Polo kinase Cdc5 associates with centromeres to facilitate the removal of centromeric cohesin during mitosis

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ABSTRACT Sister chromatid cohesion is essential for tension-sensing mechanisms that monitor bipolar attachment of replicated chromatids in metaphase. Cohesion is mediated by the association of cohesins along the length of sister chromatid arms. In contrast, centromeric cohesin generates intranastroid cohesion and sister centromeres, while highly cohesin enriched, are separated by >800 nm at metaphase in yeast. Removal of cohesin is necessary for sister chromatid separation during anaphase, and this is regulated by evolutionarily conserved polo-like kinase (Cdc5 in yeast, Plk1 in humans). Here we address how high levels of cohesins at centromeric chromatin are removed. Cdc5 associates with centromeric chromatin and cohesin-associated regions. Maximum enrichment of Cdc5 in centromeric chromatin occurs during the metaphase-to-anaphase transition and coincides with the removal of chromosome-associated cohesin. Cdc5 interacts with cohesin in vivo, and cohesin is required for association of Cdc5 at centromeric chromatin. Cohesin removal from centromeric chromatin requires Cdc5 but removal at distal chromosomal arm sites does not. Our results define a novel role for Cdc5 in regulating removal of centromeric cohesins and faithful chromosome segregation.

INTRODUCTION

Accurate chromosome segregation is important for the maintenance of genome stability during growth and proliferation of organisms (Lengauer et al., 1998; Maddox et al., 2012). Errors in chromosome segregation result in aneuploidy, which has been linked with several human diseases, such as developmental disorders and many cancers (Santaguida and Amon, 2015; Singh and Gerton, 2015). The kinetochore, composed of centromeric DNA (CEN) and associated proteins, is an essential component of the chromosomal segregation machinery (Burrack and Berman, 2012; Choy et al., 2012; Maddox et al., 2012). The assembly of kinetochore proteins at budding yeast CEN (core centromere composed of ~125 base pairs of defined DNA sequences) results in a highly ordered, unique, and topologically distinct chromatin structure in the chromosomes (Clarke and Carbon, 1980; Bloom and Carbon, 1982; Bloom et al., 1984; Saunders et al., 1990), the structural integrity of which is required for accurate chromosome segregation (Newlon, 1988; Verdaasdonk and Bloom, 2011; Haase et al., 2013; Mishra et al., 2013; Mishra et al., 2015). Budding yeast centromeric chromatin...
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Cdc5 regulates cohesin at centromeres (Yeh et al., 2008; Verdaasdonk et al., 2012). The intramolecular linkages in the C-loop and the intramolecular linkages along the length of the chromosomes promote the biorientation of kinetochores and sister chromatids toward opposite spindle pole bodies for faithful chromosome segregation.

Cohesion along the length of the chromosomes is facilitated by chromosomal association of cohesins (Hornig and Uhlmann, 2004; Gerton, 2007; Brooker and Berkowitz, 2014). The cohesin complex is composed of four subunits: Scc1/Mcd1, Scc3, Smc1, and Smc3 (Hartman et al., 2000; Laloraya et al., 2000; Nasmyth, 2002; Glyn et al., 2004; Weber et al., 2004; Gerton, 2005; Bose and Gerton, 2010; Rossi et al., 2010; Marston, 2014; Roccuzzo et al., 2015). An enrichment of cohesins is seen at cohesin-associated regions (CARs) and at centromeric chromatin (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). Removal of cohesins from chromosomes facilitates separation of sister chromatids at anaphase and cell cycle progression (Hartman et al., 2000; Marston, 2014; Guacci et al., 2015). Evolutionarily conserved Cdc5 has been shown to regulate several aspects of mitosis (Zitooni et al., 2014; Archambault et al., 2015), including sister chromatid separation, by phosphorylating Scc1/Mcd1 (Alexandru et al., 2001) to promote its proteolytic cleavage by separase (Uhlmann et al., 2000). Mutation of phosphorylatable serines to alanines in Scc1/Mcd1 by Cdc5 accelerates its cleavage following procedures described previously (Calvert and Lannigan, 2010). In agreement with previous results (Figure 1B), Western blot analysis revealed cell cycle-regulated expression of Cdc5, with increased levels observed in metaphase and anaphase cells (Figure 2B). ChIP results showed that CEN association of Cdc5 is low in cells soon after release from α-factor arrest (40 min), increasing as cells enter mitosis (60 min), maximum in anaphase (80 min), and decreasing as cells exit mitosis (100–120 min; Figure 2, A and C). We did not detect Cdc5 enrichment at a negative control region, 6K120 (Figure 2C bottom). On the basis of these results, we conclude that CEN association of Cdc5 is cell cycle dependent, with maximum enrichment in mitosis (metaphase and anaphase).

Because a previous study showed enrichment of Cdc5 at CARs (Rossio et al., 2010), we investigated whether Cdc5 localization at CARs is cell cycle dependent. We examined three CARs on chromosome III (Figure 3A, green triangles), and two non-CARs (Figure 3A, black vertical lines) previously reported to either associate or fail to associate with cohesin components, respectively (Eckert et al., 2007; Ng et al., 2009). The CARs are 114 (CEN3), 134 (peri-CEN, 20 kb from CEN3), and 261 (chromosome arm, 147 kb from CEN3), and non-CARs are 125 (peri-CEN, 11 kb from CEN3) and 266 (chromosome arm, 152 kb from CEN3; Figure 3A). Experiments performed with an untagged wild-type strain showed no significant enrichment of Cdc5 at any of the regions tested (Figure 3B). In logarithmically growing cells, Cdc5 associates specifically with CARs (Figure 3B). Cdc5 enrichment at CARs was significantly higher in mitotic cells (M) than in S-phase cells and was barely detectable in G1 (Figure 3C). The increased levels of Cdc5 at CARs in mitosis were verified by assaying cells synchronized in G1 and released into phenomere-free medium. Consistent with the increased enrichment of Cdc5 at peri-CEN and arm CARs (134 and 261, respectively) in nocodazole-treated cells (Figure 3C), we observed higher levels of Cdc5 at these regions in cells undergoing mitosis (60–100 min postrelease; Figure 4). Taken together, these results show that Cdc5 enrichment at CEN and CARs is cell cycle dependent, with maximum enrichment during mitosis.

Induced expression of Cdc5 in G1 does not result in its recruitment to centromeric chromatin and CARs

The mitotic enrichment of Cdc5 at CEN and CAR sites correlates with the protein expression levels of Cdc5, and no significant enrichment of Cdc5 was observed at these sites in G1, when protein expression of Cdc5 was not detected (Figure 1, C and D). Therefore induced expression of Cdc5 in G1 does not result in its recruitment to centromeric chromatin and CARs.
Cohesin proteins interact with Cdc5 and regulate the mitotic enrichment of Cdc5 at centromeric chromatin and CARs

The association of Cdc5 at CEN and CARs (Figures 1–4) coincides with the high levels of cohesins shown to associate at these regions (Megee et al., 1999; Laloraya et al., 2000; Glynn et al., 2004; Weber et al., 2004; Eckert et al., 2007); hence we asked whether cohesin interacts with Cdc5 in vivo and investigated its role in Cdc5 centromeric association. In budding yeast, the cohesin complex is composed of four subunits: Scc1/Mcd1, Scc3, Smc1, and Smc3 (Nasmyth, 2002). We constructed strains expressing Cdc5 and TAP-tagged Mcd1 or Cdc5 and green fluorescent protein (GFP)–tagged Smc3 from their endogenous promoters. Immunoprecipitation experiments showed in vivo interaction of Cdc5 with Mcd1 and Smc3 (Figure 6). No interaction was observed in control experiments using a strain with untagged Mcd1 or Smc3 (Figure 6).
Next we examined whether Mcd1 is required for enrichment of Cdc5 at CEN and CARs. Because cohesin subunits are essential for cell viability, we used mcd1-1, a well-characterized temperature-sensitive mutant that arrests in M phase at the nonpermissive temperature (37°C) and exhibits defects in establishment and maintenance of cohesion (Guacci et al., 1997; Noble et al., 2006; Skibbens et al., 2010; Eng et al., 2014, 2015). The expression of Cdc5 was similar in wild-type and mcd1-1 strains grown at 25 and 37°C, as measured by Western blot analysis (Figure 7A). The enrichment of Cdc5 at CEN and CARs in nocodazole-treated (M-phase arrest) wild-type and mcd1-1 cells grown at 25°C and after shift to 37°C was assayed by ChIP. The cell cycle arrest was verified by FACS assay (Figure 7B). ChIP-qPCR showed that Cdc5 levels at CEN3, as well as CARs located at peri-CEN (134) and the chromosomal arm (261), are reduced threefold to fourfold specifically in the mcd1-1 strain at 37°C (Figure 7C). There was no significant enrichment of Cdc5 at negative control region 6K120 under any condition (Figure 7C). We conclude that cohesin is required for enrichment of Cdc5 at CEN and CARs.

cdc5-99 strains exhibit persistence of cohesin at CEN and peri-CEN regions in anaphase

Given our findings that association of Cdc5 at centromeric chromatin and CARs is maximal in cells undergoing the metaphase-to-anaphase transition (Figures 2 and 4), we posited that Cdc5 might be required for the removal of chromatin-bound cohesin. In wild-type cells, cohesin is lost from both centromeric chromatin and chromosomal arms at anaphase onset (Marston, 2014). To characterize the Cdc5 dependence of cohesin loss, we monitored the localization of Smc3-GFP in anaphase cells of wild-type and cdc5-99 strains grown at permissive temperature (25°C) and after a 2.5-h shift to the nonpermissive temperature of 37°C. The well-characterized temperature-sensitive cdc5-99 mutant exhibits a defect in mitotic exit at the nonpermissive temperature of 37°C (St-Pierre et al., 2009). Spindle pole body (SPB) component Spc29–red fluorescent protein (RFP) was used as a marker for centromeric chromatin due to the close physical proximity of the SPB to the kinetochores in budding yeast. Using the distance between SPBs as a measure for spindle length, we quantitated Smc3-GFP foci in anaphase cells with a spindle length of 3–8 μm. In addition to the diffuse Smc3-GFP fluorescence observed over the separated chromatin masses in both wild-type and cdc5-99 cells, distinct Smc3-GFP foci were observed in the cdc5-99 mutant (Figure 8A). No detectable Smc3-GFP foci were ever observed in wild-type cells in anaphase. Quantification of the cdc5-99 cells with Smc3-GFP foci at one or both of the spindle poles was significantly higher (32 vs. 13%, p < 0.05) at 37°C (n = 144) than at 25°C (n = 125; Figure 8B). We next examined the localization...
number of cdc5-99 cells with Mcd1-GFP foci at one or both of the spindle poles was significantly higher (24 vs. 3%, \( p < 0.05 \)) at 37°C (\( n = 197 \)) than at 25°C (\( n = 94 \); Figure 8D).

The proximity of Smc3-GFP and Mcd1-GFP foci to Spc29-RFP in cdc5-99 strains suggested the persistence of cohesin in centromeric chromatin; hence we used ChIP to assay the association of Smc3-HA at CARs close to (CEN) or distant from the centromere (arm), using either metaphase or anaphase cells from wild-type, cdc5-99, and cdc15-2 strains. Metaphase cells were obtained by growing strains at 25°C in nocodazole, and anaphase cells were obtained after release from nocodazole at the non-permissive temperature of 37°C (Figure 9). We used cdc15-2 as a control because this strain arrests at a stage similar to cdc5 mutants during mitosis (Surana et al., 1993; St-Pierre et al., 2009). The cell cycle stage was determined by monitoring Pds1 levels (Cohen-Fix et al., 1996) by Western blotting, DNA content by FACS, and nuclear morphology by microscopy (Calvert and Lannigan, 2010). High levels of Pds1 are present in metaphase; however, levels of Pds1 are undetectable in anaphase, as degradation of Pds1 is required for anaphase onset (Cohen-Fix et al., 1996; Cohen-Fix and Koshland, 1999). Consistent with this, high levels of Pds1 were observed in metaphase but not in anaphase cells of wild-type, cdc5-99, and cdc15-2 strains (Figure 9, A and B). Large-budded cells with the nucleus at the neck or separated nuclei indicate metaphase and anaphase cells, respectively (Figure 9B). ChIP-qPCR showed enrichment of Smc3-HA at CEN (114), CARs flanking the centromere (112, 115), and the arms (261, 304, 307) in metaphase cells of wild-type, cdc5-99, and cdc15-2 strains (Figure 9, C and D). Smc3-HA levels were significantly reduced at both the centromeric chromatin (CEN (114), peri-CEN CARs (112, 115)) and the chromosomal arms (261, 304, 307) in anaphase cells of wild-type and cdc15-2 strains, and these levels were largely similar to those at non-CARs (125, 310; Figure 9D). In contrast, in anaphase cells of the cdc5-99 strain, Smc3-HA levels were significantly reduced only at CARs at chromosomal arms (261, 304, 307) and remained high at centromeric chromatin (CEN (114) and peri-CEN CARs (112, 115)). The levels of Smc3-HA at CEN and peri-CEN CARs in anaphase cells of cdc5-99 strains were not statistically different from those observed at these chromosomal regions in metaphase cells (Figure 9D). No significant enrichment of Scm3-HA was detected at non-CARs (125, 310) at either metaphase or anaphase (Figure 9D). These results suggest that Cdc5 is required for the removal of cohesin from centromeric chromatin during mitosis.

pattern of another cohesin component, Mcd1-GFP, in anaphase cells of wild-type and cdc5-99 strains using growth conditions and procedure as described earlier. The persistence of Mcd1-GFP foci was observed in anaphase cells of cdc5-99 strain, whereas no detectable Mcd1-GFP foci were observed in wild-type cells (Figure 8C). The
FIGURE 5: Induced expression of Cdc5 in G1 does not result in its recruitment to CEN and CARs. A wild-type strain containing Cdc5-HA (YMB9441) and untagged control (JG595) were grown at 30°C, arrested in G1 with α-factor, washed, and released into pheromone-free medium. α-Factor was added again 80 min after release to block the cells at the subsequent G1. Samples were taken at various time points (minutes) after G1 release. ChIP-qPCR was performed to determine the enrichment pattern of Cdc5 at CARs and non-CARs on chromosome III throughout the cell cycle. The CAR (green triangles) and non-CAR (black vertical lines) examined are shown schematically in Figure 3A. Cdc5 enrichment is shown as percentage input. Average from three biological replicates ± SE for each time point. (A) Enrichment levels of Cdc5 at non-CAR (125) located at peri-CEN3. (B) Enrichment levels of Cdc5 at CAR (134) located at peri-CEN3. (C) Enrichment levels of Cdc5 at CAR (261) located at the chromosomal arm. (D) Enrichment levels of Cdc5 at non-CAR (266) located at the chromosomal arm.

**p < 0.01; ns, not statistically significant, Student’s t test.
A previous study showed that SMC subunits of cohesin complex (e.g., Scc1) remain associated with chromosomes for ~10 min after the degradation of Scc1/Mcd1 in anaphase (Tanaka et al., 1999). Hence we investigated whether Cdc5 is also required for removal of Mcd1 from centromeric chromatin. We examined the association of Mcd1-HA at CEN and CARs in cdc5-99 and cdc15-2 grown at 25°C in nocodazole (metaphase) and after release from nocodazole at 37°C (anaphase). We detected high levels of Pds1 in metaphase but observed significantly lower levels of chromatin-associated Cdc5 in anaphase cells of cdc5-99 strains (Figure 10D). Consistent with the results for Smc3-HA, significantly reduced levels at cdc15-2 and the arms (261, 304, 307) in metaphase cells of cdc5-99 and cdc15-2 strains (Figure 10, A and B). The levels of Pds1 were slightly lower in cdc15-2 than in the cdc5-99 strain (Figure 10A). ChIP-qPCR showed enrichment of Mcd1-HA at CEN (114), CARs flanking the centromere (112, 115), and the arms (261, 304, 307) in metaphase cells of cdc5-99 and cdc15-2 strains (Figure 10, C and D). Mcd1-HA levels were significantly reduced at CEN (114), peri-CEN CARs (112, 115), and chromosomal arms CARs (261, 304, 307) in anaphase cells of cdc15-2, and these levels were largely similar to those at non-CARs (125, 31; Figure 10D). Consistent with the results for Smc3-HA, significantly reduced levels of Mcd1-HA were observed at CARs in chromosomal arms (261, 304, 307), whereas Mcd1-HA levels remained high at centromeric chromatin (CEN[114] and peri-CEN CARs[112, 115]) in anaphase cells of cdc5-99 strains. The levels of Mcd1-HA at CEN and peri-CEN CARs in anaphase cells of cdc5-99 strains were statistically different from those observed at these chromosomal regions in metaphase cells (Figure 10D). No significant enrichment of Mcd1-HA was detected at non-CARs (125, 310) at either metaphase or anaphase (Figure 10D). The persistence of both Smc3 and Mcd1 in CEN and peri-CEN chromatin in anaphase cells of the cdc5-99 strain supports a role for Cdc5 in the removal of cohesin from centromeric chromatin.

**Cdc5 is required for faithful chromosome segregation**

The cell cycle–regulated centromeric association of Cdc5 and its role in removal of centromeric cohesin led us to examine whether defects in Cdc5 would result in increased chromosome loss. We constructed a cdc5-99 strain carrying a reporter chromosome (RC) and determined the frequency of loss of the RC using a colony color assay as described previously (Spencer et al., 1990). Loss of the RC results in formation of red sectors in an otherwise white colony. Colonies that are at least half red indicate loss of the RC in the first cell division. The frequency of RC loss in cdc5-99 strains was about sixfold higher than in the wild-type strain (Figure 11A), similar to that reported for kinetochore mutants (Kastenmayer et al., 2005; Ma et al., 2012).

**DISCUSSION**

Evolutionarily conserved polo-like kinase Cdc5 and its homologues play a range of crucial and conserved roles in regulation of cell cycle events (Alexandru et al., 2001; Lee et al., 2005; St-Pierre et al., 2009; Rossio et al., 2010; Ratsima et al., 2011; Botchkarev et al., 2014; Walters et al., 2014; Zitouni et al., 2014; Archambault et al., 2015). Among its many roles, Cdc5 is required for the phosphorylation and subsequent cleavage of Scc1/Mcd1 in order to promote sister chromatid separation (Alexandru et al., 2001; Hornig and Uhlmann, 2004). Our results provide mechanistic insight into the role of Cdc5 in the removal of centromeric cohesin during mitosis. We show that Cdc5 associates with centromeric chromatin and is required for the efficient removal of cohesin at core CEN and peri-CEN regions during mitosis. These conclusions are based on results showing that Cdc5: 1) associates with CEN and CARs in a cell cycle–dependent manner, with maximum enrichment in mitosis; 2) interacts with cohesins in vivo; 3) requires cohesin for its mitotic enrichment at CEN and CARs; 4) is required for the removal of cohesin from centromeric chromatin in anaphase; and 5) is required for faithful chromosome segregation.

Enrichment of Cdc5 at centromeric chromatin was highest in mitotic cells undergoing the transition from metaphase to anaphase. Even though Cdc5 is expressed in S-phase and anaphase cells, we observed significantly lower levels of chromatin-associated Cdc5 during the S phase of the cell cycle. Moreover, ectopic expression of Cdc5 in G1 does not result in its recruitment to CEN and CARs, suggesting that the binding of Cdc5 to these chromosomal regions is regulated by the cell cycle. Association of Cdc5 flanking CEN6 and CARs was reported in a ChIP-on-chip study evaluating the role of Rsc2 in mitotic exit (Rossio et al., 2010). Our comprehensive ChIP analysis shows that the cell cycle–regulated centromeric enrichment of Cdc5 coincides with its role in removal of cohesin from this region. We discovered that cohesin components Scc1/Mcd1 interact with Cdc5 in vivo, and the interaction is required for enrichment of Cdc5 at CEN and CARs. It is of note that high-throughput proteomic studies also identified cohesin components when Cdc5 was used as bait in affinity-capture mass spectroscopy experiments (Ho et al., 2002; Snead et al., 2007; Breitkreutz et al., 2010). The cohesin-dependent enrichment of Cdc5 at CEN and CARs reflects a dynamic enzyme–substrate relationship between Cdc5 and cohesin components, resulting in preferential removal of chromatin-bound cohesin. Of most importance, our results suggest a role for Cdc5 in removal of centromeric cohesins, most likely from the intramolecular linkages in the C-loop (Yeh et al., 2008; Hu et al., 2011), but not cohesins in the arms of chromosomes (Figure 11B). The C-loop model resolved a paradox in which sister kinetochores can separate up to 800 nm in...
vivo despite high levels of cohesin at centromeres. The persistence of Smc3 and Mcd1 in anaphase of cdc5-99 cells at the nonpermissive temperature may reflect the presence of intramolecularly linked cohesin in the C-loop, whereas the absence of cohesin in chromosome arms shows that Cdc5 is dispensable for removal of intramolecularly linked cohesins. Cdc5 has been shown to regulate removal of cohesin from chromosome arms for segregation of homologous chromosomes in meiosis I but to be dispensable for meiosis II (Brar et al., 2006; Katis et al., 2010; Attner et al., 2013). Attner et al. (2013) concluded that additional kinases or substrates and/or the differential nature of centromeric versus chromosome arm cohesin regulate the removal of centromeric cohesin in meiosis II. On the basis of our results, we conclude that Cdc5 represents the first example of differential regulation of centromeric versus arm cohesin during mitosis in budding yeast.

The mitotic enrichment of Cdc5 at CEN suggests a role for Cdc5 in kinetochore function because of major changes in spatial geometry and spindle tension that occur at the kinetochore during mitosis. Consistent with this hypothesis, we observed errors in chromosome segregation in the cdc5-99 strain. Because Cdc5 is involved in many biological processes (Archambault and Glover, 2009; St-Pierre et al., 2009; Botchkarev et al., 2014; Walters et al., 2014; Zitouni et al., 2014; Roccuzzo et al., 2015), chromosome segregation errors observed in cdc5-99 strain may not be due to solely defects in cohesin removal. Previous studies showed that Cdc5 interacts with and phosphorylates kinetochore proteins in vivo (e.g., Slk19) to promote proper microtubule attachment and spindle function (Stegmeier et al., 2002; Snead et al., 2007; Park et al., 2008; Liang et al., 2009; Richmond et al., 2013). Plk1, the human homologue of Cdc5, also localizes to the CEN (Arnaud et al., 1998; Kishi et al., 2009), where it likely phosphorylates Rad21 (the human Scc1 homologue; Waizenegger et al., 2000). In addition, Plk1 phosphorylates kinetochore protein Mis18BP1, which in turn licenses the recruitment of new CENP-A to kinetochores in the G1 phase of the cell cycle (McKinley and Cheeseman, 2014). Of note, Cse4 (the budding yeast CENP-A homologue) was identified as an in vivo interacting partner of Cdc5 in a genome-wide proteomic study (Snead et al., 2007). Future studies should help us to address whether Cdc5-mediated phosphorylation of kinetochore proteins affects the removal of centromeric cohesins.

In summary, our studies provide novel insights into the role of Cdc5 in removal of centromeric cohesin but not cohesin from...
chromosomal arms. Differential regulation of cohesin removal has also been reported in mammalian cells, where removal of cohesin from chromosomal arms in prometaphase is regulated by the cohesin antagonist WAPL, whereas removal of centromeric cohesin at the metaphase-to-anaphase transition by Plk1 is achieved by a separase-dependent mechanism (Waizenegger et al., 2000; Hauf et al., 2001; Gandhi et al., 2006; Kueng et al., 2006; Haarhuis et al., 2013). Mutations and overexpression of Plk1 are observed in many cancers (Davies et al., 2005; Stephens et al., 2005; Kan et al., 2010), displaying chromosomal instability and aneuploidy (Degenhardt and Lampkin, 2010). Hence elucidation of molecular mechanisms by which polo kinases regulate cohesin removal and faithful chromosome segregation might help us identify and develop novel therapeutic targets for cancer therapy.

**MATERIALS AND METHODS**

**Strains, plasmids, and culture conditions**

Saccharomyces cerevisiae strains and plasmids used in this study are listed in Table 1. Strains were grown in 1% yeast extract/2% Bacto-peptone/2% glucose (YPD) or in yeast synthetic medium containing 2% glucose and supplements to allow for the selection of plasmids used.

**Assay for the loss of reporter chromosome**

Loss of a nonessential RC was measured using a colony color assay in which RC loss results in red sectors in an otherwise white colony (Spencer et al., 1990). Wild-type and cdc5-99 strains were grown to logarithmic phase in selective medium to maintain the RC. Cultures were diluted and plated on complete synthetic medium with limiting adenine at 30°C. The frequency of chromosome loss was measured by counting the colonies that were at least half red, indicating loss of the RC at the first cell division. At least 3000 colonies of three individual transformants were examined for each strain.

**ChiP and qPCR experiments**

ChiP assays were performed with three biological replicates as described previously (Mishra et al., 2007, 2011). Antibodies used to capture protein–DNA complexes were α-HA (A2095; Sigma-Aldrich), α-Myc (A7470; Sigma-Aldrich). ChiP-qPCR was carried out using SYBR Green Master Mix in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) following conditions used previously (Mishra et al., 2011). The enrichment values were determined as percentage input using the ΔΔC_T method (Livak and Schmittgen, 2001). Primer sequences used in this study are presented in Table 2.
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**FIGURE 9:** Cdc5 is required for the removal of Smc3 from centromeric chromatin. WT (YMB9693), cdc5-99 (YMB9709), and cdc15-2 (YMB9703) expressing Smc3-6HA and Pds1-18Myc from their endogenous promoters were used. Smc3-6HA was an internally tagged version of Smc3 (six copies of HA inserted at amino acid residue 607) using plasmid pVG466 (a gift from Vincent Guacci). Strains were grown in YPD at permissive temperature (25°C) with nocodazole (NOC, 20 μg/ml) for 2 h to synchronize cells in metaphase. Cells were then shifted to nonpermissive temperature (37°C) for 1 h, followed by release from NOC into YPD at 37°C for 45 min to enrich cells in anaphase. Samples were collected for Western blot analysis for Pds1, nuclear morphology, DNA content, and ChIP analyses. Three biological replicates were performed for each strain. (A) Western blot analysis showing high levels of Pds1 only in metaphase cells of WT, cdc5-99, and cdc15-2 strains. Antibodies used were α-Myc (Pds1) and α-Tub2 (loading control). (B) Cell cycle stages (metaphase and anaphase) were determined based on nuclear position and cell morphology by microscopic examination of 100 cells for each strain per replicate as described previously (Calvert and Lannigan, 2010; Mishra et al., 2011). DNA content was determined by FACS. (C) Schematic representation of CARs and non-CARs on chromosome III. The schematic is derived from Figure 3A with additional CARs and non-CARs. Centromere (CEN3, violet circle), CAR (green triangle), and non-CAR (black vertical line) sites. CEN is located at 114 kb; peri-CEN CARs 115 (1 kb from CEN) and 112 (2 kb from CEN), chromosome arm CAR 261 (147 kb from CEN), 304 (190 kb from CEN), 307 (193 kb from CEN), peri-CEN non-CAR 125 (11 kb from CEN), and chromosome arm non-CAR 310 (196 kb from CEN) were examined. (D) Smc3 persists at CEN and peri-CEN chromatin in cdc5-99 strains. ChIP was performed with chromatin prepared from metaphase (ME) or anaphase (A) with strains as described using α-HA antibodies. Enrichment of Smc3 at CEN3 (114), peri-CEN CARs (115, 112, 261), and chromosomal arm CARs (261, 304, 307) were determined by qPCR and is shown as percentage input. The non-CAR peri-CEN loci 125 and 310 in chromosome arms were used as a negative control. Average from three biological replicates ± SE. **p <0.01; *p <0.05; ns, not significant, Student’s t test.
FIGURE 10: Cdc5 is required for the removal of Mcd1 from centromeric chromatin. cdc5-99 (YMB9711), and cdc15-2 (YMB9712) strains expressing Mcd1-3HA, and Pds1-18Myc from their endogenous promoters were used. Strains KT046 (Tong and Skibbens, 2014) and D1469 carrying MCD1-3HA were used to construct strains YMB9711 and YMB9712, respectively. Strains were grown in YPD at permissive temperature (25°C) with nocodazole (NOC; 20 μg/ml) for 2 h to synchronize cells in metaphase. Cells were then released into YPD at nonpermissive temperature (37°C) for 2.5 h to enrich cells in anaphase. Samples were collected for Western blot analysis, nuclear morphology, DNA content, and ChIP analyses. Three biological replicates were performed for each strain. (A) Western blotting showing high levels of Pds1 only in metaphase cells of cdc5-99 and cdc15-2 strains. Antibodies used were α-Myc (Pds1) and α-Tub2 (loading control). (B) Cell cycle stages (metaphase and anaphase) were determined based on nuclear position and cell morphology by microscopic examination of 100 cells for each strain per replicate. DNA content was determined by FACS. (C) Schematic representation of CARs and non-CARs on chromosome III as detailed in Figure 9C. (D) Mcd1 persists at CEN and peri-CEN chromatin in cdc5-99 strains. ChIP experiments were performed using chromatin prepared from metaphase (ME) or anaphase (A) cells from strains as described using α-HA antibodies. Enrichment of Mcd1 at CEN3 (114), peri-CEN CARs (112, 115), and chromosomal arm CARs (261, 304, 307) were determined by qPCR and is shown as percentage input. The non-CAR peri-CEN loci 125 and 310 in chromosome arms were used as negative controls. Average from three biological replicates ± SE. **p < 0.01; *p < 0.05; ns, not significant, Student’s t test.

Immunoprecipitation and Western blotting
Immunoprecipitation assays were performed as described previously (Mishra et al., 2011). Protein extracts were prepared with the trichloroacetic acid procedure and quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein for each sample were resolved on
SDS–polyacrylamide gels and transferred to nitrocellulose membrane. Primary antibodies used were α-HA (clone 12CA5; Roche Molecular Systems, Pleasanton, CA), α-GFP (A11122; Life Technologies, Carlsbad, CA), α-TAP (CAB1001; Thermo Scientific, Waltham, MA), α-Cdc5 (11H12 and 4F10; Medimabs, Montreal, Canada), α-Myc (Z-5, sc-789; Santa Cruz Bio-technology, Dallas, TX), and α-Tub2 (Mishra et al., 2011). Secondary antibodies were horseradish peroxidase–conjugated donkey α-rabbit immunoglobulin G (IgG; NA934V, Amersham Bioscience, Amersham, United Kingdom), and HRP-conjugated sheep α-mouse IgG (NA931V, Amersham Biosciences).

Microscopy
Cultures grown to logarithmic phase at permissive temperature (25°C) and after a shift to nonpermissive temperature (37°C) were used for all imaging experiments. Anaphase cells that exhibited a spindle length of 3–8 μm were selected based on the position of spindle pole bodies (Spc29-RFP; Chen et al., 2000; Maddox et al., 2000). Cells were imaged for Smc3-GFP, Mcd1-GFP, and Spc29-RFP signals at room temperature on a Nikon TE-2000E inverted microscope equipped with a 1.4 numerical aperture/100 × Plan-Apo objective (Nikon Instruments, New York, NY) as described previously (Haase et al., 2013; Salmon et al., 2013).

Table 1: Strains and plasmids used in this study.

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<td>p344</td>
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TABLE 1: Strains and plasmids used in this study. Continued

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TABLE 2: Primers used in this study.
ACKNOWLEDGMENTS
We are highly thankful to Vincent Guacci and Robert Skibbens for strains, plasmids, and helpful suggestions. We thank Sue Biggins for plasmids and Jennifer Gerton for strains, Kathy McKinnon of the National Cancer Institute Vaccine Branch FACS Core for assistance with FACS and the Basrai laboratory for discussions. P.K.M., S.C.Y., W.C.A., L.B., L.E.D., Z.J., T.P., and M.A.B. were supported by the Intramural Research Program of the National Cancer Institute, National Institutes of Health; M.A.H. and D.R. by the National Science Foundation; E.Y. and K.B. by the National Institutes of Health (R37 GM32238); and D.D. by the Cancer Research Society and the Canadian Institutes of Health Research (MOP 82912).

REFERENCES
1 and Cdc7-Dbf4 kinase regulates cohesin cleavage by separase during meiosis. Dev Cell 18, 397–409.