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Tension Sensing by Aurora B Kinase is Independent of Survivin-Based Centromere Localization

Christopher S. Campbell[1] and **Arshad Desai**[1]

[1]Ludwig Institute for Cancer Research and Department of Cellular & Molecular Medicine University of California San Diego, La Jolla, CA 92037, USA

Abstract

Accurate segregation of the replicated genome requires chromosome biorientation on the spindle. Biorientation is ensured by Aurora B kinase, a member of the 4-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 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 attachments[3–5]. Here we show that an engineered truncation of the INCENP/Sli15 subunit of budding yeast CPC that eliminates association with the inner centromere nevertheless supports proper chromosome segregation during both mitosis and meiosis. Truncated INCENP/Sli15 suppresses the deletion phenotypes of the inner centromere-targeting proteins Survivin/Bir1, Borealin/Nbl1, Bub1 and Sgo1[6]. Unlike wildtype INCENP/Sli15, truncated INCENP/Sli15 localizes to pre-anaphase spindle microtubules. Premature targeting of full-length INCENP/Sli15 to microtubules by preventing Cdk1 phosphorylation also suppresses inviability of Survivin/Bir1 deletion. These results suggest that activation of Aurora B/Ipl1 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 Bub1[6], and direct binding of Bir1 to the Ndc10 subunit of the centromeric DNA-binding complex CBF3[7,8]. In other species, Survivin binding to histone H3 phosphorylated on threonine 3 by Haspin kinase is also implicated in CPC centromere targeting[9–11]; 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.

@: Corresponding author: abdesai@ucsd.edu, Phone:(858)-534-9575, Fax: (858)-534-7750, Address: CMM-E Rm 3052, 9500 Gilman Dr, La Jolla, CA 92093-0653.

AUTHOR CONTRIBUTIONS

C.C. and A.D. designed experiments and wrote the manuscript. C.C. performed the experiments and analyzed the data.

In agreement with the view that Bir1-directed targeting of the CPC to centromeres is critical for chromosome biorientation, the majority of *bir1*Δ spores fail to grow (Fig. 1b) and temperature-sensitive mutations in *bir1* exhibit chromosome missegregation similar to that observed in *ipl1* and *sli15* mutants[12,13]. A low frequency (10%, n=60) of *bir1*Δ spore survival is observed after extended incubation (Fig. 1b: [14]); these survivors, which we refer to as *bir1*Δ*, have high chromosome missegregation rates and harbor significant aneuploidy (Fig. S1a & not shown). To determine if the severe *BIR1* deletion phenotype is due to inability of the CPC to target to the inner centromere, we generated truncations of the Sli15 N-terminus that are predicted to eliminate the interaction of Sli15-Ipl1 with Bir1-Nbl1[15]. Surprisingly, truncations of up to 228 N-terminal amino acids of Sli15 (the longest viable truncation; referred to from now on as Sli15ΔNT; Fig. 1c) exhibited no lethality—cells harboring these truncations as the sole source of Sli15 grew indistinguishably from wildtype (Fig. 1c, 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. While Sli15ΔNT and Ipl1 localized on the anaphase spindle (Fig. 1e), Bir1 and Nbl1 were delocalized in *sli15*Δ*NT* mutant cells (Fig. 1f).

The above results show that Sli15ΔNT 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*Δ*NT* fully suppressed inviability of *bir1*Δ and *nbl1*Δ—double *sli15*Δ*NT;bir1*Δ or *sli15*Δ*NT;nbl1*Δ mutant spores formed colonies at the expected frequency with normal growth properties (Fig. 2a,b). Wild type Sli15 and Sli15ΔNT were expressed at similar levels indicating that suppression of *bir1*Δ and *nbl1*Δ lethality was not due to overexpression of mutant Sli15 (Fig. 2c). Sli15 was not detected in *bir1*Δ* and *nbl1*Δ* strains, suggesting that full length Sli15 is destabilized when complex formation is disrupted (Fig. S1b; see Suppl. Discussion). We conclude that Sli15ΔNT-Ipl1 is sufficient to perform the CPC's essential function(s) in the complete absence of Bir1 or Nbl1.

To assess the degree to which Sli15ΔNT-Ipl1 substitutes for the full CPC, we analyzed chromosome segregation and biorientation using multiple assays. First, we monitored a single tagged chromosome, which revealed high fidelity segregation for both the single *sli15*Δ*NT* and double *sli15*Δ*NT;bir1*Δ mutants (Fig. 2d); in contrast, extensive missegregation was observed in the rare *bir1*Δ* survivors (Fig. 2d; Fig. S1a) and has been previously reported for *ipl1* kinase activity-defective mutants[3,16,17]. Second, we monitored segregation of a minichromosome (non-essential chromosome III fragment) in a sensitive colony color assay, which revealed near-normal fidelity of segregation in *sli15*Δ*NT* cells (Fig. 2e); *mcm21*Δ cells, which are also viable and do not exhibit significant growth defects but reduce chromosome segregation fidelity, are shown for comparison (Fig. 2e). Third, modest defects in chromosome biorientation are enhanced by transient nocodazole treatment and removal, due to formation of multiple incorrect attachments during the recovery of collapsed spindles[18]. We therefore tested cell viability following transient nocodazole treatment. *bir1*Δ* spores exhibited rapid death following transient nocodazole treatment; in

contrast, *sli15ΔNT* and *sli15ΔNT;bir1Δ* cells behaved similarly to wild-type cells (Fig. 2f). Fourth, mutations that increase chromosome segregation errors exhibit sensitivity to microtubule depolymerizing drugs such as benomyl[18,19]. *sli15ΔNT* growth rates were similar to wildtype in the presence of benomyl (Fig. 2g). Fifth, mild defects in CPC activity, as well as deletion mutants of non-essential chromosome segregation proteins, show strong synthetic lethal/sick interactions with mutations in the spindle checkpoint (such as *mad1Δ* or *mad2Δ*) (e.g. [20]). *sli15ΔNT* mutant cells did not exhibit a synthetic lethal/sick interaction with *mad1Δ* (Fig. 2h). Sixth, *sli15ΔNT* mutant cells delayed cell cycle progression in response to lack of sister chromatid cohesion, indicating that they are competent to communicate absence of tension to the spindle checkpoint (Fig. S2a). Seventh, subtle defects in chromosome segregation are often magnified during meiosis[21], but *sli15ΔNT* cells did not exhibit increased meiotic missegregation relative to control cells (Fig. 2i). Thus, *sli15ΔNT* cells exhibit remarkably normal chromosome segregation fidelity during mitosis and meiosis in budding yeast.

We next tested if Sli15ΔNT also bypasses mutations in the Bir1 localization pathway. Bir1-dependent targeting of the CPC to the inner centromere involves recognition of phosphorylated histone H2a by Sgo1 (Fig. 3a). Chromosome biorientation errors in *bub1Δ* and *sgo1Δ* mutants lead to severe growth defects, sensitivity to benomyl, and difficulty correcting defective attachments following nocodazole washout[18]. *sli15ΔNT* suppressed the severe growth defect of both *bub1Δ* and *sgo1Δ* cells (Fig. 3b); in addition, the benomyl sensitivity, the rapid loss of viability following transient nocodazole treatment, and the chromosome missegregation of *sgo1Δ* cells were also suppressed (Fig. 3b,c; Fig. S1c). The benomyl sensitivity of *bub1Δ* was not suppressed as the spindle checkpoint function of Bub1 is independent of its role in CPC localization. While Haspin kinases contribute to CPC targeting in other organisms via creating 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 *sli15ΔNT* (Fig. 3d).

An *ipl1* temperature-sensitive mutant is synthetic lethal with deletion mutants of the Ctf19 and Mcm21 subunits of the Ctf19/COMA kinetochore complex, which provides a non-essential function in centromeric cohesion[20]. In contrast to the *bir1Δ*, *nbl1Δ*, *sgo1Δ*, and *bub1Δ* mutants, whose lethality/severe growth defects were suppressed by *sli15ΔNT*, *ctf19Δ* and *mcm21Δ* mutants exhibited synthetic lethality/sickness with *sli15ΔNT* (Fig. 3e,f; Fig. S2b,c). Combining *sli15ΔNT* with a deletion of *CTF18*, a separate non-essential mutant affecting cohesion establishment, also led to a synthetic sick phenotype (Fig. S2b,c). Thus, while Bir1-dependent CPC targeting is dispensable for chromosome biorientation and segregation, this targeted CPC pool exhibits a functional connection with cohesion (Fig. 3f; **see** Suppl. Discussion).

We next examined the localization of CPC subunits in wildtype cells and in cells expressing Sli15ΔNT. In cells arrested in metaphase by depletion of the anaphase activator Cdc20, where the chromosomes are already bioriented, significant localization of Sli15 or Ipl1 on chromatin was not detected (Fig. 4b *top row;* Fig. S3b). 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; Fig. S3a). In *sli15ΔNT* cells, localization between sister kinetochore clusters was lost for both Sli15ΔNT and Ipl1 (Fig. 4a; Fig. S3a); instead weak localization was observed coincident with kinetochore clusters (Fig. 4a; **see** Suppl. Discussion). Thus, the Sli15ΔNT-Ipl1 complex supports accurate chromosome segregation without enriching between sister kinetochores in vivo.

In wildtype 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[22–24]. However, the Sli15ΔNT-Ipl1 complex exhibited robust accumulation on the spindle in cells held in metaphase by depletion of the anaphase activator Cdc20 (Fig. 4b; Fig. S3b). The central domain of Sli15, which harbors microtubule-binding activity, is required for chromosome biorientation ([14]; Fig. 1c) and INCENP microtubule binding activates Aurora B kinase through local clustering[25–27]. We therefore hypothesized that the Sli15ΔNT mutant may rely on clustering mediated by microtubule binding to activate Ipl1, which in turn detects and corrects defective kinetochore-spindle attachments. To test this idea, we wanted to determine if prematurely clustering Sli15 on microtubules by another means bypassed the requirement for Bir1 for viability. For this purpose, we used the previously described *sli15-6A* mutant that prevents Cdk1 phosphorylation in the central domain of Sli15 and prematurely targets it to preanaphase spindle microtubules[22]. Consistent with our hypothesis, *sli15-6A* suppressed the inviability of *bir1*Δ—in contrast to *bir1*Δ alone (Fig. 1b) all double mutant *sli15-6A;bir1*Δ spores formed colonies (Fig. 4c). However, the *sli15-6A;bir1*Δ double mutant cells grew slowly and exhibited benomyl sensitivity indicating that the suppression was partial, unlike the case for *sli15ΔNT* (not shown). As with *sli15ΔNT*, the suppression of *BIR1* deletion by *sli15-6A* was not due to overexpression of the mutant protein (Fig. S3d). A Sli15 mutant that cannot be phosphorylated by Ipl1 and displays premature localization to the metaphase spindle[24] also partially rescued *BIR1* deletion (Fig. S3c).

Our finding that biorientation occurs normally in the absence of Bir1-dependent targeting of the CPC has significant implications for how the discrimination of correct (amphitelic; with tension) and incorrect (syntelic; lacking tension) attachments is achieved (Fig. 4d; **see** Suppl. Discussion). Current models for biorientation emphasize the distance between inner centromere-localized Aurora B kinase and outer kinetochore-localized phosphatase activity as being critical for this discrimination[3–5]. 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 tension[28], 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 biorientation in budding yeast.

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 described[29]. Gene truncations were made with the QuikChange 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 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 YPD and selecting for growth on 5-FOA plates. All integrations were then checked by PCR and sequencing.

Immunoprecipitation and Western Blotting

Cells in exponential growth were pelleted and resuspended in 600μL IP buffer with protease inhibitors [50mM Tris pH7.6, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 2mM PMSF, 4mM Benzamidine, cOmplete EDTA-free protease inhibitor cocktail (Roche)] and vortexed for 45 min with 400μL glass beads. Lysates were cleared at 18,000g for 10 minutes, transferred to a new tube and centrifuged again. 25 μL of antibody-bead slurry (Anti-HA rat monoclonal clone 3F10; Roche) was combined with 500 μL of cleared lysate and rotated at 4°C for one hour. Beads were washed once with IP buffer and 3 times with TBS (Tris buffer saline, pH 7.4) and then resuspended in protein sample buffer. Samples were analyzed by 8 or 10% SDS-PAGE and immunoblotted with anti-HA clone 3F10 (Roche) and anti-myc mouse monoclonal 4A6 (Millipore), followed by HRP-conjugated secondary antibodies.

Nocodazole Recovery Assay

Yeast cultures were diluted to an OD600 of 0.1 in YPD and incubated at 30°C for 1.5 hours. 10 μM α-factor was added for 2.5 hours. Cells were washed 5 times with YPD, resuspended in YPD plus 10 μg/mL benomyl and 15 μg/mL nocodazole, and incubated at 23°C. Samples were collected every 2 hours 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 days growth on plates. A minimum of 200 colonies were counted for each mutant at time zero.

Analysis of Cell Cycle Progression Following Cohesin Depletion

Overnight cultures of *GAL-MCD1* strains were diluted into fresh YPG media and arrested in G1 with 1μM α-factor. Cells were then washed five times and released into fresh medium. 1μM 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 two minutes with glass beads in sample buffer. The samples were then analyzed by SDS-PAGE and western blot.

Microscopy

Overnight cultures were diluted ~ 100-fold and grown for 5 hours. Cells were pelleted, washed once and resuspended in water, placed on 1% agar pads supplemented with complete synthetic media, covered with a cover-slip 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 100x, 1.3 NA Olympus U-PlanApo objective. 14 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 depletions, asynchronous cultures in rich media with 1% galactose and 1% raffinose were washed 3 times and switched to media containing 2% glucose for 2.5 hours before imaging. For mitotic chromosome segregation assays, cells were grown overnight in media selective for the lacO cassette and LacI-GFP and then switched to rich media for 5 hours. The cells were then fixed with 4% formaldehyde, washed once, stored in storage solution (100mM potassium phosphate pH7.5, 1M Sorbitol) at 4°C, and imaged no more than 2 days later. For meiosis microscopy, saturated cultures in YPD were pelleted, resuspended in 1% potassium acetate, and incubated with rotation for 24 hours at 23°C. For imaging following microtubule depolymerization, cultures undergoing exponential growth were treated with 10 μg/mL benomyl and 15 μg/mL nocodazole for 15 minutes, washed once with water, put on agar pads with complete synthetic media and 10 μg/mL 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 Sodium Acetate, 0.35 mg/mL Magnesium Sulfate, 1.9 mg/mL Potassium Chloride, 1.2 mg/mL Sodium Chloride, 16 mg/mL agar) and incubated at 23°C for 2–3 days. Spores were then washed with water and digested with 1 mg/mL zymolyase for 5 minutes 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 six hours. The cultures were then plated on synthetic media with low (6μg/mL) adenine to enhance the color change[30] and grown for three days at 23°C. The percent of red or sectored versus completely white colonies was then counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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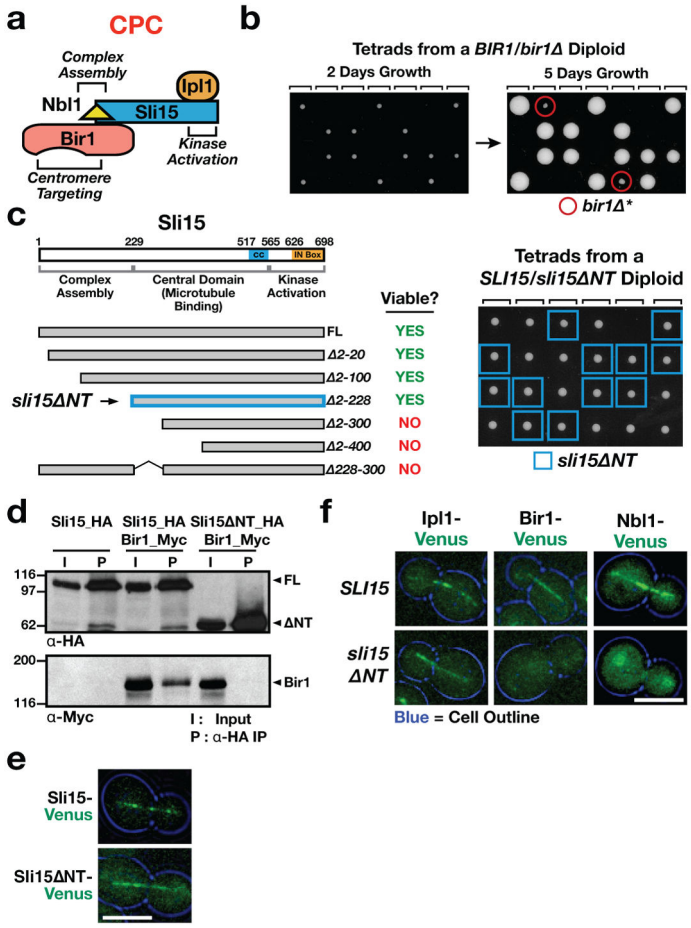

Figure 1. Deletion of the Sli15 N-terminus prevents association with Bir1 but does not affect cell viability or growth

(a) Schematic of the CPC in budding yeast. Sli15=INCENP; Bir1=Survivin; Nbl1=Borealin/Dasra and Ipl1=Aurora B kinase.

(b) Phenotype of *bir1*Δ. Tetrad dissections from a *bir1*Δ heterozygote—the 4 spores from individual tetrads are arrayed in columns. Rare survivors (referred to as *bir1*Δ*) are observed after extended growth (*right panel*).

(c) Phenotype of Sli15 truncations. Tetrad dissections from a *sli15*Δ*NT* heterozygote are shown on the right.

(d) Co-immunoprecipitation analysis of full length (FL) Sli15 or Sli15ΔNT and Bir1. 9Myc and 6HA tags were inserted at endogenous loci to generate functional C-terminally tagged proteins. Bir1 coimmunoprecipitates with FL Sli15 but not with Sli15ΔNT.

(e) Localization of Sli15-Venus and Sli15ΔN-Venus to the anaphase spindle. Scale bar is 5 μm.

(f) Localization of CPC components during anaphase in cells with either wildtype Sli15 or Sli15ΔNT. Scale bar is 5 μm.

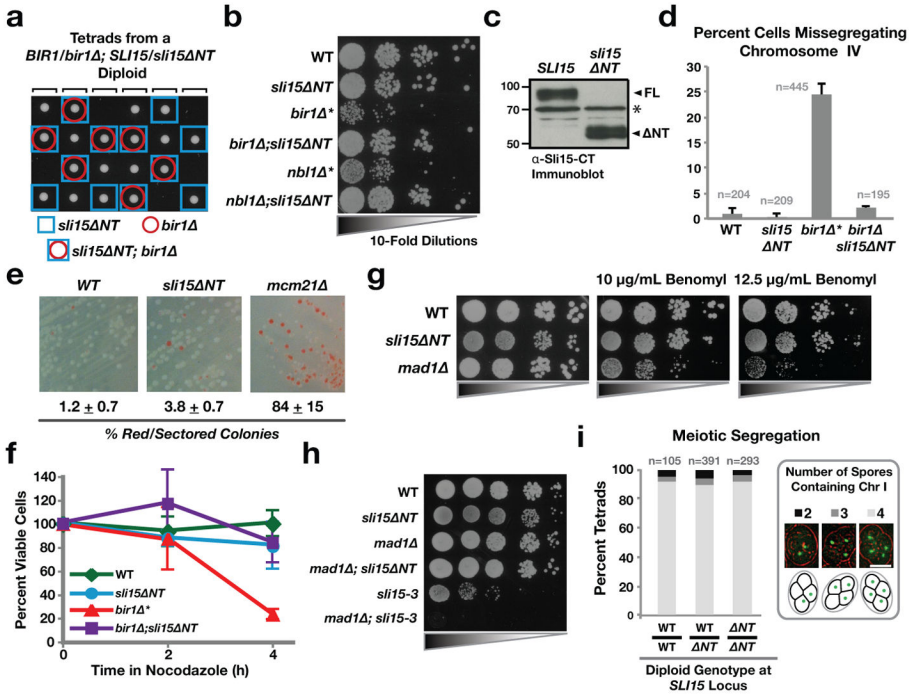

Figure 2. *sli15ΔNT* suppresses lethality of *bir1Δ* and *nbl1Δ*, and exhibits normal fidelity mitotic and meiotic chromosome segregation

(a) Tetrad dissection showing viability of *sli15ΔNT;bir1Δ* double mutant spores.

(b) 10-fold serial dilutions of cells of indicated genotypes. *bir1Δ** and *nbl1Δ** represent rare survivors recovered as shown in Fig. 1B.

(c) Immunoblot of extracts prepared from strains expressing either wild type Sli15 or Sli15ΔNT and blotted using an antibody raised against the C-terminus of Sli15. The asterisk (*) indicates a non-specific band recognized by the primary antibody that serves as a loading control.

(d) Analysis of segregation fidelity of GFP-tagged chromosome IV in cells of the indicated genotypes. The average of 3 to 5 experiments is shown; error bars represent standard error.

(e) Minichromosome loss assay. The percentage and standard error of colonies that were either red or sectored is shown.

(f) Viability following transient nocodazole treatment of cells of the indicated genotypes. The average of 2 to 4 experiments is shown; error bars represent standard error.

(g) Serial dilutions of cells with the indicated genotypes spotted on plates with different concentrations of the microtubule-depolymerizing drug benomyl.

(h) Lack of synthetic lethality/sickness following checkpoint inhibition in *sli15ΔNT* cells. Plates were incubated at 37°C. *sli15-3* is a temperature-sensitive mutant that compromises Ipl1 activation[17].

(i) Meiotic segregation following sporulation of diploid cells. The presence or absence of Chromosome I was scored for each individual spore in a tetrad. Scale bar is 5 μm.

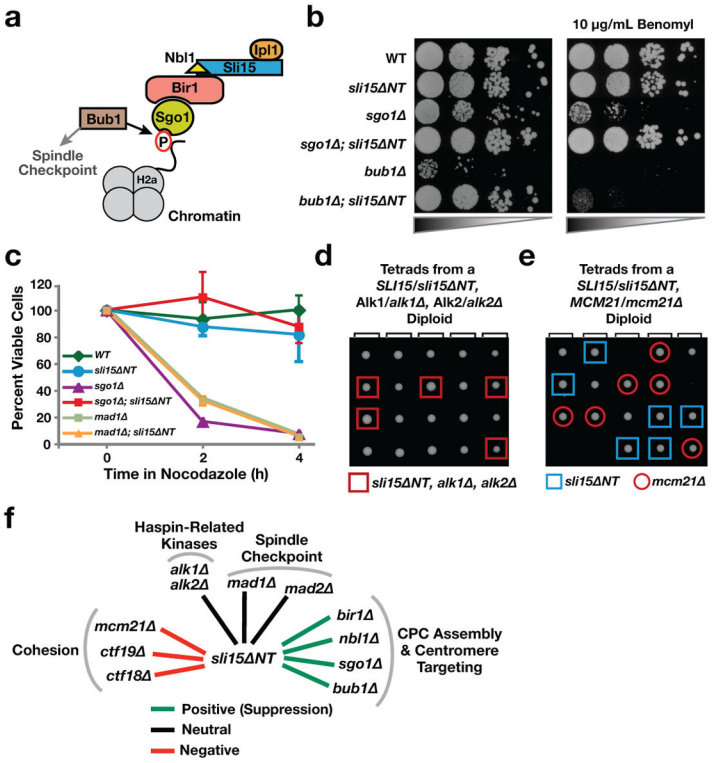

Figure 3. *sli15ΔNT* suppresses mutants in the Bir1-dependent CPC targeting pathway but is synthetically lethal with genes implicated in centromere cohesion

(a) Schematic of the CPC targeting mechanism involving Bub1 kinase and Sgo1.

(b) Suppression of *sgo1*Δ and *bub1*Δ growth phenotypes by *sli15ΔNT*. Compromised growth of *sgo1*Δ on benomyl is also suppressed by *sli15ΔNT*.

(c) Viability following transient nocodazole treatment of cells of the indicated genotypes. The average of 2 to 4 experiments is shown; error bars represent standard error. WT and *sli15ΔNT* measurements are the same as in Fig. 2f.

(d) Tetrad analysis showing no synthetic defect for cells that are triple mutants for *sli15ΔNT alk1*Δ and *alk1*Δ.

(e) Tetrad analysis showing synthetic lethality of *sli15ΔNT* and *mcm21*Δ. Similar results were observed for *ctf19*Δ (Fig. S2b).

(f) Summary of genetic interactions exhibited by *sli15ΔNT*. A positive genetic interaction (*green*) indicates suppression; negative genetic interaction (*red*) indicates synthetic lethality/sickness and neutral interaction (*black*) indicates lack of a synthetic phenotype.

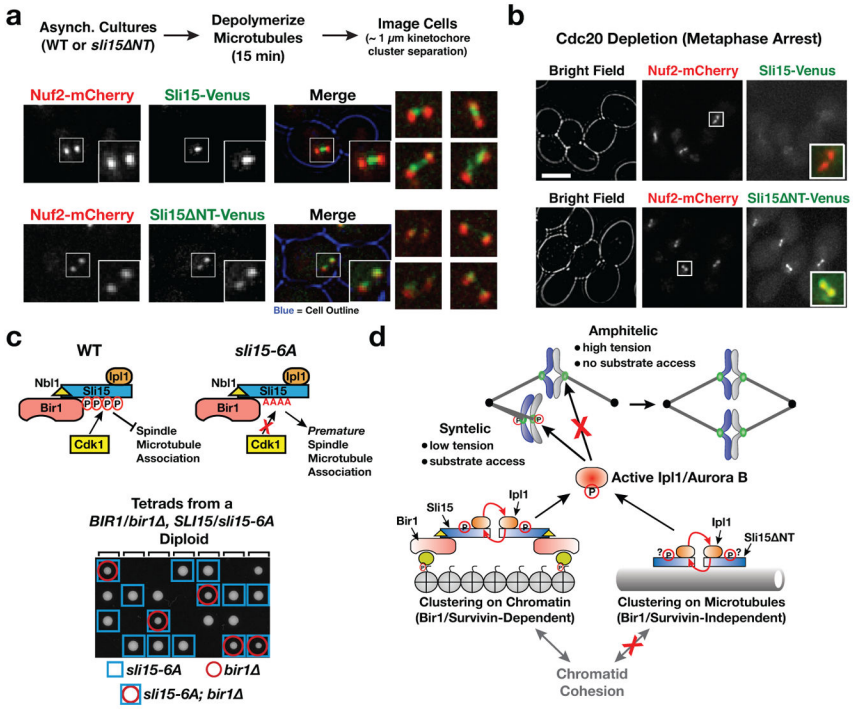

Figure 4. Localization of Sli15ΔNT and relationship between Sli15 microtubule localization and suppression of *bir1Δ*

(a) Images of cells expressing Nuf2-mCherry and either Sli15-Venus or Sli15ΔNT-Venus after brief microtubule depolymerization. A cell with ~1 μm separation of Nuf2-mCherry clusters is shown for each. Boxes are 2.1 μm square. 4 additional examples are shown on the right. See Fig. S3A for similar analysis of Ipl1 localization.

(b) Images of cells arrested in metaphase by Cdc20 depletion expressing Nuf2-mCherry and either Sli15-Venus or Sli15ΔNT-Venus. Scale bar is 5 μm; merged insets are magnified 2.5-fold. See Fig. S3B for similar analysis of of Ipl1 localization.

(c) Schematic summarizing prior work on Cdk1 regulation of Sli15 spindle localization[22] and tetrad analysis showing growth of *sli15-6A;bir1*Δ double mutant cells.

(d) Model for mechanism of chromosome biorientation. Bir1-dependent chromatin clustering of the CPC or Bir1-independent clustering on microtubules of Sli15ΔNT-Ipl1 generates active Ipl1 kinase, which is capable of discriminating between correct and incorrect attachments.