *Ycg1-1*, *ycg1-2*, and *ycs4-2* cells were then fixed and processed for IIF of tubulin and DAPI staining of chromosomes. Chromatin spreads were performed as previously described (Michaelis et al., 1997). Both mouse anti-HA and goat anti-mouse-FITC antibodies used for spreads were at 1:500. DNA was stained with DAPI. Images were obtained using a ZEISS epifluorescence microscope, and were recorded digitally with the use of a Princeton Scientific Instruments charge-coupled device with Scanalytics processing software, which allows image superimposition. To allow direct comparisons of signal between different chromatin spreads, identical exposures (1 s) were taken and background levels of the images were adjusted to the same range using the Scanalytics software. Images shown reflect what was observed directly through the microscope. Longer exposures (3 s) enhanced background signal in all of the samples including the untagged control (unpublished data). FISH was performed as previously described (Guacci et al., 1994; Lavoie et al., 2000) using digoxigenin-labeled rDNA or CEN16 proximal probes and FITC-conjugated secondary and tertiary antibodies. Chromosomes were counterstained with propidium iodide in antifade mounting medium (Intergen).

We thank Z.-F. Wang for the isolation of the *ycs4-2* allele, and P. Megee for communication of unpublished results and for providing PMY153 and pPCM87. We also thank V. Guacci (Fox Chase Cancer Center, Philadelphia, PA), B. Andrews (University of Toronto, Toronto, Canada), and P. Megee for critical reading of the manuscript.

D. Koshland is funded by the Howard Hughes Medical Institute.

Submitted: 18 September 2001

Revised: 18 January 2002

Accepted: 20 January 2002

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