Phosphorylation of the Scc2 cohesin deposition complex subunit regulates chromosome condensation through cohesin integrity

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ABSTRACT The cohesion of replicated sister chromatids promotes chromosome biorientation, gene regulation, DNA repair, and chromosome condensation. Cohesion is mediated by cohesin, which is deposited on chromosomes by a separate conserved loading complex composed of Scc2 and Scc4 in \textit{Saccharomyces cerevisiae}. Although it is known to be required, the role of Scc2/Scc4 in cohesin deposition remains enigmatic. Scc2 is a phosphoprotein, although the functions of phosphorylation in deposition are unknown. We identified 11 phosphorylated residues in Scc2 by mass spectrometry. Mutants of \textit{SCC2} with substitutions that mimic constitutive phosphorylation retain normal Scc2–Scc4 interactions and chromatin association but exhibit decreased viability, sensitivity to genotoxic agents, and decreased stability of the Mcd1 cohesin subunit in mitotic cells. Cohesin association on chromosome arms, but not pericentromeric regions, is reduced in the phosphomimetic mutants but remains above a key threshold, as cohesion is only modestly perturbed. However, these \textit{scc2} phosphomimetic mutants exhibit dramatic chromosome condensation defects that are likely responsible for their high inviability. From these data, we conclude that normal Scc2 function requires modulation of its phosphorylation state and suggest that \textit{scc2} phosphomimetic mutants cause an increased incidence of abortive cohesin deposition events that result in compromised cohesin complex integrity and Mcd1 turnover.

INTRODUCTION
The evolutionarily conserved cohesin complex is essential for the maintenance of genome integrity. Cohesins tether replicated sister chromatids together in a process called cohesion, which ensures accurate chromosome segregation to daughter cells by promoting bipolar spindle microtubule attachments (Eckert et al., 2007; Onn et al., 2008). Cohesins also facilitate DNA double-strand break repair by homologous recombination and play multiple roles in the maintenance of chromosome architecture that promote chromosome condensation in mitosis and variable diversity joining (VDJ) recombination in immunoglobulin genes in interphase cells (Guacci et al., 1997; Ünal et al., 2004; Ribeiro de Almeida et al., 2012). In addition, cohesins mediate long-range chromatin interactions that affect the transcriptional regulation of a subset of metazoan genes. Defects in this process result in human cohesinopathies such as Cornelia de Lange syndrome, a severe developmental disease affecting multiple organ systems (for review, see Liu and Krantz, 2009; Horsfield et al., 2012). Thus understanding how many critical nuclear processes are faithfully executed will require a fuller understanding of cohesin regulation.

Cohesin complexes assemble through tripartite interactions involving a heterodimer of structural maintenance of chromosomes (SMC) proteins Smc1 and Smc3 and a kleisin subunit, known in budding yeast as Mcd1/Scc1 (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998). SMCs are members of a family of ATP-binding cassette transporter-like chromosomal ATPases that mediate a
variety of chromosomal processes that include chromosome condensation and repair in addition to cohesion. Within the cohesin complex, Mcd1 binds to both SMC ATPase domains, promoting their interaction (Haering et al., 2002; Arumugam et al., 2003; Gruber et al., 2003; Weitzer et al., 2003). Of interest, Mcd1 stability is dependent on its association with both SMC subunits (Toth et al., 1999). Mcd1 is also mediates the tripartite ring’s association with the fourth cohesin subunit, Scc3/SA1, whose function in the complex remains obscure (Zhang et al., 2013; Orgil et al., 2015).

The establishment of cohesion requires cohesin deposition onto chromosomes by the evolutionarily conserved, heterodimeric Scc2/Scc4 complex in budding yeast (Ciosk et al., 2000). Cohesins copurify with Scc2/Scc4, suggesting that Scc2/Scc4 directly mediates deposition (Toth et al., 1999; Ciosk et al., 2000; D’Ambrosio et al., 2008). Scc2/Scc4 plays no role in cohesin assembly, however, as cohesin subunits accumulate and form rings normally in conditional scc2-4 or scc4-4 mutants (Ciosk et al., 2000). Although Scc2 and Scc4 protein levels remain constant through the cell division cycle, the chromatin association of Scc2/Scc4 is biphasic, reaching its highest levels in post–S-phase cells (Kogut et al., 2009; Woodman et al., 2014), suggesting involvement of unknown regulatory mechanisms. In addition, the molecular function(s) of Scc2/Scc4 in deposition also remain enigmatic but may involve stimulating cohesin’s ATPase activity, which is essential for deposition (Weitzer et al., 2003). Alternatively, Scc2/Scc4 may facilitate conformational changes in cohesin structure that promote its loading, consistent with the in vitro finding that the fission yeast Scc2 orthologue makes contacts with all four cohesin subunits (Murayama and Uhlmann, 2014; Soh et al., 2015).

Cohesin’s postdeposition chromatin association is regulated by several cohesin-associated factors. Cohesin’s initial chromatin association is inherently unstable due to an antiestablishment activity mediated by Wpl1/Wap1 (Gerlich et al., 2006; Bernard et al., 2008; McNairn and Gerton, 2009; Chan et al., 2012; Eichinger et al., 2013; Ladurner et al., 2014). However, Wpl1 activity is inhibited in part during S phase by the Eco1/Ctf7 acetyltransferase, whose modification of conserved Smc3 lysine residues stabilizes chromatin-associated cohesin complexes (Ivanov et al., 2002; Rowland et al., 2009). Wpl1 activity is also antagonized by Pds5, which associates with cohesin and promotes cohesin maintenance (Hartman et al., 2000; Stead et al., 2003). Of interest, reduced Mcd1 stability has been noted when either Wpl1 is deleted or Pds5 is inactivated (Chan et al., 2012; Tong and Skibbens, 2014). Thus Mcd1 stability is critically dependent on its interactions with other cohesin subunits and with a number of cohesin-associated proteins. Whether Mcd1 contributes to additional cohesin regulatory mechanisms remains an important unanswered question.

Evidence suggests that Scc2/Scc4 continues to play important roles even after cohesin establishment in S phase. Recent studies indicate that Scc2 participates in gene regulation, noncoding RNA biogenesis, and translational fidelity (Zuin et al., 2014; Zakari and Gerton, 2015; Zakari et al., 2015). Furthermore, Scc2 inactivation in postreplicative cells, the period when Scc2/Scc4 achieves its maximal chromatin association levels, results in decreased cell viability (Furuya et al., 1998; Woodman et al., 2014). It has been suggested that Scc2/Scc4 is a general loader of SMC complexes, and, as such, its inactivation in post–S-phase cells would adversely affect chromosome condensation due to defective condensin loading. However, Scc2/Scc4-condensin interactions have not been detected (D’Ambrosio et al., 2008), instead suggesting that post–S-phase cohesin deposition has critical roles in other aspects of mitotic chromosome structure, such as chromosome condensation.

That Scc2 and its orthologues are phosphorylated (Villén et al., 2007; Dephoure et al., 2008; Wilson-Grady et al., 2008; Woodman et al., 2014) suggests the intriguing possibility that Scc2/Scc4 activities in cohesin deposition are subject to phosphoryregulation. We showed previously that dephosphorylation of whole-cell extract leaves phosphoprotein Scc2 susceptible to regulated amino-terminal proteolysis, which disrupts Scc2–Scc4 interactions, as well as the complex’s cohesin deposition activity (Woodman et al., 2014). This observation is consistent with a recent structural study of amino-terminal Scc2 residues 1–181 with Scc4, which concluded that unfolding or proteolysis is required for Scc2–Scc4 dissociation (Hinshaw et al., 2015). To define more precisely the role of Scc2 phosphorylation in cohesin deposition, we mapped Scc2 phosphorylation sites and determined the cellular consequences of scc2 mutations that either prevent phosphorylation or constitutively mimic its effects. Our data suggest that the cohesin complex is regulated, in part, through Mcd1 protein stability and that the phosphorylation state of Scc2 plays a critical role in maintaining necessary levels of Mcd1.

RESULTS
Scc2 is phosphorylated at multiple residues
To elucidate the function of Scc2 phosphorylation, we first mapped phosphorylation sites within Scc2 using phosphopeptide enrichment and mass spectrometry (MS; see Materials and Methods). Briefly, Scc2-hexahistidine (6His)-3FLAG (hereafter referred to as Scc2-FLAG) was immunopurified from yeast cells and subjected to in-gel trypsin digestion followed by immobilized metal affinity chromatography to enrich for phosphopeptides in preparation for MS. Scc2 tryptic peptides recovered in MS analyses represented 77% of the total protein sequence, which increased an additional 6% when semitryptic peptides were included, resulting in the identification of 61 candidate phosphorylation sites throughout Scc2, as summarized in Supplemental Table S1 (MS data have been deposited with the ProteomeXchange Consortium [Vizcaino et al., 2014] via the PRIDE partner repository with the data set identifier PXD001830). Stringent criteria appropriate for enriched protein samples, described in detail in Materials and Methods, were used to select phosphosites for further analysis (Supplemental Table S1). Of 11 residues that met these criteria, eight sites (T67, S127, S157, S163, T231, T236, S305, and S320, where T and S refer to threonine and serine, respectively) cluster within Scc2’s amino terminus (Figure 1A). Of note, this region of Scc2 mediates its interaction with its binding partner, Scc4, and these interactions are essential for the chromatin association of the Scc2/Scc4 complex (Warin et al., 2006; Takahashi et al., 2008; Bermudez et al., 2012; Brahnolz et al., 2012; Woodman et al., 2014). Internal (S753) and carboxyl-terminal (S1182, S1185) sites of Scc2 is phosphorylated (Villén et al., 2007; Dephoure et al., 2008; Wilson-Grady et al., 2008; Woodman et al., 2014) suggests the intriguing possibility that Scc2/Scc4 activities in cohesin deposition are subject to phosphoryregulation. We showed previously that dephosphorylation of whole-cell extract leaves phosphoprotein Scc2 susceptible to regulated amino-terminal proteolysis, which disrupts Scc2–Scc4 interactions, as well as the complex’s cohesin deposition activity (Woodman et al., 2014). This observation is consistent with a recent structural study of amino-terminal Scc2 residues 1–181 with Scc4, which concluded that unfolding or proteolysis is required for Scc2–Scc4 dissociation (Hinshaw et al., 2015). To define more precisely the role of Scc2 phosphorylation in cohesin deposition, we mapped Scc2 phosphorylation sites and determined the cellular consequences of scc2 mutations that either prevent phosphorylation or constitutively mimic its effects. Our data suggest that the cohesin complex is regulated, in part, through Mcd1 protein stability and that the phosphorylation state of Scc2 plays a critical role in maintaining necessary levels of Mcd1.

Scc2 phosphomimetic mutants exhibit decreased viability and sensitivity to genotoxic agents
To assess directly the effects of phosphorylation at these sites, we generated scc2 mutants that either mimic or abolish phosphorylation by substituting serine/threonine residues with glutamate (E) or alanine (A), respectively. Residues were mutated in groups based on...
FIGURE 1: scc2 phosphomimetic mutants have decreased viabilities and sensitivities to genotoxic agents. (A) Immobilized metal affinity chromatography and mass spectrometry were used to identify phosphorylated Scc2 residues, indicated by amino acid residue and number in a schematic of Scc2 (drawn approximately to scale), in extracts of Scc2-FLAG cdc16-1 cells (1891-32C) arrested in mitosis at 37°C for 3 h. Vertical black lines indicate groupings of amino acids mutated simultaneously, labeled 1N, 2N, 8, and C, where N and C indicate amino- and carboxyl-terminal Scc2 residues, respectively, and 1, 2, and 8 indicate the first or second group of four amino terminal phosphosites, or all eight amino terminal phosphosites, respectively. Open boxes indicate the locations of HEAT repeat motifs, according to the UniProt database. (B) Isogenic SCC2-FLAG and scc2-FLAG phosphomutant strains (JWY179-JWY190) were inoculated on synthetic minimal medium lacking leucine or on complete synthetic medium containing 5-FOA. Plates were photographed after 3 d of growth at 23°C, except for the 5-FOA plate containing scc2 carboxyl-terminal
their proximity to one another in the linear Scc2 protein sequence (Figure 1A). Accordingly, the eight amino-terminal phosphorylated residues (T67, S127, S157, S163, T231, T236, S305, and S320) were mutated simultaneously to glutamate or alamine residues, creating alleles scc2-8E and scc2-8A, respectively. In addition, the amino-terminal residues were mutated in two groups of four substitutions, denoted scc2-1NA/E and scc2-2NA/E, where 1N and 2N indicate the first four or second four phosphorylated residues in the amino (N)-terminal group of eight residues, respectively. Similarly, carboxyl (C)-terminal residues S1182 and S1185 were mutated together (scc2-CA/E), whereas S753 was analyzed as a single-substitution mutant.

Because Scc2 is an essential protein, we first determined whether mutations that constitutively alter the phosphorylation landscape of Scc2 affect cell viability. FLAG-tagged SCC2 or scc2 phosphomutant alleles under the control of the endogenous SCC2 promoter were integrated ectopically at LEU2 in a host strain containing a deletion of the endogenous SCC2 locus and rescued with a URA3 vector containing SCC2. Phosphomutants capable of supporting viability in the absence of wild-type SCC2 were then identified by inoculating the cells on medium containing 5-fluoro-orotic acid (5-FOA; Boeke et al., 1984), which kills cells that must retain the SCC2/URA3 vector (Figure 1B). Although the scc2-8E and scc2-CE mutants grew slowly in the absence of plasmid-borne SCC2, only the scc2-S753E mutant failed to form colonies on 5-FOA. To determine whether these growth defects were caused by altered stability of the Scc2 phosphomutant proteins, we analyzed samples from all phosphomutant strains before 5-FOA exposure by immunoblot (Figure 1C). Whereas Scc2 protein levels were not reduced in any of the viable phosphomutants, they were noticeably reduced in the scc2-S753E mutant, with only trace amounts detectable upon over-exposure. Taken together, these observations indicate that a mutation that mimics the constitutive phosphorylation of S753 causes inviability through protein instability. Whether this instability reflects an increased susceptibility of the mutant Scc2-S753E protein to amino-terminal cleavage is unknown. We note, however, that S753 is located within a HEAT repeat motif that is likely to play an integral role in Scc2 structure, suggesting that its mutation may alter protein folding, leading to its degradation by a pathway that is independent of amino-terminal cleavage.

The remaining nine viable scc2 phosphomutants were further analyzed to determine whether altering Scc2 phosphorylation compromises Scc2 function, which include both S-phase and post-S-phase roles (Furuya et al., 1998; Woodman et al., 2014; Xu et al., 2015). Of interest, both plating efficiency and serial dilution growth assays demonstrate that whereas the scc2 alanine phosphomutants and the scc2-1NE phosphomimetic mutant grow indistinguishably from an isogenic wild-type control, the scc2-8E and scc2-CE phosphomimetic mutants have significant reductions in colony formation, indicating reduced cell viabilities (Figure 1, D and E, left). Moreover, these two phosphomimetic mutants, as well as scc2-2NE, exhibit extreme sensitivities to growth on medium containing low doses of methyl methanesulfonate (MMS) or hydroxyurea (HU; Figure 1E), which induce DNA damage or replication stress, respectively. The sensitivities of these mutants to the drugs were similar to, or perhaps even more pronounced than, those of a deletion mutant of DUN1, which encodes a checkpoint kinase required for DNA damaged–induced transcription of DNA repair genes (Zhou and Elledge, 1993). By contrast, none of the mutants is sensitive to the microtubule-depolymerizing agent benomyl (Figure 1E). These observations eliminate the possibility that a sensitivity to a cell cycle delay per se, such as that initiated in response to altered kinetochore–microtubule attachments, explains the sensitivity of scc2 phosphomimetic mutants to genotoxic agents. Furthermore, Rnr3 protein levels are only weakly elevated in scc2-8E and scc2-CE mutants and are undetectable in scc2-2NE mutant cells (Supplemental Figure S1A). These observations argue strongly against the possibility that the DNA damage checkpoint is constitutively active in scc2 phosphomimetic mutants, given that Rnr3 is a ribonucleotide reductase subunit that is strongly induced upon activation of the DNA damage checkpoint. Consistent with this conclusion, we find little evidence for substantial delays in cell cycle progression in phosphomutant cells when released synchronously from a G1 arrest (Supplemental Figure S1B).

With the possible exception of scc2-CE, the scc2 phosphomutants progressed through S phase normally and entered the next cell cycle indistinguishably from isogenic wild-type control cells. By contrast, scc2-CE phosphomimetic mutant cells appeared to delay slightly in G2/M, as indicated by the absence of cells in the population with 1C DNA contents at 150 min postrelease. Given that Scc2’s role in cell cycle progression is largely unaffected in the mutants, it seems likely that altering the phosphorylation state of Scc2 through phosphomimetic mutation interferes with its ability to meet the increased demand for cohesin deposition required when cells are challenged with genotoxic agents (Ünal et al., 2004; Heidinger-Pauli et al., 2010).

**scc2-8A mutant acquires novel Scc2 phosphorylation sites**

A clear implication of the observed phenotypes of the scc2 phosphomimetic mutants is that the ability to modulate the phosphorylation state of Scc2 is critical for its normal function. We therefore considered the possibility that the absence of phenotypes in the scc2 alanine substitution mutants is due to compensatory phosphorylation of alternate sites in Scc2 in these mutants. To determine whether this possibility was indeed the case, we examined the phosphorylation state of Scc2-8A, in which eight amino-terminal S/T residues were made nonphosphorylatable by alanine substitution. Of interest, we found, using MS, that neighboring residues (S43, S74, S162, and T360) within the amino terminus of Scc2-8A were phosphorylated at sites not previously found to be modified in wild-type Scc2 (Supplemental Table S2). In addition, the carboxyl terminus of Scc2-8A also had an altered phosphorylation profile. Whereas S1185 was clearly phosphorylated in Scc2-8A, there was a notable absence of S1182 phosphorylation, which had been found in eight phosphomutants (bottom right), which was photographed after 5 d. (C) Immunoblots of wild-type and mutant Scc2-FLAG proteins, with a longer exposure indicated by an asterisk, and G6PDH from scc2 phosphomutant cells before FOA shuffling (JWY179-JWY189). (D) The viabilities of scc2 phosphomutant cells (JWY216-JWY225) obtained in plating efficiency experiments shown with SDs; n = 3. Student’s t test determined the significance p; *p < 0.03, **p < 0.0001.

(E) Isogenic SCC2 and scc2 phosphomutant cells (JWY216-JWY225 and Y03798) were grown to mid log phase, counted, subjected to fivefold serial dilutions, and then spotted onto plates containing rich medium without any drug (YPD) or rich medium containing 0.03% MMS, 50 mM HU, or 5 μg/ml benomyl. Triangles indicate decreasing cell densities from left to right. Plates were photographed after 3 d of growth.
independent MS analyses of wild-type Scc2. Instead, novel sites of phosphorylation, namely S1179 and S1183, were acquired in Scc2-8A. These data are consistent with the hypothesis that compensatory patterns of phosphorylation in the alanine substitution mutants may promote normal function of the Scc2 cohesin deposition complex subunit. In addition, these results suggest the interesting possibility that amino-terminal phosphorylation of Scc2 contributes to the regulation of the phosphorylation landscape of the entire protein.

**scc2 phosphomutants interact with Scc4 and bind chromatin**

In vitro treatment of whole-cell extract with phosphatase has been shown to promote amino-terminal cleavage of Scc2, leading to the loss of Scc2–Scc4 interactions (Woodman et al., 2014). Such an approach is imprecise, however, due to its inability to target specific residues or groups of residues. Nevertheless, these observations suggest that the phosphorylation state of Scc2 may play a role in regulating Scc2–Scc4 interactions. To determine unambiguously the ability of the phosphomutants to affect this interaction, we subjected the scc2 phosphomutants to reciprocal coimmunoprecipitation analyses. SCC4-6His-13Myc (hereafter referred to as Scc4-Myc) cells with integrated SCC2-FLAG or scc2 phosphomutants were presynchronized in G1 using αf and then released into a G2/M arrest with nocodazole, at which point reciprocal coimmunoprecipitations were performed and analyzed via immunoblot (Figure 2). Overall, no obvious differences in Scc2–Scc4 interactions were observed in the scc2 phosphomutants compared with a control scc2 phosphomutant with Mcd1 coimmunoprecipitations. To do this, we first integrated SCC2 or scc2 phosphomutants ectopically at LEU2 in a host strain in which the endogenous Scc2 gene was fused to an auxin-inducible domain (AID) that promotes proteasome-mediated proteolysis of AID-tagged proteins after the addition of a plant auxin, indole-3-acetic acid (IAA), to the culture medium (Materials and Methods). These cells also contained the Lac repressor protein (LacI) fused to green fluorescent protein (GFP) and tandem Lac operators integrated at LYS4 on the arm of chromosome IV (Eng et al., 2014). Cells were staged in G1 with αf, and Scc2-AID degradation was induced in G1 by the addition of IAA to the culture medium for 1 h. Cells were then scored for the presence of one or two GFP spots after release from G1 and arrest in G2/M in media containing nocodazole and IAA. If sister chromatid cohesion is maintained under these conditions, only one GFP spot is detected, whereas two GFP spots indicate precocious sister chromatid separation. We found that cells dependent solely on scc2 phosphomimetic mutants for Scc2 function exhibited a twofold increase in separated sister chromatids relative to isogenic wild-type cells (p < 0.05). These defects are mild, however, when compared with empty vector control cells lacking any form of Scc2 (∼18 vs. ∼80% of cells with two GFP spots, respectively; Figure 4A).

We then performed ChIP of the Mcd1 cohesin subunit in cells in which the scc2 phosphomutants were the sole source of Scc2 function to determine whether specific regions of chromosomes are particularly susceptible to reduced cohesin association levels after changes in the phosphorylation state of Scc2. Cells were then processed for ChIP as described. No significant differences in Mcd1 chromatin association were observed in any of the scc2 alanine phosphomutants compared with those of an isogenic SCC2 strain (Figure 4B). Similarly, the levels of pericentromeric sequences present in Mcd1 ChPs were indistinguishable in scc2 phosphomimetic and wild-type SCC2 cells (Figure 4C, top). However, the levels of sequences corresponding to two different chromosome arm CAR sites on CHRs XII and III, were reduced approximately twofold in the Mcd1 ChPs of the scc2 phosphomimetic mutants compared with an isogenic wild-type control (Figure 4C, middle and bottom), consistent with the modest precocious separation defects we observed microscopically using a chromosome arm reporter location. Reduced Mcd1 chromatin association levels in the scc2 phosphomimetic mutants cannot be attributed to altered cohesin–Scc2–Scc4 interactions, however, as we found that Mcd1 coimmunoprecipitates with Scc2 in mitotically arrested scc2 phosphomutants at levels that are indistinguishable from those of the isogenic wild-type Scc2 control.
Although reproducible, the rather mild cohesion defect in the scc2 phosphomimetic mutants suggested that their high inviability is due to a defect in another chromosomal process. Given the (Supplemental Figure S2). Thus these results indicate that cohesion association is modestly compromised along chromosome arms in scc2 phosphomimetic mutants even though Scc2/Scc4 remains present on chromatin and associates with cohesin at wild-type levels in these mutants.

**FIGURE 3:** Scc2 chromatin association is not reduced in scc2 phosphomutants. Isogenic SCC2-3FLAG (JWY216) and scc2 phosphomutant cells (JWY217-JWY225) were arrested in mitosis with nocodazole for 3 h and processed for ChIP using anti-FLAG antibody. Scc2-FLAG distributions are shown in scc2 phosphomimetic (A) and alanine substitution mutants (B) within CHRXIV pericentromeric regions (top) and CHRXII (middle) and CHRIII (A, bottom) arm sites, respectively. The position of the CHRXIV centromere is indicated by a black oval. Quantitation of DNA in the ChIPs, expressed as the percentage of the input DNA, is plotted as a function of the locations of the midpoints of the DNA fragments based on *Saccharomyces cerevisiae* Genome Database (SGD) coordinates. ChIPs were performed at least twice, and representative data from one biological replicate are shown.

**scc2 phosphomimetic mutants have defects in chromosome condensation**

Although it was reproducible, the rather mild cohesion defect in the scc2 phosphomimetic mutants suggested that their high inviability is due to a defect in another chromosomal process. Given the...
established role of cohesins in chromosome condensation (Guacci et al., 1997; Lavoie et al., 2002, 2004) and a strong correlation between condensation defects and cellular inviability (Guacci and Koshland, 2012; Orgil et al., 2015), we monitored ribosomal DNA (rDNA) morphologies in wild-type and scc2 phosphomutant cells. In G1-staged yeast, the CHRXII rDNA repeat has an amorphous appearance, protruding from the nuclear DNA mass. However, upon entry into mitosis, the rDNA repeat locus becomes highly organized and morphologically distinct, condensing into a continuous, line-like structure that can be visualized unambiguously as a "loop" emanating from the main DNA mass (Figure 5A). As previously demonstrated, the rDNA repeat in conditional mcd1-1 or scc2-4 mutant cells incubated at the restrictive temperature in mitosis becomes dis-organized, reminiscent of the structure observed in G1 cells, rather than the continuous loop-like structure characteristic of mitotic cells (Guacci et al., 1997; D’Ambrosio et al., 2008; Figure 5A). To elucidate the role of Scc2 phosphorylation in chromosome condensation, we arrested isogenic SCC2 and scc2 phosphomutant cells lacking mitochondrial DNA in mitosis with nocodazole and fixed and processed them for rDNA visualization to analyze DNA and rDNA morphologies microscopically in the absence of potentially complicating mitochondrial DNA fluorescence (Materials and Methods). Of interest, ~90% of isogenic wild-type and scc2 alanine substitution mutants exhibited morphologically normal rDNA loop formation, whereas ≥85% of mitotically arrested scc2 phosphomimetic mutants exhibit amorphous rDNA structures with discontinuous, punctate

FIGURE 4: Mcd1 chromatin association is reduced along chromosome arms but not at pericentromeric regions. (A) Isogenic Scc2-AID cells with SCC2-3FLAG (JWY226), no ectopic SCC2 allele (JWY254), or the indicated scc2 phosphomimetic mutants integrated at LEU2 (JWY230, JWY232, JWY236, and JWY231) were arrested in G1 with αF. IAA was added to induce turnover of wild-type Scc2-AID, and cells were released into medium containing IAA and nocodazole to arrest in mitosis. Cells were fixed in paraformaldehyde, and the number of GFP spots/nucleus was counted in 200 cells from each strain. Error bars represent SD; n = 3. Student’s t test was performed to determine significance; *p < 0.05, **p < 0.002, ***p < 0.0001. (B, C) Mcd1-6HA cells containing the indicated SCC2 (JWY216) or scc2 phosphomutant alleles (JWY219, JWY221, JWY224), or scc2 phosphomimetic alleles (JWY220, JWY222, and JWY225) were arrested in mitosis with nocodazole and processed for ChIP using anti-HA antibody to determine the levels of pericentromeric (CHRXIV [B, C, top]) and chromosome arm (B, bottom; C, middle and bottom) sequences present in Mcd1-HA ChIPs. Quantitation of DNA in ChIPs, expressed as a percentage of input DNA, is plotted as a function of the midpoints of the DNA fragments based on SGD coordinates. ChIPs were performed at least twice, and representative data from one biological replicate are shown. Black oval indicates the relative position of the centromere.
FIGURE 5: Condensation defects are prevalent in scc2 phosphomimetic mutants. (A) mcd1-1 and scc2-4 cells (1813-4D and 2551-11D, respectively) were first arrested in mitosis using nocodazole at 23°C and either maintained at 23°C or shifted to 37°C for 1 h and then fixed and prepared for rDNA visualization as described in Materials and Methods. Asterisks indicate rDNA loops with continuous, line-like loop structures at 23°C, and arrows indicate amorphous, punctate structures at 37°C. White bar, 5 μm. (B) Isogenic SCC2 and scc2 phosphomutant strains lacking mitochondrial DNA (JWY247-JWY253) were arrested in mitosis using nocodazole and processed for rDNA visualization as described. White bar, 5 μm. (C) Percentage of "normal" vs. "defective" rDNA structures. For each experimental condition, 100 cells were scored with SDs; n = 2. Student’s t test determined the significance p; *p < 0.01, **p < 0.0001.
foci, frequencies similar to those observed after Mcd1 or Scc2 inactivation in the corresponding conditional mutants (Figure 5, B and C). These observations indicate that, in addition to sensitivity to growth in the presence of genotoxic agents (Figure 1E) and mildly reduced cohesin binding along chromosome arms (Figure 4), the scc2 phosphomimetic mutants have pervasive rDNA condensation defects. In addition, we note that while the scc2-2NE phosphomimetic mutant does indeed exhibit abnormal rDNA structures, this mutant retained more of the normal line-like, albeit punctate, rDNA structure as well as better cell viability than did the two other phosphomimetic mutants (Figure 1, D and E), again indicating the reduced viabilities of the scc2 phosphomimetic mutants are strongly correlated with abnormal rDNA structure.

**Mcd1 levels are reduced in scc2 phosphomimetic mutants**

Reduced cohesin association in arm, but not pericentromeric, CARS and chromosome condensation defects were observed previously when Mcd1 cohesin subunits were systematically lowered, suggesting that Mcd1 protein levels may also be reduced in the scc2 phosphomimetic mutants (Heidinger-Pauli et al., 2010). To determine whether this possibility is indeed the case, we monitored Mcd1 levels in isogenic SCC2 or scc2 phosphomimetic mutants after Scc2-AID degradation before S phase. Cell cultures were staged in G1 using αf and then split, with one-half receiving IAA for 1 h to induce Scc2-AID degradation and the other half being treated with dimethyl sulfoxide (DMSO) alone. Cells were then released from G1 into fresh medium containing either IAA or DMSO alone as before and allowed to progress synchronously through the cell cycle. Samples were taken at 30-min intervals for protein analysis upon release from G1 arrest, until cells had just reached G2/M. However, Mcd1 levels were maintained under these conditions in the scc2 phosphomimetic mutants even in absence of Scc2-AID (Supplemental Figure S3A).

Given the dramatic defects in chromosome condensation, a late cell cycle event, in the scc2 phosphomimetic mutants, we next determined whether Mcd1 stability is more vulnerable in mitotically arrested cells. The same strains described earlier were presynchronized in G1 and then released into a mitotic arrest with nocodazole, at which point Scc2-AID degradation was induced by IAA addition for 1 h. Samples for protein analyses were taken upon IAA addition and every 30 min thereafter. Of interest, while Mcd1 protein levels were only insignificantly reduced (p = 0.23) in cells lacking any Scc2 and were maintained, if not increased, in cells with integrated wild-type SCC2, Mcd1 levels were reduced approximately fourfold in phosphomimetic (scc2-2NE, scc2-8E, and scc2-CE) mutant mitotic cells treated with IAA for 90 min (Figure 6A). Although it is unclear why Mcd1 instability is specifically detected in postreplicative cells, one possibility is that Mcd1 instability increases in this cell cycle period due to the presence of higher levels of chromatin-associated Scc2/Scc4 (Woodman et al., 2014), whose dysfunction in scc2 phosphomimetic mutants appears to destabilize Mcd1. Moreover, that Mcd1 levels remain unaffected after Scc2-AID depletion in isogenic control cells lacking any other source of Scc2 suggests that Mcd1 destabilization in the scc2 phosphomimetic mutants requires the physical interaction of the mutant Scc2 proteins with cohesin. This data suggest not only that Scc2 plays a critical role in maintaining Mcd1 levels through mitosis, but also that constitutive phosphorylation of Scc2 specifically disrupts this regulatory process.

**Mcd1 instability is independent of Esp1 and Eco1**

Mcd1 is normally degraded by the Esp1/separase protease at the metaphase/anaphase transition, thereby triggering sister chromatid

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**FIGURE 6:** Mitotic Mcd1 protein levels are reduced in scc2 phosphomimetic mutants. (A) Mcd1-HA Scc2-AID cells (JWY226, JWY230, JWY232, and JWY236) containing SCC2, no SCC2 allele, or scc2 phosphomimetic mutants integrated at LEU2 (scc2-2NE, scc2-8E, scc2-CE) were arrested in mitosis with nocodazole, and Scc2-AID turnover was induced by IAA addition. Samples were prepared for protein analysis upon IAA addition (time 0) and every 30 min thereafter. Representative immunoblot of Mcd1 levels in scc2-CE mutant cells with and without IAA treatment. Quantitation of immunoblots of Mcd1-HA protein levels relative to G6PDH at 90 min after IAA addition normalized to Mcd1-HA/G6PDH at 0 min is shown with error bars indicating SD; n = 3. Student’s t test determined the significance p; *p < 0.05 and **p < 0.01. (B) Isogenic esp1-1 mutant cultures containing SCC2 or scc2 phosphomimetic mutants (JWY239-JWY242) were first arrested in mitosis with nocodazole and then split and incubated for 1 h at either 23 or 37°C. Immunoblots of Mcd1-HA and G6PDH. (C) scc2 phosphomimetic mutant cells (JWY243-JWY246) carrying a plasmid that expresses Eco1/CTF7 under the control of a galactose-inducible promoter were grown in noninducing/nonrepressing medium. Cell cultures were presynchronized in G1, released to a mitotic arrest in nocodazole, and split in half, and one-half was treated with galactose (2% final) for 1 h. All cultures were then treated with IAA, and protein samples were taken at the time of IAA treatment and every 30 min thereafter. The immunoblots show protein levels of Mcd1-HA and G6PDH at the time of IAA addition (time 0) and at 90 min of treatment.
separation (Uhlmann et al., 1999). To rule out the possibility that the reduction in Mcd1 levels in mitotically arrested scc2 phosphomimetic mutant cells is due to the premature activation of separase, we determined whether Mcd1 instability in these cells is Esp1 dependent. As a control, Mcd1 levels in isogenic ESP1 and esp1-1 mutant cells were first analyzed at 23 and 37°C to confirm that Mcd1 stability is unchanged after Esp1 inactivation in mitotically arrested cells (Supplemental Figure S3B). Conditional esp1-1 mutant cells expressing various scc2 phosphomimetic alleles as the sole source of Scc2 were then arrested in mitosis with nocodazole, at which time the cultures were split and incubated at permissive or restrictive temperatures (23 or 37°C, respectively). We observed no change in Mcd1 levels by immunoblot after Esp1 inactivation (Figure 6B), suggesting that the Mcd1 instability observed in scc2 phosphomimetic mutants is not due to premature activation of Esp1.

To test whether stabilizing cohesin complexes on chromatin is sufficient to rescue Mcd1 levels in the scc2 phosphomutants, we determined the effect of Eco1/Ct7 overexpression in the scc2 phosphomutants. Eco1 normally acetylates Smc3 to promote the establishment of sister chromatid cohesion only during S phase, but Eco1 also becomes active outside of S phase when DNA damage is incurred. Moreover, induced Eco1 expression bypasses a requirement for DNA damage to produce “cohesive” cohesin chromatin association in G2/M cells (Uñal et al., 2007). As before, Scc2-AID cells with SCC2 or scc2 phosphomutants integrated at LEU2 that also express ECO1 under the control of a galactose-inducible GAL1 promoter on a URA3 vector were grown in medium that allows rapid transcription of GAL1-regulated genes upon addition of galactose to the culture medium. Cells presynchronized in G1 using α-factor were released into a mitotic arrest using nocodazole. Once arrested, cultures were split in half, and galactose was added to one of the cultures for 1 h to induce Eco1 overexpression. IAA was then added to all cultures to induce the degradation of Scc2-AID, and aliquots of cells were taken upon IAA addition and every 30 min thereafter to monitor Mcd1 protein levels by immunoblot. Growth of control cells on galactose-containing medium promotes functional Eco1, as indicated by complementation of a conditional eco1/c7 mutant grown at the restrictive temperature (Supplemental Figure S3C). However, we found that the reduction in mitotic Mcd1 protein levels in scc2 phosphomimetic mutant cells after Scc2-AID degradation was not reversed by Eco1 overexpression (Figure 6C). Of interest, as observed previously, Mcd1 stability was maintained in cells that lack any source of Scc2 after IAA treatment, strongly suggesting that the Mcd1 instability observed in the scc2 phosphomimetic cells occurs through an active engagement of cohesin with Scc2 phosphomimetic proteins that is apparently adversely affected by changes in Scc2 phosphorylation state. Collectively these data suggest that whereas Scc2 phosphorylation clearly affects Mcd1 protein stability, it does so independently of Esp1 or Eco1.

DISCUSSION

Although large-scale proteomics studies and a more recent biochemical study demonstrate that budding yeast Scc2 and its orthologues are phosphorylated, the consequences of this posttranslational modification on Scc2 function were not addressed previously (Villén et al., 2007; Deploure et al., 2008; Wilson-Grady et al., 2008; Woodman et al., 2014). We find that although scc2 mutations that prevent phosphorylation resulted in no obvious phenotypes, possibly due to the use of alternative phosphorylation sites throughout the protein, scc2 phosphomimetic mutants exhibit sensitivity to genotoxic agents, substantial cellular inviability, and decreased cellular Mcd1 levels, demonstrating the importance of Scc2 phosphoregulation. Of importance, these scc2 phosphomimetic mutants also have extensive defects in rDNA condensation but only mild sister chromatid cohesion defects. We infer from the rather modest arm cohesion defects in the scc2 phosphomimetic mutants, as well as the existence of a cohesin-independent chromosome segregation mechanism in budding yeast (Guacci and Koshland, 2012), that the high levels of inviability in the scc2 phosphomimetic mutants are not due to defects in sister chromatid cohesion. Instead, it seems more likely that this inviability is caused by the chromosome condensation defects we describe in these mutants. This conclusion is supported by the fact that both cellular inviability and a severe condensation defect present in an mcd1 ROCC (regulation of cohesion and condensation) domain mutant are suppressed by deletion of WPL1, whereas the mutant’s cohesion defect is exacerbated despite the maintenance of normal chromosomal cohesin association levels, suggesting that the recovered viability in the double mutant is due to the restoration of chromosome condensation (Eng et al., 2014). Moreover, it was recently shown that mutants in the stromalin conserved domain of the Scc3 cohesin subunit that have a high incidence of inviability exhibit severe chromosome condensation defects but only minor cohesin association defects, again suggesting that defective condensation in the cohesin mutants is responsible for cellular inviability (Orgil et al., 2015). Finally, we note that chromosome condensation defects are frequent, but not always, associated with mutants that lower Mcd1/cohesin levels on chromosomes, specifically in cases in which Mcd1 levels were reduced either through genetic manipulation or through polysumoylation-associated degradation of Mcd1 in pds5 mutants (Hartman et al., 2000; Heidinger-Pauli et al., 2010; D’Ambrosio and Lavioe, 2014; Tong and Skibbens, 2014).

We also note that the condensation defects in the scc2 phosphomimetic mutants do not support a priori the proposal that rather than being a dedicated cohesin deposition factor, Scc2/Scc4 may instead be a general loader of SMC-containing complexes. This suggestion was derived from the appearance of DNA condensation defects in conditional scc2-4 mutants in budding yeast, as well as the colocalization on chromosomes of Scc2/Scc4 with condensin, an essential SMC-containing complex that mediates chromosome condensation (D’Ambrosio et al., 2008; Dowen et al., 2013). Several observations challenge this model, however. First, although cohesin is readily coimmunoprecipitated with Scc2/Scc4, condensin is not (D’Ambrosio et al., 2008). Furthermore, condensin’s chromosomal association is only partially reduced in scc2-4 mutants (Ciok et al., 2000; D’Ambrosio et al., 2008). Instead, the findings that cohesins promote proper condensin function during chromosome condensation and that condensin recruitment to chromosomes is independent of cohesins (Guacci et al., 1997; Lavioe et al., 2002) together suggest that condensation defects in the scc2-4 mutant, and indeed the scc2 phosphomimetic mutants, arise due to aberrant cohesin levels rather than a failure to load condensins. In fact, our results suggest that continuous cohesin loading may be required for condensation maintenance, perhaps directing condensin activity.

Our observations also confirm and extend previous findings regarding the existence of a hierarchical association of cohesins along chromosomes and further suggest that the execution of various chromosomal processes requires different in vivo concentrations of cohesins (Heidinger-Pauli et al., 2010). Although the levels of cohesin association in the scc2 phosphomimetic mutants are apparently below a threshold required for chromosome condensation, they are above the threshold required for normal pericentromeric cohesion. A mechanism that enriches Scc2/Scc4 recruitment within pericentromeric chromatin likely ensures that sufficient levels of functional
cohesins are maintained in these regions to promote chromosome biorientation (Eckert et al., 2007; Kogut et al., 2009; Natsume et al., 2013), even in the scc2 phosphomimetic mutants. By contrast, the reduced levels of arm cohesin association indicate that arm-associated cohesin is more sensitive to Sc2 phosphorylation, suggesting that cohesin association at arm CARs may be particularly amenable to remodeling by targeted Sc2 phosphorylation.

The phosphomimetic mutants described here represent a novel class of separation of function alleles of SCC2 that enable rather robust levels of sister chromatid cohesion, especially within pericentromeric regions, but fail to promote proper chromosome condensation. By contrast, the only other (conditional) scc2 alleles that have been described, scc2-4 and SCC2-AID, cause severe defects in both sister chromatid cohesion establishment and chromosome condensation (D’Ambrosio et al., 2008; Eng et al., 2014). The dichotomy in phenotypes produced by scc2 alleles may be related to differences in Mcd1 levels. Whereas cellular Mcd1 levels are significantly reduced in scc2 phosphomimetic mutants, they are unaffected by Sc2 degradation in SCC2-AID cells lacking any other source of Sc2 (this study) or in scc2-4 cells grown at a temperature that prevents cohesin deposition activity while having no effect on Sc2 stability (Ciok et al., 2000). We infer from these results, as well as our observation that Mcd1–Sc2 interactions are intact in scc2 phosphomimetic mutants, that reduced Mcd1 levels result from compromised cohesin integrity in the scc2 phosphomimetic mutants after phosphorylation promotes or stabilizes ATP hydrolysis–dependent conformational changes in cohesin subunit structure associated with cohesin deposition (Figure 7; Hopfner et al., 2000; Gruber et al., 2006; Hu et al., 2011). In such a model, scc2 phosphomimetic mutants may trap cohesin deposition intermediates that are normally resolved by Sc2 dephosphorylation. Alternatively, Sc2 dephosphorylation, along with Eco1 and Pds5, may promote the stabilization of cohesin’s chromatin association. In the latter scenario, a requirement for Sc2/Sc4 function would potentially extend beyond the cohesin deposition event per se, consistent with observations in both budding and fission yeast indicating that Sc2 inactivation after DNA replication, the interval when cohesin deposition normally occurs, reduces cellular viability (Furuya et al., 1998; Woodman et al., 2014). In both models, we postulate that scc2 phosphomimetic mutants cause an increased incidence of abortive cohesin deposition events, which compromise cohesin complex integrity, resulting in reduced Mcd1 levels. The reduction of Mcd1 levels may be more evident in mitotically arrested cells due to the higher levels of Sc2/Sc4 that normally associate with chromosome at this cell cycle stage (Woodman et al., 2014). Moreover, the inability of ectopic Eco1/Ctf7 expression to stabilize Mcd1/cohesin integrity in scc2 phosphomimetic mutants suggests that constitutive Sc2 phosphorylation reduces either the number of cohesin complexes or their suitability as substrates for acetylation, suggesting that key aspect(s) of the cohesin deposition cycle are regulated by Sc2 phosphorylation.
In summary, the ability to fine-tune cohesin function through Scc2 phosphorylation presents a previously unappreciated avenue for cohesin regulation. Evidence that Scc2 orthologues from metazoans are also phosphorylated indicates that Scc2 phosphorylation of cohesin complex integrity may be a conserved mechanism to promote refined control of cohesin function. Given that the human Scc2 orthologue, NIPBL, is mutated in ~60% of patients with the multisystem developmental disorder referred to as Cornelia de Lange syndrome, it will be of clinical interest to determine whether these mutations play a role in altering the phosphorylation profile of NIPBL and how these changes in NIPBL phosphorylation state may affect the etiology of the syndrome.

MATERIALS AND METHODS

Fe–nitrotriacetic acid phosphopeptide enrichment and identification

Scc2-6His-3FLAG was immunopurified from cells at the indicated cell cycle phases using FLAG-bound magnetic beads. Because Scc2 exhibits greater stability during mitosis (Woodman et al., 2014), cells used for phosphopeptide enrichment/mass spectrometry were first arrested in mitosis at 37°C using a temperature-sensitive cdc16 strain. Samples were prepared as MS previously described (Woodman et al., 2014). Mitotic samples were also prepared for phosphopeptide enrichment using immobilized metal affinity chromatography (catalogue number 88300; ThermoFisher Pierce, Grand Island, NY) using the manufacturer’s instructions and supplied buffers. The dry phosphopeptides were resuspended in 200 μl of binding buffer (5% acetic acid) and incubated with Fe–nitrotriacetic acid beads for 20 min at room temperature with end-over-end rotation. Two 100-μl washes with buffer A (0.1% acetic acid) were followed by two washes of 100 μl buffer B (0.1% acetic acid, 10% acetonitrile). Phosphopeptides bound to the magnetic beads were eluted three times with 50 μl of elution buffer (0.1 M ammonium bicarbonate), pooled, desalted using graphite spin columns (Pierce), and resuspended in 20 μl of 0.1% formic acid. A volume of 8 μl was analyzed for each liquid chromatography MS/MS analysis by higher-energy collisional dissociation– and collision-induced dissociation–type fragmentation. Phosphosites chosen for further analysis included those with ion scores ≥20 (five phosphorylation sites were ≥53), as well as those with lower ion scores that met additional criteria. Those criteria included common fragmentation profiles and high mass accuracy when comparing phosphorylated and nonphosphorylated forms of the same peptides. The peptides identified were manually validated using positive identification of y and b fragment ions. Mascot software algorithms indicated the likelihood that a particular residue within a fragment peptide was phosphorylated relative to neighboring phosphorylatable residues within the same peptide (percent probability; Supplemental Table S1). Mass spectrometry data were deposited with the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the data set identifier PXD001830 and 10.6019/PXD001830.

Yeast strain construction

Scc2, SCC4, and MCD1 were epitope tagged as previously described (Kogut et al., 2009). SCC2 was deleted from its chromosomal location using standard molecular biology techniques, and viability was maintained with plasmid pPCM102 (CEN/ARS/SCC2/URA3; Woodman et al., 2014). The genotypes of yeast strains used in this study are listed in Supplemental Table S3.

The scc2 phosphomutants were constructed essentially as described (Gibson et al., 2009) using double-digested pJW4 (Woodman et al., 2014) and multiple overlapping 500–base pair double-stranded DNA fragments (Integrated DNA Technologies, Coralville, IA; sequences available upon request) that span the SCC2 open reading frame. pJW4 contains SCC2-6His-3FLAG in YCplac22 (Gietz and Sugino, 1988). For amino-terminal mutants scc2-1N(A/E), scc2-2N(A/E), and scc2-8(A/E), Afe/BseRl-digested pJW4 was ligated in a single-step Isothermal reaction with three overlapping 500-base pair DNA fragments that contained the desired nucleotide substitutions. Pmel/Bar1-digested pJW4 was used to substitute nucleotides encoding for S753, and Msd/Bar1-digested pJW4 was used with DNA fragments to make scc2-CA and scc2-CE. The reassembled vectors were each confirmed by DNA sequencing. Integrating vectors containing each of the phosphomutants were generated by subcloning, using the same restriction enzymes indicated, into pMH466, a Yt1plac128 vector containing wild-type SCC2-6His-3FLAG on a NarI/BamHI fragment blunt end ligated into the unique Smal site of Yt1plac128. Vectors were linearized by Clal digestion and integrated at LEU2 in strain JWY208, whose sole copy of SCC2 resides on PCR102, a CEN/ARS plasmid carrying the URA3 locus as a selectable marker.

Sensitivity to genotoxic agents

To survey sensitivities of scc2 phosphomutants cells to genotoxic agents, cells were cultured on plates contained 0.03% MMS to induce DNA damage, 50 mM HU to cause DNA replication stress during S phase, or 5 mg/ml benomyl to affect microtubule dynamics. To survey sensitivities of scc2 phosphomutants to mitotic arrest, cells were cultured on plates contained 0.5 mM edu to cause DNA replication stress during S phase, or 3 μM αf mating pheromone to promote arrest in G1 or G2/M.

Scc2-AID turnover

IAA (Gold Biototechnology, St. Louis, MO) was added to culture medium at a final concentration of 0.5 mM in 1% DMSO to induce DNA damage, 50 mM HU to cause DNA replication stress during S phase, or 5 mg/ml benomyl to affect microtubule dynamics.

Plating efficiency measurements

Cell viabilities were determined by measuring plating efficiencies. Briefly, cell densities of cultures were first determined by hemocytometer counting. Serial dilutions of each culture were then made to enable plating of ~200 cells/plate on rich medium in triplicate. Colonies formed at 23°C after 3 d (or 7 d in the case of slowly growing strains) were counted, and plating efficiencies were then calculated as the percentage of colonies formed as a function of the original number of cells plated.

Commissn precipitation

To immunoisolate Scc2, protein A magnetic beads (ThermoFisher Invitrogen, Grand Island, NY) prebound overnight at 4°C to FLAG (Sigma-Aldrich) or Myc (Millipore, Billerica, MA) antibodies at ratios of 2–5 μg of serum to 10 μl of a 50% bead suspension were then

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incubated with whole-cell extracts at 4°C for 2 h and washed thoroughly six times with 10 min between washes in 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 mM KCl, 2.5 mM MgCl₂, 10% glycerol, 0.1% Triton, 0.1% Tween, and 300 mM NaCl plus the following protease and phosphatase inhibitors: 1 mM benzamidine, 0.5 mM sodium metabisulfite, 2.7 mg/ml pepstatin A, 4 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mM NaF, and 10 mM β-glycerophosphate (Woodman et al., 2014). Samples were then subjected to immunoblotting as described later.

Chromatin immunoprecipitation
ChIP experiments were performed as previously described (Kogut et al., 2009) without modification and with at least two biological replicates. Cells processed for ChIP were presynchronized in G1 with αf and released into fresh medium containing nocodazole for G2/M arrest.

Protein isolation and Western blotting
Proteins were isolated for analysis by treatment with 10% trichloroacetic acid or by spheroplasting and lysing of a whole-cell extract with 1 mg/ml Zymolyase and 0.25% Triton-X, with the protease and phosphatase inhibitors indicated previously.

Immunoblotting and quantitation
SDS polyacrylamide gels (6%) were used to distinguish full-length and cleaved Scc2 species. Proteins were transferred to polyvinylidene difluoride membrane at 100 V for 1 h. Primary and secondary antibody solutions contained 1% bovine serum albumin, 1% dry nonfat milk, and 0.1% Tween in phosphate-buffered saline. Mouse monoclonal FLAG (Sigma-Aldrich) and hemagglutinin (Roche, Indianapolis, IN) antibodies, rabbit glucose-6-phosphate dehydrogenase (G6PDH; Sigma-Aldrich), and mouse polyclonal Myc serum (Indianapolis, IN) antibodies, rabbit glucose-6-phosphate dehydrogenase–conjugated secondary antibodies (Bio-Rad, Hercules, CA) were used at 1:2500 and 1:10,000, respectively. Quantitation was performed using the Bio-Rad ChemiDoc System and ImageLab software.

Chromosome condensation analyses
rDNA was visualized in situ as previously described (Guacci et al., 1994). To remove potentially complicating fluorescence contributions from mitochondrial DNA, phosphomutant cells were first grown to saturation twice in 25 µg/ml ethidium bromide to evict mitochondrial DNA (Goldring et al., 1970). Strains that lacked mitochondrial DNA were identified by their inability to use glycerol as the sole carbon source. Cells were arrested in G2/M with nocodazole and fixed with 3.6% formaldehyde at 23°C for 2 h with gentle shaking. Chromosomal DNA was stained with 50 ng/ml 4′,6-diamidino-2-phenylindole and visualized using a Nikon Eclipse E800 fluorescence microscope equipped with a Plan APO 100x oil objective and a CoolSNAP HQ charge-coupled device camera (Photometrics, Tucson, AZ). Only loops or amorphous spots clearly associated with an intact spherical nuclear mass were counted in the scoring of the rDNA repeat morphologies.

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