Tension sensing by Aurora B kinase is independent of survivin-based centromere localization

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Accurate segregation of the replicated genome requires chromosome biorientation on the spindle. Biorientation is ensured by Aurora B kinase (Ipl1), a member of the four-subunit chromosomal passenger complex (CPC)1,2. Localization of the CPC to the inner centromere is central to the current model for how tension ensures chromosome biorientation: kinetochore–spindle attachments that are not under tension remain close to the inner centromere and are destabilized by Aurora B phosphorylation, whereas kinetochores under tension are pulled away from the influence of Aurora B, stabilizing their microtubule attachments1,3. Here we show that an engineered truncation of the Sli15 (known as INCENP in humans) subunit of budding yeast CPC that eliminates association with the inner centromere nevertheless supports proper chromosome segregation during both mitosis and meiosis. Truncated Sli15 suppresses the deletion phenotypes of the inner-centromere-targeting proteins survivin (Bir1), borealin (Nbl1), Cdc28 and Sgo1 (ref. 4). Unlike wild-type Sli15, truncated Sli15 localizes to pre-anaphase spindle microtubules. Premature targeting of full-length Sli15 to microtubules by preventing Cdk1 (also known as Cdc28) phosphorylation also suppresses the inviability of Bir1 deletion. These results suggest that activation of Aurora B kinase by clustering either on chromatin or on microtubules is sufficient for chromosome biorientation.

All known mechanisms targeting the CPC to centromeric chromatin, in budding yeast and elsewhere, rely on the survivin (Bir1) subunit. Budding yeast CPC (Fig. 1a) is targeted by two Bir1-dependent mechanisms: interaction of Bir1 with Sgo1, which recognizes histone H2a phosphorylated by the kinetochore-localized kinase Cdk1, and direct binding of Bir1 to the Ndc10 (also known as Ctb2) subunit of the centromeric DNA–binding Ctb3 complex5,6. In other species, survivin binding to histone H3 phosphorylated on Thr 3 by haspin kinase is also implicated in CPC centromere targeting7–9; however, deletion of the two haspin-like genes (ALK1 and ALK2) does not lead to a growth phenotype (see below), suggesting that this mechanism may not operate in budding yeast.

In agreement with the view that Bir1-directed targeting of the CPC to centromeres is critical for chromosome biorientation, the majority of bir1Δ spores fail to grow (Fig. 1b) and temperature-sensitive mutations in bir1 show chromosome missegregation similar to that observed in ip1Δ and sli15 mutants10,11. A low frequency (10%, n = 60) of bir1Δ spore survival is observed after extended incubation12 (Fig. 1b); these survivors, which we refer to as bir1Δ*, have high chromosome missegregation rates and harbour severe aneuploidy (Supplementary Fig. 1a and data not shown). To determine whether the severe Bir1 deletion phenotype is due to inability of the CPC to target to the inner centromere, we generated truncations of the Sli15 amino terminus that are predicted to eliminate the interaction of Sli15–Ipl1 with Bir1–Nbl1 (ref. 13). Surprisingly, truncations of up to 228 N-terminal amino acids of Sli15 (the longest viable truncation; referred to hereafter as Sli15(ΔNT); Fig. 1c) showed no lethality—cells harbouring these truncations as the sole source of Sli15 grew indistinguishably from wild type (Figs 1c and 2b). Further truncations that encroached on the Sli15 central domain were lethal (Fig. 1c). Immunoprecipitation experiments indicated that deleting the Sli15 N terminus eliminated the interaction with Bir1 (Fig. 1d). Analysis of CPC anaphase spindle localization, which is dependent on Sli15, confirmed this result. Whereas Sli15(ΔNT) and Ipl1 localized on the anaphase spindle (Fig. 1e), Bir1 and Nbl1 were delocalized in sli15(ΔNT) mutant cells (Fig. 1f).

The above results show that Sli15(ΔNT) is viable even though it disrupts CPC formation and disconnects the kinase activity of the CPC from the Bir1 subunit that targets it to centromeres. Consistent with this finding, sli15(ΔNT) fully suppressed inviability of bir1Δ and nbl1Δ: sli15(ΔNT) bir1Δ or sli15(ΔNT) nbl1Δ double-mutant spores formed colonies at the expected frequency with normal growth properties (Fig. 2a, b). Wild-type Sli15 and Sli15(ΔNT) were expressed at

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**Figure 1** | Deletion of the Sli15 N terminus prevents association with Bir1 but does not affect cell viability or growth. a. Schematic of the CPC in budding yeast. Bir1, survivin; Ipl1, Aurora B kinase; Nbl1, borealin/dasra; Sli15, INCENP. b. Tetrad dissections from a bir1Δ diploid. The four spores from individual tetrads are arrayed in columns. Rare survivors (bir1Δ*) are observed after extended growth (right). c. Phenotype of Sli15 truncations (left) and tetrad dissections from a sli15Δ(ΔNT) heterozygote (right). d. Co-immunoprecipitation analysis of full-length (FL) Sli15 or Sli15(ΔNT) and Bir1. 9-Myc and 6-haemagglutinin (HA) tags were inserted at endogenous loci to generate functional C-terminally tagged proteins. Bir1 co-immunoprecipitates with full-length Sli15 but not with Sli15(ΔNT). I, input; P, anti-HA immunoprecipitate. e. Localization of Sli15–Venus and Sli15(ΔNT)–Venus to the anaphase spindle. Scale bar, 5 μm. f. Localization of CPC components during anaphase in cells with either wild-type Sli15 or Sli15(ΔNT). The cell outline is in blue. Scale bar, 5 μm.

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similar levels, indicating that suppression of bir1Δ and nbl1Δ lethality was not due to overexpression of mutant Sli15 (Fig. 2c). Sli15 was not detected in bir1Δ* and nbl1Δ* strains, suggesting that full-length Sli15 is destabilized when complex formation is disrupted (Supplementary Fig. 1b and Supplementary Discussion). We conclude that Sli15(ΔNT)–Ipl1 is sufficient to perform the essential function(s) of the CPC in the complete absence of Bir1 or Nbl1.

To assess the degree to which Sli15(ΔNT)–Ipl1 substitutes for the full CPC, we analysed chromosome segregation and biorientation using multiple assays. First, we monitored a single tagged chromosome, which revealed high-fidelity segregation for both the single sli15(ΔNT) and double sli15(ΔNT) bir1Δ mutants (Fig. 2d); by contrast, extensive missegregation was observed in the rare bir1Δ* survivors (Fig. 2d and Supplementary Fig. 1a) and has been previously reported for ipl1 kinase-activity-defective mutants3,16,17. Second, we monitored the segregation of a minichromosome (non-essential chromosome 4) in a sensitive colony colour assay, which revealed high-fidelity segregation for both the single sli15(NT) and double sli15(NT) bir1Δ mutants (Fig. 2e). We conclude that Sli15(NT)–Ipl1 substitutes for the full CPC, we analysed chromosome segregation and biorientation in other organisms through the creation of a binding site suppression of the spindle checkpoint function of Bub1 is independent of its role in CPC localization. Although haspin kinases contribute to CPC targeting in other organisms through the creation of a binding site on centromeric chromatin for survivin, deletion of the two haspin homologues in budding yeast had no growth phenotype on their own or in combination with sli15(ΔNT) (Fig. 3d).

An ipl1 temperature-sensitive mutant is synthetic lethal with deletion mutants of the Ctf19 and Mcm21 subunits of the Ctf19 kinetochore complex, which provides a non-essential function in centromeric cohesion16. In contrast to the bir1Δ, nbl1Δ, sgo1Δ and bub1Δ mutants, whose lethality/severe growth defects were suppressed by sli15(ΔNT), ctf19Δ and mcm21Δ mutants showed synthetic lethality/sickness with sli15(ΔNT) (Fig. 3e, f and Supplementary Fig. 2b, c). Combining
**sli15(ΔNT)** with a deletion of **CTF18**, a separate non-essential mutant affecting cohesion establishment, also led to a synthetic sick phenotype (Supplementary Fig. 2b, c). Thus, whereas Bir1-dependent CPC targeting is dispensable for chromosome biorientation and segregation, this targeted CPC pool shows a functional connection with cohesion (Fig. 3f and Supplementary Discussion).

We next examined the localization of CPC subunits in wild-type cells and in cells expressing Sli15(ΔNT). In cells arrested in metaphase by depletion of the anaphase activator Cdc20, where the chromosomes are already bioriented, localization of Sli15 or Ipl1 on chromatin was not detected (Fig. 4b, top row and Supplementary Fig. 3b). However, localization of Sli15 and Ipl1 between sister kinetochore clusters (analogous to the inner-centromere localization in other species) was observed following brief microtubule depolymerization in asynchronously growing cells (Fig. 4a and Supplementary Fig. 3a). In sli15(ΔNT) cells, localization between sister kinetochore clusters was lost for both Sli15(ΔNT) and Ipl1 (Fig. 4a and Supplementary Fig. 3a); instead weak localization was observed coincident with kinetochore clusters (Fig. 4a and Supplementary Discussion). Thus, the Sli15(ΔNT)–Ipl1 complex supports accurate chromosome segregation without enriching between sister kinetochores in vivo.

In wild-type cells, the CPC is prevented from localizing to the spindle by Cdk1 phosphorylation until anaphase onset, when it is recruited to spindle microtubules and functions in spindle elongation. However, the Sli15(ΔNT)–Ipl1 complex showed robust accumulation on the spindle in cells held in metaphase by depletion of the anaphase activator Cdc20 (Fig. 4b and Supplementary Fig. 3b). The central domain of Sli15—which harbours microtubule-binding activity—is required for chromosome biorientation (Figs. 1c, 4d, and 5), and microtubule binding by human or frog INCENP activates Aurora B kinase through targeting pathways but is synthetically lethal with genes implicated in cohesion establishment, also led to a synthetic sick phenotype (the same as in Fig. 2f). Tetrad analysis showing no synthetic defect for cells that are triple mutants for sli15(ΔNT), alk1Δ and alk2Δ. Tetrad analysis showing synthetic lethality of sli15(ΔNT) and mcm21Δ. Similar results were observed for ctf9A (Supplementary Fig. 2b). f, Summary of genetic interactions shown by sli15(ΔNT). A positive genetic interaction (green) indicates suppression; negative genetic interaction (red) indicates synthetic lethality/sickness and a neutral interaction (black) indicates lack of a synthetic phenotype.
prematurely clustering Sli15 on microtubules by another means bypassed the requirement for Bir1 for viability. For this purpose, we used the previously described sl15-6A mutant that prevents Cdk1 phosphorylation in the central domain of Sli15 and prematurely targets it to pre-anaphase spindle microtubules2. Consistent with our hypothesis, sl15-6A suffered the inviability of bir1Δ in contrast to bir1Δ alone (Fig. 1b) all double-mutant sl15-6A bir1Δ spores formed colonies (Fig. 4c). However, the sl15-6A bir1Δ double-mutant cells grew slowly and exhibited benomyl sensitivity, indicating that the suppression was partial, unlike the case for sl15(ANT) (not shown). As with sl15(ANT), the suppression of Bir1 deletion by sl15-6A was not due to overexpression of the mutant protein (Supplementary Fig. 3d). A Sli15 mutant that cannot be phosphorylated by Ipl1 and displays premature localization to the metaphase spindle24 also partially rescued Bir1 deletion (Supplementary Fig. 3c).

Our finding that bioregion occurs normally in the absence of Bir1-dependent targeting of the CPC has considerable implications for how the discrimination of correct (amphitelic; with tension) and incorrect (syntelic; lacking tension) attachments is achieved (Fig. 4d and Supplementary Discussion). Current models for bioregion emphasize the distance between inner-centromere-localized Aurora B kinase and outer-kinetochore-localized phosphatase activity as being critical for this discrimination25–27. Our findings instead suggest that active Aurora B, generated by clustering on either microtubules or centromeric chromatin, is capable of discriminating between correct and incorrect attachments and that this discrimination is intrinsic to the kinetochore (Fig. 4d). A parsimonious explanation for how this discrimination is achieved is substrate access, with correct, tense attachments becoming less sensitive to Aurora B activity. Super-resolution imaging studies have documented structural changes in microtubule-attached kinetochores under tension versus lacking tension9, which may lead to changes in susceptibility to Aurora B phosphorylation. Defining the property that is detected by Aurora B to discriminate correct versus incorrect attachments should be facilitated by the finding that survivin-mediated centromere targeting of the CPC is not necessary for tension-sensing and chromosome bioregion in budding yeast.

METHODS SUMMARY

**Yeast strains and media.** All yeast strains used in this study are listed in Supplementary Table 1. Cells were cultured on yeast extract and peptone media with glucose unless otherwise indicated.

**Microscopy.** All microscopy was performed on a DeltaVision microscopy system (Applied Precision) and deconvolved with softWoRx software. Cells were prepared on 1% agar pads supplemented with complete synthetic media for imaging.

**Nocodazole recovery assay.** Cultures were synchronized with 2a-factor, washed and released into yeast extract peptone dextrose (YPD) with 10 μg ml⁻¹ benomyl and 15 μg ml⁻¹ nocodazole. Samples were collected every 2 h, and 100 μl of a 1:10,000 dilution was plated on YPD agar plates.

**Immunoprecipitations.** In total, 500 μl of cleared lysate was combined with 25 μl of antibody-bead slurry and rotated at 4 °C for 1 h. Beads were washed and resuspended in protein sample buffer for analysis by western blot.

**Tetrad dissection.** Spores were washed with water and digested with 1 mg ml⁻¹ zymolyase for 5 min at 30 °C and then digested onto YPD plates. Genotyping was performed by replica plating colonies onto selective media.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Yeast strains and media. All yeast strains and plasmids used in this study are listed in Supplementary Table 1. Strains were grown in either yeast extract/peptone or synthetic media at 30 °C unless otherwise indicated. Epitope and fluorescent tags were inserted at the C terminus of genes at their native loci as previously described25. Gene truncations were made with the QuickChange Mutagenesis Kit (Agilent Technologies). Truncations and point mutants were integrated either at their native loci (by digesting the plasmid with the unique NruI site in the Sli15 promoter), or at the URA3 locus (by digesting with the StuI site in the URA3 gene). For native locus integration, the wild-type copies of the gene were then excised by growing overnight in yeast extract peptone dextrose (YPD) and selecting for growth on 5-fluoroorotic acid (5-FOA) plates. All integrations were then checked by PCR and sequencing.

Immunoprecipitation and western blotting. Cells in exponential growth were pelleted and re-suspended in 600 μl immunoprecipitation buffer with protease inhibitors (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 4 mM benzamidine, Complete EDTA-free protease inhibitor cocktail (Roche)) and vortexed for 45 min with 400 μl glass beads. Lysates were cleared at 18,000 g for 10 min, transferred to a new tube and centrifuged again. A total of 25 μl of antibody-bead slurry (Anti-HA monoclonal clone 3F10, Roche) was combined with 500 μl of cleared lysate and rotated at 4 °C for 1 h. Beads were washed once with immunoprecipitation buffer and three times with Tris buffer saline, pH 7.4, and then re-suspended in protein sample buffer. Samples were analysed by 8 or 10% SDS–PAGE and immunoblotted with anti-HA clone 3F10 (Roche) and anti-Myc mouse monoclonal 4A6 (Millipore), followed by horseradish peroxidase-conjugated secondary antibodies.

Nocodazole recovery assay. Yeast cultures were diluted to an attenuation (D600nm) of 0.1 in YPD and incubated at 30 °C for 1.5 h. Next, 10 μM α-factor was added for 2.5 h. Cells were washed five times with YPD, re-suspended in YPD plus 10 μg ml⁻¹ benomyl and 15 μg ml⁻¹ nocodazole, and incubated at 23 °C. Samples were collected every 2 h, and 100 μl of a 1:10,000 dilution was plated on YPD agar plates. The percentage of colonies formed was determined by dividing the number of colonies at the indicated time point to the number at time zero after 3-day growth on plates. A minimum of 200 colonies were counted for each mutant at time zero.

Analysis of cell cycle progression after cohesin depletion. Overnight cultures of GAL-MCD1 strains were diluted into fresh YPG media and arrested in G1 phase with 1 μM α-factor. Cells were then washed five times and released into fresh medium. A further 1 μM α-factor was added again when the cells had small buds to prevent entry into the next cell cycle. Samples were taken at the indicated time points and lysed by vortexing for 2 min with glass beads in sample buffer. The samples were then analysed by SDS–PAGE and western blot.

Microscopy. Overnight cultures were diluted ~100-fold and grown for 5 h. Cells were pelleted, washed once and re-suspended in water, placed on 1% agar pads supplemented with complete synthetic media, covered with a coverslip and sealed around the edges with VALAP (a 1:1:1 mixture of vaseline, lanolin (Fisher) and paraffin (Fisher) by weight). Images were collected on a DeltaVision microscopy system (Applied Precision) using a 100×, 1.3 NA Olympus U-PlanApo objective. Fourteen z-sections were taken with 0.5-μm steps and deconvolved with softWoRx software. Further image analyses, including maximum intensity projections and contrast adjustments, were performed in ImageJ (NIH). Images within each figure were all collected under the same conditions and contrast adjusted identically. For metaphase arrest with Cdc20 deletions, asynchronous cultures in rich media with 1% galactose and 1% raffinose were washed three times and switched to media containing 2% glucose for 2.5 h before imaging. For mitotic chromosome segregation assays, cells were grown overnight in media selective for the lacO cassette and green fluorescent protein-tagged Lacl, and then switched to rich media for 5 h. The cells were then fixed with 4% formaldehyde, washed once, stored in storage solution (100 mM potassium phosphate, pH 7.5, 1 M sorbitol) at 4 °C and imaged no more than 2 days later. For meiosis microscopy, saturated cultures in YPD were pelleted, re-suspended in 1% potassium acetate, and incubated with rotation for 24 h at 23 °C. For imaging after microtubule depolymerization, cultures undergoing exponential growth were treated with 10 μg ml⁻¹ benomyl and 15 μg ml⁻¹ nocodazole for 15 min, washed once with water, put on agar pads with complete synthetic media and 10 μg ml⁻¹ benomyl, and immediately imaged.

Tetrad dissection. Diploids were sporulated by transferring patches of yeast from YPD plates grown overnight at 30 °C to sporulation plates (8.2 mg ml⁻¹ sodium acetate, 0.35 mg ml⁻¹ magnesium sulphate, 1.9 mg ml⁻¹ potassium chloride, 1.2 mg ml⁻¹ sodium chloride, 16 mg ml⁻¹ agar) and incubated at 23 °C for 2–3 days. Spores were then washed with water and digested with 1 mg ml⁻¹ zymolyase for 5 min at 30 °C and then dissected onto YPD plates. Genotyping was performed by replica plating colonies onto selective media. For Sli15 constructs integrated at their endogenous loci, a G418 resistance cassette was integrated 1.5 kilobases upstream of either the wild-type or mutant protein for genotyping purposes.

Minichromosome loss assay. Cultures were started overnight in selective (∼His) media and then diluted into YPD and grown without selection for 6 h. The cultures were then plated on synthetic media with low (6 μg ml⁻¹) adenine to enhance the colour change26 and grown for 3 days at 23 °C. The per cent of red or sectored versus completely white colonies was then counted.

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