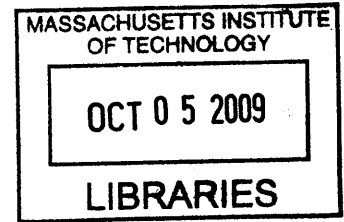# Mechanisms for maintaining genomic integrity during chromosome segregation in budding yeast

by

Ilana L. Brito

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Signature of Author:___

Department of Biology
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Certified by:_

Angelika Amon
Professor of Biology
Thesis Advisor

Accepted by:_____

Stephen P. Bell
Professor of Biology
Chairman, Committee for Graduate Students

Mechanisms for maintaining genomic integrity during chromosome segregation in budding yeast

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Submitted to the Department of Biology on July 14, 2009 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

Abstract

Maintaining genomic integrity is crucial for an organism's fitness and survival. Regulation of chromosome segregation requires complex surveillance mechanisms that vary for different loci within the genome. This thesis focuses on two complexes, monopolin (made up of Lrs4, Csm1 and Mam1) and condensin, a protein complex required for chromosome condensation, and their roles in chromosome segregation during mitosis and meiosis. During mitosis, Lrs4-Csm1 and condensin reside in the nucleolus where they regulate the maintenance and segregation of the budding yeast ribosomal DNA array, a highly repetitive and transcriptionally active locus. Here I show that Lrs4 and Csm1 bind the RENT complex at the non-transcribed space region 1 within the rDNA array and via cohesin or condensin inhibit unequal exchange between sister chromatids. This complex is released during anaphase, during which Lrs4 and Csm1 localize to kinetochores, where they play a role in mitotic chromosome segregation. Although their role in meiotic chromosome

Here we show that Lrs4 and Csm1 collaborate with condensins at kinetochores to control mitotic and meiotic chromosome segregation. During meiosis, diploid cells must first segregate homologous chromosomes before sister chromatids can separate. Lrs4-Csm1 and condensin are required during the first meiotic division to bring about the co-segregation of sister chromatids towards one pole and for the binding of monopolin subunit Mam1. In summary, I show here that condensins and Lrs4-Csm1 are required at various chromosomal locations to provide linkages between sister chromatids to promote high fidelity chromosome segregation.

Thesis Supervisor: Angelika Amon
Title: Professor of Biology

This thesis is dedicated to my mother and my grandmother.

They are the most inspiring women I have had the pleasure to have known.

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Chapter 1

Introduction

Partitioning the genome and consequences of failure

In the adult human body, there are on the order of 10^{13} (10 trillion) cells. Each time a cell goes through mitosis, the process by which somatic cells undergo division to create new replicate cells, it must coordinate the processes of DNA replication, chromosome segregation and cytokinesis, the process of cytosolic division. Given that genome integrity is essential to an organism's survival, it is crucial that during every cellular division, each resulting cell receives the correct karyotype. Errors in this process, which produce aneuploid cells defined by abnormal karyotypes, have been hypothesized to give rise to tumorigenic precursors in mammalian cells (Cahill et al., 1998; Kops et al., 2005; Torres et al., 2008).

Sexually reproducing organisms employ an additional cell division program, called meiosis, to produce gametes. Relative to mitotic chromosome segregation, errors in meiosis are more frequent, with an estimated 2% of sperm and 10-30% of ova containing more or less than the expected 23 chromosomes, resulting in aneuploid embryos in at least five percent of all clinically recognized pregnancies, and 0.3% of all births (reviewed in Hassold and Hunt, 2000). The consequences of this are staggering—Down's syndrome is the most commonly recognized outcome of aneuploid embryos, yet aneuploidy is also a leading cause of spontaneous abortion. Understanding the mechanisms that lead to errors in chromosome segregation could provide insight in preventing these afflictions.

Mitosis and bi-orientation

During mitotic cellular division, chromosomes must be faithfully replicated, collated and distributed to each pole of a cell to ensure that once the cell cytokineses, or divides, along the midzone or budneck, as in the budding yeast *S. cerevisiae*, the genome of the resulting cells will be intact. A series of checkpoints throughout the cell cycle ensures that a step is completed before the next step begins. My thesis research focuses on the mechanisms of chromosome segregation in both mitosis and meiosis. During mitosis, duplicated chromosomes (sister chromatids) must attach to the mitotic spindle in such a way that ensures equational segregation (Figure 1A). Each sister chromatid accommodates a protein-structure called the kinetochore which directionally links centromeric DNA to the mitotic spindle (Figure 1B). Each pair of sister chromatid kinetochores must be bi-oriented towards opposite poles to ensure that when the checkpoint is satisfied, chromosomes segregate accordingly. During meiosis, a single round of DNA synthesis is followed by two rounds of nuclear division. During the first meiotic division, homologous pairs of chromosomes segregate away from each other, requiring that each pair of sisters co-segregate towards one pole; their kinetochores are said to be 'co-oriented' (Figure 1B). The second meiotic division resembles mitosis in that each pair of sister chromatids is 'bi-oriented' and is ultimately segregated equationally (Figure 1B). Further explanations of how bi-orientation and co-orientation come about will be discussed in the following sections.
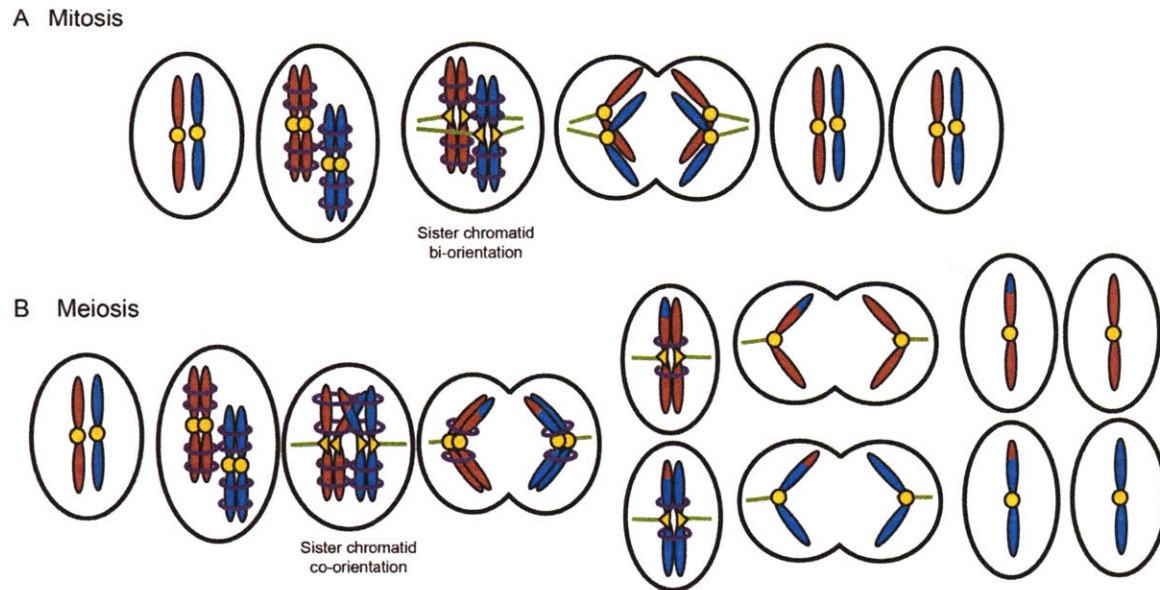
Figure 1. Mitosis and Meiosis.

(A) During mitosis, a diploid cell undergoes DNA replication and cellular division to produce two identical daughter cells. Shown in red and blue is a pair of homologous chromosomes. The kinetochores (yellow) of each pair of sister chromatids, attach to the spindle (green), and are said to be bi-oriented.

(B) During meiosis, a diploid cell undergoes one round of DNA replication and two subsequent rounds of nuclear division to produce four non-identical haploid gametes or spores. Shown in red and blue is a pair of homologous chromosomes. The kinetochores (yellow) of each pair of sister chromatids must be co-oriented in meiosis I and bi-oriented in meiosis II.

Although many of these mechanisms are conserved throughout all eukaryotes including fungi and humans, the budding yeast *S. cerevisiae* serves as a simplified model through which these concepts can be more thoroughly explored. Discrepancies between model organisms will be pointed out as they arise. This thesis focuses on the roles of the monopolin complex in segregating repetitive DNA during mitosis and how it functions to segregate homologous chromosomes during meiosis I.

A chromosome partitioning system built on tension

The processes of mitosis and meiosis require that each and every chromosome attach to the spindle. To accomplish this, sister chromatids are aligned along the metaphase plate in the center of the cell. Microtubules extend from spindle pole bodies (SPBs), the yeast equivalent of mammalian centrosomes, situated at opposite poles of the cell. They "search and capture" chromosomes at their kinetochores. Pole-ward forces exerted by the spindle on kinetochores (reviewed in Kline-Smith and Walczak, 2004) would normally drag chromosomes towards the cell's periphery, if it were not for a "glue" holding sister chromatids together preventing their premature separation. This creates a "tug-of-war" game between the two SPBs, during which surveillance mechanisms ensure that all chromosomes are part of the game, and are under tension (Figure 1). Only after this requirement is fulfilled, the cell may signal entry into anaphase. The tension is relieved and chromosomes are dragged towards opposite poles. The main components which make this possible are outlined below.

A. Cohesin

Originally, the "glue" holding sister chromatids together was thought to be either incomplete replication or catenation (tangling), between sister chromatids created by DNA replication (Murray and Szostak, 1985). Topoisomerase II, a molecule that decatenates DNA, though required for chromosome segregation, was shown not to be the "glue" between sister chromatids (DiNardo et al., 1984). Instead, studies confirmed that a tetrameric complex, called cohesin, localized to chromosomes in a cell cycle-dependent manner, and was required to hold sister chromatids together (Guacci et al., 1997; Michaelis et al., 1997). Cohesin is a highly conserved protein structure consisting of Scc3, two coiled-coil ATPase subunits (Smc1 and Smc3), and one kleisin (SMC-binding) family subunit (Scc1/Mcd1). Together, they form a ring-like structure (Gruber et al., 2003; Haering et al., 2003) that entraps sister chromatids. Cohesin is deposited onto DNA by the acetyltransferase Eco1/Ctf7 (Toth et al., 1999) at Cohesin Associated Regions (Glynn et al., 2004) and it links sister chromatids immediately post-replication. Cleavage of the cohesin subunit Scc1/Mcd1 liberates sister chromatids (Uhlmann et al., 1999; Uhlmann et al., 2000) and anaphase ensues.

B. Kinetochores

The kinetochore serves as the attachment site between the microtubule spindle and centromeric DNA. The budding yeast kinetochore is a 95MDa protein structure (larger than its ribosome and smaller than its nuclear pore complex), and although it encompasses more than eighty protein subunits (DeWulf et al., 2003; reviewed in Cheeseman and Desai, 2008), it is one of the simplest known kinetochores. It is situated on only 125 base pairs of centromeric DNA (Clarke and Carbon, 1980; Cottarel et al., 1989). The kinetochore is thus built on a single nucleosome (Furuyama and Biggins, 2007) containing the yeast centromeric histone Cse4 (Meluh et al., 1998, CENP-A homolog), and has been shown to attach to only one microtubule (Peterson and Ris, 1976; Winey et al., 1995).

The Ndc10/Cbf3 complex binds to the centromere and serves as the foundation for the kinetochore (Goh and Kilmartin, 1993; Espelin et al., 2003) to directly or indirectly recruit additional structural and regulatory complexes that modulate kinetochore-microtubule interactions (Kitamura et al., 2007). Inactivation of Ndc10 results in the abolishment of a functional kinetochore, in addition to the loss of a signaling cascade called the spindle assembly checkpoint used to halt the cell cycle in the presence of unattached kinetochores (Goh and Kilmartin, 1993; Sorger et al., 1994; He et al., 2001). Situated atop the kinetochore foundation is a layer of linker proteins that provides structural stability between the foundation and the outer-most microtubule-binding proteins (De Wulf et al., 2003).

Centromeres and kinetochores are more complex in other organisms. Fission yeast centromeres span 40-100kb containing repetitive and palindromic sequences that are not conserved across chromosomes (Steiner et al., 1993). In addition, each fission yeast chromosome makes 2-4 microtubule-kinetochore attachments (Ding et al., 1993), exacerbating the complexity of ensuring that all microtubule-kinetochore attachments are in place and oriented properly. Still, fission yeast kinetochores are relatively simpler than the kinetochores of higher eukaryotes. Human kinetochores are built upon DNA sequences which range in size and contain anywhere from 1,500 to 30,000 repeats of a 171bp sequence, which is itself neither necessary nor sufficient for centromeric function (reviewed in Karpen and Allshire, 1997). Each human chromosome

makes between 15 and 20 attachments (McEwen et al., 2003). To complicate things further, the nematode *C. elegans* and many plants have large centromeres spanning each chromosome, such that their chromosomes are said to be 'holocentric.'
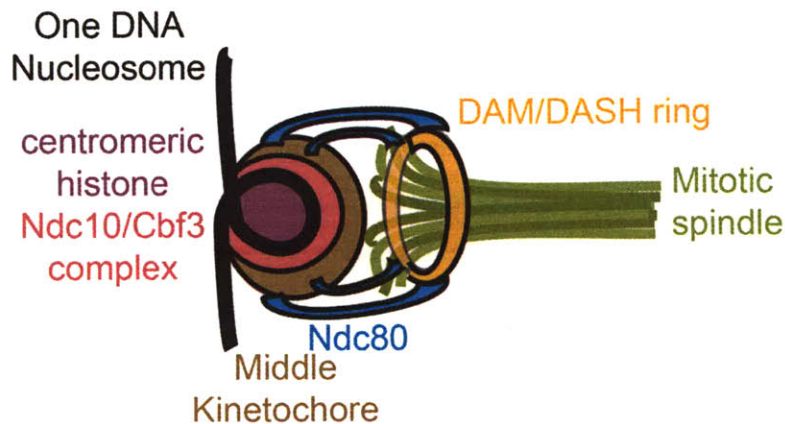
Figure 2. Kinetochore-microtubule attachments.

The centromere shown here of budding yeast encompasses a single nucleosome. The Ndc10-containing complex serves as the foundation for the kinetochore. Several linker layers are shown. The DAM/DASH complex creates a collar around a microtubule and upon microtubule fraying, is pulled towards the spindle pole body. The Ndc80 complex serves to link the kinetochore to the microfibrils created by the fraying microtubule to enhance poleward movement.

The relative structural simplicity of the budding yeast centromeric DNA and kinetochore composition when compared with higher eukaryotes makes it abundantly clear why yeast kinetochores serve as a starting point for our understanding how more complex kinetochore-microtubule interactions are coordinated. Although the various kinetochores require fundamentally different mechanisms for regulation, many components are conserved throughout many eukaryotes (reviewed in Cheeseman and Desai, 2008). Among those that are conserved are the centromeric-specific histone that binds DNA (CENP-A in humans, Cse4 in yeast; Wieland, 2004), and members of the Ndc80 complex and the Mis12-containing complex (or MIND complex), which is required for bi-orientation (PInskey et al., 2003; Kline et al., 2006).

C. Microtubule Dynamics

To generate tension on the microtubule spindle, microtubules must elicit a pole-ward force on the chromosome. Fluorescently-tagged kinetochores attached to a pair of sister chromatids will "breathe," or transiently separate as much as 0.8 microns when they are under tension (Goshima and Yanagida, 2000; He et al., 2000). Although there are several means by which microtubule dynamics affect chromosomal movement, the central mechanism stems from their depolymerization. Microtubules are dynamic, growing steadily and then quickly shrinking, but once they are captured by the kinetochore, they are stabilized (reviewed in Kline-Smith and Walczak, 2004). The DAM/DASH complex of the kinetochore multimerizes to create a collar surrounding the microtubule and serves as the interface between the kinetochore and microtubule (Westermann et al., 2006; reviewed in Tanaka and Desai, 2007; Figure 2). Fraying of the microtubule applies a pressure on this collar that, in anaphase, serves to drag the chromosome towards the SPB, the microtubule nucleation site. This is mediated by kinetochore attachments to the fraying fibrils of the microtubule by a kinetochore complex containing Ndc80 (McIntosh et al., 2008; Figure 3). During metaphase, the force generated upon the chromosomes is insufficient to overcome cohesion. Thus, tension is generated across the spindle.

D. Spindle assembly checkpoint and anaphase entry

The spindle assembly checkpoint monitors that a) all chromosomes are attached and b) that there is tension pulling sister chromatids towards opposite poles. Once these requirements are fulfilled, the key event of the metaphase-anaphase transition can occur: the cleavage of the cohesin subunit Scc1 by the protease Esp1 (Ciosk et al., 1997; Uhlmann et al., 1999). Until the spindle checkpoint is satisfied, Esp1 is held inactive by its inhibitor Pds1 (Ciosk et al., 1997; Figure 3). Upon satisfying the spindle checkpoint, metaphase proceeds. Pds1 and other cell cycle regulators, such as B-type cyclins, are degraded by ubiquitin-mediated proteolysis by the E3-ubiquitin ligase Anaphase Promoting Complex (APC) (Irniger et al., 1995; reviewed in Zachariae, 2000), and Esp1 becomes free to cleave Scc1, thereby liberating sister chromatids. Using proteolysis and cleavage to govern the metaphase-anaphase transition ensures that these steps will be irreversible.
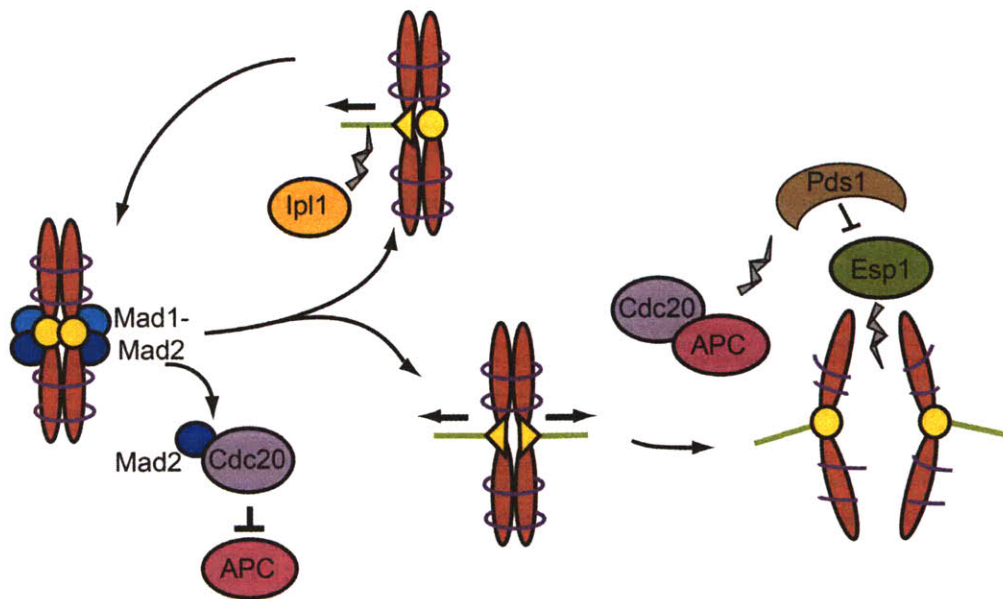
Figure 3. The spindle checkpoint and the metaphase-anaphase transition.

In the presence of an unattached kinetochore, the closed version of Mad2 binds Cdc20 and keeps it from activating the APC. In the presence of non-tensioned kinetochores, Ipl1 severs microtubule-kinetochore connections to allow them to reform. Once all of the chromosomes are attached properly, an open conformer of Mad2 liberates Cdc20, which can then bind and activate the Anaphase Promoting Complex (APC) subunits of the ubiquitin-mediated proteasome. The APC targets Pds1 for degradation liberating Esp1, a protease. Esp1 is then able to cleave the cohesin subunit Scc1 (cohesin is in purple) to relieve chromosomes of tension and allow chromosomes to separate towards opposite poles.

The spindle checkpoint is a highly conserved signaling pathway that is sensitive enough to detect a single unattached or tension-less kinetochore (Rieder et al., 1995). Although it has long been debated whether it is the unattached kinetochores or the lack of tension generated on the pair of sister chromatids that is sensed by the cell due to difficulties in teasing apart these two possibilities, it is generally accepted that both activities are at play (reviewed in Pinsky and Biggins, 2005). For example, the presence of nocodazole which depolymerizes microtubules triggers cell cycle arrest (arguing for attachment; Chen et al., 1996; Waters et al., 1998), as does the lack of a replicated sister chromatid, as in the case of *cdc6* mutants which fail to replicate their genome (arguing for tension; Stern and Murray, 2001). Mechanisms to survey merotelic

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situations, which involve different numbers of microtubule attachments per pair of sister chromatids as in fission yeast, in which weak attachments or imbalanced tension are generated by the creation of fewer attachments than necessary, are still poorly understood.

In budding yeast, the spindle assembly checkpoint is a bifurcated pathway, one branch acting to inhibit anaphase onset in the presence of unattached kinetochores and the second branch (via Bub2-Bfa1) acting to inhibit mitotic exit in the presence of a mis-positioned spindle. The first branch of the spindle checkpoint consists of five proteins which bind the kinetochore: Mad1, Mad2, Mad3, Bub1, Bub3 and Mps1 (reviewed in Pinsky and Biggins, 2005). Aside from Mps1, which is a kinase involved in spindle duplication as well as spindle checkpoint activation, the other components of the checkpoint are nonessential in the absence of spindle damage. In the presence of an unattached kinetochore, Mad2 is phosphorylated by Mps1 (Weiss and Winey, 1996) and then binds to Mad1 at the kinetochore causing a conformational change which allows it to bind and inhibit Cdc20, a metaphase-specific APC activation factor to prevent anaphase onset (Hwang et al., 1998; Mapelli et al., 2007).

To sense tension, the cell employs Ipl1, an Aurora B kinase that has multiple cell cycle roles in maintaining spindle stability rDNA compaction. Ipl1 severs microtubules attached to non-tensioned kinetochores (Tanaka et al., 2002; Dewar et al., 2004) by phosphorylating kinetochore components that most intimately interact with microtubules (Cheeseman et al., 2002). Once severed, the presence of an unattached kinetochore activates the spindle checkpoint and signaling by Mad2 and other checkpoint proteins arrest the cell cycle until new microtubule connections are made and are properly tensioned.

Genome segregation and mitotic exit

Once the genome has divided, the cell must exit mitosis, cytokinese and return to G1. The cell has an additional checkpoint to ensure that the full genome, including the rDNA which segregates in anaphase, has segregated equationally along the proper cell polarity axis (the mother-bud axis in yeast). The Clb-CDKs, which govern progression through the cell cycle, are partially degraded at the metaphase-anaphase transition by the APC, but during mitotic exit, all

mitotic CDK activity is abolished (reviewed in Murray, 2004). Cdc14, a protein phosphatase, is integral to this process: Cdc14 dephosphorylates Cdh1, the anaphase-specific APC activation factor, to activate the destruction of mitotic cyclins by the proteolytic APC (Visintin et al., 1998), marking the end of the cell cycle.

Normally, Cdc14 is sequestered in the nucleolus by Cfi1/Net1, its inhibitor (Shou et al., 1999; Visintin et al., 1999). During anaphase, the FEAR (Cdc Fourteen Early Anaphase Release) network and the Mitotic Exit Network (MEN) promote the phosphorylation of Cfi1 by Clb-CDKs which causes the partial and complete release of Cdc14 from the nucleus throughout the cell, respectively (Azzam et al., 2004; reviewed in Stegmeier and Amon, 2004). The FEAR network, which is triggered by the proteolysis of Pds1, the inhibitor of Esp1, is a non-essential signaling pathway which promotes the reliable segregation of the ribosomal DNA array, spindle midzone assembly, and stabilizing microtubules during anaphase (reviewed in D'Amours and Amon, 2004).

Since FEAR-dependent Cdc14 release is insufficient to promote exit from mitosis, the Mitotic Exit Network is required for the full release Cdc14 from the nucleolus (Shou et al., 1999; Visintin et al., 1999; Stegmeier et al., 2002; Azzam et al., 2004). The Mitotic Exit Network is a Ras-like GTP-signaling cascade that is controlled by the positioning of the mitotic spindle. The GTPase Tem1 resides at the top of this pathway is negatively controlled by the two-component GTPase-activating protein Bub2-Bfa1 which is bound to the spindle pole body (Stegmeier and Amon, 2004). Since Lte1, the putative guanine nucleotide exchange factor, localizes primarily to the bud (Seshan et al., 2002), once the Tem1-bearing spindle pole body enters the bud, the Mitotic Exit Network becomes activated (Bardin et al., 2000; Pereira et al., 2000). The activated Tem1 propagates a signal to two downstream kinases, Cdc15 and Dbf2-Mob1, resulting in the release of Cdc14 from the nucleolus (Frenz et al., 2000; Lee et al., 2001; Mah et al., 2001; Visintin and Amon, 2001). MEN mutants arrest during late anaphase with large buds and divided nuclei (Jaspersen et al., 1998; reviewed in Bardin and Amon, 2001). Although Cdc14 is conserved in higher eukaryotes, it does not by itself play as significant a role in mitotic exit as it does in budding yeast.

Meiosis I – Establishing tension across homologous chromosomes

Meiosis, the process of generating haploid gametes in sexually reproducing organisms, requires that diploid cells undergo one round of DNA synthesis followed by two rounds of chromosome segregation (Figure 1). During meiosis I, homologous pairs of chromosomes are segregated and subsequently during meosis II, sister chromatids are segregated in a similar fashion to mitosis. To ensure the correct distribution of chromosomes, the cell employs the same principles as in mitosis to each successive nuclear division: a) link each group of chromosomes that need to be separated; b) attach chromosomes that need to segregate to opposite poles; c) generate force by the spindle to elicit tension. The means by which cells are able to undergo two successive rounds of replication and segregate homologous chromosomes during meiosis I distinguish meiosis as a specialized cell division (reviewed in Marston and Amon, 2004).

Since homologous chromosomes must segregate first, it is the homologous chromosomes that must be linked. While replication-mediated attachment (cohesin) of sister chromosomes still occurs, homologous chromosomes are paired up (by a currently unknown mechanism) and physically linked by chiasmata initiated by Spo11, an endonuclease that catalyzes the formation of double strand breaks which are used to initiate recombination events (Figure 4). Cohesins must therefore be selectively removed from chromosome arms distal to crossovers to allow for homolog separation, while being preferentially maintained surrounding kinetochores so that tension can be created on the meiosis II spindle (Klein et al., 1999; Buonomo et al., 2000). To accomplish this, shugoshin, or Sgo1, protects centromeric cohesin, which contains Rec8 in meiosis rather than Scc1, at the kinetochore (Klein et al., 1999; Kitajima et al., 2004; Rabitsch et al., 2004; Kiburz et al., 2005; Klein et al., 1999). Once homologs are linked, a process that occurs during prophase, each pair of sister chromatids must make attachments towards only one pole.
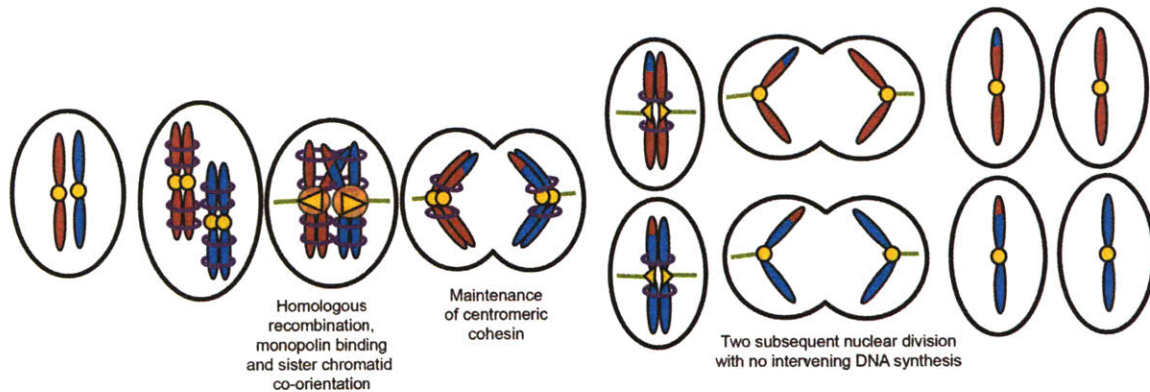
Figure 4. Meiotic modifications to the mitotic cell cycle.

During meiosis, homologous chromosomes (in red and blue) must recombine to create chiasmata to physically link homologous chromosomes. Rec8 (purple), the meiotic paralog of Scc1,-containing cohesin complexes encircle the chromosomes initially during meiosis, but must be selectively lost from the arms of chromosomes to allow homologous chromosomes to segregation and retained at the centromere to allow for tension to be created along the meiosis II spindle. Kinetochores must be co-oriented, with the help of the monopolin complex (orange) during meiosis I, and bi-oriented during meiosis II, to allow for reductional and equational segregation, respectively. Meiosis proceeds with two subsequent nuclear divisions with no intervening DNA synthesis.

Achieving Co-orientation During Meiosis I

Homologous chromosomes need to attach to the meiotic I spindle in such a way to generate tension and allow sister chromatids to co-segregate (Figure 4). Kinetochores, which attach to opposite poles during mitosis, must now be "co-oriented" towards one pole. It is worth noting that Ipl1 is operational during meiosis I and acts at the level of ensuring tension between homologous chromosomes rather than sister chromatids as in mitosis (Monje-Casas et al., 2007). Accordingly, it appears that the canonical spindle checkpoint is also active (Lacefield and Murray, 2007; McGuiness et al., 2009). Several models can explain how co-orientation comes about (Figure 5) (reviewed in Hauf and Watanabe, 2004): both kinetochores may be held in concert so they face one pole creating what are called "syntelic" attachments; one kinetochore may be masked or sterically hindered from binding to a microtubule; the kinetochores may be fused into one kinetochore binding site; or any combination thereof.
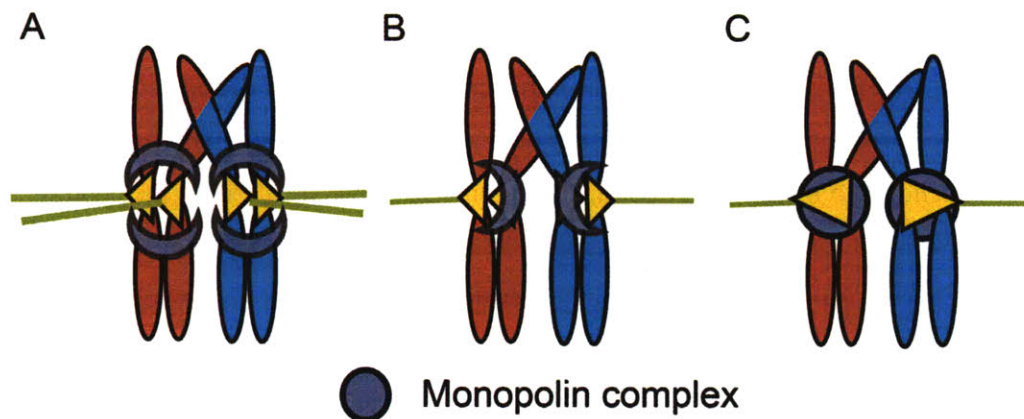
Figure 5. Models for co-orientation.

(A) Both kinetochores attach to one pole via microtubules.
(B) One kinetochore sterically masks a microtubule binding site.
(C) Fusion of the two kinetochore-microtubule attachment sites into one.

At least in budding yeast, we can rule out syntelly (Figure 6A) as the mechanism for co-orientation. Electron microscopy experiments found that each pair of sister chromatids makes only one microtubule attachment (Winey et al., 2005). In higher organisms, the evidence appears mixed: pig oocytes sister kinetochores are held adjacent to one another but still appear to each make microtubule attachments (Lee et al., 2000); *D. melanogaster* (fruit fly) sister kinetochores appear to fuse but it remains unclear whether the fused kinetochore makes one or two attachments (Goldstein et al., 1981). Evidence from mouse spermatocytes is inconclusive in metaphase but suggests that sister kinetochores are able to make two monopolar attachments during anaphase (Parra et al., 2004). Each of these organisms is burdened by the complexity of organizing not only one but multiple kinetochores per sister chromatid.

There is some evidence that sister kinetochores are oriented in such a way that precludes capture by microtubules emanating from opposite poles. One piece of evidence comes from the observation that recombination events enhance meiosis I chromosome segregation fidelity (Yamamoto and Hiraoka, 2003; Lamb et al., 1996), though are by no means necessary (Toth et al., 2000; Monje-Casas et al., 2007; Klein et al., 1999; Kitajima et al., 2003). Chiasmata between homologous chromosomes may aid in orienting kinetochore structures towards opposite poles or preclude microtubule-kinetochore attachments from one kinetochore.

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In many eukaryotes, cohesins play a role in meiosis I orientation. In *Schizosaccharomyces pombe*, fission yeast, cohesin is excluded from the core centromere during mitosis and primarily localizes to the peri-centromeric regions (Tomonaga et al., 2000), presumably allowing flexibility for kinetochores to bi-orient. By contrast, fission yeast Rec8 localizes to the central kinetochore and is essential for meiotic segregation (Watanabe et al., 2001; Sakuno et al., 2009). In addition to fission yeast, similar requirements for cohesin during meiosis I have been observed in plants, including maize and *Arabidopsis thaliana* (Yu and Dawe, 2000; Chelysheva et al., 2005). Although sister chromatid cohesion is mediated by the cohesin complex in many eukaryotes whose centromeres encompass large swaths of regional heterochromatin, point centromeres in budding yeast rely on cohensin-independent mechanisms to co-segregate sister chromatids during meiosis I (Toth et al., 2000; Monje-Casas et al., 2007).

The monopolin complex

In budding yeast, the complex responsible for the co-segregation of sister chromatids is called the monopolin complex. The monopolin complex is composed of four proteins— Mam1 (monopolar attachment in meiosis 1); Lrs4 (loss of rDNA silencing 4), Csm1 (chromosome segregation in meiosis 1), and Hrr25 (homologous recombination repair 25) (Toth et al., 2000; Rabitsch et al., 2003; Petronczki et al., 2006). Localizing to kinetochores throughout prophase and metaphase I, this complex presumably links sister kinetochores, even in the absence of centromeric cohesion (Toth et al., 2000; Monje-Casas et al., 2007). This complex is largely the focus of my thesis.

A. Identification of the monopolin complex

Mam1 was originally identified in a screen for proteins which were specifically expressed at high levels during the meiotic divisions, preferably meiosis I (Rabitsch et al., 2001), since the hypothesis was that the factor responsible for co-orientation would have this profile. Mutations of a subset of these genes resulted in chromosome mis-segregation during meiosis; Csm1 was also detected using this screen. It was discovered that sister chromatids disjoined in *mam1*Δ mutants (Toth et al., 2000). This approach was fruitful, but initially failed to identify two

other co-orientation factors that were expressed during mitosis and early meiosis: Lrs4 and Csm1.

A screen for mutants that allowed the survival of *spo11Δ spo12Δ* double mutants identified Lrs4 and Csm1 (Rabitsch et al., 2003), two coiled-coil proteins. *spo11Δ* diploids normally produce inviable aneuploid spores due to severely reduced homologous recombination (CITATION). *spo12Δ* diploids undergo only one round of meiotic meiotic chromosome segregation resulting in dyads of variable viability (Klapholz and Esposito, 1980). Although *spo11Δ spo12Δ* diploids produce inviable spores, it was thought that the additional removal of co-orientation factors would allow sister chromatids to come under tension and be segregated equationally, permitting meiosis II to occur on the meiosis I spindle. The deletion of *LRS4* and *CSM1* in this background restores viability of these dyads (Rabitsch et al., 2003). Interestingly, Lrs4 was originally noted for its nucleolar role in rDNA segregation, silencing and maintenance (Smith et al., 1999), a function shared by Csm1 (Rabitsch et al., 2003).

The fourth member of the monopolin complex, Hrr25, a casein kinase, plays a role in numerous cellular functions including vesicular trafficking, DNA repair, RNA transcription and ribosome biogenesis (Knippschild, 2005; Ray et al., 2008). Therefore, Hrr25 was not originally identified in a screen for monopolin function. Instead, tandem affinity purification identified it as a Mam1 binding partner, and an allele of Hrr25 that could no longer bind kinetochores was identified (Petronczki et al., 2006). Hrr25 is known to phosphorylate Rec8 and Mam1, but interestingly, Hrr25 kinase activity is not required for monopolin localization to kinetochores or for Hrr25-Mam1 binding, but it is indispensable for co-orientation, suggesting that Hrr25 targets kinetochore proteins for phosphorylation to promote the co-orientation of sister kinetochores (Petronczki et al. 2006).

B. Regulation of the monopolin complex

While Lrs4, Csm1 and Hrr25 are expressed in both mitotic and meiotic cells, their expression appears to increase during meiosis I and II (Chu et al., 1998; Petronckzi et al., 2006; Matos et al., 2008). Transcription of Mam1 appears to be regulated by Ndt80, a prophase-specific

transcription factor: there are two Ndt80 consensus sites upstream of Mam1 and ectopically expressed Ndt80 is able to promote Mam1 transcription (Chu et al., 1998), although this is not its only transcriptional activator (Lo et al., 2008). During pachytene, Dbf4 and Cdc7 kinase, two proteins most noted for their role in firing early origins during DNA replication, also play an indirect role in monopolin expression, via Ndt80 (Lo et al., 2008; Xu et al., 1998), although Mam1 is still up-regulated, albeit not to normal meiotic levels, when Cdc7 is inhibited (Lo et al., 2008). Further inquiry into the regulation of monopolin expression is necessary.

Monopolin loading onto kinetochores requires additional regulation (Figure 6). Cdc7-Dbf4 also act on Mam1 localization post-translationally (Matos et al., 2008) in collaboration with the polo-like kinase Cdc5 to localize the monopolin complex to kinetochores (Clyne et al., 2003; Lee and Amon, 2003). This is most likely due to the role of Cdc5 in hyper-phosphorylating Lrs4 (Matos et al., 2008; Monje-Casas et al., 2007) because the kinetochore localization of Mam1 requires that of Lrs4 and Csm1 (Rabitsch et al., 2003). Maintenance of Mam1 localization at kinetochores requires Spo13 (Lee et al., 2004; Katis et al., 2004), a meiosis-specific factor necessary to undergo two rounds of successive reductional and equational division (Klapholz and Esposito, 1980), suggesting that Spo13, which is exclusively present during meiosis I, ensures that monopolin localizes to kinetochores at this time.
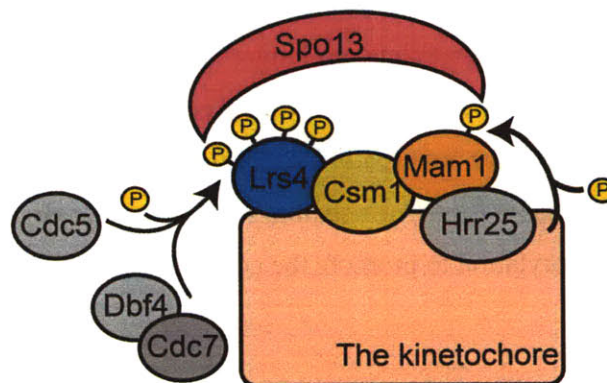
Figure 6. How monopolin localizes to kinetochores

Lrs4 binds Csm1 via the coiled-coil sections at its N-terminus. Csm1 subsequently binds Mam1. Hrr25 also binds Mam1 via its kinase domain. Lrs4 and Csm1 putatively bind kinetochore. To facilitate their localization to kinetochores, Cdc7-Dbf4 and Cdc5, with the help of Spo13, phosphorylate Lrs4. Hrr25 phosphorylates Mam1. Spo13 helps maintain Mam1 at kinetochores. All kinases are colored gray.

Neither the structure of the monopolin complex nor how it associates with kinetochores is known. Yeast-two-hybrid experiments have identified putative binding Lrs4-Csm1 partners: Dsn1, a member of the MIND kinetochore complex, and Ctf19, a member of the COMA complex, which both serve as linkers between the core and outer kinetochore complexes (Wong et al., 2007). More rigorous experiments are needed to understand how robust and how direct these interactions are. None of the monopolin components can localize to a large degree to the kinetochores alone (Rabitsch et al., 2003). Deletion analysis suggests that Lrs4 and Csm1 bind via the N-terminus of their coiled-coil regions, while Csm1 binds to Mam1 in extracts, with detectable higher-ordered interactions detections of the full complex, suggesting multimerization *in vivo* (Rabitsch et al., 2003). Hrr25 has been shown to co-immuno-precipitate Mam1 (Petronczki et al., 2006), but it is unknown whether Lrs4 and Csm1 are required to mediate this attachment. A comprehensive picture of how the monopolin complex binds kinetochores and/or the peri-centromeric region is still needed to fully understand their function

C. Function of the monopolin complex

Current data best support the "masking" and "fusing" or "clamping" models for monopolin linkage of sister chromatid kinetochores during meiosis I. First, when monopolin is over-expressed in vegetative cells arrested in nocodazole, a microtubule depolymerizing drug that normally triggers spindle checkpoint activation, approximately 35% of cells are able to escape this arrest despite normal Ipl1 activity (Monje-Casas et al., 2007). This finding would suggest that the unattached kinetochore is no longer sensed due to masking or fusing. Second, the monopolin complex holds sister kinetochores together and co-segregates pairs of sister kinetochores in the absence of cohesin, something that is not seen in temperature sensitive mutants of Ipl1 or cohesin mutants (Monje-Casas et al., 2007). This cohesin-independent linking ability that is able to subvert the spindle checkpoint, supports a "clamping" or "fusing" model for monopolin function.

Is there an equivalent monopolin complex in other organisms? Although the casein kinase Hrr25 is conserved by sequence across kingdoms, other components of the monopolin complex have only been identified in yeast species. Several yeast species from different genera encode Csm1-

like proteins. The *Schizosaccharomyces pombe* Pcs1 protein bears 22% sequence similarity with Csm1 (Rabitsch et al., 2003). Pcs1 binds Mde4, which shares some secondary structure similarities with Lrs4 (Gregan et al., 2007). Interestingly, these proteins exclusively function during mitotic chromosome segregation to promote amphitelic attachments (Rabitsch et al., 2003; Gregan et al., 2007). Due to the multiple spindle attachments made by each sister chromatid in *S. pombe*, we can loosely say that Pcs1-Mde4 "co-orient" the microtubule attachment sites of one sister chromatid in *S. pombe*.

Sequence alignment has not identified any Lrs4, Csm1 or Mam1 homologs, yet functional orthologs may still exist. A genetic screen, similar to that used to find Lrs4/Csm1, identified the *S. pombe* protein Moa1 (Yokobayashi and Watanabe, 2005). Moa1 has similar expression and localization patterns to *S. cerevisiae* Mam1: it is expressed from prophase to metaphase I and it localizes to kinetochores. The central difference between co-orientation within these two yeast species is that Moa1 has the essential role of recruiting Rec8 to core centromeric regions, to establishes syntelic (co-oriented) attachments, whereas *S. cerevisiae* Mam1 performs this function alone (Yokobayashi and Watanabe, 2005; Sakuno et al., 2008; Figure 8). Further understanding the similarities and differences between these two species may reveal how co-orientation can arise.
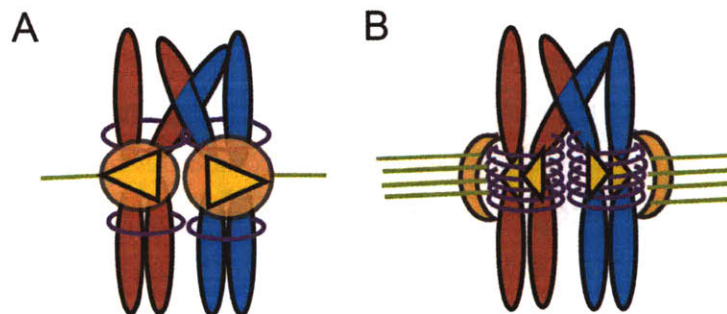
Figure 7. Co-orientation in *S. cerevisiae* and in *S. pombe*.

(A) The monopolin complex (orange) in *S. cerevisiae* fuses sister chromatids together to allow for one attachment per pair of sisters. Cohesin (purple) is present in the peri-centromeric regions (and at the arm regions, not shown) and does not contribute to co-orientation.
(B) Cohesin (purple) is primarily used to link sister chromatids during meiosis I in *S. pombe*. It is also present at peri-centromeric and arm regions, not shown) Moa1 (orange) recruits Rec8 to kinetochores and is required for syntelic (multiple co-oriented) attachments.

The rDNA array

Although chromosome segregation is largely explained by kinetochore-microtubule interactions and surveillance thereof, the rDNA locus poses an additional problem in chromosome segregation that requires additional mechanisms to maintain its integrity. The rDNA locus is an array of ~150 tandem 9.1kb repeats on one chromosome in budding yeast (and up to 10,000 repeats situated on 5 chromosomes in humans) that each encode the 35S and 5S precursor ribosomal RNAs (transcribed by RNA Polymerase I and III, respectively) (Figure 8). The rDNA is situated on chromosome 12, making it the largest chromosome in budding yeast, of up to 2Mb. Although this array makes up only 9 percent of the yeast genome, its transcription encompasses roughly 60 percent of the ongoing transcription within the cell (Woolford and Warner, 1991) in order to meet the cell's demand for ribosomes. This highly specialized segment of DNA is cordoned off into its own subnuclear compartment, the nucleolus, the site of ribosomal RNA transcription, processing and ribosome assembly (reviewed in Nomura, 2001). Since this region is highly repetitive and transcriptionally active throughout the cell cycle, including mitosis, it requires special coordination of chromosome structure, replication, recombination, and transcription, which has evolved to require fundamentally different segregation mechanisms.
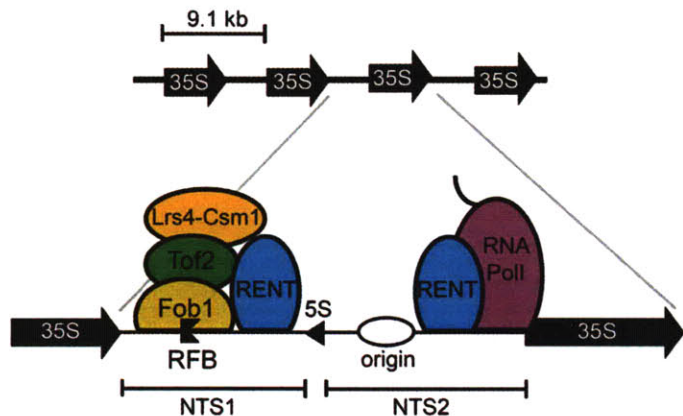
Figure 8. The rDNA array. The rDNA array is situated on chromosome 12.

Each repeat contains two transcribed genes—the 35S rRNA, transcribed by RNA Polymerase I is processed within the nucleus into the 25S, 18S and 5.8S rRNAs. The 5S rRNA is transcribed by RNA Polymerase III. Non-transcribed Spacer Region 1 (NTS1) contains the replication fork block on which Fob1 is situated, and subsequently Tof2, Lrs4-Csm1. Sir2 associates with both NTS1 and NTS2. The Non-Transcribed Spacer Region 2 contains the origin of replication.

Maintaining rDNA integrity using the Non-Transcribed Spacer region 1

The highly recombinogenic nature of the rDNA locus is a liability for the cell because gross expansion or contraction of the rDNA array can lead to a reduction in fitness. The creation of extra-chromosomal rDNA circles which arise from recombination events has been associated with premature cellular senescence (Sinclair and Guarente, 1997). Recombination must be prevented between rDNA repeats on the same chromatid and also between a pair of sister chromatids. Interestingly, recombination at the rDNA locus is lower than expected for such a highly repetitive sequence (Petes, 1980) because regulators of rDNA recombination, rDNA replication, rRNA transcription and rDNA segregation all reside at non-transcribed spacer region 1 (NTS1) within each rDNA repeat.

The RENT (regulator of nucleolar silencing and telophase exit) complex, which includes Sir2, Cdc14 and its inhibitor Cfi1/Net1, regulates recombination and silencing of Polymerase II transcripts within the rDNA. Although Cdc14 is not known to be active in the nucleolus, one role of Cfi1/Net1 is to recruit Sir2 (Straight et al., 1999). Sir2, an NAD+-dependent deacetylase, is known for regulating recombination and gene silencing at the rDNA as well as other repetitive loci including telomeres (Gottlieb and Esposito, 1989; Bryk et al. 1997; Fritze et al., 1997; Smith and Boeke 1997). Sir2 deacetylates the N-terminus of histone tails, suggesting that chromatin structure antagonizes recombination and transcription at this locus by hindering recombination machinery's access to the DNA (Imai et al., 2000).

Sir2 is also required to suppress recombination at the replication fork barrier (RFB) site within NTS1 (Kaeberein et al., 1999; Benguria et al., 2003). Fob1, a protein which binds the RFB ensures that rDNA replication is uni-directional, thereby preventing interference between transcription and replication machinery (Kobayashi et al., 1998). Collisions between replication forks and transcription machinery are thought to trigger recombination events (Takeuchi et al., 2003). Thus, the loss of Fob1 results in general instability of the number of rDNA repeats (Kobayashi et al., 1998; Kobayashi et al., 2000). It remains to be seen whether all rDNA recombination events between sister chromatids occur through Fob1-dependent intermediates.

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Other components are involved in rDNA silencing as well. The monopolin component Lrs4 was originally identified in a screen for mutants that showed decreases in rDNA silencing (Smith et al., 1999) and Csm1 was shown to have similar effects (Rabitsch et al., 2003), a function that seems distant from their function at the kinetochore during meiosis. RNA polymerase I (Rpa135) seems to be intimately involved in rDNA copy number stability (Kobayashi et al, 1998; Buck et al., 2002; Cioci et al, 2003), where it seems to interact with Sir2 (Huang and Moazed, 2003). Topoisomerase I and II, both of which affect DNA structure, supercoiling and heterochromatin at the rDNA, are both required for rDNA stability (Christman et al., 1988, 1993; Cavalli et al., 1996). Additional factors may still be identified, but clarification of how these proteins are situated, interact and function at the rDNA must be the next step to understanding how rDNA stability is achieved.

An rDNA segregation system built on compaction

As mentioned earlier, the rDNA array requires special mechanisms for segregation that are coordinated with rDNA replication, rRNA transcription, and rDNA maintenance. To do so, it segregates via a completely different system than the kinetochore-tension paradigm of mitosis and on a different schedule. Segregation of the rDNA occurs after the rest of the genome has segregated. Interestingly, ectopic cleavage of the cohesin subunits Scc1, though sufficient to allow sister chromatid segregation in mitosis, alone is insufficient to segregate the rDNA locus (Sullivan et al., 2004). In addition, microtubules are not required for the segregation of rDNA as it occurs normally in cells treated with nocodazole during anaphase (Machin, 2005). Therefore, the steps required to faithfully segregate the rDNA locus are: detanglement, condensation, and a mechanism to regulate rDNA segregation on a later timescale than the remainder of the genome.

A. Detanglement

Catenation that occurs between sister chromatids within the rDNA due to concurrent passage of the DNA machinery and transcription machinery cannot be resolved by chromosome segregation alone and requires the evolutionarily conserved decatenation enzymes topoisomerase I and II (reviewed in Wang, 2002). Specifically, sister chromatids can become intertwined due to the

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convergence of two replication forks during DNA replication, which can subsequently result in chromosomal breakage in the absence of detanglement during chromosome segregation (Sundin and Varshavsky, 1981; Spell and Holms, 1994). Topoisomerase I and II have the ability to detangle DNA by breaking single or double-stranded DNA, respectively, and, via a covalent intermediate, passing through a second strand of DNA (reviewed in Wang, 2002). Although topoisomerases are required for segregation of the full genome in *S. pombe* (Uemura et al., 1987), *S. cerevisiae*, whose chromosomes are an order of magnitude shorter, requires topoisomerase activity primarily to resolve catenation in the rDNA (D'Ambrosio et al., 2008).

B. Condensation

Chromosomal compaction, especially for the largest chromosomes, is required to prevent breakage during cytokinesis. The pentameric condensin complex was originally found in *Xenopus* oocytes for its ability to compact DNA (Hirano and Mitchison, 1994; Hirano et al., 1997) and later found in *S. pombe* because condensin mutants cytokinesed with unsegregated nuclei resulting in a *cut* phenotype (Hirano et al., 1986; Saka et al., 1994; Sutani et al., 1999). The large chromosomes of fission yeast and other higher eukaryotes undergo massive condensation during mitosis from 4-fold to up to 50-fold during mitosis, whereas the rDNA array is the only locus that compacts, albeit to a much lesser degree (around 2-fold) in *S. cerevisiae* (Guacci et al., 1994).

The condensin complex is a highly conserved complex containing: two coiled-coil ATPase subunits, Smc2 and Smc4 (Freeman et al., 2000); and a globular head made up of Ycs4 (Bhalla et al., 2002), Ycg1 and an uncleavable kleisin subunit Brn1 (Ouspenski et al., 2001). Although condensins localize to other chromosomal locations (including enrichment at centromeres throughout the cell cycle), during anaphase, *S. cerevisiae* condensins become enriched in the nucleolus where they compact the rDNA (Nakazawa et al., 2008; D'Ambrosio et al., 2008; D'Amours et al., 2004). Condensins require a hierarchy of Fob1, Tof2 and Lrs4-Csm1 for binding at the NTS1 region within the rDNA repeats (Waples et al., 2009; Johzuka and Horiuchi, 2009).

Condensin resembles cohesin, in that both complexes form rings. Through ATP binding and hydrolysis, condensin rings are able to entrap double stranded DNA (reviewed in Uhlmann and Hopfner, 2006). Condensin appears to bind distant DNA sequences into chromatin loop formations *in vitro* and provides a super-structure to DNA *in vivo* that partially remains as loops of 30nm fibre chromatin during high salt extractions (Hudson et al., 2003; reviewed in Hirano, 2006). At the rDNA, these loops facilitate chromosomal compaction, but condensin-mediated chromosomal compaction at centromeres is thought to provides structural stiffness for chromosomes to resist cytoskeletal and microtubule pulling forces (Stear and Roth, 2002; Ribierio et al., 2009; Oliviera et al., 2005).

In all eukaryotes, the process of decatenating DNA is intimately linked with the process of chromosomal compaction; the decompaction process is akin to detangling two threads, where each must be wound around a spool to prevent further entanglement. Topoisomerase is required for initial compaction of DNA in *Xenopus* oocytes, but not for the maintenance of condensed DNA, despite the reversibility of this process (Hirano and Mitchison, 1993; Lavoie et al., 2002). While condensins are required to recruit topoisomerase II, over-expression of topoisomerase is insufficient to segregate the rDNA in the absence of condensin (Adachi et al., 1001; Coelho et al., 2003). Since condensins mediate rDNA segregation after chromosome segregation has largely finished, we can imagine a model for rDNA segregation whereby condensin creates a lateral "pulling force" via compaction on the rDNA towards the kinetochore which is at the cellular periphery.

Condensin complexes have other functions outside of modulating chromosome architecture. In budding yeast, during meiosis, the condensin complex helps resolve recombination intermediates between homologous chromosomes and in assembling protein structures that facilitate this process (Yu and Koshland, 2003). Although most higher eukaryotes have two condensin complexes that each condense chromosomes during different points in the cell cycle (Hagstrom et al., 2002; Hirota 2004), a third condensin complex in *C. elegans* is involved in dosage compensation of the sex chromosomes (Chuang et al., 1994).

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C. Regulating rDNA segregation in late anaphase

rDNA segregation occurs during late anaphase, after cohesin has been removed from the chromosomes and the rest of the genome has segregated. In budding yeast, the FEAR pathway is essential for late segregation of the rDNA array (D'Amours et al., 2004; Sullivan et al., 2004). FEAR pathway mutants display rDNA segregation defects resulting in rDNA bridges, similar to condensin and topoisomerase II mutants. As a result, it was found that Cdc5, the polo-like kinase that is a member of the FEAR pathway, is required for condensin localization (St-Pierre et al., 2009). A second better conserved pathway to regulate condensin localization and subsequent rDNA segregation is via the Aurora kinase Ipl1 (Lavoie et al., 2004; Giet and Glover, 2001; Petersen et al., 2001). Interestingly, in fission yeast and higher eukaryotes, condensin localization to the rDNA is controlled by cyclin-dependent kinases (Kimura et al., 1998; Sutani et al., 1999), although in *S. cerevisiae* this is not directly the case. Decatenation, condensation and regulation of late segregation of the rDNA are intimately linked processes mediated by condensins, topoisomerases and Cdc14, to ensure that rDNA segregates without chromosomal damage.

The monopolin complex's many functions

The monopolin complex in *S. cerevisiae* and its homologs in *S. pombe* cover a wide range of functions whose mechanisms are not understood. Their role in "clamping" the kinetochores of sister chromatids during *S. cerevisiae* meiosis I and of a single sister chromatid during *S. pombe* mitosis, is not yet mechanistically linked to its role within the nucleolus. It is not clear whether the "clamping" function of Lrs4-Csm1 apparent at kinetochores uses the same protein domains as those required for binding RENT complex components, Tof2, Fob1 and condensin components. Additionally, *S. pombe* monopolin homologs Pcs4-Mde1 have recently been shown to localize to the mitotic spindle during anaphase, where they stabilize microtubules and promote microtubule elongation (Choi et al., 2009). Lrs4-Csm1 has not been observed at microtubules during mitosis nor meiosis. Further studies are required to parse out the mechanisms underlying each of the functions of monopolins in both *S. cerevisiae* and *S. pombe*. Whether this protein complex functions similarly at the rDNA locus and at kinetochores is the central question of this

thesis. Using various methods to identify binding partners and regulatory proteins, I have arrived at a preliminary model for tying together their role at the rDNA and kinetochores via their association with the condensin complex.

Conclusion

In general, chromosomes rely on tension created by kinetochore-microtubule attachments and intact surveillance mechanisms to ensure their faithful distribution to daughter cells. The processes of segregating homologs during meiosis and segregating the rDNA require additional mechanisms to proceed accurately. During meiosis, the monopolin complex, a tetrameric complex of Lrs4, Csm1, Hrr25 and Mam1, facilitate the "fusing" or "clamping" of sister chromatid kinetochore attachment sites, so that tension can be created between homologous chromosome pairs. During anaphase, condensin mediates chromosomal compaction of the rDNA so it can be discretely separated into the two daughter cells. The mitotic monopolins, Lrs4 and Csm1, also play a role at the rDNA in mitosis in rDNA silencing. The remainder of my thesis focuses on understanding the roles of monopolins and condensins in mitosis. I have investigated how these protein complexes collaborate to bring about co-orientation of sister chromatids during meiosis.

The central question that initially guided my thesis work related to what the roles of Lrs4 and Csm1 are in mitosis. We initially noted that these mitotic monopolins were released from the nucleolus during anaphase. Further experiments showed that while in the nucleolus, they interacted with the RENT complex at the NTS1 region within each rDNA repeat. Along with experimental evidence of their ability to prevent rDNA recombination and prior existing data suggesting that Lrs4-Csm1 bind cohesin, we created a model whereby Lrs4-Csm1 hold rDNA repeats in register, preventing unequal exchange of DNA between sister chromatids. During anaphase, this complex is released by the Mitotic Exit Network, thereby allowing the rDNA to segregate.

Further analysis of Lrs4 and Csm1 upon release from the nucleolus exposed their association with kinetochores during anaphase by the Mitotic Exit Network. We found that Lrs4 and Csm1

were important for chromosome segregation fidelity and, through their association with condensin, associate with kinetochores during anaphase. During meiosis, we found that condensins, like the monopolin complex, are needed for the co-orientation of sister chromosomes during the first meiotic division. In the absence of condensin function, monopolin complex components fail to associate with kinetochores. We conclude that condensins and Lrs4-Csm1 function together to provide linkages between sister chromatids at specialized chromosomal locations. The following two chapters together create a model whereby Lrs4-Csm1 contribute to larger-order chromosomal structure in order to maintain genomic integrity in both mitosis and meiosis.

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Chapter 2

Inhibition of homologous recombination by a cohesin clamp complex recruited to the rDNA recombination enhancer

Julie Huang, Ilana L. Brito, Judit Villén, Steven P. Gygi,
Angelika Amon, and Danesh Moazed

Experiments in Figures 14 and 15 were performed by I. L. B.

Abstract

Silencing within the yeast ribosomal DNA (rDNA) repeats protects the integrity of this highly repetitive array by inhibiting hyperrecombination and repressing transcription from foreign promoters. Using affinity purification combined with highly sensitive mixture mass spectrometry, we have analyzed the protein interaction network involved in suppressing homologous recombination within the rDNA locus. We show that the Net1 and Sir2 subunits of the RENT (regulator of nucleolar silencing and telophase exit) silencing complex, and Fob1, which recruits RENT to the nontranscribed spacer I (NTS1) region of rDNA, are physically associated with Tof2. In addition to RENT components and Fob1, Tof2 copurified with a two-subunit complex composed of Lrs4 and Csm1. Tof2, Lrs4, and Csm1 are recruited to the NTS1 region by Fob1 and are specifically required for silencing at this rDNA region. Moreover, Lrs4 and Csm1 act synergistically with Sir2 to suppress unequal crossover at the rDNA and are released from the nucleolus during anaphase. Together with previous observations showing that Csm1 physically associates with cohesin, these findings suggest a possible model in which RENT, Tof2, and Lrs4/Csm1 physically clamp rDNA to the cohesin ring, thereby restricting the movement of rDNA sister chromatids relative to each other to inhibit unequal exchange.

Introduction

Eukaryotic genomes contain a wide variety of repetitive DNA, including arrays of essential genes, transposons, and retroelements. Such repetitive sequences are attractive substrates for homologous recombination events, some of which may lead to unwanted chromosomal rearrangements or repeat instability due to unequal crossover between sister chromatids. Cells have therefore evolved mechanisms that protect regions such as the ribosomal DNA (rDNA) locus, a prime example of a highly repetitious segment of the genome whose stability is absolutely critical for growth and survival.

In all eukaryotes, rDNA is organized as one or more arrays containing anywhere from <100 to >10,000 repeating units, which can form one or more nucleoli where rRNA synthesis, processing, and assembly into ribosomes occur (Nomura 2001). How the integrity of rDNA is maintained is not well understood, but has been extensively studied in the budding yeast *Saccharomyces cerevisiae*, which has 100–200 copies of rDNA tandemly repeated (Petes and Botstein 1977). However, rDNA recombination rates in budding yeast are significantly lower than would be expected for such a large, repetitive locus, indicating that recombination within the array is negatively regulated (Petes 1980). Such regulation is important for suppression of unequal recombination events that cause loss of repeats or generate extrachromosomal rDNA circles, the latter of which leads to premature cellular senescence in this organism (Sinclair and Guarente 1997).

Several lines of evidence indicate that suppression of recombination at rDNA is related to gene silencing, a form of transcriptional repression analogous to the heterochromatin found at repetitive regions in larger eukaryotes. First, rDNA recombination levels in *S. cerevisiae* are down-regulated by *SIR2* (Gottlieb and Esposito 1989), an NAD+-dependent deacetylase (Imai et al. 2000; Landry et al. 2000) originally identified for its roles in transcriptional silencing at the mating-type cassettes and at telomeres (Ivy et al. 1986; Rine and Herskowitz 1987; Gottschling et al. 1990). Furthermore, *SIR2* is also required for "rDNA silencing," the suppression of both Ty1 transposition and RNA polymerase II (Pol II)-dependent transcription within rDNA (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). These and other observations, such as the altered sensitivity of rDNA to micrococcal nuclease, *dam* methylation, and psoralen cross-linking in *sir2Δ* mutants (Fritze et al. 1997; Smith and Boeke 1997), support a model in which silencing mechanisms create an altered chromatin structure at this locus that is also refractory to recombination.

The mechanism of silencing is best understood at the mating-type cassettes and at telomeres. Silencing initiates via the association of DNA-binding proteins that recruit the SIR complex, consisting of Sir2, Sir3, and Sir4 proteins (Rusche et al. 2003), with chromatin. Sir2 likely deacetylates the N-terminal tails of histones, while Sir3 and Sir4 are structural components of silent chromatin that bind to these deacetylated tails (Hecht et al. 1995). Repeated rounds of

deacetylation and histone binding result in stepwise spreading of SIR complexes along the chromatin fiber (Moazed 2001; Rusche et al. 2003). Of the Sir proteins, only Sir2 is required for rDNA silencing (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). Sir2 is part of a separate complex that regulates rDNA-specific silencing and cell cycle progression, called RENT (regulator of nucleolar silencing and telophase exit), which contains two other subunits, Net1 (also known as Cfi1) and Cdc14. Net1 is located in the nucleolus and recruits Sir2 to rDNA (Straight et al. 1999), and specific alleles of Sir2 selectively abolish binding to Net1 (Cuperus et al. 2000). Net1 also sequesters the protein phosphatase Cdc14 in the nucleolus until the RENT complex disassembles during late anaphase (Shou et al. 1999; Visintin et al. 1999). The resulting release of Cdc14 permits cells to exit from mitosis (Visintin et al. 1998; Shou et al. 1999).

Chromatin immunoprecipitation experiments show that RENT subunits are primarily associated with two regions of the rDNA unit (Huang and Moazed 2003; Stegmeier et al. 2004). In budding yeast, each 9.1-kb repeat yields a 35S precursor rRNA (transcribed by RNA Pol I) and a 5S rRNA (transcribed by RNA Pol III), separated by two nontranscribed spacers, NTS1 and NTS2 (Petes and Botstein 1977). RENT preferentially cross-links with DNA sequences within NTS1 and a region spanning the RNA Pol I promoter (TIR, transcription initiation region) and the 5'-end of the 35S coding region (Huang and Moazed 2003; Stegmeier et al. 2004). Silencing factors are recruited to these regions using distinct pathways, suggesting that silencing at these regions may regulate different biological functions (Huang and Moazed 2003).

NTS1 contains several sequence elements that stimulate recombination and also establish a polar replication fork block (RFB) (Keil and Roeder 1984; Brewer and Fangman 1988). Both the recombination and fork-blocking activities require the fork-blocking protein Fob1, which can directly bind to sequences in this region (Kobayashi and Horiuchi 1996; Kobayashi 2003; Mohanty and Bastia 2004). Deletion of *FOB1* results in both the suppression of recombination and a complete loss of rDNA silencing specifically at NTS1, and cross-linking studies indicate that association of Sir2 with this region is abolished (Huang and Moazed 2003). Furthermore, Fob1 physically interacts with the RENT complex in vivo, indicating that Fob1 not only stimulates recombination but also represses it through recruitment of the RENT complex to NTS1 (Huang and Moazed 2003). Factors responsible for recruiting the RENT complex to the

NTS2 region have not been identified, but Pol I is an attractive candidate since it is required for silencing of Pol II promoters(Buck et al. 2002; Cioci et al. 2003) and physically interacts with RENT in vivo and in vitro (Shou et al. 2001;Huang and Moazed 2003).

Once silencing factors are recruited to rDNA, it is unknown how they prevent recombination. Some observations are not fully consistent with models in which silencing renders rDNA chromatin less accessible to recombination (and transcriptional) machinery. For example, rDNA is highly active (60% of total transcription in rapidly growing yeast cells) (Woolford and Warner 1991), and Pol I transcription is not dramatically affected by *SIR2*-dependent silencing mechanisms (Shou et al. 2001; Sandmeier et al. 2002). In addition, the rate of mitotic recombination within individual rDNA units on the same chromatid is unchanged despite the deletion of *SIR2*, whereas unequal recombination between sister chromatids increases (Kobayashi et al. 2004). Rather than preventing access to DNA in a nonspecific manner, which is a general property of heterochromatic silencing mechanisms, rDNA silencing complexes may alter chromatin in ways that allow the assembly of specialized structures that regulate recombination.

Here we have analyzed the composition of core rDNA silencing complexes in budding yeast using a combination of native protein affinity purifications and highly sensitive mixture mass spectrometry analysis. We find that known rDNA silencing factors interact with Tof2, a nucleolar protein that is required for silencing specifically at the NTS1 region of rDNA. Tof2 predominantly cross-links to NTS1 sequences that overlap the binding site of the fork-blocking protein Fob1, and requires Fob1 for its association with NTS1. Purification of Tof2 shows that it interacts with Lrs4 and Csm1, which have previously been shown to play critical roles in chromosome segregation during meiosis I as subunits of the monopolin complex, which also contains the meiotic-specific protein Mam1. Lrs4 and Csm1 are both required for silencing at NTS1 and associate with the NTS1 region in a Fob1- and Tof2-dependent, but Sir2-independent, manner. Moreover, Lrs4/Csm1 are required for suppression of unequal recombination within rDNA and act synergistically with Sir2 to suppress recombination in rDNA. Finally, the mitotic exit network is required for the release of Lrs4 and Csm1 from the nucleolus during anaphase.

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Results

Affinity purification of rDNA silencing complexes

To better understand the mechanisms that regulate recombination at the rDNA, we affinity-purified factors that both stimulate and suppress rDNA recombination from budding yeast extracts using the tandem affinity purification (TAP) method (Rigaut et al. 1999). The endogenous *NET1*, *SIR2*, and *FOB1* genes were modified to produce proteins with C-terminal TAP tags, each of which was fully functional for silencing (Huang and Moazed 2003; Tanny et al. 2004). The TAP tag is a dual epitope tag consisting of a calmodulin-binding peptide (CBP) separated from two Protein A repeats by a TEVprotease cleavage site (Rigaut et al. 1999). Following purification, the complexes were visualized by SDS-PAGE followed by silver staining (Figure 1A–D). Additionally, the complexes were TCA-precipitated, digested with trypsin, and analyzed by liquid chromatography coupled with tandem mass spectrometry analysis (LC-MS/MS) (Peng and Gygi 2001; Tanny et al. 2004). Analysis of the entire mixture permits the identification of peptides from proteins that are not highly abundant and/or not readily visible on Coomassie- or silver-stained polyacrylamide gels and would therefore not be selected for individual band analysis, making it possible to identify factors that may interact transiently or peripherally with core complex components. The lists of specific proteins presented for each of the purifications in this study exclude proteins that were also found in parallel untagged/ mock purifications as well as peptides from likely contaminants, such as ribosomal subunits or heat-shock proteins, which copurify with many different types of complexes (Table 1). In general, we observe a correlation between the number of peptides that are identified by LC-MS/MS and the relative abundance of each protein in the purified mixtures, so that stoichiometric components of complexes are represented by peptides that correspond to a similar percentage of protein length (referred to as percent coverage).

A Markers

kD
220-
160-
120-
100-
90-
80-
70-
60-
50-
40-
30-
20-

B Net1

— Net1-CBP

C Sir2

— Sir2-CBP

D Fob1

— Fob1-CBP

histones
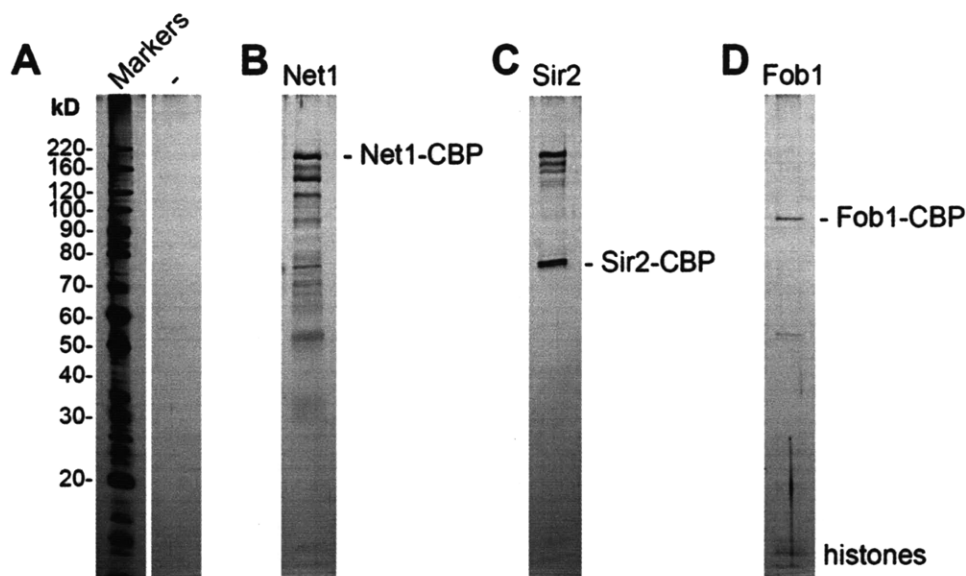
E

LC-MS/MS of total mixtures
Protein (#unique peptides, coverage)

Net1-TAP (I)		Sir2-TAP (I)		Sir2-TAP (II)		Fob1-TAP (I)		Fob1-TAP (II)	
Net1 (98, 38%)		Sir4 (77, 52%)		Sir4 (105, 64%)		**Fob1** (32, 41%)		**Fob1** (36, 42%)	
Sir2 (16, 34%)		Net1 (45, 41%)		Net1 (68, 49%)		Net1 (6, 8%)		Tof2 (9, 18%)	
Tof2 (22, 34%)		**Sir2** (27, 38%)		**Sir2** (28, 47%)		Tof2 (5, 9%)		H2B (3, 15%)	
Cdc14 (27, 58%)		Sir1 (8, 15%)		Sir1 (15, 27%)		H2B (2, 8%)		Net1 (2, 2%)	
5 pol I subunits		Cdc14 (6, 16%)		Cdc14 (12, 27%)		Cdc14 (1, 2%)		Cdc14 (2, 4%)	
		Tof2 (4, 6%)		Tof2 (9, 14%)		H1 (1, 4%)			
Net1-TAP (II)		H2B (1, 7%)		Sir3 (6, 8%)		H2A (1, 5%)			
Net1 (109, 57%)		Sir3 (1, 1%)				H4 (1, 12%)			
Sir2 (21, 37%)									
Tof2 (11, 22%)		Kap95 (16, 19%)		Kap95 (14, 18%)		Top1 (9, 14%)		Top1 (13, 18%)	
Cdc14 (10, 27%)		Srp1 (6, 15%)		Srp1 (8, 23%)		Reb1 (5, 10%)		Nog1 (4, 7%)	
		Rfm1 (3, 10%)		Rfm1 (2, 7%)		Nog1 (1, 2%)		Reb1 (1, 1%)	
Csm1 (2, 22%)				Rtn1 (1, 4%)					

F 1 ▬▬▬□□□□□□□ 771 Tof2

1 ▬▬▬□□□□□□□□□□□□ 1189 Net1
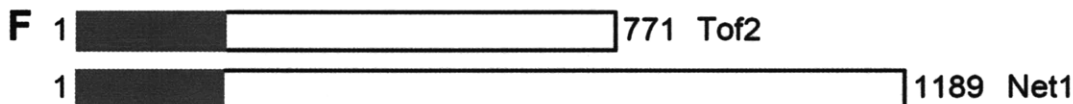
Figure 1. Affinity purifications of rDNA silencing complexes.

Silver-stained SDS-PAGE gels of complexes purified from untagged (*A*), Net1-TAP (*B*), Sir2-TAP (*C*), and Fob1-TAP (*D*) cells; 2.5% of the total purified material is shown. (*E*) The results of the total mixture analysis by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). (*F*) The protein sequence alignment of the N termini of Tof2 and Net1 indicates 30% identity and 53% similarity within the N-terminal 250 amino acids (shaded area). (CBP) Calmodulin-binding protein.

Table 1. Mock Purification Results

#unique peptides	ORF name	Common name	Mol weight
31	SSB1	YDL229W	66485
30	SSA1	YAL005C	69642
25	MYO1	YHR023W	223483
19	DED1	YOR204W	65422
18	RPS1A	YLR441C	28660
17	CDC19	YAL038W	54412
15	ATP3	YBR039W	30620
15	TEF2	YBR118W	49912
15	PAB1	YER165W	64204
14	RPS7B	YNL096C	21543
14	PDC1	YLR044C	61382
13	UBP3	YER151C	14627
13	RPS3	YNL178W	26378
13	RPL26B	YGR034W	101915
12	TDH3	YGR192C	15766
12	RPS19B	YNL302C	35612
12	SSE1	YPL106C	77235
11	RPS14A	YCR031C	14445
11	HSP60	YLR259C	58249
10	RPL31A	YDL075W	12830
10	RPL4A	YBR031W	21529
10	RPS5	YJR123W	24937
10	PGK1	YCR012W	39022
10	EFT2	YDR385W	44636
10	RPS7A	YOR096W	62865
10	CNA1	YLR433C	93145
10	YMR031C	YMR031C	93200
9	RPL25	YOL127W	15643
9	SSA2	YLL024C	26336
9	ILV2	YMR108W	43773
9	VMA4	YOR332W	64757
9	SPT5	YML010W	69342
9	TUF1	YOR187W	82532
9	RPG1	YBR079C	110198
9	YGL245W	YGL245W	115480
8	ENO2	YHR174W	15761
8	HSC82	YMR186W	19659
8	MCX1	YBR227C	27023
8	RPS6B	YBR181C	27305
8	RPS16B	YDL083C	27480
8	RPL3	YOR063W	27827
8	SUI3	YPL237W	31458
8	LAT1	YNL071W	43665
8	RPL2A	YFR031C-A	46773
8	GPM1	YKL152C	48534
8	RSM7	YJR113C	57946
8	RPL11B	YGR085C	80754
7	RPL17B	YJL177W	14354
7	RPS22A	YJL190C	14532
7	RPL23A	YBL087C	15688
7	RPS17B	YDR447C	20442
7	TIM44	YIL022W	28018
7	RPL8A	YHL033C	39174
7	YPL009C	YPL009C	42685
7	NIP1	YMR309C	82049
7	TFG1	YGR186W	92650
7	ILV5	YLR355C	93036
7	MYO2	YOR326W	119047
7	YGR130C	YGR130C	180525
6	YJL122W	YJL122W	13801
6	DBP2	YNL112W	16913
6	YHR121W	YHR121W	16947
6	ADH1	YOL086C	19169
6	RPL9A	YGL147C	21189
6	RPS18A	YDR450W	21579
6	FUN12	YAL035W	25251
6	RPS0A	YGR214W	27903
6	RPS13	YDR064W	33580
6	RPL35B	YDL136W	36743
6	RPL5	YPL131W	60874
6	CMP2	YML057W	68382
6	RPL10	YLR075W	112249
5	NOP58	YOR310C	11041
5	RPL30	YGL030W	11291
5	RPS15	YOL040C	14662
5	BRE5	YNR051C	15040
5	YOR252W	YOR252W	15245
5	RPS24A	YER074W	15394
5	VMA2	YBR127C	15914
5	RPL36A	YMR194W	16654
5	TPI1	YDR050C	21907
5	TAF61	YDR145W	22181
5	RPL13A	YDL082W	22448
5	RPL27B	YDR471W	23944
5	RPS9B	YBR189W	26661
5	YDJ1	YNL064C	27467
5	TIF34	YMR146C	27807
5	CMK2	YOL016C	28700
5	ECM1	YAL059W	33569
5	RHR2	YIL053W	37547
5	YPL077C	YPL077C	38741
5	YDR101C	YDR101C	39561

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5	YCR030C	YCR030C	41707	3	SRV2	YNL138W	29874
5	RPP0	YLR340W	44379	3	SRO9	YCL037C	35618
5	RPL14B	YHL001W	44382	3	CLC1	YGR167W	39477
5	RPC40	YPR110C	50307	3	PRP19	YLL036C	41953
5	MRPL6	YHR147C	57552	3	TUB2	YFL037W	42141
5	RPL32	YBL092W	57595	3	ATP15	YPL271W	48454
5	PSA1	YDL055C	60955	3	NOP14	YDL148C	49820
5	NSR1	YGR159C	65076	3	LYS21	YDL131W	50891
5	RPS1B	YML063W	96007	3	RPS30A	YLR287C-A	51791
5	ACT1	YFL039C	56964	3	HTA2	YBL003C	56553
4	RPL12B	YDR418W	8708	3	YDR229W	YDR229W	57381
4	TIF4631	YGR162W	9976	3	YRA1	YDR381W	57715
4	RPL19B	YBL027W	10601	3	RPS4B	YHR203C	77746
4	ATP7	YKL016C	15835	3	ARC1	YGL105W	88834
4	RPL7A	YGL076C	16610	3	TAF90	YBR198C	90595
4	ENO1	YGR254W	17646	3	CYR1	YJL005W	94169
4	RPL24A	YGL031C	18131	3	NUP2	YLR335W	227691
4	RPL15A	YLR029C	19710	2	RPS2	YGL123W	6029
4	RPL43B	YJR094W-A	19872	2	PDC6	YGR087C	11035
4	SET2	YJL168C	21323	2	RPL1B	YGL135W	12052
4	YJR083C	YJR083C	21452	2	RPL16B	YNL069C	12741
4	MRPL13	YKR006C	21605	2	TIF2	YJL138C	12845
4	SSC1	YJR045C	21605	2	YPR143W	YPR143W	13338
4	RPL20A	YMR242C	24331	2	SUI2	YJR007W	13809
4	PBP1	YGR178C	27531	2	RPS10B	YMR230W	15330
4	RPA190	YOR341W	35391	2	YKL056C	YKL056C	18252
4	PMA1	YGL008C	46679	2	SUB1	YMR039C	18388
4	TSA1	YML028W	52636	2	YJL200C	YJL200C	18594
4	RPS25A	YGR027C	68081	2	RPS8A	YBL072C	19878
4	RPL38	YLR325C	72928	2	DST1	YGL043W	20461
4	RPL21A	YBR191W	78774	2	ASC1	YMR116C	22174
4	ECM16	YMR128W	84420	2	RPP2B	YDR382W	22385
4	PRT1	YOR361C	88114	2	NUP60	YAR002W	24399
4	SWI3	YJL176C	92873	2	HOR2	YER062C	25323
4	RVS167	YDR388W	99584	2	BUD20	YLR074C	27366
4	RPL28	YGL103W	106952	2	RPA49	YNL248C	27678
4	RGA1	YOR127W	112687	2	FPR4	YLR449W	28076
4	DOT6	YER088C	144804	2	RPS26B	YER131W	34572
4	RPL6B	YLR448W	186421	2	IDH2	YOR136W	34697
3	YGR081C	YGR081C	6614	2	BFR2	YDR299W	34852
3	RPL22A	YLR061W	7003	2	YNL110C	YNL110C	37796
3	SAM2	YDR502C	13560	2	HIT1	YJR055W	40660
3	STM1	YLR150W	13870	2	RPL18B	YNL301C	43736
3	FIP1	YJR093C	22125	2	RGA2	YDR379W	44551
3	TSR1	YDL060W	23079	2	SNF12	YNR023W	46536
3	FBA1	YKL060C	23952	2	YEF3	YLR249W	49777
3	SOD2	YHR008C	24853	2	RPL40A	YIL148W	56743
3	YER006W	YER006W	26369	2	TUB1	YML085C	57731
3	YER002W	YER002W	26777	2	RPL33B	YOR234C	57753
3	RPL16A	YIL133C	29300	2	RPL6A	YML073C	58999

2	TAF60	YGL112C	59051	1	YNR053C	YNR053C	33455
2	BUD3	YCL014W	61161	1	ADE5	YGL234W	34219
2	GCD11	YER025W	61185	1	DRS1	YLL008W	34474
2	YIL105C	YIL105C	61438	1	RLP7	YNL002C	35619
2	SIK1	YLR197W	63723	1	YPL070W	YPL070W	35712
2	RRP5	YMR229C	77863	1	REX4	YOL080C	35832
2	RPS20	YHL015W	83583	1	CST6	YIL036W	36071
2	LCP5	YER127W	104088	1	YKR090W	YKR090W	36438
2	RPN2	YIL075C	113161	1	MLC1	YGL106W	36596
2	OSH2	YDL019C	115818	1	CDC33	YOL139C	36931
2	RPS12	YOR369C	145650	1	YPR169W	YPR169W	37510
2	SIF2	YBR103W	184692	1	RVS161	YCR009C	38025
2	RPL31B	YLR406C	192975	1	RPP2A	YOL039W	38568
2	PGI1	YBR196C	33096	1	PFK1	YGR240C	38700
1	SSA4	YER103W	6538	1	NHP2	YDL208W	38814
1	TIM9	YEL020W-A	6553	1	YML093W	YML093W	38992
1	EFB1	YAL003W	6604	1	YDR493W	YDR493W	41597
1	YPL146C	YPL146C	7598	1	PAM1	YDR251W	41671
1	TFG2	YGR005C	8695	1	DIM1	YPL266W	41878
1	RPL4B	YDR012W	8870	1	STE5	YDR103W	42350
1	EBS1	YDR206W	9788	1	RPS28B	YLR264W	43010
1	BMH1	YER177W	10203	1	RPA43	YOR340C	43989
1	RPP1B	YDL130W	10519	1	YMR075W	YMR075W	45212
1	RPL33A	YPL143W	10728	1	GLN3	YER040W	46363
1	TDH1	YJL052W	11283	1	RPT5	YOR117W	46389
1	INO2	YDR123C	12067	1	YNL022C	YNL022C	46473
1	YKR071C	YKR071C	13694	1	TKL1	YPR074C	48107
1	YGR002C	YGR002C	13804	1	YMR188C	YMR188C	48690
1	YMR144W	YMR144W	14120	1	LEU2	YCL018W	48983
1	YDL053C	YDL053C	14168	1	YPL105C	YPL105C	49671
1	LIP5	YOR196C	14918	1	ALD6	YPL061W	49673
1	RPL13B	YMR142C	15380	1	FAB1	YFR019W	50288
1	SEC1	YDR164C	15718	1	IPP1	YBR011C	50433
1	BRX1	YOL077C	16303	1	RRP1	YDR087C	50982
1	SSB2	YNL209W	18751	1	RPS29A	YLR388W	51464
1	MCK1	YNL307C	19768	1	TAL1	YLR354C	52442
1	RPS21B	YJL136C	20440	1	HHF1	YBR009C	54272
1	RVB2	YPL235W	20526	1	RTG2	YGL252C	55096
1	SPT16	YGL207W	22418	1	SNF2	YOR290C	55377
1	SAH1	YER043C	22500	1	DBP3	YGL078C	56189
1	NPI46	YML074C	24113	1	KRE35	YGL099W	57357
1	RPL17A	YKL180W	27513	1	PFK2	YMR205C	58707
1	RSA1	YPL193W	27842	1	HTZ1	YOL012C	63643
1	TDH2	YJR009C	29971	1	YGR103W	YGR103W	65263
1	MRPL38	YKL170W	30117	1	PDA1	YER178W	65443
1	SAM1	YLR180W	30369	1	IDH1	YNL037C	66479
1	MMD1	YIL051C	32159	1	TAF145	YGR274C	69410
1	SOD1	YJR104C	32784	1	RPS29B	YDL061C	69526
1	PHO90	YJL198W	33193	1	CMK1	YFR014C	72604
1	MYO4	YAL029C	33332	1	PET9	YBL030C	73669

1	AAC3	YBR085W	77473
1	ARF2	YDL137W	78851
1	SSE2	YBR169C	79397
1	TIF35	YDR429C	79421
1	URA2	YJL130C	83346
1	BAT1	YHR208W	84385
1	OSH7	YHR001W	84698
1	TEF4	YKL081W	92758
1	THS1	YIL078W	94354
1	PNG1	YPL096W	97684
1	STU2	YLR045C	100013
1	CRP1	YHR146W	100792
1	HCM1	YCR065W	102236
1	ADH2	YMR303C	102696

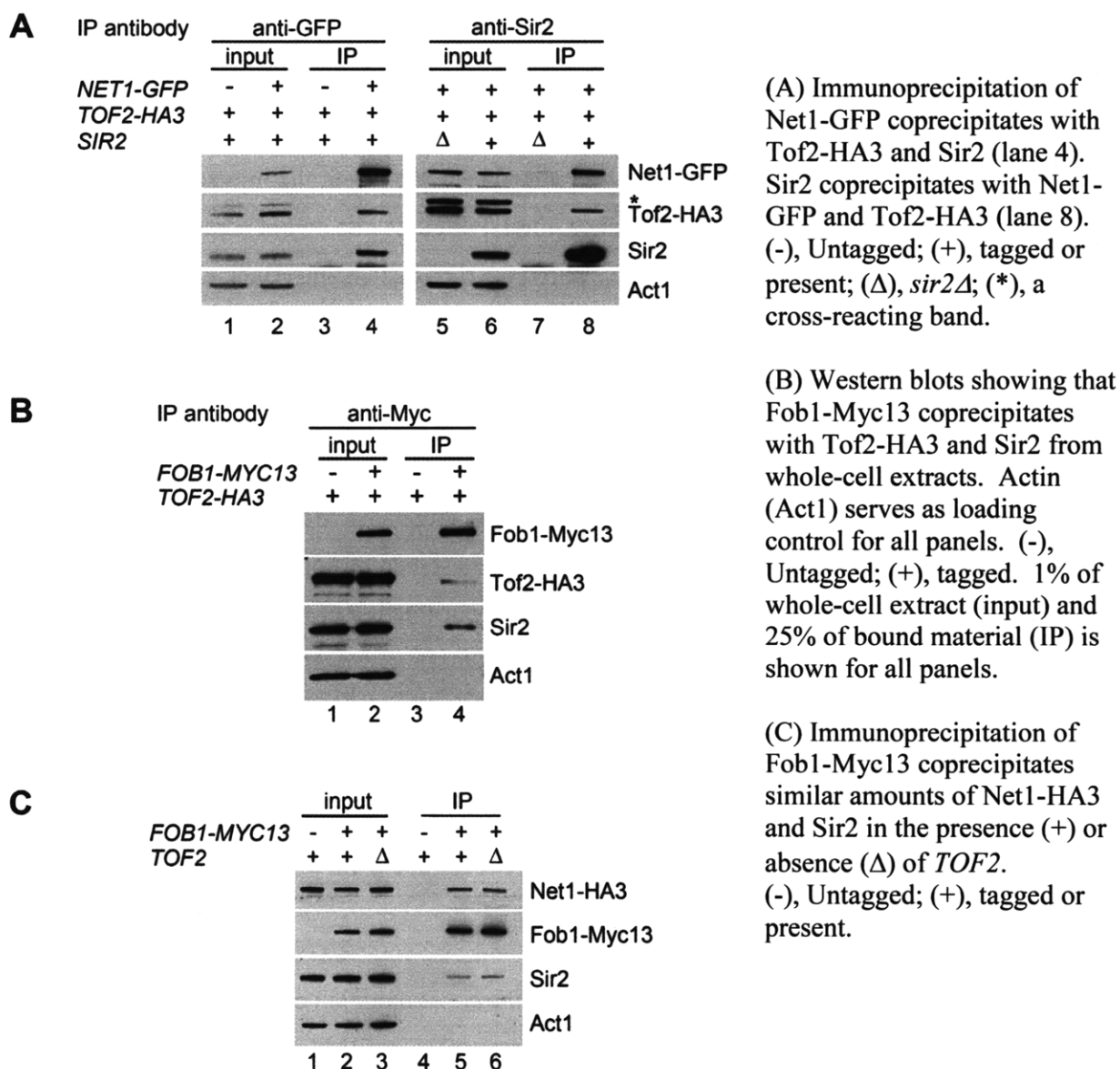| 1	RPL29	YFR032C-A	102844
1	MIS1	YBR084W	104475
1	ADH5	YBR145W	107822
1	BUR6	YER159C	118591
1	ERG10	YPL028W	120561
1	RPS0B	YLR048W	169211
1	RPL26A	YLR344W	194039
1	RPT2	YDL007W	244946
1	RPS31	YLR167W	257328
1	RPL22B	YFL034C-A	38401
1	RPS27B	YHR021C	15912
1	ADA2	YDR448W	86039
1	TUB3	YML124C	69865

As expected, Net1 complexes consisted primarily of Net1, Sir2, and Cdc14 (Figure 1B,E; Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999; Tanny et al. 2004). One purification also contained several subunits of Pol I, while in the second, Pol I subunits appeared in mock purifications from untagged strains, likely due to sheer abundance. However, the interaction between Net1 and Pol I is supported by other evidence. First, Pol I subunits do not copurify with the other complexes studied here. Second, Pol I is required for rDNA silencing (Buck et al. 2002; Cioci et al. 2003). Finally, we and others have observed that Net1 physically interacts with Pol I in vivo and in vitro (Shou et al. 2001; Huang and Moazed 2003).

Net1 and Sir2 purifications also contained a large number of peptides from Tof2. In particular, in Net1 purifications, Tof2 peptides were represented with similar abundance to Sir2 and Net1 (Figure 1E). Tof2 was originally identified in a yeast two-hybrid screen for topoisomerase I-interacting factors (Park and Sternglanz 1999), and a previous purification of RENT had also identified a small number of peptides from Tof2 (Tanny et al. 2004). However, no physiological function has been ascribed to Tof2. Tof2 and Net1 share 22% identity (40% similarity) (Park and Sternglanz 1999; Shou et al. 1999; Straight et al. 1999), with the region of highest conservation (30% identity, 53% similarity) located within the first 250 amino acids of both proteins (Figure 1F).

Supporting the finding that Tof2 copurifies with both Net1 and Sir2 complexes, immunoprecipitation of either Net1-GFP or Sir2 results in the coprecipitation of Tof2- HA3 (Figure 2A). Consistent with previous results, Sir2 purifications also yielded interacting subunits

from both the RENT complex (Net1 and Cdc14) and the SIR mating-type/telomeric silencing complex (Sir3, Sir4) (Figure 1C,E). Surprisingly, we also found a significant number of peptides from Sir1 (15%–27% amino acid sequence coverage), which binds to DNA-binding factors and recruits the other Sir proteins to mating-type silencers (Rusche et al. 2003). Sir1 has not previously been shown to copurify with the entire SIR complex, although an interaction between Sir1 and overexpressed Sir3 has been detected (Ho et al. 2002). Our findings suggest the existence of a soluble form of the SIR complex that includes Sir1.

Figure 2. Tof2 physically associates with Fob1, Net1, and Sir2

(A) Immunoprecipitation of Net1-GFP coprecipitates with Tof2-HA3 and Sir2 (lane 4). Sir2 coprecipitates with Net1-GFP and Tof2-HA3 (lane 8). (-), Untagged; (+), tagged or present; (Δ), $sir2\Delta$; (*), a cross-reacting band.

(B) Western blots showing that Fob1-Myc13 coprecipitates with Tof2-HA3 and Sir2 from whole-cell extracts. Actin (Act1) serves as loading control for all panels. (-), Untagged; (+), tagged. 1% of whole-cell extract (input) and 25% of bound material (IP) is shown for all panels.

(C) Immunoprecipitation of Fob1-Myc13 coprecipitates similar amounts of Net1-HA3 and Sir2 in the presence (+) or absence (Δ) of *TOF2*. (-), Untagged; (+), tagged or present.

Purification of Sir2 also reproducibly yielded many peptides from the α and β subunits of the importin family, Srp1 (Ima1) and Kap95 (Imb1). Importin-α and importin-β form a heterodimer that mediates bidirectional translocation of hundreds of proteins across the nuclear envelope, but each protein also participates in cellular processes unrelated to transport (Goldfarb et al. 2004). Interestingly, screens for genes that bypass the requirement for the kinase Cdc15 to exit from mitosis have identified alleles of not only *CDC14* and *NET1* (Shou et al. 1999; Visintin et al. 1999), but also of several karyopherins (*SRP1*, *MTR10*, and *KAP104*) (Asakawa and Toh-e 2002; Shou and Deshaies 2002). Finally, Sir2 purifications contained two to three peptides of Rfm1 (7%– 10% amino acid coverage), a DNA-binding protein that is required for the recruitment of Sum1 and Hst1, a yeast Sir2 homolog, to middle sporulation genes (Xie et al. 1999; McCord et al. 2003).

Purification of Fob1 yielded low amounts of Net1, Cdc14, and Tof2 peptides, consistent with previously observed physical interactions between Fob1 and RENT (Figure 1D, E; Huang and Moazed 2003) and Net1 and Tof2 (Figure 1E). In addition, Fob1 purifications contained a significant number of Top1 (topoisomerase I) peptides. The copurification of Tof2 and Top1 with Fob1 is consistent with the previous identification of Tof2 as a Top1-interacting protein in a yeast two-hybrid screen (Park and Sternglanz 1999). Furthermore, Top1 is required for rDNA silencing at NTS2 (Smith et al. 1999) and NTS1 (Figure 8), prevents Pol II transcription-dependent Ty1 transposition (Bryk et al. 1997), suppresses mitotic recombination within rDNA, and modulates rDNA chromatin structure (Christman et al. 1988, 1993; Cavalli et al. 1996). Fob1 purifications also contained a small number of peptides from Reb1, the 35S rRNA transcription termination factor (Lang et al. 1994), and Nog1, a nucleolar GTPase that is involved in ribosome biogenesis and nuclear transport (Park et al. 2001; Jensen et al. 2003; Kallstrom et al. 2003; Saveanu et al. 2003). These results are consistent with the observation that the RFB region overlaps the site of Pol I transcription termination, where Reb1 binds (Morrow et al. 1989), and with previous two-hybrid interactions detected between Fob1 and Nog1 (Ito et al. 2001).

Since Tof2 peptides were present in Fob1 complexes, it seemed likely that physical interactions, direct or indirect, between these proteins is responsible for recruitment of Tof2. To confirm this,

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we performed coimmunoprecipitation experiments from extracts prepared from yeast strains in which the endogenous copies of both *FOB1* and *TOF2* were modified to express Fob1-Myc13 and Tof2-HA3. Immunoprecipitation of Fob1-Myc13 resulted in the coprecipitation of both Tof2 and Sir2 (Figure 2B). In contrast, Fob1-Myc13 did not interact with actin, an abundant cytoskeletal protein.

Tof2 is an NTS1-specific rDNA silencing factor

The presence of Tof2 in Fob1, Net1, and Sir2 complexes strongly suggested that Tof2 might play a role in rDNA silencing. To test this possibility, we deleted *TOF2* in strains carrying an *mURA3* reporter gene integrated into one of three sites: outside the rDNA array at the *LEU2* gene and within the rDNA unit at two locations exhibiting strong silencing (NTS1 and NTS2 reporters) (Figure 3A; Huang and Moazed 2003). Cells were 10-fold serially diluted and spotted on complete medium as a plating control and on medium lacking uracil to monitor expression of the reporter genes.

**Figure 3. Tof2 is a nucleolar protein required specifically for rDNA silencing at NTS1.**
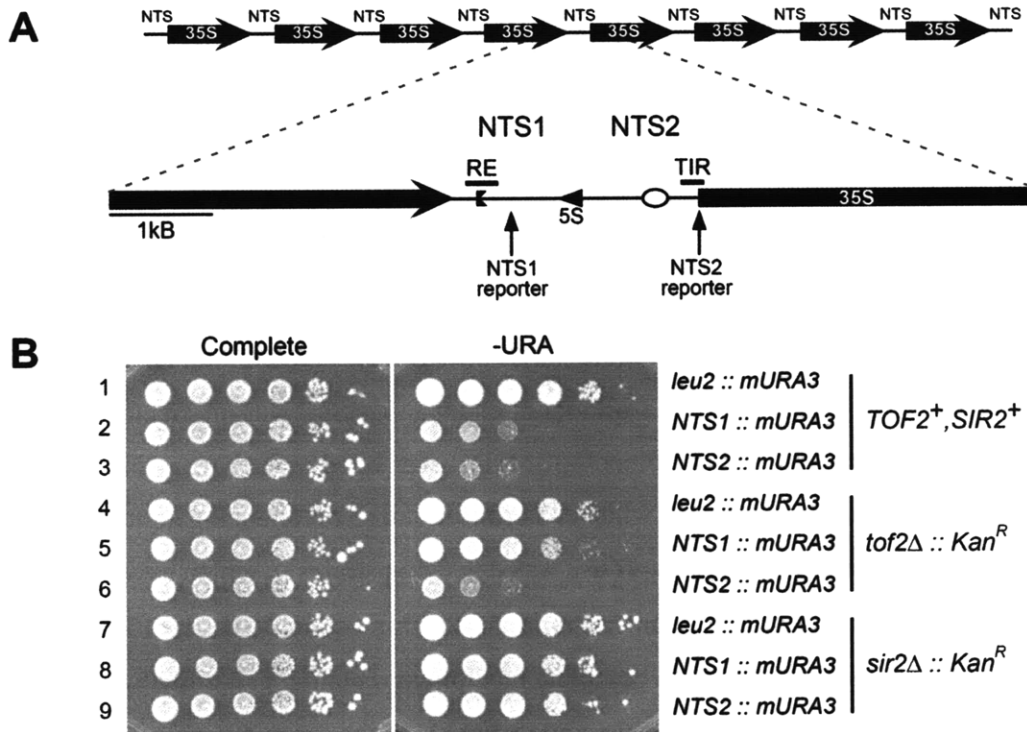
(A) The physical structure of the tandemly repeating *RDN1* locus of *S. cerevisiae* is shown *above*, and a single 9.1-kb rDNA unit is shown expanded *below*. Each repeat yields a Pol I-transcribed 35S precursor rRNA (shown as a divided thick arrow) and a Pol III-transcribed 5S rRNA (arrowhead). The 35S coding regions are separated by an NTS, which is divided by the 5S gene into NTS1 and NTS2. Solid bars indicate the recombination enhancer (RE) region and the Pol I TIR. The locations of the RFB (◄) and autonomously replicating sequences (○) are indicated. Vertical arrows indicate insertion sites of the NTS1 and NTS2/TIR silencing reporters.

(B) *TOF2* is required for rDNA silencing at NTS1 but not at NTS2/TIR. Silencing within rDNA was assessed by monitoring the growth of 10-fold serial dilutions of cells plated on −URA medium. SC medium was used as a plating control. *TOF2* is required specifically for silencing only at NTS1, unlike *SIR2*, which is required for silencing at both NTS1 and NTS2. Locations of rDNA reporter genes are as indicated in Figure 3A.

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Consistent with previous observations, the reporter gene was strongly silenced at either the NTS1 or NTS2 sites, as indicated by poor growth on −URA medium compared with the same reporter inserted at a euchromatic locus (Figure 3B, cf. rows 1 and 2,3; Huang and Moazed 2003). As expected, silencing was dependent on the presence of the *SIR2* gene (Figure 3B, cf. rows 2,3 and 8,9). Similar to the region-specific silencing gene *FOB1*, deletion of *TOF2* resulted in complete derepression of the NTS1 reporter (Figure 3B, cf. rows 2 and 5) but had no effect on silencing of the NTS2 reporter (Figure 3B, cf. rows 3 and 6). Loss of NTS1 silencing was specific to *TOF2*, since addition of a single-copy plasmid containing the *TOF2* gene under the control of its native promoter restored silencing to *tof2Δ* cells (Figure 4A). Furthermore, Western blot analysis of whole-cell extracts (WCEs) showed that the amount of endogenous Sir2 was unaffected by deletion of *TOF2*, arguing that the loss of silencing at NTS1 was not due to changes in Sir2 protein levels (Figure 4B). We next tested whether Tof2 is required for silencing at other heterochromatic regions, such as telomeres. *TOF2* was deleted in strains in which a *URA3* reporter gene was integrated within the telomeric repeats of Chromosome VIIL or <15 kb away at the *ADH4* locus. Cells were spotted onto complete medium as a plating and growth control or onto medium supplemented with 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing *URA3*. As expected, wild-type cells fully express *URA3* at the *ADH4* locus and were unable to grow on 5-FOA medium (Figure 4C, row 1). In contrast, the telomeric *URA3* reporter was silenced efficiently, permitting growth on 5-FOA medium (Figure 3A, row 2) in a *SIR2*-dependent manner (Figure 4C, cf. rows 2 and 4; Aparicio et al. 1991). However, the absence of either *TOF2* or *FOB1* had no effect on telomeric silencing (Figure 4C cf. rows 2 and 6,8). Therefore, Tof2, like Fob1, is an NTS1-specific rDNA silencing factor.

Figure 4. Silencing in *tof2Δ* cells is specific to the rDNA and is rescued by ectopic expression of *TOF2*
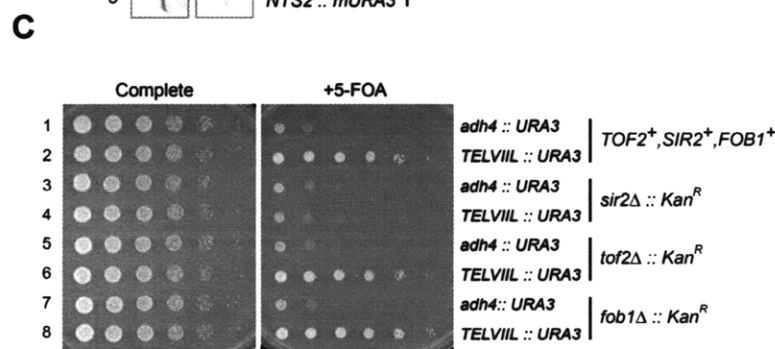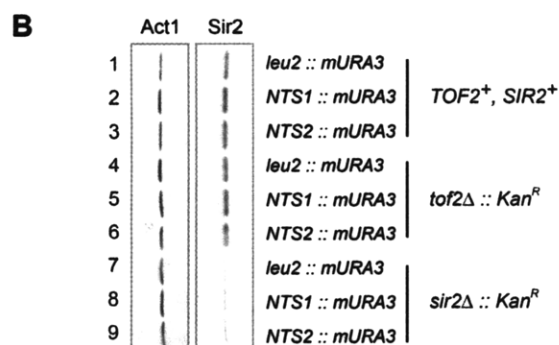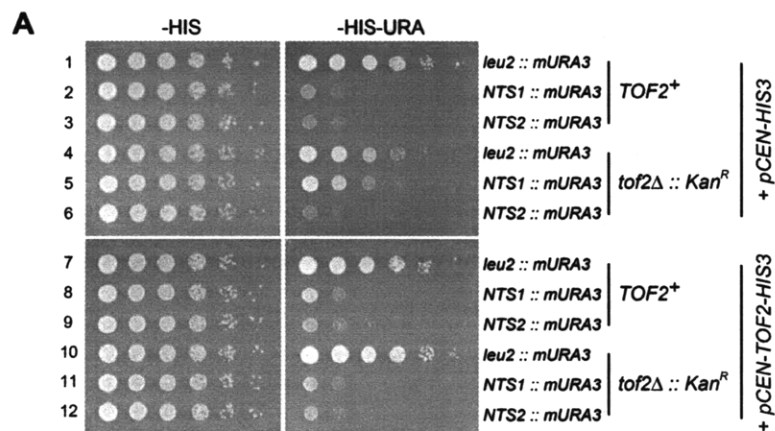
(A) Silencing was assessed as described in Figure 3B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 3A. Cells were plated on synthetic complete media lacking histidine (-HIS) to maintain *CEN* plasmids, which expressed either no gene (*pCEN-HIS3*) or *TOF2* (*pCEN-TOF2-HIS3*). Silencing was assayed on synthetic complete media lacking histidine and uracil (-HIS-URA).

(B) The level of Sir2 protein does not change in the absence of *TOF2* as shown by Western blotting of whole-cell extracts prepared from cells shown in (A). Act1 is shown as a loading control.

(C) *TOF2* and *FOB1* are not required for telomeric silencing. Silencing was assessed by monitoring the growth of 10-fold serial dilutions of cells on SC (synthetic complete) medium supplemented with 5-FOA. SC medium was used as a plating control. The *URA3* reporter gene was integrated either adjacent to the telomeric repeats of Chromosome VIIL (*TELVIIL*) or <15 kb away, at the *ADH4* locus.

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To examine the subcellular location of Tof2, we performed immuno-fluorescence microscopy on cells in which the endogenous *TOF2* gene was modified to produce a protein with a C-terminal HA3 epitope tag (Tof2- HA3). Cells expressing Tof2-HA3 exhibited wild-type levels of silencing, suggesting that the tagged protein was functional (Figure 5A). For comparison, we also analyzed the localization of Net1-GFP, a well characterized nucleolar marker (Straight et al. 1999). Similar to Net1-GFP, Tof2-HA3 formed compact and crescent-shaped structures and did not colocalize with the DNA-specific dye DAPI, which is typically excluded from the nucleolus (Figure 5C). Merged images showed strong colocalization of Tof2-HA3 and Net1-GFP, even in cells actively undergoing mitosis (Figure 5C, arrows), demonstrating that Tof2 is a bona fide nucleolar protein.

Figure 5. Silencing and the localization of Tof2 tagged with a C-terminal HA3 or TAP epitope is normal.
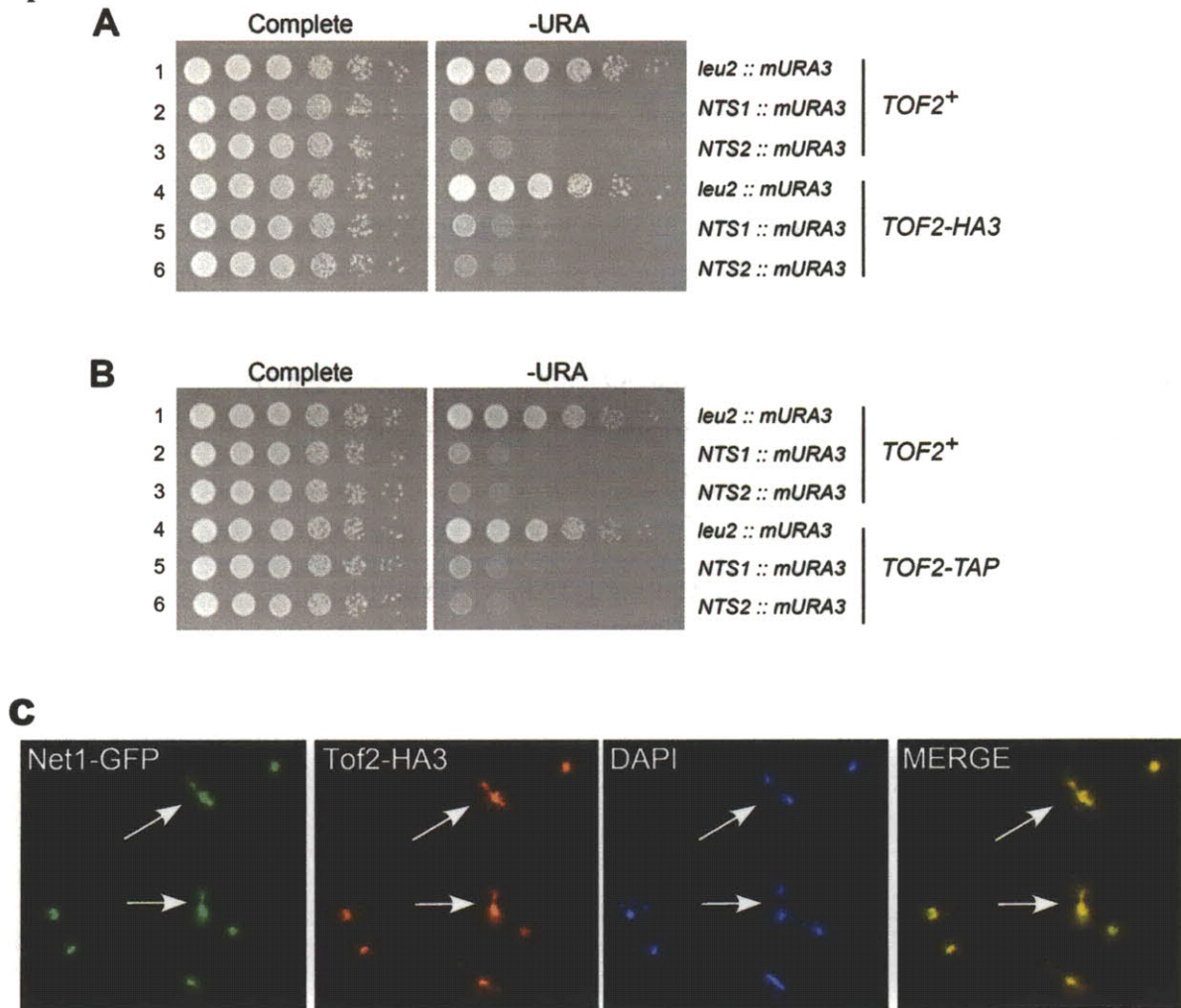

(A-B) Silencing was assessed as described in Figure 3B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 3A. Cells in which the endogenous copy of *TOF2* was modified to express either *TOF2-HA3* (A) or *TOF2-TAP* (B) maintained wild-type levels of silencing.

(C) Tof2 colocalizes with nucleolar marker Net1. Immunofluorescence images show the subcellular localization of Net1-GFP (green), Tof2-HA3 (red), and DAPI-stained DNA (blue). The merged image shows that Net1-GFP and Tof2-HA3 colocalize to nucleolar domains that are nonoverlapping with the rest of the genome (yellow). Arrows indicate dividing cells.

Tof2 primarily associates with the NTS1 region of rDNA in a Fob1-dependent but Sir2-independent manner

The silencing phenotype, nucleolar localization, and physical interaction with Fob1 and RENT subunits suggested that Tof2 should be associated with rDNA. To test this possibility, we mapped the association of Tof2 with rDNA by chromatin immunoprecipitation (ChIP). The endogenous *TOF2* gene was modified to produce a protein with a C-terminal TAP tag (Tof2-TAP), which was fully functional for rDNA-NTS1 silencing (Figure 5B). Cells were cross-linked with formaldehyde, and Tof2-TAP was immunoprecipitated using IgG-Sepharose from WCEs containing sheared chromatin. WCE and immunoprecipitated (IP) chromatin from untagged and Tof2-TAP strains served as template DNA in quantitative PCR. rDNA was amplified to produce the PCR products schematically diagrammed in Figure 6B and as described previously (Huang and Moazed 2003). In parallel, Net1-TAP and Fob1-TAP, whose profiles of rDNA association have already been characterized, were also immunoprecipitated (Huang and Moazed 2003).
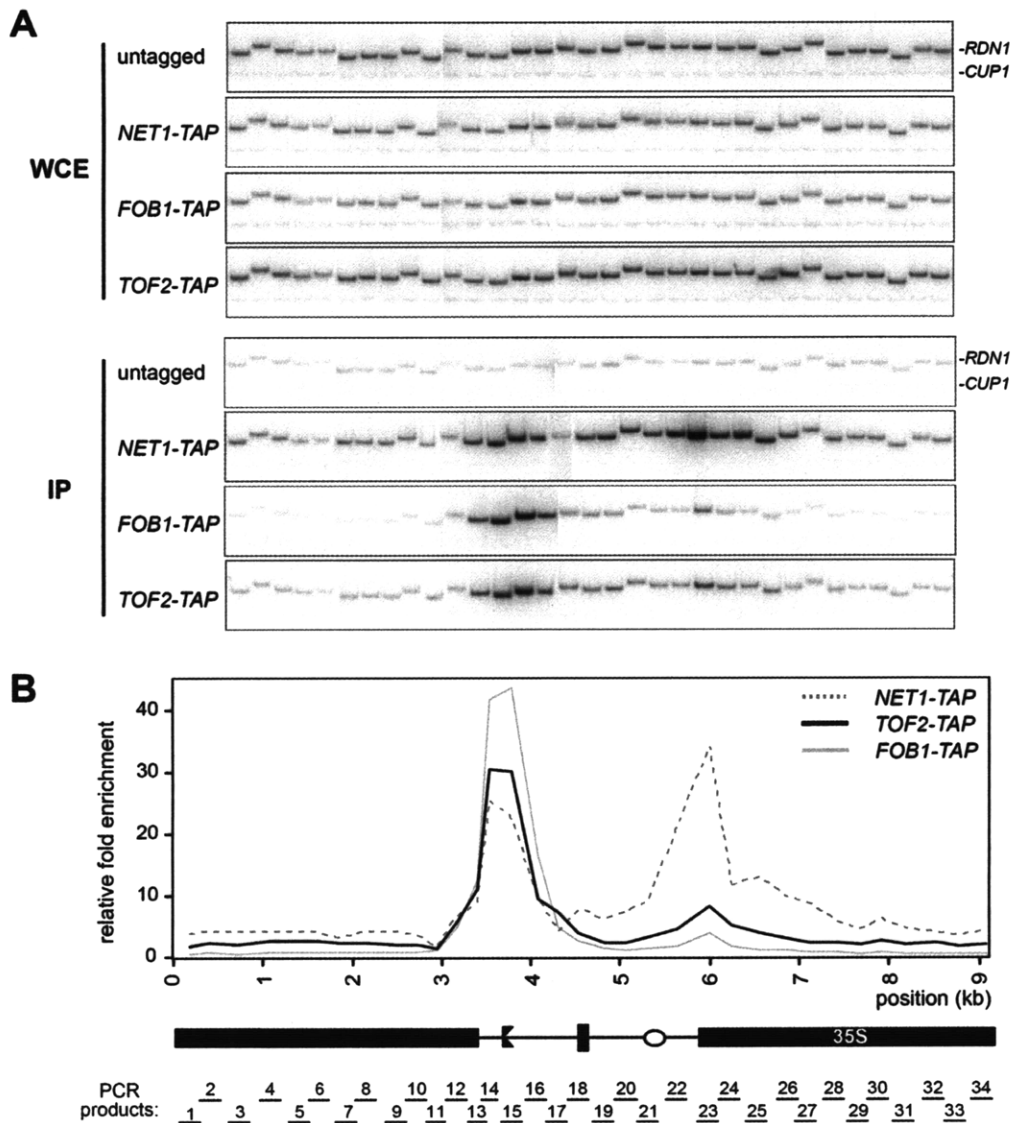
Figure 6. Tof2 associates primarily with the NTS1 region of rDNA.

(*A*) PCR products amplified from WCE (*upper* panels) and IP (*lower* panels) chromatin. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 1–4 and 6–34 are shown.

(*B*) Representative graph showing the relative fold association of Tof2-TAP (solid black line), Net1-TAP (dashed gray line), and Fob1-TAP (solid gray line) across the rDNA repeat. A schematic representation of the rDNA unit is shown *below* the graph, with significant features shown as in Figure 3A and PCR products depicted *below*. Most of the Tof2-TAP is concentrated within NTS1, with a smaller peak observed near the border of NTS2 and the 35S rRNA coding region.

The overall association profile of Tof2-TAP with rDNA closely resembled that of Fob1-TAP (Figure 6A,B; Huang and Moazed 2003). Tof2 primarily associated with the region of rDNA that precisely overlaps the RFB of NTS1, consistent with its specific requirement in NTS1 silencing (Figure 3B). Also similar to what has been observed for Fob1, we observed a much smaller peak of Tof2 association with the TIR, although neither protein was required for silencing at this location (Figure 3B; Huang and Moazed 2003). In contrast, Net1 associates with both NTS1 and NTS2/TIR regions (Figure 6A,B; Huang and Moazed 2003).

To better understand the assembly of silencing complexes at NTS1, we examined the association of Tof2 with rDNA in the absence of both Fob1 and Sir2. Data from a representative experiment are shown in Figure 7A with quantification of relative fold enrichment of rDNA shown in Figure 7B. In the absence of *FOB1*, the association of Tof2 with the NTS1 region was completely abolished, while association with the NTS2/TIR region was unaffected. We conclude that, similar to what was observed for Net1 and Sir2 (Huang and Moazed 2003), *FOB1* is absolutely required for the localization of Tof2 specifically to NTS1, but there exists a *FOB1*-independent mechanism for localization of Tof2 to the TIR. However, in contrast to NTS1, the association for Tof2 with the TIR region is not required for silencing since deletion of *TOF2* has no effect on the silencing of a reporter gene inserted at NTS2 (Figure 3B). Notably, the physical interaction detected between Sir2 and Tof2 is not required for the recruitment of Tof2 to rDNA. In the absence of *SIR2*, the association of Tof2-TAP with rDNA was completely unaffected (Figure 7A,B). Thus, Tof2 is recruited to NTS1 primarily by Fob1.
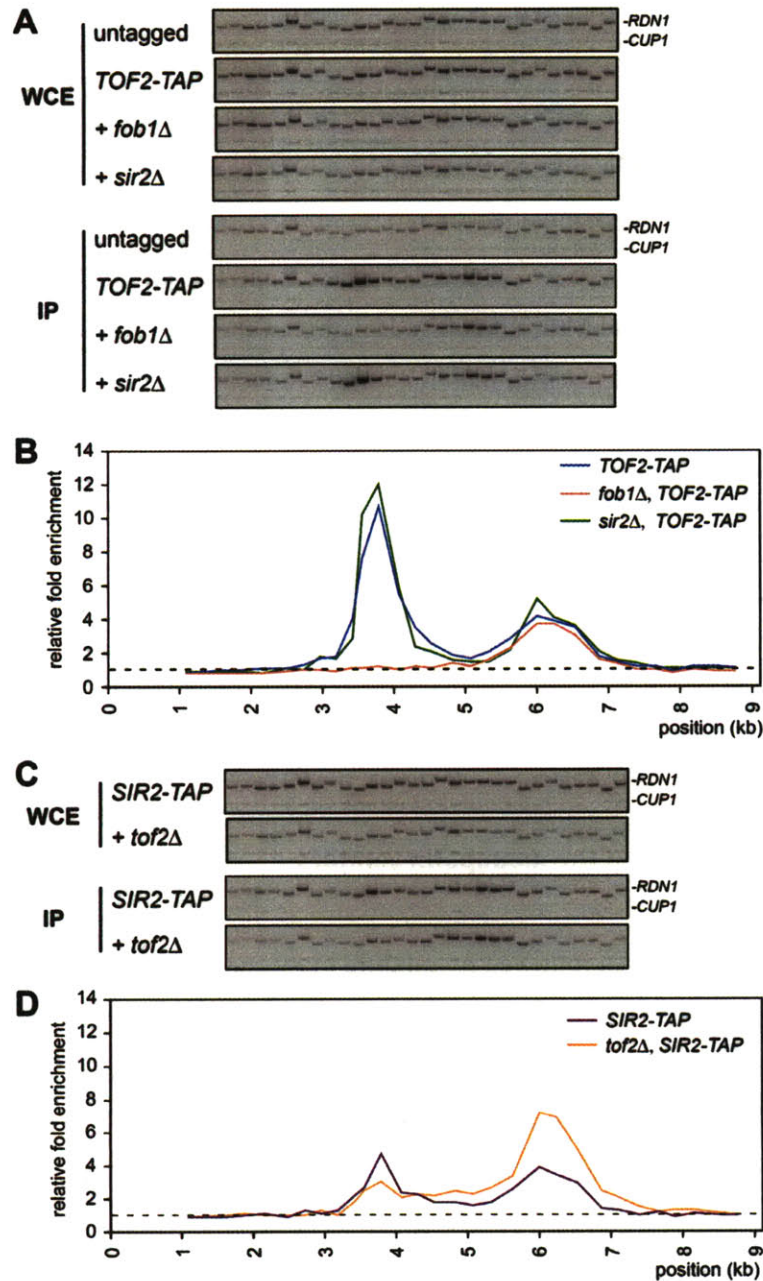
Figure 7. Tof2-TAP requires *FOB1* but not *SIR2* for association with NTS1.

(*A,C*) Examples of ChIP data showing PCR products amplified from WCE and IP DNA. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 4 and 6–33 are shown.

(*B*) Representative graphs showing the association of Tof2-TAP across an rDNA repeat in wild-type (blue), *fob1Δ* (red), or *sir2Δ* (green) cells.

(*D*) Sir2-TAP association with rDNA is largely Tof2 independent.

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Role of Tof2 in the association of Sir2 with NTS1

The localization of Tof2 primarily to NTS1 and its ability to localize to rDNA in the absence of Sir2 led us to ask whether Tof2 influences Sir2 assembly at NTS1. A representative example of these data is shown in Figure 7C with quantification in Figure 7D. As previously observed, the mapping profile of Sir2-TAP displayed two regions of enrichment, located at NTS1 and NTS2/TIR (Figure 7C,D; Huang and Moazed 2003). Deletion of the *TOF2* gene resulted in a partial loss of Sir2-TAP from NTS1 (approximately twofold reduced relative fold enrichment), while at NTS2/TIR, the association of Sir2 slightly increased (approximately twofold increased relative fold enrichment). These findings suggest that Sir2 does not require Tof2 for association with rDNA. Furthermore, the deletion of *TOF2* did not noticeably affect the amount of Net1-HA3 or Sir2 that associates with Fob1-Myc13, suggesting that Tof2 is also not required for the stability of RENT-Fob1 interactions (Figure 2C). We conclude that Tof2 represents a new NTS1-specific rDNA silencing factor that is recruited to NTS1 via Fob1 and contributes to rDNA silencing independently of Sir2 localization.
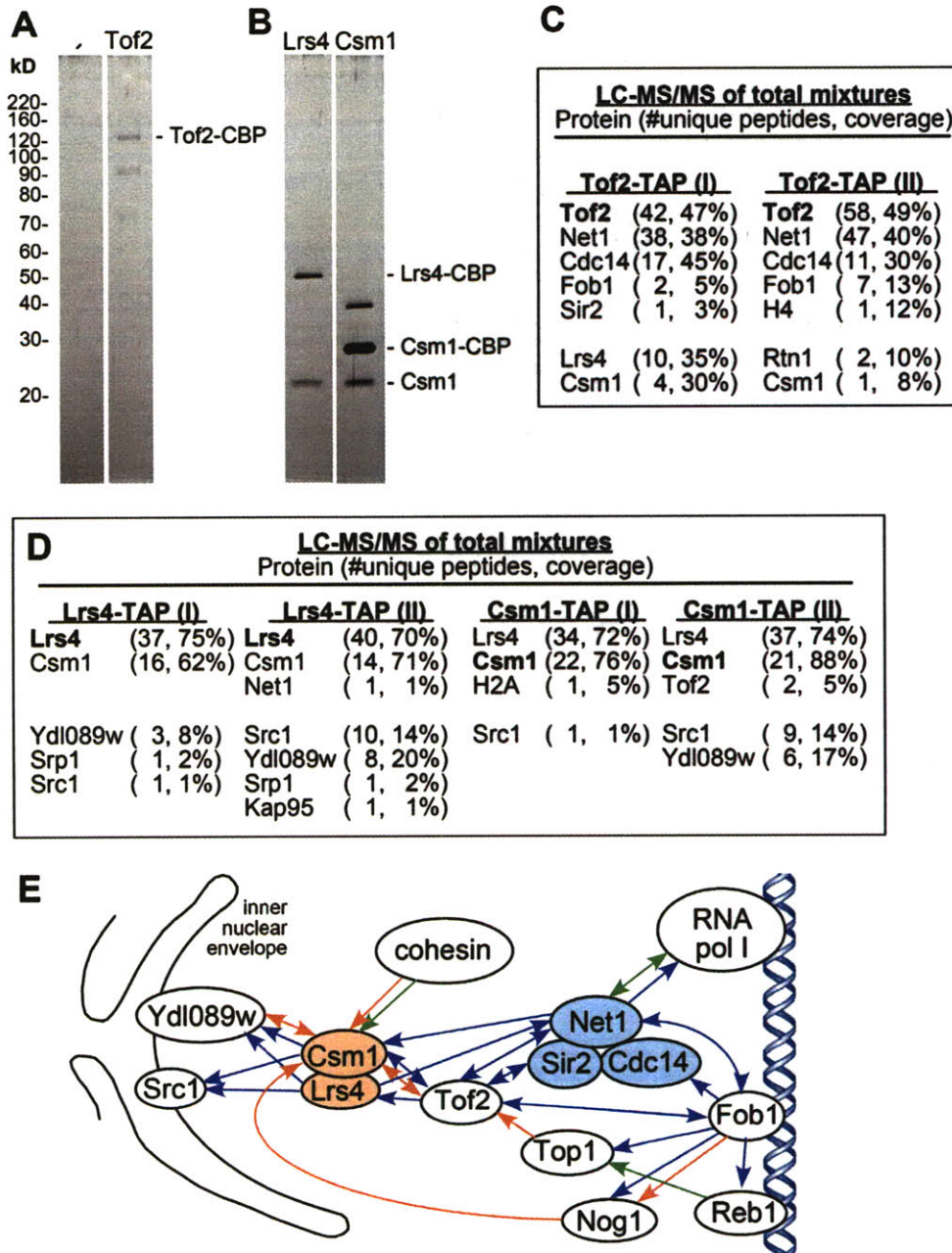
Tof2 is associated with subunits of the monopolin complex

Purification of Tof2-TAP complexes followed by mixture mass spectrometry analysis yielded peptides from Net1, Cdc14, and Sir2 (Figure 9A,C). In addition, consistent with the observation that Fob1 physically interacts with Tof2 in vivo, we recovered several Fob1 peptides in Tof2 purifications. However, Top1, which has previously been shown to interact with Tof2 in a two-hybrid assay, did not copurify with Tof2 (Figure 9C), raising the possibility that Tof2 and Top1 interact transiently or via Fob1 (see Figure 1D,E). When we tested the requirement for *TOP1* in transcriptional silencing of the *mURA3* reporter gene located at either NTS1 or NTS2, we found that *TOP1* was required for silencing at NTS1 to the same extent as *SIR2* and, consistent with previous results (Bryk et al. 1997; Smith et al. 1999), loss of *TOP1* partially derepressed the silencing marker at NTS2 (Figure 8).
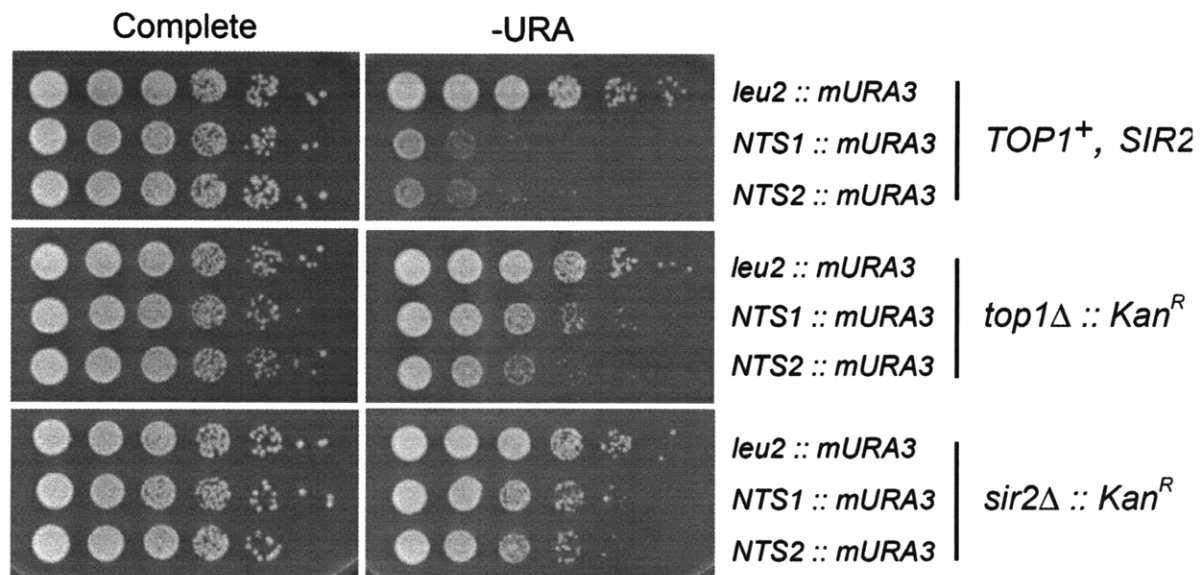
Figure 8. *TOP1* **is required for silencing at both NTS1 and NTS2**

Silencing was assessed as described in Figure 3B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 3A. Cells were plated on synthetic complete media as a plating and growth control and on synthetic complete media lacking uracil (-URA) to assay silencing.

A

B

C

LC-MS/MS of total mixtures			
Protein (#unique peptides, coverage)			
Tof2-TAP (I)		**Tof2-TAP (II)**	
Tof2	(42, 47%)	**Tof2**	(58, 49%)
Net1	(38, 38%)	Net1	(47, 40%)
Cdc14	(17, 45%)	Cdc14	(11, 30%)
Fob1	(2, 5%)	Fob1	(7, 13%)
Sir2	(1, 3%)	H4	(1, 12%)
Lrs4	(10, 35%)	Rtn1	(2, 10%)
Csm1	(4, 30%)	Csm1	(1, 8%)

D

| LC-MS/MS of total mixtures							
Protein (#unique peptides, coverage)							
Lrs4-TAP (I)		**Lrs4-TAP (II)**		**Csm1-TAP (I)**		**Csm1-TAP (II)**	
Lrs4	(37, 75%)	**Lrs4**	(40, 70%)	Lrs4	(34, 72%)	Lrs4	(37, 74%)
Csm1	(16, 62%)	Csm1	(14, 71%)	**Csm1**	(22, 76%)	**Csm1**	(21, 88%)
		Net1	(1, 1%)	H2A	(1, 5%)	Tof2	(2, 5%)
Ydl089w	(3, 8%)	Src1	(10, 14%)	Src1	(1, 1%)	Src1	(9, 14%)
Srp1	(1, 2%)	Ydl089w	(8, 20%)			Ydl089w	(6, 17%)
Src1	(1, 1%)	Srp1	(1, 2%)				
		Kap95	(1, 1%)				

E

Figure 9. Affinity purifications of native Tof2, Lrs4, and Csm1 complexes.

Silverstained SDS-PAGE gels of native complexes purified from Tof2-TAP (*A*) and Lrs4-TAP and Csm1-TAP (*B*); 2.5% of total purified material is shown. (*C,D*) The results of the total mixture analysis by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). (*E,F*) Summaries of the protein–protein interaction network of silencing factors. Arrows indicate physical interactions determined by affinity purifications from this work (blue) or by others (green), or by yeast two-hybrid (red). Direction of the arrowheads is from bait toward interactor.

In addition to the above proteins, Tof2 copurified with Lrs4 and Csm1, two of three subunits previously identified as the monopolin complex, which co-orients sister chromatids during meiosis I (Figure 9C; Toth et al. 2000; Rabitsch et al. 2003). The third subunit of monopolin, Mam1, is expressed only during meiosis I (Toth et al. 2000) and therefore would be absent from our mitotic extracts. Consistent with copurification with Tof2, both Lrs4 and Csm1 localize to the nucleolus during mitosis and most of meiosis (Rabitsch et al. 2003). Furthermore, each has been identified previously as required for rDNA silencing (Smith et al. 1999; Rabitsch et al. 2003). In addition, two-hybrid analysis detected an interaction between Tof2 and Csm1 (Ito et al. 2001; Wysocka et al. 2004), and one of our Net1 purifications yielded trace amounts of Csm1 (Figure 1E).

We next purified the Lrs4 and Csm1 proteins via the TAP tag. While deletion of either gene results in slow growth on glucose (Smith et al. 1999; Rabitsch et al. 2003), modification of either protein with the TAP tag did not result in any obvious growth defects (J. Huang, unpubl.), and cells expressing either modified protein were competent for transcriptional silencing at rDNA (Figure 10), suggesting that these proteins were functional. Purification of either protein resulted in a complex consisting primarily of Lrs4 and Csm1 in stoichiometric amounts as shown by silver staining (Figure 9B). Notably, the Csm1-TAP purification yielded not only the tagged form of Csm1, but also a faster migrating form that was identified by band analysis to be Csm1 lacking the C-terminal epitope tag (Figure 9B). This observation suggests the presence of at least two Csm1 protomers in the Lrs4/Csm1 complex. The truncated form of Csm1-CBP is likely to result from proteolytic cleavage during purification. The Lrs4 and Csm1 purifications yielded trace amounts of both Net1 and Tof2 (Figure 9D), further supporting the existence of a network of protein–protein interactions that link these proteins to the RENT complex. Lrs4-TAP purifications also yielded minor amounts of the importins Srp1 and Kap95 as well as significant numbers of peptides from two putative inner nuclear envelope proteins: Src1, which shares homology with the human Lap2 protein (Figure 11) and Ydl089w (Figure 9D; Huh et al. 2003). A summary of the interactions uncovered by our purifications and those described in the literature is presented in Figure 9, E and F.
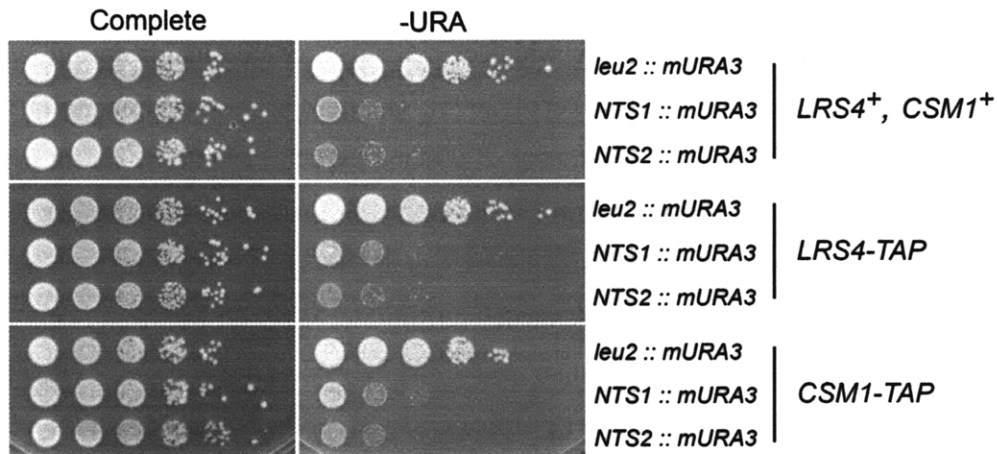
Figure 10. Expression of *LRS4* or *CSM1* modified with a C-terminal TAP epitope tag does not affect silencing.

Silencing was assessed as described in Figure 3B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 3A. Cells were plated on synthetic complete media as a plating and growth control and on synthetic complete media lacking uracil (-URA) to assay silencing.

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S.c. Src1    13  D P N S M K V A T L R R I L V E N N V D F P S - N A R K N A L V G L F D E K V K P Q  53
S.c. Ydr458c  7  D P K T L K V S Q L R R V L V E N D V A F P A - N A R K P V L V K L F E E K V R Q R  47
X.l. Lap2     7  D P S V L T K E K L K S E L V A N N V T L P S G E Q R K D V Y V Q L Y L Q H L T S Q  48
H.s. Lap2    40  D P S V L T K D K L K S E L V A N N V T L P A G E Q R K D V Y V Q L Y L Q H L T A R  80
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Figure 11. Protein sequence homology of the LEM domains of Src1, its *S. cerevisiae* homologue Ydr458c, and the Lap2 proteins of *Xenopus laevis* and *Homo sapiens*.

Identical and similar residues are indicated by gray shading. LEM domains are approximately 40 amino acids in length and primarily found in inner nuclear membrane proteins of metazoans. Although it is presumed that no LEM domain proteins exist in yeast, and thus far, no yeast inner nuclear envelope proteins have been identified (Cohen et al. 2001; Bengtsson and Wilson 2004; Segura-Totten and Wilson 2004), GFP fusions of Src1 and its *S. cerevisiae* homologue Ydr458c localize to the nuclear envelope (Drees et al. 2001; Huh et al. 2003) (data not shown).

Associations with Top1 and nuclear envelope proteins

Our purifications provide links between rDNA silencing factors and several other nuclear proteins that may regulate rDNA structure or subnuclear localization. The association of Topoisomerase I (TopI) with Fob1 provides support for a direct role for Top1 in silencing, as first suggested by genetic experiments (Christman et al. 1988; Bryk et al. 1997; Smith et al. 1999), and its Fob1-dependent recruitment to the NTS1 region (Vogelauer et al. 1998; Di Felice et al. 2005). Furthermore, purifications of both Sir2 and the Lrs4/Csm1 complex also yield karyopherins, suggesting a possible mechanism for subnuclear localization of silent chromatin. Finally, rDNA-specific silencing factors Tof2 and Lrs4/Csm1 interact with two putative inner nuclear envelope proteins, Src1 and Ydl089w (Huh et al. 2003), raising the possibility that the NTS1 region of rDNA may also play a role in tethering rDNA to and positioning the nucleolus along the nuclear periphery. Notably, Src1 and Ydl089w contain putative transmembrane domains as well as LEM domains, which, are frequently found in metazoan inner nuclear envelope proteins and interact with BAF, a highly conserved metazoan protein which has roles in chromatin and nuclear organization (Bengtsson and Wilson 2004; Segura-Totten and Wilson 2004). A possible role for nuclear envelope proteins in the regulation of rDNA remains to be determined.

Lrs4 and Csm1 associate primarily with NTS1 in a Tof2- and Fob1-dependent manner and are required for silencing at NTS1

We next tested whether the previously observed presence of Lrs4/Csm1 in the nucleolus (Rabitsch et al. 2003) was due to their association with rDNA by performing ChIP assays using Lrs4-TAP and Csm1-TAP. Examples of PCR products amplified from WCE or IP material and quantifications of these data are shown in Figure 12. The results showed that Lrs4-TAP and Csm1-TAP IP sequences primarily from the NTS1 region, overlapping the RFB. A slight enrichment was also observed at the NTS2/TIR region, similar to what has been observed for Fob1 and Tof2 proteins. In the absence of *TOF2*, neither protein precipitated NTS1 sequences significantly above background, suggesting that their recruitment to this region may require physical interactions with Tof2 (Figure 12). Furthermore, *FOB1* was also required for the

localization of both subunits to NTS1, either through additional physical interactions or indirectly through the requirement for Fob1 to properly localize Tof2 to the region (Figure 12). However, in the absence of Sir2, both proteins still associated with the NTS1 region, although Csm1 associated with NTS1 less efficiently. These findings suggest that Sir2 may help stabilize Lrs4/Csm1 at this region but is not absolutely required.
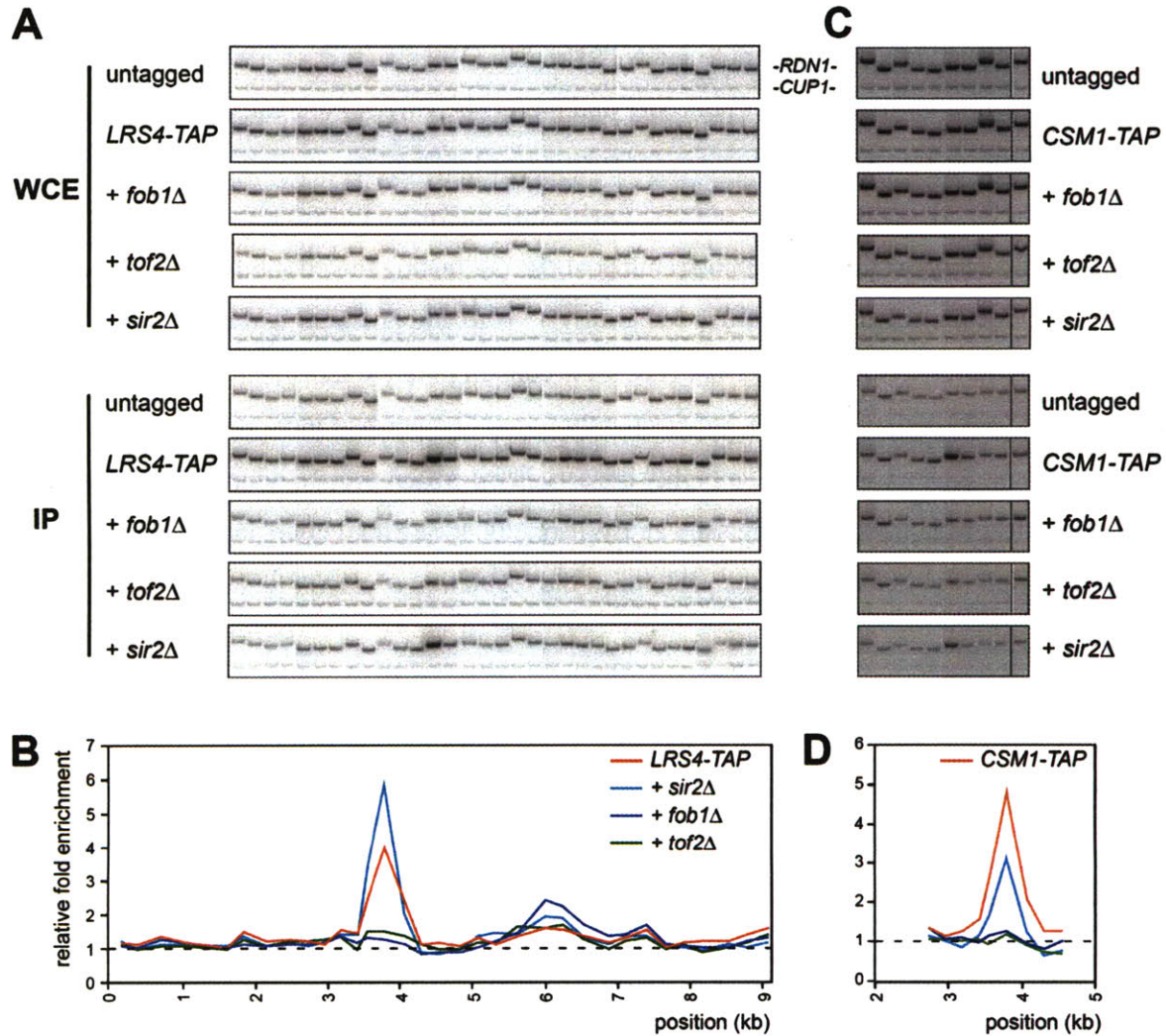

Figure 12. Lrs4-TAP and Csm1-TAP associate with NTS1 in an *FOB1*- and *TOF2*-dependent manner.

Examples of ChIP data showing PCR products amplified from WCE and IP DNA associated with Lrs4- TAP (*A*) and Csm1-TAP (*C*). Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 2–4, 6–34, and 1 (*A*) and 10–18 and 23 (*C*) are shown. Quantifications of these data are shown in *B* and *D*. Both Lrs4-TAP and Csm1-TAP associate significantly with NTS1 but not with the NTS2/35S region.

Previously, *LRS4* and *CSM1* were shown to be required for silencing using *mURA3* (in NTS2) or *MET15* (in 35S) reporter genes, respectively (Smith et al. 1999; Rabitsch et al. 2003). We tested whether *LRS4* and *CSM1* were required for silencing of the *mURA3* reporter gene integrated at either NTS1 or NTS2. We found that the NTS1 reporter was completely derepressed in both deletion mutants (Figure 13A, cf. rows 5,8 and 4,7, respectively). However, deletion of either *LRS4* or *CSM1* had only a weak effect on the NTS2/TIR reporter, which was silenced <103-fold better than the NTS1 reporter (Figure 13A, cf. rows 6,9 and 5,8). The difference in silencing defects at NTS1 compared with NTS2/TIR in these mutant backgrounds is consistent with our observations that Tof2, Lrs4, and Csm1 are primarily localized to the NTS1 region of rDNA (Figures 6, 12).

Figure 13. *LRS4* and *CSM1* are required for NTS1 silencing and unequal sister chromatid exchange.
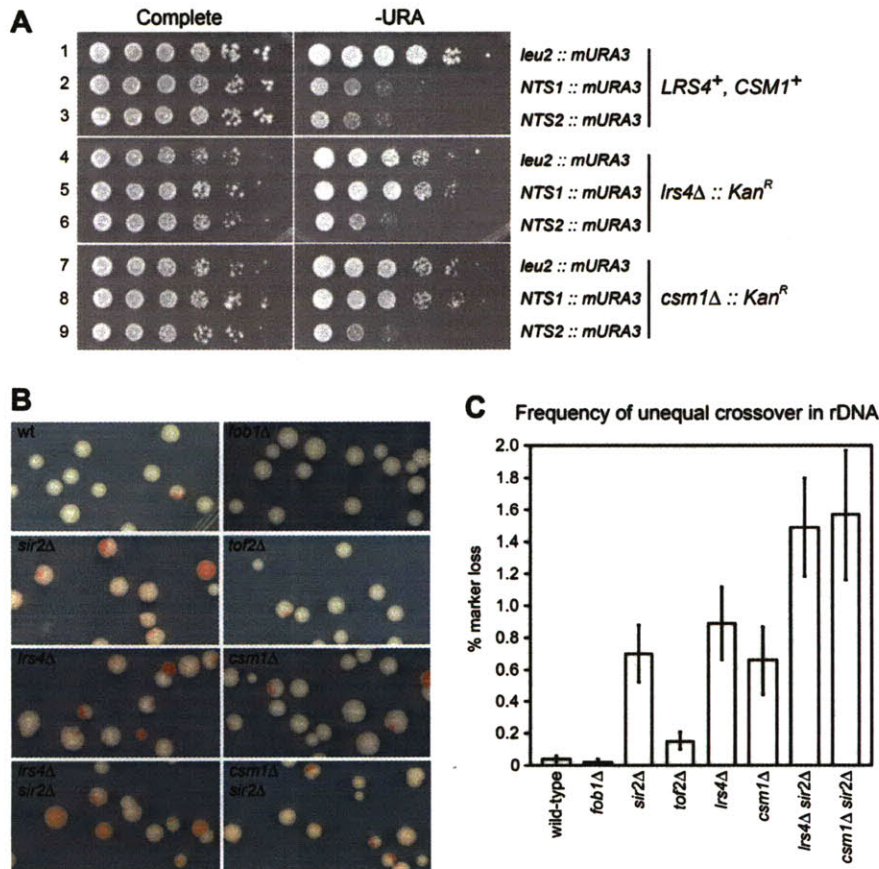
(*A*) Silencing was assayed as described in Figure 3B, and locations of reporters are shown in Figure 3A. Cells lacking *LRS4* or *CSM1* exhibit a complete loss of silencing at NTS1 but wild-type levels of silencing at NTS2.

(*B*) Unequal sister chromatid exchange is monitored by loss of the *ADE2* gene located within the rDNA array. Cells expressing *ADE2* are white, while cells lacking the *ADE2* gene are red. Half-sectored colonies represent loss of the marker during the first division upon plating. Entirely red colonies are descended from a cell that has lost the marker prior to plating.

(*C*) Unequal sister chromatid exchange is represented as percent marker loss, calculated as the ratio of half-sectored colonies to the total number of colonies, excluding entirely red colonies.

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LRS4 and _CSM1_ are required for suppression of rDNA recombination

We hypothesized that Tof2, Lrs4, and Csm1 may assemble at the NTS1 region to cooperate with the RENT complex in generating a chromatin structure that inhibits unequal recombination within the rDNA. To test this, we measured the rate of _ADE2_ marker loss from the rDNA array in cells lacking each of the NTS1-specific silencing factors, described above, as well as Sir2. Colonies in which the _ADE2_ marker has been lost accumulate a red pigment, while colonies that maintain and express _ADE2_, which is only weakly silenced in rDNA, remain white. The rate of unequal recombination or exchange is determined by the number of half-sectored colonies compared with the total number of colonies present (Kaeberlein et al. 1999). Half-sectored colonies signify a marker loss event during the first division after plating. Entirely red colonies have lost the marker prior to plating and thus were excluded from the total number of colonies. Loop-out events (recombination between repeats on the same chromosome) do not produce half-sectored colonies because the excised _ADE2_ marker lacks a centromere and is preferentially retained in the mother cell (Murray and Szostak 1983). Examples of colonies are shown in Figure 13B, and quantification of unequal crossover in rDNA is presented in Figure 13C.

As expected, deletion of _FOB1_ decreased the rate of marker loss by half compared with wild-type cells, while deletion of _SIR2_ greatly increased it (>20-fold) (Figure 13C; Kaeberlein et al. 1999). Similarly, deletion of either _LRS4_ or _CSM1_ caused dramatic increases in marker loss rate (29- and 21-fold, respectively) (Figure 13C). Combining either the _lrs4_ or _csm1_ mutation with deletion of _SIR2_ had an additive effect, with double mutants exhibiting a nearly 50-fold increase in marker loss compared with wild-type cells (Figure 13C). Thus, Lrs4/Csm1 and Sir2 may regulate recombination via independent mechanisms. However, although Tof2 was required for the association of Lrs4/Csm1 with rDNA-NTS1, the deletion of _TOF2_ caused a smaller increase in recombination rates (less than fivefold) (Figure 13C), suggesting that, like Fob1, Tof2 may be required for both rDNA recombination and silencing.

Lrs4 and Csm1 are released from the nucleolus during anaphase

The RENT complex disassembles during anaphase, releasing the phosphatase Cdc14 (as well as Sir2) from the nucleolus and promoting exit from mitosis (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999). To test whether the Lrs4/Csm1 complex is also released from rDNA during mitosis, we examined the subcellular localization of each protein throughout one complete cell cycle. Cells carrying an HA-tagged version of Lrs4 (Lrs4- 6HA) were arrested with α-factor in G1 followed by release into medium lacking pheromone. Lrs4-6HA was released from the nucleolus and became dispersed throughout the nucleus during anaphase (Figure 14A,B). We saw full release of Lrs4 and Csm1 in 40% of cells (Figure 14E), in contrast to Cdc14, which is released in all anaphase cells (Shou et al. 1999; Visintin et al. 1999). Whether Lrs4/Csm1 are retained in the nucleolus by means other than the RENT complex is not known. However, similar to what is observed for Cdc14, nucleolar localizations of Lrs4-6HA and Csm1-9Myc depended on *NET1*, as both proteins were dispersed throughout the nucleus at every stage of the cell cycle in *net1Δ* cells (Figure 14F; data not shown).

Figure 14. Lrs4-6HA is released from the nucleolus during anaphase.
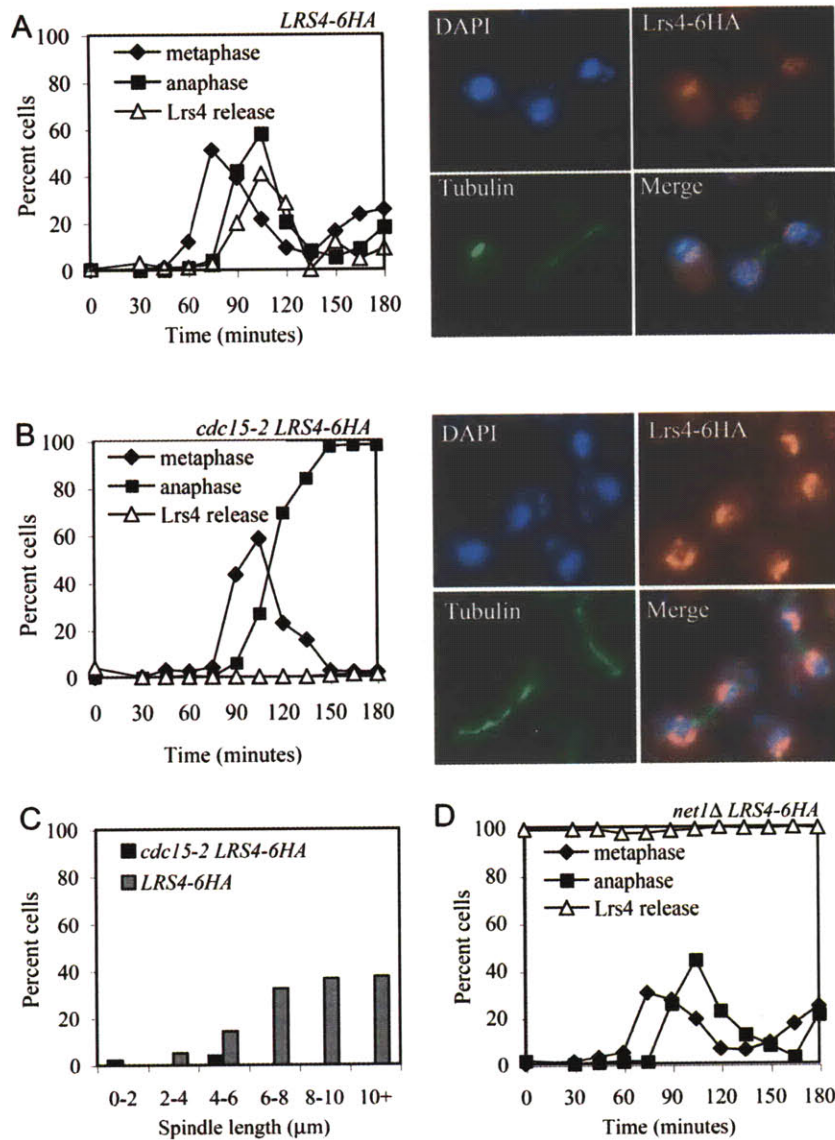
(*A*) Wild-type cells (A13838) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/mL). When arrest was complete, cells were released into YEPD medium lacking pheromone at 23°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase and anaphase spindles, as well as the percentage of cells with Lrs4-6HA released from the nucleolus.

(*B*) An example of Lrs4 release in anaphase cells. Lrs4-6HA is shown in red, microtubules in green, and DNA in blue.

(*C*) *cdc15-2* cells (A13839) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/mL). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated times, samples were taken to determine the percentages of cells with metaphase and anaphase spindles and the percentage of cells with Lrs4-6HA released from the nucleolus.

(*D*) An example of Lrs4 localization in *cdc15-2* cells. Lrs4 is shown in red, microtubules in green, and DNA in blue.
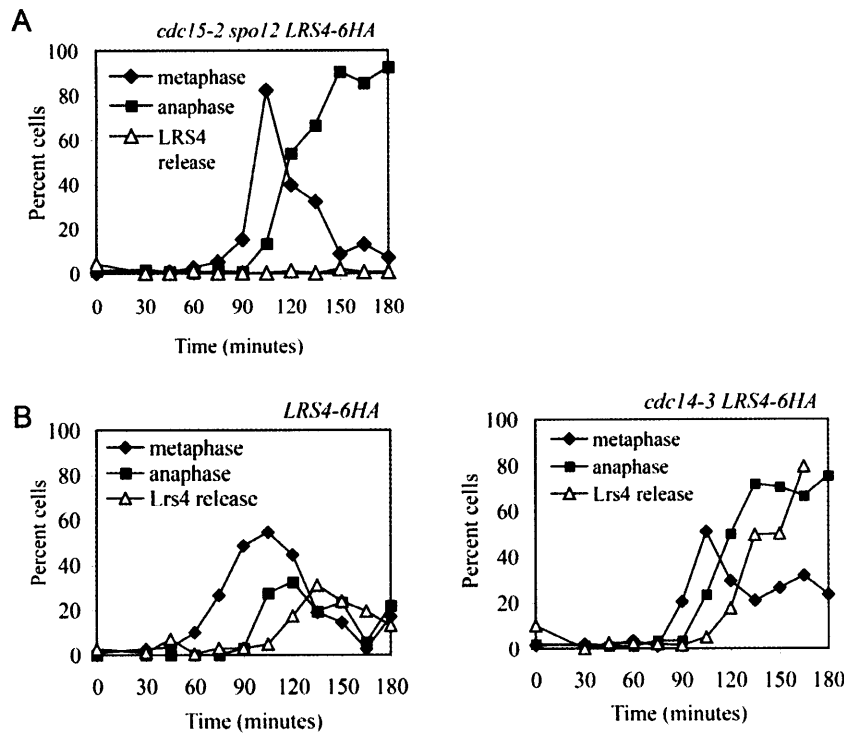
(*E*) Wild-type (A13838) and *cdc15-2* cells (A13839) were grown as described in *A*, and the localization of Lrs4- 6HA was determined with respect to the length of the mitotic spindle as described in Stegmeier et al. (2004).

(*F*) Wild-type (A13838) and *net1*Δ cells (A14568) were grown as described in *A*. At the indicated times, samples were taken to determine the percentages of cells with metaphase and anaphase spindles, and the percentage of cells with Lrs4-6HA released from the nucleolus.

We next determined whether the dispersal of Lrs4 and Csm1 required the two regulatory networks that promote Cdc14 release from the nucleolus during early and late anaphase: the Cdc14 early-anaphase release network (FEAR network) and the mitotic exit network (MEN), respectively (for review, see Stegmeier and Amon 2004). We did not detect FEAR network-dependent release of Lrs4 and Csm1 during early anaphase (Figure 15A). Furthermore, release of Csm1 and Lrs4 was in-dependent of Cdc14 (Figure 15B; data not shown). Whether this is due to Lrs4 and Csm1 release from the nucleolus being independent of the FEAR network or whether the release is too transient to be detected is at present unknown. We did find that release of Lrs4 and Csm1 depended on Cdc15, which is required for activation of the mitotic exit network (Figure 14C–E; data not shown). Therefore, we conclude that the Lrs4/Csm1 complex is released from the nucleolus during anaphase and that disassembly is regulated by the mitotic exit network.

## Figure 15: Lrs4-6HA localization in FEAR network and *cdc14* mutants.

A

cdc15-2 spo12 LRS4-6HA

- ◆ metaphase
- ■ anaphase
- ▵ LRS4 release

Percent cells / Time (minutes)

B

LRS4-6HA

- ◆ metaphase
- ■ anaphase
- ▵ Lrs4 release

Percent cells / Time (minutes)

cdc14-3 LRS4-6HA

- ◆ metaphase
- ■ anaphase
- ▵ Lrs4 release

Percent cells / Time (minutes)

(A) *cdc15-2 spo12Δ* cells (A14158) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α factor (5 µg/ml). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated times samples were taken to determine the percentages of cells with metaphase and anaphase spindles, as well as the percentage of cells with Lrs4-6HA released from the nucleolus. *SPO12* is a component of the FEAR network that is required for the release of Cdc14 from the nucleolus during early anaphase (Stegmeier et al. 2002).

(B) Wild type (A13838), *cdc14-3* (A14204), and *cdc14-1* cells (A14566) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α factor (5 µg/ml). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated times samples were taken to determine the percentages of cells with metaphase and anaphase spindles, and the percentage of cells with Lrs4-6HA released from the nucleolus.

Discussion

Our findings reveal an extensive network of protein–protein interactions that regulate rDNA silencing and recombination. In particular, several factors specifically localize to the NTS1 region of rDNA, underscoring the importance of this region as a recombination control center. The requirement for the NTS1-specific factors Tof2 and the Lrs4/Csm1 complex in suppression of rDNA recombination indicates that this region is a key target of *trans*-acting factors that regulate rDNA recombination levels. Furthermore, the association of Lrs4/ Csm1 with both chromatin (this study) and the cohesin complex (Newman et al. 2000; Graumann et al. 2004) suggests a novel mechanism for the assembly of a bridge or clamp complex that inhibits unequal sister chromatid exchange by restricting the movement of rDNA repeat units on different chromatids relative to each other (Figure 16). Below we discuss the implications of these results for the regulation of rDNA recombination and its link to the pathways that regulate mitotic exit.

Figure 16. Model for a protein bridge that inhibits recombination by unequal crossover.
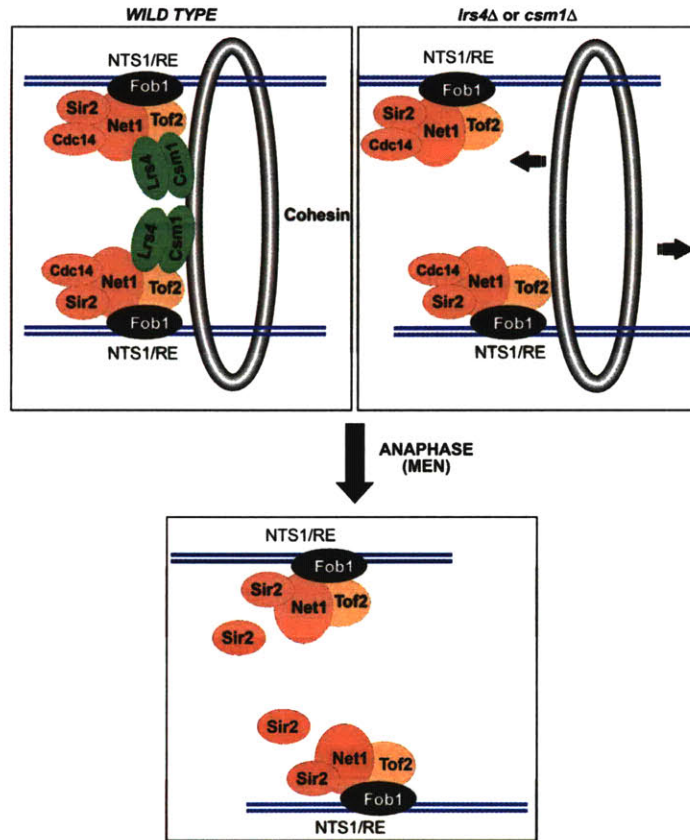
Within NTS1, RE sequences are bound by Fob1, which is required for the recruitment of the RENT complex, consisting of Net1, Sir2, and Cdc14. Fob1 also recruits Tof2, which is required for the association of Lrs4/Csm1 with RE sequences. Lrs4/Csm1 may form a protein bridge that clamps sister chromatids together, either directly or through association with cohesin. Lrs4/Csm1–cohesin association would clamp rDNA to the cohesin ring, thereby restricting the movement of sister chromatids relative to each other to inhibit unequal exchange. (*Bottom*) The bridge is disassembled during mitosis by the release of cohesin and Lrs4/Csm1. (*Right* side) In *lrs4Δ* and *csm1Δ* mutant cells, cohesin is no longer clamped to rDNA, allowing unrestricted movement of sister chromatids relative to each other.

A recombination control and pairing center at the NTS1 region of rDNA

Previously, it had been shown that the transcriptional silencing components of the RENT complex, Net1 and Sir2, associate with both the NTS1 and the NTS2 regions (Huang and Moazed 2003). Silencing factors are recruited to the NTS1 region by the replication fork-blocking protein Fob1 (Huang and Moazed 2003), which binds specifically to sequences within NTS1 that constitute a recombination hotspot (Keil and Roeder 1984; Kobayashi 2003; Mohanty and Bastia 2004). Both Fob1 and the sequences bound by it are required for all recombination within rDNA, indicating that this region is influenced by both inhibitory and stimulatory mechanisms. Affinity purifications of these regulatory factors have now identified a large network of interacting proteins that are specifically required for silencing at the NTS1 region, preferentially localize to NTS1, and collaborate to inhibit recombination. One such new factor is Tof2, which is recruited to rDNA-NTS1 via Fob1. Tof2 appears to act as an adaptor that recruits the Lrs4 and Csm1 proteins, strong negative regulators of unequal sister chromatid exchange that are required for NTS1-specific silencing. However, deletion of *TOF2* results in a smaller increase in recombination rates compared with deletion of *SIR2*, *LRS4*, or *CSM1*, suggesting that Tof2, like Fob1, may be required for stimulation of rDNA recombination as well as for the recruitment of inhibitors of recombination.

Prior studies had established a role for Lrs4 and Csm1 in the regulation of chromosome segregation during meiosis, when they form the "monopolin" complex with a meiosis-specific factor, Mam1 (Toth et al. 2000; Rabitsch et al. 2003; Lee et al. 2004). During prophase I and metaphase I, this complex localizes to centromeric regions to form a "sister chromatid clamp" that somehow bridges and co-orients sister chromatids to ensure proper segregation toward the same pole, resulting in a reductional division characteristic of meiosis I (Toth et al. 2000; Rabitsch et al. 2003). Throughout other stages of meiosis as well as mitosis, Lrs4 and Csm1 are found in the nucleolus (Rabitsch et al. 2003), but where these proteins localize within the nucleolus and how they function in rDNA silencing have been unknown. Our data show that Lrs4 and Csm1 form a stable, two-subunit complex that primarily associates with the NTS1 region. The Lrs4/Csm1 complex is recruited to this site by two NTS1-specific factors, Fob1 and Tof2, and physically associates with Tof2 and NTS1-associated RENT. Here, we have shown

that these proteins not only inhibit RNA Pol II-dependent transcription but also suppress mitotic recombination. Since Lrs4/Csm1 are present in the nucleolus throughout most of meiosis, they are likely to collaborate with Sir2 to repress meiotic recombination as well (Gottlieb et al. 1989).

Lrs4/Csm1 and Sir2 may represent two independent pathways that suppress recombination. While all three proteins are required for suppression of recombination, our data show that they can localize to the NTS1 region independently. Deletion of either *LRS4* or *CSM1* has little or no effect on the localization of Sir2 to the NTS1 region (Figure 17), suggesting that these proteins function in a step downstream from Sir2 localization to silence rDNA. Similarly, deletion of *SIR2* has little effect on Lrs4/Csm1 or Tof2 localization (Figure 12). In this model, the Tof2-dependent recruitment of Lrs4/ Csm1 to rDNA contributes to the regulation of silencing and recombination independently of Sir2 recruitment.

Figure 17. Sir2 localization to rDNA does not require *LRS4/CSM1*

(A) Examples of chromatin immunoprecipitation data showing PCR products amplified from whole-cell extract (WCE) and immunoprecipitated (IP) DNA. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 12-17 and 21-26 are shown.
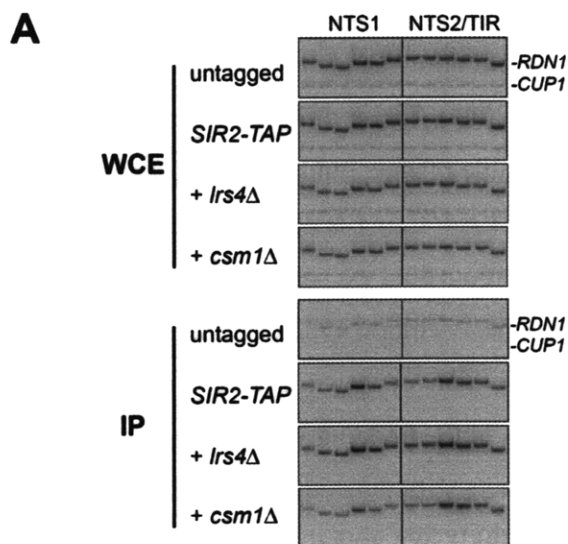
(B) Representative graph showing the association of Sir2-TAP at rDNA in wild-type (solid black line), *lrs4*D (solid gray line), or *csm1*D (dashed gray line) cells.

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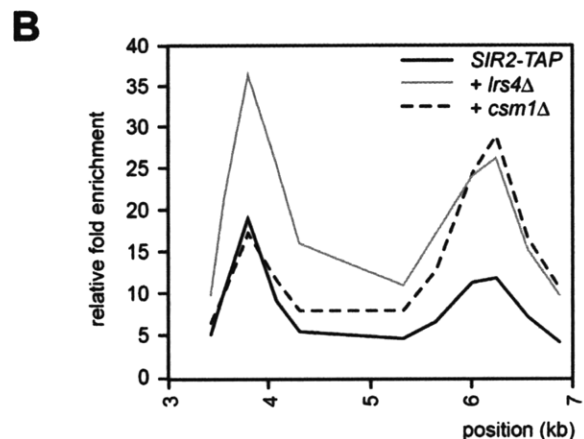
Our analysis suggests two possible mechanisms by which Lrs4/Csm1 inhibit unequal crossover (Figure 16). One possibility is that Lrs4 and Csm1 form a bridge across sister chromatids through homotypic and/or heterotypic interactions to restrict the movement of rDNA sister chromatids relative to each other. Both Lrs4 and Csm1 contain extensive coiled-coil regions that may be involved in such interactions (Newman et al. 2000; Rabitsch et al. 2003), and our purifications of Csm1 complexes contain both epitope-tagged and untagged forms of Csm1, suggesting that it can form at least homodimers. However, an Lrs4/Csm1 sister chromatid bridge on its own is unlikely to impede chromosome segregation because both proteins appear to be released from the nucleolus in late anaphase after chromosome segregation is already completed (Figure 14A; data not shown). A second possibility is that Lrs4/Csm1 physically associate with the cohesin complex to inhibit unequal crossover. The cohesin complex on its own should be unable to restrict the movement of sister chromatids relative to each other, as cohesin forms topological rings that embrace sister chromatids (Haering et al. 2002; Chang et al. 2005; Ivanov and Nasmyth 2005), but allow DNA and chromatin to move freely within the ring (Glynn et al. 2004; Lengronne et al. 2004). These findings demonstrate that cohesin does not interact with DNA or chromatin in a stable manner, and thus, we propose that the association of cohesin with chromatin-bound Lrs4/ Csm1 is necessary to "fix" two sister chromatids relative to each other and thereby inhibit unequal exchange (Figure 16). Notably, purification of the Scc1/Mcd1 subunit of the cohesin complex uncovered multiple peptides of Csm1 (Graumann et al. 2004). Furthermore, an independent two-hybrid study testing pairwise interactions between all predicted coiled-coil motifs in the budding yeast proteome showed that the coiled-coil domain of Csm1 specifically interacts with the coiled-coil region of the Smc1 subunit of the cohesin complex (Newman et al. 2000). We note that our purifications of Lrs4 and Csm1 were devoid of cohesin subunits (Figure 9D), although in some experiments we observed a weak enrichment of Scc1/Mcd1 in both Csm1 and Lrs4 immunoprecipitations (J. Huang, unpubl.). This discrepancy may be due to the transient or temporally regulated nature of the monopolin– cohesin interaction, which may only occur stably on chromatin.

An Lrs4/Csm1-cohesin clamp model is consistent with the observation that Sir2 is required for maximal association of cohesin with the NTS1 recombination enhancer region (Kobayashi et al. 2004). Previously, Sir2- dependent silencing mechanisms have been proposed to prevent

recombination by suppressing transcription from divergent RNA Pol II promoters in this region, which would otherwise promote removal of cohesin rings (Kobayashi and Ganley 2005). However, as mentioned above, the presence of cohesin alone is unlikely to prevent unequal sister chromatid exchange, and therefore, Lrs4/Csm1 may be required to clamp rDNA to the cohesin ring. Recruitment of cohesin may be a feature that the rDNA-NTS1 region shares with other heterochromatin- like domains, such as silent chromatin at the budding yeast mating type loci (Chang et al. 2005) and heterochromatin surrounding fission yeast centromeres (Bernard et al. 2001; Nonaka et al. 2002).

Relationship between rDNA silencing and cell cycle proteins

 The first connection between rDNA silencing and the cell cycle came from the discovery that the Net1 protein is responsible for the recruitment of Sir2 to rDNA as well as the sequestration of Cdc14 in the nucleolus to prevent premature exit from anaphase during mitosis or anaphase I during meiosis (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999; Buonomo et al. 2003; Marston et al. 2003). However, it is unclear why proteins that regulate the cell cycle and rDNA silencing form subunits of the same complex and assemble together on rDNA chromatin. We suggest that clamping of sister chromatids in close alignment from S phase until the onset of anaphase must be coordinated with DNA replication such that, as new daughter DNA strands emerge, corresponding repeat units are held together in register (Figure 16). In this model, the cohesin component of the clamp is released from chromosomes at the onset of anaphase, while the release of Lrs4/Csm1 occurs later in anaphase after the completion of chromosome segregation. The release of Lrs4/Csm1 during mitosis may be required to allow a resetting of the Lrs4/Csm1-cohesin clamp during S phase.

Although some rDNA silencing and cell cycle factors are preferentially associated with NTS1, nearly all also localize to the NTS2 region. For example, both Fob1 and Tof2 localize to NTS2, although neither is required for silencing there. The requirements for the association of RENT and other factors with these two regions are likely to be distinct. For example, the association of RENT subunits and other factors with NTS1 is Fob1-dependent, unlike association with NTS2. Moreover, in the absence of *FOB1*, the kinetics of Cdc14 release from the nucleolus is not

affected (Stegmeier et al. 2004), suggesting that a Fob1-independent mechanism(s) maintains nucleolar sequestration of Cdc14 and prevents premature exit from mitosis. The NTS2/35S region may serve as a secondary binding site for regulatory factors such as Cdc14 and Lrs4/Csm1 if the NTS1 site is perturbed during either mitosis or meiosis. Since Lrs4/Csm1 ensure a reductional division of chromosomes only during meiosis I by dynamic relocalization to centromeres (Toth et al. 2000; Rabitsch et al. 2003), inappropriate loss of association with rDNA may be catastrophic. Thus, nucleolar sequestration of Lrs4 and Csm1 and meiosis-specific expression of the third monopolin subunit Mam1 may be redundant mechanisms, one spatial and one temporal, to ensure the fidelity of chromosome segregation.

Materials and methods

Yeast strains and plasmids

Yeast strains are listed in Table 2. *NET1*, *FOB1*, *TOF2*, *LRS4*, *and CSM1* genes were deleted or modified with the C-terminal TAP, MYC13, HA3, or GFP epitope tags as described (Longtine et al. 1998; Rigaut et al. 1999; Huang and Moazed 2003). The *TOF2* gene was modified with the C-terminal HA3 tag by integration of plasmid pDM240.

The m*URA3* gene contains the *TRP1* promoter followed by the *URA3* open reading frame (Smith and Boeke 1997). Plasmids for integrating the NTS1 and NTS2 m*URA3* reporters and reporter yeast strains have been previously described (Huang and Moazed 2003). All transformations were performed with the lithium acetate method (Guthrie and Fink 1991), and proper integration was confirmed by PCR. Telomeric silencing strains were a gift from A. Rudner.

pDM240 was constructed by using primers DM267 (CGG GGT ACC TTG CCA ATG CTG GGA AAC) and DM268 (GAT GCG GCC GCC CTG GTC GTC TTC ATC ACT) to amplify a 0.5 kb *Asp*718-*Eag*I fragment of *TOF2* from genomic DNA. This fragment was ligated into the Yplac111d vector to generate pDM240, which was cut with *Msc*I to integrate at *TOF2*. pDM749 (*pCEN-TOF2-HIS3*) was constructed by ligation of a ~2.8 kb *Xho*I-*Eag*I PCR product containing the *TOF2* gene into pRS313 (*pCEN-HIS3*). The *TOF2* gene was amplified from genomic DNA

using primers JH362 (TAC ctc gag TTT CCG GGA AAA CAT GTC) and JH363 (ATT cgg ccg ATA TGG TTG AGA GAT CCC).

Table 2. Yeast strains

Strain	Genotype	Reference
SF1	JRY2334, *Mat a ade2-1 can1-100 his3-11 leu2-3.112 trp 1 ura3-1 GAL*	J. Rine
SF3	SF1 *sir2Δ::HIS3*	J. Rine
DMY631	SF1 *NET1-HA3-LEU2*	Huang and Moazed 2003
DMY1427	W303a *NET1-GFP-KAN^R*	This work
DMY2733	SF1 *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2735	DMY631 (*NET1-HA3-LEU2*) with *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2737	DMY633 (*sir2Δ::HIS3, NET1-HA3::LEU2*) with *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2889	SF1 *TOF2-HA3-LEU2*	This work
DMY2893	DMY1427 (*NET1-GFP-KAN^R*) with *TOF2-HA3-LEU2*	This work
DMY2909	SF3 (*sir2Δ::HIS3*) with *NET1-GFP-KAN^R* and *TOF2-HA3-LEU2*	Huang and Moazed 2003
DMY2946	DMY2889 (*TOF2-HA3-LEU2*) with *FOB1-MYC13-KAN^R*	This work
DMY2798	W303a *leu2::mURA3*	This work
DMY2804	W303a *RDN1-NTS1::mURA3*	This work
DMY2800	W303a *RDN1-NTS2::mURA3*	This work
DMY2845	DMY2798 (*leu2::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2847	DMY2804 (*RDN1-NTS1::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2849	DMY2800 (*RDN1-NTS2::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2827	DMY2798 (*leu2::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2835	DMY2804 (*RDN1-NTS1::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2831	DMY2800 (*RDN1-NTS2::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2982	DMY2798 (*leu2::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2983	DMY2804 (*RDN1-NTS1::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2984	DMY2800 (*RDN1-NTS2::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2987	DMY2798 (*leu2::mURA3*) with with *TOF2-TAP-K.l-TRP1*	This work
DMY2988	DMY2804 (*RDN1-NTS1::mURA3*) with *TOF2-TAP-K.l-TRP1*	This work
DMY2989	DMY2800 (*RDN1-NTS2::mURA3*) with *TOF2-TAP-K.l-TRP1*	This work
DMY3143	DMY2798 (*leu2::mURA3*) with *lrs4Δ::KAN^R*	This work
DMY3145	DMY2804 (*RDN1-NTS1::mURA3*) with *lrs4Δ::KAN^R*	This work

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DMY3147	DMY2800 (*RDN1-NTS2::mURA3*) with *lrs4Δ::KAN*[R]	This work
DMY3149	DMY2798 (*leu2::mURA3*) with *csm1Δ::KAN*[R]	This work
DMY3151	DMY2804 (*RDN1-NTS1::mURA3*) with *csm1Δ::KAN*[R]	This work
DMY3153	DMY2800 (*RDN1-NTS2::mURA3*) with *csm1Δ::KAN*[R]	This work
DMY2895	W303a *adh4::URA3*	A. Rudner
DMY2896	W303a *TELVIIL::URA3*	A. Rudner
DMY2841	DMY2985 (*adh4::URA3*) with *sir2Δ::KAN*[R]	This work
DMY2839	DMY2986 (*TELVIIL::URA3*) with *sir2Δ::KAN*[R]	This work
DMY2897	DMY2985 (*adh4::URA3*) with *fob1Δ::KAN*[R]	This work
DMY2899	DMY2986 (*TELVIIL::URA3*) with *fob1Δ::KAN*[R]	This work
DMY2901	DMY2985 (*adh4::URA3*) with *tof2Δ::KAN*[R]	This work
DMY2903	DMY2986 (*TELVIIL::URA3*) with *tof2Δ::KAN*[R]	This work
DMY3010	W303a *RAD5+* with *RDN1::ADE2*	L. Guarente
DMY3011	DMY3010 (*RDN1::ADE2*) with *sir2Δ::TRP1*	L. Guarente
DMY3012	DMY3010 (*RDN1::ADE2*) with *fob1Δ::URA3*	L. Guarente
DMY3022	DMY3010 (*RDN1::ADE2*) with with *tof2Δ::KAN*[R]	This work
DMY3200	DMY3011 (*RDN1::ADE2, sir2Δ::TRP1*) with *lrs4Δ::KAN*[R]	This work
DMY3202	DMY3011 (*RDN1::ADE2, sir2Δ::TRP1*) with *csm1Δ::KAN*[R]	This work
SF10	BJ5459, *Mat a ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*	E. Jones
DMY1690	SF10 *NET1-TAP::K.l-TRP1*	Huang and Moazed 2003
DMY1704	SF10 *SIR2-TAP::K.l-TRP1*	Hoppe et al 2002
DMY3173	DMY1704 (SF10 *SIR2-TAP::K.l-TRP1*) with *tof2Δ::KAN*[R]	This work
DMY2334	SF10 *FOB1-TAP::K.l-TRP1*	Huang and Moazed 2003
DMY2883	SF10 *TOF2-TAP::K.l-TRP1*	This work
DMY2924	DMY2883 (*TOF2-TAP::K.l-TRP1*) with *fob1Δ::KAN*[R]	This work
DMY3163	DMY2883 (*TOF2-TAP::K.l-TRP1*) with *sir2Δ::KAN*[R]	This work
DMY3047	SF10 *LRS4-TAP::K.l-TRP1*	This work
DMY3051	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *fob1Δ::KAN*[R]	This work
DMY3053	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *tof2Δ::KAN*[R]	This work
DMY3165	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *sir2Δ::KAN*[R]	This work

DMY3049	SF10 *CSM1-TAP::K.l-TRP1*	This work
DMY3055	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *fob1Δ::KAN^R*	This work
DMY3057	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *tof2Δ::KAN^R*	This work
DMY3167	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *sir2Δ::KAN^R*	This work
A13838	W303, MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, LRS4-6HA*	This work
A13839	A13838 with *cdc15-2*	This work
A14158	A13839 with *spo12Δ*	This work
A14204	A13838 with *cdc14-3*	This work
A14566	A13838 with *cdc14-1*	This work
A14568	A13838 with *net1Δ*	This work

Purification and identification of native complexes

Cells were grown at 30°C to late log phase (optical density at 600 nm of ~4.0) in YEP media containing 4% glucose. Cells were harvested, washed once with water, and frozen in liquid nitrogen. Approximately 8-15 g of frozen cells was combined with an equal volume of 2X ice cold buffer L (12 mM Na_2HPO_4, 8 mM $NaH_2PO_4 \cdot H_2O$, 0.2% NP-40, 300 mM NaCl, 4 mM EDTA, 2 mM EGTA, 100 mM NaF, 0.2 mM Na_3VO_4, 40 mM β-mercaptoethanol, 2 mM PMSF, 4 mM benzamidine, and 2 mM each of leupeptin, bestatin, and pepstatin). All subsequent steps were performed at 4°C unless stated otherwise.

An equal volume of cold glass beads was added to the lysate, and the mixture was bead-beat for ten pulses of 10 sec each in a small chamber bead-beater (BioSpec Products Inc.). The extract was centrifuged at 30,000 g for 25 min, and the supernatant was incubated with 300 μl of a 50% slurry of pre-washed IgG-sepharose beads (GE) for 2 to 3 hrs. Beads were transferred to a Poly-Prep chromatography column (BioRad) and washed three times with 10 ml each of buffer W (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, and 1mM DTT), followed with one wash with 10 ml of TEV-C buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 5% glycerol, and 1mM DTT). Beads were washed with 200 μl of TEV-C buffer

containing 5 μg/ml HIS6-TEV protease purified from E. coli, followed by an overnight incubation with 1 ml of TEV-C buffer containing 5 μg/ml TEV protease.

After cleavage, eluate was transferred to a new PolyPrep column and combined with two 1 ml washes of the IgG-sepharose beads with TEV-C buffer. To the TEV cleavage eluate and washes, 6 ml of binding buffer CAM-B (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 5% glycerol, and 10 mM β-mercaptoethanol), 9 μl of 1M $CaCl_2$, and 250 μl of a 50% slurry of pre-washed calmodulin-sepharose beads (GE) was added and nutated for 2 to 3 hr. The beads were washed three times each with 1.5 ml of CAM-B buffer and eluted as five 250 μl fractions with elution buffer CAM-E (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 10 mM EGTA, 5% glycerol, and 10 mM β-mercaptoethanol). Ten percent of the peak fraction was run on a 10-20% SDS-PAGE gradient gel and silver stained. Half of the peak fraction was precipitated in 20% TCA on ice for 20 min and centrifuged at maximum speed at 4°C for 20 min. The pellet was washed with cold (-20°C) acetone, centrifuged at 4°C for 30 min, and air-dried. Mixture mass spectrometry analysis was performed on 5 to 10% of precipitated samples as previously described (Tanny et al. 2004).

Silencing assays

Silencing assays were performed as described (Huang and Moazed 2003). Cells lacking *LRS4*, *CSM1*, or *TOP1* were plated in parallel with wild-type strains but were photographed later due to slower growth compared to wild-type strains. We observed that *tof2Δ* cells consistently formed smaller colonies on –URA medium compared with wild-type or *sir2Δ* cells. Telomeric silencing was assayed by plating cells onto synthetic complete (SC) or SC supplemented with 0.8g/L 5-FOA.

Immunofluorescence microscopy

Immunofluorescence assays were performed essentially as described (Guthrie and Fink 1991). Images were collected and processed using a Nikon Eclipse 80i upright microscope and MetaMorph (Version 6.0) software at the Nikon Imaging Center at Harvard Medical School.

Five milliliter cultures were grown in liquid YEPD at 30°C to an optical density at 660 nm (OD_{660}) of 0.5, fixed by adding 0.7 ml of 37% formaldehyde for 1 hr. Cells were washed twice with water and resuspended in 1 ml SP buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.0). Cells were spheroplasted for 15-30 min in 1 µl β-mercaptoethanol and 20 µl of lyticase (1mg/ml in 1M sorbitol) per 0.5 ml of cells. Cells were washed with 1 ml of SP and resuspended in 0.5-1 ml of SP. Fifteen microliters of cell suspension was adhered to each well of pre-coated 10-well slides (Polysciences, Inc.) for 5 min. Wells were aspirated, washed three times with PBS, and dried for 10 min at room temperature. Slides were pre-coated with polylysine by rinsing with water and drying, followed by incubation with 15 µl of polylysine (1 mg/ml) per well for 10 min at room temperature. After aspiration of excess polylysine, slides were dried, rinsed with water, and incubated at 37°C for 15 min.

Wells were blocked and permeabilized with 15 µl of blocking buffer (PBS with 1% BSA and 0.1% Triton X-100) for 1 hr, followed by four washes with PBS. Primary antibodies in antibody binding buffer (1% BSA in PBS) were spun for 15 min at 13K and incubated with cells for 1 hr at room temperature or overnight at 4°C (15 µl per well), followed by three washes with PBS. Mouse anti-Nop1 (gift from P. Silver), mouse anti-HA (HA11, BabCO), and rabbit anti-GFP (gift from A. Rudner) were used at 1:1000, 1:1000, and 1:5000 dilutions, respectively. Cells were incubated with secondary antibodies for 1-2 hrs in the dark. FITC-conjugated goat anti-rabbit or Texas Red Rhodamine-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Labs) were used at 1:500 dilutions in antibody binding buffer. Wells were washed three times with PBS and twice with water, followed by incubation with 15 µl DAPI (1 ng/ml) for 5 min at room temperature. Wells were washed once with water and covered by a coverslip after addition of mounting media and sealed with clear nail polish.

Indirect in situ immunofluorescence methods and antibody concentrations for Lrs4-6HA nucleolar release assays were as previously described (Visintin et al. 1999; Stegmeier et al. 2004).

Immunoprecipitation assays

Assays were performed essentially as described (Straight et al. 1999). Fifty-milliliter cultures of yeast cells were grown to an optical density at 600 nm (OD_{600}) of 1.5-1.8. Cells were harvested, washed once with cold TBS (20 mM Tris-HCl at pH 7.6 and 150 mM NaCl), and frozen at −80°C. Cell pellets were resuspended in 400 µl of lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM EDTA, 2 mM Benzamidine, 1 mM PMSF, and 1 µg/ml each of pepstatin, leupeptin, and bestatin), and bead-beat with glass beads (beads and Mini Beadbeater, Biospec Products) twice for 30 sec. Lysates were centrifuged at 13,000 rpm for 5 and 15 min. Clarified extract was incubated with 1 µg of rabbit anti-GFP antibody (gift from A. Rudner), mouse anti-HA (HA11, BabCO), or mouse anti-Myc (9E10) at 4°C for 2 h. Thirty microliters of a 50% slurry of pre-washed ProteinA Sepharose beads (GE) was added and incubated for an additional for 2 h. Beads were washed once with 1 ml of lysis buffer, twice with 1 ml wash buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, and 1 mM EDTA), and resuspended in 2X SDS sample buffer. One percent of input whole-cell extract or 25% of bound fractions was run on 4-12% gradient gels (NuPage, Invitrogen) or 8% SDS-PAGE gels and blotted to nitrocellulose membranes for Western analysis. Membranes were probed using 1:5000 dilutions of rabbit anti-Sir2, mouse anti-Myc (9E10), and mouse anti-HA (HA.11) and 1:10,000 dilution of mouse anti-Act1 antibodies in TBS with 0.1% Tween-20 and 5% milk.

Whole cell protein analysis

Seven hundred microliters of a saturated culture was harvested by centrifugation, resuspended in 150 µl of 1.5X SDS sample buffer supplemented with 2 mM PMSF and 5 mM benzamidine, and bead-beat with glass beads (beads and Mini Beadbeater, Biospec Products) twice for 90 sec. Lysates were centrifuged briefly at 13,000 rpm and heated at 95°C for 5 min. Five microliters of sample was run on an 8% SDS-PAGE gel and blotted to nitrocellulose for Western analysis. Sir2 and Act1 proteins were detected using 1:5000 and 1:10,000 dilutions of rabbit anti-Sir2 and mouse anti-Act1 (Chemicon International) antibodies, respectively, in TBS with 0.1% Tween-20 and 5% milk.

ChIP assays

ChIP assays and quantification of the data were performed essentially as previously described (Huang and Moazed 2003). Relative fold enrichment was determined by calculating the ratio of rDNA to *CUP1* enrichment in the IP material and comparing this with the ratio of rDNA to *CUP1* enrichment in the WCE material. This is represented in the following calculation: [rDNA(IP)/*CUP1*(IP)/rDNA(WCE)/*CUP1*(WCE)]. *CUP1* is a repetitive, non-silenced locus that serves as a negative control and a control for PCR efficiency. In Figure 6, the amount of *CUP1* sequences in the immunoprecipitated material was below the linear range of *RDN1* quantitation, and the *CUP1* value used was an average of *CUP1* values from all of the multiplex PCR reactions for each yeast strain within a single experiment.

Table 3. ChIP primer sets

RDN1 (5′→3′)

1)	AAAAGAAACCAACCGGGATT	CCACCCACTTAGAGCTGCAT
2)	GGGAATGCAGCTCTAAGTGG	ATGGATTTATCCTGCCACC
3)	TGCGACGTAAGTCAAGGATG	CTGGCTTCACCCTATTCAGG
4)	TCCCTCAGGATAGCAGAAGC	GTGGTGTCTGATGAGCGTGT
5)	CCGAATGAACTAGCCCTGAA	CGACTAACCCACGTCCAACT
6)	AAAGGTTCCACGTCAACAGC	AGCCATAAGACCCCATCTCCG
7)	ATCCGGAGATGGGGTCTTAT	CTGACCAAGGCCCTCACTAC
8)	TTGTAGACGGCCTTGGTAGG	ATGACGAGGCATTTGGCTAC
9)	CTAGCGAAACCACAGCCAAG	AATGTCTTCAACCCGGATCA
10	ATTGTCAGGTGGGGAGTTTG	TGTCGCTATGAACGCTTGAC
11)	TGGCAGTCAAGCGTTCATAG	CAGCCGCAAAAACCAATTAT
12)	TAATTGGTTTTTGCGGCTGT	ATGATTTATCCCCACGCAAA
13)	TTTGCGTGGGGATAAATCAT	CATGTTTTTACCCGGATCAT
14)	CCGGGGCCTAGTTTAGAGAG	ACCCATCTTTGCAACGAAAA
15)	AGGGCTTTCACAAAGCTTCC	TCCCCACTGTTCACTGTTCA
16)	TGATGATGGCAAGTTCCAGA	CTTATTCCTTCCCGCTTTCC
17)	GGAAAGCGGGAAGGAATAAG	CGATTCAGAAAAATTCGCACT
18)	GTGCGAATTTTTCTGAATCG	CCCTCATATCACCTGCGTTT
19)	GAGGTGTTATGGGTGGAGGA	GCCACCATCCATTTGTCTTT
20)	TGCAAAAGACAAATGGATGG	GCACCTTTTCCTCTGTCCAC
21)	AGAGGAAAAGGTGCGGAAAT	TTTCTGCCTTTTTCGGTGAC
22)	GTTGGTTTTGGTTTCGGTTG	TCGCCGAGAAAAACTTCAAT
23)	GGGAGGTACTTCATGCGAAA	AAGATGCCCACGATGAGACT
24)	AGTCTCATCGTGGGCATCTT	TCCGTCACCATACCATAGCA
25)	GGCAGCAGAGAGACCTGAAA	GAGCCATTCGCAGTTTCACT
26)	TCGACCCTTTGGAAGAGATG	GCCTTCCTTGGATGTGGTAG
27)	AAACGGCTACCACATCCAAG	GGCCCAAAGTTCAACTACGA

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28)	CCTTGAGTCCTTGTGGCTCT	TGAAAACGTCCTTGGCAAAT
29)	GGGGATCGAAGATGATCAGA	TTGTGTCTGGACCTGGTGAG
30)	CTCACCAGGTCCAGACACAA	CCAGAACGTCTAAGGGCATC
31)	AGCCAGCGAGTCTAACCTTG	TTGTCCAAATTCTCCGCTCT
32)	TGTTTTGGCAAGAGCATGAG	CTCGAATGCCCAAAGAAAAA
33)	GGCCCAGAGGTAACAAACAC	GGAAATGACGCTCAAACAGG
34)	CTGGCCTTTTCATTGGATGT	ATCCCGGTTGGTTTCTTTTC

CUP1 (5′→3′)

TGAAGGTCATGAGTGCCAAT   TTCGTTTCATTTCCCAGAGCA

Unequal sister chromatid exchange assays

Assays were performed as previously described (Kaeberlein et al. 1999). Cells were grown to an OD_{600} of 0.4-0.8, sonicated briefly to prevent aggregation, and plated at a density of ~400 cells per SC plate. Cells were incubated at 30°C for 2-5 days and transferred to 4°C for 1-3 days to enhance color development. The unequal sister chromatid crossover rate was calculated by dividing the number of half-red/half-white colonies by the total number of colonies. Red colonies were excluded from all calculations. At least 12,000 colonies total from 3-5 independent isolates were examined for each genotype except for *csm1Δ sir2Δ*, for which at least 8,500 colonies were counted.

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Chapter 3

Condensins collaborate with the monopolin complex to promote co-orientation of sister chromatids in budding yeast.

Ilana L. Brito, Fernando Monje-Casas, Hong-Guo Yu and Angelika Amon

All experiments performed by I. L. B. except the experiment depicted in Figure 8B.

Abstract

rDNA maintenance and segregation is, in part, regulated by monopolin complex components Lrs4 and Csm1, and condensins, protein complexes required for chromosome condensation. We show here that these two complexes also collaborate at kinetochores to control both mitotic and meiotic chromosome segregation. Lrs4 and Csm1 are important for chromosome segregation fidelity in mitosis and, together with condensin, associate with kinetochores during anaphase. During meiosis, condensins, like the monopolin complex, are needed for faithful co-orientation of sister chromosomes during the first meiotic division. In the absence of condensin function, sister kinetochores are bi-oriented during meiosis I and association of the monopolin complex subunit Mam1 with kinetochores is decreased. Our studies indicate that condensins and Lrs4-Csm1 function together to provide linkages between sister chromatids at specialized chromosomal locations.

Introduction

Meiosis is a cellular division consisting of a single DNA synthesis phase followed by two chromosome segregation phases and employed in the generation of gametes. During the first meiotic division, homologous chromosomes segregate, requiring that each pair of sister chromatids co-segregate towards one pole (co-orientation); during the second meiotic division, sister chromatids separate towards opposite poles (bi-orientation). In budding yeast, the monopolin complex is crucial for co-orienting sister chromatid kinetochores to allow only one microtubule attachment per pair of sisters (Winey et al., 2005; reviewed in Marston and Amon, 2004). The monopolin complex is composed of four components: Mam1, expressed only during meiosis, localizes to kinetochores from late pachytene until metaphase I (Toth et al, 2000); Lrs4 and Csm1, two nucleolar components which are released from the nucleolus during prophase I and targeted to kinetochores by the polo-like kinase Cdc5 (Clyne et al, 2003; Lee and Amon, 2003; Rabitsch et al, 2003); and Hrr25, a casein kinase (Petronczki et al, 2006). The monopolin complex is thought to clamp sister kinetochores through a cohesin-independent mechanism and

fuse the two sister kinetochores into a single microtubule attachment site to facilitate co-orientation (Monje-Casas et al., 2007). Deletion of the monopolin complex results in the bi-orientation of sister chromatids during meiosis I (Toth et al., 2000; Rabitsch et al, 2003; Lee and Amon, 2003; Petronczki et al., 2006).

During the mitotic cell cycle, the mitotically expressed monopolin complex subunits Lrs4 and Csm1 are involved in linking sister chromatids at the rDNA locus. Lrs4 and Csm1 reside in the nucleolus where they are part of the RENT (Regulator of nucleolar silencing and telophase exit) complex that binds to the replication fork barrier site (RFB) within the non-transcribed region NTS1 in the ribosomal DNA repeat. This complex promotes rDNA segregation, mediates transcriptional silencing and prevents unequal sister chromatid exchange within the repetitive rDNA array, by presumably joining sister chromatid rDNA repeats (Waples et al., 2009; Huang et al., 2006). Lrs4 and Csm1 together with another RENT complex subunit, Tof2, bind to and recruit condensins to the rDNA, where they participate in rDNA segregation (Johzuka and Horiuchi, 2009). The condensin complex is a conserved pentameric complex best known for its role in chromosomal compaction (reviewed in Hirano, 2005). Like the RENT complex, condensin is also required for rDNA maintenance, gene silencing within the rDNA, and rDNA segregation (Freeman, 2000; Lavoie et al., 2002; D'Amours et al. 2004), which has led to the idea that Lrs4 and Csm1 regulate rDNA functions by recruiting condensins to the rDNA.

During mitosis, Lrs4 and Csm1 are not only located at the rDNA. At late anaphase, the Mitotic Exit Network, a signaling pathway that triggers exit from mitosis by promoting the release of the protein phosphatase Cdc14 from the nucleolus, also promotes the dissociation of Lrs4 and Csm1 from the rDNA and their dispersal throughout the nucleus (Huang et al., 2006). The mitotic roles of Lrs4 and Csm1 once they are released from the nucleolus are not understood. We show here that Lrs4 and Csm1 associate with kinetochores upon their release from the nucleolus during anaphase. Condensins are also not only enriched at the rDNA but are found to accumulate at kinetochores in budding yeast (Wang et al., 2005; D'Ambrosio et al., 2008) and in fission yeast too (Nakazawa et al., 2008) raising the possibility that the two complexes may also function together at this chromosomal location. We find that this is indeed the case. Condensins and the monopolin complex collaborate at centromeric regions to facilitate key aspects of mitotic and

meiotic chromosome segregation. During mitosis, Lrs4, Csm1 and condensins are required for accurate chromosome segregation. Our studies of the function of the monopolin complex and condensins during meiosis uncovered a requirement for condensin in co-orienting sister chromatids during meiosis I by promoting the localization of Mam1 to kinetochores. Our results suggest that condensins and monopolins form a higher order complex whose function is to link sister chromatids. At the rDNA, this is necessary to prevent unequal exchange between sister chromatids; at meiotic kinetochores, it facilitates co-orientation of sister kinetochores.

Results

**The monopolin complex localizes to kinetochores during anaphase of mitosis.**

Our previous studies showed that during mitosis, Lrs4 and Csm1 become released from the nucleolus during anaphase (Huang et al. 2006). To determine whether, as in meiosis, the mitotic monopolin complex localized to kinetochores after its release from the nucleolus, we analyzed Lrs4 localization on chromosome spreads prepared from cells progressing through the cell cycle in a synchronous manner. To identify kinetochores in these cells, cells carried a GFP-tagged version of the kinetochore component Ndc80, in addition to an epitope-tagged version of Lrs4 (Lrs4-6HA.) We found that, during anaphase, a portion of Lrs4 molecules remained associated with the rDNA as judged by Lrs4-6HA localization between the DNA lobes of anaphase cells (note that the rDNA is one of the last genomic regions to segregate; Figure 1B). Interestingly, a fraction of Lrs4 was also found to co-localize with Ndc80-GFP (Figure 1B). This localization appeared to only occur during anaphase, when a fraction of Lrs4 is released from the nucleolus (Figures 1A-C). Lrs4 and Csm1 nucleolar localization was found to be interdependent (Figure 2A, D, E) and Lrs4 protein expression required *CSM1* (Figure 2B).
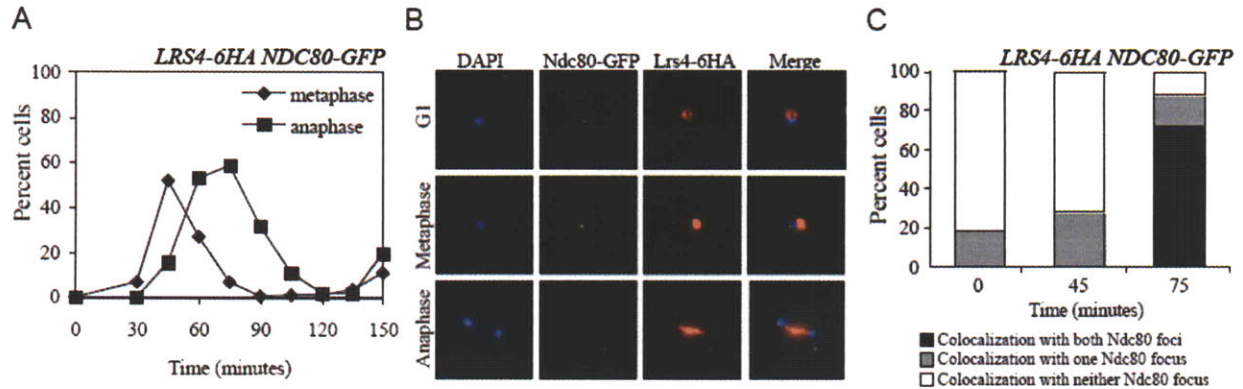
Figure 1: Lrs4 and Csm1 localize to kinetochores during mitotic anaphase.

(A - C) Wild-type cells carrying an Lrs4-6HA and an Ndc80-GFP fusion (A15127) were arrested in G1 using α-factor pheromone (5μg/ml) and released into medium lacking the pheromone at 25°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase (squares) spindles (A) and the percentage of cells showing co-localization of Lrs4-6HA with both, one or neither Ndc80-GFP-marked spindle pole body (C).

The micrographs in (B) show Ndc80-GFP (green) and Lrs4-6HA (red) localization on chromosome spreads at 0, 45 and 75 minutes after release from the G1 arrest. DNA is shown in blue.

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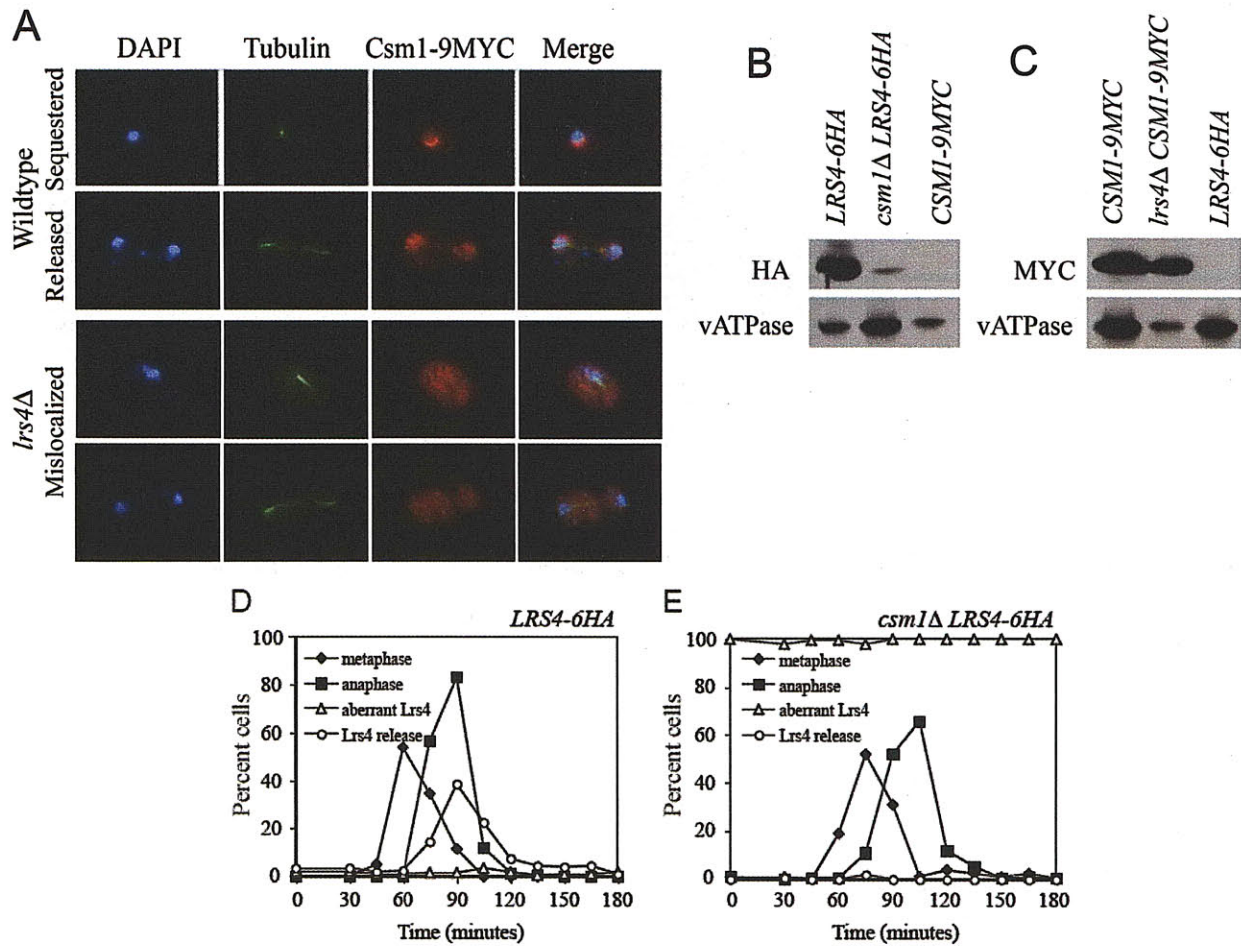
**Figure 2: Lrs4 and Csm1 localization are interdependent.**

(A) The micrographs show examples of localization of Csm1-9MYC (red) and tubulin (green) in wild-type cells (A15087) (top panels) and *lrs4Δ* cells (A15976) (bottom panels). DNA is shown in blue. The uppermost row shows nucleolar sequestration during G1, whereas the second row shows nuclear release during anaphase.

(B) Levels of Lrs4-6HA in cycling cultures of wild-type (A13838) and *csm1Δ* (A15974) cells at 25°C. vATPase was used as a loading control.

(C) Levels of Csm1-9MYC in cycling cultures of wild-type (A15087) and *lrs4Δ* (A15976) cells at 25°C. vATPase was used as a loading control.

(D-E) Wild-type (A13838; D) and *csm1Δ* (A15974; E) cells carrying an Lrs4-6HA fusion were released from a pheromone-induced G1 arrest at 25°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase spindles (squares) and the percentage of cells showing the release of Lrs4-6HA from the nucleolus (open circles) or aberrant Lrs4-6HA localization (open triangles).

During anaphase, kinetochores are closely associated with spindle pole bodies (SPBs), the yeast equivalent of centrosomes (Guacci et al., 1997). Using light microscopy, it is therefore not possible to distinguish a kinetochore-localized from an SPB-localized protein. To assess whether Lrs4 and Csm1 bind spindle poles or kinetochores, we analyzed Lrs4 localization in relation to Spc42–marked spindle pole bodies in cells that lack *NDC10*, a gene that encodes a central kinetochore component. Cells carrying a temperature-sensitive *ndc10-1* allele progress through mitosis but cannot segregate chromosomes because kinetochore structures are absent (Goh and Kilmartin, 1993). To capture cells in anaphase, when Lrs4 is fully released from the nucleolus, we conducted this analysis in *cdc14-3* mutants (Huang et al., 2006). In *ndc10-1 cdc14-3* double mutants, which arrest as single lobed cells with anaphase-like spindles, Lrs4 no longer co-localized with Spc42 (Figure 3A). The residual co-localization observed in the *ndc10-1 cdc14-3* mutant is likely due to the incomplete penetrance of the *ndc10-1* mutant as evidence from 12% of whole cells that displayed elongated spindles and divided nuclei. We conclude that Lrs4 and Csm1 localize to kinetochores during anaphase.
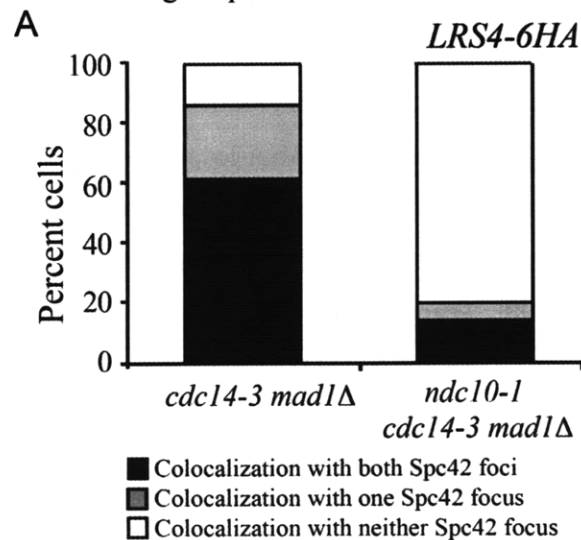
Figure 3. Lrs4 localizes to kinetochores, not spindle pole bodies.

(A) *cdc14-3* (A16802) and *ndc10-1 cdc14-3* (A17569) cells carrying an Spc42-GFP fusion were released from a pheromone-induced G1 arrest at 37°C. Chromosome spreads were performed 105 minutes after release and the percentage of cells with Lrs4 co-localized with both or one Spc42-GFP signal was determined.
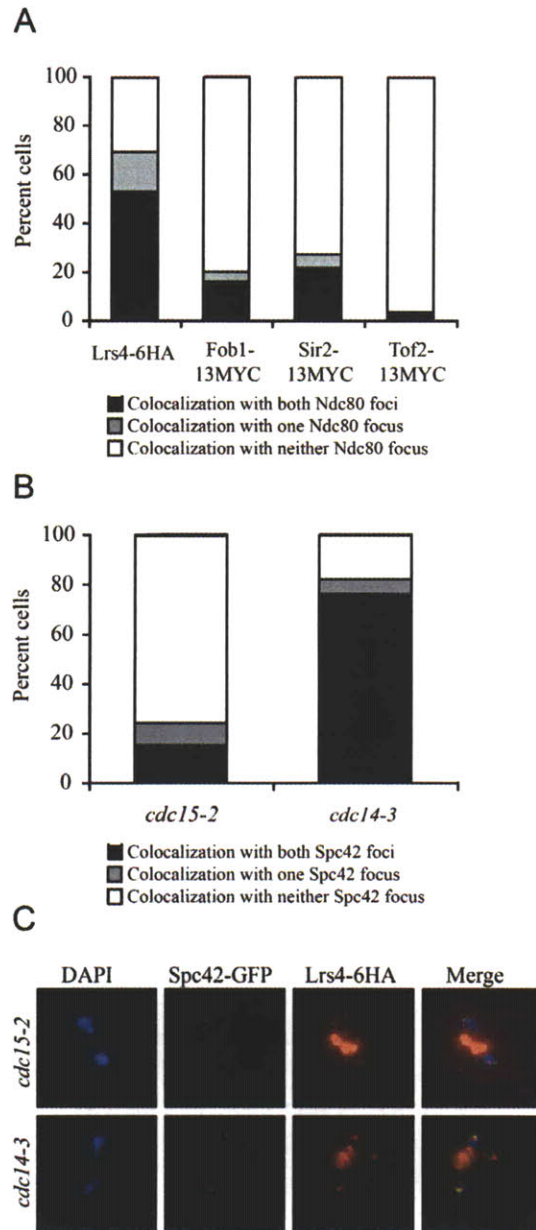
The Mitotic Exit Network targets the monopolin complex and condensins to kinetochores during anaphase.

Lrs4 and Csm1 are not the only nucleolar proteins whose localization changes during anaphase. In addition, the RENT complex that contains Cdc14 and its inhibitor Cfi1/Net1; Sir2, a protein required for gene silencing; Tof2, a protein required for rDNA silencing and condensation; and the replication fork-binding protein Fob1, disassembles during anaphase, releasing Cdc14, Lrs4-Csm1 and Sir2 from the nucleolus (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999). Condensins, which interact with Lrs4-Csm1, associate with chromosomes in early mitosis but become enriched in the nucleolus during early anaphase (Johzuka and Horiuchi, 2009; Bhalla et al., 2002; D'Amours et al., 2004) and are released by the Mitotic Exit Network (Varella et al., 2009). To test whether other RENT complex components or condensins associate with kinetochore during anaphase, we examined the localization of the RENT complex components Tof2, Sir2 and Fob1 in chromosome spreads of anaphase cells. Although Lrs4 co-localized with the kinetochore component Ndc80 in over 50% of anaphase cells, fewer than 20% of anaphase cells showed co-localization of Fob1, Sir2 and Tof2 with Ndc80 (Figure 4A). Despite evidence from fission yeast of Cdc14-homolog Clp1p localization to kinetochores during mitosis (Trautmann et al., 2004), we have not observed Cdc14-3HA localization to kinetochores using immuno-fluorescence (data not shown). By contrast, we found condensins to be enriched at kinetochores during anaphase (Figure 5), which is consistent with previous results in budding and fission yeast (Nakazawa et al., 2008, Wang et al., 2005; D'Ambrosio et al., 2008). In *cdc14-3* mutants, the two condensin subunits Ycs4 and Smc4 co-localized with Ndc80-GFP (Figure 5). Our results indicate that only a subset of nucleolar-enriched proteins, namely the mitotic monopolin complex components Lrs4 and Csm1 and condensins associate with kinetochores during anaphase.

Figure 4: The Mitotic Exit Network is required for Lrs4-Csm1 association with kinetochores during anaphase.

(A) Cells carrying Ndc80-GFP and either Lrs4-6HA (A15127), Fob1-13MYC (A20431), Sir2-13MYC (A20432) and Tof2-13MYC (A20433) fusions were released from a pheromone-induced G1 arrest at 25°C. The percent of anaphase cells showing co-localization of the tagged proteins with Ndc80-GFP was determined.

(B, C) *cdc15-2* (A16755) and *cdc14-3* (A16802) cells carrying an Spc42-GFP and a Lrs4-6HA fusion were released from a pheromone-induced G1 arrest at 37°C. Chromosome spreads were performed on samples taken 150 minutes after release and the percentage of cells with Lrs4-6HA co-localized with Ndc80-GFP was determined (B). The micrographs in (C) show Lrs4-6HA (red) and Spc42-GFP (green) localization in *cdc14-3* and *cdc15-2* mutants.

A

■ Colocalization with both Ndc80 foci
▨ Colocalization with one Ndc80 focus
☐ Colocalization with neither Ndc80 focus

B

■ Colocalization with both Spc42 foci
▨ Colocalization with one Spc42 focus
☐ Colocalization with neither Spc42 focus

C

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Figure 5: Accumulation of the condensin subunits Ycs4 and Smc4 at kinetochores during anaphase is mediated by the Mitotic Exit Network.
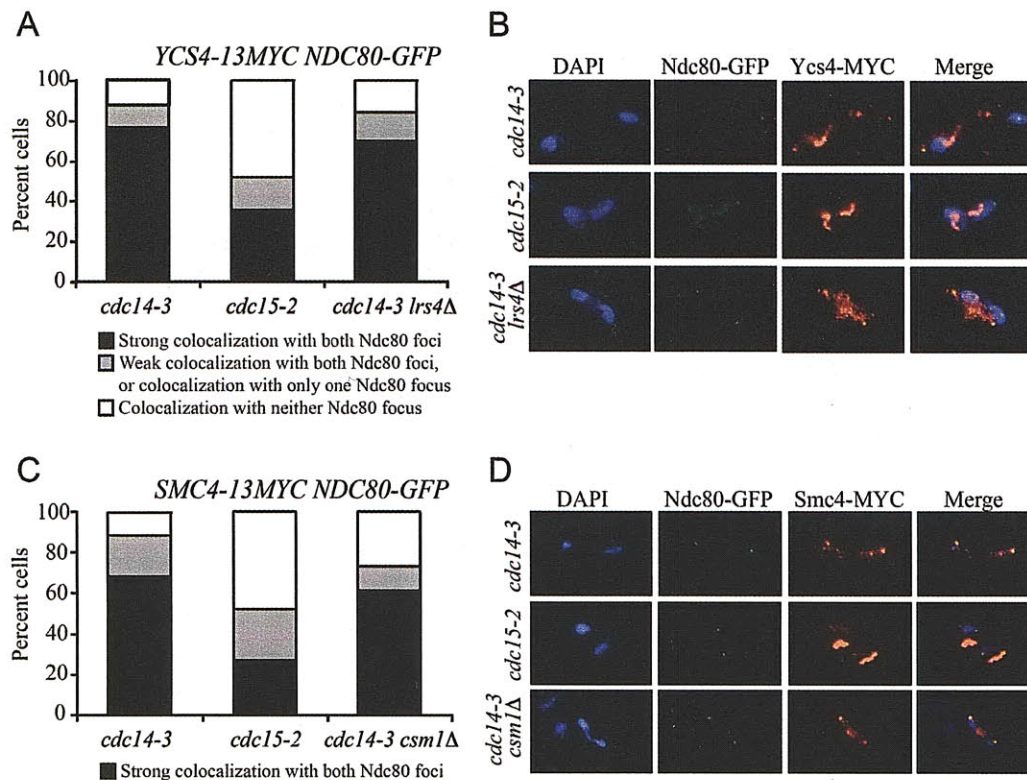
(A-B) *cdc14-3* (A20336), *cdc15-2* (A20328) and *cdc14-3 lrs4Δ* (A21607) cells carrying Ndc80-GFP and Ycs4-13MYC fusions were released from a pheromone-induced G1 arrest at 37°C. Chromosome spreads were performed on samples taken 150 minutes after release to determine the percentage of cells showing strong, weak or no co-localization of Ycs4-13MYC with Ndc80-GFP (A). Strong co-localization refers to Ycs4 staining at Ndc80 foci that is at or above the level of Ycs4 staining at the rDNA. Weak co-localization refers to Ycs4 signal at either only one Ndc80 focus or Ycs4 signal to both Ndc80 foci that is weaker than Ycs4 signal at the rDNA. The micrographs in (B) show Ycs4-13MYC (red) and Ndc80-GFP (green) localization in *cdc14-3*, *cdc15-2* and *cdc14-3 lrs4Δ* mutants.

(C-D) *cdc14-3* (A21860), *cdc15-2* (A21858), and *cdc14-3 csm1Δ* (A21861) cells carrying Ndc80-GFP and Smc4-13MYC fusions were released from a pheromone-induced G1 arrest at 37°C. Chromosome spreads were performed on samples taken 150 minutes after release to determine the percentage of cells showing strong, weak or no co-localization of Smc4-13MYC with Ndc80-GFP (C). Strong co-localization refers to Smc4 staining at Ndc80 foci that is at or above the level of Smc4 staining at the rDNA. Weak co-localization refers to Smc4 signal at either only one Ndc80 focus or Smc4 signal at both Ndc80 foci that is weaker than Smc4 signal at the rDNA. The micrographs in (D) show Smc4-13MYC (red) and Ndc80-GFP (green) localization in *cdc14-3*, *cdc15-2* and *cdc14-3 csm1Δ* mutants.

The release of Lrs4 and Csm1 from the nucleolus temporally coincides with their localization at kinetochores. Cdc15, a protein kinase that functions in the Mitotic Exit Network to release Cdc14 from the nucleolus, is required for the release of both Lrs4 and Csm1, while Cdc14 activity is not (Huang et al., 2006). *CDC15* was also required to target Lrs4 to kinetochores but *CDC14* was not. Whereas 80% of anaphase-arrested cells in *cdc14-3* mutants showed co-localization between Lrs4-6HA and Spc42-GFP, only 20% showed co-localization in anaphase-arrested *cdc15-2* cells (Figures 4B, C). To determine whether condensin deposition at kinetochores during anaphase was also regulated by the MEN, chromosome spreads were performed on *cdc15-2* and *cdc14-3* arrested cells carrying MYC-tagged versions of the condensin subunits Ycs4 (Figures 5A, B) and Smc4 (Figures 5C, D). In *cdc14-3* anaphase-arrested cells, approximately 80% of cells showed Ycs4 and Smc4 enrichment at kinetochores, as compared with approximately 30% of *cdc15-2* anaphase-arrested cells. The residual fraction of *cdc15-2* cells with condensin enrichment at kinetochores is at least in part due to the overlap of the fully divided nucleolar region and kinetochores in *cdc15-2* mutants.

Kinetochore enrichment of condensin subunits was not dependent on *LRS4* and *CSM1*, as the percentage of cells with kinetochore enrichment of condensin components did not change in the absence of monopolin subunits (Figure 5). We were not able to determine whether the release of Lrs4 and Csm1 from the nucleolus or their association with kinetochores depended on condensin because temperature-sensitive condensin alleles are lethal in the presence of tagged versions of the monopolin complex components (data not shown; Waples et al., 2009). Our results indicate that association of monopolin complex components and enrichment of condensins at kinetochores depend on the MEN.

The monopolin complex is required for faithful chromosome segregation during mitosis.
Defects in centromere separation have been observed in temperature-sensitive mutants of condensin subunits Ycs4 (Bhalla et al, 2002), Brn1 (Oupenski et al., 2000), and the ATPase subunit Smc2 (Yong-Gonzalez et al., 2007). Similar observations have been observed in *S. pombe* homologs of Smc2 and Smc4 (Saka et al., 1994). Do *LRS4* and *CSM1*, which also associate with kinetochores during anaphase also play a role in mitotic chromosome segregation? To address this question we measured the loss rates of a centromere-containing plasmid in

lrs4Δ and *csm1Δ* single and double mutants using fluctuation analysis. We observed a 2.5- to 4-fold increase in plasmid loss rates in the two mutants (Figure 6A). This increase in plasmid loss was comparable to that observed in cells carrying deletions of genes encoding non-essential kinetochore components. For example, cells lacking Mcm21, a non-essential member of the kinetochore COMA sub-complex, or Chl4, an outer-kinetochore component showed plasmid loss rates within this range. Loss rates of plasmids carrying a replication origin (ARS) but not a centromere were not increased in *lrs4Δ* and *csm1Δ* single and double mutant (Figure 6B) indicating that it is kinetochore defects that bring about the CEN plasmid segregation defect observed in the mutants. Condensins have also previously been implicated in mitotic chromosome segregation, independent of their role at the rDNA locus. We conclude that Lrs4-Csm1 and condensins participate in faithful chromosome segregation.
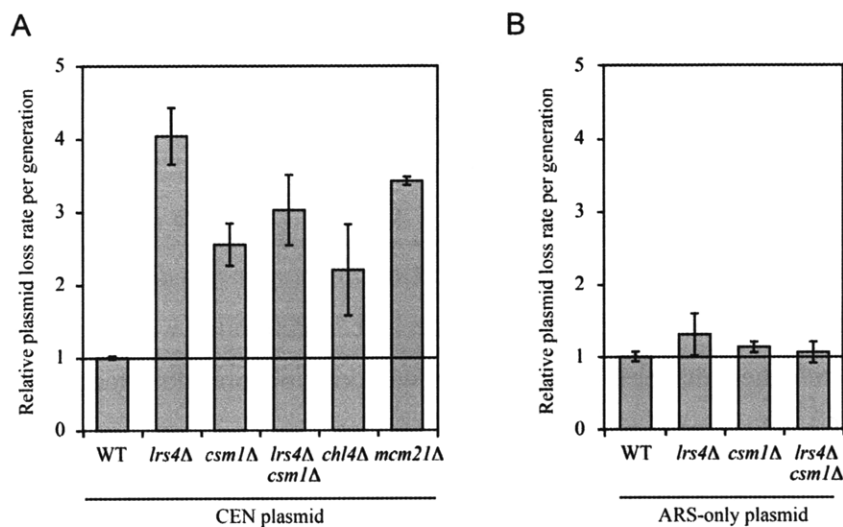
Figure 6: Lrs4 and Csm1 are required for the faithful segregation of CEN plasmids.

(A) Fluctuation analysis was performed to determine the rate of plasmid loss per generation (Experimental Procedures) of wild-type (A18996; WT), *lrs4Δ* (A18998), *csm1Δ* (A19000), *lrs4Δ csm1Δ* (A19002), *chl4Δ* (A20435), or *mcm21Δ* (A20436) mutants carrying a centromeric plasmid (CEN). Bars represent standard error of the mean.

(B) Fluctuation analysis was performed to determine the rate of plasmid loss per generation on wild-type (A19004; WT), *lrs4Δ* (A19005), *csm1Δ* (A19006) and *lrs4Δ csm1Δ* (A19007) cells carrying a plasmid that contains only an autonomous replication sequence (ARS-only). Bars represent standard error of the mean.

Condensins are required for sister kinetochore co-orientation induced by *CDC5* and *MAM1*.

The co-regulation of Lrs4–Csm1 and condensins (Figures 4, 5) and the observation that the two complexes function together at the rDNA (Johzuka and Horiuchi, 2009) prompted us to test the possibility that these proteins also collaborate at kinetochores. The main function of Lrs4 and Csm1 at kinetochores is to co-orient sister kinetochores during meiosis (Rabitsch et al., 2003). To determine whether condensins are required for this process, we first tested the requirement for the protein complex in a system developed to induce sister kinetochore co-orientation during mitosis. We have previously shown that overexpression of the meiosis-specific co-orientation factor Mam1 and the Polo kinase Cdc5 was sufficient to induce sister kinetochore co-orientation during mitosis, leading to co-segregation of sister chromatids during anaphase (Monje-Casas et al., 2007). To follow the segregation of a single pair of sister chromatids, a tandem array of tetO sequences was integrated proximal to the centromere of chromosome IV and a tetR-GFP fusion protein, which binds the tetO sequence, was expressed to visualize the repeats (Michaelis et al., 1997). Overexpression of *CDC5* and *MAM1* led to co-segregation of sister chromatids during anaphase in 22% of cells at 34°C (Figure 7A; Monje-Casas et al., 2007). Deletion of *LRS4* or *CSM1* reduces co-segregation by approximately 50%, whereas deletion of both mitotic components of the monopolin complex almost completely suppressed the co-segregation of sister chromatids induced by high levels of Cdc5 and Mam1 (Figure 7A; Monje-Casas et al., 2007).

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Figure 7: Condensin is required for sister chromatid co-orientation induced by high levels of Mam1 and Cdc5.
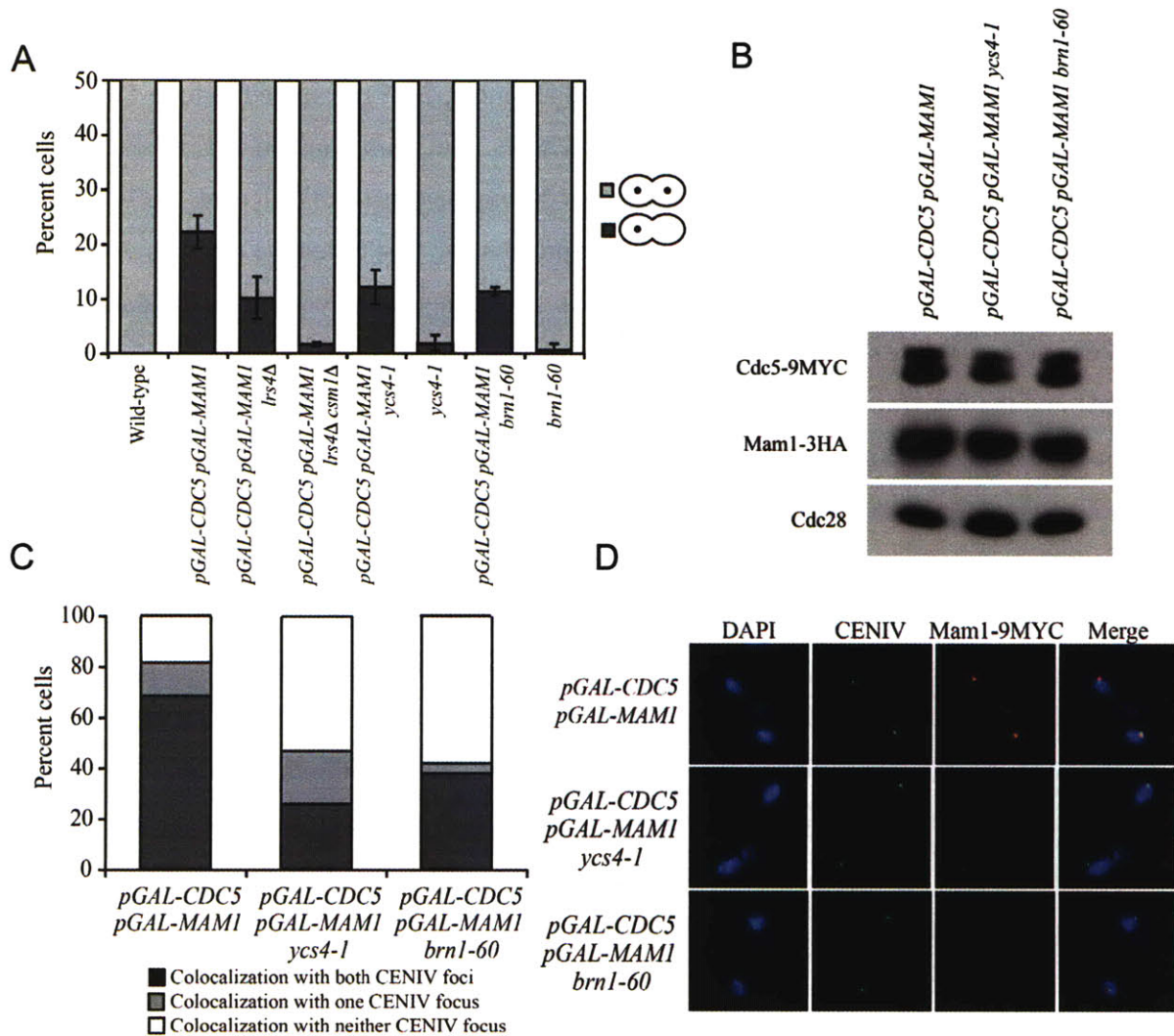
(A) Wild-type (A5244), *pGAL-CDC5 pGAL-MAM1* (A12312), *pGAL-CDC5 pGAL-MAM1 lrs4Δ* (A15910), *pGAL-CDC5 pGAL-MAM1 lrs4Δ csm1Δ* (A21128), *pGAL-CDC5 pGAL-MAM1 ycs4-1* (A20739), *ycs4-1* (A21818), *pGAL-CDC5 pGAL-MAM1 brn1-60* (A21712), and *brn1-60* cells (A21688), all carrying CENIV GFP dots, were arrested in G1 using 5 µg/ml α-factor in YEP medium containing 2% raffinose. One hour prior to release, galactose (2%) was added to induce *MAM1* and *CDC5* expression. Cells were released into YEP containing 2% raffinose and 2% galactose at 34°C. The percentage of anaphase cells in which GFP dots co-segregated (dark grey bars) was determined.

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(B) Exponentially growing wild-type (A12312), *ycs4-1* (A20739) and *brn1-60* (A21712) cells carrying *pGAL-3HA-MAM1* and *pGAL-3MYC-CDC5* fusions were treated with galactose for 1 hour at 25°C and then shifted to 34°C. Samples were taken after 90 minutes to determine the levels of Cdc5 and Mam1 protein. Cdc28 was used as a loading control.

(C-D) *pGAL-CDC5 pGAL-MAM1* (A12312), *pGAL-CDC5 pGAL-MAM1 ycs4-1* (A20739), and *pGAL-CDC5 pGAL-MAM1 brn1-60* (A21712) were grown as described in (A) to determine the co-localization of Mam1-9MYC with both, one or neither CENIV GFP dots by chromosome spreads of anaphase cells (B). The micrographs in (C) show Mam1-9Myc (red) and CENIV-GFP (green) localization.

Inactivation of *YCS4* or *BRN1* reduced *CDC5* and *MAM1* induced sister kinetochore co-segregation by approximately 50% (Figure 7A). This reduction in co-segregation is likely to be an underestimation of the effect of condensin on sister kinetochore co-orientation. Co-segregation of sister chromatids was analyzed only in cells with fully divided nuclei. Cells with stretched or non-divided nuclei, which is indicative of a more complete inactivation of condensin function, were not included in this analysis as co-segregation of sister chromatids cannot be unambiguously determined in such cells. Furthermore, we observed that *ycs4-1* and *brn1-60* mutants show low levels of non-disjunction (Figure 7A), thus reducing the expected increase in cells with segregated CEN IV dots. Finally, because the *GAL1-10* promoter does not function as well at 37°C compared to 25°C, we performed the experiment at 34°C when the temperature-sensitive condensin alleles may not be completely inactivated. Indeed, at 34°C, both *ycs4-1* and *brn1-60* mutants exhibited intermediate phenotypes with respect to chromosome segregation during mitosis (Ouspenski, 2000; Bhalla, 2002). Nevertheless, our results indicate that condensins are required for full sister kinetochore co-orientation induced by Cdc5 and Mam1 overproduction. This loss of co-segregation in the absence of condensin was not due to reduced levels of Cdc5 and Mam1 produced in condensin mutants (Figure 7B) indicating that condensin was required for the function of co-orientation factors rather than their production.

To determine how condensins affect co-orientation, we analyzed the ability of overexpressed Mam1 to associate with kinetochores in condensin mutants by chromosome spreads. In cells overexpressing *CDC5* and *MAM1* during mitosis, Mam1 co-localizes with centromeric GFP dots in approximately 70% of cells with divided nuclei (Figure 7C, D). In contrast, cells carrying the temperature-sensitive condensin allele *ycs4-1* or *brn1-60* exhibited reduced Mam1-9MYC

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localization to kinetochores. Only 25% of anaphase *ycs4-1* cells and 37% of anaphase *brn1-60* cells were able to target Mam1 to kinetochores at 34°C (Figures 7C, D). Our results show that condensins are required for full Cdc5/Mam1-induced co-orientation of sister chromatids during mitosis.

Condensins are required for full sister kinetochore co-orientation during meiosis I.
Examination of the role of condensin in co-orienting sister chromatids during meiosis I is confounded by its additional meiotic functions. Condensin functions during prophase I in processing of double-strand breaks and resolving recombination-dependent chromosome linkages (Yu and Koshland, 2003). To isolate the effect of condensin on co-orientation, we analyzed cells arrested in metaphase I, by depleting the Anaphase Promoting Complex activator Cdc20. In this arrest sister kinetochores are tightly associated. When the centromere of one homolog is GFP-tagged (heterozygous GFP dots), the pair appears as one focus in the arrest (Lee and Amon, 2003). By contrast, when co-orientation is disrupted, as occurs when *MAM1* is deleted, sister chromatids bi-orient in metaphase I and tension exerted by the meiosis I spindle allows two GFP dots to become visible (Lee and Amon, 2003). To examine the consequences of inactivating condensins on sister kinetochore co-orientation, we analyzed the separation of heterozygous GFP dots in cells carrying temperature-sensitive alleles of *YCG1* and *YCS4*, two genes encoding condensin subunits. Cells were transferred into sporulation-inducing medium; one hour later, they were shifted to 34°C. 60% of Cdc20-depleted cells lacking *MAM1* arrested in metaphase and approximately half of these cells exhibited separated CENV GFP dots (Figure 8A; Lee and Amon, 2003). In Cdc20-depleted strains carrying either the *ycg1-2* or *ycs4-2* alleles only 40% of cells reached metaphase, yet, similar to Cdc20-depleted cells lacking *MAM1*, approximately half of the cells showed CENV GFP dot separation (Figure 8A).

Figure 8. Condensin is required for Mam1 localization to kinetochores and sister chromatid co-orientation during meiosis I.
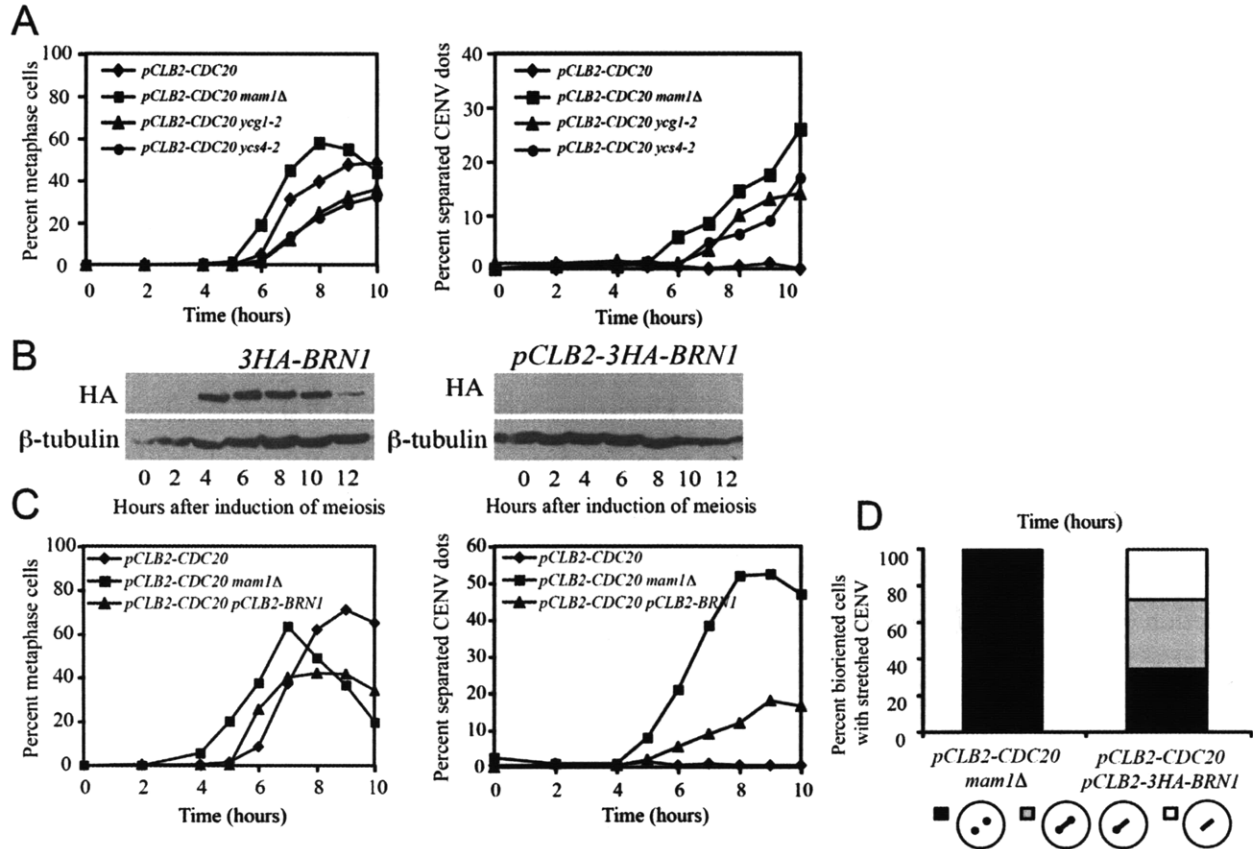
(A) *pCLB2-CDC20* (A7118), *pCLB2-CDC20 mam1Δ* (A7316), *pCLB2-CDC20 ycg1-2* (A23218), and *pCLB2-CDC20 ycs4-2* (A23220) cells containing heterozygous CENV GFP dots were induced to sporulate at 25°C. One hour after transfer into sporulation medium, cells were shifted to 34°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase I spindles (left graph) and separated CENV GFP dots (right graph).

(B) *3HA-BRN1* (HY1143) and *pCLB2-3HA-BRN1* (3069C) cells were induced to sporulate and 3HA-Brn1 protein levels were examined in wild-type (left) and *pCLB2-3HA-BRN1* (right) cells. β-tubulin was used as a loading control.

(C, D) *pCLB2-CDC20* (A7118), *pCLB2-CDC20 mam1Δ* (A7316), *pCLB2-CDC20 pCLB2-BRN1* (A22520) diploid cells containing heterozygous CENV GFP dots were induced to sporulate at 30°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase I spindles (C, left graph) and separated CENV GFP dots (C, right graph). 10 hours after transfer into sporulation medium, the percentages of cells displaying stretched CENV GFP signal was determined (D).

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We also examined sister kinetochore co-orientation in cells depleted of condensins. We generated a depletion allele of the condensin component Brn1 by placing the gene encoding it under the control of the mitosis-specific *CLB2* promoter. Whereas epitope-tagged Brn1 can be detected throughout meiosis, when placed under the *CLB2* promoter, Brn1 levels are undetectable after transfer to sporulation medium (Figure 8B). In cells depleted of Brn1 sister kinetochore co-orientation was impaired but the defect was not as severe as that observed in temperature-sensitive condensin mutants. 40% of cells reached metaphase I and 15% showed CENV GFP dot separation. A fraction of cells depleted of Brn1 exhibited stretching of the tetO array represented by lines of tetR-GFP signal (Figure 8D), which has been observed in bi-oriented cells lacking structural integrity at the kinetochores (Oliveira et al. 2005; Gerlich et al., 2006; Warsi et al., 2008). These observations suggest a requirement for condensin in establishing structural integrity at kinetochores during meiosis. The difference in penetrance between temperature-sensitive condensin alleles and the depletion allele could be due to incomplete depletion of Brn1 in *CLB2-BRN1* cells and/or due to high temperature exaggerating the co-orientation defect of condensin mutants.

Condensins are required for Mam1 but not Lrs4-Csm1 localization to kinetochores.
Based on the evidence that condensins were required to localize Mam1 in mitotic cells overexpressing Cdc5 and Mam1, we asked whether condensin was also needed for Mam1 association with kinetochores during meiosis I. Mam1 protein accumulation was not affected by the inactivation of condensin (Figure 9A). However, Mam1 association with kinetochores was. Mononucleate cells were viewed 6 hours after transfer into sporulation medium when approximately 30% of wild-type and 20% of Brn1-depleted cells were in metaphase I (Figure 9B). 52% of wild-type cells showed full co-localization between tagged versions of Mam1 and Ndc10. Another 27% of cells showed partial co-localization, as defined by a minority of Ndc10 foci displaying Mam1 co-localization (Figures 9C, D). In cells depleted of Brn1, Mam1 associated with chromatin but only 18% of cells showed full co-localization and 17% showed partial co-localization with Ndc10 foci (Figures 9C, D). We can conclude that condensin is required for full association of Mam1 with kinetochores and to bring about co-orientation during meiosis I.

**Figure 9: Cell cycle progression of cells depleted of Brn1.**
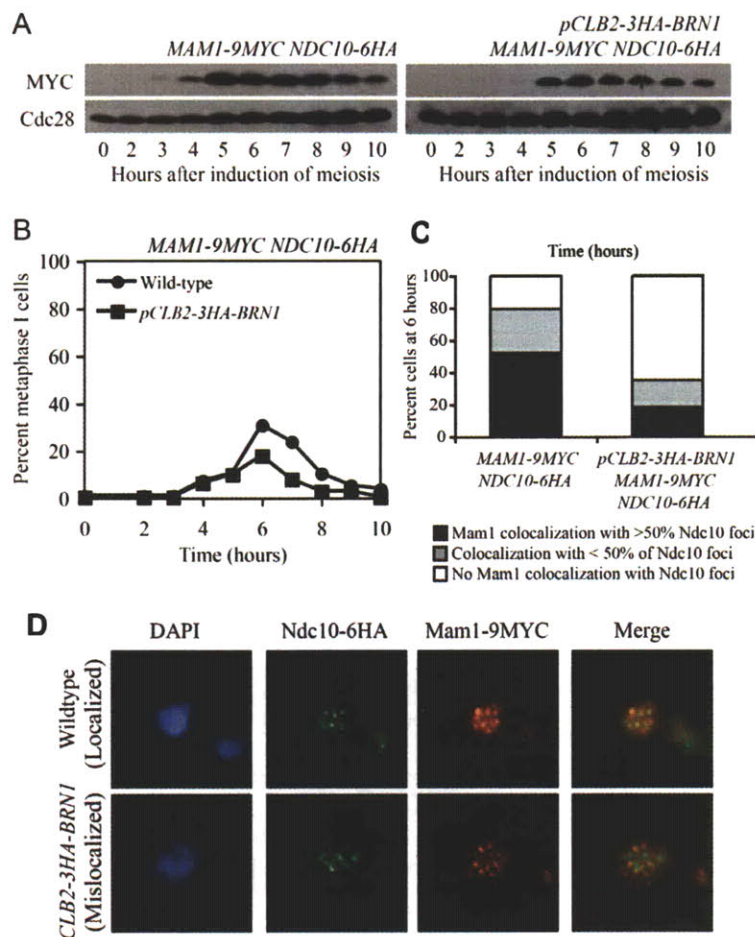
(A) Wild-type (A7097) and *pCLB2-3HA-BRN1* (A22517) cells carrying Mam1-9MYC and Ndc10-6HA fusions were induced to sporulate at 30°C. At the indicated times, samples were taken to determine the levels of Mam1-9MYC. Cdc28 was used as a loading control.

(B) Wild-type (A7097) and *pCLB2-3HA-BRN1* (A22517) cells carrying Mam1-9MYC and Ndc10-6HA fusions were induced to sporulate at 30°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase I spindles.

(C, D) Wild-type (A7097) and *pCLB2-3HA-BRN1* (A22517) cells carrying Mam1-9MYC and Ndc10-6HA fusions were induced to sporulate at 30°C. Chromosome spreads were performed on cells 6 hours after transfer into sporulation medium. The percentage of cells showing over 50%, less than 50% or no co-localization of the Mam1-9MYC (red) and Ndc10-6HA (green) foci was determined (C). The micrographs in (D) show examples of Mam1 association with kinetochores (top panels), taken from wild-type cells, and Mam1 association with chromosomes but not kinetochores (bottom panels) taken from Brn1-depleted cells.

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The reduction of Mam1 at kinetochores in cells depleted for condensin during meiosis could simply be explained if condensin was required to release Lrs4 and Csm1 from the nucleolus. Six hours after transfer into sporulation medium, Lrs4 co-localized with Ndc10 in approximately 65% of mononucleate cells in wild-type cells and cells carrying a meiotic depletion allele of Brn1 (Figure 10 A-C). In addition, Lrs4 was released normally from the nucleolus in mitotic cells depleted for condensin components (Chapter 4). Condensin is therefore not required for Lrs4-Csm1 release or localization.

Figure 10. Condensins are not required for Lrs4 localization to kinetochores during meiosis I.
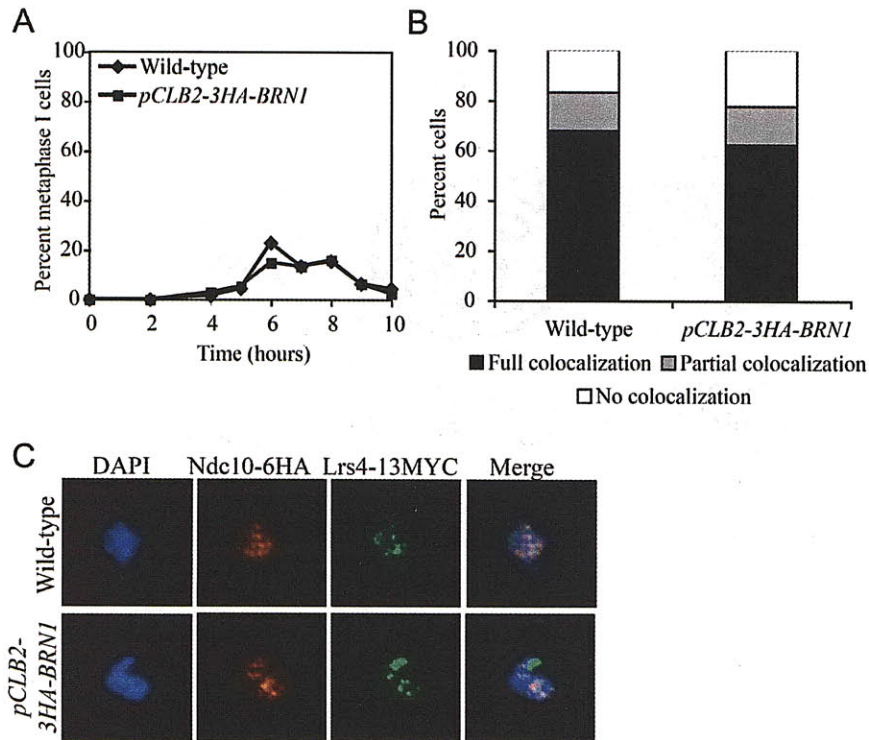
(A-C) Wild-type (A9043) and *pCLB2-3HA-BRN1* (A23861) cells carrying Lrs4-13MYC and Ndc10-6HA fusions were induced to sporulate at 30°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase I spindles.

(B) Chromosome spreads were performed on cells 6 hours after transfer into sporulation medium. The percentage of cells showing over 50%, less than 50% or no co-localization of the Lrs4-13MYC (green) and Ndc10-6HA (red) foci was determined

(C) The micrographs show examples of Lrs4 association with kinetochores in wild-type (top panels) and Brn1-depleted cells (bottom panels).

Discussion

Our studies and those of others indicate that members of the condensin complex and the mitotic components of the monopolin complex, Lrs4 and Csm1, bind to and function together at specialized genomic sites to link sister chromatids. At the rDNA, Lrs4 and Csm1 recruit condensins to inhibit the unequal exchange between sister chromatids (Huang et al., 2006; Johzuka and Horiuchi, 2009), a function most simply explained by a role in linking sister chromatids so that movement of the repeats is restricted with respect to one another (Figure 11A). At meiotic sister kinetochores, condensins recruit monopolins where they provide cohesive properties to sister kinetochores, putting steric constraints on the two sister kinetochores, hence favoring attachment of sister kinetochore to microtubules emanating from the same spindle pole.

Figure 11: A speculative model for how condensins and Lrs4-Csm1 collaborate to prevent unequal recombination of rDNA repeats and sister kinetochore co-orientation.
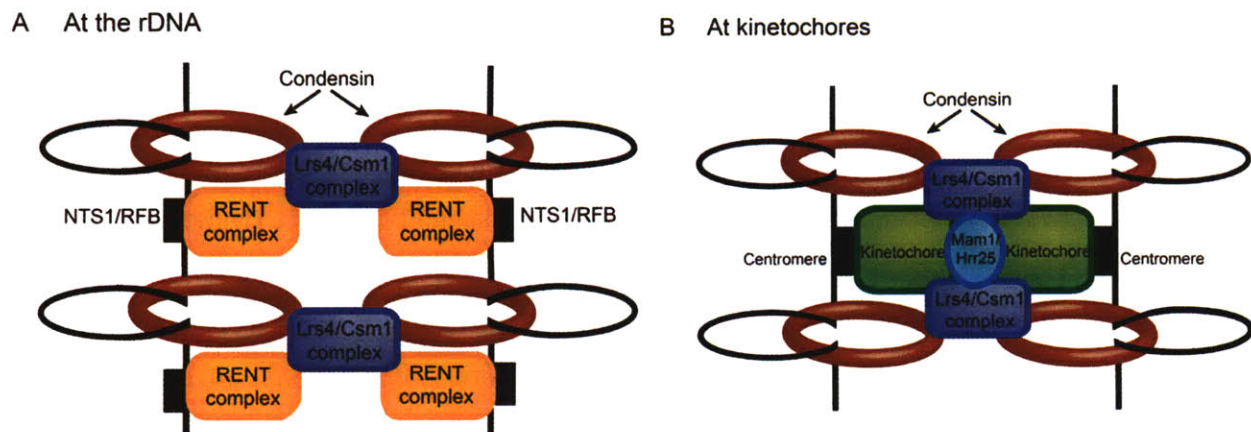
In the nucleolus, Lrs4-Csm1, are recruited to rDNA repeats by Fob1 and other RENT complex components. The two proteins in turn, bind to condensins and recruit them to the rDNA, where they bring about higher-order chromosome structure. We speculate that Lrs4 and Csm1 "zip up" condensin complexes or restrict their movement with respect to each other, thereby preventing the interaction of rDNA repeats with repeats that are not at the homologous position (A). Lrs4 and Csm1 could perform a similar function at kinetochores. There, the recruitment hierarchy is reversed, with condensins recruiting monopolin complex components. We speculate that once recruited, these protein complexes could function in an analogous manner to bring about the fusion of sister kinetochores. This leads to physical constraining of the two sister kinetochores, thus favoring their attachment to microtubules emanating from the same pole (B).

Condensins promote sister kinetochore co-orientation during meiosis I.

Our results indicate that condensins are required for Mam1 localization to kinetochores in order to promote the co-orientation of sister chromatids during meiosis I. How could condensin bring about the association of co-orientation factors? It is unlikely that Mam1 fails to associate with kinetochores due to a loss of overall centromeric and pericentromeric structure in condensin mutants. First, kinetochore assembly does not appear to be affected in condensin mutants, as evident by the sister chromatids' ability to separate in metaphase I-arrested cells in condensin mutants. This activity requires kinetochores to have captured a microtubule and be under tension. Second, inactivation of another SMC chromosome structure complex, the cohesin complex, does not interfere with the association of the monopolin complex with kinetochores (Monje-Casas et al., 2007). We believe that condensins create a higher-ordered chromatin structure at the kinetochore, which not only provides a scaffold for the recruitment of co-orientation factors but that also contributes to the co-orientation process (Figure 11B). Once at kinetochores, monopolins, perhaps together with condensins, establish linkages between sister kinetochores that apply steric constraints which favor attachment of both kinetochores to microtubules emanating from the same spindle pole.
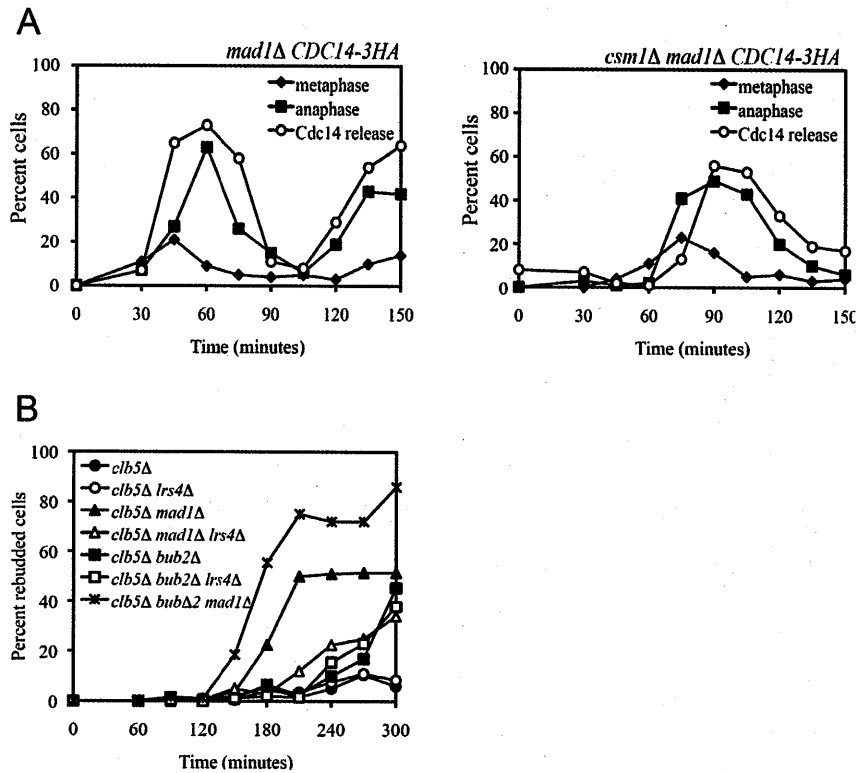
Condensins do not appear to be involved in meiosis I sister kinetochore co-orientation in other eukaryotes. *D. melanogaster* mutants of a non-SMC condensin subunit DCAP-G, (homologous to *S. cerevisiae* Ycg1) do not show defects in co-orientation (Resnick et al., 2009). *C. elegans* mutants in *hcp-6*, a non-SMC condensin II subunit, show defects in chromosome segregation during meiosis I (Chan et al., 2004) but whether sister kinetochore co-orientation is affected in the mutant is not clear. Instead it appears that in most species, the other SMC-containing chromosome structure complex, the cohesin complex, facilitates sister kinetochore co-orientation. In fission yeast, meiotic cohesin complexes associate with the core centromere where they, together with the co-orientation factor Moa1, facilitate a kinetochore geometry that favors sister kinetochore co-orientation (Yokobayashi and Watanabe, 2005; Sakuno et al., 2009). In maize and Arabidopsis, cohesins are also essential for sister kinetochore co-orientation (Yu and Dawe; 2000; Chelysheva et al., 2005). Clearly, SMC protein-containing complexes play critical roles in establishing co-orientation. In species with large, heterochromatic regional centromeres,

cohesins promote sister kinetochore co-orientation, while in budding yeast, whose chromosomes carry point centromeres, condensins appear to be important for this process.

A new role for Lrs4 and Csm1 during mitosis.

What are Lrs4 and Csm1's functions during vegetative growth? Lrs4 and Csm1 are essential for rDNA segregation and maintenance (Huang et al., 2006; Waples et al., 2009). Our localization studies further raised the possibility that the protein complex has additional functions during anaphase. Our observation that Lrs4 and Csm1 are released from the nucleolus concomitantly with Cdc14 and that the release of all three proteins depends on the mitotic exit network raises the possibility that Lrs4-Csm1 function to control Cdc14 activity. Our data argue against this idea. Cdc14 release from the nucleolus is not affected by deletion of *LRS4* or *CSM1* (Figure 12A) nor are the proteins involved in restraining Cdc14 activity (Figure 12B). Instead our data implicate Lrs4 and Csm1 function in chromosome segregation fidelity. Lrs4 and Csm1 localize to kinetochores during anaphase. Furthermore the two proteins have been shown to interact with members of two different sub-kinetochore complexes, Ctf19 and Dsn1, in two-hybrid screens (Wong et al., 2007), and that Dsn1 co-immunoprecipitates with Lrs4 in *cdc14-3*-arrested cells (Chapter 4). The localization of Lrs4 and Csm1 to kinetochores after their release from the nucleolus may reflect their natural affinity for kinetochore components but lack functional importance. However, we provide evidence to suggest otherwise; *lrs4*Δ and *csm1*Δ single and double mutants show elevated rates of centromeric plasmid loss that is comparable to that observed in cells deleted for non-essential kinetochore components.

Figure 12: Lrs4 and Csml do not regulate Cdc14.

A

mad1Δ CDC14-3HA

- metaphase
- anaphase
- Cdc14 release

csm1Δ mad1Δ CDC14-3HA

- metaphase
- anaphase
- Cdc14 release

B

- clb5Δ
- clb5Δ lrs4Δ
- clb5Δ mad1Δ
- clb5Δ mad1Δ lrs4Δ
- clb5Δ bub2Δ
- clb5Δ bub2Δ lrs4Δ
- clb5Δ bubΔ2 mad1Δ

(A) *mad1Δ* (A2853) and *mad1Δ csm1Δ* (A13981) cells carrying a Cdc14-3HA fusion were released from a pheromone-induced G1 arrest at 25°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase (squares) spindles and the percentage of cells showing partial and full release of Cdc14-3HA from the nucleolus (open circles).

(B) *clb5Δ* (A1794; circles), *clb5Δ lrs4Δ* (A14561; open circles), *clb5Δ mad1Δ* (A8604; triangles), *clb5Δ mad1Δ lrs4Δ* (A14964; open triangles), *clb5Δ bub2Δ* (A8605; squares), *clb5Δ bub2Δ lrs4Δ* (A14962; open squares), *clb5Δ bub2Δ mad1Δ* (A8606; asterisks) cells were released from a pheromone-induced G1 arrest at 25°C into media containing nocodazole (15μg/ml). Wild-type cells arrest as large budded cells due to activation of the spindle assembly checkpoint. Cells carrying more than 1 bud indicates bypass of the checkpoint arrest (Stegmeier et al., 2004) and is scored as a "rebudded cell". Preventing Cdc14 release from the nucleolus is required to maintain the spindle checkpoint arrest induced by nocodazole. Deletion of the gene encoding the S-phase cyclin Clb5, an antagonist of Cdc14 activity, leads to a bypass of the checkpoint in few cells. If *clb5Δ* mutants are deleted for *MAD1* and/or *BUB2*, two spindle checkpoint genes that modulate Cdc14 release by the FEAR (Cdc14 Early Anaphase Release) network and the MEN, respectively, enhance this bypass (Stegmeier et al., 2004). Deletion of *LRS4* or *CSM1* did not.

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The function of Lrs4 and Csm1 at kinetochores remains elusive. Dynamic localization of kinetochore components during anaphase has rarely been observed. Fission yeast Scm3 and human hMis18 both localize to kinetochores during anaphase to promote the incorporation of specialized centromeric histone, CENP-A (Fujita, 2007; Jansen, 2007). Lrs4 and Csm1 are not involved in loading the budding yeast homolog of CENP-A onto DNA (I. L. B., unpublished observations). It is possible that condensins and monopolin, analogous to their roles in meiosis, promote clustering of kinetochores or their tethering to spindle poles during anaphase, which in turn could be necessary for high fidelity chromosome transmission. Alternatively, based on evidence that condensins function to provide structural rigidity to centromeres during mitosis in mammals (Ribeiro et al., 2009), condensins and monopolins could create centromeric rigidity.

The logic of meiosis.

It is generally believed that meiosis is a modulation of the mitotic division with meiosis-specific factors bringing about this transformation (reviewed in Marston and Amon, 2004). The analysis of sister kinetochore co-orientation may shed light on this transformation. Aspects of sister kinetochore co-orientation, the Cdc5-dependent release of Lrs4 and Csm1 from the nucleolus occur during both mitosis and meiosis but at different times. In meiosis, Cdc5 promotes the association of the monopolin complex with kinetochores during prophase I (Lee and Amon, 2003), during mitosis this does not occur until anaphase (Huang et al., 2006; this study). It thus appears that establishing meiosis I-specific sister kinetochore co-orientation requires the transposition of anaphase events to prophase, by modifying the regulation of the polo kinase Cdc5. Changes in chromosome compaction that occur during prophase I also resemble those that take place during late stages of mitosis (Kleckner, 2004) raising the possibility that other meiosis I chromosome events are also the result of transposing anaphase events into earlier stages of meiosis. It will be interesting to determine whether other meiotic events, such as chromosomal compaction associated with prophase, are also mediated by the earlier activation of Cdc5.

Materials and Methods

Strains and growth conditions

Derivatives of W303 are described in Table1 ; derivatives of SK1 strains in Table 2. Proteins were tagged using the PCR-based method described in Longtine et al., 1998. GFP dots were constructed from the integration of an array of bacterial TET operator sites 2 kb from the centromere on CENIV in the W303 strains, or 1.4 kb from the centromere of one homolog of chromosome V, as in the diploid SK1 strains (Toth et al., 2000). Conditions for growth and release are as described in Amon, 2002. α-factor was re-added to all cultures 90 min after release from the G1 arrest to prevent cells from entering the next cell cycle. Growth conditions for individual experiments are described in the Figure legends.

Table 1. Strain derivatives of W303 used in this study

Note: All strains are derivatives of W303 and share the same markers as A2587 unless otherwise noted.

Strain	Relevant genotype
A2587	MATa, *ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+,*
A1794	MATa, *clb5::URA3, CDC14-3HA*
A2853	MATa, *mad1::URA3, CDC14-3HA*
A5244	MATa, *pURA3-TetR-GFP::*LEU2, CenIV::tetOx448::URA3
A8604	MATa, *clb5::URA3, mad1::URA3, CDC14-3HA*
A8605	MATa, *clb5::URA3, bub2::HIS3, CDC14-3HA*
A8606	MATa, *clb5::URA3, mad1::URA3, bub2::HIS3, CDC14-3HA*
A12312	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3, pGAL-3HA-MAM1::KanMX6, pGAL-3MYC-CDC5::URA3*
A13838	MATa, *LRS4-6HA::HIS3MX6*
A13981	MATa, *mad1::URA3, csm1::KANMX, CDC14-3HA*
A14561	MATa, *clb5::URA3, lrs4::KANMX6, CDC14-3HA*
A14564	MATa, *clb5::URA3, mad1::URA3, lrs4::KANMX, CDC14-3HA*
A14962	MATa, *clb2::URA3, bub2::HIS3, lrs4::KANMX, CDC14-3HA*
A15087	MATa, *csm1::CSM1-9MYC::TRP1*

A15127	MATa, *LRS4-6HA-HIS3::MX6, Ndc80-GFP::URA3*
A15910	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3, pGAL-3HA-MAM1::KanMX6, pGAL-3MYC-CDC5::URA3 lrs4::HIS3MX6*
A15974	MATa, *LRS4-6HA::HIS3MX6, csm1Δ::KanMX6*
A15976	MATa, *csm1::CSM1-9MYC::TRP1,lrs4Δ::KanMX6*
A16755	MATa, *LRS4-6HA::HIS3MX6, SPC42-GFP::TRP1, cdc15-2*
A16802	MATa, *SPC42-GFP::TRP1, LRS4-6HA::HIS3MX6, cdc14-3*
A17569	MATa, *SPC42-GFP::TRP1, lrs4::LRS4-6HA::HIS3MX6, cdc14-3, ndc10-1*
A18996	MATa, / YCplac 33 *CEN4, ARS1, URA3*
A18998	MATa, *lrs4::KanMX* / YCplac 33 *CEN4, ARS1, URA3*
A19000	MATa, *csm1::kanMX* / YCplac33 *CEN4, ARS1, URA3*
A19002	MATa, *csm1::kanMX, lrs4::KanMX* / YCplac33 *CEN4, ARS1, URA3*
A19004	MATa / YRp17 *ARS1, TRP1, URA3*
A19005	MATa, *lrs4::KanMX6* / YRp17 *ARS1, TRP1, URA3*
A19006	MATa, *csm1::KanMX6* / YRp17 *ARS1, TRP1, URA3*
A19007	MATa, *lrs4::KanMX6, csm1::KanMX6* / YRp17 *ARS1, TRP1, URA3*
A20328	MATa, *YCS4-13MYC::KanMX, NDC80-GFP-URA3, cdc15-2*
A20336	MATa, *YCS4-13MYC::KanMX, NDC80-GFP-URA3, cdc14-3*
A20431	MATa, *FOB1-13MYC::KanMX, NDC80-GFP::URA3*
A20432	MATa, *SIR21-13MYC::KanMX, NDC80-GFP::URA3*
A20433	MATa, *TOF2-13MYC::KanMX, NDC80-GFP::URA3*
A20435	MATa, *chl4::KanMX* / YCplac33 *CEN4, ARS1, URA3*
A20436	MATa, *mcm21::KanMX* / YCplac33 *CEN4, ARS1, URA3*
A20739	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3, pGAL-3HA-MAM1::KanMX6, pGAL-3MYC-CDC5::URA3, ycs4-1*
A21607	MATa, *YCS4-13MYC::KanMX, NDC80-GFP-URA3, cdc14-3, lrs4::KanMX6*
A21128	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3,*

	pGAL-3HA-MAM1::KanMX6, pGAL-3MYC-CDC5::URA3 *lrs4::HIS3MX6 csm1::KanMX6*
A21688	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3, brn1-60*
A21712	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3,* *pGAL-3HA-MAM1::KanMX6, pGAL-3MYC-CDC5::URA3, brn1-60*
A21818	MATa, *pURA3-TetR-GFP::LEU2, CenIV::tetOx448::URA3, ycs4-1*
A21858	MATa, *SMC4-13MYC::HIS3MX6 NDC80-GFP::URA3, cdc15-2*
A21860	MATa, *SMC4-13MYC::HIS3MX6, NDC80-GFP-URA3, cdc14-3*
A21861	MATa, *SMC4-13MYC::HIS3MX6, NDC80-GFP-URA3, cdc14-3,* *csm1::KanMX*

Table 2. Strain derivatives of SK1 used in this study

Note: All strains are derivatives of SK1 and share the same markers as A4962 unless otherwise noted.

Strain	Relevant genotype
A4962	MATa/α, *ho::LYS2/ho::LYS2, ura3/ura3, leu2::hisG/leu2::hisG,* *trp1::hisG/trp1::hisG*
A7097	MATa/α, *NDC10-6HA::HIS3MX6/NDC10-6HA::HIS3MX6, MAM1-* *9MYC::TRP1/ MAM1-9MYC::TRP1*
A7118	MATa/α, *pCLB2-CDC20::KanMX6/pCLB2-CDC20::KanMX6,* *leu2::pURA3-TetR-GFP::LEU2/+, CENV::tetOx224::HIS3/+*
A7316	MATa/α, *pCLB2-CDC20::KanMX6/pCLB2-CDC20::KanMX6,* *leu2::pURA3-TetR-GFP::LEU2/+, CENV::tetOx224::HIS3/+,* *mam1::TRP1/mam1::TRP1*
A9043	MATa/α, *NDC10-6HA::HIS4MX6/NDC10-6HA::HIS4MX6, LRS4-* *13MYC::KanMX6/LRS4-13MYC::KanMX6*
A22517	MATa/α, *NDC10-6HA::HIS3MX6/NDC10-6HA::HIS3MX6, MAM1-* *9MYC::TRP1/MAM1-9MYC::TRP1, pCLB2-3HA-* *BRN1::KANMX4/pCLB2-3HA-BRN1::KANMX4*

A22520	MATa/α, pCLB2-CDC20::KanMX6/pCLB2-CDC20::KanMX6, leu2::pURA3-TetR-GFP::LEU2/+, CENV::tetOx224::HIS3/+, pCLB2-3HA-BRN1::KanMX4/pCLB2-3HA-BRN1::KanMX4
A23218	MATa/α, pCLB2-CDC20::KanMX6/pCLB2-CDC20::KanMX6, leu2::pURA3-TetR-GFP::LEU2/+, CENV::tetOx224::HIS3/+, ycg1-2::KanMX4/ycg1-2::KanMX4
A23220	MATa/α, pCLB2-CDC20::KanMX6/pCLB2-CDC20::KanMX6, leu2::pURA3-TetR-GFP::LEU2/+, CENV::tetOx224::HIS3/+, ycs4-2/ycs4-2
A23861	MATa/α, pCLB2-3HA-BRN1::KanMX4/pCLB2-3HA-BRN1::KanMX4, NDC10-6HA::HIS3MX6/NDC10-6HA::HIS3MX6, LRS4-13MYC::KanMX6/LRS4-13MYC::KanMX6
HY1143	MATa/α, his3:KAN/his3:KAN, BRN1-3HA::HIS5/ BRN1-3HA::HIS5
3069C	MATa/α, brn1::pCLB2-3HA-BRN1::Kan/ brn1::pCLB2-3HA-BRN1::KanMX

Sporulation conditions

Cells were grown to saturation in YPD (YEP + 2% glucose) for 24 hours, diluted into YPA (YEP + 2% KAc) at OD_{600} = 0.3 and grown overnight. Cells were then washed with water and resuspended in SPO medium (0.3% KAc [pH = 7.0]) at OD_{600} = 1.9 at 30°C to induce sporulation.

Localization techniques

Indirect *in situ* immunofluorescence was carried out as described in Visintin et al., 1999 for tubulin, HA-, and MYC-tagged proteins. CEN GFP dot visualization was performed as described in (Monje-Casas et al., 2004). Two hundred cells were scored for each time point. Chromosomes were spread as described in Nairz and Klein, 1997. HA-tagged proteins were detected with a mouse α-HA.11 antibody (Covance) at a 1:500 dilution. MYC-tagged proteins were detected with a mouse anti-MYC 9E10 antibody (Babco) at a 1:500 dilution. Both were followed by a secondary anti-mouse CY3 antibody (Jackson ImmunoResearch) at a 1:1000 dilution. Endogenous luminescence was sufficient for visualization of Ndc80-GFP and Spc42-GFP on

chromosome spreads. In spreads done on meiotic cells carrying NDC10-6HA and Mam1-9MYC, mouse anti-HA and rabbit anti-MYC were used at 1:500 dilution, followed by anti-mouse FITC antibody and anti-rabbit CY3 antibody, also used at 1:500 dilution. In each experiment, at least fifty cells per strain.

Western blot analysis

Cells were harvested, and incubated in 5% trichloroacetic acid (TCA) and lysed as described in Moll et al., 1991. Immunoblots were performed as described in Cohen-Fix et al., 1996. HA-tagged proteins were detected with a mouse α-HA.11 antibody (Covance) at a 1:500 dilution. MYC-tagged proteins were detected with a mouse anti-MYC 9E10 antibody (Babco) at a 1:1000 dilution. Pgk1 was detected with a mouse anti-PGK1 antibody (Molecular Probes) at a 1:20,000 dilution. vATPase was detected using a mouse anti-vATPase antibody (Molecular Probes) at a 1:2000 dilution. The secondary antibody used was a goat anti-mouse antibody conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch) at a 1:2000 dilution. Cdc28 was detected using a rabbit anti-Cdc28 antibody at a 1:1000 dilution. The secondary antibody used was a donkey anti-rabbit antibody conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch) at a 1:2000 dilution.

Plasmid loss experiments

Standard fluctuation analysis (Lea and Coulson, 1949) was used to determine the percentage of cells that lose a plasmid per generation. Cells carrying either the YCPlac33 (*CEN4, ARS1, URA3*) or YRp17 (*ARS1, URA3, TRP1*) plasmids were grown in –URA media overnight. To begin the experiment, cells were counted using a Coulter Counter and then plated on YPD and plates containing 5-FOA to monitor plating efficiency and the percentage of the starting population that contained the plasmid. After 24 hours in YPD medium, cells were re-counted and plated on YPD and plates containing 5-FOA. There were no large discrepancies regarding viability. All cultures were grown for the same number of generations. Plating efficiency and the percentage of cells at the start of the experiment that had lost the plasmid were taken into account. Three or more replicates of each strain were grown on three or more separate occasions.

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Chapter 4

Discussion and Future Directions

Genomic segregation of different loci requires precise processes specialized for each specific locus. In this thesis, I report progress made in understanding how the nucleolus segregates during mitosis and how homologous chromosomes segregate during meiosis. Both of these processes, despite their largely different mechanisms, require two complexes: monopolins and condensins. In chapter 2, I discussed how the monopolin complex links complexes at the rDNA with cohesin rings to hold them in register to prevent recombination events between sister chromatids and subsequent gross expansions or contractions of this array. In chapter 3, I developed a model by which the condensin complex recruits monopolins to potentially provide structural scaffolding to promote co-orientation in meiosis I. The remainder of this chapter will focus on the questions and implications garnered by these findings.

From the architecture of one NTS1 region to the architecture of the nucleolus

The roles of monopolins at the rDNA have been well-tested: Lrs4 and Csm1 bind to rDNA components (Chapter 2; Johzuka and Horiuchi, 2009); and cells lacking *LRS4* or *CSM1* show increased marker loss rates (Smith et al., 1999; Chapter 2) and changes in rDNA copy number (Mekhail et al., 2008.) Yet, a clear picture is still needed of how these components influence the

larger structure of the rDNA via binding to chromosomal architecture proteins, including both cohesin and condensin complexes.

The model (Chapter 2) that explains how monopolins prevent unequal sister chromatid exchange relies on their putative binding with cohesin rings. Despite the fact that Csm1 was found to bind Scc1, the cleavable kleisin subunit of cohesin, and Smc1, an ATPase subunit, via tandem affinity purification and yeast-two-hybrid interactions, respectively (Graumann et al., 2004; Newman et al., 2000), cohesin subunits have not been detected in our purifications of either Lrs4 or Csm1. (Chapter 2). In addition, chromatin immuno-precipitation (ChIP) of cohesin components to the rDNA suggests that cohesin binds the non-transcribed spacer region 2 (Laloraya et al., 2000), whereas Lrs4-Csm1 and condensin bind preferentially to replication fork block (RFB) site at NTS1 (Chapter 2; Johzuka and Horiuchi, 2009). Additionally, cohesin and condensin localization do not overlap (D'Ambrosio et al., 2008) and they likely do not interact (Strunnikov et al. 1995; Bhalla et al. 2002). If there was evidence that cohesin required Lrs4-Csm1 to bind rDNA repeats, as does condensin (Johzuka and Horiuchi, 2009), the highly speculative model proposed at the end of chapter 2 would be supported. Rather, it appears that condensin may play the role of anchoring Lrs4-Csm1-bound rDNA repeats between sister chromatids.

Experiments trying to tease out how Lrs4-Csm1 affects the larger nucleolar structure and the mechanisms governing which complexes Lrs4 and Csm1 bind would provide insight into how rDNA structure and segregation is regulated physiologically. Lrs4 and Csm1 have many proposed binding partners: the RENT complex components, Tof2 and Cfi1/Net1 (Chapter 2); condensin (Johzuka and Horiuchi, 2009), cohesin (although the evidence is less robust; Graumann et al., 2004; Newman et al., 2000); Heh1 and Nur1, two inner nuclear membrane proteins (Mekhail et al., 2008); and potentially histones (H2A, H2B and H4; Chapter 2; Mekail et al., 2008). The specifics about whether these binding interactions are direct or indirect and how exclusive each interaction is remains unclear. How often Lrs4 and Csm1 anchor repeats to the nuclear membrane versus how often they bind condensin or cohesin complexes within the rDNA array is unknown, as is the number of repeats to which Lrs4 and Csm1 are bound. One possible model is shown in Figure 1.
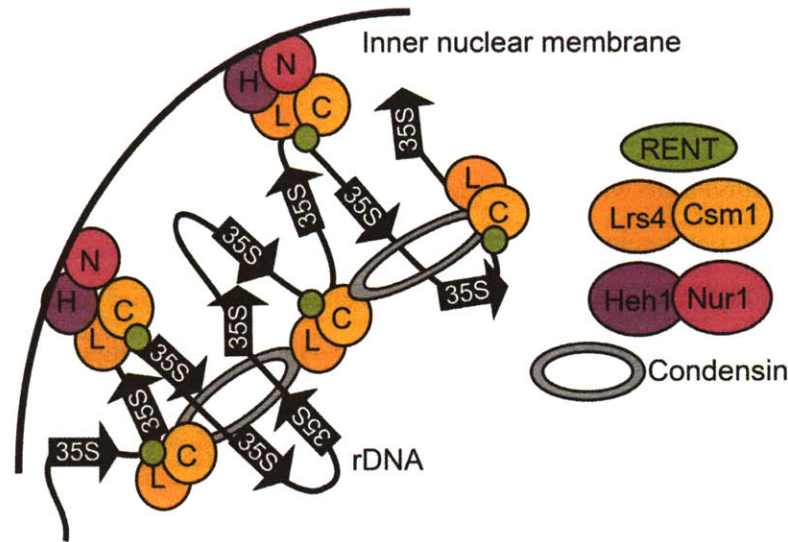
Figure 1. A model for nucleolar organization mediated by Lrs4-Csm1.
Lrs4 and Csm1 bind the RENT complex at various rDNA repeats, but these complexes either bind inner nuclear membrane components or bind condensin. Condensin may bind distant rDNA repeats to facilitate large-scale rDNA condensation. It is currently unknown whether these interactions are exclusive and how often within the rDNA array each occurs. In addition, it is unknown how often the RENT and Lrs4-Csm1 complexes bind within the rDNA. The inter-molecular architecture of condensin rings with each other (not shown) may also contribute to rDNA condensation (Hirano, 2006).

What role do these interactions with Lrs4 and Csm1 play in the structure and regulation of the nucleolus? The loss of Lrs4-Csm1 binding to the nuclear-membrane via Heh1 and Nur1 resulted in a loss of silencing but no longer a loss of rDNA stability, suggesting two different mechanisms for promoting these two outcomes (Mekhail et al., 2008).. By placing ectopic RFB sites or individual rDNA repeats within the genome or on plasmids and varying the spacing or the composition of the inter-repeat region, capturing condensin-mediated structures using cross-linking, and then analyzing the resulting structures (similarly to the methods used in Haering et al., 2008 to decipher cohesin structure), it may be possible to gain knowledge about how these interactions affect the larger nucleolar structure and its regulation. Ectopic rDNA repeats, present on a plasmid, are sufficient to form nucleoli (Oakes et al., 2006) and recruit condensin (Johzuka and Horiuchi, 2009). This suggests that they may also be sufficient to recruit RENT complex components and Lrs4-Csm1 as well. Answering these questions will be essential towards gaining a larger picture of the interplay between rDNA maintenance, rRNA transcription and rDNA segregation, and the overall structure of the nucleolus.

Potential roles for monopolin at other genomic loci

The fact that Lrs4 and Csm1 are found at the rDNA as well as kinetochores like Sir2 and condensin, begs the question as to whether they are present at other genomic loci where Sir2 and condensin are found, namely the telomeres and at tRNA-encoding genes (Smith et al., 1998; Wang et al., 2005). Although Sir2-containing complexes encompass different sets of proteins at these two loci and at the rDNA (Smith et al., 1998,) it appears that several mutants which were isolated in the screen in which Lrs4 was identified, showed both a loss of rDNA silencing and increased telomere lengths (Smith et al., 1999). Although Csm1 was not identified in this screen and Lrs4 did not show increased telomere lengths, Csm1 was later found in another screen to have slightly longer telomeres than wild-type cells, on the order of an additional 50bp (Askree et al., 2004) suggesting they may play a minor role suppressing recombination at this locus. Csm1 was also found in a screen for suppressors of a temperature-sensitive allele of *CDC13*, a gene involved in telomere capping (Addinall et al., 2008). Although these observations were not verified more rigorously than in high throughput screens, it is curious that Csm1 appeared in both independent experiments, which may warrant further study.

tRNA genes cluster in the nucleolus along with the rDNA, which results in RNA Polymerase II gene silencing in adjacent loci (Haeusler et al., 2008), suggesting that other nucleolar RNA Polymerase II-silencing proteins would be present at these loci as well. In fact, condensin was found to bind tRNA genes through ChIP-chip assays (D'Ambrosio et al., 2008). tRNA gene clusters abandon the nucleolus, but still remain in clusters upon treatment with nocodazole (Haeusler et al., 2008), indicating spindle-independent mechanisms for tRNA-coding gene clustering which could implicate Lrs4-Csm1 and their ability to bind inner nuclear membrane proteins. Again, this could easily be examined by analyzing genome-wide binding sites of Lrs4 and Csm1.

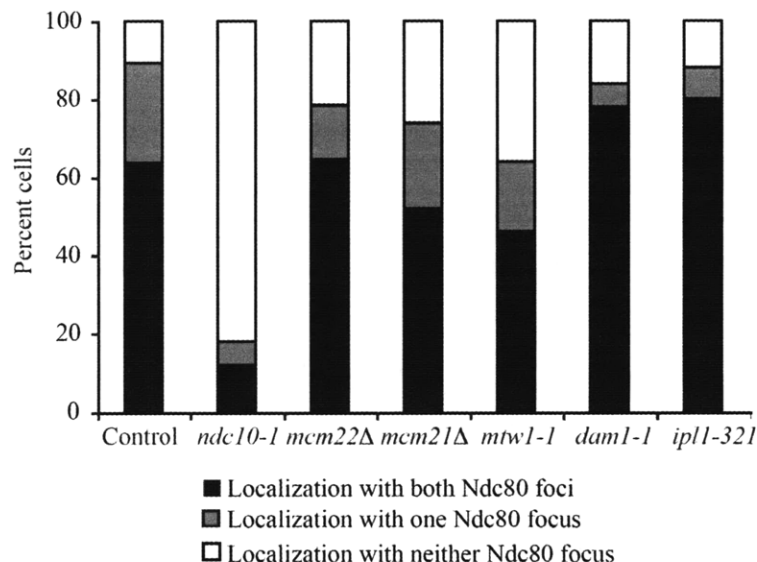## The architecture of the monopolin and condensin complex at the kinetochore and peri-centromeric regions

The architecture of the co-oriented kinetochore remains elusive. It is unknown how many complexes of Lrs4-Csm1-Mam1 are required for co-orientation and in what stoichiometry. Aside from crude secondary structure showing that Lrs4 is a coiled-coil protein with a lysine/arginine-rich cluster and a region of low-complexity and that Csm1 is also a coiled-coil protein with a globular region, little is known about how this protein binds to the kinetochore or to RENT complex components or condensins. Ultimately, understanding the structure of the Lrs4-Csm1 and Lrs4-Csm1-Mam1 complexes would be invaluable to gaining insight as to how these proteins influence kinetochore structure and function. Lrs4 and Csm1 are small proteins (40KDa and 22KDa, respectively) producing rather short coiled-coils, when compared to condensin's coiled-coil ATPase subunits, Smc2 and Smc4 (134KDa and 162KDa, respectively). Knowing how these proteins interact at kinetochores will open doors to experiments to probe their function.

The search for binding partners for this complex has largely used immuno-precipitation followed by tandem mass spectrometry analysis (Chapter 2; Petronczki et al., 2006; Mekhail et al., 2008). Since these techniques have been fruitful, repeating these experiments in a mutant with a shorter rDNA array may boost the kinetochore-rDNA ratio of protein to aid in identifying other potential regulatory or structural binding partners at the kinetochore. Additionally, performing these experiments during mitosis in cdc14-3 arrested cells, or in pCLB2-CDC20 meiotic cells, when the monopolin complex is at the kinetochore (Chapter 3; Lee and Amon, 2003), could additionally aid in capturing monopolin complexes bound to kinetochores.

To begin characterizing Lrs4-Csm1 binding to kinetochores, I analyzed the ability for Lrs4-6HA to co-localize with Ndc80-GFP-marked kinetochores when individual kinetochore components were absent or inactive. This experiment was performed in cells carrying the cdc14-3 allele which arrests cells at a point in the cell cycle when Lrs4 and Csm1 are localized at kinetochores (Chapter 3). In addition, they lack MAD1, to prevent spindle checkpoint activation due to faulty kinetochore-microtubule attachments that would be induced by inactivation of kinetochore

components, and allow cells to progress to anaphase. As previously shown, Lrs4-6HA no longer co-localizes with Ndc80-GFP in *ndc10-1* mutants (Chapter 3). Whereas inactivation of components of the DAM-DASH complex which creates a collar around microtubules produced no change in Lrs4 localization, inactivation of Mtw1 (*Mis twe*lve-like), a member of the inner kinetochore MIND complex, using a temperature-sensitive allele produced the next largest effect after Ndc10 inactivation (Figure 2). Since temperature-sensitive alleles of kinetochore components may still localize properly, though they lack another essential function, the effect on Lrs4 binding may be subtle. These preliminary results suggest that Lrs4-Csm1 binds the MIND complex, a middle-kinetochore structural complex, of which Mtw1 is a member. This observation is further supported by yeast-two-hybrid evidence that Csm1 binds Dsn1 (Wong et al., 2007) and by co-immuno-precipitation experiments that I performed using Lrs4-6HA as a bait and probing for Dsn1-9MYC in *cdc14-3*-arrested cells (Figure 3). Further research must be performed to determine the significance of this putative Lrs4-Csm1-MIND complex binding.

■ Localization with both Ndc80 foci
■ Localization with one Ndc80 focus
☐ Localization with neither Ndc80 focus

Figure 2. Lrs4-6HA is dependent on kinetochore proteins to localize properly to kinetochores.

*cdc14-3 mad1*Δ cells carrying Lrs4-6HA fusion proteins (A18441) and either deletions of Mcm22 (A18497) and Mcm21 (A18422) or temperature-sensitive alleles of Mtw1 (A19468), Dam1 (A19466) or Ipl1 (A19469) were arrested in G1 using α-factor pheromone (5µg/ml) and released into medium lacking pheromone at 37°C. Cells were harvested 150 minutes after release and chromosome spreads were performed to determine the percentage of cells with co-localization of Lrs4-6HA to both, one or neither Ndc80-GFP-marked kinetochore.
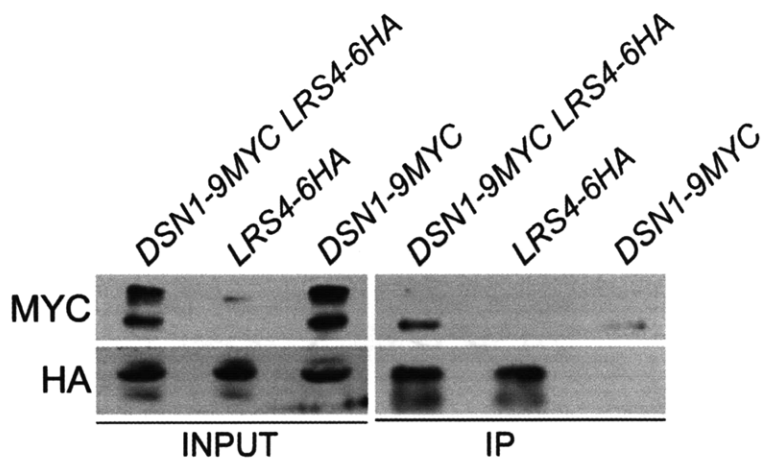
Figure 3. Lrs4-6HA binds Dsn1-9MYC in whole cell lysates.
cdc14-3 cells carrying either an Lrs4-6HA or Dsn1-9MYC fusion protein or both were arrested in G1 using α-factor pheromone (5μg/ml) and released into medium lacking pheromone at 37°C. Lrs4-6HA was immuno-precipitated from whole cell lysates made from cells harvested 150 minutes after release. Western blots were performed to determine the presence of Dsn1-9MYC.

Although during meiosis I, Mam1 requires condensin to bind kinetochores, it is not clear whether the same holds true for Lrs4 and Csm1 during mitosis. At the rDNA, it appears that condensin requires Fob1, Tof2 and Lrs4-Csm1 to bind, creating a "hierarchy" of protein assembly at the NTS1 region of the rDNA repeat (Johzuka and Horiuchi, 2009). Due to a synthetic lethal interaction between tagged copies of monopolin subunits and condensin temperature-sensitive alleles (Waples et al., 2009; data not shown), ubiquitin-conjugated condensin subunits were placed under a galactose-inducible promoter in order to test whether Lrs4-Csm1 localization to kinetochores was dependent on condensin. These strains exhibited growth defects when plated on glucose-containing medium (Figure 4A) and the disappearance of detectable condensin subunit 3HA-Smc4 occurred within 90 minutes after post-transfer into glucose containing medium (Figure 4B). Cells depleted for condensin subunits Smc4 and Ycs4 exhibited normal release of Lrs4 and Csm1 (Figure 4C). We therefore anticipate that Lrs4 and Csm1 will localize to kinetochores independently of condensin.

Figure 4. Lrs4 and Csm1 are released from the nucleolus in cells depleted for condensin subunits Smc4 and Ycs4.
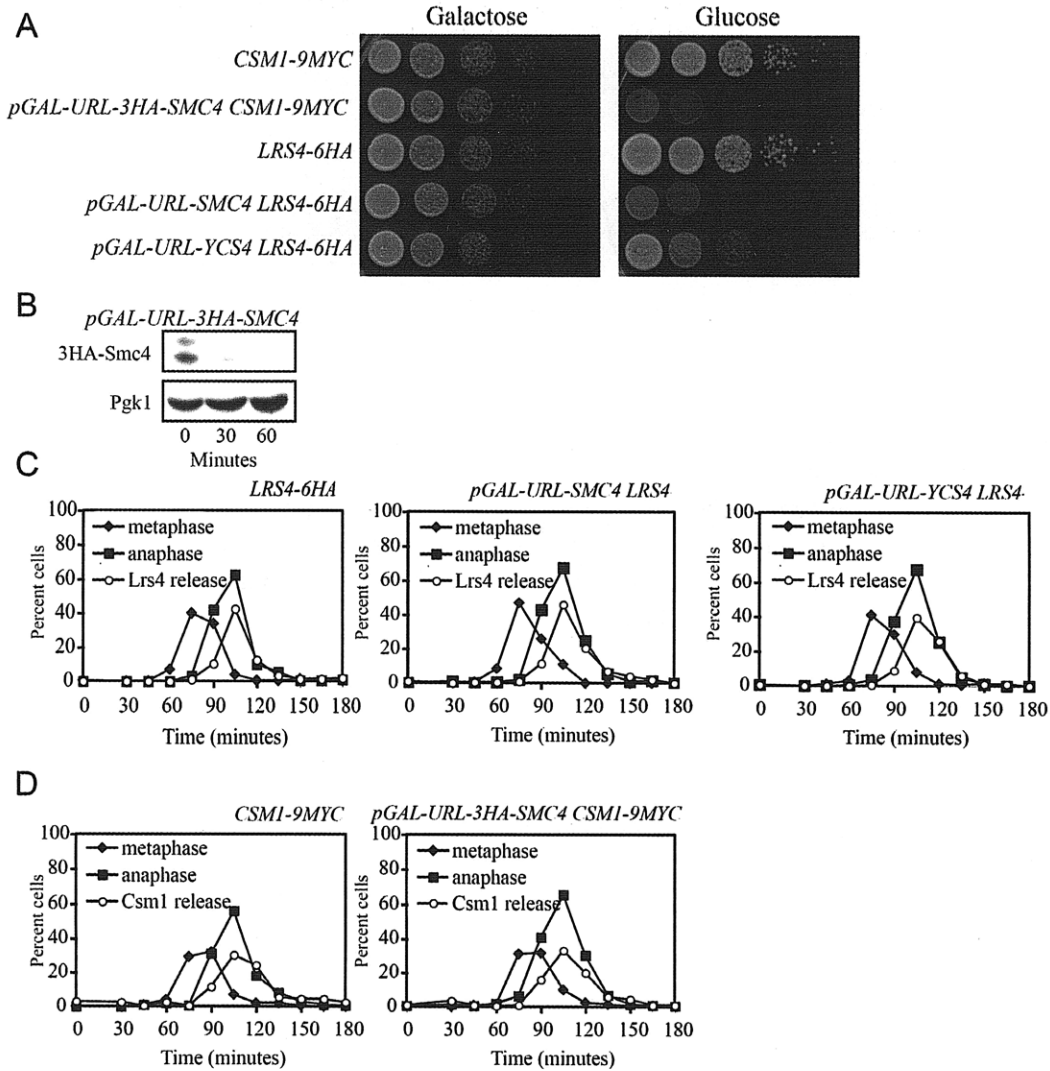
(A) Wild-type (A15087) and *pGAL-URL-3HA-SMC4* (A23709) cells containing Csm1-9MYC fusions; and wild-type (A13838), *pGAL-URL-SMC4* (A23707) and *pGAL-URL-YCS4* (A23708) cells containing Lrs4-6HA fusions were spotted in identical serial dilutions on YEP plates containing either 2% raffinose 2% galactose or 2% glucose.

(B) *pGAL-URL-3HA-SMC4* (A23709) cells were arrested in G1 using α-factor pheromone (5µg/ml) in 2% raffinose 2% galactose-containing medium and released into medium containing 2% glucose lacking pheromone at 25°C. One hour prior to release, glucose (2% final concentration) was added to the medium. Samples were taken to determine the levels of Smc4. Cdc28 was used as a loading control.

(C) Wild-type (A15087) and *pGAL-URL-3HA-SMC4* (A23709) cells containing Csm1-9MYC fusions; and wild-type (A13838), *pGAL-URL-SMC4* (A23707) and *pGAL-URL-YCS4* (A23708) cells containing Lrs4-6HA fusions were arrested in G1 using α-factor pheromone (5μg/ml) in 2% raffinose 2% galactose-containing medium and released into medium containing 2% glucose lacking pheromone at 25°C. One hour prior to release, glucose (2% final concentration) was added to the medium. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase spindles (squares); and the percentage of cells showing the release of Lrs4-6HA or Csm1-9MYC (open circles) from the nucleolus.

How does condensin impact the architecture of the kinetochore and/or peri-centromeric regions? This thesis work has exposed a novel requirement for condensins in co-orientation, implying that these structural proteins directly or indirectly modify kinetochore architecture. Using ChIP-chip to determine where condensin binds on meiotic chromosomes and whether its distribution on chromosomes changes during anaphase in mitotic cells when compared to its localization in G1 and metaphase cells (D'Ambrosio et al., 2008) may provide a hint at how condensin affects centromeric regions. Likewise, it would be interesting to see whether condensin binding is dependent on Ndc10, an inner kinetochore component. How condensin loading is regulated at *S. cerevisiae* centromeres during meiosis is unknown. At the rDNA, condensin is temporally regulated via sumoylation, the FEAR pathway and the Ipl1 kinase (D'Amours et al., 2004; Lavoie et al., 2004), the latter being important for centromeric localization of condensin during mitosis in *S. pombe* (Nakazawa et al., 2008). Knowing the regulation and profile of condensin binding to centromeric regions during meiosis may provide insight to how co-orientation is facilitated.

Does condensin binding at centromeres affect other proteins involved in kinetochore orientation? Sgo1, which plays the dual role of protector of centromeric cohesin (Kiburz et al., 2005) during meiosis I and also biasing kinetochores towards bi-orientation (Kiburz et al., 2008), localizes to a 50kb region flanking kinetochores (Kiburz et al., 2005). Spo13, which maintains monopolin binding to kinetochores also localizes to the kinetochores (Lee et al., 2004; Katis et al., 2004). By observing Sgo1 and Spo13 localization in cells depleted of condensins during meiosis, we can test whether the presumed centromeric scaffolding provided by condensin promotes or antagonizes the functions of other factors involved in kinetochore orientation. It is not known whether monopolin binds strictly kinetochore components or whether monopolin binds larger

regions of the DNA as well. These experiments could help determine whether condensin-monopolin collaboration at the kinetochore in "fusing" kinetochores versus applying geometric constraints, are separable or unavoidably linked.

Acquiring a broader picture of how budding yeast achieves co-orientation at kinetochores via the condensin and monopolin complexes, as well as Spo13, Sgo1, Ipl1, spindle checkpoint proteins and other regulatory proteins, will hopefully promote an understanding of this mechanism in humans. Segregation defects in meiosis I presumably lead to the majority of human chromosomal non-disjunction events (Hassold and Hunt, 2001). Eventually, we may come to terms, conceptually, with the mechanisms that increase the incidence of human meiotic errors when compared with other eukaryotes. Meiotic errors in humans account for over 5 percent of clinically recognized pregnancies, whereas only 1-2% of mouse zygotes are aneuploid (Hassold and Hunt, 2001).

What role do monopolins and condensins play at kinetochores during mitosis?

The fact that Lrs4 and Csm1 localize to kinetochores during mitotic anaphase is highly unusual. This interaction at kinetochores is of value in maintaining the genomic integrity of the cell as *lrs4Δ csm1Δ* single and double mutants display modest but statistically significant centromeric plasmid loss rates (Chapter 3). Spindle checkpoint proteins dynamically localize to kinetochores during metaphase, but the purpose of anaphase-specific kinetochore localization once chromosomes have segregated is puzzling. Protein fusions between Lrs4 or Csm1 and kinetochore components could force their kinetochore function during other times of the cell cycle which may in turn reveal their anaphase-specific function. The mechanism for maintaining genomic stability via these interactions at kinetochores during anaphase is currently unknown, though several possibilities seem likely.

Kinetochore clustering occurs in *S. cerevisiae* during anaphase (Guacci et al., 1997). Whether this is a result of merely having shortened microtubule-kinetochore attachments or whether a microtubule-independent mechanism ensures that chromosomes are held solidly at the cell periphery before and during cytokinesis is unknown. The compaction role that condensin plays at

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the rDNA may apply to pericentromeric regions during anaphase. The mitotic monopolins, akin to their "kinetochore fusing" function in meiosis, may provide a microtubule-independent linkage between kinetochores during mitosis. Alternatively, they may help stabilize the short microtubules between spindle pole bodies and kinetochores that exist during anaphase, suggesting that cells lacking mitotic monopolin components would be hypersensitive to microtubule depolymerizing drugs like nocodazole or benomyl. Another means by which the same outcome could come about is by providing a spindle-pole-independent anchoring of chromosomes in the nuclear membrane. Analysis of Lrs4-Csm1 binding partners in *cdc14-3* versus *cdc15-2*-arrested cells via mass spectrometry would indicate whether Nur1 and Hec1, two inner membrane proteins, are still present. It may identify other binding partners at the kinetochore to further support this observation as well as potentially providing a clue to their kinetochore function.

Condensin co-localization with kinetochores may indicate a requirement for structural rigidity at centromeric regions during mitosis. There is prior evidence from mammalian cells that condensin-mediated structural rigidity at centromeres is important for normal function (Ribiero et al., 2009). In budding yeast, chromatin surrounding the metaphase kinetochore is elastic (Bouck and Bloom, 2007). This elasticity is thought to allow tension created on the metaphase spindle to be distributed within the centromeric region to prevent chromosome breakage. In anaphase, this is no longer needed, and a more compact form of centromeric DNA might be preferred to retain chromosomes at the periphery. Whereas this elasticity is dependent on histone deposition, it would be interesting to follow up on potential interactions between Lrs4-Csm1 and histone subunits H4, H2A and H2B (Chapter 2; Mekhail et al., 2008). Further cytological analysis of the mechanical properties of anaphase kinetochores (as well as meiotic kinetochores) will be undoubtedly informative.

Modifications of Lrs4 in mitosis and meiosis

The phosphorylation of Lrs4, by Cdc7-Dbf4, is thought to be important for monopolin binding to kinetochores (Matos et al., 2008). Yet, in mitosis, Lrs4 appears to be phosphorylated to a much smaller degree (Figure 5; Petronckzi et al., 2006). Although we cannot rule out the possibility that hyper-phosphorylated forms of Lrs4 drive high-affinity kinetochore binding, phosphorylation during mitosis is neither required nor correlated with its localization at kinetochores. Lrs4 gets phosphorylated in both *cdc14-3* and *cdc15-2* cells (Figure 5A, B) in which Lrs4 either localizes to the kinetochores or the nucleolus, respectively (Chapter 2, 3). In addition, Lrs4 is phosphorylated in a *cdc5-1* mutant (Figure 5C, D), suggesting that Cdc5 is not absolutely required to phosphorylate Lrs4 in mitosis. In summary, the phosphorylation of Lrs4 during mitosis does not correlate with its localization and analysis of phosphorylation and phospho-site analysis are needed to determine the functional significance of phosphorylation towards rDNA recombination or meiosis I chromosome segregation.
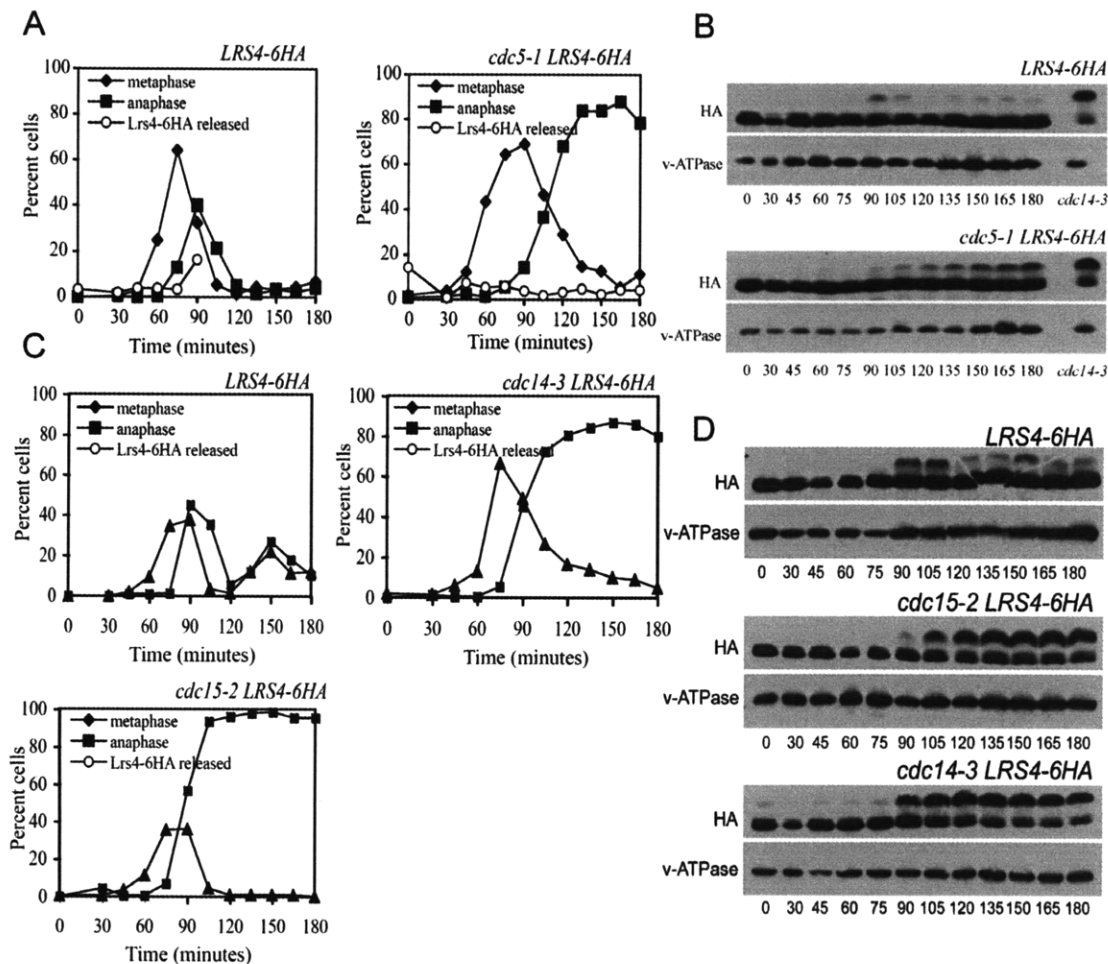
**Figure 5. Lrs4 is phosphorylated during mitosis.**

(A) Wild-type (A13838) and *cdc5-1* (A21713) cells carrying Lrs4-6HA fusions were arrested in G1 using α-factor pheromone (5μg/ml) and released into medium at 37°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase spindles (squares); and the percentage of cells showing the release of Lrs4-6HA (open circles) from the nucleolus.

(B) Samples were taken at the indicated times to determine the levels of Lrs4-6HA. v-ATPase was used as a loading control.

(C) Wild-type (A13838), *cdc14-3* (A14204), and *cdc15-2* (A13839) cells carrying Lrs4-6HA fusions were arrested in G1 using α-factor pheromone (5μg/ml) and released into medium at 37°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase (diamonds) and anaphase spindles (squares); and the percentage of cells showing the release of Lrs4-6HA (open circles) from the nucleolus.

(D) Samples were taken at the indicated times to determine the levels of Lrs4-6HA. v-ATPase was used as a loading control.

Additionally, experiments on cells over-expressing *LRS4* and *CSM1* produced no specific cell cycle progression defect (Figure 6C), although cells over-expressing both *LRS4* and *CSM1* did display a proliferative disadvantage. The localization of these components within the rDNA or kinetochores was not studied, but it is unlikely that they localized to kinetochores or influenced normal microtubule-kinetochore binding since these cells generally proliferated normally (Figure 6). An effect at the rDNA would likely not arise without the over-expression of Tof2 and Fob1, which are required for rDNA binding. Likewise, ectopic localization to kinetochores may not have occurred due to the lack of meiotic modifications as discussed above. In addition, the proposed anaphase-specific functions for Lrs4-Csm1 at kinetochores (kinetochore clustering, anchoring and promoting centromeric stability) may also not be sensitive to over-expression of these components.
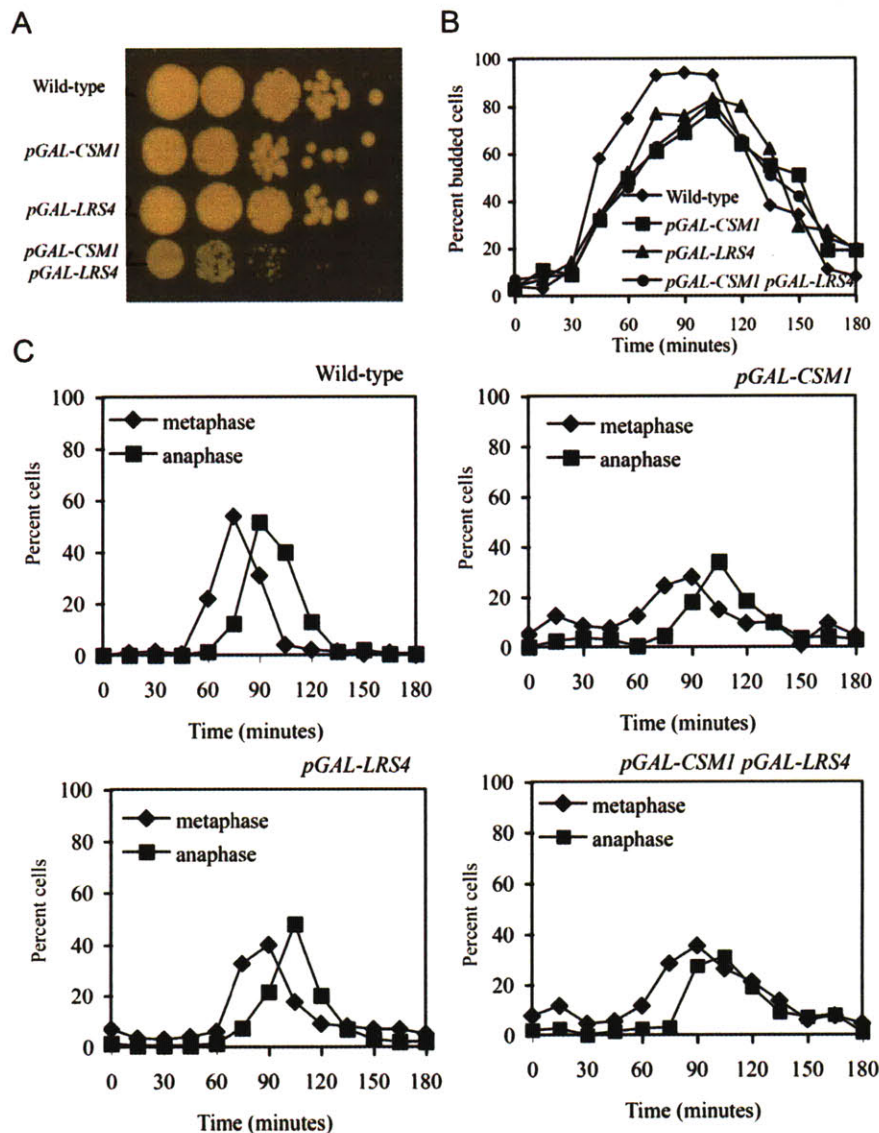
Figure 5. Over-expression of *LRS4* and *CSM1* did not affect cell cycle progression.

(A) Wild-type (A2587), *pGAL-LRS4* (A18122), *pGAL-CSM1* (A18121) and *pGAL-LRS4 pGAL-CSM1* (A18142) cells were spotted in serial dilutions onto plates containing 2% raffinose and 2% galactose.

(B-C) Wild-type (A2587), *pGAL-LRS4* (A18122), *pGAL-CSM1* (A18121) and *pGAL-LRS4 pGAL-CSM1* (A18142) cells grown in medium containing 2% raffinose were arrested in G1 using α-factor pheromone (5μg/ml) and released into medium containing 2% raffinose 2% galactose at 37°C. One hour prior to release, galacatose was added to the medium (2% final concentration). At the indicated times, samples were taken to determine the percentage of budded cells (B) and the percent of cells displaying metaphase (diamonds) and anaphase spindles (squares).

Monopolin's contribution to co-orientation

Using chromosome spreads, we find that Mam1 localization to the kinetochore is dependent on the condensin complex in both meiosis and in mitotic cells induced to co-segregate sister chromatids (Chapter 3). The benefit of using such an experimental set-up is that we were able to test the individual contributions of different meiotic regulators in the process of co-orientation. Over-expression of *MAM1* and *CDC5* was sufficient to promote sister chromatid co-segregation during mitosis (Monje-Casas et al., 2007). Condensin was required to achieve their full effects (Chapter 3). This experiment was very telling about monopolin and condensin function. Yet, one question that arises is why the greatest level of co-orientation seen using this assay is only around 35 percent?

There are likely additional levels of regulation of the monopolin complex that are not accounted for when merely over-expressing *MAM1* and *CDC5*. One could test the contribution of several factors that have already been identified in post-translationally modifying monopolins or promoting their localization to kinetochores: Spo13, and three kinases: Cdc5, Cdc7 and Hrr25. Spo13, though required to maintain monopolin at kinetochores during meiosis I, produced no additive effect with Mam1 and Cdc5 over-expression (Monje-Casas et al., 2007). Cdc7 and Dbf4, proteins involved in firing early replication origins, were recently found to promote co-orientation, in conjunction with Cdc5 and Spo13, by hyperphosphorylating Lrs4 (Matos et al., 2008). Additionally, Hrr25 phosphorylates Mam1 (Petronczki et al., 2006), although this phosphorylation event has unknown consequences. This hyper-phosphorylation of Lrs4 and/or the phosphorylation of Mam1 may be required for high-affinity binding to the kinetochore during metaphase.

Do Lrs4, Csm1 and Mam1 act synergistially or redundantly? In cells induced to co-orient sister chromatids by over-expressing *CDC5* and *MAM1*, the deletion of both *LRS4* and *CSM1* results in full restoration of bi-oriented sister chromatids, whereas the loss of each of these components separately reduces the percent of co-oriented cells by only half, suggesting that there is some functionally redundancy between monopolin complex members, or that they each play additional roles in chromosomal architecture. Curiously, it seems that during meiosis, tetrads formed from

diploids lacking any one of the monopolin components show the same segregation patterns of a marked pair of homologous chromosomes (Toth et al., 2000; Rabitsch et al., 2003). Interestingly, temperature-sensitive condensin alleles individually introduced into the *GAL-CDC5 GAL-MAM1* background show only a fifty percent reduction in co-orientation as well (Chapter 3), which may only suggest that these alleles remain partially active. Although this assay does not represent physiological conditions present during meiosis, these data suggest that it may be used to test individual components' contributions to co-orientation.

Conclusion

The mechanisms governing chromosome segregation are highly complex and rely on proteins that modify chromosome architecture, including the monopolin and condensin complexes. In this thesis, I have explored the way monopolins bring about both high fidelity rDNA segregation and, with condensin, meiosis I sister chromatid co-segregation. Whereas in other eukaryotes, it appears that the cohesin complex is indispensable for meiosis I chromosome segregation, orthologs to the monopolin complex and the condensin contribution to meiosis I chromosome segregation may warrant further inquiry. These endeavors may shed light on the occurrence non-disjunction events that lead to unfortunate aneuploidy-related complications that occur in humans.

Materials and Methods

Strains and growth conditions

Derivatives of W303 are described in Table 1. Proteins were tagged using the PCR-based method described in Longtine et al., 1998. Conditions for growth and release are as described in Amon, 2002. α-factor was re-added to all cultures 90 min after release from the G1 arrest to prevent cells from entering the next cell cycle. Growth conditions for individual experiments are described in the Figure legends.

Localization techniques

Indirect *in situ* immunofluorescence was carried out as described in Visintin et al., 1999 for tubulin, HA-, and MYC-tagged proteins. Chromosomes were spread as described in Nairz and Klein, 1997. HA-tagged proteins were detected with a mouse α-HA.11 antibody (Covance) at a

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1:500 dilution. MYC-tagged proteins were detected with a mouse anti-MYC 9E10 antibody (Babco) at a 1:500 dilution. Both were followed by a secondary anti-mouse CY3 antibody (Jackson ImmunoResearch) at a 1:1000 dilution. Endogenous luminescence was sufficient for visualization of Ndc80-GFP on chromosome spreads. In each experiment, fifty or more cells were counted per strain.

Western blot analysis

Cells were harvested, and incubated in 5% trichloroacetic acid (TCA) and lysed as described in Moll et al., 1991. Immunoblots were performed as described in Cohen-Fix et al., 1996. HA-tagged proteins were detected with a mouse α-HA.11 antibody (Covance) at a 1:500 dilution. vATPase was detected using a mouse anti-vATPase antibody (Molecular Probes) at a 1:2000 dilution. The secondary antibody used was a goat anti-mouse antibody conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch) at a 1:2000 dilution.

Co-Immuno-Precipitation

Cells were arrested in G1 using α-factor (5μg/ml) and released into media at 37°C. 50 mls of culture (OD of 0.8) were harvested, washed with 10 mM Tris (pH 7.5), frozen in liquid nitrogen, and stored at −80°C overnight. Cell pellets were thawed on ice and resuspended in 200 μl of NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM TRIS (pH 7.5), 1 mM dithiothreitol (DTT), 60 mM β-glycerophosphate, 1 mM NaVO$_3$, 2 μM Microcystin-LR (EMD Biosciences), and complete EDTA-free protease inhibitor cocktail (Roche)). Cells were disrupted with ~100 μl glass beads in a FastPrep FP120 (Savant) homogenizer for 3 cycles of 45 sec (6.5 m/s). 1–5 mg of extract (in 120 μl of NP40 buffer) was used for immunoprecipitations. 24 μl of protein G-coupled sepharose beads were added to each sample and incubated with rotation for 2 hrs at 4°C. Samples were washed five times with NP40 buffer, boiled in SDS-based sample buffer, and run on SDS-PAGE gels for subsequent Western blot analysis.

Table 1. List of yeast strains

All strains are isogenic to wild-type W303 (A2587).

Strain number	Genotype
A2587	MATa,
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, mcm22Δ*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, mcm21Δ*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, ndc10-1*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, mtw1-1*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, dam1-1*
	MATa, *cdc14-3, mad1::URA3, LRS4-6HA, ipl1-321*
	MATa, *LRS4-6HA, DSN1-9MYC, cdc14-3*
A14204	MATa, *LRS4-6HA, cdc14-3*
	MATa, *DSN1-9MYC, cdc14-3*
A14204	MATa, *cdc14-3, LRS4-6HA*
A13839	MATa, *cdc15-2, LRS4-6HA*
A21813	MATa, *cdc5-1, URA3, LRS4-6HA*
A13838	MATa, *LRS4-6HA*
A18122	MATa, *pGAL-LRS4*
A18121	MATa, *pGAL-CSM1*
A18142	MATa, *pGAL-LRS4, pGAL-CSM1*
A23708	MATa, *LRS4-6HA, pGAL-URL-YCS4*
A23707	MATa, LRS4-6HA, pGAL-URL-SMC4
A15087	MATa, CSM1-9MYC
A23709	MATa, CSM1-9MYC, pGAL-URL-3HA-SMC4

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Appendix

The reduced ability for disomic yeast strains to segregate a Yeast Artificial Chromosome

Introduction

Two common characteristic of cells in solid tumors are abnormal karyotypes (reviewed in Storchova and Kuffer, 2008) and genomic instability (reviewed in Geigl et al., 2007). During tumorigenesis, cells undergo significant genomic changes including gaining oncogenes or losing tumor suppressor genes, but the mechanism that drives these changes is unknown. Whether aneuploidy is a cause or a consequence of tumorigenesis remains a debate since the first observations that tumor cells contain aberrant karyotypes (Boveri, 1902).

One possibility is that aneuploidy is a late event of tumorigenesis. Mutations and the inactivation of the tumor suppressor gene p53 have been known to induce tetraploidy and genomic instability in cells (Cross et al., 1995; Bunz et al., 2002; Duelli et al., 2005), although p53 mutations can occur late in tumorigenesis and diploid cells can tolerate p53 mutations and retain a normal karyotype (Vogelstein and Kinzler, 2004). Mutations in spindle checkpoint proteins also lead to aneuploidy and eventually tumorigenesis (Michel et al., 2001; Schliekelman et al., 2009; Sotillo

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et al., 2007; reviewed in Vogelstein and Kinzler, 2004). An alternative hypothesis is that aneuploidy tips the balance of a cell towards tumorigenesis. Small ademomas and aypical breast tissue show low levels of loss of heterozygosity (Bomme et al., 1998, 2001; Shih et al., 2001; Larson et al., 2006), which may suggest a role for aneuploidy in tumorigenesis. Still, the evidence is mixed: for example, Down's syndrome patients develop fewer solid tumors but more leukemias (Hasle et al., 2000) and cells carrying mutations in a cell cycle regulated motor protein result in high levels of aneuploidy, a higher incidence of leukemia *in vivo*, but lower rates of tumorigenic growth (Weaver et al., 2007). The relationship between aneuploidy and tumorigenesis needs further attention.

Interestingly, aneuploidy, by itself, confers no proliferation advantage. Morphological defects, infertility, inviability, and restricted growth have been observed in all model organisms (fruit flies, nematodes, fission yeast, jimsom weed and mice) used to study aneuploidy (Williams et al., 2008; reviewed in Torres et al., 2008). Aneuploidy in human organisms is generally lethal and is the largest cause for spontaneous abortions (Hassold and Hunt, 2001), with the only exception being trisomy of the 21st chromosome which results in Down's Syndrome. This fundamental difference between tumors and aneuploid cells exposes the requirement for additional protective mutations that allow annueploid cells to proliferate or allow tumor cells to tolerate aneuploidy.

How then, might aneuploidy confer a proliferation advantage? One common trait between all of the disomic yeast strains is a characteristic stress response (Torres et al., 2007). It appears that this stress response is due to protein imbalances caused by thea additional chromosome being active (Torres et al., 2007). Cancer cells are also sensitive to several translation inhibitors (Easton et al, 2006; Whitesell et al., 2005). There is growing evidence that cellular stress induces chromosomal instability (reviewed in Galhardo et al., 2007). One model is that cellular stress may result in gene amplification, translocation, and mutation events that allow aneuploid cells to acquire advantageous mutations and evolve towards a higher proliferative capacity.

Results

Chromosomal instability is defined loosely by increased mutation rates, chromosomal rearrangements, microsattellite instability, and aberrant methylation (Geigl et al., 2007). To monitor mutation in a population over time, we wish to employ several assays derived from the Lea and Coulson fluctuation assays (1949) using rates of marker gain and loss as a phenotypic readout for different chromosomal arrangements. For example, resistance to canavanine, an arginine analog due to mutations in *CAN1*, an arginine transporter, is generally used as a method for testing mutation rate, and the looping out of chromosomal markers can be used to test recombination rates (Prado and Aguilera, 2004). Aneuploid strains were crossed with wild-type strains carrying markers used for these assays and then selected for the novel marker, and both copies of the marked disomic chromosome to perform these assays (Table 1).

To test the rate of chromosomal instability, a 340kb yeast artificial chromosome (YAC) carrying a portion of chromosome 7 from humans and marked with *URA3* and *TRP1* cassettes at the end of both arms of the chromosome (yWSS1572-1 as described in Huang and Koshland, 2003), were introduced into disomic yeast strains by traditional crosses and fluctuation assays were performed to determine the loss rate per generation. Most of the disomes exhibited increased rates of YAC loss, with disomes of chromosomes 11, 15 and 16 having the largest increases (Figure 1). Surprisingly, the disomic strains with the most severe proliferative delays do not necessarily correspond to those with the highest YAC loss rates. Disome 16 cells exhibit a large cell cycle delay (Torres et al., 2007) and has the highest observed YAC loss rate, but Disome 15 has only a slight cell cycle delay, yet presents the third highest YAC loss rate. These chromosomes are also relatively similar in size, suggesting that additional DNA content alone is not an indicator that a disomic strain will have greater chromosome segregation difficulty.
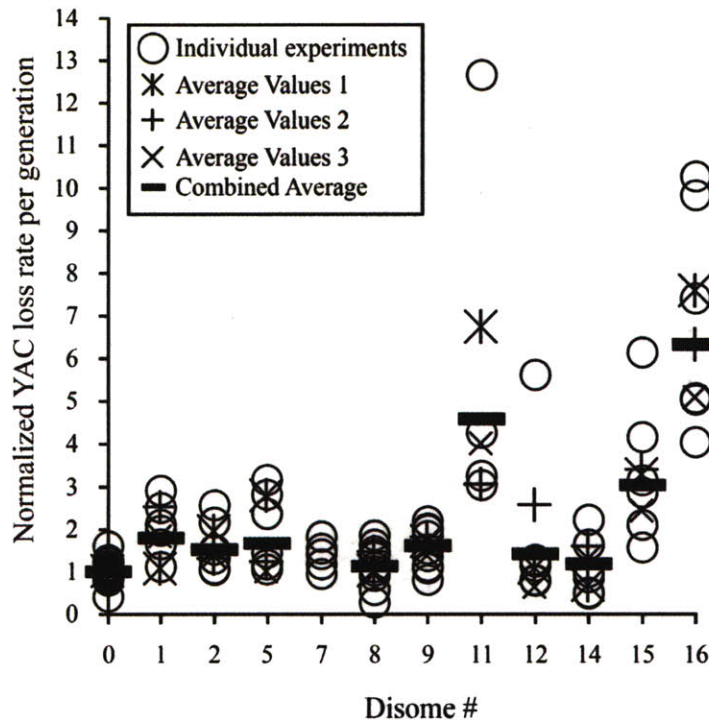
Figure 1. Disomic yeast strains exhibit elevated rates of YAC loss.
Fluctuation analysis was performed to determine the rate of YAC loss per generation
(Methods) of disomic yeast strains relative to wild-type carrying a YAC.
Genotypes and strain numbers are listed in Table 2.

Table 1. YAC loss rates per generation.
Values listed for each disome strain represent the YAC loss rate per generation relative to
wild-type loss rates and corrected for the plating efficiency and the starting population
that lost the YAC. The final column represents the percent of colonies that lost the *URA3*
marker but retained the *TRP1* marker.

Genotype	Expt. #1	Expt. #2	Expt. #3	Combined Average	Percent of cells that were *TRP1* but *ura3-* (Expt. 3 only)
WT	1.00	1.00	1.00	1.00	7.74%
Disome 1	1.05	2.51	1.82	1.80	3.46%
Disome 2	1.93	1.29	1.36	1.53	2.70%
Disome 5	2.78	1.23	0.99	1.67	0.58%
Disome 8	1.01	1.45	0.93	1.13	3.20%
Disome 9	1.70	1.79	1.36	1.62	3.07%
Disome 11	6.73	3.04	3.99	4.59	3.72%
Disome 12	1.06	2.56	0.61	1.41	4.87%
Disome 14	0.66	1.58	1.28	1.17	5.22%
Disome 15	3.27	3.39	2.42	3.03	0.96%
Disome 16	7.57	6.32	5.08	6.32	4.42%

In one replicate, the final plates were replicated onto plates lacking tryptophan to see whether there was an increase in mitotic recombination and the cells only retained a portion of the YAC. The wild-type strains exhibited the highest portion of cells able to grow on medium lacking tryptophan. The strain background for the disomic strains includes the *trp1-1* allele which contains one amber suppressor and can frequently revert back to a wild-type phenotype. Therefore, it is unclear what the starting frequency of *TRP1* revertants, the level of gene repair between the *trp1-1* allele and the *TRP1* contained on the YAC, and the amount of mitotic recombination. To determine if the full YAC is still intact, pulsed field gel electrophoresis can be used. Regardless, it is interesting to note that the rate of growth on medium lacking tryptophan was not increased among the disomic strains.

Discussion

Further analysis of the mechanism that causes YAC loss in these disomic strains is needed, but the result is intriguing. Do aneuploid yeast strains carrying multiple copies of 2 or more chromosomes exhibit further increases in YAC loss rates? Tetraploid cells exhibit exacerbated loss rates, above 200-fold YAC loss rates in diploid cells, and it was found that this is due to an inability to scale the size of spindle pole bodies and the mitotic spindle (Storchova et al., 2006). This suggests that the defect is due to extra microtubule-spindle attachments rather than the size of the chromosome. It remains to be seen whether aneuploid cells also exhibit spindle pole morphological differences. The challenge for cells containing a full extra genome versus a small portion of the genome is fundamentally different: the transcriptome of tetraploid and diploid cells is merely scaled whereas disomic strains experience a significant transcriptional imbalance due to the excess open reading frames present on the duplicated chromosome.

One line of inquiry is whether the spindle checkpoint is functioning properly in disomic cells. Preliminary observation of Pds1 degradation showed normal degradation of Pds1, or securin, which serves as a read-out of spindle checkpoint inactivation (data not shown). Pds1 is an inhibitor of the metaphase-anaphase transition which is normally degraded once the spindle checkpoint is satisfied. In the event of spindle checkpoint activation, it is stabilized. It can also be stabilized in the presence of DNA damage during anaphase (Tinker-Kulberg and Morgan,

1999). Nevertheless, of the 5 aneuploid strains tested, none showed a delay in Pds1 degradation nor stabilization of Pds1 when compared to wild-type. The segregation difficulty the cell faces due to the presence of the extra chromosome and the YAC may be too subtle to trigger Pds1 stabilization, given that the disomic yeast strains do not display a metaphase delay (Torres et al., 2007).

Further experiments into mutation rates, recombination rates, microsatellite instability and other readouts for genetic instability will need to be performed to determine whether aneuploid strains have specific problems with chromosome segregation or whether general mechanisms for genomic instability are increased.

Materials and methods

Yeast strains

All of the yeast strains were generated as described in Torres et al., 2007. The creation of a strain carrying the original YAC is described in Huang and Koshland, 2003. Strains carrying the wild-type *CAN1* allele or epitope-tagged Pds1 were created by crossing the disomic strains to an isogenic strain carrying either of these alleles. Disomic strains carrying the YAC were created by a normal mating between the disomic yeast strain and the YAC-containing strain, isogenic to the disomic strains. Disomic strains carrying the YAC were grown on media containing G418 and lacking histidine, uracil and tryptophan to select for the two homologous chromosomes marked by the *KanMX6* and *HIS3* markers and to select for the YAC which is marked by *URA3* and *TRP1*. The presence of the YAC in the original strain was confirmed by Pulsed Field Gel Electrophoresis and Southern Blotting using a probe for *URA3* (Torres et al., 2007).

Table 2. Yeast strains generated

Disome	Strain number	Genotype
Wildtype + YAC	A23744	MATa, *ade1::HIS3, lys2:KanMX6*
Disome I + YAC	A23745	MATa, *ade1::HIS3, ade1::KanMX6*
Disome II + YAC	A23746	MATa, , *lys2::HIS3, lys2::KanMX6*
Disome V + YAC	A23747	MATa, *can1::HIS3, intergenic region (187520-187620) between YER015W and YER016W::KanMX6*
Disome VIII + YAC	A23748	MATa, *intergenic region (119778-119573) between YHR006W and YHR007C::HIS3, intergenic region (119778-119573) between YHR006W and YHR007C::KanMX6*
Disome IX + YAC	A23749	MATa, *intergenic region (341900-34200) between YIL009W and YIL008W::HIS3, intergenic region (341900-34200) between YIL009W and YIL008W::KanMX6,*
Disome XI	A23750	MATa, , *intergenic region (430900-431000) between YKL006C-A and YKL006W::HIS3, intergenic region (430900-431000) between YKL006C-A and YKL006W::KanMX6*
Disome XII + YAC	A23751	MATa, *ade16::HIS3, ade16::KanMX6*
Disome XIV + YAC	A23752	MATa, *intergenic region (622880-622980) between YNL005C and YNL004W::HIS3, intergenic region (622880-622980) between YNL005C and YNL004W::KanMX6*
Disome XV + YAC	A23753	MATa, *leu9::HIS3, leu9::KanMX6*
Disome XVI + YAC	A23754	MATa, *met12::HIS3, met12::KanMX6*

Comparative Genome Hybridization.

The karyotype of each strain was determined by comparative genome hybridization after each cross. 15ml cultures of cells were grown to saturation in medium containing G418 and lacking histidine, uracil and tryptophan. Cells were incubated for 60 minutes at 37°C in 1.5 mls of 1 M Sorbitol, 10 mM Na-phosphate, pH 7.0, 10 mM EDTA, 200µg/ml zymolase and 150µ-mercaptoethanol. Cells were pelleted and incubated in 1.5 mls of 50 mM EDTA, pH 8.0, 0.3% SDS, 200µg/ml proteinase K and incubated for another 60 minutes at 65oC. 0.6mls of 5 M KOAc was added and incubated on ice for 30 minutes. After centrifugation, the supernatant was

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subjected to a phenol/chloroform extraction and DNA was precipitated. The DNA was RNAse treated at 37°C for 2 hours (10 mM Tri-HCl, 1 mM EDTA, pH 7.5, 1 mg/ml RNAse), followed DNA purification (Qiagen). Samples were sonicated three times, each for 10 seconds and labeled with Cy3- or Cy5-dCTP using Klenow polymerase. Yield and dye incorporation were checked with a Nanodrop spectrophotometer. 200 ng differentially labeled DNA from the reference strain and the strain of interest were mixed, combined with control targets and hybridization buffer, boiled for 5 minutes, and applied to a microarray consisting of 60mer probes for each yeast open reading frame (Agilent). Microarrays were rotated at 60°C for 17 hours in a hybridization oven (Agilent). Arrays were then washed according to the Agilent SSPE wash protocol, and scanned on an Agilent scanner. The image was processed using the default settings with Agilent Feature Extraction software. All data analysis was performed using the resulting $\log_2$ ratio data, and filtered for spots called as significantly over background in at least one channel.

YAC loss assay

Standard fluctuation analysis (Lea and Coulson, 1949) was used to determine the percentage of cells that lose the YAC per generation. Cells carrying *HIS3* and *KanMX* cassettes as well as the YAC (*URA3, TRP1*) were grown in medium containing G418 and lacking uracil, histidine, tryptophan media overnight. To begin the experiment, cells were counted using a Coulter Counter and then plated on plates containing G418 and lacking histidine (+G418 –HIS); as well as plates containing 5-FOA and G418 and lacking histidine (+G418 +5-FOA –histidine), to monitor plating efficiency and the percentage of the starting population that contained the YAC. After 24 hours in medium that was selective for the disomic chromosome but not for the YAC (+G418 –HIS), cells were re-counted and plated onto both +G418 –HIS plates and +G418 +5-FOA –HIS plates. There were no large discrepancies regarding viability. All cultures were grown for roughly the same number of generations (note that some strains exhibit slower growth in –HIS +G418 medium (Torres et al., 2007) and were therefore grown for a longer length of time). Plating efficiency and the percentage of cells at the start of the experiment that had lost the YAC were taken into account. Three replicates of each strain were grown on three separate occasions.

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