The Monopolin Complex Crosslinks Kinetochore Components to Regulate Chromosome-Microtubule Attachments

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The Monopolin Complex Crosslinks Kinetochore Components to Regulate Chromosome-Microtubule Attachments

Kevin D. Corbett,1 Calvin K. Yip,2 Ly-Sha Ee,3 Thomas Walz,2,4 Angelika Amon,3,4 and Stephen C. Harrison1,4,*

1Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115, USA
2Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
3David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
4Howard Hughes Medical Institute
*Correspondence: harrison@crystal.harvard.edu
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SUMMARY
The monopolin complex regulates different types of kinetochore-microtubule attachments in fungi, ensuring sister chromatid co-orientation in Saccharomyces cerevisiae meiosis I and inhibiting merotelic attachment in Schizosaccharomyces pombe mitosis. In addition, the monopolin complex maintains the integrity and silencing of ribosomal DNA (rDNA) repeats in the nucleolus. We show here that the S. cerevisiae Csm1/Lrs4 monopolin subcomplex has a distinctive V-shaped structure, with two pairs of protein-protein interaction domains positioned ~10 nm apart. Csm1 presents a conserved hydrophobic surface patch that binds two kinetochore proteins: Dsn1, a subunit of the outer-kinetochore MIND/Mis12 complex, and Mif2/CENP-C. Csm1 point-mutations that disrupt kinetochore-subunit binding also disrupt sister chromatid co-orientation in S. cerevisiae meiosis I. We further show that the same Csm1 point-mutations affect rDNA silencing, probably by disrupting binding to the rDNA-associated protein Tof2. We propose that Csm1/Lrs4 functions as a molecular clamp, crosslinking kinetochore components to enforce sister chromatid co-orientation in S. cerevisiae meiosis I and to suppress merotelic attachment in S. pombe mitosis, and crosslinking rDNA repeats to aid rDNA silencing.

INTRODUCTION
Mitosis and meiosis are related processes in which duplicated eukaryotic chromosomes segregate to daughter cells or gametes (Lee and Amon, 2001; Marston and Amon, 2004; Nasmyth, 2001). In mitosis, chromosomes replicate and the resulting sister-chromatid pairs are held together along their length by cohesin complexes. Associated with the centromere of each chromatid is a kinetochore, a specialized protein assembly that captures microtubules (MTs) of the mitotic spindle. In early mitosis, each sister chromatid pair becomes “bi-oriented” when its kinetochores capture MTs extending from opposite spindle poles. Once all chromatid pairs are properly attached, cleavage of the cohesin links between sisters allows chromosome segregation and subsequent cell division.

DNA replication and cell division strictly alternate in mitosis, but in meiosis, DNA replication is followed by two successive divisions to yield four haploid gametes. During meiotic prophase, homologous chromosomes align and form crossovers that hold them together. This organization allows for bi-orientation and segregation of homologs in meiosis I, followed by segregation of sister chromatids in meiosis II (Lee and Amon, 2001; Marston and Amon, 2004; Nasmyth, 2001). Thus, although sister chromatids bi-orient and segregate from each other in mitosis and meiosis II, they instead co-orient and segregate together in meiosis I.

In the budding yeast Saccharomyces cerevisiae, sister chromatid co-orientation in meiosis I depends on the four-protein monopolin complex (Mam1, Csm1, Lrs4, and Hrr25/casein kinase 1), which localizes to centromeres from meiotic prophase through metaphase I (Monje-Casas et al., 2007; Petronczki et al., 2006; Rabitsch et al., 2003; Toth et al., 2000). Mam1 is expressed specifically in meiosis (Toth et al., 2000) and associates at centromeres with the ubiquitous kinase Hrr25 (Petronczki et al., 2006). The remaining subunits, Csm1 and Lrs4, form a complex that resides in the nucleolus during interphase and relocates to centromeres during meiotic prophase, accompanied by phosphorylation of Lrs4 (Huang et al., 2006; Katis et al., 2004; Lo et al., 2008; Matos et al., 2008; Rabitsch et al., 2003). Robust centromeric localization of Csm1/Lrs4 requires Mam1 (Rabitsch et al., 2003). It has been proposed that the monopolin complex crosslinks and/or co-orient sister kinetochores in meiosis I, so that they attach to MTs extending from the same spindle pole (Monje-Casas et al., 2007). Although monopolin complex subunits have not been identified outside of fungi, the concept of sister kinetochore “fusion” in meiosis I may have parallels in higher eukaryotes: in maize meiosis I, for example, inner kinetochores of sister chromatids can be
resolved by fluorescence microscopy, whereas their outer kinetochores appear fused (Li and Dawe, 2009).

Orthologs of the monopolin subunits Csm1 and Lrs4 are present in the fission yeast Schizosaccharomyces pombe (Pcs1 and Mde4, respectively) and also cycle between the nucleolus and kinetochores (Gregan et al., 2007; Rabitsch et al., 2003). These proteins inhibit merotelic attachment (capture of a single kinetochore by MTs from opposite spindle poles) during mitosis, but they do not contribute to sister chromatid co-orientation in meiosis I. (An unrelated protein, Moa1, is important for ensuring meiosis I sister co-orientation in S. pombe, probably by modifying cohesion-complex function near centromeres [Yokobayashi and Watanabe, 2005]). Although S. cerevisiae Csm1 interacts with the nucleolar protein Tof2 through the so-called Heh1 and Nur1 (Mekhail et al., 2008), and the condensin complex (Johzuka and Horiuchi, 2009), in addition to Sir2 (Huang et al., 2006; Smith and Boeke, 1997; Smith et al., 1999). With the exception of Sir2, which independently contributes to USCE suppression, these proteins appear to tether rDNA repeats to the nuclear periphery, sequestering them from recombination factors (Mekhail et al., 2008), and they may also clamp sister chromatids together in register (Brito et al., 2010; Johzuka and Horiuchi, 2009). Thus, although Csm1/Lrs4 contributes to both rDNA silencing and USCE suppression, it acts with distinct sets of proteins in these different processes, raising the question of whether a common mechanism underlies these activities.

The regulatory functions of monopolin described above suggest that Csm1/Lrs4 and the orthologous Pcs1/Mde4 are molecular crosslinkers, joining MT-binding elements at kinetochores and rDNA repeats in the nucleolus. We report here that S. cerevisiae Csm1 and Lrs4 form a complex with a distinctive “V” shape, which positions two pairs of protein-protein interaction domains ~10 nm apart. We find that a conserved surface patch on these domains binds two kinetochore subunits: Dsn1, a subunit of the outer-kinetochore MIND/Mis12 complex, and Mif2/CENP-C. Point-mutations in this conserved surface disrupt both Dsn1 and Mif2 binding in vitro and cause bi-orientation of sister chromatids in meiosis I. These data are consistent with a model of monopolin as a crosslinker that clamps kinetochores together to enforce co-orientation in S. cerevisiae meiosis I and inhibit merotelic attachment in S. pombe mitosis. We also find that Csm1 interacts with the nuclear protein Tof2 through the same conserved surface that interacts with Dsn1 and Mif2, and that mutating the Csm1 surface patch also disrupts rDNA silencing. These mutations do not, however, affect the rate of unequal sister chromatid exchange, demonstrating that Csm1/Lrs4 has two biochemically separate roles in the maintenance of rDNA. Overall, our data show that Csm1/Lrs4 is a molecular crosslinker that regulates kinetochore-microtubule attachment and helps preserve rDNA integrity.

RESULTS

Structure of Csm1

We purified full-length S. cerevisiae Csm1 and S. pombe Pcs1 proteins, as well as truncations lacking the bulk of their N-terminal regions, which are predicted to form coiled-coils (Gregan et al., 2007; Rabitsch et al., 2003). By sedimentation equilibrium analytical ultracentrifugation, we found that both constructs of Csm1 and Pcs1 are homodimers in solution (Table 1). We obtained crystals of both full-length S. cerevisiae Csm1 and its isolated C-terminal domain (residues 69–181 of 190). We determined the structure of the C-terminal domain to 2.35 Å resolution using anomalous diffraction methods with selenomethionine-derivatized protein (see Table S1, available online, for crystallographic statistics), and we then determined the structure of the full-length protein to 3.4 Å resolution by molecular replacement. The structures show that Csm1 has a 12-nm long, N-terminal coiled-coil (residues 3–82), and a

<table>
<thead>
<tr>
<th>Protein/Complex</th>
<th>Observed MW (kDa)</th>
<th>Calculated MW (kDa)</th>
<th>Oligomeric State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csm1 full-length</td>
<td>43.2 ± 0.9</td>
<td>43.5</td>
<td>2 Csm1</td>
</tr>
<tr>
<td>Csm1 1–181</td>
<td>43.4 ± 1.2</td>
<td>41.2</td>
<td>2 Csm1</td>
</tr>
<tr>
<td>Csm1 69–190</td>
<td>29.1 ± 2.3</td>
<td>28.2</td>
<td>2 Csm1</td>
</tr>
<tr>
<td>Pcs1 full-length</td>
<td>53.4 ± 1.7</td>
<td>51.8</td>
<td>2 Pcs1</td>
</tr>
<tr>
<td>Pcs1 85–222</td>
<td>30.1 ± 2.3</td>
<td>32.2</td>
<td>2 Pcs1</td>
</tr>
<tr>
<td>Csm1/Lrs4 full-length</td>
<td>172.3 ± 4.7</td>
<td>165.6</td>
<td>4 Csm1 + 2 Lrs4</td>
</tr>
<tr>
<td>Csm1/Lrs4 1–130</td>
<td>120.4 ± 3.1</td>
<td>117.6</td>
<td>4 Csm1 + 2 Lrs4</td>
</tr>
<tr>
<td>Csm1/Lrs4 1–102</td>
<td>108.5 ± 3.0</td>
<td>111.0</td>
<td>4 Csm1 + 2 Lrs4</td>
</tr>
<tr>
<td>Csm1 1–181/Lrs4 2–30</td>
<td>91.3 ± 3.1</td>
<td>89.7</td>
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</tr>
<tr>
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<td>164.2 ± 7.8</td>
<td>198.9</td>
<td>4 Pcs1 + 2 Mde4</td>
</tr>
<tr>
<td>Pcs1/Mde4 1–231</td>
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<tr>
<td>Pcs1/Mde4 1–125</td>
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<td>108.2 ± 4.1</td>
<td>121.0</td>
<td>4 Pcs1 + 2 Mde4</td>
</tr>
</tbody>
</table>

*Calculated MW is the expected molecular mass of a complex with the stoichiometry listed in “Oligomeric State.”*
C-terminal globular domain (residues 83–181) containing three α helices and five β strands (Figure 1A). The β sheet of each monomer wraps around the N-terminal α helix of its dimer mate, forming an intimate dimer interface.

Csm1 is a structural relative of the kinetochore proteins Spc24 and Spc25 (Figures 1B and 1C) (Wei et al., 2006). These proteins are paralogs that form a heterodimer similar to the Csm1 homodimer and constitute the inner half of the conserved Ndc80 kinetochore complex (Joglekar et al., 2006; Wei et al., 2005). The similarity of the tertiary and quaternary structures of Csm1 and Spc24/Spc25 imply a common evolutionary origin, despite their very low sequence identity (<15%). The C-terminal globular domains of Spc24 and Spc25 are thought to connect the Ndc80 complex with proteins of the inner kinetochore, suggesting that this domain of Csm1 may also be a protein interaction module. Inspection of amino acid conservation among 41 fungal Csm1/Pcs1 orthologs reveals a conserved surface patch on the face directly opposite the coiled-coil, bordering α-3, β-5, and α-4 (Figure 1D; Figure S1). The patch faces away from the dimer interface, so that the Csm1 dimer has two conserved surfaces centered ~3 nm from each other. Because both α-3 and β-5 are located in a Csm1-specific insertion in the globular domain, this surface is not present in Spc24/Spc25. Therefore, it is unlikely that Csm1 and Spc24/Spc25 have a common binding site at kinetochores, despite their overall structural similarity.

Structure of the Csm1/Lrs4 Complex
S. cerevisiae Csm1 and Lrs4 are known to interact through their N-terminal coiled-coil regions (Rabitsch et al., 2003). Coexpression of Csm1 with full-length Lrs4 (347 residues) or with an isolated N-terminal segment (residues 1–130 or 1–102) yields a complex with four copies of Csm1 and two copies of Lrs4 (Table 1). Coexpression of S. pombe Pcs1 and Mde4 also yields a complex with 4:2 stoichiometry, with the N-terminal region of Mde4 (residues 1–77 of 421 is the smallest segment we have tested) sufficient for complex formation (Table 1; Figure S2).

We obtained crystals of the complex between S. cerevisiae Csm1 and residues 1–102 of Lrs4, which diffracted anisotropically...
to between 6.0 Å (along the a* and b* reciprocal unit-cell axes) and 3.9 Å (along c*) resolution, and we determined the structure by molecular replacement. We located two Csm1 homodimers per asymmetric unit and found that the N-terminal coiled-coils of these two dimers sandwich two closely packed, parallel, 30 amino acid α helices, creating a V-shaped complex that positions the two pairs of Csm1 globular domains ~10 nm apart (Figures 2A and 2B).

Our electron density maps indicated that two copies of a ~30 amino acid region of Lrs4 might be sufficient for complex formation with Csm1, but the low resolution precluded direct assignment of sequence to this electron density. From sequence conservation and secondary structure predictions, it appeared that the density probably represented two N-terminal segments of Lrs4 residues 34–102, with the remainder of the protein (approximately residues 34–102) disordered in our crystals. Mixing Csm1 with a peptide containing Lrs4 residues 2–30 results in a stable complex with the expected molecular mass for a 4:2 complex (Table 1; Figure S2). This complex also forms crystals isomorphous to those of the complex with Lrs4 1–102 (data not shown),

Figure 2. Structure of the Csm1/Lrs4 Complex

(A) Diagram of Csm1 and Lrs4 polypeptide chains. Domains of Csm1 are colored as in Figure 1; residues 1–33 of Lrs4 are in green. For Lrs4, predicted coil-coil (residues 54–62) is in gray, and the motif conserved between Lrs4 and S. pombe Mde4 (Gregan et al., 2007) in red; a blue arrowhead indicates a lysine/arginine-rich motif (K/R). The gray arrow indicates the interacting regions of the proteins.

(B) Orthogonal views of the (Csm1) 4:(Lrs4) 2 complex, colored as in (A). Residue numbers of the two Lrs4 α helices are marked. Although Lrs4 residues 34–102 were present in the complex as crystallized, they were disordered in the electron density maps. In the crystals, the two Lrs4 α helices extend into a solvent channel large enough to accommodate these disordered regions (not shown).

(C) Electron density surrounding the Lrs4 α helices. Refined 2Fo−Fc density (1.2 σ) is in gray, and anomalous difference density from an Lrs4 Leu8→Met selenomethionine (Se-Met) dataset (4.0 σ), is in red. The Cz-atom of Leu8 is shown as a sphere. There is a single strong anomalous difference-density peak directly between the helices, which probably represents the anomalously scattering Se atoms of both Se-Met residues in the Lrs4 dimer.

(D) Electron micrograph of negatively stained Csm1/Lrs4 1–102 complex, with representative particles circled.

(E) Electron micrograph of the full-length nucleolar Csm1/Lrs4 complex, with representative particles circled.

(F) Representative class averages of the full-length Csm1/Lrs4 complex are shown side-by-side with matched resolution-filtered projections of the Csm1/Lrs4 1–102 crystal structure. For more information on the assembly and purification of Csm1/Lrs4 and S. pombe Pcs1/Mde4 complexes, see Figure S2. See also Figure S2 and Table S1.
supporting the inference that Lrs4 residues 34–102 are disordered in the latter crystals and hence do not contribute to the diffraction intensities. We established the orientation and sequence register of the Lrs4 α helices using selenomethionine anomalous scattering from a construct containing a L8→M mutation in Lrs4. We observed a single anomalous difference peak in the electron density maps, directly between the two Lrs4 α helices (Figure 2C); at this resolution (6.0/3.9 Å), the single peak probably represents the anomalous scattering of both Se atoms in the Lrs4 dimer. This assignment indicates that the disordered C-terminal region of Lrs4 extends outward from the base of the observed V.

We also examined the architecture of the native Csm1/Lrs4 complex using negative-stain electron microscopy. We purified Csm1/Lrs4 from mitotically cycling S. cerevisiae by TAP-tagging Lrs4, thus obtaining a near-native nucleolar form of Csm1/Lrs4. Individual particles of this complex, as well as of the recombinant Csm1/Lrs4 1–102 complex, show a clear V shape (Figures 2D and 2E), and class averages of the native full-length complex can be matched with 2D projections of the X-ray structure (Figure 2F). We can draw two conclusions from the close correspondence of the projections and class averages. First, the conformation of the complex is stiff and not grossly affected by the packing in our crystals. Second, the bulk of the two Lrs4 subunits is either largely disordered in solution or flexibly linked to the rest of the complex. This conclusion is consistent with our observation that residues 34–102 are disordered in the crystal structure of Csm1/Lrs4 1–102. The C-terminal region of Lrs4 is nonetheless crucial for regulation of monopolin: phosphorylation and dephosphorylation of Lrs4 (and its S. pombe ortholog Mde4) is needed for localization and activity at kinetochores (Choi et al., 2009; Katis et al., 2004; Lo et al., 2008; Matos et al., 2008), and even a small C-terminal deletion of Lrs4 (23 residues) compromises function in the nucleolus (Johzuka and Horichi, 2009).

Csm1 binds two kinetochore subunits

Previous attempts to identify direct binding partners of Csm1/Lrs4 using negative-stain electron microscopy have not identified any kinetochore subunits, which are present in relatively low abundance in the cell (Huang et al., 2006; Petronczki et al., 2006). The kinetochore proteins Ctf19 and Dsn1 were recently identified as potential Csm1 binding partners by a large-scale two-hybrid screen focused on S. cerevisiae kinetochore proteins (Wong et al., 2007). Ctf19 is part of the COMA complex, which is not well-conserved in higher eukaryotes, whereas Dsn1 is a component of the highly conserved MIND/Mis12 complex (Cheeseman and Desai, 2008). In addition, a recent study identified an interaction between S. pombe Psc1 and the inner-kinetochore protein Cnp3/CENP-C (S. cerevisiae Mif2), also by yeast two-hybrid analysis (Tanaka et al., 2009). We tested binding of in vitro-translated [35S]-labeled Dsn1, Ctf19, and Mif2 to several constructs of Csm1, using a Ni2+-affinity pull-down assay. Although Ctf19 did not bind Csm1 (data not shown), both Dsn1 and Mif2 bound to full-length Csm1 and to the isolated C-terminal globular domain, but not to the isolated N-terminal coiled-coil region (Figure 3A). Because Csm1 has previously been shown to interact with the monopolin subunit Mam1 (Rabitsch et al., 2003), we also tested binding of this protein to our Csm1 constructs and found that Mam1 binds specifically to the C-terminal globular domain of Csm1 (Figure 3A). We next tested whether Dsn1, Mif2, or Mam1 binding was affected by point mutations in the conserved hydrophobic surface patch on the Csm1 globular domain. None of the mutations tested affected Mam1 binding (Figure 3A), but three of them, Y156→E, L161→D, and L161→K, substantially reduced binding of both Dsn1 and Mif2 (Figure 3A). These results indicate that both Dsn1 and Mif2 contact the conserved surface patch on Csm1 and may even compete for a common binding surface, whereas Mam1 probably binds elsewhere on the Csm1 C-terminal domain.

To confirm and extend these results, we purified the four-protein MIND (Mtw1 including Nsi1, Nnf1, and Dsn1) kinetochore complex (De Wulf et al., 2003), which forms an extended ~25-nm-long structure containing one copy of each subunit (Table S2; Figure S3). In the Ni2+-affinity pull-down assay, this purified complex bound full-length Csm1 and the isolated C-terminal globular domain, and mutating Csm1 residues Y156 and L161 disrupted the interaction (Figure S3). Using size exclusion chromatography, we observed that the Csm1/Lrs4 complex comigrated with purified MIND complex, although some dissociation occurred during the course of the experiment (Figures 3B and 3C). When the two complexes were present in a 1:4 ratio (Csm1/Lrs4:MIND), essentially all of the Csm1/Lrs4 comigrated with MIND. When the complexes were present at 1:2 or 1:1 ratios, some Csm1/Lrs4 did not comigrate with MIND. Thus, each Csm1/Lrs4 complex can probably interact with up to four MIND complexes, meaning that each conserved surface patch in the complex can independently interact with a partner protein (Figure 3C).

Because the conserved Csm1 surface patch is shared in all fungal Csm1/Pcs1 orthologs (Figure S1), Dsn1 and/or Mif2 may represent conserved binding partners for these proteins. To test this idea, we performed Ni2+-affinity pull-down assays using S. pombe Pcs1 as bait, and the S. pombe Dsn1 and Mif2 orthologs, Mis13 and Cnp3, as prey. We found that the S. pombe Pcs1 C-terminal domain interacts with Mis13, and with both full-length Cnp3 and the minimal Pcs1-binding fragment identified previously, amino acids 130–270 (Figure 3D) (Tanaka et al., 2009). Binding to both Mis13 and Cnp3 was disrupted by mutations to Pcs1 residues Y197 and I202 (Figure 3D), which correspond to S. cerevisiae Csm1 residues Y156 and L161, respectively (Figure S1). Thus, S. cerevisiae Csm1 and S. pombe Pcs1 bind orthologous kinetochore subunits through the conserved surface patch on their C-terminal globular domains.

Csm1 point mutations disrupt sister chromatid co-orientation in meiosis I

Deletion of any single monopolin subunit in S. cerevisiae results in mis-segregation of chromosomes in meiosis I (Petronczki et al., 2006; Rabitsch et al., 2003; Toth et al., 2000). In order to observe sister chromatid MT attachment geometry in meiosis I, we used an S. cerevisiae strain bearing an array of TET operator sequences inserted at the chromosome V centromere and also expressing a fusion of GFP and the Tet repressor protein, which binds to the operator sites (referred to as CENV-GFP) (Lee and Amon, 2003). We introduced CSM1 point-mutations into a strain

heterozygous for CENV-GFP and with the endogenous promoter of CDC20 replaced by that of CLB2, to arrest the cells in metaphase I (Lee and Amon, 2003). The heterozygous CENV-GFP marker allows a simple readout of sister chromatid attachment geometry: when the monopolin complex is functioning properly to co-orient sister kinetochores, the GFP signals from the two marked sister centromeres should overlap. If the monopolin complex is compromised, sister kinetochores become bi-oriented, and spindle forces pull the marked sister centromeres far enough apart to form two resolved foci (Lee and Amon, 2003; Monje-Casas et al., 2007; Toth et al., 2000). We introduced the three Csm1 point mutations that disrupt Dsn1/Mif2 binding in vitro: Y156→E, L161→D, and L161→K. Two of these, L161→D and L161→K, result in 45% and 41% GFP dot separation, respectively, matching the severity of a MAM1 deletion (40% separation, see Figure 4A). The third mutation, Y156→E, does not cause elevated levels of sister chromatid bi-orientation in vivo, suggesting that the Y156→E mutant retains some affinity for its binding partners at the kinetochore that is not detected by our pull-down assay.

We next used strains carrying CENV-GFP on both homologs and the wild-type CDC20 promoter (no metaphase I arrest) to examine chromosome segregation fidelity through a complete meiosis, by counting GFP signals in the spores of tetrads. In 95% of wild-type tetrads, all four spores contained GFP foci, indicating that each spore had received a single copy of...
mutations (as noted) were arrested in metaphase I (Lee and pCLB2-CDC20 562 Cell)

We found a similar range in the / (16.5% faulty segregation) and L161 severity, with the Y156 increases in faulty segregation over wild-type, but varied in CSM1 point-mutations all showed statistically significant chromosome V (5% faulty segregation; Figure 4B and Table S3). See also Table S3.

Table S3).

to spores (2 stars, p < 0.01; 3 stars, p < 0.001) and for spore viability (data in CENV Amon, 2003 ), and scored for sister chromatid bi-orientation (gray) versus CENV (A) Yeast strains with heterozygous Figure 4. Effects of Csm1 Point Mutations on Chromosome Segregation

(B) Yeast strains with indicated as stars (3 stars, p < 0.001). In contrast, a CSM1 deletion resulted in nearly 80% of tetrads with GFP dots in only three, two, or one spore, in close agreement with previous studies (Rabitsch et al., 2003). The three CSM1 point-mutations all showed statistically significant increases in faulty segregation over wild-type, but varied in severity, with the Y156→E mutation having the mildest effect (16.5% faulty segregation) and L161→D the strongest (58% faulty segregation; Figure 4B). We found a similar range in the effects of the point mutations on spore viability: 88% of spores were viable in the Y156→E mutant, 34% in L161→K, and only 12% in L161→D (wild-type = 93% viable, Δcsm1 = 4% viable; Figure 4B and Table S3). This range of phenotypes indicates that each point mutation affects Csm1-kinetochore interactions to a different degree, and that none of the mutations completely eliminates kinetochore binding. Overall, however, our results show that mutations to the conserved surface patch of Csm1 cause significant errors in meiotic chromosome segregation, most likely by disrupting Csm1-kinetochore interactions in metaphase I.

Csm1 Point Mutations Affect rDNA Silencing

In addition to acting at kinetochores, Csm1 and Lrs4 localize to the nucleolus during interphase, where they participate in rDNA silencing and the suppression of USCE (Huang et al., 2006; Huang and Moazed, 2003). Our structure of the Csm1/ Lrs4 complex and our studies of its interactions with kinetochore components suggest that it may also crosslink proteins associated with rDNA, potentially through interactions in the conserved surface patch of Csm1. We therefore studied the effects of Csm1 conserved-patch mutations in two assays, one measuring reporter gene silencing and another measuring USCE. To measure rDNA silencing, we compared the growth of yeast strains with an mURA3 reporter gene inserted at either the leu2 locus (which is not silenced) or at two locations in the nontranscribed regions of the rDNA repeat (NTS1 or NTS2; Figure 5A), on either complete media or media lacking uracil. This assay has previously shown that the Sir2 histone deacetylase is required for silencing throughout the rDNA, while a network of proteins associated with the replication-fork block sequence (RFB in Figure 5A), including Fob1 and Tof2 as well as Csm1/ Lrs4, is required for silencing specifically in the NTS1 region (Huang et al., 2006; Huang and Moazed, 2003). We found that mutations to the conserved surface patch of Csm1 had the same effect as a CSM1 deletion: silencing was completely lost at NTS1 and partially lost at NTS2 (Figure 5B).

We next tested the rate of recombination resulting in USCE, which is strongly inhibited in wild-type cells through multiple mechanisms: a Sir2-mediated mechanism presumably dependent on the assembly of silenced chromatin, and tethering of rDNA repeats to the nuclear periphery mediated by Csm1/ Lrs4, the inner nuclear membrane proteins Heh1 and Nur1, and possibly condensins (Huang et al., 2006; Huang and Moazed, 2003; Johzuka and Horiuchi, 2009; Kaeberlein et al., 1999; Mekhail et al., 2008). We measured the rate of loss of an ADE2 reporter gene embedded within the rDNA array and found a greater than three-fold increase in USCE when either CSM1 or LRS4 was deleted (Figure 5C; Table S4), in agreement with previous results (Huang et al., 2006). In contrast, we found that none of the CSM1 point-mutations significantly increased USCE (Figure 5C).

These results indicate that the Csm1/Lrs4 complex may have multiple, biochemically distinct roles in rDNA regulation. The behavior of the CSM1 point mutants resembles that of a deletion of TOF2, another member of the RFB-bound protein network. A TOF2 deletion results in loss of rDNA silencing at NTS1, but has much more modest effects on USCE than deletions of CSM1, LRS4, or SIR2 (Huang et al., 2006; Figure 5C). Because previous biochemical purifications of rDNA-associated protein complexes (Huang et al., 2006) and two-hybrid screens (Wong et al., 2007; Wysocka et al., 2004; Yu et al., 2008) had identified Tof2 as a potential binding partner of Csm1, we tested this interaction in vitro using Ni2+-affinity pull-downs. We found that
full-length Tof2 specifically interacts with the Csm1 C-terminal domain and that this binding is modestly affected by mutations to the conserved surface patch (Figure 5D). Because full-length Tof2 may be poorly behaved in solution, we also looked for truncations of Tof2 that bind Csm1. We identified a region (residues 251–500) that interacts strongly with wild-type Csm1 and binding of which is disrupted by mutations to conserved-patch residues Y156, L161, and K174 (Figure 5D). The effect of the Csm1 K174→E mutation on Tof2 binding is much more pronounced than its effect on binding of either Dsn1 or Mif2 (Figure 3A). This result, along with the finding that the Y156→E mutation strongly affects Csm1/Lrs4 function at rDNA but has more modest effects at kinetochores, suggests that although Csm1 binds its multiple partners through a common surface, the details of each interaction probably differ.

Figure 5. Csm1 Binds the rDNA Protein Tof2, and Csm1 Mutations Affect rDNA Silencing

(A) Diagram of a single rDNA repeat, with positions of mURA3 markers inserted into the array at NTS1 and NTS2 noted. 35S and SS refer to ribosomal genes, RFB; replication fork block sequence, rARS; autonomously replicating sequence.

(B) Silencing of an inserted mURA3 marker (at leu2, NTS1, or NTS2 as noted) was assessed by growth on synthetic complete media or media lacking uracil as previously described (Huang et al., 2006).

(C) The rate of loss through unequal recombination of an ADE2 marker inserted into the rDNA array was measured as described previously (Huang et al., 2006; Kaeberlein et al., 1999). For exact values, see Table S4.

(D) In vitro-expressed and [35S]-labeled Tof2 constructs were incubated with His-tagged wild-type and point mutant Csm1 proteins, and analyzed as in Figure 3.

(E) The localization of HA-tagged Lrs4 was examined in interphase, in wild-type and strains with CSM1 point-mutations or a TOF2 deletion. The characteristic nucleolar localization of Lrs4 is lost in all of the mutant strains, although some residual nucleolar enrichment is visible in the TOF2 deletion strain. See also Table S4.
assays, indicates that Csm1/Lrs4 and Tof2 probably function together to aid rDNA silencing. This idea fits with the observation that Csm1/Lrs4 depends on Tof2 for specific association with the NTS1 region of rDNA repeats, as measured by chromatin immunoprecipitation (Huang et al., 2006). Nonetheless, the lack of effect on USCE in the CSM1 point mutant strains indicates that there is probably a Tof2-independent function for Csm1/Lrs4 in suppressing rDNA recombination. To determine whether Csm1/Lrs4 nucleolar localization is maintained upon disruption of the Csm1-Tof2 interaction, we examined Lrs4 localization during interphase in strains with either a deletion of TOF2 or with CSM1 point mutations (Y156/E or L161/D). Wild-type cells showed a pattern of Lrs4 staining characteristic of nucleolar localization (Figure 5E). In contrast, the TOF2 deletion and CSM1 point mutant strains showed mislocalization of Lrs4, with the protein found dispersed throughout the nucleus (Figure 5E). In the TOF2 deletion strain, but not the CSM1 point mutant strains, some cells showed visible enrichment of Lrs4 in a nuclear region that stained poorly with DAPI, suggesting that Csm1/Lrs4 may still be partially localized to the nucleolus in the absence of interactions with Tof2. The more complete dispersal of Lrs4 in the CSM1 point mutant strains as compared to the TOF2 deletion also suggests that the Csm1 conserved surface patch may mediate interactions with multiple partners in the nucleolus, as it does at kinetochores.

**DISCUSSION**

The eukaryotic kinetochore is a large multiprotein structure that creates a dynamic connection between chromosomes and MTs. The most likely candidate for direct MT binding in the outer kinetochore is the ~57-nm-long Ndc80 complex (Ciferri et al., 2008; Wei et al., 2007), of which there are 6-8 copies in each S. cerevisiae kinetochore (Joglekar et al., 2006). These complexes cooperate to bind a single MT and extend outward from a “linker” layer of protein complexes, including the MIND/Mis12 complex, which in turn bind inner kinetochore components, including the specialized Cse4/CENP-A histone and the DNA-binding Mif2/CENP-C protein (Cheeseman et al., 2006; Joglekar et al., 2006; Wei et al., 2007; Wei et al., 2005). A single MT is 25 nm wide, and the V-shaped Csm1/Lrs4 complex presents two pairs of kinetochore-binding globular domains separated by ~10 nm. A plausible model for the mechanism of sister chromatid co-orientation by monopolin, then, is that these two pairs of globular domains bind across sister kinetochores, bringing them so close together that they effectively fuse and create a single, composite MT-binding site (Figures 6A and 6B). This picture of kinetochore fusion is consistent with measures of spindle MT numbers indicating that, in meiosis I, each pair of sister kinetochores probably attaches to only a single MT (Winey et al., 2005). We cannot currently speculate about the exact geometry of sister kinetochore fusion: each Csm1/Lrs4 complex presents two pairs of kinetochore-binding globular domains separated by ~10 nm. A plausible model for the mechanism of sister chromatid co-orientation by monopolin, then, is that these two pairs of globular domains bind across sister kinetochores, bringing them so close together that they effectively fuse and create a single, composite MT-binding site (Figures 6A and 6B).
identified kinetochore binding partners, Dsn1 and Mif2, remains uncertain. Finally, it is also unknown how Mam1, which binds the Csm1 globular domain and is required for robust Csm1/Lrs4 localization to kinetochores (Rabitsch et al., 2003), might regulate or strengthen specific interactions between Csm1 and its binding partners at the kinetochore.

*S. pombe* Pcs1 has overall sequence similarity to *S. cerevisiae* Csm1, with high conservation in the kinetochore-binding surface patch. We have shown that the Pcs1/Mde4 complex has the same general architecture as *S. cerevisiae* Csm1/Lrs4 and that it interacts with orthologous binding partners (Dsn1/Mis13 and Mif2/Cnp3). From this evidence, we envision a crosslinking mechanism for monopolar in *S. pombe* that is conceptually similar to its mechanism in *S. cerevisiae*, but functioning within a single kinetochore rather than across sister chromatids. The kinetochores of *S. pombe* are larger than those of *S. cerevisiae*, and they attach to 2–4 MTs through about 20 Ndc80 complexes (Ding et al., 1993; Joglekar et al., 2008). In this context, monopolin-mediated crosslinking of inner kinetochore elements could organize the outer kinetochore and force co-orientation of adjacent MT-binding sites.

Our structural and functional data also help clarify the functions of Csm1/Lrs4 in maintaining the integrity of rDNA. Together with previous data (Huang et al., 2006), our results indicate that the Csm1/Lrs4 complex is recruited to the NTS1 region of rDNA repeats through a direct interaction with Tof2, and that this interaction is important for rDNA silencing. Because a deletion of Tof2 does not significantly affect association of Sir2 with the rDNA (Huang et al., 2006), the loss of rDNA silencing in our mutants cannot be due to simple loss of Sir2 localization. We propose that Csm1/Lrs4 crosslinks multiple rDNA repeats (up to four per Csm1/Lrs4 complex) through interactions with rDNA-associated Tof2, thereby assisting localized Sir2 in silencing these now closely juxtaposed/clustered repeats (Figure 6C). This rDNA repeat clustering/crosslinking by Csm1/Lrs4 and Tof2 must not, however, directly suppress unequal sister chromatid exchange, because disturbing the Csm1–Tof2 interaction does not affect the rate of USCE. We propose that, instead, Csm1/Lrs4 may control recombination through interactions with a different set of proteins: the inner nuclear membrane proteins Heh1 and Nur1 (Mekhall et al., 2008) and condensin complexes (Johzuka and Horiiuchi, 2009). These interactions mediate the tethering of rDNA repeats to the nuclear periphery and may also link sister chromatids together in-register to suppress unequal exchange (Figure 6D). There is probably some functional interplay between these distinct protein networks controlling rDNA silencing and USCE. The structures and interactions we have described now provide a basis for determining how Csm1/Lrs4 contributes to the activities of each of these protein networks and to their interactions at rDNA.

**EXPERIMENTAL PROCEDURES**

For detailed methods, see the Extended Experimental Procedures.

**Protein Expression and Purification**

All proteins were expressed in *Escherichia coli* with TEV-protease cleavable His6 tags, and purified by Ni²⁺ ion-exchange, and gel filtration chromatography. For sedimentation equilibrium analytical ultracentrifugation, proteins were spun in a Beckman Optima XL-A centrifuge at three speeds, which varied with the expected molecular weight of the protein/complex.

**Crystallization and Structure Determination**

Protein crystallization is described in the Extended Experimental Procedures. All datasets were collected on NE-CAT beamlines 24ID-C and 24ID-E at the Advanced Photon Source at Argonne National Laboratory. The structure of Csm1 69–181 was determined by single-wavelength anomalous dispersion (SAD) in space group R3 at 2.6 Å resolution, from a selenium-methionine-labeled crystal of Csm1 69–181 (L157M), then determined by molecular replacement in space group P2₁2₁2₁ at 2.35 Å resolution. The structure of full-length Csm1 was determined by molecular replacement in space group P3,21 to 3.4 Å resolution. The Csm1 1–181/Lrs4 1–102 (L38–44) complex structure was determined by molecular replacement. Because of the low resolution (6.0/3.9 Å) of the data, refinement was limited to rigid-body and restrained B-factor refinement (see Table S1 for data and refinement statistics).

**Electron Microscopy**

Native Csm1/Lrs4 was purified as described (Huang et al., 2006), adsorbed to glow-discharged carbon-coated copper grids, and stained with 0.75% (w/v) uranyl formate (Ohi et al., 2004). Images were collected with a Tecnai T12 electron microscope (FEI, Hillsboro, OR) operated at 120 kV using low-dose procedures, and processed using SPIDER software (Frank et al., 1996).

**Ni²⁺ Affinity Pull-Down Assay**

Bait proteins were purified as described above with His6-tags left intact, and 35S-labeled prey proteins were produced using an in vitro coupled transcription/translation kit (Promega). Pull-down assays were performed essentially as described (Rabitsch et al., 2003).

**Yeast Strains, Sporulation, and Immunofluorescence**

Strains were generated with PCR-based methods as described (Longtine et al., 1998). For spore viability, cells were grown on YPD agar and then patched onto SPO medium (1% KOAc) for 48–72 hr. Forty tetrads (160 spores) were dissected for each strain. For metaphase I bi-orientation, cells were grown in YPD, then diluted into BYTA (YPE plus 1% KOAc/50 mM potassium phthalate) at an OD₆₀₀ of 0.3, grown overnight, then washed and resuspended in SPO medium (0.3% KOAc, pH 7.0) at an OD₆₀₀ of 2.0 at 30°C to induce sporulation. Samples were removed hourly for 10 hr, fixed, and stained with DAPI (bi-orientation was assayed at 7 hr); arrested cells were scored for one (indicating co-orientation) or two (indicating bi-orientation) CEN-V-GFP foci per nucleus. For CEN-V-GFP segregation to spores, cells were patched onto SPO medium for 48–72 hr, then fixed and stained with DAPI. Tetrad analysis revealed specific GFP foci in DAPI masses. Samples were compared to wild-type using an independent two-sample t test. Indirect immunofluorescence and chromosome spreads were performed as described previously (Visintin et al., 1999).

**rDNA Assays**

rDNA silencing and unequal sister chromatid exchange assays were performed essentially as described (Huang et al., 2006; Huang and Moazed, 2003; Kaeberlein et al., 1999).

**ACCESSION NUMBERS**

Coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 3N4R (Csm1 69–181, R3 form), 3NAS (Csm1 69–181, P2₁2₁2₁ form), 3NAX (Csm1 full-length), and 3N7N (Csm1/Lrs4 1–102).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, three figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2010.07.017.
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