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Sister Chromatid Separation in Budding Yeast

by

Needhi Bhalla

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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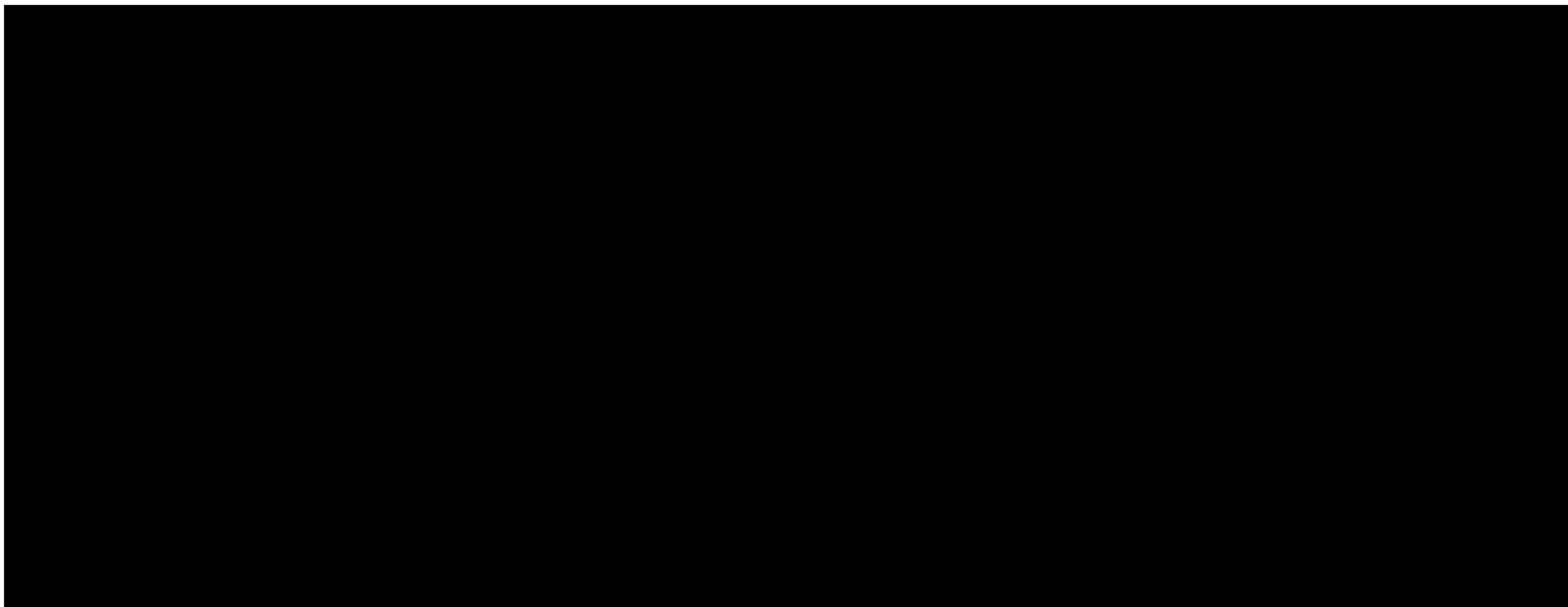

This work is dedicated to my family: my parents, Susheel and Asha Bhalla and my sister, Naina Bhalla. Everything that I am and all that I have accomplished is a testament to their love and support.

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ABSTRACT

Cell survival depends on the accurate transmission of a cell's genetic material to its daughters. Coordinating chromosome behavior with the cell cycle machinery ensures that the products of cell division are two viable and genetically identical progeny. Chromosomes replicate to produce two sister chromatids that are held together by topological and protein-mediated linkages. At anaphase, the protein and topological connections between sisters are quickly resolved to allow the separation and segregation of sister chromatids away from each other to opposite poles of the mitotic spindle.

We undertook two genetic screens to identify proteins required for sister chromatid cohesion and separation. We visually screened a temperature sensitive collection of mutants that contained a GFP marked chromosome for defects in sister chromatid cohesion in metaphase (precocious dissociation of sisters, *pds*) and sister chromatid separation at anaphase (loss of cohesion, *loc*). We identified fifteen *PDS* complementation groups and nine *LOC* complementation groups.

The budding yeast *YCS4* gene encodes a conserved regulatory subunit of the condensin complex. We isolated an allele of this gene in the *LOC* screen. The phenotype of the *ycs4-1* mutant is similar to topoisomerase II mutants and distinct from the *esp1-1* mutant: the topological resolution of sister chromatids is compromised in *ycs4-1* despite normal removal of cohesins from mitotic chromosomes. However, the *ycs4-1* mutant exhibits additional defects in chromosome behavior aside from those observed during sister chromatid separation; inter-repeat recombination in the rDNA array is elevated and transcriptional silencing is defective at the permissive temperature. Taken together, our

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CHAPTER ONE

Introduction

A successful mitotic cell cycle produces two genetically identical, viable daughter cells from the division of a mother cell. To ensure this, cells must faithfully replicate and transmit their chromosomes. Errors in chromosome segregation can result in genetic instability and the loss of tumor suppressor genes, hallmarks of tumor progression. One round of replication produces duplicate sister chromatids that are held together by topological and protein-mediated linkages. At the onset of mitosis, the chromatids condense into discrete bodies, converting the chromatids into physically strong, rod-shaped structures short enough to segregate away from each other. The condensed sister chromatids then attach to the mitotic spindle via a *cis*-acting DNA sequence, the centromere, and the proteins that assemble upon it, the combination of which is termed the kinetochore. During anaphase, the linkages between sisters are readily dissolved so that the sister chromatids can separate and segregate to opposite poles of the spindle. The anaphase spindle in yeast is 10 μm long, implying that the longest chromosome arm (1Mb) must be compacted at least 60 fold relative to the length it would occupy as naked DNA to allow full segregation of chromosome arms. Furthermore, this chromosome cycle must be coordinated with the cell cycle machinery so that cell division can be temporarily suspended in response to defects in some of these processes.

Sister Chromatid Separation and the Cell Cycle

The entry into mitosis is dependent on the activation of MPF, the maturation-, or mitosis-, promoting factor, originally identified in embryonic cell extracts and later shown to be the cyclin dependent kinase (CDK) complexed with its cyclin partner

(reviewed in (Morgan 1995). The exit from mitosis requires the inactivation of this complex, which is rapidly and irreversibly accomplished by the degradation of the cyclin B subunit (Murray, Solomon et al. 1989). The destruction of cyclin B is mediated by the cyclosome/ anaphase promoting complex (APC), a highly specific ubiquitin ligase that recognizes a short sequence in the substrate protein, termed the destruction box (Glotzer, Murray et al. 1991), and polyubiquitinates the protein, thus targeting it for proteolysis by the 26S proteasome (King, Peters et al. 1995; Sudakin, Ganoth et al. 1995). Initially, it was believed that cyclin B destruction, and thus MPF inactivation, provided the impetus for anaphase sister chromatid separation (Murray and Kirschner 1989; Shamu and Murray 1992). Additional investigation contradicted this hypothesis. Sister chromatid separation actually occurs independent of cyclin B degradation in both *Xenopus laevis* egg extracts (Holloway, Glotzer et al. 1993) and budding yeast (Surana, Amon et al. 1993; Straight, Belmont et al. 1996). Yet, APC activity is still fundamental to promoting sister separation. Sister chromatid separation will occur in the presence of high cyclin levels and active MPF in both *in vitro* extracts (Holloway, Glotzer et al. 1993) and yeast (Surana, Amon et al. 1993), but only if APC activity is not compromised. This point was elegantly illustrated when the addition of competitive inhibitors of proteolysis to *in vitro* egg extracts resulted in delays in sister chromatid separation (Holloway, Glotzer et al. 1993). These observations suggest the existence of at least one additional factor whose ubiquitin-mediated proteolysis is required for the separation of sister chromatids at anaphase.

The identity of this factor was revealed with the discovery of the anaphase inhibitor Pds1p (Yamamoto, Guacci et al. 1996; Yamamoto, Guacci et al. 1996), also

known as securin. Earlier studies in fission yeast had identified two essential genes, *CUT1* and *CUT2*, whose functions were required for sister chromatid separation (Funabiki, Kumada et al. 1996). The analysis of the Cut1 and Cut2 proteins offered the paradigm of an anaphase inhibitor, Cut2, whose destruction is required to release a sister separating activity, Cut1 (Funabiki, Yamano et al. 1996). Although *PDS1* shares no homology with *CUT2* and is essential only at 37°, the similarities are difficult to deny. Indeed, the initial incompatibility of the *pds1Δ* phenotypes at the non-permissive temperature makes sense in light of this model. The precocious dissociation of sister chromatids if cells are kept in metaphase for a long period of time is explained by the ultimate activation of the sister separating activity despite the absence of Pds1p (Yamamoto, Guacci et al. 1996). The defect in sister separation observed during a continuous cell cycle is the result of a delay in activation of the sister separating activity (Ciosk, Zachariae et al. 1998). This confusing paradox was hinted at by discovery that the *cut2Δ* and mutations in *CUT2* that prevent its destruction both exhibit an inability to separate sister chromatids (Funabiki, Yamano et al. 1996).

THE LINKAGE BETWEEN SISTER CHROMATIDS

Catenation

The topological linkage between sister chromatids is formed during DNA replication, most likely as a consequence of the collisions between replication forks that terminate DNA synthesis (Sundin and Varshavsky 1980; Sundin and Varshavsky 1981). It was hypothesized that the catenation of the newly replicated chromatid with the

template chromatid might provide the basis for sister chromatid cohesion (Murray and Szostak 1985). At anaphase, DNA topoisomerase II enzyme would resolve this intertwining so sisters can fully separate from each other. Indeed, observing chromosome dynamics in the absence of topoisomerase II supported this hypothesis; the inability to fully decatenate sister chromatids resulted in chromosome segregation defects, such as non-disjunction and chromosome breakage (DiNardo, Voelkel et al. 1984; Holm, Goto et al. 1985; Uemura, Ohkura et al. 1987; Shamu and Murray 1992). However, the importance of catenation in holding sister chromatids together may have been overestimated; minichromosomes introduced into budding yeast are not catenated after DNA replication nor at metaphase, even though they segregate with high efficiency at anaphase (Koshland and Hartwell 1987). These data suggest that catenation is neither necessary nor sufficient for accurate segregation. Recent technology allowing the visualization of the chromosomes and minichromosomes (Straight, Belmont et al. 1996) has brought the conclusions of this study into question. Minichromosomes, although accurately segregated, may not be true representatives of linear chromosome dynamics. Visual analysis reveals that minichromosomes separate prematurely in the presence of spindle forces (Tanaka, Cosma et al. 1999).

Protein Linkage: Cohesins

The cohesin complex is the protein linkage between sister chromatids (Guacci, Koshland et al. 1997; Michaelis, Ciosk et al. 1997) (for review see (Biggins, Severin et al. 1999; Nasmyth, Peters et al. 2000). It consists of two coiled-coil ATPases, Smc1p and Smc3p, and the regulatory subunits, Mcd1p/ Scc1p and Scc3p (Guacci, Koshland et al.

1997; Michaelis, Ciosk et al. 1997; Losada, Hirano et al. 1998; Toth, Ciosk et al. 1999; Tomonaga, Nagao et al. 2000). Mutations in any of these subunits result in defects in the establishment and maintenance of sister chromatid cohesion and inviability due to the massive chromosome missegregation that follows (Guacci, Koshland et al. 1997; Michaelis, Ciosk et al. 1997; Toth, Ciosk et al. 1999; Tomonaga, Nagao et al. 2000). The complex associates with chromosomes along the arms and is heavily concentrated at the centromeres (Blat and Kleckner 1999; Megee and Koshland 1999; Tanaka, Cosma et al. 1999), perhaps even directing association along chromosomes from its initial deposition at the centromeres (Megee, Mistrot et al. 1999). Indeed, centromere function and sister chromatid cohesion appear to be intimately involved as cohesin loading at centromeres requires functional kinetochore activity (Megee and Koshland 1999; Tanaka, Cosma et al. 1999) and the presence of cohesion at the centromeres ensures that kinetochores attach to the spindle microtubules correctly (Tanaka, Cosma et al. 1999).

In vitro biochemical analysis of cohesin activity indicates that it induces the formation of large protein-DNA aggregates and stimulates the formation of inter-molecular interactions between DNA species (Losada and Hirano 2001), as opposed to the inter-molecular interactions mediated by a similarly organized protein complex involved in condensation (see below) (Kimura, Rybenkov et al. 1999). However, the *in vitro* biochemical activities associated with the cohesin complex appear to be ATP-independent (Losada and Hirano 2001).

PDS5 is an additional player that cooperates with the cohesin complex to accomplish sister chromatid cohesion (Hartman, Stead et al. 2000; Panizza, Tanaka et al. 2000). The ability of *PDS5* to bind chromosomes and link sister chromatids is dependent

on the activity of cohesin (Hartman, Stead et al. 2000; Panizza, Tanaka et al. 2000). There is some confusion over whether the inverse is true.

Cohesion is established between sister chromatids during replication (Uhlmann and Nasmyth 1998; Skibbens, Corson et al. 1999; Toth, Ciosk et al. 1999). If cohesin is not provided until after replication, cohesion cannot be established despite its binding to chromosomes (Uhlmann and Nasmyth 1998), suggesting that the complex, in and of itself, cannot identify homology between sister chromatids and replication might provide a window during which sister chromatids are in close proximity. Proteins that establish the linkage between replicating chromosomes are required only during S phase; they are obsolete once S phase is completed and sister chromatids are linked to each other (Skibbens, Corson et al. 1999; Toth, Ciosk et al. 1999; Ciosk, Shirayama et al. 2000). Two proteins, Scc2p and 4p, function in securing cohesion between sister chromatids by loading the cohesin complex on duplicating chromosomes (Ciosk, Shirayama et al. 2000). Other genes, such as *ECO1/ CTF7*, although required for the establishment of cohesion, do not affect the loading of the cohesin complex (Toth, Ciosk et al. 1999), indicating that the presence of cohesins on chromosomes during S phase is not sufficient for cohesion.

There are several lines of evidence that suggest that DNA replication and the establishment of cohesion between sister chromatids is functionally as well as temporally coupled. An early indication was the discovery that multiple copies of *POL30*, which encodes the DNA replication processivity factor proliferating cell nuclear antigen (PCNA), suppress the temperature sensitivity of an *eco1/ ctf7* mutation (Skibbens, Corson et al. 1999). PCNA forms a doughnut-like sliding clamp that is loaded behind DNA polymerases at the replication fork (Krishna, Kong et al. 1994); it has also been

implicated in chromatin remodeling and as a landing pad for proteins not directly involved in DNA replication (Kelman 1997). A more direct link between sister chromatid cohesion and DNA replication has been presented with the report that functional DNA polymerase κ, encoded by the *TRF4* gene, is necessary for sister chromatid cohesion (Wang, Castano et al. 2000). The authors offer a model in which a polymerase switching event precedes the replication of cohesin-associated sites during S phase, analogous to the polymerase switching (from DNA polymerase α to DNA polymerase δ) accomplished by replication factor C (RFC). Such a model holds considerable merit, as an alternate RFC complex composed of Ctf8p, Dcc1p, Ctf18p, Rfc2p, Rfc3p, Rfc4p and Rfc5p, is required for sister chromatid cohesion as well (Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001).

Mitotic Chromosome Condensation

A complex similar to the cohesin complex is responsible for the compaction of mitotic chromosomes to facilitate their segregation. The condensin complex was isolated biochemically from *Xenopus* egg extracts and is required for mitotic chromosome condensation (Hirano and Mitchison 1994; Hirano, Kobayashi et al. 1997; Cubizolles, Legagneux et al. 1998). Like the cohesins, the condensin complex is composed of two coiled-coil ATPases of the SMC family, Smc2p and Smc4p, and three regulatory subunits, Brn1p, Ycs4p and Ycg1p, although the latter show no obvious homology between cohesins and condensins (Hirano 1999). The condensins can form loops in DNA molecules (Kimura and Hirano 1997; Kimura, Rybenkov et al. 1999) and promotes

renaturation of single stranded DNA *in vitro* (Sutani and Yanagida 1997). The idea that condensins accomplish condensation by the active reconfiguration of chromatin conforms to observations that condensation requires ATP hydrolysis (Kimura and Hirano 1997) and that members of the SMC family have predicted secondary structures resembling motor proteins that convert chemical energy into movement (Strunnikov, Larionov et al. 1993; Hirano and Mitchison 1994).

In frogs and fission yeast, the condensin complex associates only with mitotic chromosomes, consistent with its role in mitotic chromosome condensation. In *Xenopus*, this cell cycle regulation is imposed by the Cdc2 phosphorylation of the regulatory subunits of the complex (Kimura, Hirano et al. 1998). Fission yeast employs a similar mechanism to regulate condensin activity and access to its substrate: phosphorylation of Smc4p by Cdc2 enables nuclear import of the complex and its localization to chromosomes (Sutani, Yuasa et al. 1999). In budding yeast, however, this level of regulation is absent as most of the condensin subunits associate with chromosomes throughout the cell cycle (Freeman, Aragon-Alcaide et al. 2000; Bhalla, Biggins et al. 2002), suggesting that either the condensin complex plays roles in chromosome behavior in addition to the condensation of mitotic chromosomes or that budding yeast chromosomes do not undergo dramatic chromosomal rearrangement during mitosis.

Sister Chromatid Separation

Decatenation of Sister Chromatids

At the metaphase to anaphase transition, sister chromatids must be topologically resolved from one another. The physical unwinding of sister DNA molecules from each other to allow their separation depends on the action of the topoisomerase II enzyme (Holm, Goto et al. 1985; Uemura, Ohkura et al. 1987; Holm, Stearns et al. 1989; Shamu and Murray 1992). Depending on the state of the substrate DNA, topoisomerase II can either catenate or decatenate circular DNA molecules. What changes to favor decatenation at anaphase? We can exclude two obvious possibilities, microtubule-dependent forces and increased topoisomerase II activity. Sisters can separate in the absence of microtubules (Straight, Belmont et al. 1996; Straight, Marshall et al. 1997), and topoisomerase activity falls as *Xenopus* extracts enter anaphase (Shamu and Murray 1992).

An alternate possibility is that increasing DNA condensation favors decatenation, because two compact DNA molecules are less likely to collide with each other and become catenated than two extended DNA molecules (Holmes and Cozzarelli 2000). Thus condensation could promote sister separation by affecting the amount or directionality of topoisomerase II activity. Studies on the bacterial SMC homolog, *MukB*, support the latter possibility (Sawitzke and Austin 2000). Sawitzke and Austin found that the chromosome partitioning defects of the *mukB, mukE,* and *mukF* mutants in *E. coli* were suppressed by mutations in the bacterial topoisomerase I gene, *topA*. Reducing topoisomerase I activity allows DNA gyrase activity to increase the negative supercoiling of the nucleoid; in the absence of Muk function, this increased negative supercoiling provided a level of chromosome organization that allowed proper segregation of the nucleoid. In eukaryotes, it is possible that the action of the condensin

complex contributes to the decatenation of sister chromatids by introducing the higher-level organization typical of mitotic condensation (for reviews see (Koshland and Strunnikov 1996; Holmes and Cozzarelli 2000).

Removal of the Cohesin Complex

In addition to the decatenation of sister chromatids, the protein linkage between sister chromatids must be removed to allow the rapid separation of sister chromatids. A proteolytic cascade accomplishes this in budding yeast. The anaphase-promoting complex (APC) mediates destruction of the anaphase inhibitor Pds1p (Cohen-Fix, Peters et al. 1996). This liberates the sister separating activity of the budding yeast separin, Esp1p (Ciosk, Zachariae et al. 1998), a highly specific protease (Uhlmann, Wernic et al. 2000). Esp1p cleaves a cohesin subunit, Mcd1p/ Scc1p, resulting in the removal of the complex from the chromosomes and sister chromatid separation (Uhlmann, Lottspeich et al. 1999). Mutations in *MCD1/ SCC1* that prevent its cleavage phenotypically mimic mutation of *ESP1*, suggesting that the primary target of *ESP1* action during sister chromatid separation is *MCD1/ SCC1* (Uhlmann, Lottspeich et al. 1999). Furthermore, cleavage of *MCD1/ SCC1* completely dictates cohesin removal and anaphase progression since mutation of the *MCD1/SCC1* to a form that can be cleaved by an alternate, exogenous protease drives sister chromatid separation and spindle elongation (Uhlmann, Lottspeich et al. 1999).

In animal cells, the picture is slightly more complex as there are two points in the cell cycle when the cohesin complex is removed from duplicated, linked sister chromatids: during prophase and at anaphase. The removal of cohesin at anaphase,

primarily from centromeres, is dependent on securin degradation, separin activation (Waizenegger, Hauf et al. 2000) and Scc1p cleavage (Hauf, Waizenegger et al. 2001), indicating conservation of that aspect of the pathway. However, the bulk of cohesin is removed prior to anaphase during prophase and pro-metaphase (Losada, Yokochi et al. 2000; Waizenegger, Hauf et al. 2000) and this event does not involve cohesin cleavage (Waizenegger, Hauf et al. 2000). Rather it seems to depend on the phosphorylation of members of the cohesin complex that destabilize its interaction with chromosomes (Losada, Yokochi et al. 2000; Sumara, Vorlaufer et al. 2002). One study implicates Cdc2 as the kinase (Losada, Yokochi et al. 2000) while another focuses on Polo-like kinase (Sumara, Vorlaufer et al. 2002). This latter report is especially interesting given the recent revelation that the yeast Polo-like kinase, Cdc5p, is involved in phosphorylating Mcd1p/ Scc1p in preparation for its cleavage by Esp1p (Alexandru, Uhlmann et al. 2001). Elements of this pathway appear be conserved between vertebrate and yeast cells in modified forms. These modifications may reflect the differences in cell cycle timing that exist between yeast and animal cells (i.e. lack of substantive G2 in budding yeast cells).

Recent years have seen an explosion in the identification of players required for mitotic chromosome structure and behavior. Whether they directly affect sister chromatid cohesion or condensation, sister chromatid separation or segregation, it is obvious that there must be some interplay between these proteins, the various functions they perform on chromosomes and the structural nature of the chromosomes themselves. A goal of my graduate research was to gain an understanding of how these multiple

processes might individually and collectively contribute to the final outcome of a mitotic division: two genetically, identical viable daughter cells. To accomplish this, I, in collaboration with Sue Biggins, a post-doctoral fellow in the laboratory, undertook two genetic screens to isolate mutants defective in sister chromatid cohesion (precocious dissociation of sisters, *pds*) and sister chromatid separation (loss of cohesion, *loc*). I characterized the mutation of a conserved condensin subunit in budding yeast and determined the role the wildtype gene product played in sister chromatid resolution during anaphase, providing additional support of a model that postulated a role for chromosome condensation in resolving the topological linkage between sister chromatids. My thesis work also offered the surprising result that members of the budding yeast condensin complex influence non-mitotic chromosome behavior.

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CHAPTER TWO

Genes Involved in Sister Chromatid Cohesion in the Budding Yeast *Saccharomyces cerevisiae*

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Abstract

Proper chromosome segregation during mitosis requires that duplicated sister chromatids remain linked to one another until anaphase, at which time they must readily separate from one another to segregate to opposite poles of the mitotic spindle. To identify genes involved in sister chromatid cohesion, we microscopically screened a temperature sensitive collection of budding yeast mutants that contain a GFP-marked chromosome. We identified fifteen *PDS* (precocious dissociation of sisters) complementation groups. We determined that six *pds* complementation groups exhibit defects in the establishment rather than the maintenance of sister chromatid cohesion. We also compared the penetrance of the sister chromatid separation phenotype if the cells were arrested in metaphase by activation of the spindle assembly checkpoint or by inactivation of a subunit of the anaphase promoting complex. We isolated two new alleles of the *PDS1* complementation group and one allele each of the *IPL1* complementation group and *MPS1* complementation group. We cloned and partially subcloned sequences that complement six of the complementation groups

INTRODUCTION

Cell survival depends on the accurate transmission of a cell's genetic material to its daughters. Coordinating chromosome behavior with the cell cycle machinery ensures that the products of cell division are two viable and genetically identical progeny. Chromosomes replicate to produce two sister chromatids that are held together by topological and protein-mediated linkages. At anaphase, the protein and topological connections between sisters are quickly resolved to allow the separation and segregation of sister chromatids away from each other to opposite poles of the mitotic spindle.

The cohesin complex is required to hold sisters together (Guacci, Koshland et al. 1997; Michaelis, Ciosk et al. 1997) (for review see (Biggins, Severin et al. 1999; Nasmyth, Peters et al. 2000). It consists of two coiled-coil ATPases, Smc1p and Smc3p, and the regulatory subunits, Mcd1p/ Scc1p and Scc3p (Guacci, Koshland et al. 1997; Michaelis, Ciosk et al. 1997; Losada, Hirano et al. 1998; Toth, Ciosk et al. 1999; Tomonaga, Nagao et al. 2000); these proteins are loaded onto replicating chromosomes (Uhlmann and Nasmyth 1998; Toth, Ciosk et al. 1999). The establishment of cohesion during replication depends on a variety of proteins (Skibbens, Corson et al. 1999; Toth, Ciosk et al. 1999; Ciosk, Shirayama et al. 2000; Wang, Castano et al. 2000; Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001), some of which directly link the replication machinery to the cohesion machinery (Skibbens, Corson et al. 1999; Wang, Castano et al. 2000; Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001), suggesting that DNA replication and the establishment of cohesion between sister chromatids is functionally as well as temporally coupled. The topological linkage between sisters is also formed

during S phase, most likely as a consequence of the collisions between replication forks that terminate DNA synthesis (Sundin and Varshavsky 1980; Sundin and Varshavsky 1981).

In budding yeast, a proteolytic cascade results in sister separation at anaphase. The anaphase-promoting complex (APC) mediates destruction of Pds1p (Cohen-Fix, Peters et al. 1996), an inhibitor of a highly specific protease, Esp1p (Ciosk, Zachariae et al. 1998; Uhlmann, Wernic et al. 2000). Esp1p cleaves a cohesin subunit, Mcd1p/ Scc1p, driving the removal of the complex from the chromosomes and sister chromatid separation (Uhlmann, Lottspeich et al. 1999). At this point in the cell cycle, DNA topoisomerase II enzyme is also actively resolving the catenation between sisters so they can fully separate from each other (DiNardo, Voelkel et al. 1984; Holm, Goto et al. 1985; Uemura, Ohkura et al. 1987; Shamu and Murray 1992).

We report the identification of mutants that affect sister chromatid cohesion in budding yeast using strains whose chromosome IV is marked by the binding of a GFP-Lac repressor fusion to a tandem array of Lactose operators. We isolated temperature sensitive (ts) mutants and examined them microscopically to identify mutants that appear to precociously separate their sister chromatids (*PDS*, precocious dissociation of sister chromatids). We identified fifteen *PDS* complementation groups and determined whether they affected the establishment or maintenance of sister chromatid cohesion.

MATERIALS AND METHODS

Microbial techniques: Media and genetic and microbial techniques were essentially as described (Sherman, Fink et al. 1974; Rose, Winston et al. 1990). Stock solutions of inhibitors were made in DMSO and stored at -20°: 30 mg/ml benomyl (DuPont) in DMSO, 10 mg/ml nocodazole (Sigma) in DMSO, 10 mg/ml α-factor (Biosynthesis) in DMSO. For benomyl/nocodazole experiments, cells were released into 30 μg/ml benomyl and 15 μg/ml nocodazole at 37° unless otherwise noted. To visualize sister chromatids, copper sulfate was added to media at a final concentration of 0.5-0.25 mg/ml to induce the GFP-lacI fusion protein that is under the control the copper promoter.

Yeast strain constructions: Yeast strains are listed in Table 2-1 and were constructed by standard genetic techniques. Diploids were isolated on selective media at 23° and subsequently sporulated at 23°. The galactose-inducible, non degradable mitotic cyclin (*pGAL-Δ176-CLB2*) that is contained in some strains is not expressed in glucose media. The strains XL1-Blue and DH5α were used for all bacterial manipulations. The strain used for the screen was constructed by first deleting the *LYS2* gene in SBY3 by integrating pAR88 (gift of Adam Rudner) digested with *Xba* I. The *URA3* gene was then selected against on 5-FOA plates to obtain SBY181, which contains an unmarked *lys2Δ*. SBY181 was subsequently integrated with the following plasmids, respectively, to generate SBY215: *pGAL-Δ176-CLB2:LYS2* (pSB102) that was digested with *Bsp* EI, *pCUP-GFP12-LacI:HIS3* that was digested with *Nhe* I (pSB116) and *lacO:TRP1* (256 lactose operators on plasmid pAFS52) that was digested with *Eco* RV. All *cdc23-1pds* double mutants were constructed by crosses.

Isolation of *pds* mutants: The bank of temperature sensitive mutants generated for the loss of cohesion (*loc*) screen (Biggins, Bhalla et al. 2001) was simultaneously screened for mutants that exhibited precocious dissociation of sister chromatids (*pds*) as follows. We directly screened each ts mutant strain by microscopy to identify the *pds* phenotype. Microtiter dishes with media containing 10μg/ml nocodazole were inoculated from fresh patches of cells that were grown on plates at 23°. The microtiter dishes were shifted to 37° for four hours and placed on ice while we directly screened the cells by microscopy for GFP signals, ruling out mutants that exhibited re-budded cell morphology consistent with spindle assembly checkpoint mutants. We isolated 286 potential mutants during this primary screen. We next screened these mutants more carefully at the non-permissive temperature to determine the degree of precocious sister chromatid separation; the mutants that exhibited a quantitative defect of greater than 50% of metaphase arrested cells with separated sister chromatids were set aside for more intensive study; sixty-six strains passed this test. Since diploidization of the mutant strains was a possibility, we screened these sixty-six during α-factor arrest for two GFP signals. This analysis resulted in a final count of thirty-six *pds* mutants.

The *pds* mutant strains were crossed to SBY238 and the resulting diploids were tested for the ts phenotype and all were recessive. They were then backcrossed at least three times to SBY215 and re-tested for the sister chromatid cohesion defect by microscopy. *MATa* and *MATα* strains generated during backcrossing were used for complementation testing which determined that there were fifteen complementation groups.

***pds* linkage testing:** Two mutants fell into the *PDS1* complementation group. A linkage test was done on one, *pds1-294*, by crossing the mutant with a strain deleted for *PDS1* (*pds1Δ::LEU2*), sporulating the diploid and observing that the ts phenotype always segregated away from the *LEU2* phenotype.

***pds* mutant cloning:** The *pds* mutants were cloned by complementation of the ts phenotype using a centromere-based yeast genomic library as described (Hardwick and Murray 1995). Mutant strains that contained the plasmid pRS316 were cured of this plasmid by growth on 5-FOA media before transformation with the library. Plasmid DNA from colonies that grew at 37° was isolated and transformed into bacteria. The DNA was retransformed into each corresponding *pds* mutant and plasmids that conferred temperature resistance were sequenced. We identified the complementing region of the clones by subcloning various regions of the plasmids and testing for complementation of the ts phenotype.

Plasmid constructions: To determine the minimal complementing region of the genomic clones that suppressed each mutant, we tested either previously described plasmids or constructed subclones of the genomic plasmids. For *pds1-294* and *pds1-76*, a *PDS1* clone, pAY53 (gift of V. Guacci, Fox Chase Cancer Center), complemented the ts phenotypes. For *pds17-1*, genomic clone pNB8 was digested with *Eco* RI to eliminate 2940 base pairs of genomic DNA and the backbone vector and religated to create pNB19, which does not complement *pds17-1*. For *pds18-1*, genomic clone pNB9 was digested with *Sac* II and *Sph* I to eliminate 3950 base pairs of genomic DNA and the backbone vector, filled in with the Klenow fragment of DNA polymerase and religated to create

pNB16 which complements *pds18-1*. For *pds19-1*, an *IPL1* clone, pSB148 complements the ts phenotype. For *pds20-1*, genomic clone pNB10 was digested with *Hga* I and the 3795 base pair fragment was filled in with the Klenow fragment of DNA polymerase and ligated into YCp50 digested with *Nru* I. The resulting clone, pNB15, does not complement *pds20-1*. For *pds22-1*, the genomic clone pNB11 was digested with *Sph* I to eliminate 2400 base pairs of genomic DNA and backbone vector and religated to create pNB18, which complements *pds22-1*.

Microscopy: Microscopy to analyze sister chromatids was performed as described (Biggins, Severin et al. 1999).

RESULTS

Isolation of *pds* mutants: We performed microscopy on a bank of temperature sensitive yeast mutants with a GFP marked chromosome to isolate mutants defective in sister chromatid cohesion. A tandem repeat of lactose operators (lacO) was integrated at the *TRP1* locus, 12 kb from the centromere of chromosome IV, the largest chromosome. A GFP fusion to the lactose repressor (GFP-lacI) was expressed in these cells to allow visualization of chromosome IV. We generated a temperature sensitive (ts) bank of conditional yeast mutants in this strain by mutagenizing cells with EMS or UV and screening for lack of growth at 37°. We isolated approximately 2000 ts mutants that were subsequently screened for chromosome behavior defects using fluorescence microscopy.

The visual screen was conducted by examining cells arrested in metaphase. When treated with the microtubule-depolymerizing drugs nocodazole or benomyl, wildtype cells arrest in metaphase due to activation of the spindle assembly checkpoint (Hoyt, Trotis et al. 1991; Li and Murray 1991). This arrest is characterized by unseparated sister chromatids, so a single GFP signal is observed in a large budded cell. If a mutant is defective in establishing or maintaining sister chromatid cohesion, it will arrest as a large-budded cell in media containing nocodazole but will exhibit separated sister chromatids. Therefore we screened for large budded cells with two GFP signals instead of one. Mutants that are defective in activation of the spindle assembly checkpoint will also separate their sister chromatids when treated with media containing nocodazole because of the continuation of the cell cycle despite the absence of a spindle (Minshull, Straight et al. 1996; Straight, Belmont et al. 1996). To avoid the identification of checkpoint mutants, we also observed the presence of re-budding cells, indicating entry into the next cell cycle (Hoyt, Trotis et al. 1991; Li and Murray 1991; Weiss and Winey 1996). Any strains that exhibited re-budded cell morphology were not taken further. Each ts strain was shifted to the non-permissive temperature in media containing nocodazole (37°) for four hours and then screened by fluorescence microscopy for the number of GFP signals in large budded cells. In this primary screen, we isolated 286 mutant strains from a total of 2000 ts mutant strains. Sixty-six of these were mutants where 50% or more of the large budded cells contained two GFP signals.

Two GFP signals can be observed in a metaphase arrested cell if the strain has diploidized and now contains two copies of the GFP marked chromosome. To rule out this possibility we treated each strain with mating pheromone (α-factor) to arrest it in G1.

If the mutant is defective in sister chromatid cohesion, the strain should have a single GFP signal in α-factor arrest and two in metaphase arrest. Thirty-six mutants passed this test. We tested whether the mutants were recessive or dominant by crossing each to a wild type strain and testing each resulting diploid for temperature sensitivity. All of the mutants were recessive. We backcrossed each *pds* mutant at least three times and re-tested the mutants for the sister chromatid cohesion defect after each backcross to ensure that the phenotype was linked to the temperature sensitivity and linked to a single genetic locus. Eighteen mutants remained after these criteria. Some mutants exhibited phenotypes weaker than the initial screening phenotype upon the completion of backcrossing but were maintained (Table 2-2). Others lost the ability to arrest well in media containing nocodazole, which may be an indication of the sickness of the strain upon shift to the non-permissive temperature (Table 2-2). *MATa* and *MATα* strains generated during backcrossing were used for complementation testing between each other and with genes known to be involved in sister chromatid cohesion (*PDS1* and *5*, *MCD1/SCC1* and *SMC1* and *3*). We had identified sixteen complementation groups: fourteen were unique *PDS* complementation groups. Two mutants did not complement a *pds1Δ* mutant and were complemented by a plasmid containing *PDS1*. We isolated 2 alleles each of the *PDS12* and *15* complementation groups and single alleles of the other complementation groups (Table 2-2).

Genes involved in sister chromatid cohesion can be involved in the establishment of the linkage during replication, the maintenance of the linkage until anaphase or both. We sought to determine whether any of the new *pds* mutants fell into the former category; mutants that affect both would be hard to differentiate from mutants that

affected only maintenance. We treated the strains with media containing benomyl and nocodazole for two hours at the permissive temperature; the strains arrest in metaphase with unseparated sister chromatids because the mutant gene product is functional at the permissive temperature. After these two hours, we shift the arrested cells to the non-permissive temperature. If the cells exhibit separated sister chromatids in this experiment (large budded cells with two GFP signals), it suggests the gene product is required to maintain the linkage between sister chromatids. If the sister chromatids remain linked (large budded cells with a single GFP signal), it indicates the gene product is required to establish but not maintain the cohesion between sisters. The linkage between the sisters is established during the initial two hour incubation at the permissive temperature. With the temperature shift to the non-permissive temperature, there is no longer a requirement for the gene product, functional or not. Table 2-2 illustrates the identification of several mutants that appear defective in the establishment of sister chromatid cohesion: *pds10-1, 12-1, 13-1, 14-1, 15-1* and *17-1.*

APC mediated proteolysis of the anaphase inhibitor Pds1p (Cohen-Fix, Peters et al. 1996) promotes sister chromatid separation by releasing the separin, Esp1p (Ciosk, Zachariae et al. 1998; Uhlmann, Wernic et al. 2000) to cleave the cohesin subunit Mcd1p/Scc1p and drive cohesin removal and sister chromatid separation (Uhlmann, Lottspeich et al. 1999). *CDC23* is a subunit of the APC (Lamb, Michaud et al. 1994; Zachariae and Nasmyth 1996); mutations in this gene arrest in metaphase with unseparated sister chromatids. We constructed *pdscdc23-1* double mutants to observe the defects in sister chromatid cohesion in the absence of APC activity and compare these data with the phenotype displayed in when cells were treated with nocodazole (Table 2-

2). In general, the penetrance of the phenotype was comparable whether the cells were arrested in metaphase by inactivation of APC function or activation of the spindle assembly checkpoint. This is not surprising given that the metaphase arrest that results from checkpoint activation is product of the binding and inhibition of the APC activator Cdc20p by the checkpoint component Mad2p (Hwang, Lau et al. 1998). However, some mutants consistently exhibited a more penetrant phenotype when arrested in metaphase by APC inactivation, for example *pds12-2* (although *pds12-1* does not exhibit this), *pds15-2* (*pds15-1* also does not exhibit this), *pds19-1* and *pds20-1*.

Identification of the genes encoding the *pds* mutants: To continue characterization of the *pds* mutants, we cloned a subset of them by complementation of the ts phenotype using a centromere-based genomic library. Genomic clones were subcloned to isolate the minimal complementing region of DNA. We determined that:

pds17-1 was complemented by an incomplete copy of *UGO1*, *RPL278*, *SNR13* or *TRS31*.

pds18-1 was complemented by an incomplete copy of *AIR1*, *THS1*, or *YIL077C*.

pds19-1 was complemented by a plasmid containing *IPL1*.

pds20-1 was complemented by an incomplete copy of *YGR046W* or an incomplete copy of *UFD1*.

pds22-1 was complemented by an incomplete copy of *RPL37A*, *EMG1*, or an incomplete copy of *YLR187W*.

pds23-1 was complemented by a genomic clone that contained *MPS1*. No further subcloning was attempted given the poor metaphase arrest of this mutant in nocodazole (Table 2-2), indicating a defect in the spindle assembly checkpoint.

Discussion

We identified fifteen *PDS* complementation groups by screening temperature-sensitive strains containing a GFP-marked chromosome for defects in sister chromatid cohesion. We classified whether these mutants were involved in establishment or maintenance of sister chromatid cohesion. We compared the penetrance of the mutant phenotype when the cells are arrested in metaphase by spindle checkpoint activation (treatment with the mictrotubule-depolymerizing drug nocodazole) with cells arrested in metaphase by APC inactivation (*pds* mutants combined with the *cdc23-1* mutant). One complementation group is complemented by *PDS1*, suggesting that we have identified two additional alleles of this gene. One complementation group (*pds19*) is complemented by *IPL1* and another (*pds23*) by *MPS1*, suggesting our attempts to rule out checkpoint mutants were not entirely successful.

We identified 6 *pds* mutants that appear defective in the establishment of sister chromatid cohesion. Once cohesion is established and the cells are shifted to the non-permissive temperature, there is no longer a cohesion defect in these mutants. Cohesion between sister chromatids is established during DNA replication; therefore it is possible that the wildtype gene products either play a role in replication or link the replication machinery to the cohesion machinery. The DNA content of the mutants at the non-permissive temperature was not analyzed but the presence of two GFP signals in metaphase at the non-permissive temperature (the primary screen) suggests that there is no defect in replication, at least of a centromere-proximal locus. Of the six, one, *pds17-1*,

was cloned. The minimal complementing sequence thus far reveals no information regarding its role in replication or cohesion.

The link that exists between APC activity and sister chromatid separation in anaphase led us to analyze the degree of the cohesion defects of the mutants we identified in a mutant background that lacked APC activity, *cdc23-1*. Most of the mutants exhibited comparable penetrance of the cohesion defect if arrested in metaphase by checkpoint activation or APC inactivation. However, a small subset exhibited a greater defect in the *cdc23-1* background. Mutants that affect the spindle assembly checkpoint have been shown to bypass the metaphase arrest of APC mutants (A. Rudner and B. Stern, personal communication). In fact, when we combined the *cdc23-1* mutation with a deletion of the spindle assembly checkpoint component *MAD1* (Li and Murray 1991), there was an increase in sister chromatid separation (43%) at the non-permissive temperature (data not shown). The inclusion of *pds19-1*, a potential *ipl1* mutant, in this group and the recent report of *IPL1*'s role in the checkpoint (Biggins and Murray 2001) raises the possibility that the *pds* mutants that exhibit greater sister chromatid separation in the *cdc23-1* arrest play a role in the spindle assembly checkpoint. The fact that some (*pds19-1* and *pds20-1*) arrest well in nocodazole may indicate that they are involved in the alternate tension-sensing branch of the checkpoint. *ipl1* mutants provide such an example (Biggins and Murray 2001). Furthermore, that the remaining two (*pds12-2* and *pds15-2*) have alternate alleles (*pds12-1* and *pds15-1*) that do not arrest well in nocodazole suggests that they may truly be defective in checkpoint function and the two alleles in each complementation group may represent separation of function alleles that specify the kinetochore attachment and tension sensing branches of the checkpoint. The sister chromatid

separation observed in *pds12-1* and *pds15-1* cells arrested in nocodazole may represent the continuation of the cell cycle despite the absence of a spindle, a hallmark of checkpoint mutants (Hoyt, Trotis et al. 1991; Li and Murray 1991; Minshull, Straight et al. 1996; Straight, Belmont et al. 1996; Weiss and Winey 1996). An interesting re-approach to this experiment would be to determine if Pds1p, the anaphase inhibitor and a target of APC-mediated proteolysis (Cohen-Fix, Peters et al. 1996), is absent in the nuclei of *pdscdc23-1* double mutants that separate their sister chromatids. This would provide insight into whether the sister chromatid separation we observe at the non-permissive temperature is a consequence of a true cohesion defect or the bypass of the metaphase *cdc23-1* arrest.

A subset of the mutants was cloned. The sequence of the minimal complementing regions thus far has not proved informative. *pds20-1* is complemented by a sequence that includes the C-terminal fragment of *YGR046W* (the last 591 base pairs of the gene) and the N-terminal fragment of *UFD1* (the first 506 base pairs of the gene). It is possible that this latter fragment complements the mutant. It was recently reported that the N-end rule degradation pathway targets the cleaved Mcd1p/ Scc1p fragments for destruction and mutations in this pathway affect chromosome stability (Rao, Uhlmann et al. 2001). The ubiquitin fusion degradation pathway (UFD) and the N-end rule pathway share components (Johnson, Ma et al. 1995). *UFD1*, a member of the UFD pathway involved in post-ubiquitination steps (Johnson, Ma et al. 1995), may be contributing to the process of sister chromatid cohesion. However, it is not readily apparent how the loss of a component of the Mcd1p/ Scc1p degradation pathway might result in precocious dissociation of sister chromatids.

Our preliminary analysis thus far suggests that multiple gene products influence sister chromatid cohesion. Further investigation is required to reveal whether the novel *PDS* complementation groups identified in this screen contribute directly or indirectly to sister chromatid cohesion.

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Table 2-1. Yeast strains used in this study

Strain	Genotype
SBY3	*MAT**a** ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 bar1Δ*
SBY181	*MAT**a** ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
SBY186	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1*
SBY214	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ*
SBY215	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2*
SBY238	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ* [pRS316]
SBY286	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds12-1 (7A6G)*
SBY287	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds12-1 (7A6G)*
SBY288	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds12-1 (7A6G)*
SBY289	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds12-1 (7A6G)*
SBY294	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds1-294 (7A1F)*
SBY295	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds1-294 (7A1F)*
SBY296	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds1-294 (7A1F)*
SBY297	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds1-294 (7A1F)*
SBY298	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds13-1 (8A4E)*

SBY299	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds13-1 (8A4E)*
SBY300	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds13-1 (8A4E)*
SBY301	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds13-1 (8A4E)*
SBY302	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds14-1 (8B5F)*
SBY303	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds14-1 (8B5F)*
SBY304	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds14-1 (8B5F)*
SBY305	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds14-1 (8B5F)*
SBY306	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds15-1 (9B4H)*
SBY307	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds15-1 (9B4H)*
SBY308	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds15-1 (9B4H)*
SBY309	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds15-1 (9B4H)*
SBY311	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds10-1 (6A2G)*
SBY312	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds10-1 (6A2G)*
SBY313	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds10-1 (6A2G)*
SBY314	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds10-1 (6A2G)*

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SBY316	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds11-1 (6B1E)*
SBY317	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds11-1 (6B1E)*
SBY318	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds11-1 (6B1E)*
SBY339	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds10-1 (6A2G)*
SBY340	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds1-295 (7A1F)*
SBY341	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds12-1 (7A6G)*
SBY342	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds13-1 (8A4E)*
SBY346	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds15-1 (9B4H)*
SBY347	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds11-1 (6B1E)*
NBY76	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds1-76 (16B4B)*
NBY78	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds1-76 (16B4B)*
NBY156	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds17-1*
NBY157	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds17-1*
NBY158	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds18-1*
NBY159	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds18-1*

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NBY160	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds19-1(ipl1-205)*
NBY161	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds19-1(ipl1-205)*
NBY162	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds16-1*
NBY163	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds16-1*
NBY164	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds15-2*
NBY165	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds15-2*
NBY166	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1 pds 21-1*
NBY167	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds21-1*
NBY168	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds22-1*
NBY169	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds22-1*
NBY170	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1*
NBY171	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc23-1*
NBY194	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds17-1 (12A4C)* [pRS315] [pRS316]
NBY195	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds17-1 (12A4C)* [pRS316]
NBY196	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds17-1 (12A4C)* [pRS316]

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NBY197	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds18-1 (12A6G)*
NBY198	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds18-1 (12A6G)* [pRS316]
NBY199	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds18-1 (12A6G)*
NBY200	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds18-1 (12A6G)*
NBY201	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds20-1 (13B2H)*
NBY202	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds20-1 (13B2H)*
NBY203	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds20-1 (13B2H)* [pRS316]
NBY204	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds20-1 (13B2H)*
NBY205	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds19-1 (ipl1-205/ 13B5C)*
NBY206	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds19-1 (ipl1-205/ 13B5C)*
NBY207	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 pds16-1 (14A3A)*
NBY208	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds16-1 (14A3A)* [pRS316]
NBY209	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds16-1 (14A3A)* [pRS316]

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NBY225	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds23-1 (mps1-224/ 21A6F)*
NBY227	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23- 1 pds20-1*
NBY228	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23- 1 pds20-1*
NBY229	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ: pGAL-Δ176-CLB2:LYS2 cdc23-1 pds12-2*
NBY230	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23- 1 pds12-2*
NBY231	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds23-1 (mps1-224)*
NBY232	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 pds23-1 (mps1-224)*

All strains are isogenic with the W303 background. Plasmids are indicated in brackets. All strains were constructed for this study unless indicated.

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Table 2-2. *pds* mutants

		+ noc, 4 hrs 37°		+ noc, 2 hrs 23° 2 hrs 37°		*pdscdc23-1* double mutants	
Mutant	Allele?	% large-budded	% separated sisters[2]	% large-budded	% separated sisters[2]	% large-budded	% separated sisters[2]
WT		good/ 86	13	81	14		
cdc23-1						85	17
pds1-76[1]	16B4B	good	61				
pds1-294	7A1F	good	61	73	40	73	80
pds10-1	6A2G	poor	52	72	27	66	60
pds11-1	6B1E	poor	63	55	61		
pds12-1	7A6G	poor	60	69	19	72	62
pds12-2	14A5F	61	25	78	20	77	53
pds13-1	8A4E	poor	73	58	23	72	77
pds14-1	8B5F	poor	64	62	23		
pds15-1	9B4H	poor	68	70	16	74	55
pds15-2	18B2G	85	20	83	21	85	48
pds16-1	14A3A	86	70	89	45	85	50
pds17-1	12A4C	79	51	81	27	84	51
pds18-1	12A6G	59	62	82	57	67	55
pds19-1	*ipl1-205*[1]	77	32	81	29	80	50
pds20-1	13B2H	82	37	80	32	84	57
pds21-1	19A2G	88	44	86	41	82	53
pds22-1	20B5F	92	39	83	35	82	47
pds23-1	*mps1-224*[1]	33	60	38	50	42	

[1]allelism has not been demonstrated

[2]sister chromatid separation in large-budded cells only (not rebudded cells)

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CHAPTER THREE

Genes Involved in Sister Chromatid Separation and Segregation in the Budding Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Accurate chromosome segregation requires the precise coordination of events during the cell cycle. Replicated sister chromatids are held together while they are properly attached to and aligned by the mitotic spindle at metaphase. At anaphase, the links between sisters must be promptly dissolved to allow the mitotic spindle to rapidly separate them to opposite poles. To isolate genes involved in chromosome behavior during mitosis, we microscopically screened a temperature sensitive collection of budding yeast mutants that contain a GFP-marked chromosome. Nine *LOC* (loss of cohesion) complementation groups that do not segregate sister chromatids at anaphase were identified. We cloned the corresponding genes and performed secondary tests to determine their function in chromosome behavior. We determined that three *LOC* genes, *PDS1*, *ESP1* and *YCS4*, are required for sister chromatid separation and three other *LOC* genes, *CSE4*, *IPl1*, and *SMT3*, are required for chromosome segregation. We isolated alleles of two genes involved in slicing, *PRP16* and *PRP19*, which impair alpha tubulin synthesis thus preventing spindle assembly, as well as an allele of *CDC7* that is defective in DNA replication.

INTRODUCTION

Accurate cell division depends upon the proper segregation of chromosomes into daughter cells. When chromosomes replicate during S phase, cohesion between the sister chromatids is established and must be maintained while chromosomes condense and align on the mitotic spindle. Chromosomes attach to the mitotic spindle by their kinetochores, specialized protein structures that are assembled on centromeric DNA sequences. Once all the chromosomes are correctly aligned on the mitotic spindle, the cohesion between sister chromatids must dissolve promptly at anaphase to allow the sister chromatids to rapidly segregate to opposite poles of the mitotic spindle. Accurate chromosome segregation depends on precisely timed sister chromatid separation (the destruction of the linkage between sisters) and chromosome segregation (the movement of the separated sister chromatids to opposite poles of the spindle).

A number of proteins involved in sister chromatid cohesion and separation have been identified (for review, see (NASMYTH et al. 2000)). A conserved complex, cohesin, is required to establish and maintain the link between sisters. The cohesin complex contains two homologous ATP binding proteins, Smc1 and Smc3, in addition to the Scc3/Irr1 and Scc1/Mcd1 proteins (GUACCI et al. 1997; MICHAELIS et al. 1997). The budding yeast cohesins associate with chromosomes during DNA replication and remain bound until the metaphase to anaphase transition (MICHAELIS et al. 1997; UHLMANN and NASMYTH 1998). At the onset of anaphase, the Esp1 protein cleaves Scc1/Mcd1p, leading to sister chromatid separation (UHLMANN et al. 1999; UHLMANN et al. 2000). Prior to anaphase, Esp1 is inactive due to binding of the Pds1 protein, which inhibits its activity (CIOSK et al. 1998). Anaphase is initiated by the ubiquitin-mediated proteolysis of Pds1p, which is triggered by activating the cyclosome/APC complex (COHEN-FIX

et al. 1996). In addition to the cohesins, additional proteins have been identified that are required to establish cohesion during DNA replication (CIOSK et al. 2000; SKIBBENS et al. 1999; TOTH et al. 1999).

After establishing cohesion, chromosomes must condense to ensure accurate chromosome transmission (for review, see (HIRANO 2000)). Condensation is regulated at least in part by the condensins, a conserved complex related to the cohesin complex (HIRANO et al. 1997; HIRANO and MITCHISON 1994). The budding yeast condensin complex contains two related Smc protein homologs, Smc2 and Smc4, as well as three regulatory proteins, Brn1, Ycs4 and Ycs5 (FREEMAN et al. 2000; LAVOIE et al. 2000; OUSPENSKI et al. 2000; STRUNNIKOV et al. 1995). The condensin complex is essential for chromosome condensation in higher eukaryotes. In budding yeast, it is essential for condensation of the repetitive ribosomal DNA (rDNA) (FREEMAN et al. 2000). The yeast condensin complex localizes to the nucleolus and is required to segregate the rDNA at mitosis. There is a link between budding yeast chromosome condensation and cohesion because two proteins required for cohesion, Pds5 and Scc1/Mcd1, have defects in condensation (GUACCI et al. 1997; HARTMAN et al. 2000).

Chromosome segregation also depends on mitotic spindle and kinetochore functions (for review, see (PIDOUX and ALLSHIRE 2000)). A protein complex called CBF3, which contains four components (Skp1p, Ndc10p, Ctf13p and Cep3p), assembles onto budding yeast centromeric DNA and is essential for kinetochore function (KAPLAN et al. 1997; LECHNER and CARBON 1991; SORGER et al. 1995; STEMMANN and LECHNER 1996). In addition to CBF3, the yeast kinetochore contains homologs of higher eukaryotic centromere proteins such as the Cse4, Mif2 and Ipl1 proteins (MELUH and KOSHLAND 1995; MELUH and KOSHLAND 1997; MELUH et al. 1998)(S. B. and A. W. M., unpublished data). Cse4p is a conserved Histone H3 variant that is

the homolog of CENP-A in mammalian cells and is thought to form a specialized nucleosome structure at the kinetochore (STOLER et al. 1995). Cse4p localizes to yeast kinetochores and *cse4* mutants have defective kinetochore function and altered centromeric chromatin structure (MELUH et al. 1998; STOLER et al. 1995). The Mif2 protein, a homolog of CENP-C in higher eukaryotes, is also required for kinetochore function and localizes to kinetochores (BROWN et al. 1993; MELUH and KOSHLAND 1995; MELUH and KOSHLAND 1997). The *IPL1* gene encodes a conserved protein kinase called *Aurora* in higher eukaryotes that regulates kinetochore function in budding yeast (BIGGINS et al. 1999; FRANCISCO et al. 1994). Ipl1p phosphorylates the CBF3 component Ndc10p *in vitro*, suggesting that it may regulate kinetochore function at least in part via Ndc10p phosphorylation (BIGGINS et al. 1999). The fidelity of kinetochore function is monitored by the spindle assembly checkpoint, which arrests cells in metaphase until defects in chromosome atttachment are corrected (for review, see (GARDNER and BURKE 2000)).

We report the identification of mutants that affect chromosome separation and segregation using strains whose chromosome IV is marked by the binding of a GFP-Lac repressor fusion to a tandem array of Lactose operators. We isolated temperature sensitive (ts) mutants and examined them microscopically to identify mutants that appear to be defective in the separation of sister chromatids at anaphase (*LOC*, loss of cohesion). We identified 9 *LOC* complementation groups and used secondary tests to determine whether they affect either sister chromatid separation or segregation.

MATERIALS AND METHODS

Microbial techniques: Media and genetic and microbial techniques were essentially as described (ROSE et al. 1990; SHERMAN et al. 1974). All experiments where cells were released from a G1 arrest were carried out by adding 1 μg/ml α-factor at the permissive temperature (23°) for four hours, washing cells twice in α-factor-free media and resuspending them in pre-warmed media at 37°. When cells started to bud, α-factor was added back to prevent cells from entering the next cell cycle. All experiments were repeated at least twice with similar results and at least 100 cells were counted at each time point. Stock solutions of inhibitors were made in DMSO and stored at -20°: 30 mg/ml benomyl (DuPont) in DMSO, 10 mg/ml nocodazole (Sigma) in DMSO, 10 mg/ml α-factor (Biosynthesis) in DMSO, 5 mg/ml doxycycline (Sigma). For benomyl/nocodazole experiments, cells were released into 30 μg/ml benomyl and 15 μg/ml nocodazole at 37°. For the *CSE4* repression experiments, 5 μg/ml doxycyline was added when cells were released from G1. To visualize sister chromatids, copper sulfate was added to media at a final concentration of 0.25 mg/ml to induce the GFP-lacI fusion protein that is under the control the copper promoter.

Yeast strain constructions: Yeast strains are listed in Table 3-1 and were constructed by standard genetic techniques. Diploids were isolated on selective media at 23° and subsequently sporulated at 23°. The galactose-inducible, non degradable mitotic cyclin (*pGAL-Δ176-CLB2*) that is contained in some strains is not expressed in glucose media. The strains XL1-Blue and DH5α were used for all bacterial manipulations. The strain used for the screen was constructed by first deleting the *LYS2* gene in SBY3 by integrating pAR88 (gift of Adam Rudner) digested with *Xba* I. The *URA3* gene was then selected against on 5-FOA plates to obtain SBY181, which contains an unmarked *lys2Δ*. SBY181 was subsequently integrated with the following

plasmids, respectively, to generate SBY215: *pGAL-Δ176-CLB2:LYS2* (pSB102) that was digested with *Bsp* EI, *pCUP-GFP12-LacI:HIS3* that was digested with *Nhe* I (pSB116, (BIGGINS et al. 1999)) and *lacO:TRP1* (256 lactose operators on plasmid pAFS52, (STRAIGHT et al. 1996)) that was digested with *Eco* RV. All *mad2Δ loc* and *cdc23-1 loc* double mutants were constructed by crosses. The intronless tubulin strains were constructed by integrating pSB273 (*TUB1 in*) that had been digested with *Afl* II into SBY186, SBY837 and SBY473 to create SBY808, SBY809 and SBY850, respectively. A strain containing the YSC4-HA3 was created by PCR integration. Primers LOC7-3 (5'-GTC/ ACT/ GCA/ TTA/ TTG/ GAG/ CAA/ GGT/ TTC/ CAA/ GGT/ TGT/ ATC/ CGC/ AAA/ AGA/ AAG/ GGA/ ACA/ AAA/ GCT/ GG-3') and LOC7-4 (5'-TAA/ TAA/ CAT/ ATA/ ATA/ TAA/ AAC/ GGA/ AGA/ AAC/ GGG/ TAA/ ACG/ TCA/ GTT/ CGA/ TTA/ CTA/ TAG/ GGC/ GAA/ TTG/ G-3') were used to PCR amplify DNA from plasmid RTK (gift of Rachel Kulberg) which was integrated into SBY215 to create NBY302.

We used a *cse4Δ* strain that was kept alive by a doxycycline repressible *CSE4* gene for the analysis of sister chromatid separation in a *mad2Δ cse4* double mutant. This was required because of difficulty arresting the *cse4* mutant strains with α-factor. To construct this strain, we first deleted *CSE4* in a diploid strain by the PCR integration method. Primers SB67 (5'- CAG/ AAG/ AAG/ GAC/ TGA/ ATA/ TAG/ AAA/ GAA/ TAC/ TAA/ TAT AAC/ ATA/ ATC/ CGG/ ATC/ CCC/ GGG/ TTA/ ATT/ AA-3') and SB64 (5'- CCG/ AAA/ AAG/ GGA/ AAA/ ATC/ GGC/ TCC/ AGC/ CCT/ GAA/ GCA/ CAA/ ATA/ TCA/ CTA/ TCG/ ATG/ AAT/ TCG/ AGC/ TCG/ TT-3') were used to PCR amplify pFA6a-kanMX6 (LONGTINE et al. 1998) and the PCR product was transformed into SBY516 to generate strain SBY597. This strain was transformed with pSB233, a plasmid containing doxycycline repressible *CSE4* which had been digested with

Afl II. The resulting strain, SBY599, was sporulated to generate a haploid *cse4Δ* strain (SBY601) covered by the repressible *CSE4* which was then crossed to SBY468. The resulting diploid was sporulated to isolate the *cse4Δ mad2Δ* double mutant (SBY626). When analyzed by Western blotting, there is no detectable Cse4 protein in SBY626 after treatment with doxycycline (data not shown). For the *cse4-327 cdc23-1* experiment, we used *GFP-TUB1* (pAFS125, gift of Aaron Straight) to visualize spindles because a large number of cells lysed during the indirect immunofluorescence procedure.

Isolation of *loc* mutants: A temperature sensitive bank of yeast mutants was generated as follows. Strain SBY215 was mutagenized with EMS or UV to 50% killing as described (ROSE et al. 1990). Mutagenized cells were plated at the permissive temperature (23°) until colonies formed and then replica printed to the non-permissive temperature (37°). After one day at 37°, plates were scored and putative ts mutants were isolated from a plate maintained at 23° and re-tested at 37° for growth. Mutant strains were subsequently colony purified and re-tested twice at 37°. Finally, mutants that did not grow on glycerol as the sole carbon source were eliminated to ensure the mutants were not petites. We isolated 2000 ts mutant strains from approximately 800,000 mutagenized strains.

We directly screened each ts mutant strain by microscopy to identify the *loc* phenotype. Microtiter dishes were inoculated from fresh patches of cells that were grown on plates at 23°. The microtiter dishes were shifted to 37° for four hours and placed on ice while we directly screened the cells by microscopy for GFP signals. We isolated 283 potential mutants out of the 2000 ts strains in this primary screen. We next screened the mutants for sister chromatid separation at anaphase. Non-destructible Clb2p was overexpressed by shifting cells to 37° for

two hours and then adding galactose to 2% final concentration for an additional two hours. Cells were screened by microscopy for a qualitative defect of 50% or less sister chromatid separation in the large-budded cells. Mutants that passed this test were then analyzed for a *cdc* phenotype. Cells were shifted to 37° for four hours and quantified for the number of large-budded cells. Mutant strains containing less than 70% large-budded cells in the population were then tested for rapid death, an indication of chromosome breakage, at 37°. Asynchronously growing mutant strains were shifted to 37° and plated for viability at 23° zero, two and four hours later. Mutant strains that decreased viability by 50% or greater during the four hour temperature shift were then tested for rescue by nocodazole/benomyl treatment. Asynchronously growing mutant strains were shifted to 37° in the presence of benomyl/nocodazole and plated for viability at 23° zero, two and four hours later. Mutant strains with increased viability in the presence of benomyl/nocodazole were retained.

The eleven *loc* mutant strains were crossed to SBY238 and the resulting diploids were tested for the ts phenotype and all were recessive. They were then backcrossed once to SBY238 to generate ***MATa*** and *MATα* strains that were used to generate diploids for complementation testing which determined there were nine complementation groups. They were then backcrossed four times to SBY215 and re-tested for the lack of a cdc phenotype and for sister chromatid separation by microscopy. We did not repeat the other secondary tests on the backcrossed strains.

loc **mutant cloning and linkage tests:** The *loc* mutants were cloned by complementation of the ts phenotype using a centromere-based yeast genomic library as described (HARDWICK and MURRAY 1995). Plasmid DNA from colonies that grew at 37° was isolated and transformed into

bacteria. The DNA was retransformed into each corresponding *loc* mutant and plasmids that conferred temperature resistance were sequenced. We identified the complementing region of the clones by subcloning various regions of the plasmids and testing for complementation of the ts phenotype.

We performed linkage tests to ensure that the cloned genes corresponded to the mutations and not suppressing genes. For *loc1*, SBY575 containing *pGAL-CSE4-myc13:URA3* was crossed to SBY323 and SBY327 and in 18 tetrads the *URA3* marker always segregated away from the *loc1* ts phenotype. The *loc2* linkage tests were previously described (BIGGINS et al. 1999). For *loc3*, we determined that the *smt3-331* ts phenotype is linked to the *GIN4* gene by crossing an *smt3-331* mutant strain to a *GIN4*-marked strain (RA5, gift of Doug Kellogg, UC Santa Cruz, CA). For *loc4*, pTW004 containing the *CDC7* gene marked with *URA3* (gift of J. Li, UCSF) was digested with *Bam* HI and integrated into SBY181. The resulting strain was crossed to SBY358 and the diploids were sporulated and in 22 tetrads the *loc4* ts phenotype always segregated away from the *URA3* marker. For *loc5*, we crossed SBY712 to UCC712 that contains a *URA3* marked *SIR1* gene and found that *URA3* always segregated away from the *loc5* ts phenotype in 20 tetrads. We also determined that the *loc5-1* allele does not complement a *prp16-2* allele (SBY748). For the *loc6* linkage test, the *loc6* allele (SBY528) was crossed to a strain with a marked *PDS1-myc18:LEU2* allele (SBY760) and in 22 tetrads the ts phenotype always segregated away from the *LEU2* marker. For the *loc7* linkage test, NBY302 containing *URA3*-marked *YSC4-HA3* was crossed to NBY290 and the resulting diploid was sporulated. Out of 22 tetrads dissected, the *URA3* marker always segregated away from the *loc7* ts phenotype. For the *prp19* linkage test, pSB194 was digested with *Bgl* II and integrated into SBY214. The resulting strain was crossed to SBY155 and 22 tetrads were analyzed and determined that the ts

phenotype always segregated away from the marked plasmid. In addition, we determined that the *loc8-1* allele does not complement a *prp19* allele. For *loc9*, we crossed SBY482 to SBY382 containing *ESP1-HA3:URA3* and found that the *loc9* ts phenotype segregated away from the *URA3* marker in 20 tetrads.

Plasmid constructions: To determine the minimal complementing region of the genomic clones that suppressed each mutant, we tested either previously described plasmids or constructed subclones of the genomic plasmids. For *loc1*, a *CSE4-HA* subclone previously described (STOLER et al. 1995) complemented the ts phenotype. The *loc2* data was previously described (BIGGINS et al. 1999). For *loc3*, genomic clone pSB333 was digested with *Nar* I and *Nhe* I and the 1020-bp fragment was ligated into pRS316 that was digested with *Cla* I and *Spe* I. The resulting clone containing *SMT3*, pSB150, complemented the *loc3* mutant phenotype. A clone containing *pGAL-SMT3* (pSB231) as the sole source of yeast DNA also complemented *loc3* mutant cells. For *loc4*, plasmid pTW004 containing the *CDC7* complemented the ts phenotype. For *loc5*, the genomic clone pNB4 was digested with *Sph* I to eliminate 2 kb of genomic DNA and the backbone vector containing the *PRP16* gene was ligated to create pNB13 which complements the *loc5* mutation. To determine that *PRP16* was the complementing gene on this plasmid, pNB13 was digested with *Xba* I and filled in with the Klenow fragment of DNA polymerase to create pNB20 that contains a frame-shift mutation in the *PRP16* gene. This clone did not complement *loc5*. For *loc6*, a *PDS1* clone, pAY53 (gift of V. Guacci, Fox Chase Cancer Center), complemented the ts phenotype. For *loc7*, DNA encoding just the *YSC4* gene was PCR amplified using primers LOC7-1 (5'-GCG/ CGC/ GGAT/ CCC/ GCG/ TTG/ TTT/ TCT/ TGT/ CG-3') and LOC7-2 (5'-GCG/ CGC/ GGC/ CGC/ GGG/ TAA/ ACG/ TCA/ GTT/ CGA-3') that

had *Bam* HI and *Not* I sites engineered, respectively. The PCR product was digested with *Bam* HI and *Not* I and ligated into pRS316 to create pNB27 which complemented the *loc7* ts phenotype. For *loc8*, the *Afl* II site in genomic clone pSB190 was filled in with the Klenow fragment of DNA polymerase to create a frame-shift mutation in the *PRP19* gene. This clone did not complement *loc8*.

We constructed clones to conduct linkage tests as needed. To confirm *PRP19* linkage to the *loc8* mutation, pSB194 was constructed by PCR amplifying the *UBI4* gene from pSB190 using primers SB15 (5'-TCG/ ATC/ GGA/ TCC/ GAG/ GGC/ GGT/ TCC/ TCC-3') and SB16 (5'-GAT/ CGA/ TCT/ AGA/ GAA/ AAT/ ATT/ GCG/ AGG/ ACT/ G-3') that had *Bam* HI and *Xba* I sites engineered, respectively. The PCR product was digested with *Bam* HI and *Xba* I and ligated into pRS306 digested with the same enzymes. The clone was digested with *Bgl* II for integration into yeast.

The intronless tubulin clone, pSB273, was constructed by digesting pRS415/ilTUB (gift of John Wagner and Jon Abelson) with *Bam* HI and *Spe* I and the 2.3 kb fragment containing intronless *TUB1* was ligated into pRS305 digested with *Bam* HI and *Spe* I. The *pGAL-Δ176-CLB2* plasmid, pSB102, was constructed by digesting pAR39 (gift of Adam Rudner) with *Stu* I and ligating this to the 5 kb *Pvu* II fragment isolated from pRS317. The repressible *CSE4* clone, pSB233, was constructed by PCR amplification of *CSE4* using primers SB82 (5'- GAT/ CGA/ TCT/ GCA/ GGA/ TGT/ CAA/ GTA/ AAC/ AAC/ AAT/ GG-3') and SB83 (5'- GAT/ CGA/ TCG/ CGG/ CCG/ CCT/ AAA/ TAA/ ACT/ GTC/ CCC/ TG-3') that had *Pst* I and *Not* I sites, respectively, engineered. The PCR product was digested with *Pst* I and *Not* I and ligated into pNB32 (N. B. and A. W. M, in preparation) that was digested with *Pst* I and *Not* I to create pSB233 containing 7 tetracycline operators upstream of the *CSE4* gene.

DNA flow cytometry: Approximately 10^7 cells were harvested before and after a four hour temperature shift to 37° and fixed in 70% EtOH. Cells were prepared for flow cytometry as described (BISHOP et al. 2000) and 20,000 cells from each sample were scanned with a FACScan machine (Becton-Dickinson).

Microscopy: Microscopy to analyze sister chromatids was performed as described (BIGGINS et al. 1999). Indirect immunofluorescence was carried out as described (ROSE et al. 1990). DAPI was obtained from Molecular Probes and used at 1 μg/ml final concentration. Anti-tubulin antibodies, yol 1/34, were obtained from Accurate Chemical and Scientific Corp and used at a 1:1000 dilution. Cy3 secondary antibodies were obtained from Jackson Immunoresearch and used at a 1:2000 dilution. .

RESULTS

Isolation of *loc* mutants: We performed microscopy on a bank of temperature sensitive yeast mutants with a GFP marked chromosome to isolate mutants defective in chromosome behavior. A tandem repeat of lactose operators (lacO) was integrated at the *TRP1* locus, 12 kb from the centromere of chromosome IV, the largest chromosome. A GFP fusion to the lactose repressor (GFP-lacI) was expressed in these cells to allow visualization of chromosome IV. We generated a temperature sensitive (ts) bank of conditional yeast mutants in this strain by mutagenizing cells with EMS or UV and screening for lack of growth at 37°. We isolated

approximately 2000 ts mutants that were subsequently screened for chromosome behavior defects using fluorescence microscopy.

The visual screen was conducted by examining cells in anaphase. When wild type cells complete anaphase, the sister chromatids have separated to opposite poles, so two GFP signals are visualized by microscopy (Figure 3-1). To isolate mutants that are defective in the loss of cohesion (*loc* mutants) at anaphase, we screened ts mutant strains for large budded cells that contain one GFP signal instead of two signals. Each ts mutant strain was shifted to the non-permissive temperature (37°) for four hours and then screened by fluorescence microscopy for the number of GFP signals in large budded cells. In this primary screen, we isolated 283 mutant strains from a total of 2000 ts mutant strains where 50% or more of the large budded cells contained one GFP signal.

A variety of defects in addition to sister chromatid separation defects will result in large budded cells containing one GFP signal. The largest class of mutants will be those that arrest in metaphase instead of proceeding into anaphase, such as mutants that activate the spindle assembly checkpoint, the DNA damage or synthesis checkpoints and mutants that are defective in the anaphase promoting complex. We therefore performed a number of secondary screens designed to eliminate mutants where a single GFP dot was due to a metaphase delay or arrest.

First, we analyzed sister chromatid separation in an anaphase arrest where wild type cells separate sister chromatids. The ubiquitin-mediated proteolysis of the major mitotic budding yeast cyclin, Clb2p, is required for cells to exit from anaphase but not for cells to separate sister chromatids. We therefore overexpressed a non-degradable version of Clb2p in each mutant in an attempt to obtain a population of cells enriched in anaphase. Although the overexpression of non-degradable Clb2 will not drive metaphase arrested cells into anaphase, we reasoned that it

might increase the fraction of sister separation in cells that delay in metaphase. Cells were shifted to the non-permissive temperature for four hours in the presence of galactose to induce the expression of non-degradable Clb2p and subsequently screened for sister chromatid separation by microscopy. We eliminated mutants that exhibited 50% or greater sister chromatid separation in this test.

We next eliminated mutants that arrested in metaphase. Metaphase arrested cells show a cell division cycle (*cdc*) phenotype in which greater than 70% of the population arrest as large budded cells. We therefore analyzed the morphological distribution of the mutant strains after shifting them to the non-permissive temperature (37°) for four hours and eliminated mutant strains in which 70% or more of the population contained large budded cells.

We performed additional secondary tests to enrich for mutants defective in the loss of cohesion rather than other mitotic defects. Although topoisomeraseII (*top2*) mutant cells do not separate sister chromatids, the spindle elongates and attempts to pull the sister chromatids apart. The force of the mitotic spindle leads to chromosome breakage and cell death. We therefore expected that mutants defective in sister chromatid separation would rapidly lose viability as they pass through mitosis at the non-permissive temperature. To test the mutants for rapid death, they were shifted to the non-permissive temperature (37°) and plated to the permissive temperature (23°) zero, two hours and four hours later to measure viability. Mutants whose viability decreased by at least 50% were retained as *loc* mutants. We reasoned that preventing the lethal anaphase event might rescue the rapid death of the mutants, so we repeated the rapid death experiment in the presence of nocodazole/benomyl which causes cells to arrest in prometaphase due to activation of the spindle assembly checkpoint. Mutants were shifted to the non-permissive temperature in the presence of nocodazole/benomyl and plated for viability zero,

two and four hours later. Mutants whose decline in viability was fully or partly suppressed by depolymerizing the spindle were retained.

The remaining eleven *loc* mutant strains fit our original criteria for mutants defective in the loss of cohesion. We tested whether the mutants were recessive or dominant by crossing each to a wild type strain and testing each resulting diploid for temperature sensitivity. All of the mutants were recessive. They were then backcrossed once to a wild type parent strain to generate *MATa* and *MATα* strains for complementation testing. Each *loc* mutant strain was crossed to all of the other mutants as well as the *esp1-1* and *top2-4* mutants and tested for growth at the non-permissive temperature. Complementation testing determined that there were 8 unique *LOC* complementation groups and an allele of the *ESP1* gene (Table 3-2). We isolated 2 alleles of the *LOC1* and *LOC2* complementation groups and single alleles of the other complementation groups.

We backcrossed each *loc* mutant five times and then re-tested the mutants for sister chromatid separation after four hours at the non-permissive temperature (37°). All of the mutants had a defect where only one GFP signal was observed in 40% or greater of the large budded cells (Table 3-2). We observed that two mutants had a very similar phenotype in which there was a clear segregation of the bulk of the DNA but pairs of sister chromatids traveled to one pole (for example, see *loc1-1* in Figure 3-1). The other mutants did not show as much segregation of the bulk of DNA (for example, see *loc3-1* in Figure 3-1). We next analyzed the cell cycle distribution at the non-permissive temperature to confirm that the mutants were not arresting as large budded cells. Mutant strains were shifted to the non-permissive temperature for four hours and the budding index was determined by microscopy. None of the mutants

exhibited a classical *cdc* phenotype of more than 70% large budded cells, although *loc3-1* and *loc4-1* were enriched for large budded cells (Table 3-2).

We next examined DNA content in the *loc* mutants to ensure that the phenotype was not due to a lack of DNA replication or a metaphase arrest. Mutant cells were shifted to the non-permissive temperature for four hours and then analyzed by flow cytometry analysis for DNA content before and after the temperature shift (Figure 3-2). Wild type and *loc5-1* mutant cells showed no significant change in DNA content after the shift to 37°. The *loc4-1* mutant was defective in DNA replication, consistent with the enrichment of large budded yeast cells reported in Table 3-2. The *loc3-1* and *loc8-1* mutants exhibited an enrichment of cells with a G2 DNA content. The remaining mutants exhibited very heterogeneous FACS profiles that included cells with increased and decreased ploidy, indications of severe defects in chromosome segregation.

Identification of the genes encoding the *loc* mutants: To continue characterization of the *loc* mutants, we cloned them by complementation of the ts phenotype using a centromere-based genomic library. Genomic clones were subcloned to isolate the minimal complementing region of DNA. We subsequently confirmed the identity of the genes by linkage analysis and determined that we had isolated mutations in the *CSE4* (*LOC1*), *IPL1* (*LOC2*), *SMT3* (*LOC3*), *CDC7* (*LOC4*) *PRP16* (*LOC5*), *PDS1* (*LOC6*), *YCS4* (*LOC7*) and *PRP19* (*LOC8*) genes in addition to the *ESP1* (*LOC9*) gene (Table 3-2). These genes are involved in a variety of processes. We previously determined that the *ipl1-321* mutants we isolated in the screen have defective kinetochores (BIGGINS et al. 1999). The *CSE4* and *SMT3* genes are also implicated in kinetochore function in budding yeast (BROWN et al. 1993; MELUH and KOSHLAND 1995; STOLER et al. 1995). We found that the *cse4-323* and *cse4-327* alleles we isolated in this screen

were different than previously reported *cse4* alleles that exhibit a metaphase arrest with unsegregated DNA (STOLER et al. 1995). Instead, the *cse4* alleles we isolated are more similar to *ipl1* mutants that segregate pairs of sister chromatids to a single pole (BIGGINS et al. 1999). The *PDS1* and *ESP1* genes are required for sister chromatid separation (CIOSK et al. 1998) and the *YCS4* gene is a component of the condensin complex that is required for chromosome condensation (FREEMAN et al. 2000). The *PRP16* and *PRP19* mutants are required for RNA splicing (BURGESS et al. 1990; CHENG et al. 1993). The *CDC7* gene is required for the initiation of DNA replication (HOLLINGSWORTH and SCLAFANI 1990), consistent with our FACS analysis on the *loc4-1 allele* that showed a defect in DNA replication. Since the function of Cdc7 has been extensively studied, we did not characterize the *loc4* mutant further.

Analysis of sister chromatid separation in *loc* mutants: Since the *LOC* genes are implicated in a variety of processes, we next determined whether the *loc* mutants we isolated are truly defective in sister chromatid separation. We previously found that the small size of the yeast nucleus relative to the resolution limit of light microscopy does not allow us to distinguish whether a single GFP spot is due to a pair of sister chromatids that are still linked or sister chromatids that are separated but in such close proximity that they cannot be resolved. We therefore used a test that we previously used to determine that *ipl1-321* mutants do not have defects in sister chromatid separation (BIGGINS et al. 1999). We analyzed sister chromatid separation in the absence of a spindle, a condition in which separated sisters slowly diffuse apart. Since the absence of a spindle activates the spindle checkpoint thus inhibiting sister chromatid separation, we did this analysis in a checkpoint mutant.

We constructed double mutants containing *loc* mutations and *mad2Δ*, which destroys the spindle checkpoint. The double mutant strains were arrested in G1 with α-factor and then released into nocodazole/benomyl at the non-permissive temperature (37°). An hour after the release α-factor was added back to prevent the cells from entering the next cell cycle. We analyzed sister chromatid separation three hours after release from G1 (Figure 3-3). Although the spindle checkpoint keeps wild type cells from separating their sister chromatids, about 60% of the *mad2Δ* cells contain two visible GFP dots. Since there is no spindle to pull the sister chromatid away from each other, sister chromatid separation never reaches 100% in *mad2Δ* cells that lack a spindle. Like wild type cells, all of the *loc* mutant strains maintain sister chromatid linkage in the presence of the spindle checkpoint (Figure 3-3, solid bars). When we analyzed sister chromatid separation in *loc mad2Δ* double mutant strains (Figure 3-3, open bars), we found that the mutants fell into 2 classes. The *esp1-478*, *ycs4-1*, *pds1-176* and *prp19-153* mutant strains failed to fully separate their sister chromatids even in the absence of the spindle checkpoint. The *prp16-186*, *smt3-331* and *cse4Δ* mutations all allow sisters to separate when the checkpoint is inactivated. We conclude that four of the *LOC* genes are required for the separation of sister chromatids while the others are required to satisfy the spindle checkpoint or to segregate the separated sisters to opposite spindle poles.

Consistent with our data, the Esp1 and Pds1 proteins have roles in sister chromatid separation (CIOSK et al. 1998). The role of Ycs4p in sister chromatid separation will be described elsewhere (N.B. and A.W.M, in preparation). We therefore wanted to test whether the remaining protein, Prp19p, is also directly involved in sister chromatid separation. Since *PRP19* is involved in DNA repair (SCHMIDT et al. 1999), it seemed possible that the *prp19-153 mad2Δ* double mutant did not separate sister chromatids due to activation of the DNA damage

checkpoint. We therefore examined sister chromatid separation in a *prp19-153 mad2Δ* strain that was also deleted for the *RAD9* gene, which is essential for the DNA damage checkpoint (Figure 3-3B). We arrested cells in G1 and then released them into nocodazole plus benomyl at the non-permissive temperature and analyzed sister chromatid separation after three hours. As expected, wild type, *rad9Δ* and *prp19-153* control strains do not separate sister chromatids. Although *mad2Δ* cells separate their sisters, we found that a *mad2Δ rad9Δ* control strain does not separate sister chromatids to the same levels as a *mad2Δ* strain. This may be due to a slower cell cycle in the *mad2Δ rad9Δ* strain (data not shown). Strains containing *prp19-153*, *prp19-153 mad2Δ* and *prp19-153 rad9Δ* mutations do not separate sister chromatids. However, a *prp19-153 mad2Δ rad9Δ* triple mutant strain separated sister chromatids to levels similar to the *mad2Δ rad9Δ* control strain. These experiments show that the *prp19-153-153* mutant does not have direct defects in sister separation and instead prevents sister separation by activating the DNA damage checkpoint.

Analysis of spindle function in *loc* mutants: We previously determined that *ipl1-321* mutants have defective kinetochores which lead to the *loc* mutant phenotype (BIGGINS et al. 1999). Pairs of sister chromatids are often segregated to the same pole leading to a single GFP dot at one pole instead of a pair of dots at opposite poles. It therefore seemed possible that the *loc* mutants that did not affect sister chromatid separation might have defective kinetochores and/or spindles which would lead to spindle abnormalities. We therefore analyzed spindle length and morphology in *loc* mutants arrested at metaphase. We constructed double mutants between the *loc* mutant strains and the *cdc23-1* mutant strain that arrests cells in metaphase due to a defect in the ubiquitin-mediated proteolysis of Pds1p. Cells were shifted to the non-permissive

temperature for four hours and then analyzed by indirect immunofluorescence for spindle length. The mutants fell into three classes. The first class consists of the *smt3-331 cdc23-1* and *ysc4-1 cdc23-1* double mutant strains that have an average spindle length of 4 μm that is similar to *cdc23-1* single mutant strains (data not shown). In addition, the spindle morphology is indistinguishable from *cdc23-1* single mutant strains. The second class of mutants, consisting of the *pds1-176 cdc23-1* and *cse4-327 cdc23-1* double mutant strains, contained a fraction of cells with spindles that were longer or shorter than the *cdc23-1* single mutant. Although this was only a fraction of the cells, these data are consistent with spindle and/or kinetochore defects in these strains. The third class, *prp16-186 cdc23-1* and *prp19-153 cdc23-1*, contained a majority of cells with either extremely short or undetectable spindles (Figure 3-4). We did not test the *esp1-478* mutant since *ESP1* has known roles in sister chromatid separation and spindle function (CIOSK et al. 1998; JENSEN et al. 2001; UHLMANN et al. 2000).

Since the mutants in the third class are both involved in splicing, we considered the possibility that the phenotype was a consequence of a defect in the splicing of one or more transcripts. One likely candidate is the major alpha tubulin gene, *TUB1*, which encodes one of the subunits of the tubulin dimer that polymerizes to form microtubules. Imbalances between the expression of alpha and beta tubulin lead to defects in microtubule polymerization and spindle assembly. To test this possibility, we integrated a copy of *TUB1* that did not contain an intron into the *prp16-186 cdc23-1* and *prp19-153 cdc23-1* double mutant strains. We shifted cells to the non-permissive temperature for four hours and then performed indirect immunofluorescence to analyze spindles in the mutant strains with and without intronless tubulin (Figure 3-5). Although there was little or no tubulin polymer in the *cdc23 prp* mutant strains containing only wild type *TUB1*, the addition of the intronless tubulin gene restored microtubules and completely

suppressed the spindle defect in these strains. Since intronless tubulin suppressed the lack of spindles in the *prp cdc23* double mutants, we tested whether it suppressed the growth defects of these strains. When cells were struck onto plates at 23, 30 or 37° there was no difference between the growth of strains with intron-containing or intronless *TUB1* (data not shown), consistent with the presence of additional essential transcripts that need to be spliced for viability.

DISCUSSION

We isolated mutants in nine *LOC* genes by screening temperature sensitive strains containing a GFP-marked chromosome for defects in sister chromatid separation. By analyzing sister chromatid separation in the mutant strains in the absence of a spindle, we classified the mutants for direct versus indirect effects on sister chromatid separation. We determined that the *esp1-478*, *pds1-176* and *ycs4-1* mutants strains are defective in sister chromatid separation whereas the *cse4*, *ipl1*, and *smt3-331* mutant strains affect chromosome segregation. The *prp16-186* and *prp19-153* mutant strains are defective in processing the *TUB1* transcript, leading to an apparent *loc* phenotype.

Genes in involved in sister chromatid separation and segregation: Although the *LOC* screen was originally designed to isolate mutants specifically defective in sister chromatid separation, secondary tests determined that mutants defective in sister chromatid separation exhibit phenotypes similar to mutants that cause non-disjunction of sister chromatids. We previously found that the *ipl1* alleles isolated in the screen have defective kinetochores that

frequently result in both sister chromatids segregating to a single pole. Since both chromosomes are close to the spindle pole they are rarely separated by more than the resolution limit of the light microscope. By depolymerizing the spindle, we allow the sisters to drift apart making a clear distinction between sister separation and sister segregation mutants. Applying this test revealed that the only three *loc* mutants directly required for sister chromatid separation encode the *ESP1*, *YCS4* and *PDS1* genes. The Esp1 and Pds1 proteins have previously been shown to be required for sister chromatid separation(CIOSK et al. 1998). Additional work from our lab on Ycs4p has also revealed a role in sister chromatid separation (N.B. and A.W.M., in preparation). *PDS1* appears to have dual roles in regulating sister chromatid separation. Although Pds1p inhibits the activation of the Esp1 protein, a complete deletion of the *PDS1* gene does not lead to precocious sister chromatid separation (CIOSK et al. 1998). Instead, *pds1Δ* strains are delayed in sister chromatid separation, indicating that Pds1 has a positive role in promoting separation in addition to a negative role inhibiting Esp1p. However, if *pds1Δ* strains are held in nocodazole for extended periods of time, they eventually separate sister chromatids due to defects in inhibiting Esp1p activity (GUACCI et al. 1997). Elimination of the fission yeast Pds1 homolog, Cut2, completely prevents sister separation (FUNABIKI et al. 1996). The *pds1-176* allele we isolated is phenotypically more similar to the phenotype of *cut2Δ* cells in fission yeast. We do not know if the difference between our results and previous experiments on *pds1Δ* strains reflects a difference in experimental procedures or the ability of our *pds1-176* allele to interfere with the function of Esp1 more severely than the complete absence of Pds1p. The *esp1-478* mutation we isolated behaves like *esp1-1* in the tests in this paper. Our screen suggests that there may not be many genes directly required for sister separation. Although the screen was far from saturated, two of the three mutants that affected sister separation identified previously identified genes, an

outcome that would be unlikely if there were many other genes that could easily mutate to prevent sister separation.

We analyzed spindles during a metaphase arrest as a secondary test to determine whether the *loc* mutants that were not directly defective in sister chromatid separation have defects in spindle function or morphology. The *smt3-331* and *ysc4-1* mutant strains did not exhibit any spindle defects in the metaphase arrest. This is consistent with the role of Ysc4p as a component of the condensin complex that regulates an aspect of chromosome structure that is unrelated to kinetochores or spindles (FREEMAN et al. 2000)(N. B. and A. W. M, in preparation). This is also consistent with the isolation of *SMT3* as a suppressor of mutations in *MIF2*, a known kinetochore component (MELUH and KOSHLAND 1995). If Smt3p regulates kinetochores, it must do so in a manner that does not affect kinetochore/microtubule attachments during a metaphase arrest leading to changes in spindle length or morphology. We found that a fraction of *cse4-327* and *pds1-176* mutants exhibit defects in maintaining spindle length in a metaphase arrest. Since Cse4p is localized to kinetochores and *cse4* mutant strains have defects in kinetochore function, it is likely that *cse4-327* mutants exhibit defects due to defective kinetochores. It was previously found that *pds1* mutant strains elongate spindles in a metaphase arrest due to lack of cohesion between sister chromatids. Our allele also behaves differently in this test. We found a large distribution in spindle length in the *pds1-186 cdc23-1* mutant strain arrested at metaphase. Although some of the cells elongate spindles, some of them have shorter spindles than cells arrested in metaphase. This suggests that this *pds1-176* allele may affect kinetochore or spindle function in addition to sister chromatid separation, consistent with a recent report showing that Pds1 mediates Esp1p localization to spindles (JENSEN et al. 2001).

Genes involved in splicing: We isolated mutations in two genes involved in RNA splicing in the *loc* screen: *PRP16* and *PRP19*. Although both genes are essential for splicing, the *PRP19* gene is also required for DNA repair (SCHMIDT et al. 1999). Since these genes have well-established roles in RNA processing, we reasoned that it was likely we isolated them as a secondary consequence of defects in splicing the transcripts of one or more genes important for chromosome segregation. There are only 270 transcripts in budding yeast that are spliced, and the most obvious candidate for a gene that could lead to a lack of spindles in a metaphase was the major alpha tubulin gene, *TUB1*. We found that an intronless version of *TUB1* that did not need to be spliced completely rescued the spindle defect during the metaphase arrest. However, the intronless tubulin did not change temperature sensitivity of these mutants, consistent with there being other essential intron-containing transcripts, such as the ribosomal protein genes.

Our evidence suggests that the *prp19-153* allele we isolated is defective in the DNA damage checkpoint. We had to delete both the spindle checkpoint and the DNA damage checkpoint in this mutant to detect sister chromatid separation in the absence of a spindle. This was not true for the *prp16-186* allele which separated sister chromatids in the absence of a spindle. Therefore, the *prp19-153* mutant has an additional defect. It is likely that this mutant activates the DNA damage checkpoint due to its role in DNA repair.

Our analysis suggests that the control of mitotic chromosome behavior is complex and that many genes are involved in the process. The alleles of *CSE4* and *PDS1* we isolated appear to behave qualitatively differently than previously identified alleles. More detailed analysis of the alleles we have isolated will help to determine the roles of these genes and others (*SMT3*, *YCS4*) in mitotic chromosome behavior.

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Table 3-1. Yeast strains used in this study

Strain	Genotype
SBY3	*MAT***a** *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 bar1Δ*
SBY15	*MAT***a***/MATα ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11/his3-11 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100 bar1Δ/bar1Δ*
SBY153	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ prp19-153*
SBY155	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 barΔ lys2Δ:pGAL-Δ176-CLB2:LYS2 prp19-153*
SBY181	*MAT***a** *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
SBY186	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1*
SBY214	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ*
SBY215	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2*
SBY238	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ* [pRS316]
SBY322	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ ipl1-321*
SBY323	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cse4-323*
SBY327	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cse4-327*
SBY329	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cse4-327*
SBY331	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ smt3-331*
SBY333	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 smt3-331*

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SBY355	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc7-355*
SBY358	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 barΔ lys2Δ:pGAL-Δ176-CLB2:LYS2 cdc7-355*
SBY382	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 ESP1-HA3:URA3:HA3*
SBY458	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ mad2::URA3 esp1-478*
SBY468	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3*
SBY473	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 prp19-153*
SBY478	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ esp1-478*
SBY482	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ esp1-478*
SBY516	*MAT**a**/MATα ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3/his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1/ trp1-1:lacO:TRP1 ade2-1/ade2-1 can1-100/can1-100 bar1Δ/bar1Δ lys2Δ/ lys2Δ:pGAL-Δ176-CLB2:LYS2*
SBY528	*MATα ura3-1 leu2,3-112 his3-11pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds1-186*
SBY530	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ top2-4* [pRS316]
SBY567	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3 smt3-331*
SBY571	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ prp19-153 rad9::LEU2*
SBY572	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3 rad9::LEU2*
SBY575	*MAT**a** ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 bar1Δ CSE4:pGAL-CSE4-myc13::URA3::KAN*

SBY597	*MAT**a**/MATα ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3/his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1/ trp1-1:lacO:TRP1 ade2-1/ade2-1 can1-100/can1-100 bar1Δ/bar1Δ lys2Δ/ lys2Δ:pGAL-Δ176-CLB2:LYS2 CSE4/cse4::KAN*
SBY599	*MAT**a**/MATα ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3/his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1/ trp1-1:lacO:TRP1 ade2-1/ade2-1:pTET-CSE4:ADE2 can1-100/can1-100 bar1Δ/bar1Δ lys2Δ/ lys2Δ:pGAL-Δ176-CLB2:LYS2 CSE4/cse4::KAN*
SBY601	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1:pTET-CSE4:ADE2 can1-100 bar1Δ lys2Δ cse4::KAN*
SBY626	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1:pTET-CSE4:ADE2 can1-100 bar1Δ lys2Δ mad2::URA3 cse4::KAN*
SBY710	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ pds1-186*
SBY712	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ prp16-176*
SBY713	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ ipl1-182*
SBY740	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3 prp19-153*
SBY804	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3 pds1-186*
SBY805	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2Δ ycs4-1 hml::LEU2*
SBY808	*MAT**a** ura3-1 leu2,3-112:TUB1(in):LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1* [pSB273]
SBY809	*MAT**a** ura3-1 leu2,3-112:TUB1(in):LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 prp16-176* [pSB273]
SBY817	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1*
SBY828	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ ycs4-1*

Strain	Genotype
SBY833	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2::URA3 prp16-176*
SBY837	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 prp16-176*
SBY840	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2Δ pr19-153 rad9::LEU2*
SBY849	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ mad2Δ rad9::LEU2*
SBY850	*MAT***a** *ura3-1 leu2,3-112:TUB1(in):LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ cdc23-1 prp19-153* [pSB273]
RA5[1]	*MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 gin4::LEU2*
YS78-2[2]	*MAT***a** *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 lys2 prp16::LYS2 prp16-2:HIS3*
UCC712[3]	*MATα ura3Δ0 leu2Δ0 his3Δ200 trp1Δ63 ade2Δ::hisG lys2Δ0 met15Δ0 sir1::URA3*
NBY302	*MAT***a** *ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 YSC4-HA3:URA3:HA3*
NBY290	*MATα ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ ycs4-1 hml::LEU2*

All strains are isogenic with the W303 background. Plasmids are indicated in brackets. All strains were constructed for this study unless indicated.

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Table 3-2. *loc* mutants

Mutant	Allele	% Unbudded	% Small Budded	% Large Budded	% Re-Budded	% Sister Separation[1]
WT		40	26	32	2	92
loc1-1	*cse4-323*	27	8	37	28	38
loc1-2	*cse4-327*	19	5	43	33	54
loc2-1	*ipl1-182*	50	19	27	4	56
loc2-2	*ipl1-321*	53	14	26	7	47
loc3-1	*smt3-331*	30	14	52	3	28
loc4-1	*cdc7-355*	11	11	66	12	17
loc5-1	*prp16-186*	36	27	34	4	14
loc6-1	*pds1-176*	47	18	33	2	61
loc7-1	*ycs4-1*	29	22	45	4	18
loc8-1	*prp19-153*	26	26	45	2	22
loc9-1	*esp1-478*	47	13	24	16	45
top2	*top2-4*	49	8	37	6	28

[1]Sister separation in large budded cells

Figure 3-1. Examples of *loc* mutant sister chromatid phenotypes. Wild type (SBY214), *loc1-1* (SBY323, *cse4-323*) and *loc3-1* (SBY331, *smt3-331*) strains were shifted to the non-permissive temperature (37°) for four hours and fixed for microscopy. Phase contrast microscopy is shown in the left panels and GFP fluorescence is shown in the right panels. Wild type cells separate sister chromatids to opposite poles so there is a GFP signal in each bud. In *loc1-1* cells, there is only one GFP signal in the large budded cell. In *loc3-1* cells, there is only one GFP signal even though the large budded cell is re-budding. Bar, 10 microns.

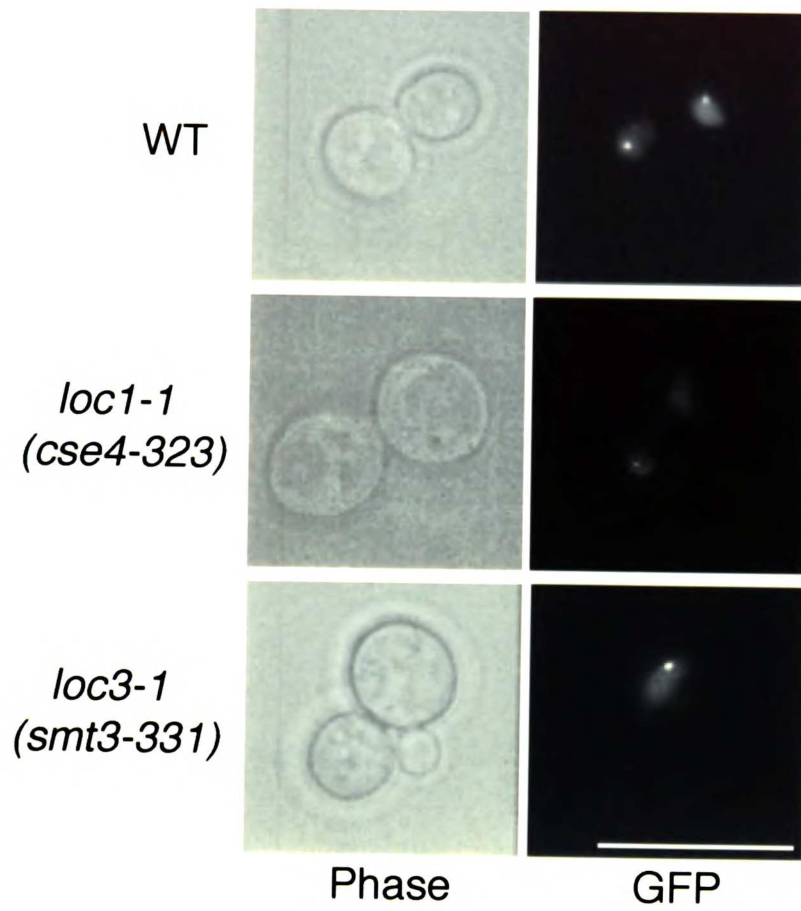
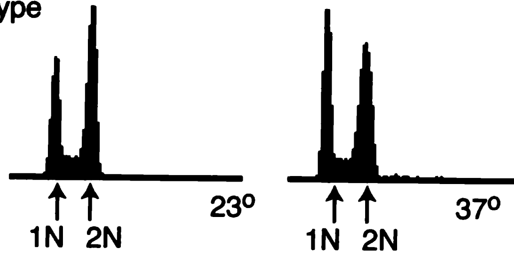
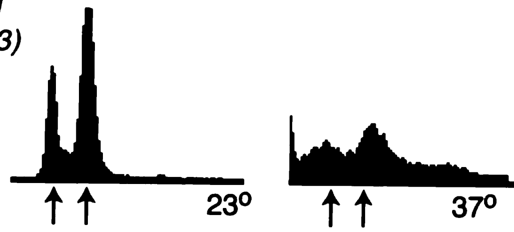
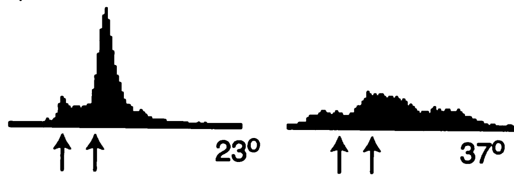
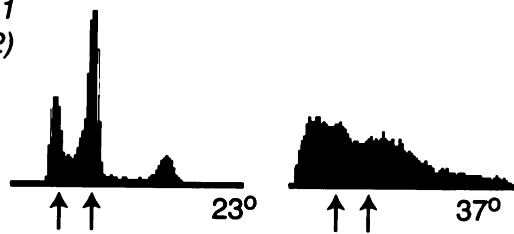

WT
loc1-1
(cse4-323)
loc3-1
(smt3-331)
Phase
GFP

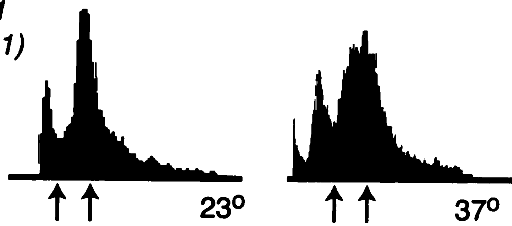
Figure 3-2. DNA content of *loc* mutant strains. Flow cytometry analysis was performed on *loc* mutant strains at the permissive temperature (23°) and after incubation for four hours at the non-permissive temperature (37°) as indicated. The arrows indicate the 1N (G1) and 2N (G2/M) DNA content. Wild type (**A**, SBY214) and *loc5* mutant cells (**G**, SBY712, *prp16-186*) do not show altered DNA content while the *loc1* (**B**, SBY323, *cse4-323* and **C**, SBY329, *cse4-327*), *loc2* (**D**, SBY713, *ipl1-182*), *loc3* (**E**, SBY331, *smt3-331*), *loc6* (**H**, SBY710, *pds1-176*), *loc7* (**I**, SBY828, *ycs4-1*), *loc8* (**J**, SBY153, *prp19-153*) and *loc9* (**K**, SBY478, *esp1-478*) mutant cells exhibit heterogeneous profiles indicative of chromosome segregation defects. The *loc4* (**F**, SBY355, *cdc7-355*) mutant arrests with unreplicated DNA. The FACS profile for *loc2-2* is similar to *loc2-1* and therefore is not shown.

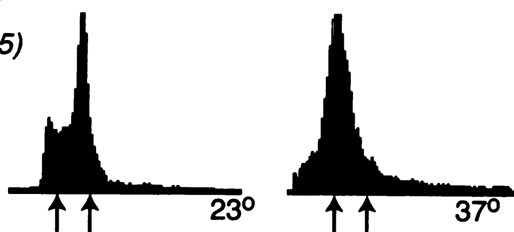

A. Wildtype
23°
1N 2N
37°
1N 2N


B. *loc1-1*
(cse4-323)
23°
37°

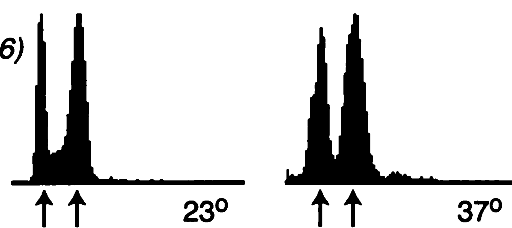

C. *loc1-2*
(cse4-327)
23°
37°


D. *loc2-1*
(ipl1-182)
23°
37°

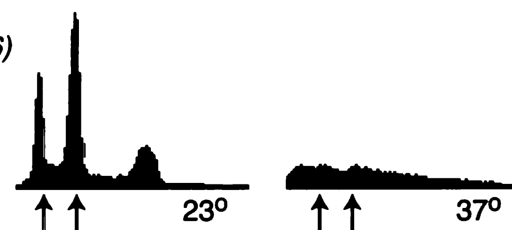

E. *loc3-1*
(smt3-331)
23°
37°


F. *loc4-1*
(cdc7-355)
23°
37°

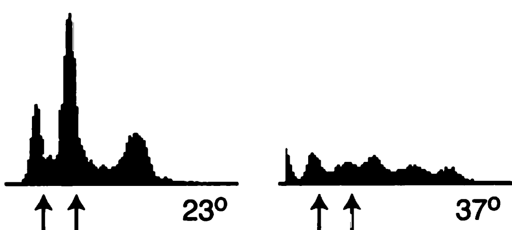
G. *loc5-1*
(prp16-186)



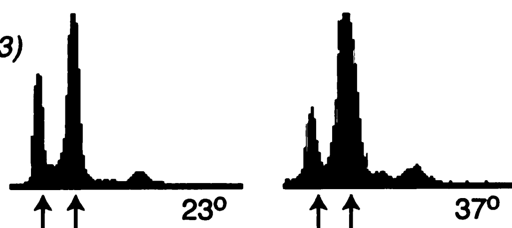
H. *loc6-1*
(pds1-176)



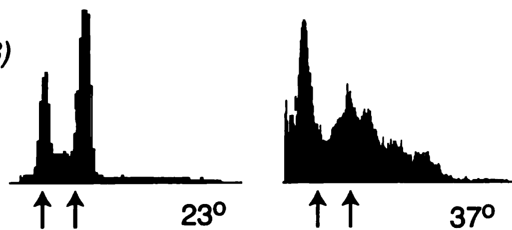
I. *loc7-1*
(ycs4-1)



J. *loc8-1*
(prp19-153)



K. *loc9-1*
(esp1-478)



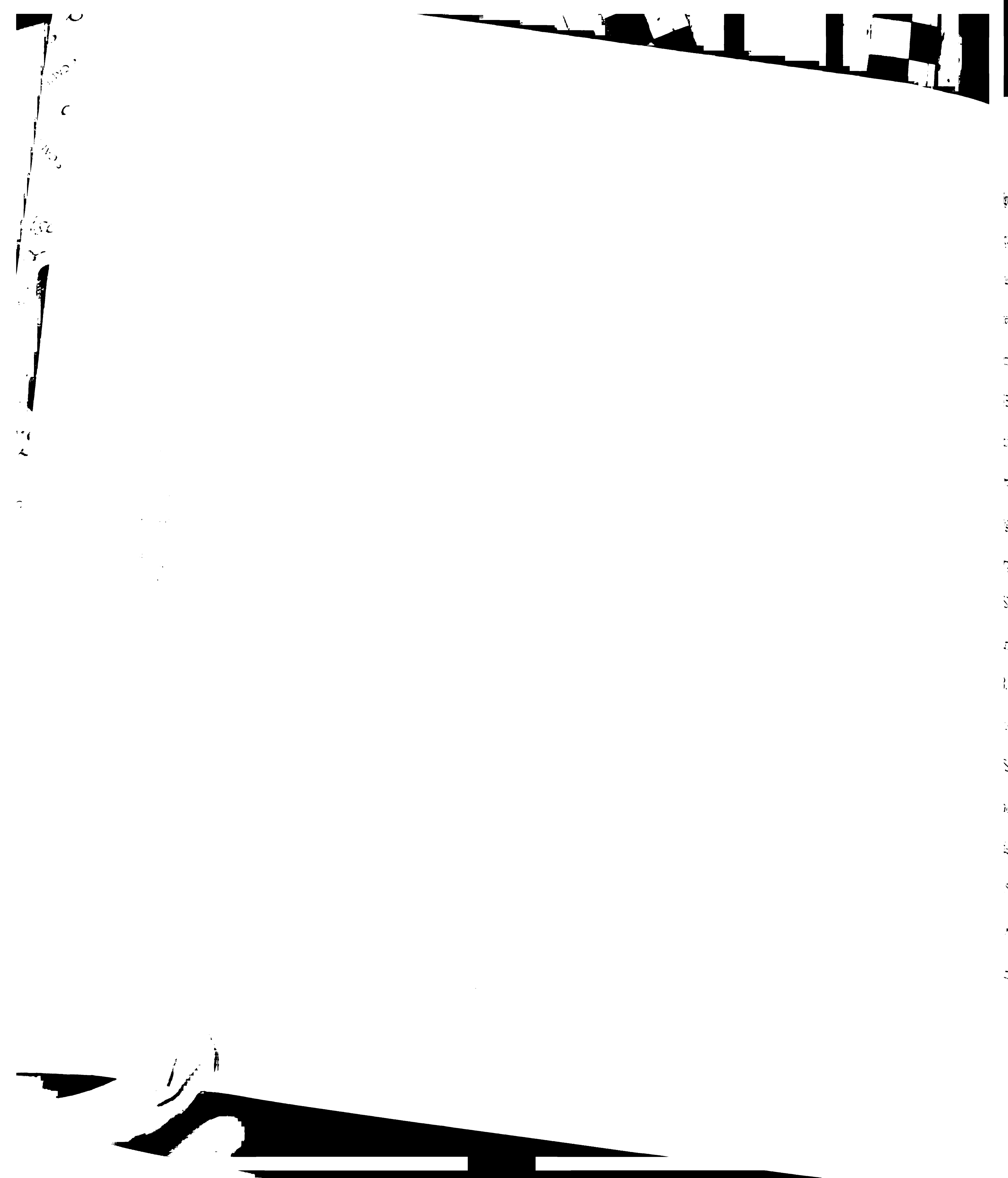
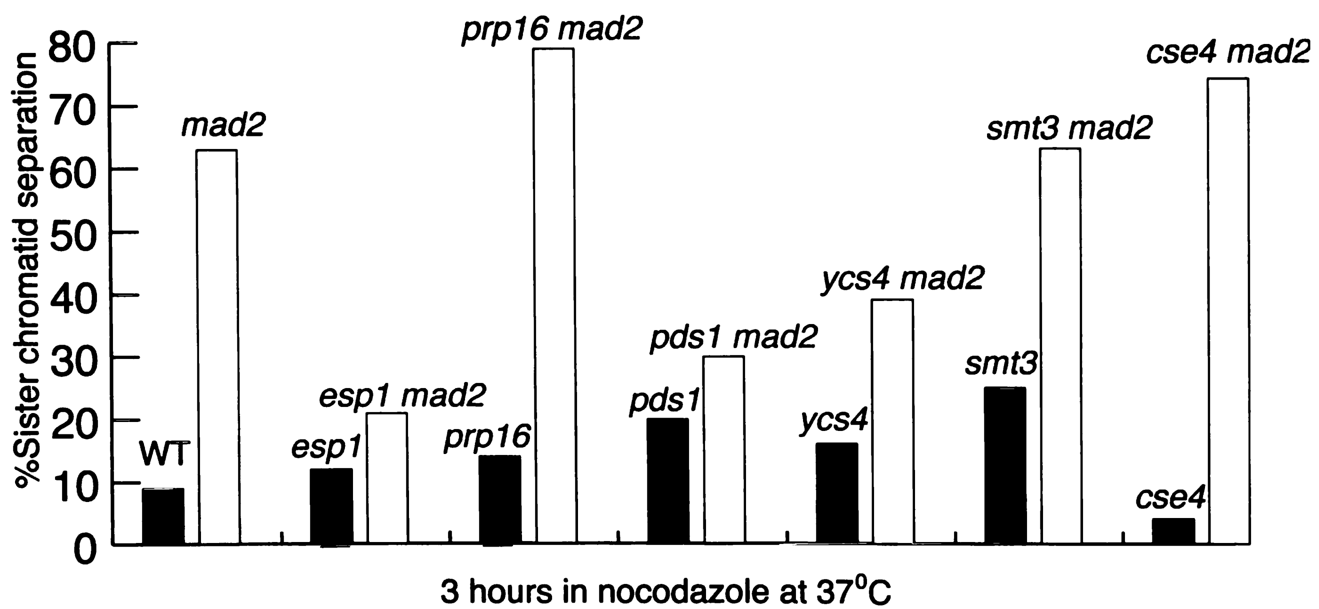


Figure 3-3. Analysis of sister separation in *loc* mutants. **A.** Comparison of sister chromatid separation between *loc* (solid bars) and *loc mad2Δ* (open bars) strains. The number of GFP dots was analyzed 3 hours after cells were released from G1 into nocodazole/benomyl containing-medium at the non-permissive temperature (37°). After one hour at the non-permissive temperature, α-factor was added to prevent cells from entering the next cell cycle. Wild type (SBY214), *esp1* (SBY468), *prp16* (SBY712), *pds1* (SBY710), *ycs4* (SBY828), *smt3* (SBY331) and *cse4 esp1-478* (SBY468), *prp16-186* (SBY712), *pds1-176* (SBY710), *ycs4-1* (SBY828), *smt3-331* (SBY331) and *cse4*Δ (SBY329) cells arrest in metaphase with sister chromatids held together (solid bars). When *mad2* was deleted from these cells, sister chromatids separated in *prp16* (SBY833), *ysc4* (SBY805), *smt3* (SBY567), *cse4 prp16-186* (SBY833), *smt3-331* (SBY567), *cse4Δ* (SBY626) and *mad2Δ* (SBY468) cells and did not fully separate in (SBY458) and *pds1 esp1-478* (SBY458), *ysc4* (SBY805), and *pds1-176* (SBY804) mutant cells (open bars). **B.** *prp19-153* cells activate the DNA damage checkpoint at the non-permissive temperature. Sister chromatid separation was analyzed in wild type (SBY214), *mad2Δ* (SBY468), *rad9Δ* (SBY572) and *mad2Δ rad9Δ* (SBY849) mutant cells 3 hours after release from G1 into nocodazole/benomyl media at the non-permissive temperature and revealed that *rad9Δ* mutants delay sister separation in *mad2Δ* mutant cells. *prp19-153* (SBY153), *prp19-153 mad2Δ* (SBY740) and *prp19-153 rad9Δ* (SBY571) cells do not separate sister chromatids. A *mad2Δ rad9Δ prp19-153* strain (SBY840) separated sister chromatids similar to *mad2Δ rad9Δ* cells indicating that *prp19-153* mutant cells activate the DNA damage checkpoint.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70
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30
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esp1
esp1 mad2
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prp16 mad2
pds1
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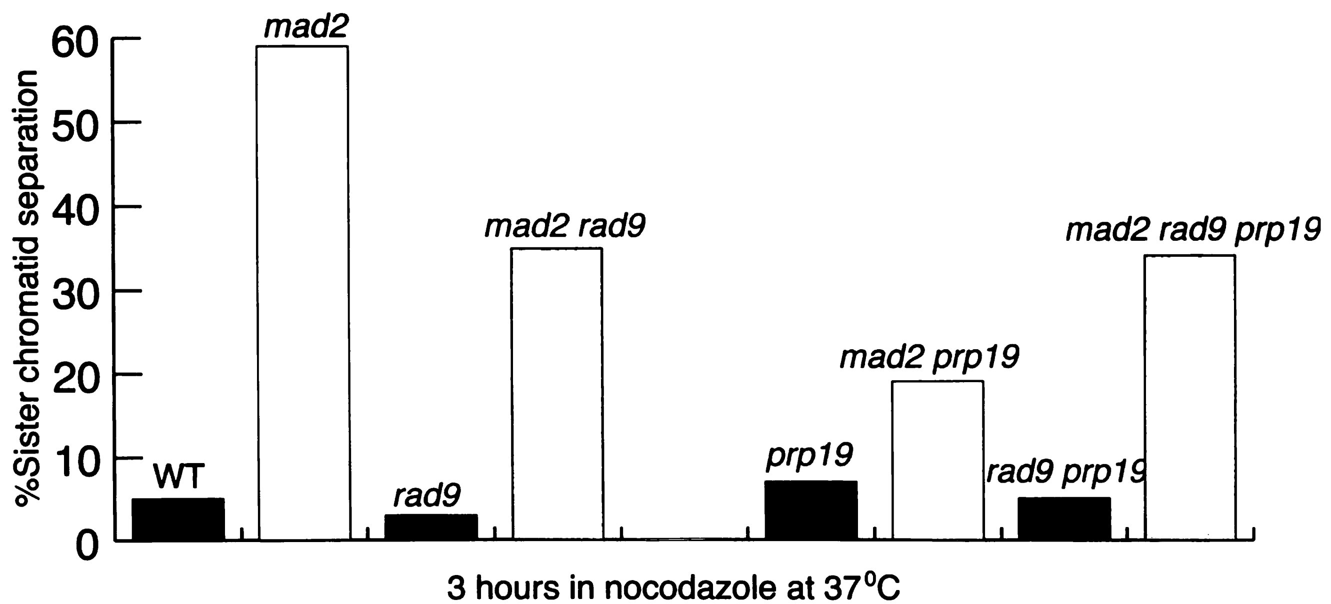
ycs4
ycs4 mad2
smt3
smt3 mad2
cse4
cse4 mad2
3 hours in nocodazole at 37°C


B.
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prp19
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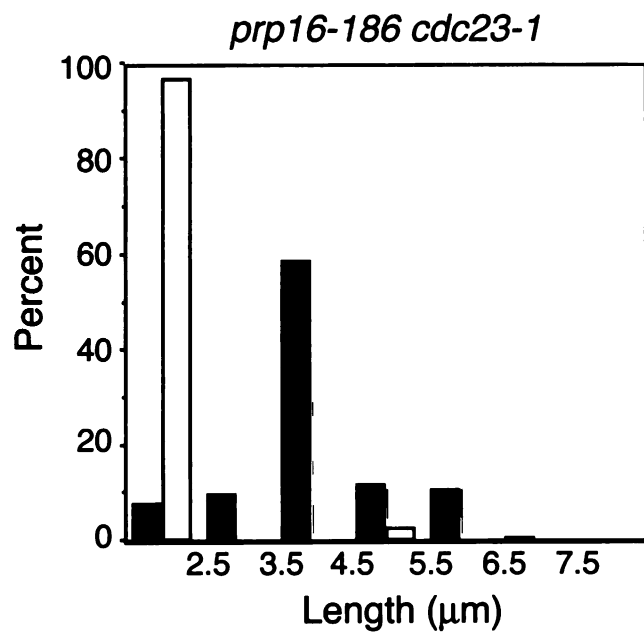
rad9 prp19
mad2 rad9 prp19
3 hours in nocodazole at 37°C

Figure 3-4. Analysis of spindle length and morphology in *prp16-186* and *prp19-153* mutant strains arrested at metaphase. Indirect immunofluorescence was performed on *cdc23-1 prp* double mutants using anti-tubulin antibodies on cells shifted to the non-permissive temperature (37°) for four hours and spindle length was measured in 100-200 cells. The percentage of cells at various spindle lengths (μm) is graphed. *prp16-186 cdc23-1* (SBY837) and *prp19-153 cdc23-1* (SBY473) double mutant strains (open bars) had either extremely short or a complete lack of spindles relative to *cdc23-1* mutant strains (SBY186, solid bars).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Percent
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Length (µm)

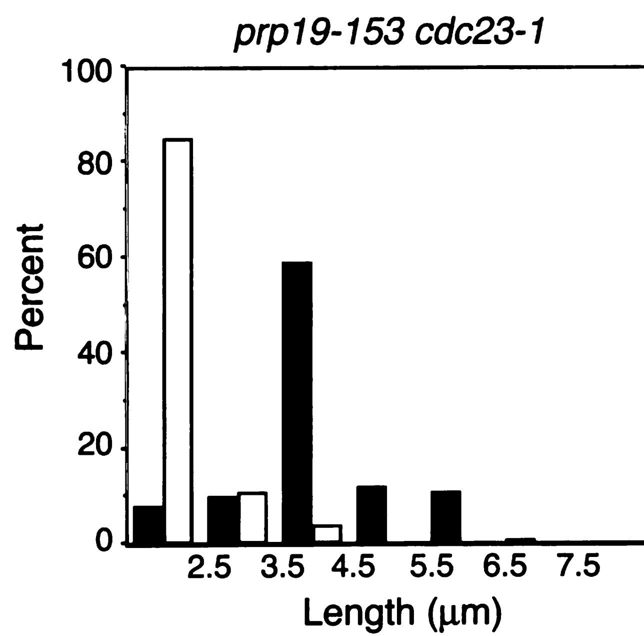
prp19-153 cdc23-1
100
80
60
40
20
0
Percent
2.5 3.5 4.5 5.5 6.5 7.5
Length (µm)

Figure 3-5. Intronless tubulin alleviates the spindle defect in the *prp* mutants. Indirect immunofluorescence was performed on *cdc23-1* (SBY186), *cdc23-1 prp16-186* (SBY837) and *cdc23-1 prp19-153* (SBY473) mutant strains without intronless tubulin (left panels) and *cdc23-1* (SBY809), *cdc23-1 prp16-186* (SBY810) and *cdc23-1 prp19-153* (SBY850) mutant strains with intronless tubulin (right panels). Cells were shifted to the non-permissive temperature (37°) for four hours. DAPI staining is shown on the left and anti-tubulin staining is shown on the right of each panel. Bar, 10 microns.

DNA Tubulin DNA Tubulin

cdc23-1

cdc23-1
prp16-186

cdc23-1
prp19-153

-intronless tubulin +intronless tubulin

CHAPTER FOUR

Mutation of *YCS4*, a budding yeast condensin subunit, affects mitotic and non-mitotic chromosome behavior

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The work in this chapter was primarily done by Needhi Bhallaand was published in *Molecular Biology of the Cell*, 13: 632-645.

ABSTRACT

The budding yeast *YCS4* gene encodes a conserved regulatory subunit of the condensin complex. We isolated an allele of this gene in a screen for mutants defective in sister chromatid separation or segregation. The phenotype of the *ycs4-1* mutant is similar to topoisomerase II mutants and distinct from the *esp1-1* mutant: the topological resolution of sister chromatids is compromised in *ycs4-1* despite normal removal of cohesins from mitotic chromosomes. Consistent with a role in sister separation, *YCS4* function is required to localize DNA topoisomerase I and II to chromosomes. Unlike its homologs in *Xenopus* and fission yeast, Ycs4p is associated with chromatin throughout the cell cycle; the only change in localization occurs during anaphase when the protein is enriched at the nucleolus. This relocalization may reveal the specific challenge that segregation of the transcriptionally hyperactive, repetitive array of rDNA genes can present during mitosis. Indeed, segregation of the nucleolus is abnormal in *ycs4-1* at the non-permissive temperature. Inter-repeat recombination in the rDNA array is specifically elevated in *ycs4-1* at the permissive temperature, suggesting that the Ycs4p plays a role at the array aside from its segregation. Furthermore, *ycs4-1* is defective in silencing at the mating type loci at the permissive temperature. Taken together, our data suggest that there are mitotic as well as non-mitotic chromosomal abnormalities associated with loss of condensin function in budding yeast.

INTRODUCTION

Cell survival depends on the accurate transmission of a cell's genetic material to its daughters. Coordinating chromosome behavior with the cell cycle machinery ensures that the products of cell division are two viable and genetically identical progeny. Chromosomes replicate to produce two sister chromatids that are held together by topological and protein-mediated linkages. At the onset of mitosis, chromosomes condense into discrete bodies, converting the chromatids into physically strong, rod-shaped structures short enough to segregate away from each other. At anaphase, the protein and topological connections between sisters are resolved and they separate and segregate away from each other to opposite poles of the mitotic spindle. The anaphase spindle in yeast is 10 um long, implying that the longest chromosome arm (1Mb) must be compacted at least 60 fold relative to the length it would occupy as naked DNA to allow full segregation of chromosome arms.

The cohesin complex is required to hold sisters together (Guacci et al. 1997; Michaelis et al. 1997) (for review see (Biggins and Murray 1999; Nasmyth et al. 2000). It consists of two coiled-coil ATPases, Smc1p and Smc3p, and additional regulatory subunits (Guacci et al. 1997; Michaelis et al. 1997; Losada et al. 1998; Toth et al. 1999; Tomonaga et al. 2000); these proteins are loaded onto replicating chromosomes (Uhlmann and Nasmyth 1998; Toth et al. 1999). In budding yeast, a proteolytic cascade results in sister separation at anaphase. The anaphase-promoting complex (APC) mediates destruction of securin (Pds1p)(Cohen-Fix et al. 1996), an inhibitor of a highly specific protease, separase (Esp1p) (Ciosk et al. 1998; Uhlmann et al. 2000). Esp1p

cleaves a cohesin subunit, Mcd1p/ Scc1p, driving the removal of the complex from the chromosomes and sister chromatid separation (Uhlmann et al. 1999). The topological linkage between sisters is also formed during S phase, most likely as a consequence of the collisions between replication forks that terminate DNA synthesis (Sundin and Varshavsky 1980; Sundin and Varshavsky 1981). At anaphase, DNA topoisomerase II enzyme resolves these intertwinings so sisters can fully separate from each other (DiNardo et al. 1984; Holm et al. 1985; Uemura et al. 1987; Shamu and Murray 1992).

The condensin complex induces mitotic chromosome condensation. Like the cohesins, the condensin complex is composed of two coiled-coil ATPases of the SMC family, Smc2p and Smc4p, and three regulatory subunits, although the latter show no obvious homology between cohesins and condensins (Hirano 1999). The condensins were isolated biochemically from *Xenopus* egg extracts, are required for mitotic chromosome condensation (Hirano and Mitchison 1994; Hirano et al. 1997; Cubizolles et al. 1998) and can form loops in DNA molecules *in vitro* (Kimura and Hirano 1997; Kimura et al. 1999). The idea that condensins accomplish condensation by the active reconfiguration of chromatin conforms with observations that condensation requires ATP hydrolysis (Kimura and Hirano 1997) and that members of the SMC family have predicted secondary structures resembling motor proteins that convert chemical energy into movement (Strunnikov et al. 1993; Hirano and Mitchison 1994).

Experiments in budding and fission yeasts support a role for the condensin complex in chromosome condensation and provide additional insights into their contribution to chromosome segregation (Saka et al. 1994; Strunnikov et al. 1995; Sutani et al. 1999; Freeman et al. 2000; Lavoie et al. 2000; Ouspenski et al. 2000). The fission

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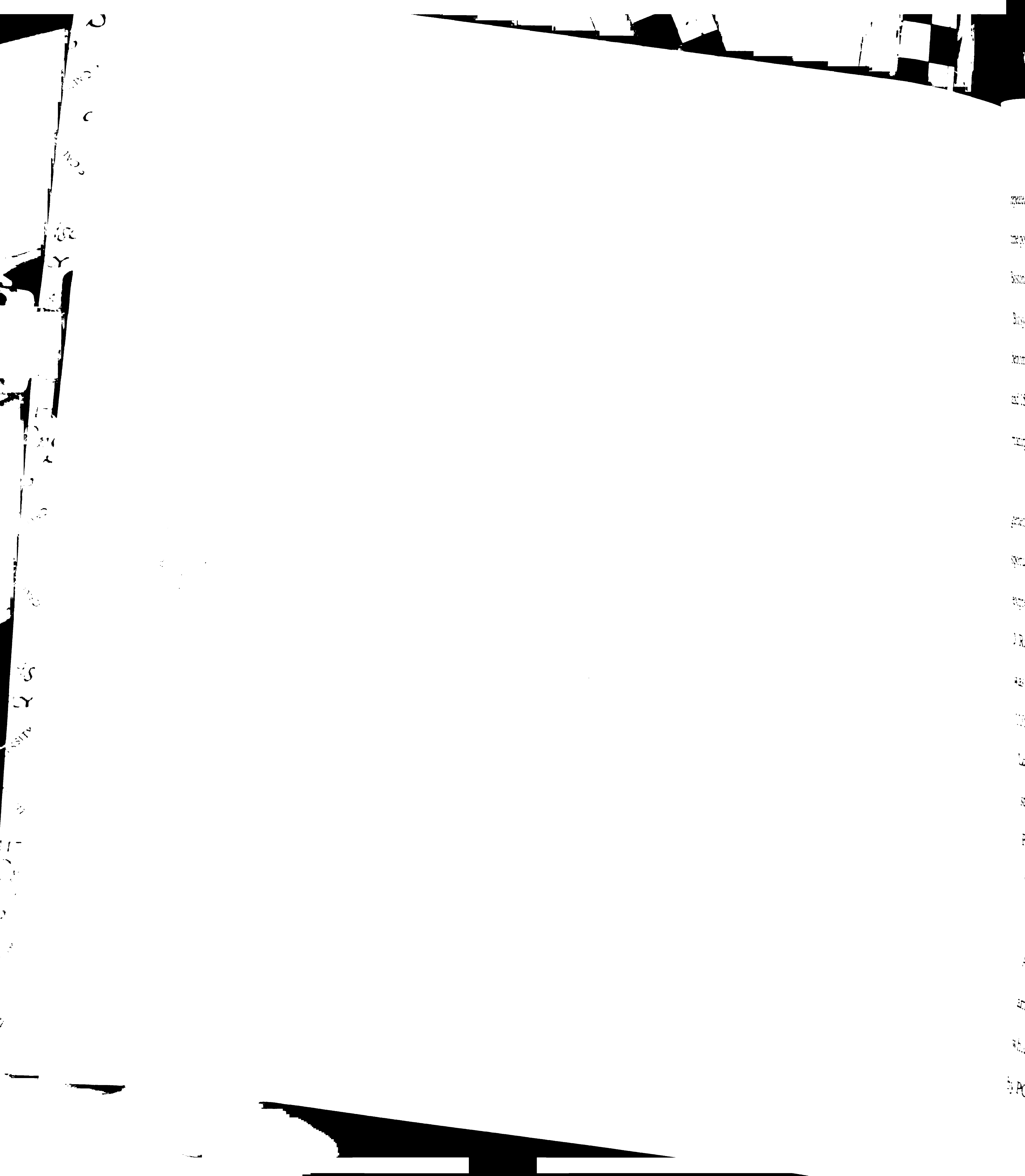
yeast complex can anneal single stranded DNA, an activity that may contribute to higher-ordered supercoiling consistent with condensation (Sutani and Yanagida 1997). In *S. cerevisiae*, reducing condensin function impairs transmission of the rDNA (Freeman et al. 2000). Recently, the essential role the 3 non-SMC subunits play has been illustrated in both *S. pombe* and *S. cerevisiae* (Sutani et al. 1999; Freeman et al. 2000). One of these subunits, *YCS4*, is the *S. cerevisiae* homolog of the *Xenopus* XCAPD2 (Kimura et al. 1998) and the *S. pombe CND1* (Sutani et al. 1999). We isolated a mutant of *YCS4* in a screen for defects in chromosome separation or segregation. Our analysis reveals role for the condensins in sister chromatid separation and the recruitment of core chromosomal proteins such as topoisomerases. Interestingly, *ycs4-1* expresses information from the silent mating type loci, whose transcription is normally repressed by the action of a number of chromosomal proteins.

MATERIALS AND METHODS

Microbial techniques and yeast strain construction

Media and genetic and microbial techniques were essentially as described (Sherman et al. 1974; Rose et al. 1990). All cytological experiments were carried out by arresting cells in 1 μg/ml α-factor at the permissive temperature (23°C) for four hours, washing cells twice in pre-warmed α-factor free media and resuspending them in media at the non-permissive (37°C) temperature. After one hour, α-factor was added back to the media to prevent cells from entering the next cell cycle. All experiments were



repeated at least twice with similar results. In all experiments, at least 100 cells for each time point were counted. Stock solutions of inhibitors were: 60 mg/ml benomyl (DuPont, Boston, MA), 10mg/ml nocodazole (Sigma, St. Louis, MO), 10 mg/ml α-factor (Biosynthesis, Lewisville, TX), all in DMSO. All stocks were stored at –20°C. For benomyl/nocodazole experiments, cells were released into media with 30μg/ml benomyl and 15μg/ml nocodazole at 37°C. The strain DH5α was used for all bacterial manipulations.

Yeast strains are listed in Table 4-1. Yeast strains were constructed by standard genetic techniques. Diploids were isolated on selective media at 23°C and subsequently sporulated at 23°C. The *pGAL-Δ176-CLB2* fusion that is contained in some strains is not expressed in dextrose media. The *HML* locus was deleted by integrating pJR826 (gift of J. Rine) and verifying the deletion by PCR. The marking of the arm of Chromosome IV was accomplished by integrating pAFS163 (gift of A. Straight) at intergenic region 1100000-1102221 of Chromosome IV into a strain containing only the *pCUP1-GFP12-LacI12::HIS3* fusion; microscopy verified the integration of the Lac operator repeats. A strain containing the epitope-tagged allele *YCS4-3XHA* was created by PCR integration. Primers LOC7-3 (5' GTC/ ACT/ GCA/ TTA/ TTG/ GAG/ CAA/ GGT/ TTC/ CAA/ GGT/ TGT/ ATC/ CGC/ AAA/ AGA/ AAG/ GGA/ ACA/ AAA/ GCT/ GG 3') and LOC7-4 (5' TAA/ TAA/ CAT/ ATA/ ATA/ TAA/ AAC/ GGA/ AGA/ AAC/ GGG/ TAA/ ACG/ TCA/ GTT/ CGA/ TTA/ CTA/ TAG/ GGC/ GAA/ TTG/ G 3') were used to PCR amplify DNA from plasmid pMPY-3XHA ((Schneider et al. 1995), gift of R. Kulberg) which was integrated into SBY215 to create NBY302. *YCS4-13Xmyc* was also created by PCR integration. Primers LOC7-10 (5' GAC/ GTC/ ACT/ GCA/ TTA/ TTG/ GAG/

CAA/ GGT/ TTC / AAG/ GTT/ GTA/ TCC/ GCA/ AAA/ GAA/ CGG/ ATC/ CCC/ GGG/ TTA/ ATT/ AA3') and LOC7-8 (5'ATA/ TAA/ TAA/ CAT/ ATA/ ATA/ TAA/ AAC/ GGA/ AGA/ AAC/ GGG/ TAA/ ACG/ TCA/ GTT/ CGA/ GAA/ TTC/ GAG/ CTC/ GTT/ TAA/ AC 3') were used to PCR amplify DNA from pFA6a-13Myc-kanMX6 (Longtine et al. 1998) which was integrated into NBY8 to create NBY333. Strains containing *TOP2-3XHA:HIS3* and *pGAL-TOP2-3XHA:LEU2* were a gift of C. Cuomo. *TOP1-3XHA* was created by PCR amplifying DNA from pFA6-3HA-His3MX6 (Longtine et al. 1998) using primers TOP1-1 (5'ATA/ AAA/ AAA/ ATC/ TAA/ AGG/ GAG/ GGC/ AGA/ GCT/ CGA/ AAC/ TTG/ AAA/ CGC/ GTA/ AAA/ CGG/ ATC/ CCC/ GGG/ TTA/ ATT/ AA 3') and TOP1-2 (5' AAC/ TTG/ ATG/ CGT/ GAA/ TGT/ ATT/ TGC/ TTC/ TCC/ CCT/ ATG/ CTG/ CGT/ TTC/ TTT/ GCG/ GAA/ TTC/ GAG/ CTC/ GTT/ TAA/ AC 3') and integrating the product into NBY8. *TOP1* was deleted by PCR integration using primers TOP1-2 and TOP1-3 (5'AGA/ GAA/ AAA/ TTC/ AAA/ TGG/ GCC/ ATA/ GAA/ TCG/ GTA/ GAT/ GAA/ AAT/ TGG/ AGG/ TTT/ CGG/ ATC/ CCC/ GGG/ TTA/ ATT/ AA 3') to PCR amplify DNA from pFA6-kanMX6 (Longtine et al. 1998), which was integrated into NBY8.

Plasmid construction

DNA encoding only the *YCS4* gene (plus 500 basepairs upstream, presumably containing the endogenous promoter) was PCR amplified using primers LOC7-1 (5' GCG/ CGC/ GGA/ TCC/ CGC/ GTT/ GTT/ TTC/ TTG/ TCG 3') and LOC7-2 (5' GCG/ CGC/ GGC/ CGC/ GGG/ TAA/ ACG/ TCA/ GTT/ CGA 3') that had *Bam* HI and *Not* I

sites engineered at the 5' and 3' ends, respectively. The PCR product was digested with *Bam* HI and *Not* I and ligated into the centromeric vector pRS316 (Sikorski and Hieter 1989) to create pNB27 which complemented the *loc7* ts phenotype. pSB10 was constructed by digesting pAFS78 (gift of A. Straight) with *Bam* HI and ligating the 1 kb lacI gene into pGEX-2T digested with *Bam* HI to create a GST-lacI fusion protein. pSB14 was constructed by ligating the 1 kb lacI *Bam* HI fragment from pAFS78 into the pQE-9 vector to generate 6HIS-lacI.

Isolation and identification of *YCS4*

The *LOC* screen was performed on the mutagenized parent strain SBY215 and the details of the screen are published elsewhere (Biggins et al. 2001). To confirm that *YCS4* was linked to the *loc7* mutation, we performed linkage analysis. NBY302 containing *URA3*-marked *YSC4-HA3* was crossed to NBY290 and the resulting diploid was sporulated. Out of 22 tetrads dissected, the *URA3* marker always segregated away from the *loc7* ts phenotype. In addition, a centromeric plasmid (pNB27) containing only the PCR amplified *YCS4* complemented the *loc7* temperature sensitive mutation, further confirming that the *YCS4* gene corresponds to *LOC7*.

Generation of lacI antibodies

LacI antibodies were generated against a GST-lacI fusion protein, pSB10 expressed and purified from bacteria. The protein was purified as described (Kellogg and

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Immu[illegible]

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Top1-3[illegible]
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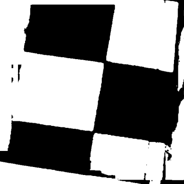
Murray 1995) and 0.5 mg protein was injected into rabbits at Babco, followed by 100 μgram boosts. The antibodies were affinity purified by first coupling a 6HIS-lacI fusion protein, pSB14 expressed and purified from bacteria, to affi-gel as described (Kellogg and Murray 1995). Antibodies were purified on the affinity column as described (Harlow and Lane 1988) and subsequently dialyzed into PBS.

Immunofluorescence and microscopy

Microscopy was performed as described (Biggins et al. 1999). $CuSO_4$ was added to a final concentration of .25-.5 mM to all experiments to induce expression of the GFP-LacI fusion. Immunofluorescence was performed as described (Rose et al. 1990). Monoclonal 9E10 anti-myc (Babco, Berkeley, CA) and rabbit polyclonal anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were pre-incubated with an untagged spheroplasted strain two times for 10 minutes each at 23°C and used at 1:1000 dilution. Anti-Nop1 antibodies were kindly provided by J.P. Aris and used at 1:5000 dilution. Anti-tubulin antibodies, yol 1/34, (Accurate Chemical and Scientific Corp., Westbury, NY) were used at 1:1000 dilution. DAPI (Molecular Probes, Eugene, OR) was used at 1μg/ml final concentration. Chromosome spreads were performed as described (Michaelis et al. 1997; Loidl et al. 1998). Monoclonal 16B12 anti-HA antibodies (Babco, Berkeley, CA) were similarly pre-incubated against an untagged strain and used at 1:1000 dilution for Mcd1-3XHAp chromosome spreads and 1:500 for Top2-3XHAp and Top1-3XHAp spreads. Anti-LacI antibodies were used at a dilution of 1:200. Lipsol was obtained from Lip Ltd. (Shipley, England)



Determining mitotic recombination frequencies

The strains to assay mitotic recombination at the rDNA and the *LEU2* locus were kindly provided by R. Rothstein (Gangloff et al. 1996; Smith and Rothstein 1999). They were crossed to the appropriate mutant and sporulated to isolate a spore that contained both the mutant allele and the construct to assay recombination. Because we are working with known and hypothesized hyper-recombinant mutants, we maintained the identified spores on –URA media to insure that the starting colony for the experiment had not already recombined out the marker. Single colonies were inoculated into YPD and allowed to grow until mid-log phase. Cultures were then diluted and plated onto YPD solid media. After growth, colonies were counted and the plates replica-plated to –URA solid media. Recombination frequencies were calculated by counting the number of colonies that failed to grow on –URA and dividing that number by the total number of colonies that grew on YPD.

Fluorescence *in situ* hybridization

In situ hybridization was performed as described (Dernburg and Sedat 1998). The digoxegenin-labeled rDNA probe was a gift of A. Rudner. Rhodamine-conjugated anti-digoxegenin antibodies (Boehringer Mannheim, Germany) were used at 1:500 dilution. Z-stacks were taken spanning ~4 um and the optical sections converted into a stacked image with Metamorph software.

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RESULTS

Sister chromatid separation in *ycs4-1*

We generated a temperature-sensitive collection of mutants and visually screened them for defects in mitotic chromosome behavior. Chromosomes were marked with green fluorescent protein (GFP): an array of Lac operator (LacO) repeats were integrated at the *TRP1* locus (~12 kb away from the centromere of chromosome IV) in a strain that expressed a GFP-Lac repressor (GFP-LacI) fusion (Straight et al. 1996). We isolated nine complementation groups (*loc1-9*) that appeared defective in sister chromatid separation or segregation (Biggins et al. 2001). *LOC7* was cloned by complementing the recessive temperature-sensitive phenotype and identified as hypothetical ORF YLR272C, the putative XCAPD2 homolog (Kimura et al. 1998). Recent studies have verified that this gene is a regulatory subunit of the condensin complex and it has been named *YCS4* (Freeman et al. 2000).

We used GFP-marked chromosomes to analyze sister chromatid separation in the *ycs4-1* mutant (Figure 4-1A). We constructed strains that combined the mutant or wildtype copy of the gene with the Lac operator array integrated near the centromere (at the *TRP1* locus), on the arm, or at the telomere of chromosome IV. Cells were arrested in G1 by treating them with α–factor at the permissive (23°C) temperature and released into media at the non-permissive temperature (37°C) in the absence of α-factor. Figure 4-1A shows that in wildtype cells, sister chromatid separation began 80 minutes after release

from G1 and was complete by 120 minutes. As in wildtype, *ycs4-1* cells began sister separation at 80 minutes, indicating that the onset of anaphase was normal, but a only a fraction of the cells had managed to separate their sisters by 120 minutes. As the position of the Lac operator array was further from the centromere, the defect became more pronounced: sister separation at the *TRP1* locus occurred in 77% of the cells while only 49% of the cells managed to separate the arms of sister chromatids and 29% the telomeres. The phenotype of *ycs4-1* is reminiscent of that of the *top2-4* mutant, in which the inability to decatenate sister chromatids presents a topological block to sister separation (DiNardo et al. 1984; Holm et al. 1985). In *top2-4*, spindle forces acting at the centromeres pull sisters apart, resulting in chromosome loss and breakage that lead to cell death (Uemura et al. 1987; Holm et al. 1989). We compared the phenotypes of the two mutants and found that although they are qualitatively similar, *top2-4* exhibits more severe sister separation defects than *ycs4-1*, particularly at the arm and telomere of chromosome IV (Figure 4-1A).

To determine if the sister chromatid separation in *ycs4-1* was a product of spindle forces, we analyzed chromosome separation in the absence of a spindle. This experiment must be performed in spindle checkpoint mutants because wildtype cells activate the checkpoint to prevent cells from separating their sister chromatids in the absence of microtubules. *mad* and *bub* mutants inactivate this checkpoint (Hoyt et al. 1991; Li and Murray 1991), allowing activation of the APC in the absence of a spindle. Under these conditions, sister chromatids diffuse apart from each other without the aid of microtubules (Straight et al. 1996; Marshall et al. 1997; Straight et al. 1997). If sister separation in *ycs4-1* requires microtubule-dependent forces, a *ycs4-1mad2Δ* double

mutant should not separate sisters in nocodazole to the degree that a *mad2Δ* mutant would.

Wildtype, *ycs4-1, top2-4*, *mad2Δ,ycs4-1mad2Δ* and *top2-4mad2Δ* strains were arrested in G1 in medium with α-factor at 23°C; all strains carried the Lac operator array at the *TRP1* locus of chromosome IV. We released them into medium containing nocodazole and benomyl at 37°C. Figure 4-1B shows that wildtype and the *top2-4* and *ycs4-1* single mutants activated the spindle checkpoint and arrested in metaphase with unseparated sister chromatids. The *mad2Δ* single mutant bypassed the checkpoint, continued cycling in the absence of a spindle and separated sister chromatids in 60% of the cells within 2 hours after release from G1. We believe that the sisters were separated in the remainder of the cells, but lie too close to each other to be resolved by the light microscope. Even in the absence of the checkpoint, sister separation was strongly inhibited in *top2-4mad2Δ*. The *ycs4-1* mutant showed an intermediate phenotype. In the absence of microtubules, the *ycs4-1mad2Δ* double mutant separated its sisters but did so more slowly than the *mad2Δ*. Two hours after release from G1, the double mutant separated sisters in only 24% of its cells and required an additional hour and a half to achieve sister separation comparable to that of *mad2Δ* cells at two hours after release. Therefore, in the absence of spindle forces, the resolution of sister chromatids is compromised in *ycs4-1*. The slow sister chromatid separation we observe in *ycs4-1* in the absence of microtubules is dependent upon topoisomeraseII activity, as a *ycs4-1top2-4mad2Δ* triple mutant in nocodazole does not separate its sister chromatids (our unpublished results).

Cohesins are loaded onto and removed from chromosome spreads in *ycs4-1* as in wildtype

Sister separation mutants fall into two classes; these are defined by *esp1-1*, which cannot remove cohesins from chromosomes (Ciosk et al. 1998), and *top2-4*, which removes cohesins normally (our unpublished results). We classified *ycs4-1* by monitoring the loading and removal of a cohesin subunit, Mcd1p/ Scc1p, (Guacci et al. 1997; Michaelis et al. 1997) as cells passed through mitosis. Wildtype and *ycs4-1* strains with an epitope-tagged *MCD1/ SCC1* gene were arrested in G1 with α-factor at 23°C and released into media at 37°C. Samples were treated with detergent and fixative simultaneously to remove soluble nuclear proteins and retain chromatin-associated proteins, which were then visualized by indirect immunofluorescence. Figure 4-2 illustrates that the association of Mcd1p/ Scc1p with chromatin in *ycs4-1* is qualitatively and quantitatively indistinguishable from wildtype. The staining pattern and the kinetics of chromatin association and dissociation of Mcd1p/ Scc1p is the same in the two strains. Thus, sister chromatid separation in *ycs4-1* mutants is defective despite the removal of cohesins from chromosomes in anaphase. The similarity to the phenotype of *top2-4* suggests that the condensin complex, which contains Ycs4p, may be required for the rapid resolution of the topological linkage between sister chromatids; alternatively, the condensins may be responsible for the abolition of a previously unsuspected, cohesin-independent, proteinaceous linkage.

***YCS4* Regulates the Localization of Topoisomerase I and II**

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Because the lack of *YCS4* function results in a *top2-4*-like phenotype, we asked if *YCS4* function was required to localize topoisomerase II. *YCS4* and *ycs4-1* strains that contained epitope-tagged *TOP2* were arrested in G1 at 23°C and released into fresh media at 37°C. Images of the chromosome spreads are shown in Figure 4-3A and the data is quantified in Figure 4-3B. Wildtype nuclei maintained a punctate Top2p association throughout the cell cycle (Figure 4-3A and B). However, over half of the *ycs4-1* nuclei lost their Top2p staining within 30 minutes of the temperature shift to 37°C (Figure 4-3A and B). Immunoblotting of cell lysates verified that the Top2p protein was still present in *ycs4-1* despite the loss of the protein from chromsome spreads (our unpublished results). We also observed a loss of Topoisomerase I from *ycs4-1* chromosome spreads at the non-permissive temperature (Figure 4-3C). The pattern of topoisomerase I staining on chromosome spreads is similar to that of topoisomerase II staining, punctate and coincident with DNA staining (our unpublished results).

However, the *ycs4-1* phenotype cannot be fully explained by the loss of topoisomerase II from chromosomes. Overexpression of Top2p does not suppress the temperature sensitivity or sister separation phenotype of *ycs4-1* despite restoration of Top2p to chromosomes as visualized by chromosome spreads (our unpublished results). A simple interpretation of this failure is that the condensin complex has general effects on mitotic chromosome structure and that Top2 is only one of several proteins whose chromosomal localization and function has been compromised.

Ycs4p is nuclear throughout the cell cycle and is enriched at the rDNA at anaphase

In order to gain a greater understanding of *YCS4*'s role in mitotic chromosome behavior, we localized Ycs4p by indirect immunofluorescence on both whole cells (Figure 4-4) and chromosome spreads (our unpublished results). In frogs and fission yeast, the condensin complex is only associated with chromatin or in the nucleus during mitosis (Hirano et al. 1997; Sutani et al. 1999). Our experiments reveal that Ycs4p was present in the nucleus (Figure 4-4A and B) and associated with chromatin (our unpublished results) throughout the budding yeast cell cycle. This is consistent with the findings of Freeman and her colleagues (Freeman et al. 2000). The only observable shift in localization occurred at anaphase when a general staining of the nucleus was replaced by specific staining of the nucleolus (detected by the nucleolar marker Nop1p (Aris and Blobel 1988)) (Figure 4-4C); cells arrested in metaphase by overexpression of Mps1p did not exhibit this subnuclear localization (our unpublished results). In cells in which the chromosomal rDNA had been deleted and replaced with a single copy of the repeat on a 2-micron plasmid (*rdn*Δ) (Nierras et al. 1997), there was no anaphase relocalization and Ycs4p was diffusely nuclear throughout the cell cycle (Figure 4-4D). This effect is not due to the presence of the plasmid borne rDNA, since anaphase nucleolar enrichment is restored in a strain that contained the rDNA array on chromosome XII as well as the 2-micron plasmid (our unpublished results). Despite Ycs4p's variation from the behavior of the *Xenopus* and fission yeast condensin complexes, its localization supports a role for Ycs4p in chromosome structure and suggests a specialized role at the rDNA.

ycs4-1 mutants exhibit defects in rDNA condensation, function and segregation

Since Ycs4p is not localized to the chromatin specifically during mitosis, we asked if the protein is required for normal mitotic chromosome structure. We monitored mitotic condensation by fluorescent *in situ* hybridization (FISH) using probes against the highly repetitive rDNA array. Condensation defects are assayed at the rDNA primarily because of the ease of interpreting the fluorescence *in situ* hybridization signal. We arrested wildtype and *ycs4-1* cells in G1 with α-factor and released them into fresh media containing benomyl and nocodazole at 37°C to yield cells arrested in pro-metaphase. The loops, bars and horseshoe shapes observed by *in situ* hybridization to the rDNA during mitosis have been interpreted as condensed rDNA while an amorphous signal at the periphery of nucleus has been interpreted as decondensed rDNA. We saw the latter structure of the rDNA in 69% of the *ycs4-1* cells compared with the intact loops and crescents seen in 95% of wildtype cells in pro-metaphase (Figure 4-5A, B). This observation suggests that *YCS4* has a role in maintaining chromosome structure in mitosis and that its functions at the rDNA are not restricted to anaphase. We have not assayed condensation at single copy sequences but other studies have illustrated the cell cycle dependence of the specific rDNA morphology associated with condensation and its correlation with condensation at single copy loci (Guacci et al. 1994; Freeman et al. 2000; Lavoie et al. 2000).

We asked if *YCS4* plays a role in the stability of the rDNA locus. Topoisomerase I and II have been implicated in maintaining the stability of the rDNA array by suppressing mitotic recombination at the locus (Christman et al. 1988; Kim and Wang 1989). Since *ycs4-1* impairs topoisomerase I and II's association with chromosomes, we

measured recombination within the rDNA array by measuring the loss of a *URA3* marker inserted into the rDNA locus (Gangloff et al. 1996). Control strains, in which the *URA3* marker was integrated between a pair of direct repeats of the *LEU2* locus, were used to determine whether effects were specific for the rDNA locus (Smith and Rothstein 1999). Table 4-2 illustrates the frequency of loss of the *URA3* marker in *top1Δ*, *top2-4*, *top1Δtop2-4* double and *ycs4-1* mutants at both the rDNA and the *LEU2* locus. The single and double topoisomerase mutants showed higher rates of mitotic recombination at the rDNA locus (38 fold higher for *top1Δ* and 83 fold higher for *top1Δtop2-4*) than wildtype with substantial but smaller increases in recombination at the *LEU2* locus. *ycs4-1* cells grown at the permissive temperature had a much more specific defect: a 63 fold elevation in recombination at the rDNA locus with only a 2 fold increase in recombination at *LEU2*.

A requirement for the budding yeast condensin complex has been implicated in rDNA segregation during mitosis (Freeman et al. 2000). We examined the segregation of the rDNA locus in synchronized cells passing through anaphase. In wildtype, 90% of the cells have segregated the nucleolar marker Nop1p to both mother and bud, and only 10% of cells contained Nop1p only in the mother (identified by the pheromone-induced shmoo morphology) (Figure 4-5C). In *ycs4-1* cells undergoing anaphase, 45% of the cells exhibited Nop1p only in the mother (Figure 4-5C). Furthermore, these cells had a perturbed nucleolar structure: the nucleolus is not bar- or crescent-shaped as in wildtype, but diffuse and amorphous, consistent with the *in situ* hybridization results (Figure 4-5D). The remaining 55% of the cells had segregated their nucleoli and exhibited normal Nop1p staining.

ycs4-1 is defective in silencing of the silent mating type locus

Initial attempts to arrest *ycs4-1 MATa* cells in media containing α-factor at the permissive temperature failed. When these cells were plated on YPD plates containing α-factor, they did not respond to the pheromone and continued to grow (Figure 4-6A). *ycs4-1*'s α-factor resistance was overcome when the silent mating locus *HML α* was deleted (Figure 4-6A), suggesting that the mutant was defective in silencing at the mating type loci. Silencing defects were not observed at the telomere at the permissive temperature (Figure 4-6B) and silencing at the rDNA could not be assayed as the integration of the reporter construct (Smith and Boeke 1997) at the rDNA is synthetically lethal with the *ycs4-1* mutation (our unpublished results).

DISCUSSION

We have shown that *YCS4*, a regulatory subunit of the condensin complex, is required for accurate sister chromatid separation; the mutant phenotype resembles that of *top2-4*, suggesting that *ycs4-1* mutants have a topological block to sister separation. Consistent with the sister separation phenotype, Top2p and Top1p are absent from chromosome spreads prepared from *ycs4-1* cells at the non-permissive temperature. Ycs4p is intimately associated with the array of rDNA genes on chromosome XII: the protein localizes to the nucleolus in anaphase cells, nucleolar structure and segregation is abnormal in *ycs4-1* and inter-repeat recombination in the rDNA array is specifically

elevated in *ycs4-1*. The mutant exhibits defects in silencing at the silent mating type loci at the permissive temperature, suggesting that yeast condensins function at all stages of the cell cycle and influence processes other than mitotic chromosome condensation.

Condensin function is required to separate sister chromatids

The phenotype of *ycs4-1* resembles that of topoisomerase II mutants; sister chromatid separation becomes more defective as the distance from the centromere increases. In *top2-4*, the separation observed near the centromere requires microtubule-dependent forces and the inability to fully resolve the catenated sister chromatids leads to lethal events such as non-disjunction and chromosome breakage (Holm et al. 1989). In *ycs4-1*, the sister chromatids have difficulty separating but this block can eventually be resolved, even in the absence of spindle forces. This observation may explain why chromosome loss phenotypes are difficult to detect in condensin mutants, especially given the small size of reporter constructs used in such assays (Hieter et al. 1985; Spencer et al. 1990). We suggest that condensins establish and maintain mitotic chromosome structure, which in turn facilitates the resolution of topological linkage between sister chromatids. In the absence of full condensin function, the decatenation, separation and proper segregation of sister chromatids are impaired, despite the normal timing of cohesin removal at anaphase.

Depending on the state of the substrate DNA, topoisomerase II can either catenate or decatenate DNA circular DNA molecules. Increasing DNA condensation favors decatenation, because two compact DNA molecules are less likely to collide with each

other and become catenated than two extended DNA molecules (Holmes and Cozzarelli 2000). Thus condensins could promote sister separation by affecting the amount or directionality of topoisomerase II activity. Studies on the bacterial SMC homolog, *MukB*, support the latter possibility (Sawitzke and Austin 2000). Sawitzke and Austin found that the chromosome partitioning defects of the *mukB, mukE,* and *mukF* mutants in *E. coli* were suppressed by mutations in the bacterial topoisomerase I gene, *topA*. Reducing topoisomerase I activity allows DNA gyrase activity to increase the negative supercoiling of the nucleoid; in the absence of Muk function, this increased negative supercoiling provided a level of chromosome organization that allowed proper segregation of the nucleoid. In eukaryotes, it is possible that the action of the condensin complex contributes to the decatenation of sister chromatids by introducing the higher-level organization typical of mitotic condensation (for review see (Holmes and Cozzarelli 2000).

Catenation of eukaryotic chromosomes is believed to arise as replication forks collide at the completion of DNA replication (Sundin and Varshavsky 1980; Sundin and Varshavsky 1981) and topoisomerase II activity is required during anaphase to allow sister chromatid separation (Holm et al. 1985; Uemura et al. 1987; Holm et al. 1989; Shamu and Murray 1992). What changes to favor decatenation at anaphase? We can exclude two obvious possibilities, microtubule-dependent forces and increased topoisomerase II activity. Sisters can separate in the absence of microtubules (Straight et al. 1996; Straight et al. 1997), and topoisomerase activity falls as Xenopus extracts enter anaphase (Shamu and Murray 1992).

We suggest that the extent of chromosome condensation reflects a dynamic balance between the activities of cohesins and condensins. We speculate that the complete removal of cohesins at anaphase allows condensins to induce further DNA compaction that makes anaphase chromosomes more condensed than metaphase ones. In this scenario, cohesins and condensins have opposing effects on chromosome condensation. This idea explains the relationship between cohesin behavior, topoisomerase activity, and chromosome condensation as vertebrate cells enter mitosis. Unlike budding yeast, most cohesin leaves vertebrate chromosomes as the cells enter mitosis, corresponding to an increase in chromosome condensation, which requires topoisomerase II activity. The removal of cohesin would allow condensin to increase chromosome compaction, thus driving topoisomerase II to remove topological linkages that would interfere with full chromosome condensation. Opposing roles of condensin and cohesin are not easily reconciled with the condensation defects observed in budding yeast cohesin mutants. We cannot exclude the possibility that there may be some collaboration between cohesin and condensin function in preparing condensed mitotic chromosomes for segregation in vertebrate cells.

Condensins are required to localize Topoisomerase I and II

We found that the condensin complex is required to localize topoisomerase I and II to chromosomes. This observation differs from those of Hirano et. al. who showed that immunodepletion of the condensin complex from *Xenopus* frog egg extracts did not affect the association of topoisomerase II with chromosomes (Hirano et al. 1997). There

are a number of differences between the experiments. First, the frog egg extract was made from cells in metaphase of meiosis II and yeast cells were studied in mitosis. Second, chromosomes in the egg extracts had not gone through replication. Third, there are large stockpiles of numerous essential proteins in the extract. A high concentration of topoisomerase II may allow condensin-independent binding to chromosomes. Indeed, we may be recapitulating such a scenario when we overexpress Top2p; under these conditions, Top2p binds to chromosomes despite defects in *YCS4*.

Studies on the barren mutant in *Drosophila* suggested an interaction between the condensin complex and topoisomerase II. Barren is the fly counterpart of *Xenopus* XCAP-H, budding yeast *BRN1* and fission yeast *CND2*. The fly protein colocalized, biochemically associated with and enhanced the enzymatic activity of topoisomerase II (Bhat et al. 1996). Attempts to recapitulate these findings in yeast and *Xenopus* have been unsuccessful (Hirano et al. 1997; Lavoie et al. 2000). Our investigations reveal that a relationship between the complex and topoisomerase II does exist; condensin function is required to localize the protein to chromosomes. However, we do not observe a biochemical interaction between Ycs4p and Top2p (our unpublished results), suggesting that yeast condensins stimulate topoisomerase binding indirectly.

Do condensins recruit other chromosomal proteins other than topoisomerases? The normal binding and displacement of Mcd1p/Scc1p indicates that at least one protein binds normally in the absence of condensins. However, condensins may recruit additional chromatin associated proteins required for mitotic chromosome behavior, some of which may collaborate with condensins to condense chromosomes and drive sister chromosome separation and segregation.

Ycs4p is localized to chromatin throughout the cell cycle

The behavior of the budding yeast condensin complex differs from that of the complexes characterized in *Xenopus* egg extracts and fission yeast. In frogs, phosphorylation of a subset of the regulatory subunits by the mitotic Cdc2/Cyclin B complex controls the association of the complex with chromatin at mitosis (Hirano et al. 1997) and activation of its supercoiling activity (Kimura and Hirano 1997; Kimura et al. 1998). The fission yeast complex is regulated by compartmentalization; nuclear import, and thus access to the chromatin, is limited to mitosis. Import depends on the phosphorylation of Cut3p, the *SMC4* homolog, by the Cdc2/ CyclinB complex (Sutani et al. 1999). The *S. cerevisiae* complex, specifically Smc2p and 4p, associate with chromatin throughout the cell cycle; strikingly, the only change in localization occurs at pro-metaphase when Smc4p and Ycs5p, another condensin regulatory subunit, concentrate at the rDNA (Freeman et al. 2000). We observe a similar dramatic shift in localization with Ycs4p. However, our analysis of the protein's localization indicates that its exclusive binding at the rDNA occurs only during anaphase; cells arrested in metaphase exhibit the nuclear and general chromatin localization observed in every other stage of the cell cycle. Could this shift in localization be a modification of the mitosis-specific chromatin association observed in fission yeast and *Xenopus*? Or does the nucleolar association we observe in anaphase indicate a budding yeast-specific-requirement for condensin function in the decatenation, separation and proper segregation of the chromosomal rDNA array?

Condensins play a special role at the chromosomal rDNA array

Freedman et al recently illustrated a special role for the condensin complex at the rDNA array (Freeman et al. 2000). They provided evidence that strongly suggests that the complex is required for the mitotic transmission of rDNA. Here, we show that the condensin complex affects the structure and stability of the chromosomal array as well as its segregation during mitosis. We observed three defects specific to the rDNA array. First, mitotic recombination at the rDNA array is increased 63 fold over wildtype in the *ycs4-1* mutant at the permissive temperature. Second, integration of a reporter construct designed to assay transcriptional silencing at the rDNA is synthetically lethal with the *ycs4-1* mutation (our unpublished results). Third, the anaphase structure and segregation of the nucleolus is abnormal in *ycs4-1* cells. When we used Nop1p to visualize segregation of the rDNA array in *ycs4-1*, we saw two phenotypes. In 55% of cells, the nucleolus had segregated normally and had a normal condensed, crescent-shaped structure, whereas 45% of cells contained a single amorphous mass that stained with Nop1p antibodies and remained in the mother. We do not know whether defects in nucleolar structure lead to defects in nucleolar segregation or vice versa. The defects in nucleolar segregation in condensin mutants (Freeman et al. 2000) suggest that nucleolar enrichment of condensin subunits during anaphase could be an attempt of the cell to facilitate the separation and segregation of this heterochromatin-like locus (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997)

The rDNA differs from the remainder of the genome in two ways: it is present as a large array of tandem repeats and a fraction of the repeats are transcribed at very high rates. Transcription produces topological effects that may interfere with proper chromosome segregation. Plant and animal cells deal with this problem by shutting down transcription during mitosis, but in budding yeast, transcription continues during mitosis, which can occupy a large fraction of the cell cycle. We speculate that the presence of condensin at the nucleolus relieves the topological constraints produced by transcription, thus facilitating separation and segregation of the rDNA.

Condensin also appears to be required for the stability of artificial chromosomes containing repetitive satellite DNA (Freeman et al. 2000), which are probably not transcribed, suggesting that repetitive DNA presents additional challenges to chromosome segregation that require condensin function. Annealing of single stranded regions from one repeat to another repeat within the same array will form structures that stimulate recombination, leading to repeat loss, repeat gain, and breaks within the array. Such single stranded DNA could appear during DNA replication or as a result of topological stress induced by transcription. The observed strand annealing activity of condensins (Sutani and Yanagida 1997) may help to prevent the formation of single stranded intermediates that could trigger such dangerous reactions. This role in DNA metabolism may explain the observed localization of condensin subunits to specific regions of chromatin during interphase in human cells (Schmiesing et al. 1998) and fruit flies (Lupo et al. 2001). Recruiting condensins to repeated DNA sequences during interphase could be the basis of heterochromatin formation.

YCS4 is required for silencing at the silent mating loci

We observed defects in silencing at the mating type loci in *ycs4-1* at the permissive temperature: *ycs4-1* cells arrest in response to α-factor only when the *HMLα* locus is deleted, suggesting that defects in condensin function interfere with silencing. At the permissive temperature these defects are mild; the loss of silencing at *HMLα* is not severe enough to prevent mating (see (Whiteway and Szostak 1985)) and we could not detect derepression of a reporter gene integrated at the telomere, although this assay may lack the sensitivity of the assay at *HMLα.* Furthermore, the silencing defects we observe may be less severe because we must assay for them at the permissive temperature; the loss of silencing may be more dramatic if we could assay it with the complete lack of *YCS4* function.

Recently, topoisomerase II and barren have been implicated in regulating epigenetic gene expression in fruit flies (Lupo et al. 2001). A *YCS4* homolog, DPY-28, is required for dosage compensation in *C. elegans* (Meyer 2000), making it tempting to infer a direct requirement for members of the condensin complex in silencing in budding yeast, perhaps with other partners. Indeed, this may explain its association with chromatin throughout the cell cycle. However, the silencing defect may be one more indirect consequence of the requirement for condensin function to maintain chromosome architecture throughout the cell cycle in budding yeast; like topoisomerase I and II, proteins required for silencing that may be lost from chromosomes as a result of perturbed chromosome structure. Two observations argue against this: 1) indirect immunofluorescence on chromosome spreads against Sir2p reveal no gross loss of this

chromosome-associated silencing factor from chromatin (our unpublished results) and 2) mutants containing temperature-sensitive alleles of *SMC2* do not exhibit the alpha factor resistance phenotype while the *smc4-1* mutant does (our unpublished results). In addition to the resolution of sister chromatids, our investigations have revealed a role for the condensins in regulating the behavior of budding yeast chromosomes throughout the cell cycle.

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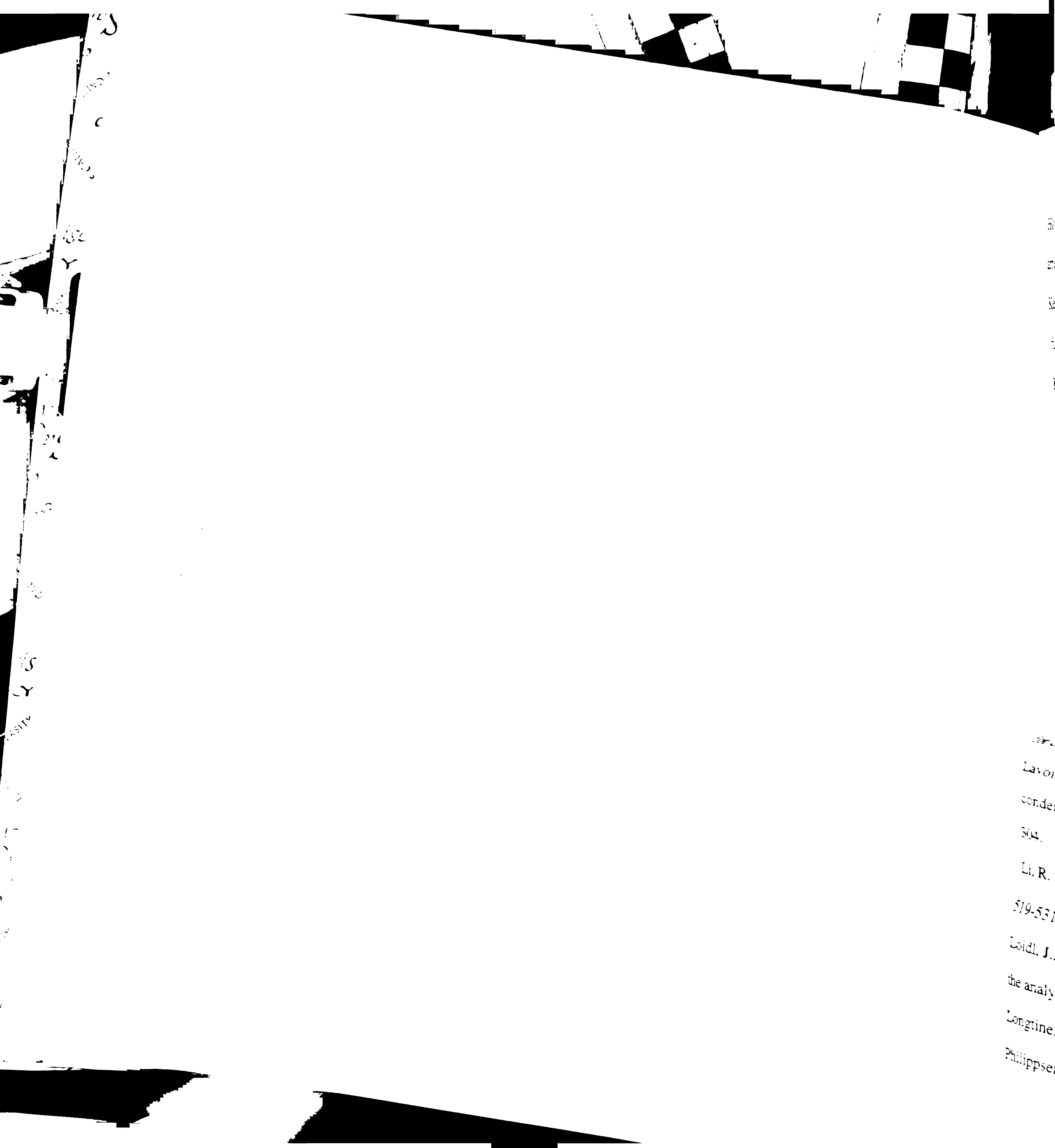

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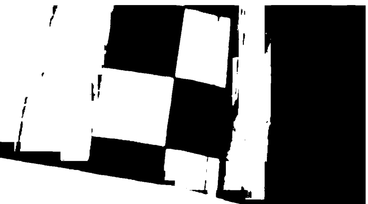
Table 4-1. Yeast strains used in this study

Strain	Genotype
SBY215	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2*
SBY376	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-lacI12::HIS trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 MCD1:3XHA:URA3:3XHA*
NBY8	*MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
NBY92	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 top2-4*
NBY113	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 mad2Δ::URA3*
NBY241	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 hmlΔ::LEU2 ycs4-1*
NBY258	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 mad2Δ::URA3 top2-4*
NBY259	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ armIV::LacO::URA3 top2-4*
NBY275	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 hmlΔ::LEU2 mad2Δ::URA3 ycs4-1*
NBY284	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-lacI12::HIS trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 MCD1:3XHA:URA3:3XHA hmlΔ::LEU2 ycs4-1*
NBY290	*MATα ura3-1 leu2,3-112 his3-11::pCUP1-GFP12-lacI12::HIS trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ hmlΔ::LEU2 ycs4-1*
NBY291	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ armIV::LacO::URA3*
NBY292	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ telIV::LacO::LEU2*
NBY302	*MAT**a** ura3-1 leu2,3-112 his3-11::pCUP1-GFP12-lacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 YCS4:3XHA:URA3:3XHA*



NBY316	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ ycs4-1*
NBY319	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ hmlΔ::LEU2 ycs4-1*
NBY322	*MATa ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ TOP2-3XHA::HIS3 hmlΔ::LEU2 ycs4-1*
NBY323	*MATa ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ telIV::LacO::LEU2 hmlΔ::LEU2 ycs4-1*
NBY327	*MATa ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ armIV::LacO::URA3 hmlΔ::LEU2 ycs4-1*
NBY333	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ YCS4-13Xmyc::KAN*
NBY374	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ ADH4::mURA3::tel ppr1Δ::LYS2*
NBY377	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ ADH4::mURA3::tel ppr1Δ::LYS2 ycs4-1*
NBY455	*MATa ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ telIV::LacO::LEU2 top2-4*
NBY474	*MATa ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ TOP2-3XHA::HIS3*
NBY479	*MATa ura3-1 leu2-3,112::pGAL-TOP2-3XHA::LEU2 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ hmlΔ::LEU2 ycs4-1*
NBY480	*MATa ura3-1 leu2-3,112::pGAL-TOP2-3XHA::LEU2 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ hmlΔ::LEU2*
NBY496	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ TOP1-3XHA::HIS3 hmlΔ::LEU2*
NBY498	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ TOP1-3XHA::HIS3 hmlΔ::LEU2 ycs4-1*
NBY507	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ YCS4-13Xmyc::KAN* {p*RDN-URA3*}
NBY508	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ YCS4-13Xmyc::KAN rdnΔ* {p*RDN-URA3*}
NBY513	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3*
NBY514	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3 top1Δ::KAN top2-4*
NBY515	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3 top1Δ::KAN*
NBY516	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3 top2-4*
NBY518	*MATα ura3-1 leu2ΔBstEII::URA3-HOcs::leu2ΔEcoRI his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ top1Δ::KAN top2-4*
NBY519	*MATα ura3-1 leu2ΔBstEII::URA3-HOcs::leu2ΔEcoRI his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ top1Δ::KAN*

NBY520	*MATα ura3-1 leu2ΔBstEII::URA3-HOcs::leu2ΔEcoRI his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ top2-4*
NBY521	*MATα ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3 ycs4-1*
NBY522	*MATα ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ RDN::URA3*
NBY523	*MATα ura3-1 leu2ΔBstEII::URA3-HOcs::leu2ΔEcoRI his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ ycs4-1*
NBY524	*MATα ura3-1 leu2ΔBstEII::URA3-HOcs::leu2ΔEcoRI his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
NBY585	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ hmlΔ::LEU2*

All strains are isogenic with the W303 strain background. Plasmids are indicated in brackets.

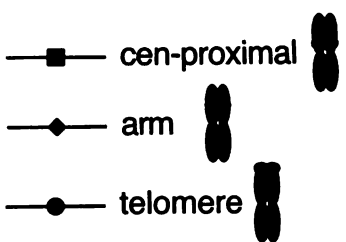
Table 4-2. Mitotic frequency of marker loss

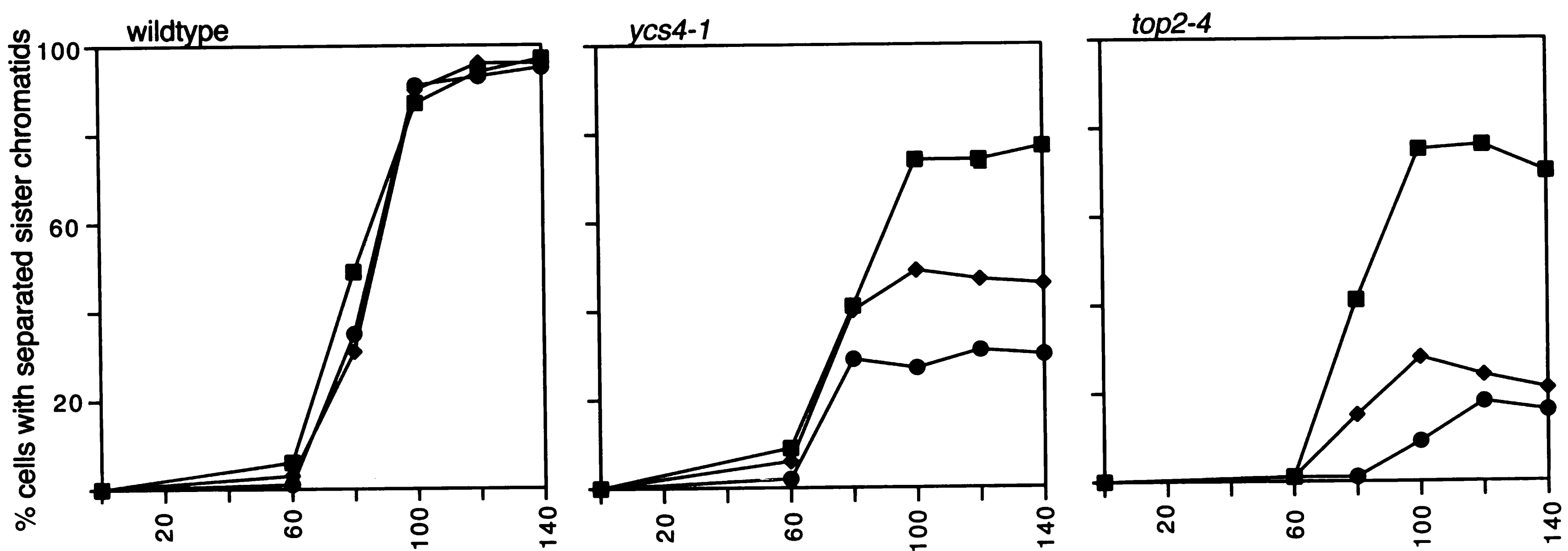
Genotype	*RDN::URA3*		*leu2::URA3::leu2*	
	Recombination frequency $X10^{-3}$	Fold increase	Recombination frequency $X10^{-3}$	Fold increase
wildtype	.4 ± .3	1	.3 ± .3	1
top1Δ	15 ± 3	38	5 ± 3	17
top2-4	1 ± 1	3	.8 ± .2	3
top1Δtop2-4	33 ± 5	83	8 ± 4	27
ycs4-1	25 ± 4	63	.5 ± .5	2

The recombination frequencies were determined as described in Materials and Methods. Values are reported as the means and standard deviations and were determined on at least 3 independent trials for each genotype.

Figure 4-1. *YCS4* is required for sister chromatid separation. **A.** The sister chromatid separation phenotypes of *ycs4-1* and *top2-4* are similar. Wildtype, *ycs4-1* and *top2-4* cells released from α-factor arrest (t=O) into media at the non-permissive temperature (37°) were scored for sister separation by microscopy at 3 loci along chromosome IV over time. After one hour, α-factor was added back to the media to prevent cells from entering the next cell cycle. Strains contained Lac operator repeats integrated at the *TRP1* locus near the centromere (cen-proximal) (wildtype, SBY215, *ycs4-1*, NBY241, *top2-4*, NBY92), on the arm (wildtype, NBY291, *ycs4-1*, NBY327, *top2-4*, NBY259) and at the telomere (wildtype, NBY292, *ycs4-1*, NBY323, *top2-4*, NBY455) of the chromosome. Although sister chromatid separation is normal at all 3 loci in wildtype, the *ycs4-1* mutants, like the *top2-4* mutants, show differential ability to separate loci based on the proximity to the centromere. **B.** In *ycs4-1*, sister chromatid separation is compromised in the absence of spindle forces. Wildtype (SBY215), *ycs4-1* (NBY241), *top2-4* (NBY92), *mad2Δ* (NBY113), *ycs4-1mad2Δ* (NBY275) and *top2-4mad2Δ* (NBY258) strains were released from α-factor arrest (t=O) into media with benomyl and nocodazole at the non-permissive temperature (37°); all strains contained the Lac operator array at the *TRP1* locus of chromosome IV. After one hour, α-factor was added back to the media to prevent cells from entering the next cell cycle. Sister chromatid separation was scored over time. In the absence of microtubules, sister chromatid separation is delayed in *ycs4-1*.

A





B

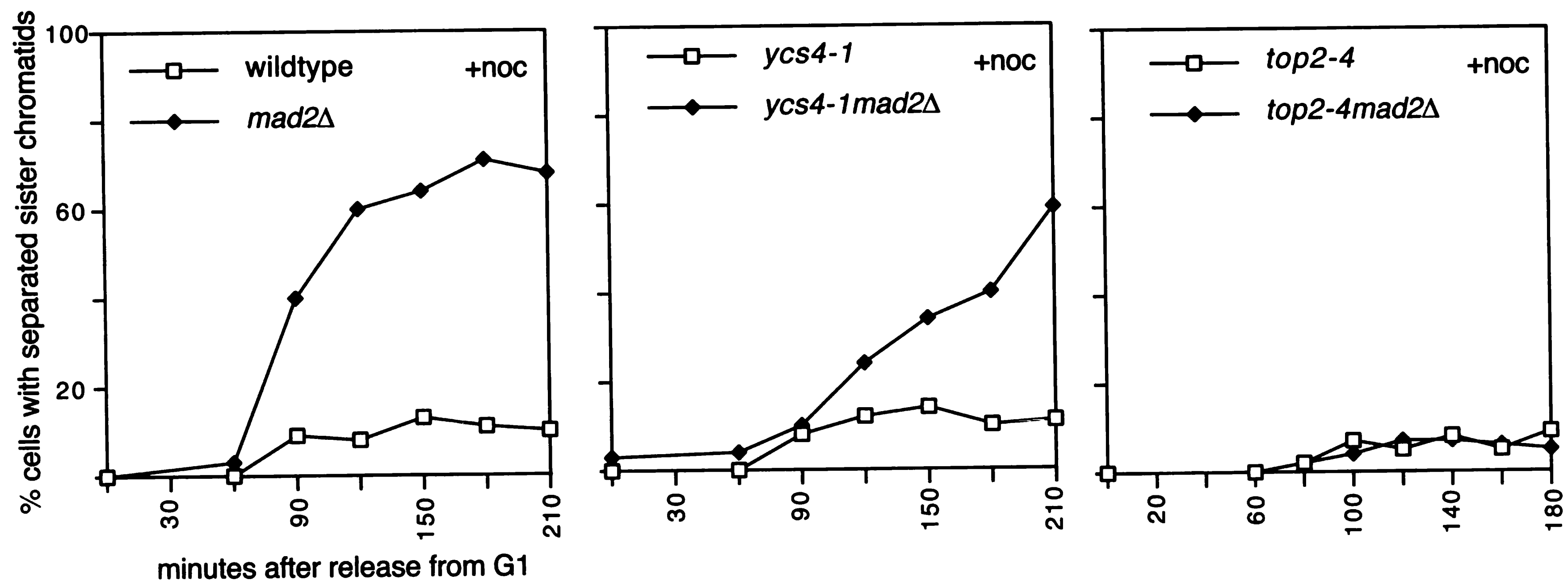
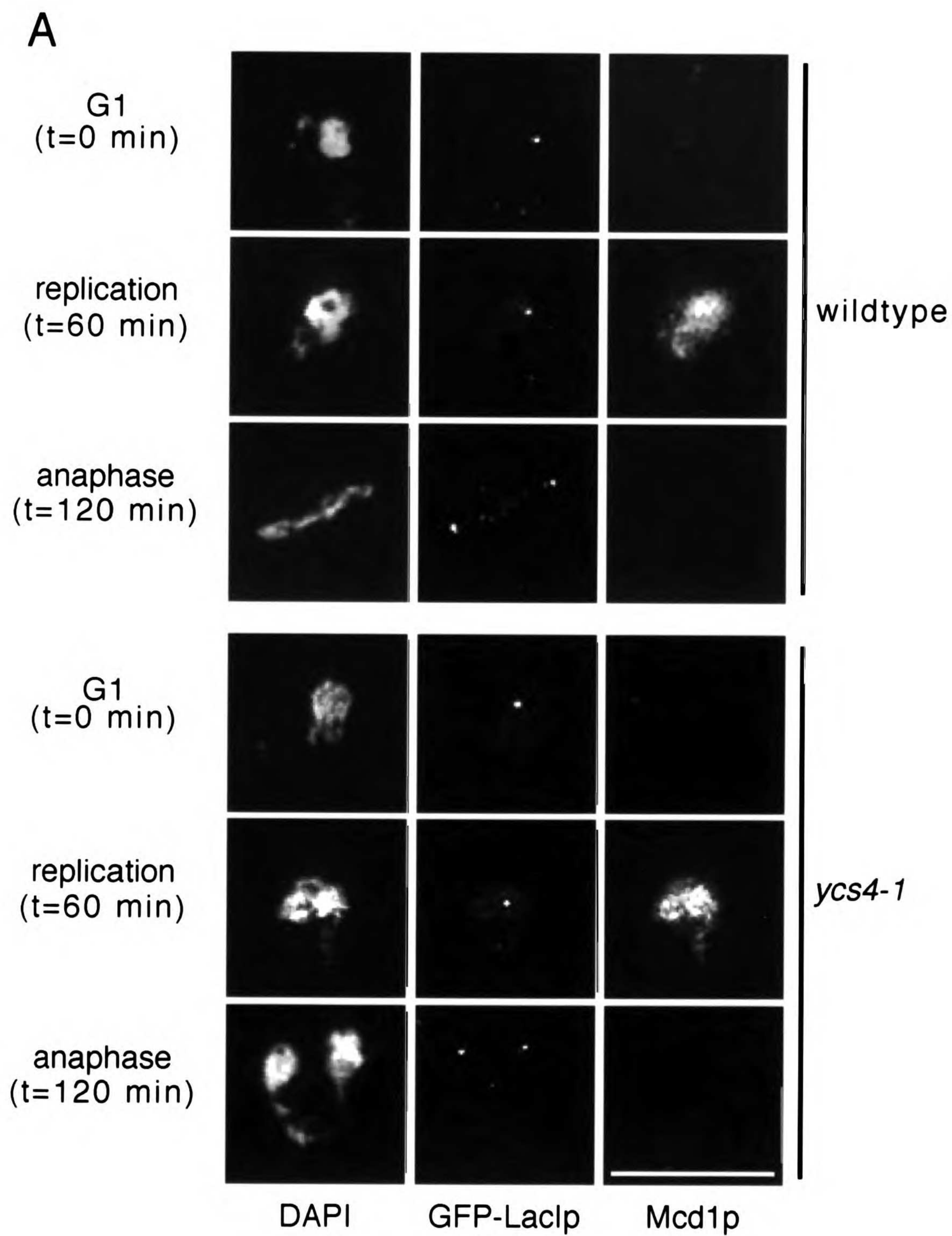
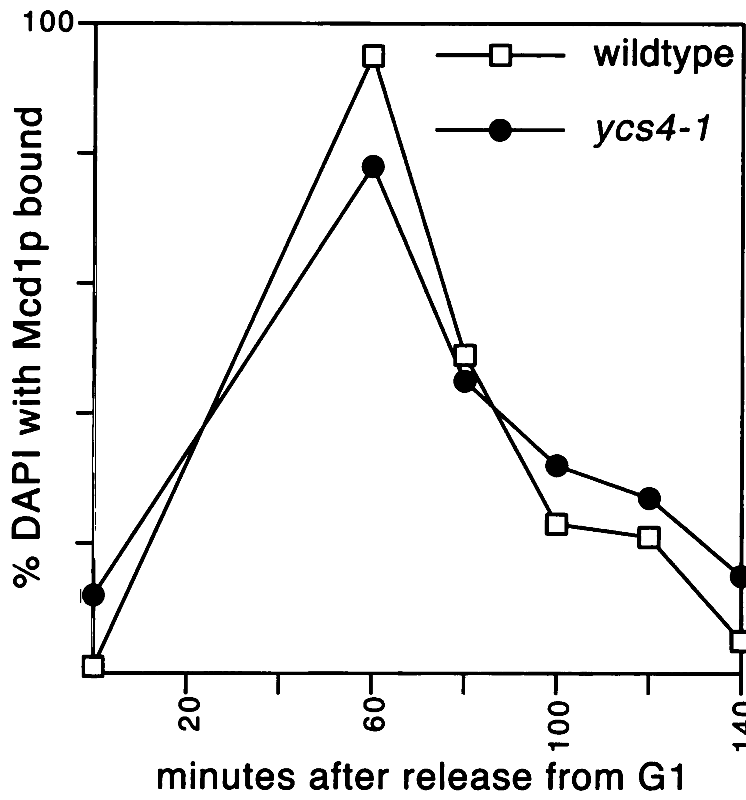


Figure 4-2. Cohesin binding and displacement is normal in *ycs4-1*. **A.** The cohesin subunit Mcd1p/ Scc1p was localized by indirect immunofluorescence on chromosome spreads in wildtype (SBY376) and *ycs4-1* (NBY284) strains containing 3XHA-epitope tagged Mcd1p/ Scc1p. Samples were fixed and stained at the indicated times during a synchronous cell cycle at the non-permissive temperature (37°). DNA-staining (DAPI) is shown in the left panels, anti-LacI antibody staining is shown in the middle panels and anti-HA antibody staining is shown in the right panels. G1, S phase and anaphase spreads prepared from wildtype (top) and *ycs4-1* (bottom) cells are shown. Mcd1p is absent from G1 and anaphase spreads but is present on spreads from cells in S phase in both wildtype and *ycs4-1*. Bar, 10μm. **B.** Quantified results of Mcd1p localization to chromosomes. The percentage of chromosomes with Mcd1p localized to chromosome spreads is represented versus time for wildtype (SBY376) and *ycs4-1* (NBY284).


A
G1
(t=0 min)
replication
(t=60 min)
anaphase
(t=120 min)
wildtype
G1
(t=0 min)
replication
(t=60 min)
anaphase
(t=120 min)
ycs4-1
DAPI
GFP-LacIp
Mcd1p

B



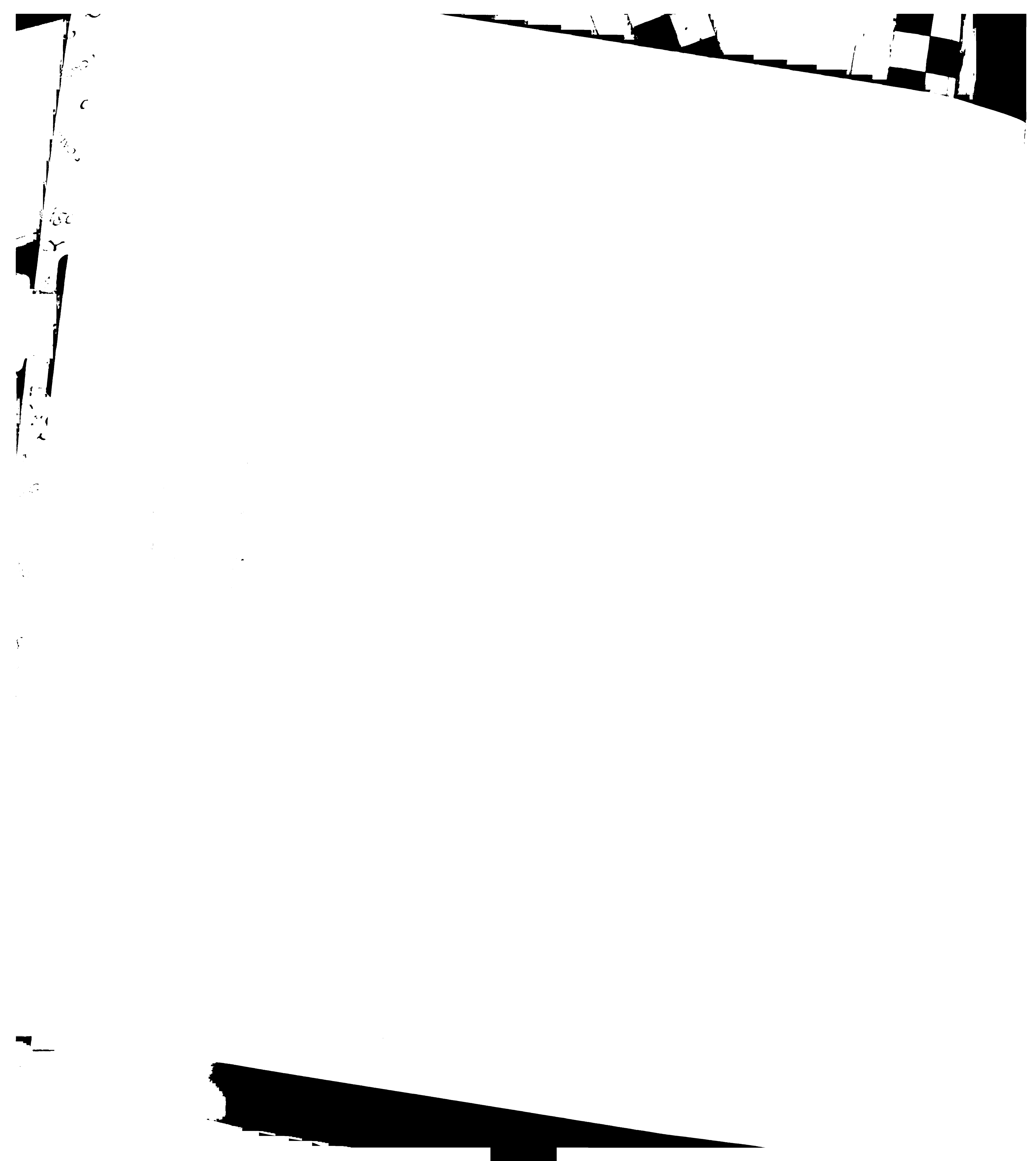
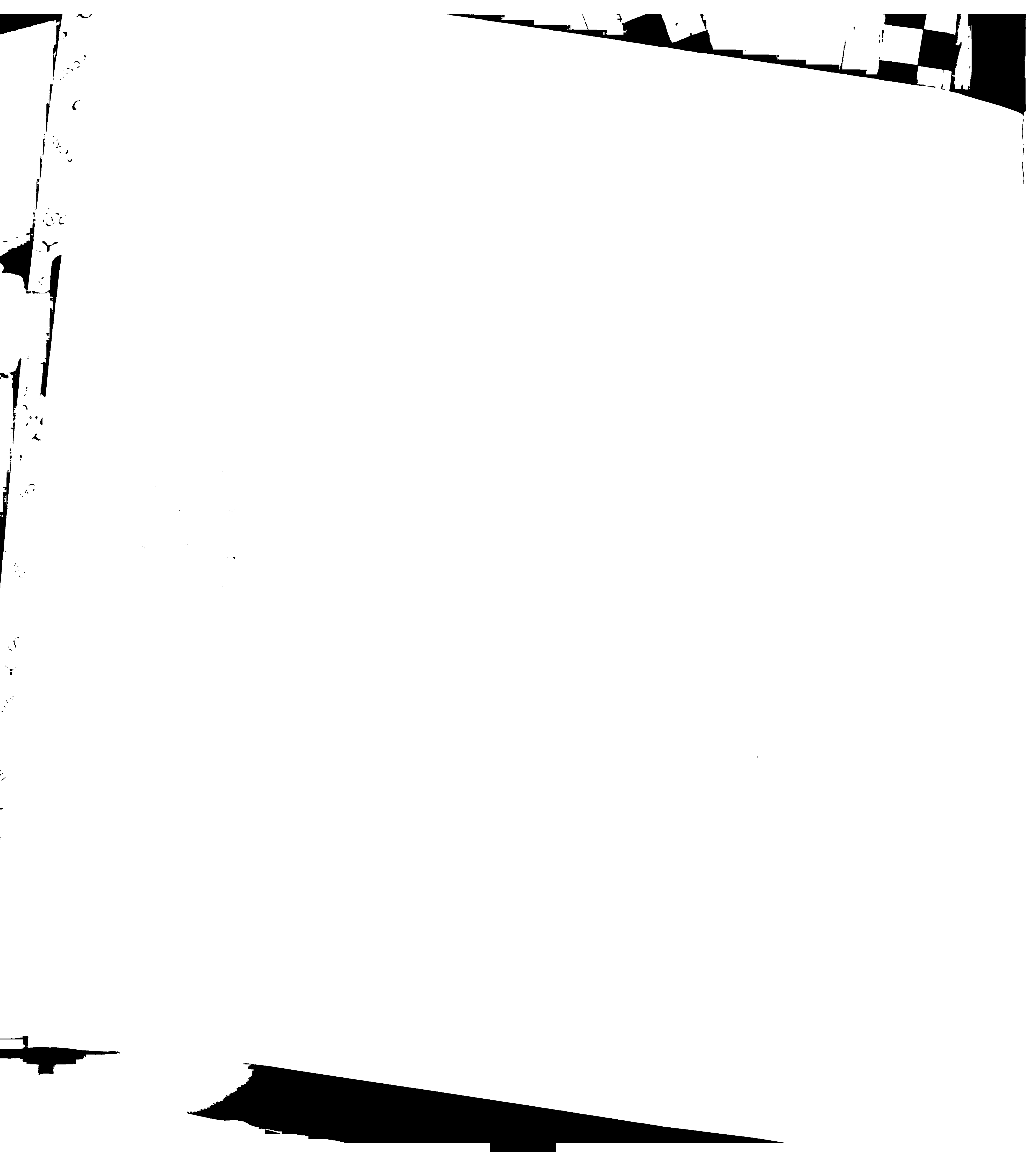
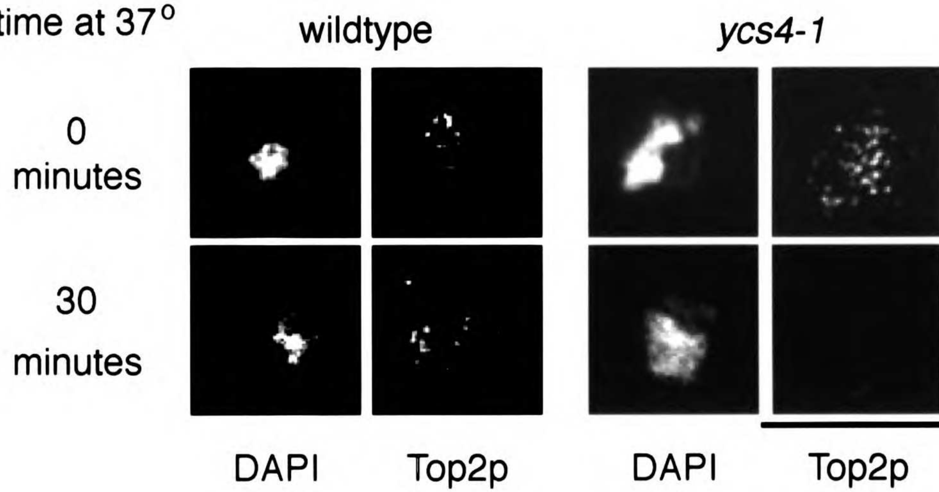
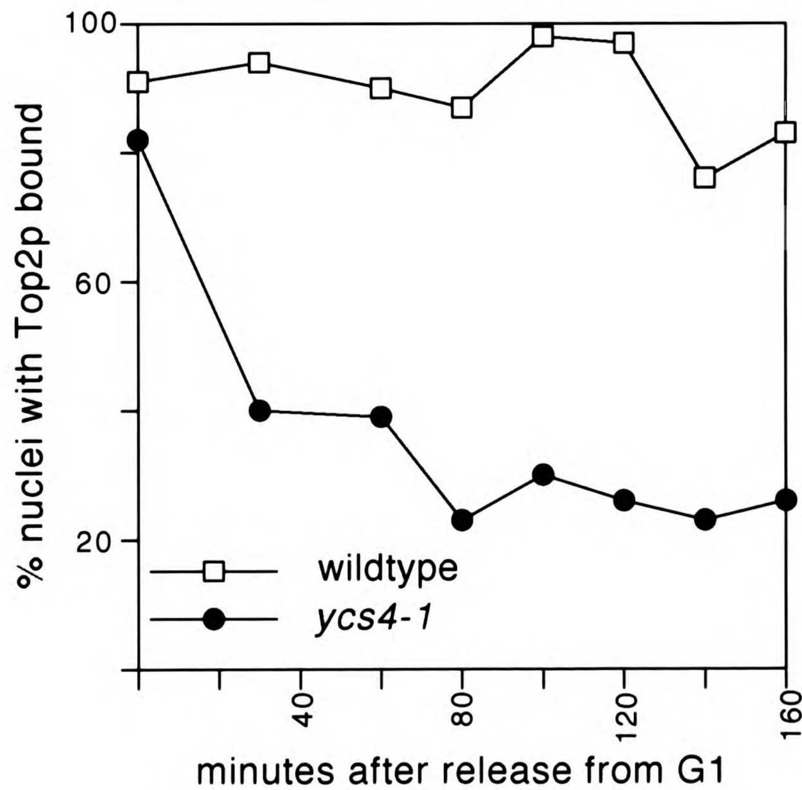


Figure 4-3. DNA topoisomerases I and II are absent from chromosomes in *ycs4-1*. **A.** Top2p was localized by indirect immunofluorescence on chromosome spreads in wildtype (NBY474) and *ycs4-1* (NBY322) strains containing 3XHA-epitope tagged Top2p during a synchronous cell cycle at the non-permissive temperature (37°). Wildtype chromosome spreads are on the right and *ycs4-1* spreads are on the left; 0 and 30 minute timepoints are shown. For each, DNA staining (DAPI) is shown on the left and anti-HA antibody staining is shown on the right. Wildtype spreads maintain the punctate Top2p. There is a dramatic loss of Top2p from chromosomes in *ycs4-1* spreads within 30 minutes of the shift to 37°. Bar, 10μm. **B.** Quantified results of Top2p localization. The percentage of chromosomes with Top2p versus time is represented for wildtype (NBY474) and *ycs4-1* (NBY322). **C.** Quantified results of Top1p localization. Top1p was localized by indirect immunofluorescence on chromosome spreads in wildtype (NBY496) and *ycs4-1* (NBY498) strains containing 3XHA-epitope tagged Top1p during a synchronous cell cycle at the non-permissive temperature (37°).




A
time at 37°
wildtype
ycs4-1
0
minutes
30
minutes
DAPI
Top2p
DAPI
Top2p


B
% nuclei with Top2p bound
100
60
20
wildtype
ycs4-1
40
80
120
160
minutes after release from G1

C

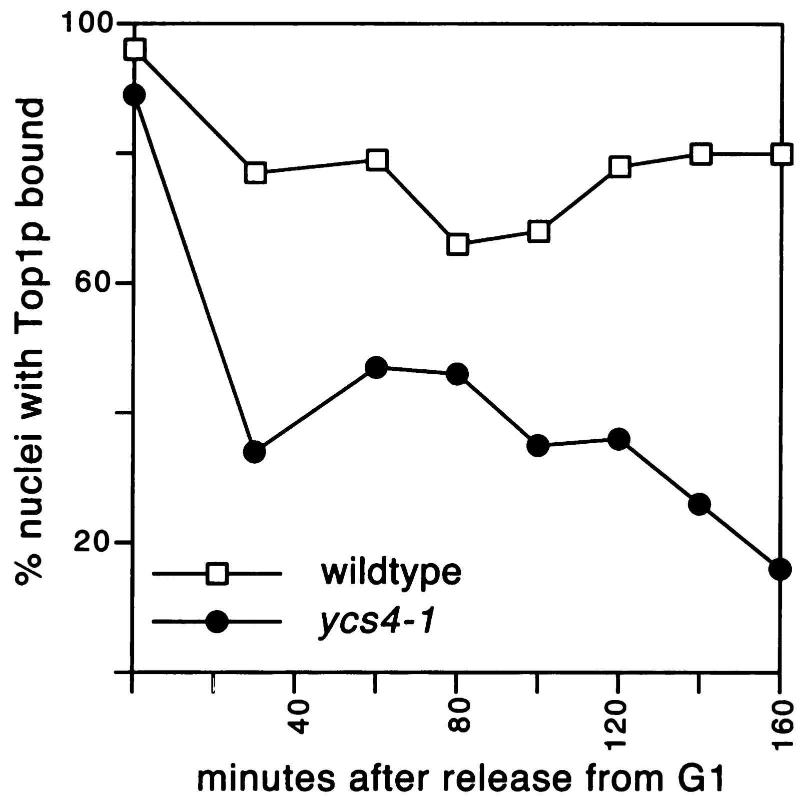
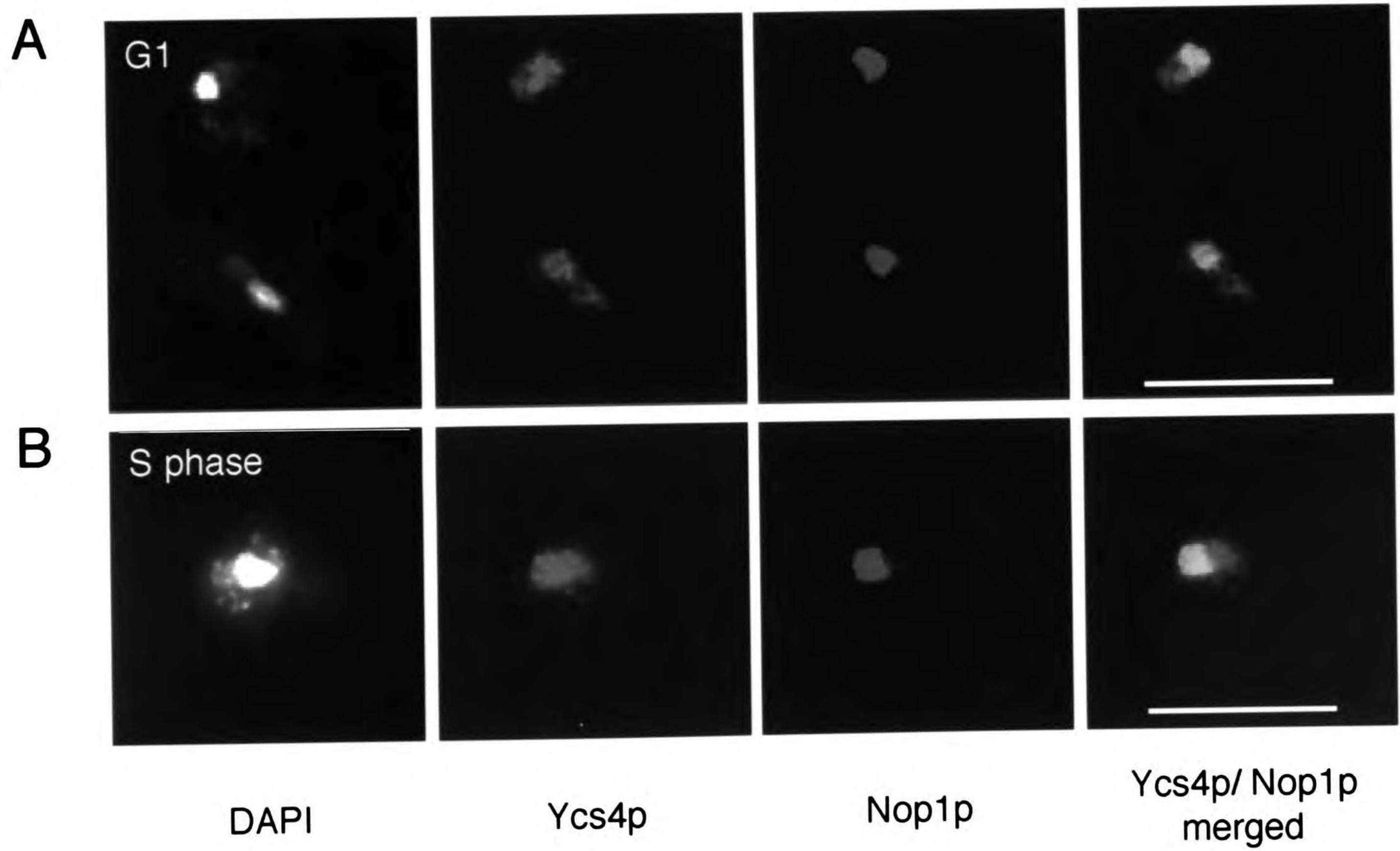
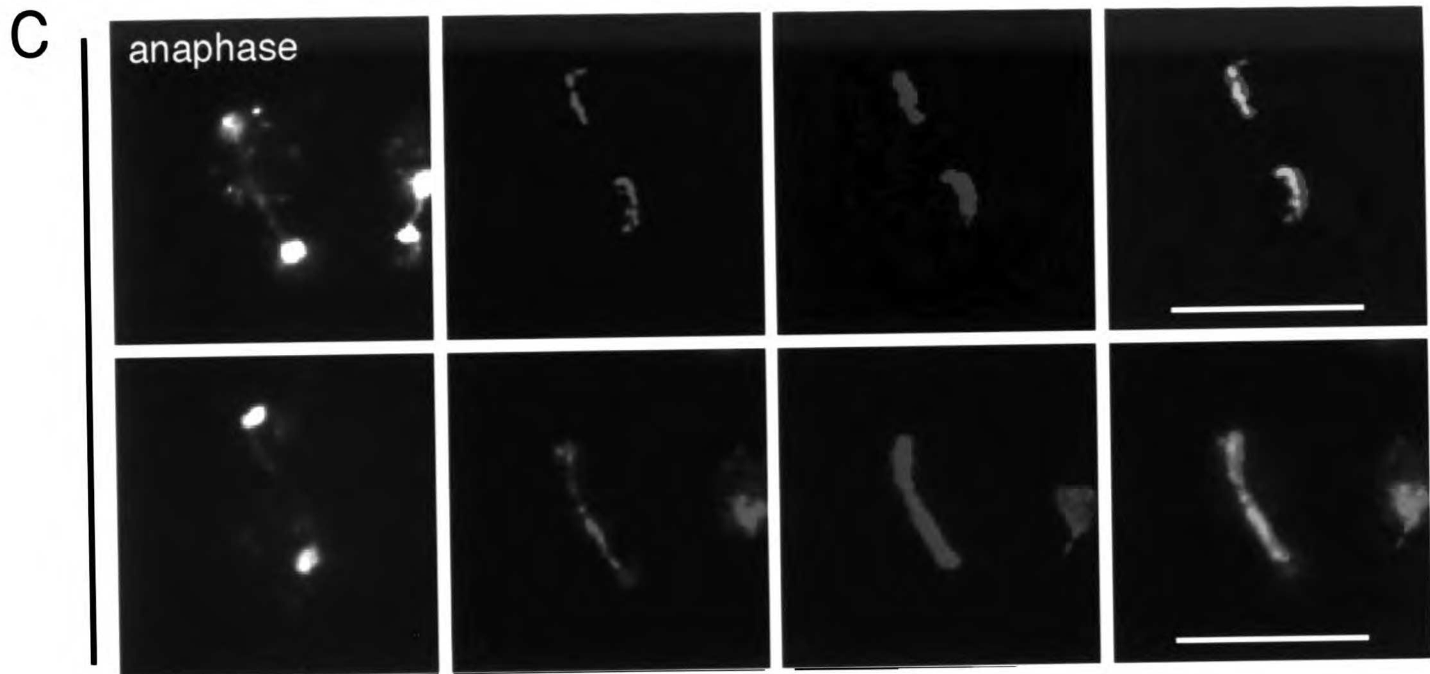


Figure 4-4. Ycs4p is nuclear protein throughout the cell cycle and becomes enriched at the nucleolus at anaphase. Indirect immunofluorescence was performed on Ycs4p-13Xmyc in cells with the chromosomal rDNA array (NBY333) and without, *rdnΔ* (NBY508). DNA staining (DAPI) is shown in the panels in the first set of vertical panels, anti-myc antibody staining that recognized Ycs4p-13Xmyc in the second set, anti-Nop1p staining in the third set and a merge of the Ycs4p and Nop1p staining in the final set of panels. Bar, 10μm.


A
G1
B
S phase
DAPI
Ycs4p
Nop1p
Ycs4p/ Nop1p
merged


C
anaphase
D
rdnΔ
DAPI
Ycs4p

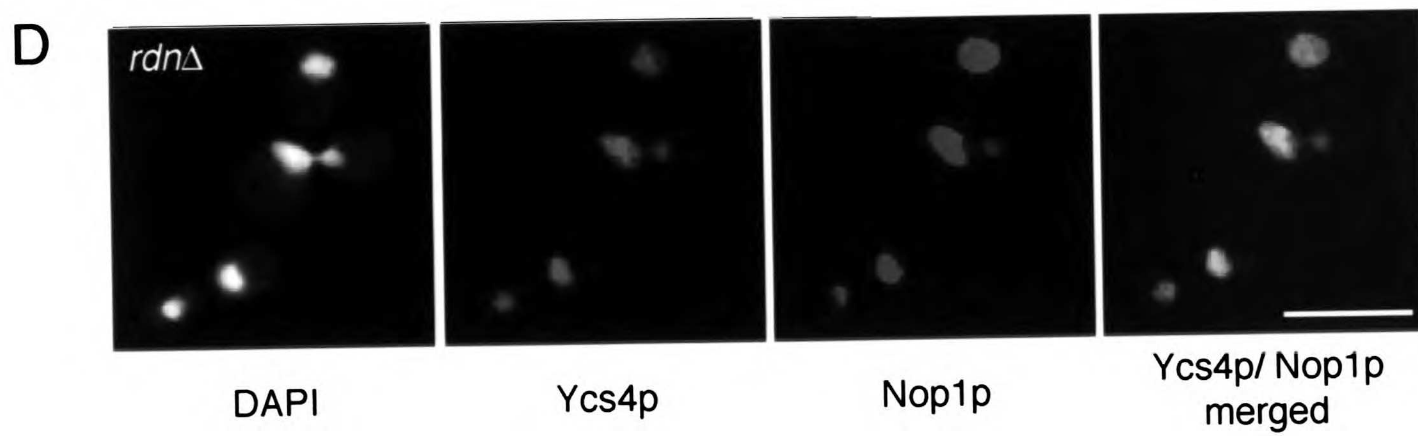
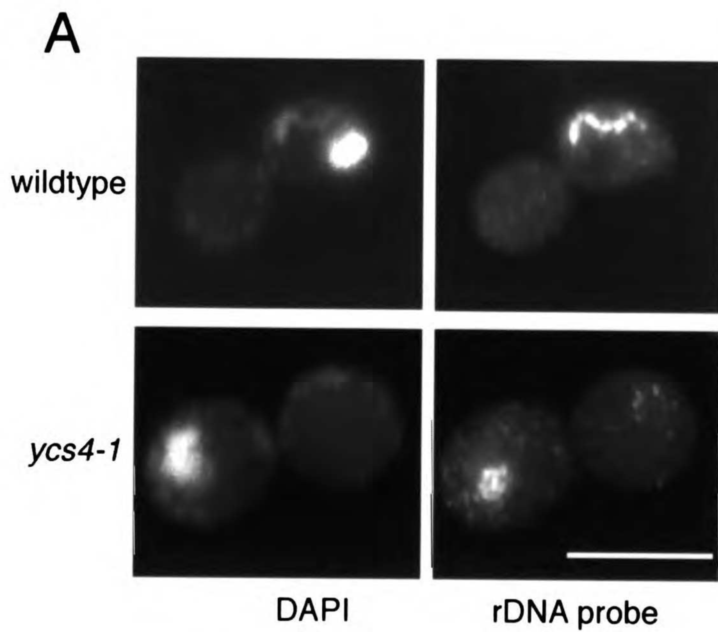
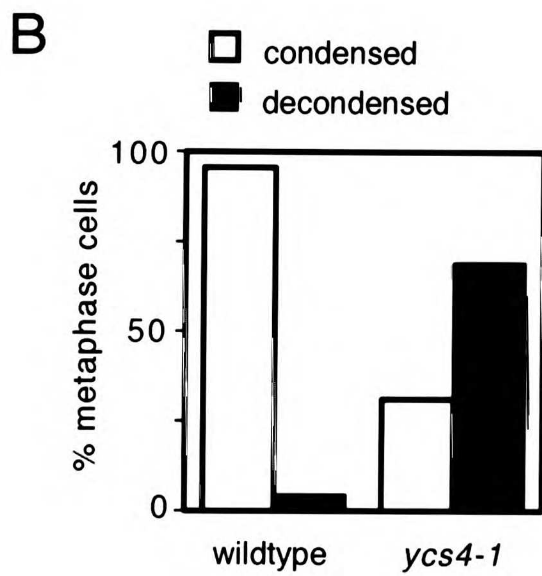
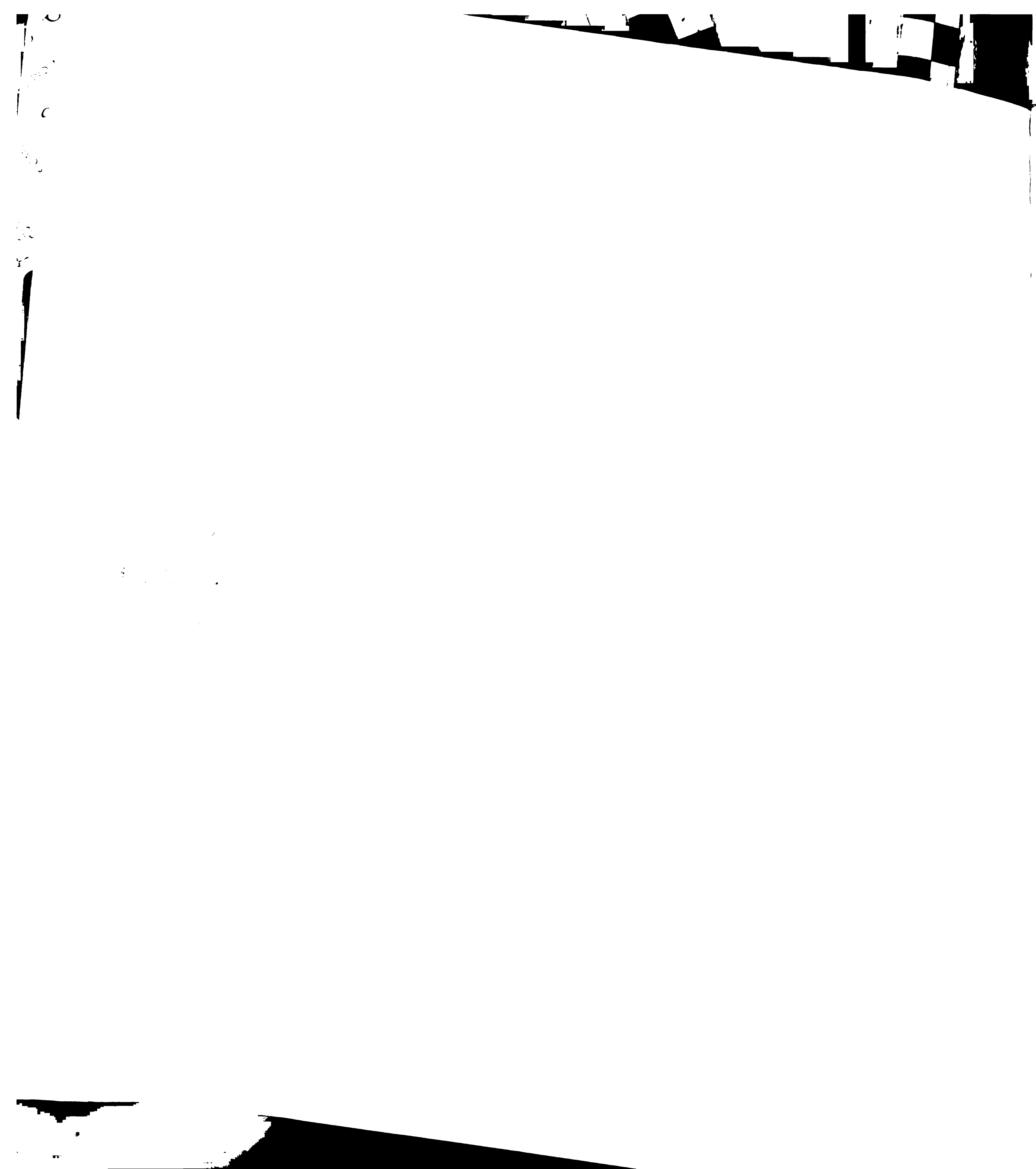
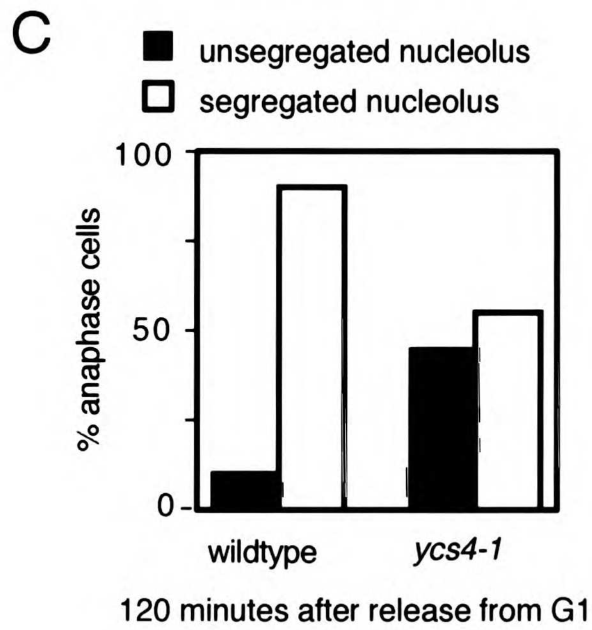

Nop1p
Ycs4p/ Nop1p merged

Figure 4-5. *YCS4* is required for rDNA structure, stability and segregation. **A.** The rDNA is decondensed in *ycs4-1* mutants. Fluorescent in situ hybridization (FISH) with probes against the rDNA was performed on wildtype (NBY8) and *ycs4-1* (NBY319) arrested in metaphase at the non-permissive temperature (37°). Compared to the loops present in wildtype rDNA, the rDNA in *ycs4-1* cells appears collapsed into an amorphous mass at the periphery of the DNA staining. Bar, 10μm. **B.** The results of in situ hybridization were quantified. In 69% of *ycs4-1* cells arrested in metaphase, the rDNA appears decondensed, as compared to 4.5% of wildtype cells. **C.** Segregation of the nucleolus is defective in *ycs4-1*. Indirect immunofluorescence for the nucleolar marker Nop1p was performed on wildtype (NBY8) and *ycs4-1* (NBY319) cells in anaphase (120 minutes after release from G1). The percentage of cells with segregated or unsegregated nucleoli was scored and graphed. Only 10% of wildtype cells in anaphase have not segregated their nucleolus; 45% of *ycs4-1* cells with elongated spindles still contain the nucleolus in the mother bud (as identified by the persistence of the α-factor induced shmoo). **D.** The loss of nucleolar structure may inhibit its segregation. A wildtype (NBY8) cell is shown on the left and a *ycs4-1* mutant (NBY319) cell on the right. DNA staining (DAPI) is shown in the top pair of panels, anti-tubulin antibody staining in the middle, and anti-Nop1p antibody staining on the bottom. The wildtype cell has segregated its crescent-shaped nucleolus into each cell body. The nucleolus in the *ycs4-1* cell has lost its structure and is found in the mother cell body; the amorphous Nop1p-containing mass is typical of the 45% of cells with an unsegregated nucleolus. Bar, 10μm.


A
wildtype
ycs4-1
DAPI
rDNA probe


B
condensed
decondensed
100
50
0
% metaphase cells
wildtype
ycs4-1




C
unsegregated nucleolus
segregated nucleolus
100
50
0
% anaphase cells
wildtype
ycs4-1
120 minutes after release from G1

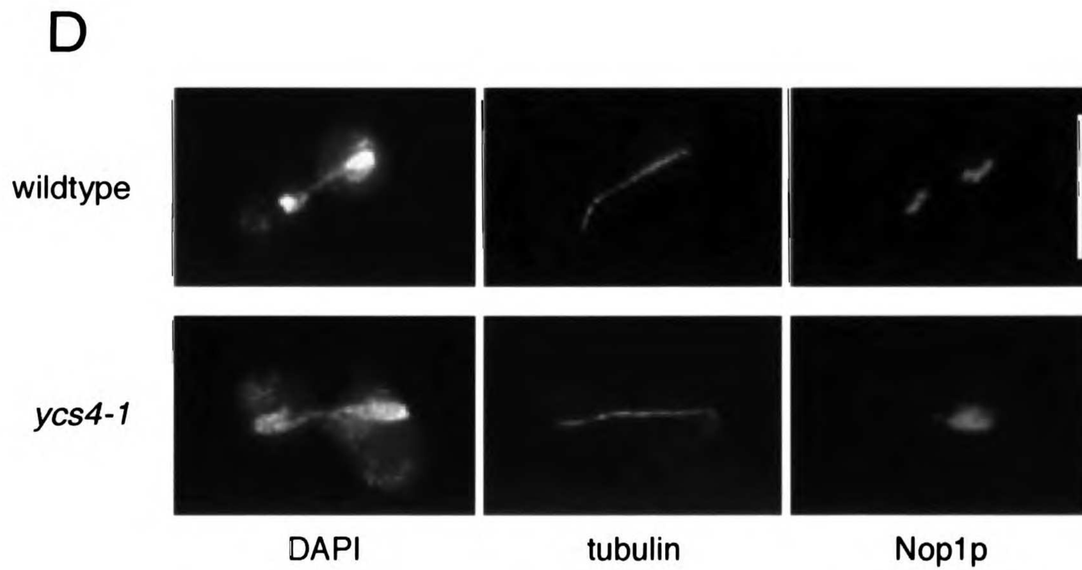
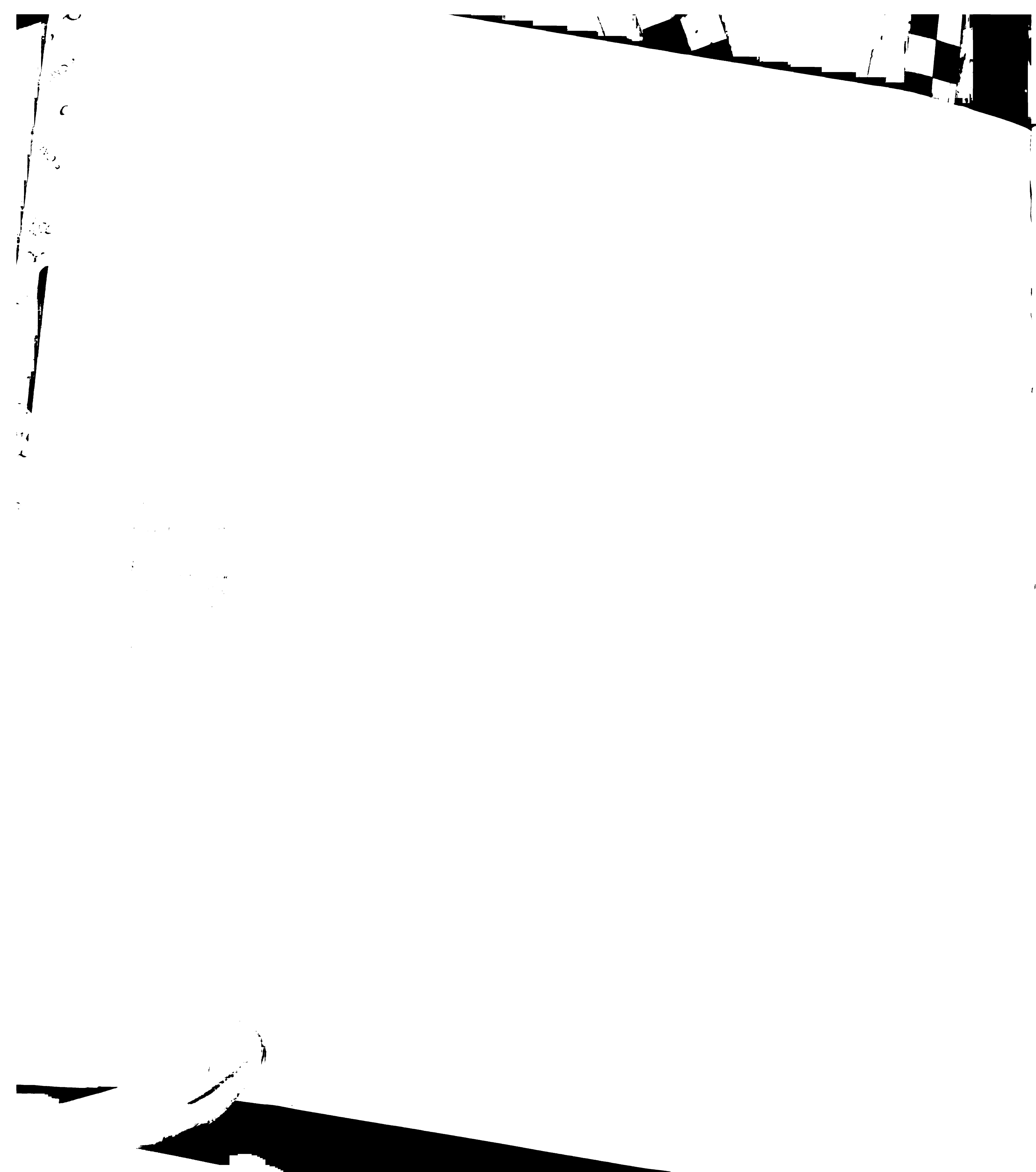
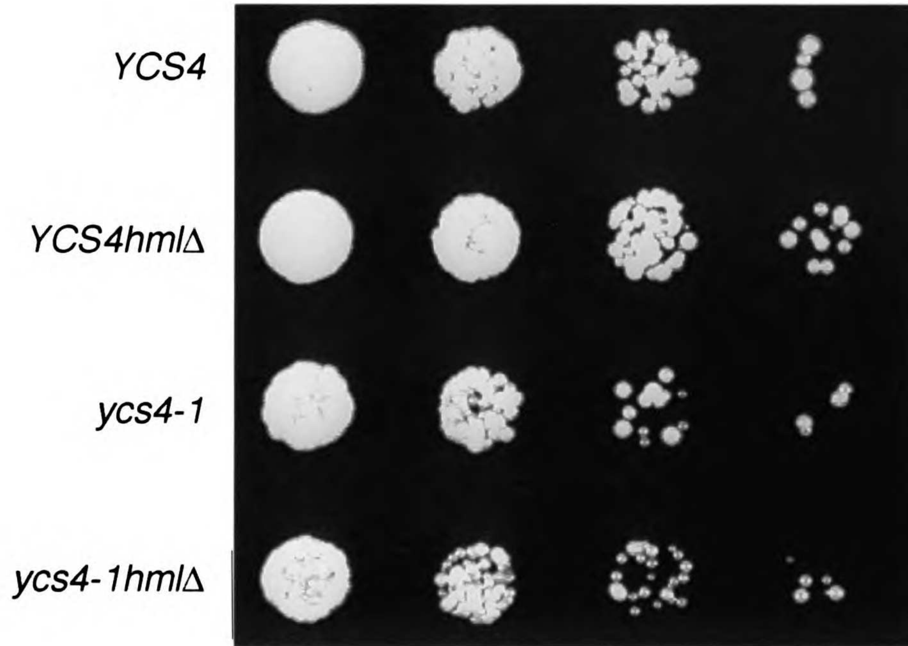
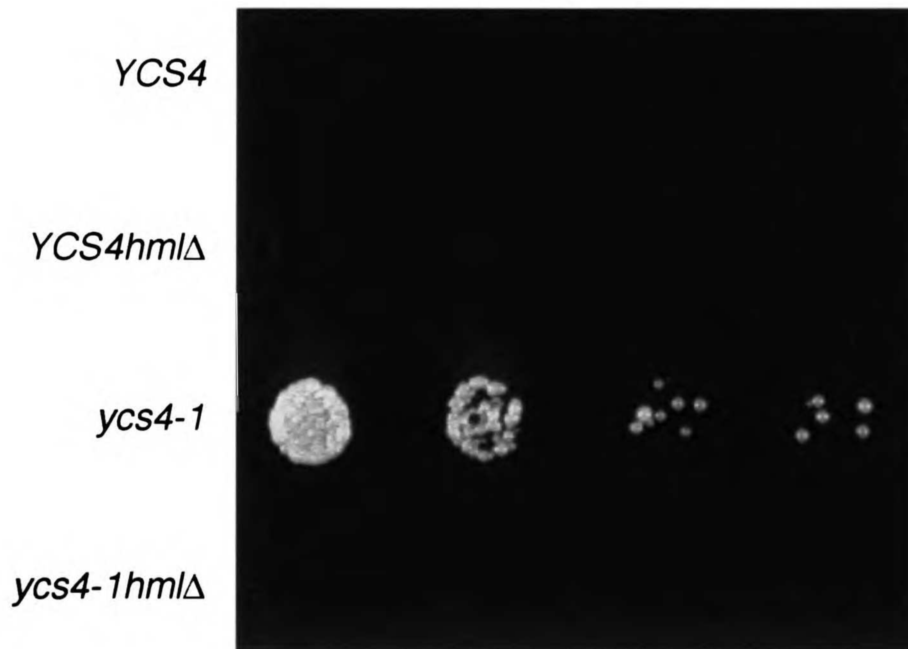

D
wildtype
ycs4-1
DAPI
tubulin
Nop1p

Figure 4-6. *ycs4-1* is defective in silencing at the silent mating type loci but not at the telomeres. **A.** Deletion of the *HMLα* locus suppresses *ycs4-1*'s growth on media containing α-factor. Serial dilutions of wildtype (NBY8), wildtype with the *HMLα* silent mating type locus deleted (*hmlΔ*) (NBY585), *ycs4-1* (NBY316), *ycs4-1* with the *HMLα* silent mating type locus deleted (*hmlΔ*) (NBY319) on YPD with and without 1μg/ml α-factor (all strains are *bar1Δ*). Deletion of the *HMLα* locus suppresses ycs4-1's growth on media containing α-factor. **B.** Silencing at the telomeres is unaffected by the *ycs4-1* mutation at the permissive temperature. Serial dilutions of wildtype (NBY374) and *ycs4-1* (NBY377), both containing a *URA3* reporter construct at the telomere to assay silencing



A





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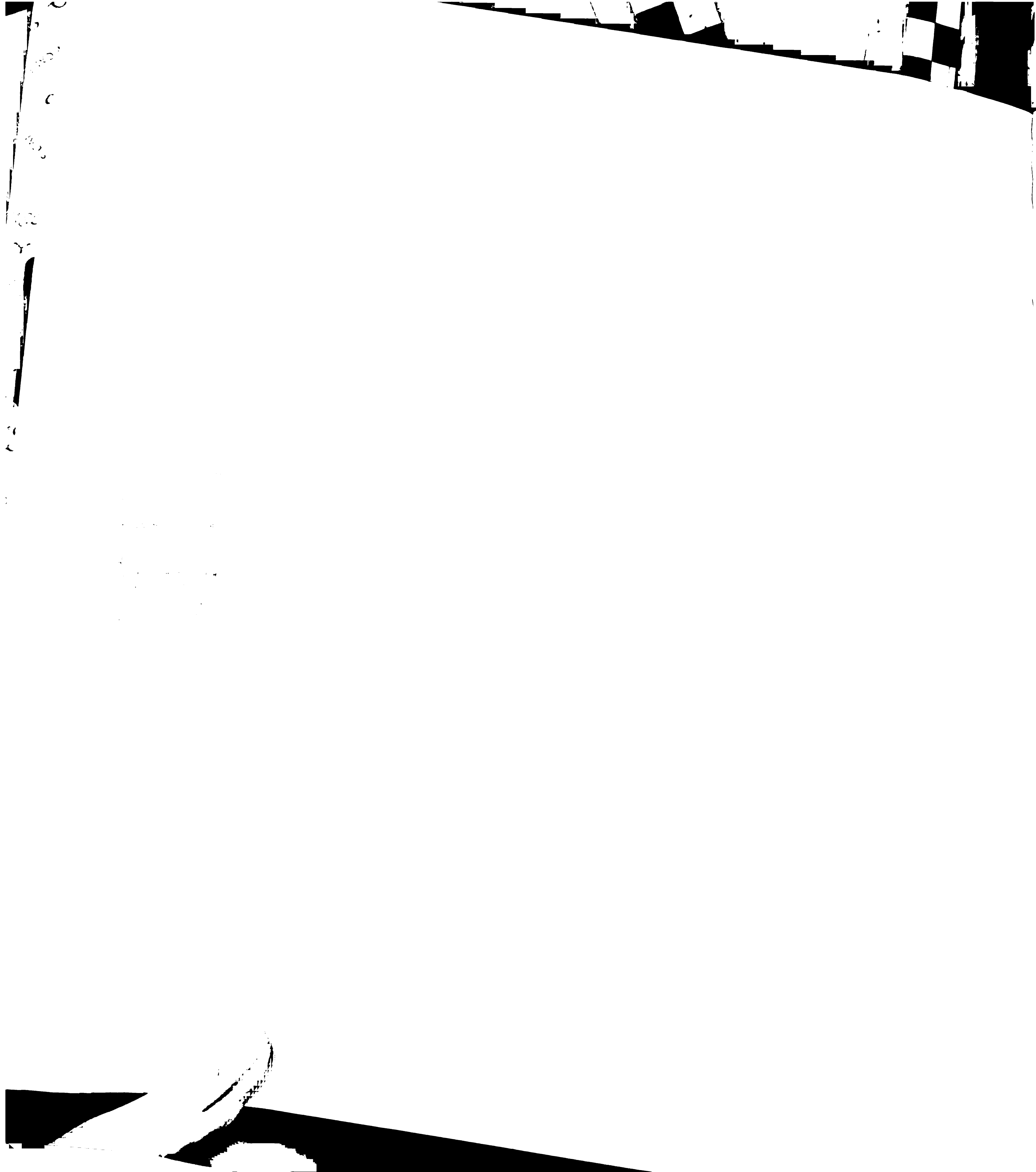
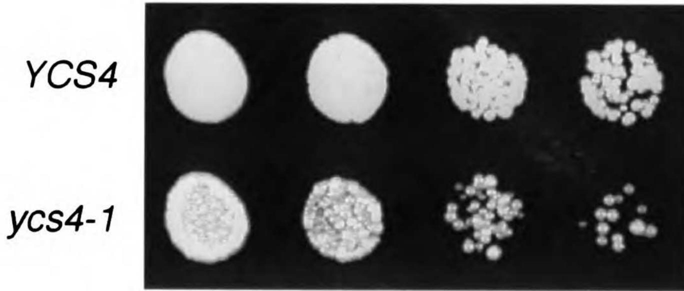


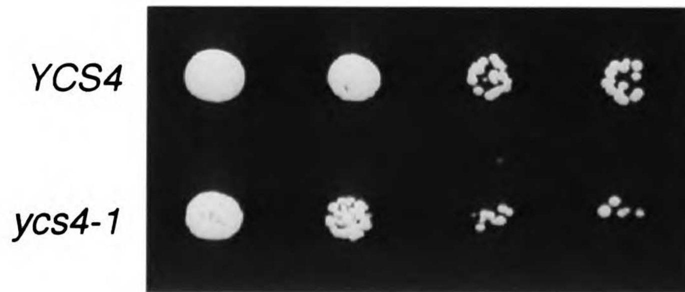
B

YPD



-uracil

5-FOA



CHAPTER FIVE

Discussion

The two genetic screens we performed identified numerous genes involved in sister chromatid cohesion, separation and segregation in budding yeast. We isolated a mutant allele of the *YCS4* gene in the *LOC* (loss of cohesion) screen; the budding yeast *YCS4* gene encodes a conserved regulatory subunit of the condensin complex. Our analysis revealed a requirement for *YCS4* function in the topological resolution of sister chromatids, the localization of core chromosomal proteins, such as topoisomerase I and II and rDNA stability and segregation. We also determined that the *ycs4-1* mutant exhibits defects in the transcriptional repression of the mating type loci and the suppression of recombination at the repetitive rDNA array. These data indicates that members of the budding yeast condensin complex play important roles in non-mitotic as well as mitotic chromosome behavior.

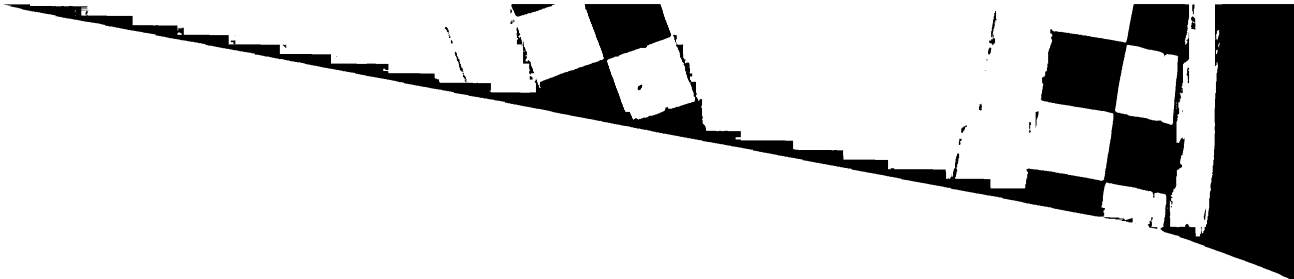
DOES THE CONDENSIN COMPLEX FACILITATE THE RESOLUTION OF TOPOLOGICAL LINKAGES BETWEEN SISTER CHROMATIDS?

Our studies into the function of *YCS4*, a budding yeast condensin subunit, indicate that the condensin complex is required to help topologically resolve sister chromatids. Topological resolution requires the topoisomerase II enzyme, which can either catenate or decatenate circular DNA molecules, depending on the state of the substrate DNA. As condensins package DNA to prepare chromosomes for mitotic division, the compaction favors decatenation of sister chromatids by topoisomerase II since two compact DNA molecules are less likely to collide with each other and become catenated than two extended DNA molecules (Holmes and Cozzarelli 2000). Recent data in *C. elegans*

supports this hypothesis (Hagstrom, Holmes et al. 2002). However, there has been no direct demonstration that the condensin complex assists topoisomerase II in this manner. Circular minchromosomes are not catenated after replication or at metaphase (Koshland and Hartwell 1987) but these experiments were done with *cdc* mutants that had been arrested at points in the cell cycle for several hours and may not represent the topological nature of minichromosomes during an unperturbed cell cycle. Alternatively, we could undertake a timecourse during which the topological structure of a circular minichromosome is assayed as cells go through a single synchronous cell cycle at the non-permissive temperature. A comparison between a wildtype strain and the *ycs4-1* mutant might reveal defects in the amount of time it takes to decatenate a newly-replicated minichromosome in the mutant, offering direct proof of a role of a condensin subunit in the topological resolution of sister DNA molecules. My attempts to do such experiments were beset by technical difficulties but offered promising possibilities.

Could Replication Factors Be Establishing the Topological Linkage Between Sister Chromatids?

The link between DNA replication and the establishment of cohesion has been elegantly illustrated (reviewed in (Carson and Christman 2001). Our experiments indicate that the post-replicative loading of the cohesin complex in combination with keeping duplicated sister chromatids artificially linked in G2 is not enough to establish cohesion (see Appendix B). Thus, replication appears to be functionally as well as temporally linked to the establishment of cohesion. This is consistent with reports that



implicate replication factors in cohesion establishment (Skibbens, Corson et al. 1999; Wang, Castano et al. 2000; Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001). However, mutation of these factors does not affect the loading of the cohesin complex (Toth, Ciosk et al. 1999). Cohesin loading during replication is dependent upon the Scc2p/ Scc4p complex (Ciosk, Shirayama et al. 2000), and so far no functional link between these proteins and replication has been found. Therefore, there may be two distinct pathways influencing the establishment of sister chromatid cohesion, one that involves the cohesin complex and one that does not. The prevailing model seeks to integrate the two pathways, arguing that the replication factors may act to make the cohesin complex competent to link sisters (Carson and Christman 2001). This has yet to be illustrated.

The paradigm of two pathways effecting cohesion establishment immediately raises the possibility that replication factors may be involved in establishing the topological linkage between sister chromatids. It has been assumed but never demonstrated that catenation between sisters results from the collision of replication forks during S phase. Perhaps the intertwining of sister chromatids is a more active process that involves DNA polymerase κ (*TRF4*) and the alternate RFC complex. In this light, the inclusion of the *trf4Δ* mutant in the above-mentioned assay for catenation of a minichromosomes might prove enlightening.

The replication factors required for cohesion establishment are not essential, despite a significant sister chromatid cohesion defect when the mutants are arrested in metaphase (Wang, Castano et al. 2000; Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001). FACS analysis of the mutants suggests that replication of the genome is complete but FACS analysis may be too gross an assay for subtle defects in replication, for

example the regions where switching to DNA polymerase κ is required for cohesion establishment. It is possible that the missing regions of the genome produce small, possibly intergenic gaps that allow the topological resolution of sister chromatids when held in metaphase but not during a normal cell cycle, thus explaining the viability of these mutants.

How Does the Condensin Complex Influence Gene Expression?

Our analysis of *YCS4* function revealed the surprising observation that some subunits of the condensin complex are required for the transcriptional repression of specific loci. Mutation of *YCS4* and *SMC4*, but not *SMC2*, interferes with the transcriptional silencing of the mating type loci. *YCS4* function was not required for the repression of other silenced loci, such as the telomere, but this may reflect the stringency of the reporter assay used to assay telomere silencing; in contrast, our assay for defects in silencing at the mating type locus, resistance to α-factor, is very sensitive. One way to clear up this discrepancy would be to use the same reporter construct at the mating type locus and see if a silencing defect is observable. In addition, the defects in silencing we observe are at the permissive temperature. Therefore, less sensitive assays may not reveal defects at the permissive temperature that may be quite apparent with complete loss of *YCS4* or *SMC4* function. A silencing assay that is not growth dependent, such as a GFP reporter construct, would help in this case. Another less attractive possibility is that there are different requirements for silencing at different loci and the condensins are not involved in silencing at the telomere.

We were not able to assay silencing at the rDNA because of the inviability of the strain containing both the *ycs4-1* mutation and the reporter construct integrated in the rDNA. However, the *ycs4-1* mutant exhibits higher rates of recombination at the rDNA array than at a control locus (*LEU2*), indicating instability in this locus independent of its segregation during mitosis. This influence on non-mitotic rDNA behavior leads us to speculate that silencing at the rDNA may also be affected with the loss of *YCS4* function.

We do not think that the loss of silencing in *ycs4-1* and *smc4-1* is a general result of perturbed chromosome structure because the *smc2-8* mutant does not exhibit a defect in silencing. A direct comparison between the *smc2-8* and *smc4-1* mutants revealed that the *smc2-8* mutation is more severe, even at the permissive temperature, suggesting that it is not simply a case of allele strength (Freeman, Aragon-Alcaide et al. 2000). What we would argue, instead, is that there is an alternate complex, playing a role in establishing and/ or maintaining silencing. This complex would include Ycs4p and Smc4p and possibly the other members of the condensin complex, Ycs5p and Brn1p, but not Smc2p. This "silencing complex" may require an additional SMC subunit to partner with Smc4p to effect its role in transcriptional regulation. Several alternate SMCs exist in the budding yeast genome (Rad50p, Rhc18p, YOLO34W). Such a paradigm exists in *C. elegans*, where the dosage compensation complex is required for controlling gene expression of the X chromosome. It shares members with the condensin complex but each has unique subunits of their own to carry out their specialized purposes (Meyer 2000).

The biochemical activity of the condensin complex may provide clues to how this alternate complex may be regulating transcription. The condensins utilize ATP

hydrolysis to compact DNA into a mitotic chromosome by introducing ordered, global, positive writhe (Kimura, Rybenkov et al. 1999). Heterochromatic, silenced regions of the genome are often hyper-condensed, thus limiting the access of factors required for transcription. Recruiting condensins or an alternate complex composed of some condensin subunits to specific regions of DNA during interphase could be the basis of heterochromatin formation and gene silencing. In fruit flies, barren, a subunit of the condensin complex, has been found to associate with DNA in interphase and this association is required for the epigenetic control of gene expression (Lupo, Breiling et al. 2001). Furthermore, additional chromatin remodeling factors involved in transcription have recently been shown to generate superhelical torsion to manipulate chromatin structure, presumably as the means by which gene regulation is accomplished (Havas, Flaus et al. 2000). The condensin complex, or a similar complex composed of select subunits, could very well repress the transcription of genes by the active reconfiguration of DNA.

If there is some unifying theme to the twists and turns of my graduate research, it would have to be a fundamental interest in what role the architecture of chromosomes may play in the myriad of functions DNA is essential for. For example, how a chromosome, exploiting its own structural nature and the proteins that assemble it and on it, contributes to the process of faithful duplication and segregation during mitosis seems the most beautiful of collaborations for so practical a goal. The awareness that errors in this collaboration can result in tumorigenesis and cancer progression presents a realistic application of the knowledge gained thus far.

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APPENDIX A

Re-screening for *loc* Mutants

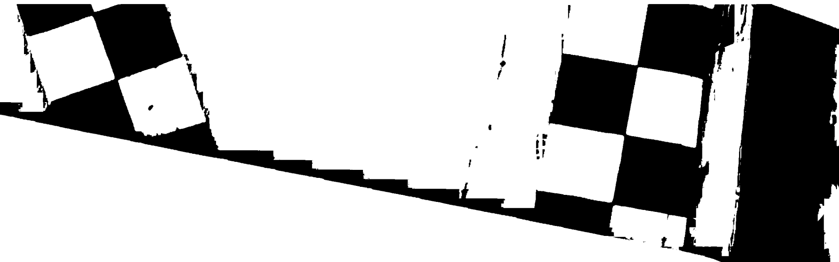
ABSTRACT

In the process of doing the loc screen, *ESP1* was identified as a *PDS1* binding partner required for sister chromatid separation in budding yeast. Our initial paradigm for a *loc* mutant was the *top2-4* mutant; therefore, our secondary screens were primarily based on the characterization of this mutant. Analysis of the *esp1-1* sister chromatid separation defect revealed qualitative differences between it and *top2-4*. Simultaneously, or so it seemed, Sue Biggins and I realized that we actually had identified two classes of mutants in our screen: those that affect sister chromatid separation and those that affect sister chromatid segregation. Before I decided to characterize the *loc7-1* mutation (*ycs4-1*) and unattracted to its α-factor resistance, I decided to rescreen my half of the temperature sensitive bank of mutants based on this new knowledge. I hoped to identify new *loc* mutants that were intimately involved in sister chromatid separation. I isolated 8 new *loc* mutants. In addition, as proof of principle, two mutants we subsequently characterized as affecting sister chromatid separation, *ysc4-1* and *pds1-478*, were identified in my screen and two mutants we subsequently characterized as affecting segregation, *cse4-327* and *ipl1-182*, were not.

INTRODUCTION

In the process of screening the temperature sensitive mutant collection, the Esp1p/ Pds1p complex was identified in budding yeast (Ciosk, Zachariae et al. 1998). Analogies were easily drawn between the role of this complex in sister chromatid separation and the Cut1/ 2 complex in fission yeast (Funabiki, Kumada et al. 1996; Funabiki, Yamano et al. 1996). Further investigation illustrated the requirement of *ESP1* function for the removal of the cohesin complex from chromosomes and thus, sister chromatid separation at anaphase (Ciosk, Zachariae et al. 1998). Additional studies have revealed that Esp1p is actually a protease which cleaves the cohesin member, Mcd1p/ Scc1p, driving cohesin removal from chromosomes and sister separation (Uhlmann, Lottspeich et al. 1999; Uhlmann, Wernic et al. 2000).

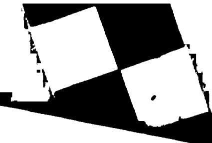
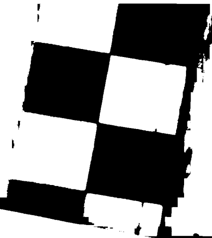
Our initial paradigm of a mutant defective in sister chromatid separation was based on the *top2-4* mutant and thus, our secondary screens (i.e. rescue by nocodazole arrest, rapid death at the non-permissive temperature, continuation of the cell cycle despite the sister chromatid separation defect) were largely the conclusion of the characterization of this mutant (Holm, Goto et al. 1985; Uemura, Ohkura et al. 1987; Holm, Stearns et al. 1989). However, growing familiarity with sister separation defects made us realize that we might have made some hasty assumptions regarding the screen. Although *esp1-1* fulfilled most of these requirements, analysis of the *esp1-1* mutant revealed additional hallmarks that might have provided better guidance for the screening of *loc* mutants (Baum, Yip et al. 1988; McGrew, Goetsch et al. 1992; Ciosk, Zachariae et al. 1998). In addition, Sue Biggins's characterization of the *ipl1-321* mutant and our



subsequent cloning and characterization of the other genes we had identified highlighted a major weakness of our screen: we had isolated mutants defective in sister chromatid segregation as well as separation (Biggins, Bhalla et al. 2001). Therefore, I attempted to rescreen the half of the bank I was responsible for screening, making some adjustments in the secondary tests that followed the primary screen.

RESULTS

The primary screen identified 284 potential mutants; I had identified 158 of these. I screened these 158 again, looking for large-budded cells with a single GFP dot once more. However, analysis of *esp1-1* had indicated that the budding index of an asynchronous population might also be informative. Due to the inability to separate sister chromatids, *esp1-1* mutants often produced two classes of progeny: diploidized mothers and anucleate daughters; this resulted in a population of cells at the non-permissive temperature that were largely unbudded or large-budded with few competent to enter the next cell cycle and form small buds (Baum, Yip et al. 1988; McGrew, Goetsch et al. 1992). Therefore, my screening procedure involved screening not only the GFP signal but the budding index as well. This allowed me to simultaneously disregard mutants that exhibited the *cdc* metaphase arrest (greater than 70% large-budded cells) that was an important secondary screen in our initial screen. The forty-nine mutants that satisfied these criteria were then subjected to the rapid death assay, the rationale being that defects in sister chromatid separation would be lethal to cells undergoing cell division at the non-permissive temperature. The secondary test that initially accompanied the rapid death

test in our first screen, rescue of the rapid death phenotype by arrest in nocodazole, was based on the assumption that the mutant protein would be functional once the cells were shifted back down from the non-permissive temperature to the permissive temperature. It was entirely possible that we had thrown out potentially interesting mutants simply because the proteins were unable to function at the permissive temperature after the temporary incubation at the non-permissive temperature. I also opted not to screen the sister chromatid separation defect of the potential mutants in the anaphase arrest accomplished by overexpressing the non-destructible Clb2p (*clb2Δ176*) because of the inconsistency of this arrest at the non-permissive temperature. Thus, once I determined which strains died rapidly at the non-permissive temperature, I had identified 8 additional mutants defective in the loss of cohesion at the metaphase to anaphase transition (*loc10-18*) (see Tables A-1 and A-2). The rescreening procedure had also identified two *loc* mutants identified in the original screen, *ysc4-1* and *pds1-478*. I had not identified any of the *loc* mutants subsequently illustrated to be defective in sister chromatid segregation (*cse4-327* and *ipl1-182*).

None of these strains have been backcrossed to determine if the phenotype is linked to a single genetic locus. Complementation has not been undertaken to ascertain if two or more of the newly identified *loc* mutants belong to the same or known complementation groups.

DISCUSSION

Although *esp1-1* and *top2-4* are both defective in sister chromatid separation, they manifest this phenotype in different ways, primarily because of the different linkages the wildtype protein products are required to resolve. Topoisomerase II (*TOP2*) decatenates sister chromatids from each other; the inability to accomplish this results in an attempt by the anaphase spindle to pull intertwined sister chromatids apart. Therefore, if one observes sister chromatid separation at a locus near the centromere, sister chromatid separation may appear nominally perturbed since the locus is near the site of spindle attachment to the chromosome and the catenation between sisters may be able to travel some distance along the chromatid. However, as one observes sister chromatid separation further from the centromere, the defect is more pronounced. This is not true of *esp1-1* mutants. Because *esp1-1* mutants do not remove the protein linkage between sister chromatids, there is a block along the entire chromatid to the pulling forces of the mitotic spindle; if one observes the defect at a cen-proximal, arm or telomeric locus, there is little difference in the quantitative degree of the defect.

In the original strain construction, the Lac operator array was integrated at the *TRP* locus, ~12 kb away from the centromere of Chromosome IV. This is not close enough to the centromere to observe the pre-anaphase sister centromere separation reported (Goshima and Yanagida 2000; He, Asthana et al. 2000; Tanaka, Fuchs et al. 2000; Pearson, Maddox et al. 2001) but it is close enough for roughly 65-70% of *top2-4* mutant cells to appear as if they have properly segregated this locus in a synchronous cell cycle. In an asynchronous cell cycle, 24% of large budded cells appear to have segregated this locus; 10% have separated their sister chromatids but they remain in the mother bud. I score this as separated but not segregated sister chromatids. This is in

contrast to the *esp1-1* mutant cells, which exhibit only 8% sister chromatid segregation in large budded cells in an asynchronous population. However, 23% of large budded cells have separated sister chromatids in the mother bud. There fore, although both mutants exhibit a roughly equal quantitative defect (~30% large-budded cells with separated sister chromatids), the *top2-4* mutant appears to have segregated Chromosome IV in most of these cells while *esp1-1* has not. If this is taken as additional criteria, one could further classify the 8 additional *loc* mutants into two categories: those that affect the resolution of topological linkage between sister chromatids (i.e. *loc11*, *13* and *16*) and those that affect the resolution of the protein linkage between sister chromatids (i.e. *loc10*, *12* and *15*). In the *loc14* and *17* mutants, the difference in large-budded cells exhibiting separated versus segregated sister chromatids is not significant enough to hazard a classification.

The analysis of the *ycs4-1* mutation reveals that there is a link, at least in budding yeast, between proteins required for mitotic chromosome behavior and the maintenance of a chromosome structure for chromosome function(s) independent of their segregation during mitosis. Whether this is a merely a consequence of condensin association with yeast chromosomes throughout the cell cycle or an indication that yeast chromosomes undergo little rearrangement during mitosis, in comparison to the chromosomes of animal and plant cells, remains to be seen. Interestingly, two of the new *loc* mutants, *loc12* and *loc15* are α-factor resistant. It has not been shown whether this is a consequence of the loss of silencing of the silent mating loci. However, given the precedence of *ycs4-1* and *smc4-1*, it is possible that defects influencing chromosome separation may be affecting the maintenance and/ or establishment of silencing.

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Table A-1. Yeast strains used in this study

Strain	Genotype
SBY215	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2*
NBY92	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 top2-4*
NBY118	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 esp1-1*
NBY192	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 ycs4-1*
NBY233	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc10(1A1C)*
NBY234	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc11(1B3G)*
NBY235	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc12(5A2B)*
NBY236	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc13(5A2G)*
NBY237	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc14(5B3H)*
NBY238	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc15(6B6B)*
NBY239	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc16(7A1C)*
NBY240	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP1 ade2-1 can1-100 bar1Δ lys2Δ::pGAL-Δ176-CLB2::LYS2 loc17(8A5E)*

Table A-2. new *loc* mutants

strain	mutant	% unbudded	% small-budded	% large-budded	% separated sisters[1]	% segregated sisters[1]	% total sister separation[1]
NBY215	WT	40	26	34	5	81	86
NBY92	*top2-4*	49	8	43	10	24	34
NBY118	*esp1-1*	35	14	51	23	8	31
NBY92	*ycs4-1*	29	22	49	11	7	18
NBY233	*loc10*	38	34	28	29	4	33
NBY234	*loc11*	36	29	35	12	25	37
NBY235[2]	*loc12*	50	24	26	29	18	47
NBY236	*loc13*	25	35	40	6	24	30
NBY237	*loc14*	39	14	47	16	9	25
NBY238[2]	*loc15*	25	22	53	35	7	42
NBY239	*loc16*	60	19	21	4	37	41
NBY240	*loc17*	68	7	25	13	9	22

[1]in large budded cells

[2]α-factor resistant

APPENDIX B

An Attempt to Establish Cohesion in G2

Abstract

Recent evidence strongly suggests that DNA replication and the establishment of cohesion between the duplicated sister chromatids are functionally as well as temporally linked. However, there is a possibility that replication primarily provides a window during which sister chromatids are in close proximity and can be linked to each other by the loading of the cohesin complex. To investigate this hypothesis, we temporally uncoupled replication from cohesin loading and asked whether keeping sister chromatids in close proximity in metaphase allowed the establishment of cohesion after the completion of replication. Our data suggests that the proximity between sister chromatids achieved by protein-protein interactions cannot duplicate that achieved during replication and therefore, does not allow for the establishment of sister chromatid cohesion in G2.

INTRODUCTION

There is a strong body of evidence that indicates the coincidence of DNA replication and the establishment of cohesion is functional as well as temporal (reviewed in (Carson and Christman 2001). An early indication was the discovery that multiple copies of *POL30*, which encodes the DNA replication processivity factor proliferating cell nuclear antigen (PCNA), suppress the temperature sensitivity of an *eco1/ctf7* mutation (Skibbens, Corson et al. 1999). A more direct link between sister chromatid cohesion and DNA replication has been presented with the report that functional DNA polymerase κ, encoded by the *TRF4* gene, is necessary for sister chromatid cohesion (Wang, Castano et al. 2000). The authors offer a model in which a polymerase switching event precedes the replication of cohesin-associated sites during S phase, analogous to the polymerase switching (from DNA polymerase α to DNA polymerase δ) accomplished by replication factor C (RFC). Such a model holds considerable merit, as an alternate RFC complex composed of Ctf8p, Dcc1p, Ctf18p, Rfc2p, Rfc3p, Rfc4p and Rfc5p, is required for sister chromatid cohesion as well (Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001).

However, strains that contain a deletion of one or more of a majority of genes listed above are viable, despite the observation of a sister chromatid cohesion defect in these mutants (Wang, Castano et al. 2000; Hanna, Kroll et al. 2001; Mayer, Gygi et al. 2001). *ECO1/CTF7* is the only essential gene that links cohesion to replication and the *eco1/ctf7* mutant does not prevent the loading of cohesins, the protein linkage between sister chromatids (Toth, Ciosk et al. 1999). In fact, mutants that fail to replicate all

together manage to load the cohesin complex onto the unduplicated chromatids (Uhlmann and Nasmyth 1998). The Scc2p and Scc4p proteins, both essential, are the only known gene products that are required for cohesin loading during replication; mutation of either of these genes result in a sister chromatid cohesion defect (Ciosk, Shirayama et al. 2000). These data suggest that at least two pathways may influence the establishment of cohesion during replication. To date, no genetic interactions between these two pathways have been reported but such investigations may prove informative. Therefore, the possibility exists that the coincidence of replication and cohesin loading primarily presents a window in the cell cycle during which sister chromatids are in close proximity to one another; this spacial adjacency is stabilized by the loading of the cohesin complex, which links sister chromatids by protein-protein interactions.

We tested this hypothesis by temporally uncoupling cohesin loading from DNA replication. We kept one pair of sister chromatids in close proximity with each other in metaphase via an alternate protein-protein interaction (the tetramerizing Lac repressor) and induced expression of a member of the cohesin complex (Mcd1p/ Scc1p). We essentially created a scenario following DNA replication in which cohesin loading was simultaneous with closely associated sister chromatids in G2 to determine if this was enough to establish cohesion. Our results suggest that it is not: the proximity between sister chromatids achieved by protein-protein interactions cannot substitute for whatever is achieved during replication and does not allow for the establishment of cohesion during G2.

Materials and Methods

Microbial techniques: Media and genetic and microbial techniques were essentially as described (Sherman, Fink et al. 1974; Rose, Winston et al. 1990). The strain DH5α was used for all bacterial manipulations. Stock solutions of inhibitors were: 30 mg/ml benomyl (DuPont), 10 mg/ml nocodazole (Sigma), 10 mg/ml α-factor (Biosynthesis), all in DMSO. A stock solution of 1M 3-aminotriazole (Sigma) was made with water. All stock solutions were stored at –20°C. A 1M solution of IPTG (US Biological) was made fresh in water for every experiment. Experiments were carried out at 30°. Cells were grown overnight in minimal media lacking histidine (to achieve strong induction of the GFP-LacI fusion protein that is under the control of the *HIS* promoter) and containing raffinose and galactose (to induce expression of the essential gene *MCD1/ SCC1*). α-factor was added to a final concentration of 1 μg/ ml and cells were arrested for three hours. Cells were then washed twice in -his media containing raffinose and resuspended in -his media containing raffinose, 1 μg/ ml α-factor and 10mM 3-aminotriazole. After one and a half hours, cells were washed with YEP media containing raffinose and released into YEP containing raffinose, 30 μg/ml benomyl and 15 μg/ml nocodazole. After three and a half hours, galactose was added to a final concentration of 4% to half of the culture. After an additional hour, IPTG (20mM) was added to all cultures. After one hour, cells were washed with YPD media containing benomyl and nocodazole and resuspended in YPD containing benomyl and nocodazole. After one and a half hours, cells were harvested for microscopy. 100 cells were counted for each time point and all experiments were performed at least twice with similar results.

Yeast strain constructions: Yeast strains are listed in Table B-I and were constructed by standard genetic techniques. Diploids were isolated on selective media at 23° and subsequently sporulated at 23°. NBY87 was transformed with pNB36 digested with *Bgl* II to direct integration to the intergenic region of Chromosome IV 793927-796231 and create NBY335; microscopy verified the integration of the LacO repeats. Strains containing *pGAL-MCD1/SCC1-3xHA* were constructed by integrating pMAS112 digested with *Eco* RV to direct integration at the LEU2 locus. NBY8 and NBY102 were transformed with pMAS112 to create NBY611 and NBY612, respectively. NBY611 was crossed to NBY102 to create NBY613. *MCD1/ SCC1* was deleted by PCR integration: DNA from pFA6-kanMX6 (Longtine, McKenzie et al. 1998) was PCR amplified using primers MCD-F1 (5' GTC/ AAA/ GAA/ AAG/ ACA/ ACT/ CAA/ TTG/ CAC/ AAT/ TAC/ TTT/ ACA/ AGA/ AAC/ ACG/ ACA/ CGG/ ATC/ CCC/ GGT/ TAA/ TTA/ A 3') and MCD-R1 (5' ATG/ CAT/ CAG/ CTT/ ATT/ GGG/ TCC/ ACC/ AAG/ AAA/ TCC/ CCT/ CGG/ CGT/ AAC/ TAG/ GTT/ GAA/ TTC/ GAG/ CTC/ GTT/ TAA/ AC 3'). The PCR product was transformed into NBY613 and NBY612 to create NBY614 and NBY615, respectively; the deletions were verified by PCR. NBY614 was sporulated to produce NBY618. NBY618, containing the GFP12-LacI12 fusion protein (non-tetramerizing) under the copper inducible promoter, was crossed to AFS286, containing the tetramerizing GFP-LacI fusion protein under the control of the *HIS* promoter. The diploid was sporulated and the spores microscopically screened for induction of GFP-LacI in media containing copper to identify NBY623 and NBY624. NBY624 was crossed to NBY291 and sporulated to produce NBY627 and NBY628. NBY624 was crossed to NBY335 and sporulated to produce NBY631. NBY615 was crossed to

NBY627 and NBY631 to produce NBY635 and NBY636, respectively. NBY623 was crossed to YBS432 and sporulated to produce NBY637.

Plasmid construction: pAFS148 was digested with *Bam* HI and *Sal* I and the resulting 5kb fragment was ligated into pAFS159 digested with *Bam* HI and *Sal* I to create pNB36.

Western blotting: Western blotting was performed as described (Kellogg, Kikuchi et al. 1995). Monoclonal 12CA5 anti-HA antibodies (Babco) were diluted 1:1000 and protein was detected using chemiluminescence (ECL, Amersham).

Immunofluorescence and microscopy: Microscopy was performed as described (Biggins, Severin et al. 1999).

In the absence of spindle forces, the wildtype tetramerizing Lac repressor bound to an array of Lac operator sites at a single locus on a chromosome can maintain sister chromatid cohesion when sister chromatids should separate (Straight, Belmont et al. 1996). We constructed strains in which the only copy of the cohesin subunit, *MCD1/ SCC1*, was present under the control of the inducible galactose promoter. In the absence of Mcd1p, other members of the cohesin complex fail to associate with chromosomes (Michaelis, Ciosk et al. 1997). The strains also contained a fusion protein of GFP with the wildtype Lac repressor (GFP-LacI[tet+]) that bound an array of 256 Lac operator repeats integrated at a site on the arm of Chromosome IV. One strain had the array integrated ~330kb away from the centromere of Chromosome IV (lower arm) while

another had it integrated ~660kb away from the centromere of Chromosome IV (upper arm). We arrested the two strains in media containing α-factor and galactose for three hours. We repressed transcription of *MCD1* by transferring the cells to media containing α-factor and raffinose for an additional ninety minutes. The ninety minute incubation in raffinose resulted in loss of most of the Mcd1p (Figure B-1A). Cells were released into media containing raffinose, benomyl and nocodazole for three and a half hours until a metaphase arrest was achieved. Sister chromatid separation was observed in only 21% of cells with the LacO on the lower arm and in 24% of the cells with the LacO array on the upper arm (Figure B-1B), illustrating the ability of GFP-LacI(tet+) to link sister chromatids together in the absence of cohesin. In cells that expressed a non-tetramerizing version of the Lac repressor (in which the last 11 amino acids have been removed), sister chromatid separation was substantially higher, consistent with a sister chromatid separation defect in the absence of cohesin (data not shown). Galactose was added to half of the culture for one hour to induce *MCD1* expression (Figure B-1A); the other half continued growing in raffinose containing media to observe sister chromatid separation in the absence of cohesin loading in G2. Mcd1p expressed during metaphase loads onto chromosomes and appears indistinguishable from wildtype association (Uhlmann and Nasmyth 1998). Isopropyl β-D-thiogalactopyranoside (IPTG) was added to all cultures for the next hour to remove GFP-LacI(tet+) from Chromosome IV. The binding of IPTG to the Lac repressor reduces its affinity for the Lac operator 1000 fold (Riggs, Newby et al. 1970). The GFP signal in the IPTG-treated cells was nuclear and diffuse (data not shown). The cells were then washed extensively and resuspended in media containing dextrose, benomyl and nocodazole for ninety minutes, after which the GFP signal was

again observed as a single dot or pair of dots. At the end of the experiment, the cells have been in media containing benomyl and nocodazole for a total of seven hours, raising the possibility that any sister chromatid separation that is observed might be the product of exit from the arrest. We consider this unlikely because of two reasons: 1) the slower doubling time in media containing galactose and raffinose (as compared to media containing dextrose, in which cells exit a nocodazole induced arrest after 5-6 hours) and 2) strains did not exhibit substantially greater sister chromatid separation whether arrested in media containing raffinose, galactose, benomyl and nocodazole for three and a half hours or seven hours (data not shown).

In the cells that did not induce *MCD1* expression, sister chromatid separation was observed in over 50% of the cells at the end of the experiment (Figure B-1B). The addition of IPTG and the subsequent release of tetramerizing GFP-LacI from the chromosomal locus allowed the unlinked sisters to separate, exhibiting two GFP signals upon removal of the ITPG and rebinding of the GFP-LacI to the chromosome. If cohesion could be established in G2 by the loading of the cohesin complex onto sister chromatids held together by the tetramerizing GFP-LacI, the strains that induced *MCD1* expression would exhibit a lower percentage of cells with separated sister chromatids at the end of the experiment. This is not the case. In cells in which *MCD1* expression was induced, sister chromatid separation was observed in 41% of cells, whether the locus was on the lower or upper arm. These data suggest the proximity achieved by protein-protein interactions at a single chromosomal locus in the presence of cohesin loading during G2 is not enough to establish cohesion between sister chromatids.

RESULTS

However, the sister chromatid separation observed in cells that induced *MCD1* expression was reproducibly lower than that in cells that did not express *MCD1*. Therefore, we postulated that the presence of multiple LacO arrays on the same chromosome might allow the establishment of cohesion in the presence of cohesin in G2. Cohesin binds all along chromosomes (Blat and Kleckner 1999; Megee and Koshland 1999; Tanaka, Cosma et al. 1999, Laloraya, 2000 #127) and therefore, may require multiple points of contact between sister chromatids to establish cohesion. We generated strains that contained two LacO arrays on Chromosome IV (Figure B-2A). One array was at the *TRP* locus, ~12 kb away from the centromere of Chromosome IV, and the other at one of the two arm loci (upper or lower). Since cohesin deposition at centromeres is particularly heavy (Blat and Kleckner 1999; Megee and Koshland 1999; Tanaka, Cosma et al. 1999, Laloraya, 2000 #127) and may direct association along chromosomes (Megee, Mistrot et al. 1999), we wanted a LacO array at or near the centromere. Also, due to a fortuitous recombination event, the LacO array at the *TRP* locus was dimmer in intensity than those at the arm loci, enabling accurate visualization of sister chromatid separation. If I observed two GFP signals of equal intensity, I scored the cell as having separated sister chromatids. I also did this if I observed greater than two GFP signals.

The experiment was carried out with the same regime described above. After three and a half hours, prior to the addition of galactose, sister chromatid separation was observed in 20-30% of metaphase arrested cells with LacO arrays at the TRP locus and at

one of the two arm loci (Figure B-2B). After induction of *MCD1* expression and IPTG addition and removal (seven hour timepoint), sister chromatid separation was observed in 40-55% of metaphase arrested cells, comparable to cells in which *MCD1* expression had never been induced.

We also constructed a strain that contained the two LacO arrays at the two arm loci used individually in the previous experiments (Figure B-2A). In this strain, the GFP signals are of equal intensity. Therefore, I simply scored the number of GFP signals to determine the degree of sister chromatid separation. At three and a half hours into the experiment, 93% of the cells exhibit a single or pair of GFP dots, indicative of cells with unseparated sister chromatids (Figure B-2C). Some of the cells with two GFP signal may actually have separated sister chromatids since the two LacO arrays on a single chromosome are not always able to be resolved (as evidenced by the 48% of cells that only exhibit a single GFP signal). At the end of the experiment, 45% of cells that had induced *MCD1* expression during G2 had greater than 2 GFP signals, suggestive of sister chromatid separation (Figure B-2C). Similar numbers were observed with cells in which Mcd1p was never expressed during the experiment (Figure B-2C).

Taken together, our data indicate that two points of linkage between sister chromatids via protein-protein interactions during cohesin loading cannot mimic the interaction achieved during replication. Cohesion is not established in G2 when we hold a pair of sister chromatids together at two loci with tetramerizing GFP-LacI, even when one of the two loci is a centromere-proximal locus.

The LacO array integrated at the *TRP* locus, despite being centromere proximal, does not exhibit the pre-anaphase centromere separation (Straight, Belmont et al. 1996)

that has been observed with arrays integrated hundreds of basepairs away from the centromere (Goshima and Yanagida 2000; He, Asthana et al. 2000; Tanaka, Fuchs et al. 2000; Pearson, Maddox et al. 2001). Cohesin is heavily concentrated at the centromeres (Blat and Kleckner 1999; Megee and Koshland 1999; Tanaka, Cosma et al. 1999, Laloraya, 2000 #127) and de novo deposition at the centromeres may be an initial and required step to drive association along chromosome arms (Megee, Mistrot et al. 1999; Laloraya, Guacci et al. 2000). Therefore, we generated a strain that contained a LacO array at the centromere of Chromosome XV (Goshima and Yanagida 2000) as well as GFP-LacI(tet+) and a single copy of *MCD1* under the galactose promoter. The same experimental protocol was carried out. At three and a half hours, 16% of metaphase arrested cells had separated sister chromatids. After seven hours, induction of *MCD1* expression, IPTG treatment and removal, sister chromatid separation was observed in 47% of cells, as compared to the 56% of cells that exhibited separated sister chromatids without galactose induction (Figure B-3A). The exogenous linkage of sister chromatids at centromeres during G2 in combination with the post-replicative loading of the cohesin complex cannot establish cohesion.

DISCUSSION

The conclusion we are forced to draw is that the linkage provided by the tetramerizing GFP-LacI, while capable of keeping sister chromatids linked together in the absence of spindle forces, cannot recreate the association achieved during replication. Therefore, even if the close proximity of sister chromatids is maintained in the absence of

the cohesin complex and the cohesins are provided in G2 after replication, cohesion cannot be established. This data suggests that replication provides some advantage to the establishment of cohesion aside from simple proximity between sister chromatids. It is possible that the presence of two sites of artificial linkage between sister chromatids is not enough to allow the cohesin complex to establish cohesion but working with greater than two LacO arrays with GFP-LacI bound would be more confusing than informative.

Thus far our data suggests that the biochemical process of replicating the genome contributes to the establishment of cohesion. The information available so far suggests that cohesion establishment may involve two pathways: cohesin loading and another catalyzed by replication factors. Is there any interplay between the two? Does the cohesion dependent reaction(s) catalyzed by the replication factors involve some modification of the cohesin complex, making it competent to link sister chromatids together? This is the prevailing model. Or do replication factors contribute to cohesion in a totally independent pathway, perhaps by producing the topological linkage between sisters? It is assumed that catenation between sister chromatids is a natural byproduct of the collision of replication forks but this has only been shown with circular chromosomes *in vitro* (Sundin and Varshavsky 1980; Sundin and Varshavsky 1981). Might there be an active intertwining of sister chromatids during replication? What role, if any, might the cohesins play in this model?

One approach to this question is to utilize replication intermediates between sister chromatids as the topological linkage between them in the absence of the cohesin complex. We would prolong replication with low doses of hydroxyurea so that replication overlaps with spindle assembly and attachment to kinetochores. We already

know that this is not enough to ensure proper sister chromatid segregation (data not shown) but what about in combination with a protein-protein interaction, such as the tetramerizing GFP-LacI? Would the combination of intertwined sister chromatids, as accomplished by the delayed replication intermediates, and protein-protein linkage, as accomplished by the tetramerizing GFP-LacI, recapitulate sister chromatid cohesion in the absence of the cohesin complex? Would it recreate it faithfully enough to allow proper segregation of sister chromatids?

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Table B-1. Yeast strains used in this study

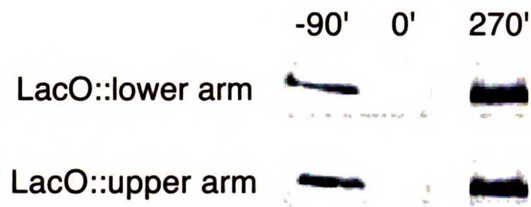
Strain	Genotype
NBY8	*MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
NBY102	*MATα ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP ade2-1 can1-100 bar1Δ lys2Δ* {pRS316}
NBY291	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ upper armIV::LacO::URA3*
NBY335	*MAT**a** ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ lower armIV::LacO::URA3*
NBY611	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ*
NBY612	*MATα ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11:: :pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP ade2-1 can1-100 bar1Δ lys2Δ*
NBY613	*MAT**a**/α ura3-1/ ura3-1 leu2-3,112/ leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11/ his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1/ trp1-1::LacO::TRP ade2-1/ ade2-1 can1-100/ can1-100 bar1Δ/ bar1Δ lys2Δ/ lys2Δ*
NBY614	*MAT**a**/α ura3-1/ ura3-1 leu2-3,112/ leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11/ his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1/ trp1-1::LacO::TRP ade2-1/ ade2-1 can1-100/ can1-100 bar1Δ/ bar1Δ lys2Δ/ lys2Δ mcd1Δ::KAN/MCD1*
NBY615	*MATα ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1::LacO::TRP ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY618	*MATα ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY623	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY624	*MATα ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY627	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN upper armIV::LacO::URA3*
NBY628	*MATα ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN upper armIV::LacO::URA3*

NBY631	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN lower armIV::LacO::URA3*
NBY633	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN lower armIV::LacO::URA3 upper armIV::LacO::URA3*
NBY635	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1::LacO::TRP ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN upper armIV::LacO::URA3*
NBY636	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1::LacO::TRP ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN lower armIV::LacO::URA3*
NBY637	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN cenXV::LacO::URA3*
AFS268	*MAT**a** ura3-1 leu2-3,112 his3-11::pHIS3-GFP-LacI(tet+)::HIS3 trp1-1 ade2-1 can1-100 BAR1 LYS2*
YBS432	*MATα ura3-1 leu2-3,112 his3-11::pCUP1-GFP12-LacI12::HIS3 trp1-1 ade2-1 can1-100 BAR1 LYS2 cenXV::LacO::URA3*

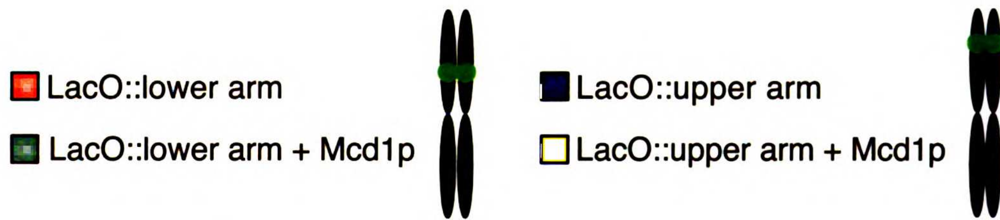
All strains are isogenic with the W303 strain background. Plasmids are indicated in brackets.

Figure B-1. Maintaining sister chromatid linkage with the tetramerizing GFP-LacI bound to a chromosomal locus does not allow sister chromatid cohesion to be established in G2/ metaphase. **A.** Western blot of Mcd1-3xHAp. A ninety minute incubation in media containing raffinose effectively represses protein expression. The 0' timepoint refers to release from G1 arrest. Galactose was added back at 210 minutes from G1 release; the Mcd1-3xHAp levels are significant after sixty minutes (270'). **B.** The induction of Mcd1p does not allow the establishment of cohesion. Sister chromatid separation in metaphase arrested cells was scored at three and a hlaf hours after G1 release and just prior to galactose induction. Sister chromatids separate in 21% and 24% of cells, whether GFP-LacI(tet+) is bound to the lower arm locus NBY631) or upper arm locus (NBY627), respectively. Sister chromatid separation was again scored at seven hours, after one half of the culture received galactose to induce Mcd1p expression and both halves were subjected to IPTG treatment and removal. There is no quantitative difference between whether the culture received galactose in the percentage of cells that exhibit sister chromatid separation.

A



B



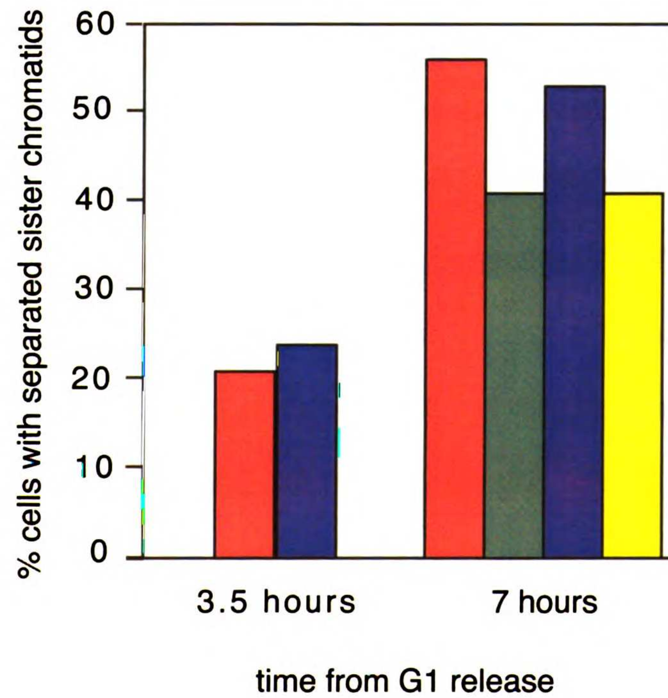
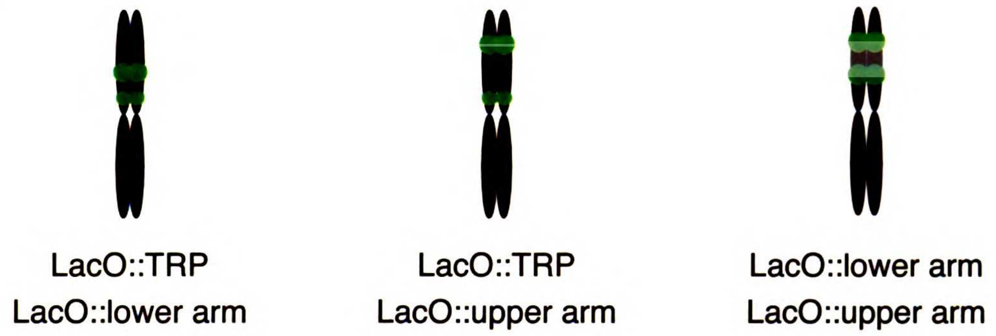
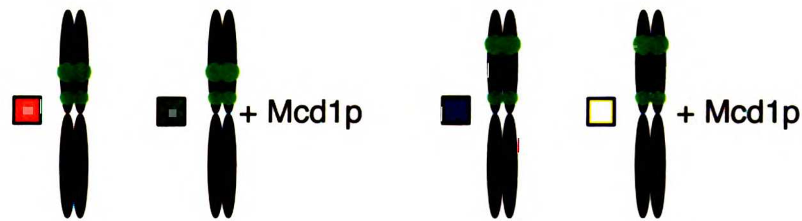


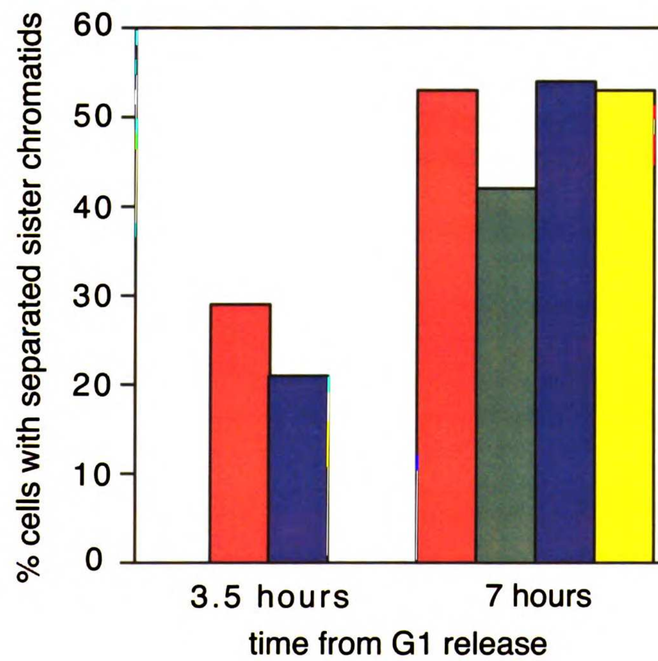
Figure B-2. Increasing the number of chromosomal loci that bind GFP-LacI(tet+) does not allow cohesion to be established in G2/ metaphase. **A.** Cartoons of the three combinations of LacO arrays on a single chromosome. **B.** The combination of a cen-proximal LacO array with an arm LacO array does not allow cohesion to be establshed. Sister chromatid separation in metaphase arrested cells was scored at three and a hlaf hours after G1 release and just prior to galactose induction. Sister chromatids separate in 21% of cells with the LacO arrays at the TRP locus and on the lower arm (NBY636) and 29% of cells with the LacO arrays at the TRP locus and on the upper arm (NBY635). Sister chromatid separation was again scored at seven hours, after one half of the culture received galactose to induce Mcd1p expression and both halves were subjected to IPTG treatment and removal. There is no quantitative difference between whether the culture received galactose in the percentage of cclls that exhibit sister chromatid separation. **C.** The presence of two LacO arrays on the arm of Chromosome IV does not allow sister chromatid cohesion to be established in G2. A strain expressing GFP-LacI(tet+) with LacO arrays at both lower and upper arm loci was used (NBY633). Because the GFP signals are of equal intensity, we simply scored the number of GFP signals we observed. At three and a half hours only 7% of the cells exhibit three dots; the remaing 93% have a single or two GFP signals. After seven hours, 45% of cells have three or four GFP signals, whether the cells have induced *MCD1* expression or not.

A



B





C

LacO::lower arm
LacO::upper arm

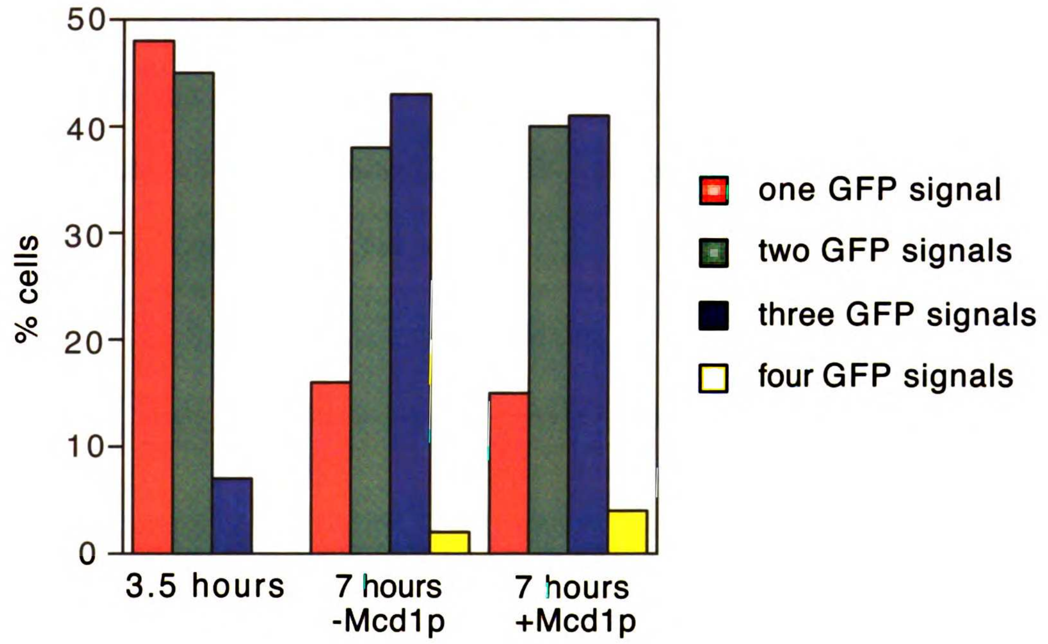
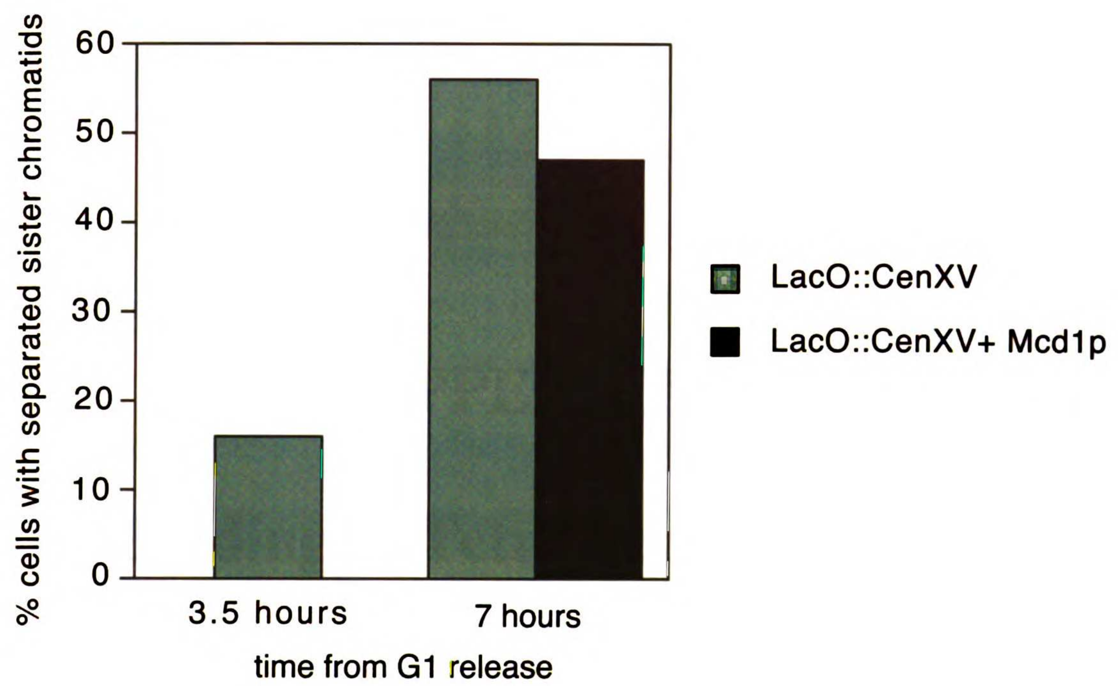


Figure B-3. LacO arrays that bind GFP-LacI(tet+) at the centromere of Chromosome XV do not allow the establishment of cohesion in G2/ metaphase. A strain expressing GFP-LacI(tet+) with LacO arrays at centromere XV was used (NBY637). Sister chromatid separation in metaphase arrested cells was scored at three and a half hours after G1 release (just prior to galactose induction) and again at seven hours, after one half of the culture received galactose to induce Mcd1p expression and both halves were subjected to IPTG treatment and removal. Sister chromatids separate in 16% of cells at three and a half hours. After seven hours, sister chromatids separate in 47% of cells that induced *MCD1* expression and 56% that did not.


% cells with separated sister chromatids
60
50
40
30
20
10
0
3.5 hours
7 hours
time from G1 release
LacO::CenXV
LacO::CenXV+ Mcd1p

APPENDIX C

The Binding of Tetramerizing Lac Repressor at the Centromere of Chromosome IV Activates the Spindle Assembly Checkpoint

ABSTRACT

The separation of sister centromeres is believed to be a requirement for the establishment of biorientation: the attachment of sister kinetochores to microtubules from opposite spindle poles. Defects in biorientation are sensed by the spindle assembly checkpoint and the conserved protein kinase *IPL1*. We attempted to link sister centromeres together with the tetramerizing GFP-LacI and observe the effects, if any, on pre-anaphase centromere separation and cell cycle progression. When GFP-LacI(tet+) was bound to a LacO array at the centromere of Chromosome IV in cells, pre-anaphase sister centromere separation was reduced and the spindle assembly checkpoint was transiently activated. However, this phenomenon was specific to the LacO array integrated at centromere IV because GFP-LacI(tet+) bound to a LacO array at centromere XV did not activate the checkpoint.

INTRODUCTION

During mitosis, the centromeres of sister chromatids separate before anaphase due to the pulling forces of the mitotic spindle (Goshima and Yanagida 2000; He, Asthana et al. 2000; Tanaka, Fuchs et al. 2000; Pearson, Maddox et al. 2001). This separation precedes Pds1p destruction (Tanaka, Fuchs et al. 2000), cohesin removal (He, Asthana et al. 2000) and separation of the arms of sister chromatids (Goshima and Yanagida 2000). A current model suggests that this pre-anaphase separation is the cell's attempt to monitor biorientation of the sister chromatids: the attachment of the sisters via the kinetochore to microtubules from opposite spindle poles. Biorientation produces tension on the kinetochore as a result of the opposing forces of microtubule pulling forces and sister chromatid cohesion. The conserved protein kinase, Ipl1p, has been implicated in monitoring biorientation. Mutation of *IPL1* (Biggins, Bhalla et al. 2001) and some spindle assembly checkpoint genes (*MAD1* and *MAD2*) (Stern and Murray 2001) result in an inability to sense defects in biorientation, suggesting that tension might be monitored by the spindle assembly checkpoint. Consistent with this hypothesis, Ipl1p localizes to kinetochores in budding yeast (Biggins, Bhalla et al. 2001) and to the space between holocentric sister chromatids .in *C. elegans* (Oegema, Desai et al. 2001). These observations offer the possibility that the establishment of biorientation and the separation of sister centromeres effectively remove Ipl1p substrates at the kinetochore from the physical presence of the kinase between sister centromeres, thus preventing checkpoint activation. An alternate model exists. Ipl1p is observed to have microtubule destabilizing activity (Biggins, Severin et al. 1999) and may act to destabilize spindle

microtubules at kinetochores that are not under sufficient tension to pull sister centromeres apart and away from the kinase (Tanaka, Rachidi et al. 2002). The destabilization of kinetochore microtubules would lead to an unattached kinetochore, a known activator of the spindle assembly checkpoint (Wells and Murray 1996). We were interested to see if the presence of the tetramerizing GFP-Lac repressor fusion protein at the centromere could resist the pre-anaphase separation of sister centromeres; if so, would there be any response from the cell cycle? Could a characterized protein-protein interaction between sister centromeres effectively mimic a defect in biorientation and delay the cell cycle by activation of the spindle assembly checkpoint?

MATERIALS AND METHODS

Microbial techniques and yeast strain construction: Media and genetic and microbial techniques were essentially as described (Sherman, Fink et al. 1974; Rose, Winston et al. 1990). Cells were cultured at 30°. All cytological experiments were carried out by arresting cells in media lacking histidine (to induce strong expression of the GFP-LacI fusion protein under the control of the *HIS3* promoter) containing 1 μg/ml α-factor for three hours, washing cells twice in α-factor free media and resuspending them in -his media containing 10mM 3-aminotriazole. After one hour, α-factor was added back to the media to prevent cells from entering the next cell cycle. All experiments were repeated at least twice with similar results. In all experiments, at least 100 cells for each time point were counted. A stock solution of 10 mg/ml α-factor (Biosynthesis) was made with

DMSO. A stock solution of 1M 3-aminotriazole (Sigma) was made with water. All stock solutions were stored at –20°C.

Yeast strains are listed in Table C-I. Yeast strains were constructed by standard genetic techniques. Diploids were isolated on selective media at 23°C and subsequently sporulated at 23°C. MAA730 was crossed to NBY621 and NBY623 and the diploids sporulated to produce NBY643 and NBY644, respectively. NBY643 was crossed to YBS439 and the diploid was sporulated to produce NBY647 and NBY648. NBY644 was crossed to YBS439 and the diploid was sporulated to produce NBY649 and NBY650. NBY650 was crossed to NBY652 and the diploid was sporulated to produce NBY655 and NBY656. NBY648 was crossed to NBY651 and the diploid sporulated to produce NBY658.

Immunofluorescence and microscopy: Microscopy was performed as described (Biggins, Severin et al. 1999). Immunofluorescence was performed as described (Rose, Winston et al. 1990). Monoclonal 9E10 anti-myc antibodies (Babco, Berkeley) were used at a 1:500 dilution. DAPI (Molecular Probes) was used at 1μg/ml final concentration.

RESULTS

We constructed strains with an array of LacO repeats integrated ~1kb away from the centromere of Chromosome IV (M. Shonn, unpublished observation) expressing either non-tetramerizing or tetramerizing versions of the GFP-LacI fusion protein under

the control of the HIS3 promoter. Cells were arrested in G1 by treating them with α-factor and released into media in the absence of α-factor. To observe progress through a single cell cycle, α-factor was added back to the media when small-budded cells appeared in the population. Figure C-1 shows that the presence of tetramerizing GFP-LacI at the centromere of Chromosome IV results in a roughly twenty minute delay in sister chromatid separation and the exit from mitosis, as illustrated by the continued presence of budded cells toward the end of the cell cycle. The pre-anaphase separation of this locus was also lower in the cells that expressed GFP-LacI(tet+) in comparison to cells expressing GFP-LacI(tet-) (data not shown).

We wanted to determine if this delay was the result of spindle assembly checkpoint activation. Therefore, we deleted the spindle assembly checkpoint component, *MAD2*, in strains with the LacO array the centromere IV expressing either non-tetramerizing or tetramerizing GFP-LacI. To more closely monitor the delay, we decided to observe the anaphase inhibitor, Pds1p, in cells progressing through a single cell cycle. In response to spindle damage and unattached kinetochores, the spindle assembly checkpoint directly impinges upon Pds1p destruction to prevent the metaphase to anaphase transition (Hwang, Lau et al. 1998). We employed indirect immunofluorescence to visualize Pds1p in the nuclei of cells. Figure C-2 illustrates that the twenty minute cytological delay initially observed in cells expressing GFP-LacI(tet+) is reflected in a delay in Pds1p destruction. This delay is lost when the spindle assembly checkpoint is abrogated by deletion of *MAD2*; the rate of Pds1p destruction more closely resembles that of cells expressing the non-tetramerizing GFP-LacI (Figure C-2).

We wanted to ensure that the phenomenon we were observing was a general one and not one specific to the integration of LacO at the centromere of Chromosome IV. Therefore, we generated a strain expressing the tetramerizing GFP-LacI which contained the LacO array at the centromere of Chromosome XV. We observed Pds1p destruction in a single cell cycle by indirect immunofluorescence of this strain in comparison with strains with the LacO array at centromere IV either expressing the non-tetramerizing or tetramerizing GFP-LacI. Figure C-3 shows that the binding of GFP-LacI(tet+) to the LacO array at centromere XV does not result in a cell cycle delay; the rate of Pds1p destruction practically overlaps with that of cells with GFP-LacI(tet-) bound to centromere IV.

DISCUSSION

Our data suggest that the binding of the tetramerizing GFP-LacI to a LacO array integrated at the centromere of Chromosome IV activates the spindle assembly checkpoint. This phenomenon is specific to this centromere, since GFP-LacI(tet+) bound to a LacO array at centromere XV does not activate the checkpoint. The two arrays are roughly the same distance, although the LacO array at centromere XV is 800 additional base pairs away from the canonical centromere (Goshima and Yanagida 2000). Other abnormalities have been observed with the cenIV:LacO strain, namely that a diploid strain homozygous for cenIV::LacO and expressing a non-tetramerizing GFP-LacI fusion protein does not undergo meiosis (M. Shonn, unpublished observation). Another strain with the LacO array integrated ~1kb away from the centromere of Chromosome VIII

does not exhibit this defect (M. Shonn, unpublished observation), suggesting that the closer proximity of the LacO array to centromere IV than XV cannot explain the phenomenon associated with this strain.

Sister chromatid cohesion is resolved by a slightly different program in meiosis than mitosis. The resolution of sister chromatid arms allows the segregation of homologous chromosomes in the first division; sister centromeres are held together by a meiotic specific cohesin complex containing Rec8p instead of Mcd1p. The segregation of sister chromatids during the second meiotic division occurs after sister centromeres are unlinked by the removal of the cohesin complex (Klein, Mahr et al. 1999). The observations that strains homozygous for cenIV::LacO fail to sporulate and, when combined with GFP-LacI(tet+), transiently activate the spindle assembly checkpoint in mitosis, may indicate that the presence of the array at this centromere presents a challenge to cells with specialized protein-protein interactions at centromeres. The spindle assembly checkpoint is required for a normal meiotic division, unlike mitotic division (Shonn, McCarroll et al. 2000). The inability of cenIV:LacO homozygous diploids to sporulate may be the product of permanent activation of the spindle assembly checkpoint in meiosis.

Why does the presence of a LacO array at centromere IV in a strain expressing GFP-LacI(tet+) activate the spindle assembly checkpoint? An attractive possibility is that Ipl1p substrates at the kinetochore are brought into contact with the kinase between sister centromeres by the GFP-LacI(tet+) bound to the centromeric LacO array. The cell effectively believes that this pair of centromeres is not properly bi-oriented and activates the checkpoint. We were unable to test if the cell cycle delay was *IPL1* dependent due to

the temperature sensitivity of the GFP-LacI fusion proteins used in these experiments (thus far, only temperature sensitive mutants of *IPL1* are available). However, we already know that the protein-protein interactions of the tetramerizing GFP-LacI are not strong enough to withstand the pulling force of the mitotic spindle [Straight, 1996 #57; data not shown]. Therefore, a more realistic possibility is that GFP-LacI(tet+) bound to the LacO arrays at centromere IV interferes with microtubule attachment, thus activating the checkpoint. This may explain the lower incidence of pre-anaphase centromere separation in cells with GFP-LacI(tet+) bound to cenIV::LacO; we do not observe this in cells GFP-LacI(tet+) bound to cenXV::LacO (data not shown). This model may also involve *IPL1*, in its capacity as a microtubule-destabilizing factor.

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Table C-1. Yeast strains used in this study

Strain	Genotype
NBY621	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY623	*MAT**a** ura3-1 leu2-3,112::pGAL-MCD1-3xHA::LEU2 his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 bar1Δ lys2Δ mcd1Δ::KAN*
NBY643	*MAT**a** ura3-1 leu2-3,112 his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP*
NBY644	*MAT**a** ura3-1 leu2-3,112 his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP*
NBY647	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP*
NBY648	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP mad2Δ::KAN*
NBY649	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP*
NBY650	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP mad2Δ::KAN*
NBY651	*MATα ura3-1 leu2-3,112 his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 bar1Δ lys2Δ cenXV::LacO::URA3*
NBY652	*MATα ura3-1 leu2-3,112 his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 bar1Δ lys2Δ cenXV::LacO::URA3*
NBY655	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 bar1Δ LYS2 cenXV::LacO::URA3*
NBY656	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet+) trp1-1 ade2-1 can1-100 bar1Δ LYS2 cenIV::LacO::TRP*
NBY658	*MAT**a** ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11::pHIS3-GFP-LacI(tet-) trp1-1 ade2-1 can1-100 bar1Δ LYS2 cenXV::LacO::URA3*
MAS730	*MATα ura3-1::pCYC1-GFP12-LacI12::URA3 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 BAR1 LYS2 cenIV::LacO::TRP*
YBS439	*MATα ura3-1 leu2-3,112::PDS1-18xMYC::LEU his3-11 trp1-1 ade2-1 can1-100 BAR1 LYS2 mad2Δ::KAN*

All strains are isogenic with the W303 strain background.

Figure C-1. Cells with GFP-LacI(tet+) bound to a Lac operator array integrated at the centromere of Chromosome IV delay the cell cycle. Cells containing a LacO array at centromere IV and expressing either GFP-LacI(tet-) (NBY643) or GFP-LacI(tet+) (NBY644) were released from α-factor arrest (t=O) and scored for budding and sister chromatid separation. After one hour, α-factor was added back to the media to prevent cells from entering the next cell cycle. Cells expresing the tetramerzing form of GFP-LacI delay sister chromatid separation and the exit from mitosis by twenty minutes.

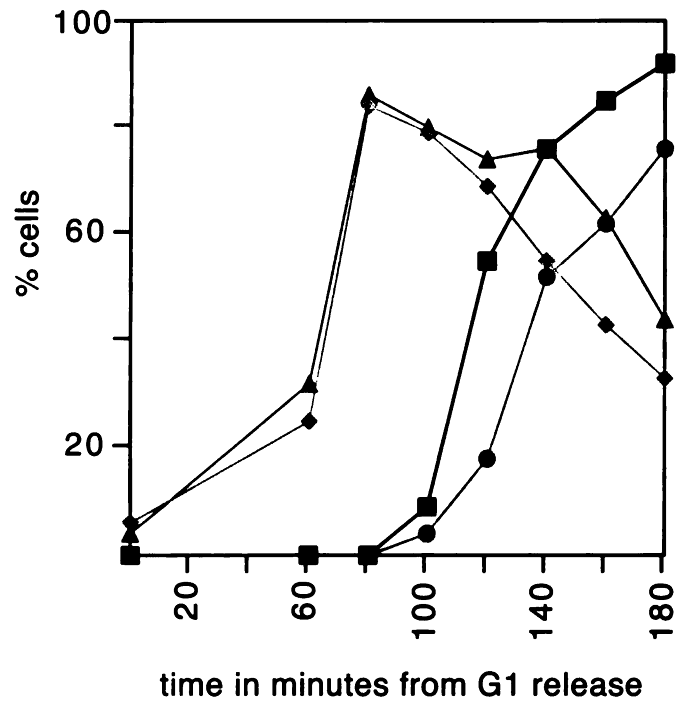

GFP-LacI(tet-) budded cells
GFP-LacI(tet+) budded cells
GFP-LacI(tet-) cells with separated sisters
GFP-LacI(tet+) cells with separated sisters
100
60
20
% cells
20
60
100
140
180
time in minutes from G1 release

Figure C-2. Cells with GFP-LacI(tet+) bound to a Lac operator array integrated at centromere IV activate the spindle assembly checkpoint. GFP-LacI(tet-) (NBY647), GFP-LacI(tet-)*mad2* (NBY648), GFP-LacI(tet+) (NBY649), and GFP-LacI(tet+)*mad2* (NBY650) strains were released from α-factor arrest (t=O) and subjected to indirect immunofluorescence to visualize Pds1p-18xMyc through the cell cycle. The delay in Pds1p destruction is lost when *MAD2* is deleted in cells expressing GFP-LacI(tet+).

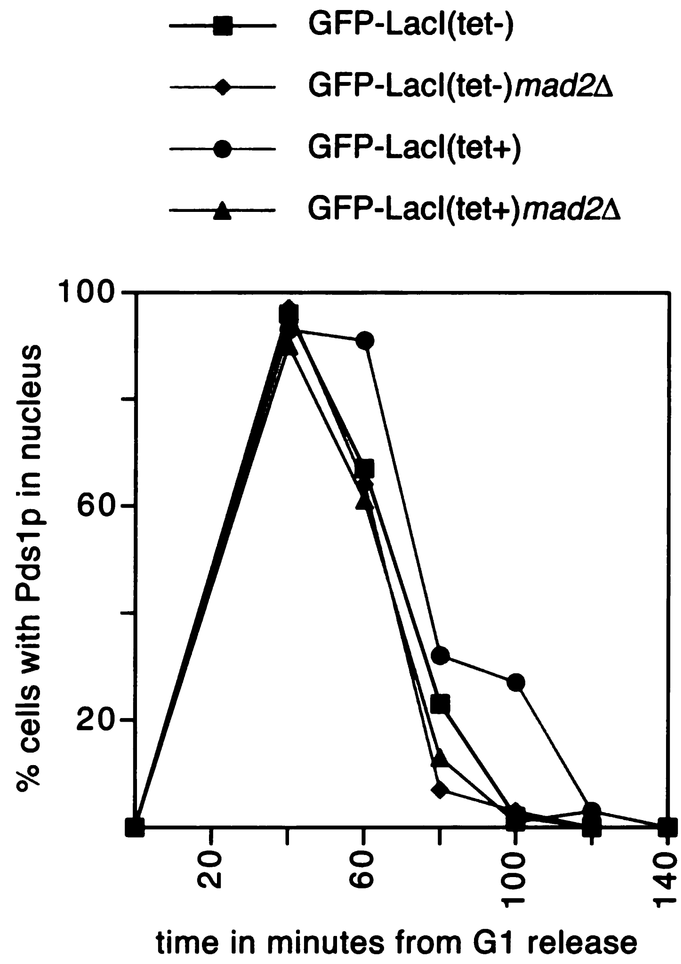
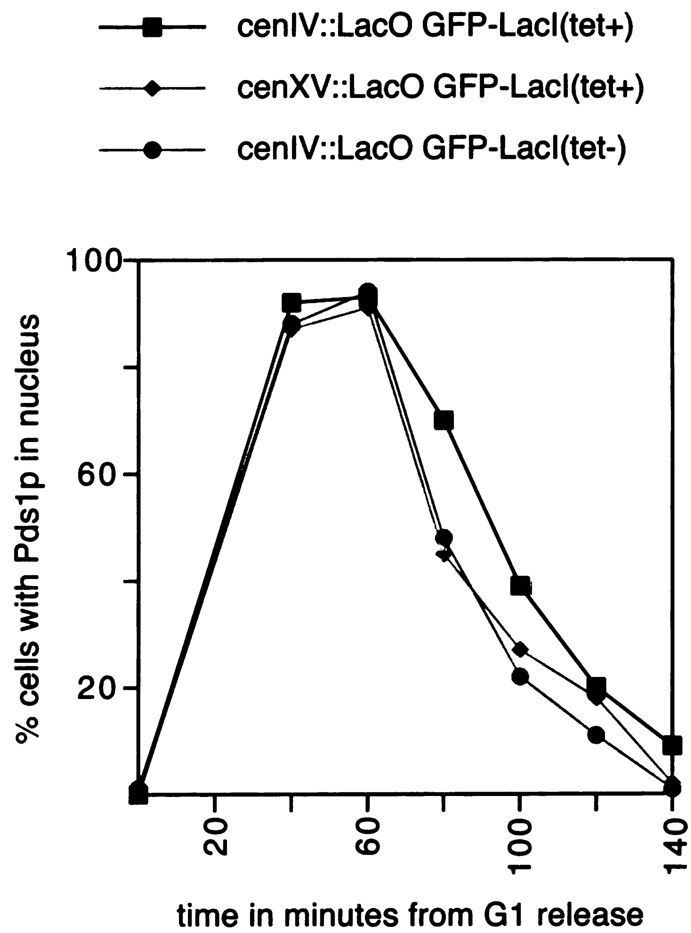
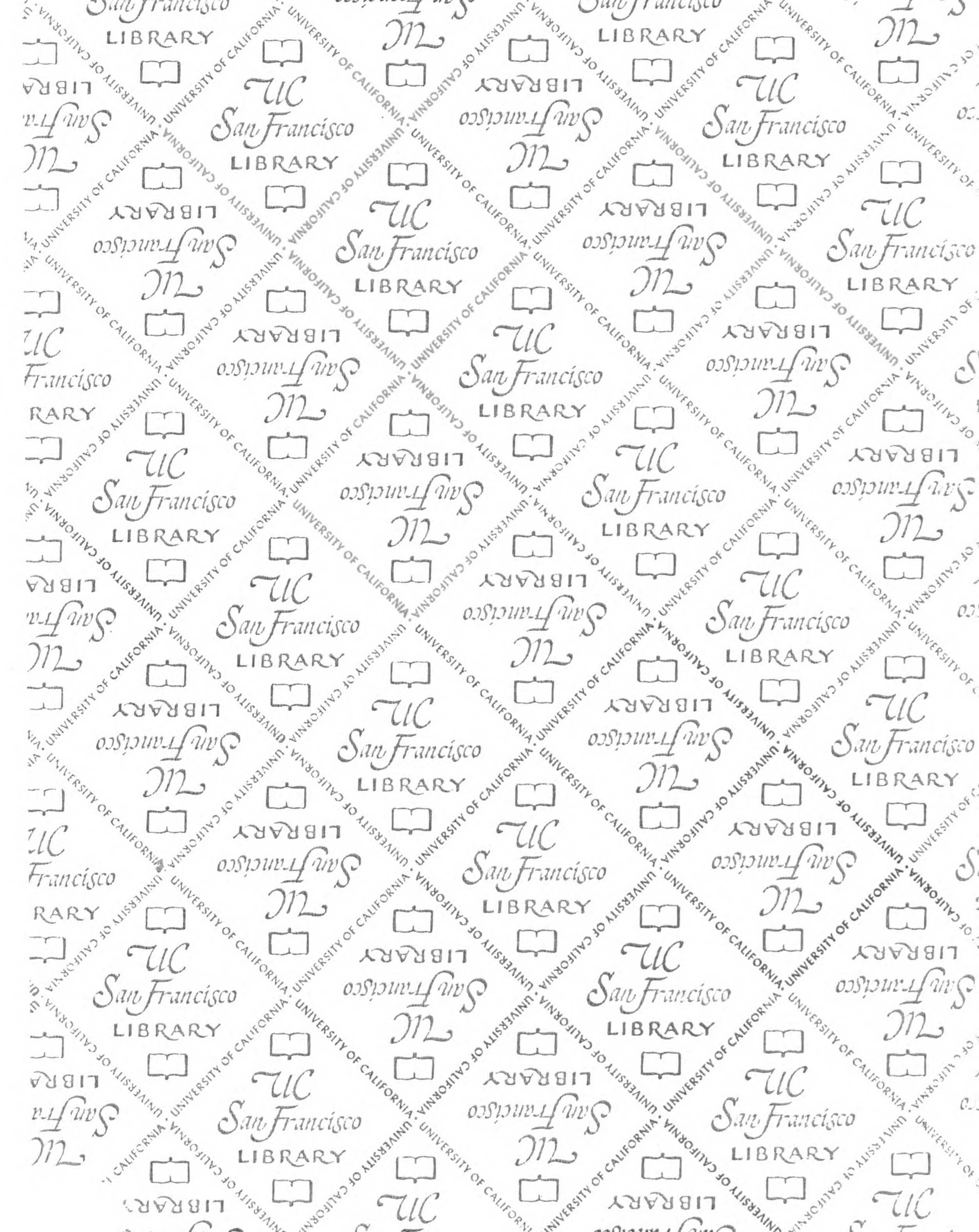

GFP-LacI(tet-)
GFP-LacI(tet-)*mad2*Δ
GFP-LacI(tet+)
GFP-LacI(tet+)*mad2*Δ
100
60
20
% cells with Pds1p in nucleus
20
60
100
140
time in minutes from G1 release

Figure C-3. Cells with GFP-LacI(tet+) bound to a Lac operator array integrated at the centromere of Chromosome XV do not activate the spindle assembly checkpoint. GFP-LacI(tet+)cenIV::LacO (NBY656), GFP-LacI(tet+)cenXV::LacO (NBY655), and GFP-LacI(tet-)cenIV::LacO (NBY658) strains were released from α-factor arrest (t=O) and subjected to indirect immunofluorescence to visualize Pds1p-18xMyc through the cell cycle. There is no delay in Pds1p destruction in cells that have GFP-LacI(tet+) bound to a LacO array at centromere XV.


cenIV::LacO GFP-LacI(tet+)
cenXV::LacO GFP-LacI(tet+)
cenIV::LacO GFP-LacI(tet-)
100
60
20
% cells with Pds1p in nucleus
20
60
100
140
time in minutes from G1 release



For reference

Not to be taken from the room.

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