Cell

A Bir1-Sli15 Complex Connects Centromeres to Microtubules and Is Required to Sense Kinetochore Tension

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DOI 10.1016/j.cell.2006.09.049

SUMMARY

Proper connections between centromeres and spindle microtubules are of critical importance in ensuring accurate segregation of the genome during cell division. Using an in vitro approach based on the sequence-specific budding yeast centromere, we identified a complex of the chromosomal passenger proteins Bir1 and Sli15 (Survivin and INCENP) that links centromeres to microtubules. This linkage does not require Ipl1/Aurora B kinase, whose targeting and activation are controlled by Bir1 and Sli15. Ipl1 is the tension-dependent regulator of centromere-microtubule interactions that ensures chromosome biorientation on the spindle. Elimination of the linkage between centromeres and microtubules mediated by Bir1-Sli15 phenocopies mutations that selectively cripple Ipl1 kinase activation. These findings lead us to propose that the Bir1-Sli15-mediated linkage, which bridges centromeres and microtubules and includes the Aurora kinase-activating domain of INCENP family proteins, is the tension sensor that relays the mechanical state of centromere-microtubule attachments into local control of Ipl1 kinase activity.

INTRODUCTION

During mitosis, kinetochores assemble on the centromeric regions of each sister chromatid to act as the primary chromosomal attachment sites for spindle microtubules (Cleveland et al., 2003; Kline-Smith et al., 2005; Maiato et al., 2004). Kinetochores exhibit both end-on and lateral interactions with microtubules. End-on connections between the outer kinetochore and microtubule plus ends couple chromosome motility to changes in the polymerization and depolymerization of bound microtubules (Inoue and Salmon, 1995). Lateral interactions between kinetochores and spindle microtubules are associated with poleward as well as equatorial chromosome movements that facilitate the establishment of stable end-on connections (Kapoor et al., 2006; Rieder and Alexander, 1990; Tanaka et al., 2005).

In addition to their mechanical role in segregation, kinetochores serve as signaling hubs that inhibit anaphase onset until every chromosome in the cell is properly connected. The kinetochore-based mitotic checkpoint pathway relays the presence of any unattached kinetochores into inhibition of the ubiquitin protein ligase that triggers sister chromatid separation and mitotic exit (Cleveland et al., 2003; Nasmyth, 2005; Pinsky and Biggins, 2005). Classic micromanipulation studies in insect spermatocytes as well as recent work using chromosome engineering in budding yeast have highlighted the importance of tension in selectively stabilizing correctly bioriented chromatid pairs (Dewar et al., 2004; Li and Nicklas, 1995; Nicklas and Koch, 1969; Stern and Murray, 2001). Biorientation places kinetochore-microtubule connections under tension, whereas incorrect syntelic attachments, where the kinetochores on both sisters are connected to the same spindle pole, do not. The conserved Aurora B kinase is required to eliminate syntelic attachments, facilitating new connection attempts until the correct configuration is achieved (Biggins and Murray, 2001; Dewar et al., 2004; Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002; Lampson et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). The action of Aurora B generates unoccupied kinetochores that in turn signal via the mitotic checkpoint pathway to prevent anaphase onset (Pinsky et al., 2006). Thus, the choreography of chromosome segregation is comprised of an intimate feedback between the mechanics of kinetochore-microtubule connections and localized signaling pathways.

Efforts to reconstitute the mechanical and regulatory functions of kinetochores in vitro have been limited by the complexity of the underlying centromeric DNA (Cleveland et al., 2003). An exception to this complexity is budding yeast, where centromeres consist of a well-defined ~125 base-pair region (Clarke, 1998; McAinsh et al., 2003). The biochemical identification of CBF3, the protein complex that directly binds the key *cis*-acting CDEIII domain (Lechner and Carbon, 1991), provided further impetus for analyzing kinetochore-microtubule interactions in vitro. Previous studies have demonstrated that budding yeast centromeric (CEN) DNA will bind to microtubules following incubation in a cell extract (Hyman et al., 1992; Kingsbury and Koshland, 1991; Severin et al., 1997; Sorger et al., 1994). This interaction requires the CBF3 complex and is subject to regulation by Ipl1, the budding yeast Aurora B kinase, and the counteracting phosphatase PP1/Glc7 (Biggins et al., 1999; Sassoon et al., 1999). However, CBF3 is not sufficient, indicating that other factor or factors are necessary to link CBF3-CEN DNA to microtubules (Sorger et al., 1994). Here, we extend this in vitro approach to biochemically identify the missing factor(s). Our results reveal that a complex of two chromosomal passenger proteins, Bir1/Survivin and Sli15/INCENP, connects CBF3-CEN DNA to microtubules in vitro. This connection is independent of Ipl1, whose activation and targeting are controlled by Bir1 and Sli15. In vivo analysis of Sli15 mutants that eliminate the in vitro activity leads us to propose that the Bir1-Sli15-mediated linkage between CBF3-CEN DNA and microtubules acts as a tension sensor that activates Ipl1 in the vicinity of incorrect syntelic attachments.

RESULTS

A Quantitative In Vitro Assay for the Interaction of CBF3-Bound CEN DNA with Microtubules

In the presence of a yeast cell extract, fluorescent beads coupled to CEN DNA will bind to immobilized microtubules adsorbed to a coverslip surface. Although required, CBF3 is not sufficient for binding, indicating the presence of additional factor or factors in the extract that connect CBF3-bound CEN DNA to microtubules (Sorger et al., 1994). To identify these factor(s), we adapted the bead assay to enable rapid, reproducible testing of column fractions (Figure 1A). As expected, beads coated with CEN DNA exhibited robust microtubule binding in the presence of wild-type extract, whereas beads coated with a mutant CEN DNA that renders it nonfunctional in vivo failed to bind (Figure 1B).

To generate a source of CBF3 for in vitro complementation assays, we optimized a partial purification using CEN DNA band shift to monitor CBF3 activity (Figure 1C). This procedure yielded ~50-fold partially purified (PP)CBF3 that exhibited a robust band shift (Figure 1C) but did not support binding of CEN DNA beads to microtubules on its own (Figure 1D). Extracts prepared from strains harboring mutations in CBF3 lacked CEN DNA band shift but complemented (PP)CBF3 in the bead-microtubule-binding assay (data not shown, Sorger et al., 1994). Trypsin treatment indicated that the complementing activity is protease sensitive (see Figure S1 in the Supplemental Data available with this article online). Taken together, these results confirmed the existence of an unknown factor that connects CBF3-bound CEN DNA to microtubules in vitro and established a robust assay that could be used for its identification.

A Conventional Purification Strategy Identifies Bir1 as a Candidate for the Activity that Links CBF3-Bound CEN DNA to Microtubules

To identify the protein or proteins that connect CBF3-bound CEN DNA to microtubules, we utilized two strategies. First, we tested CBF3-complementing activity in extracts either prepared from mutant strains or immunodepleted of candidate kinetochore-localized or microtubule-binding proteins. At the budding yeast kinetochore, the Dam1 ring complex plays an important role in bioriented microtubule attachments and the Mis12, Ctf19, and Ndc80 complexes are suggested to direct assembly of the microtubule-binding interface (reviewed in De Wulf et al. [2003], McAinsh et al. [2003], and Tanaka et al. [2005]). However, neither mutations in nor immunodepletions of these complexes perturbed the in vitro linkage between CEN DNA and microtubules (Table S1). Other candidates, including motor and nonmotor microtubule-binding proteins, were similarly excluded. These results indicated the presence of a CBF3-dependent linkage between CEN DNA and microtubules that did not involve any of the obvious candidates suggested by prior studies.

Parallel to the candidate analysis, we pursued an unbiased conventional purification, using the quantitative in vitro assay (Figure 2A). Complementing activity was measured relative to a standard curve generated by serially diluting the starting material for the purification step into a constant amount of (PP)CBF3 (Figures 2B and 2C). Negative controls in which mutant CEN DNA beads were used, or (PP)CBF3 was omitted, verified the specificity of binding. By combining three purification steps in series, we enriched the complementing activity ~400-fold (Figure 2D). However, attempts at additional purification resulted in a significant loss of specific activity that did not appear to be due to separation of different components.

Since the purification did not achieve sufficient enrichment to directly correlate complementing activity with copurifying proteins, we identified candidates that could be functionally tested by performing mass spectrometry (Washburn et al., 2001) on the highest specific activity material—the Mono S cation-exchange pool (Figures 2E and 2F). This approach identified 247 polypeptides with greater than 10% sequence coverage in the Mono S pool (Table S2). Database functional annotations identified a single protein within this large set previously implicated in centromere function (Figure 2G; Table S2). This protein was Bir1, the budding yeast homolog of the Survivin subunit of the chromosomal passenger complex. This

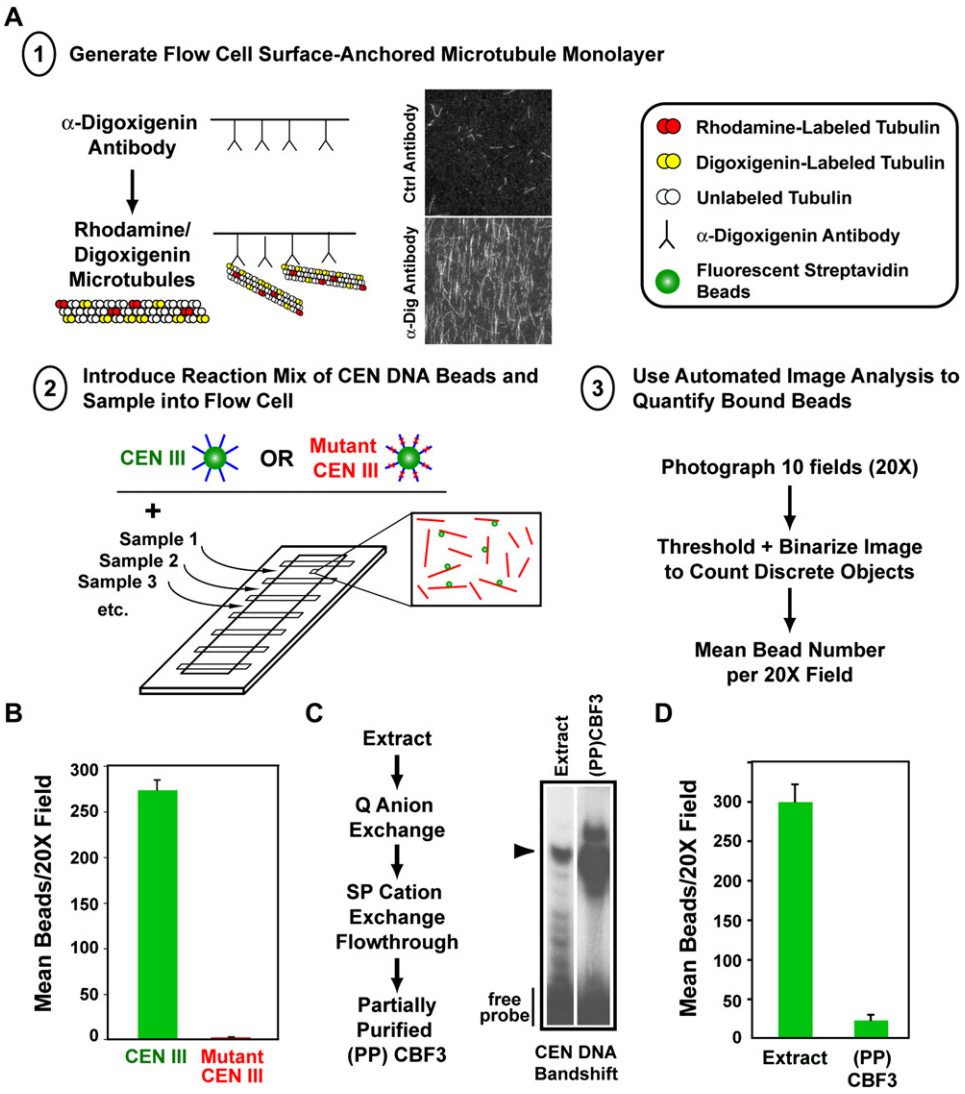

Figure 1. A Quantitative In Vitro Assay for the Binding of Budding Yeast CEN DNA to Microtubules

(A) Schematic of the in vitro assay. Adaptations to the original scheme important for quantitative biochemical analysis are emphasized here and include the following: (1) stable adsorption of microtubules using tubulin covalently modified with digoxigenin, (2) multiplexing of flow cells on a single slide, and (3) automated image analysis to measure number of bound beads. For quantitation, ten fields at 20× magnification are photographed per sample and averaged.

(B) Linkage of beads to microtubules is observed with wild-type but not mutant CEN DNA. The mutant harbors a deletion of the central CCG in CDEIII that prevents binding of the CBF3 complex and abolishes centromere activity in vivo. Error bars show SD.

(C) Partial purification of CBF3 using a CEN DNA gel shift assay. The flow chart describes the chromatography steps, and the gel panel shows enrichment of the CEN DNA band shift relative to starting extract in the partially purified (PP) fraction. The arrowhead marks the CBF3-CEN DNA complex.

(D) Partially purified CBF3 does not link CEN DNA beads to microtubules. Note that the volume of starting extract used to prepare the CBF3 added to the (PP)CBF3 reaction is ~25-fold greater than that assayed in the extract reaction. If equivalent extract volumes are assayed, no binding is observed with (PP)CBF3. Error bars show SD.

finding led us to focus on Bir1 as a candidate for the CBF3-complementing activity.

Bir1 Is an Essential Component of the CBF3-Complementing Activity

To test whether Bir1 is required to link CEN DNA to microtubules in vitro, we generated a strain in which *bir1* was deleted. Sporulation of heterozygous diploids yielded two wild-type and two apparently inviable spores (n = 105 tetrads), as expected for an essential gene. However, consistent with previous conflicting studies describing *bir1* as both essential (Li et al., 2000; Widlund et al., 2006) and nonessential (Uren et al., 1999; Yoon and Carbon, 1999), ~10% of *bir1Δ* spores formed tiny colonies

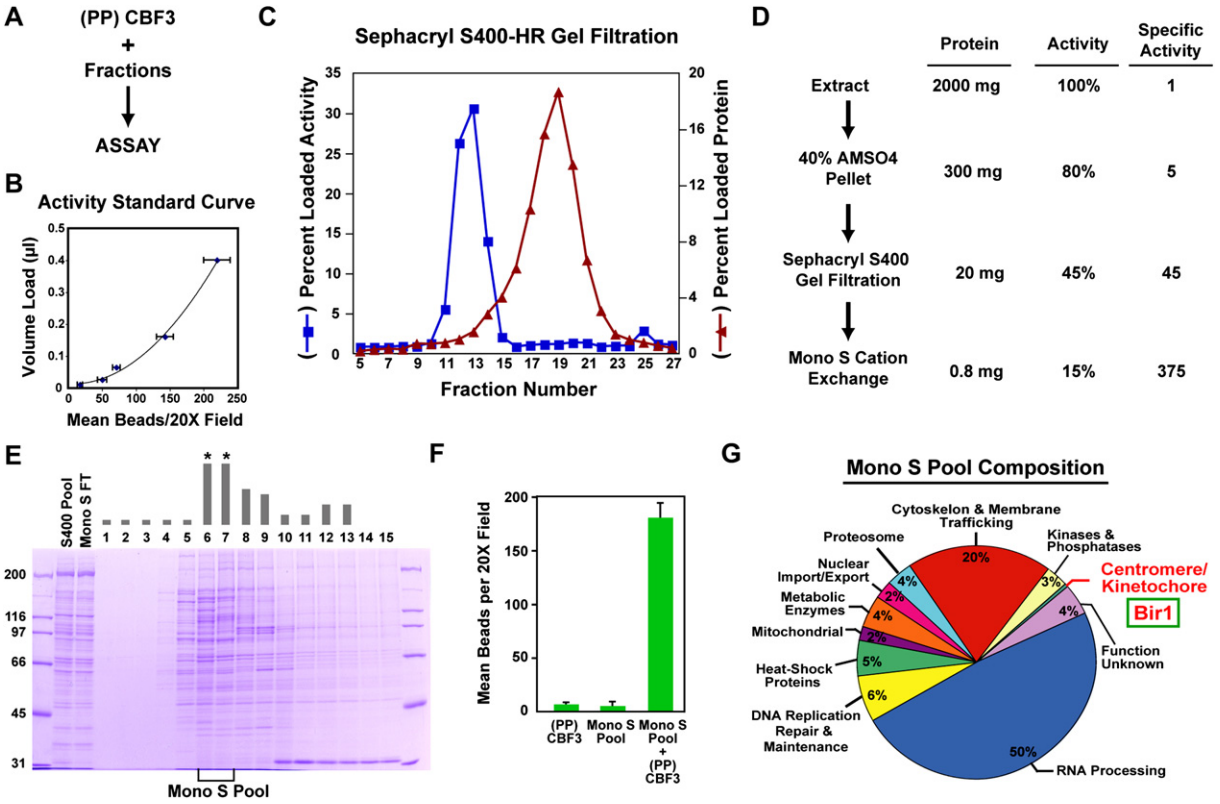

Figure 2. Conventional Purification of an Activity that Complements CBF3 in the In Vitro Assay

(A) Schematic of the in vitro complementation approach.

(B) Example of a standard curve used to quantitatively monitor fractionation of the complementing activity. The starting material, in this case the gel filtration load, is serially diluted into a constant amount of (PP)CBF3, and the points are fitted to a polynomial curve. Complementing activity in each fraction measured after adding the same amount of (PP)CBF3 is converted to a percentage of total loaded activity. Error bars show SD.

(C) Column profile of Sephacryl S400HR gel filtration. The percentage of loaded activity calculated from the standard curve and the percentage of total loaded protein are plotted for each fraction.

(D) Summary of the complementing-activity purification. The activity column lists the percentage yield, relative to the starting extract, after each step.

(E) Fractions from the Mono S gradient elution stained with Coomassie blue. The complementing activity is indicated with the gray bars above each fraction. No activity is detected in the column flowthrough. Asterisks denote the two fractions that constitute the Mono S Pool.

(F) The Mono S Pool complements (PP)CBF3. Error bars show SD.

(G) Annotation-based classification of proteins identified by mass spectrometry of the Mono S pool. The 247 proteins that showed >10% sequence coverage are represented in the pie chart (see also Table S2).

after extended incubation. The *bir1Δ* cells derived from these colonies grew but had aberrant, polyploid DNA content (data not shown). Similar aberrant cells are obtained if the deletion is covered with a centromeric plasmid expressing Bir1 and the plasmid is removed from the deleted haploid using negative selection after tetrad dissection, indicating that neither passage through meiosis nor haploinsufficiency in the diploid state is required for the observed phenotype. Immunoblotting using an affinity-purified anti-Bir1 antibody confirmed that a series of bands present in wild-type extracts, likely reflecting differentially phosphorylated forms of Bir1, is absent in *bir1Δ* extracts (Figure 3A).

Extracts prepared from *bir1Δ* cells exhibit no activity in the in vitro CEN DNA-microtubule interaction assay (Figure 3B), whereas the ability of the CBF3 to bind CEN DNA was unaffected (Figure 3C). Addition of (PP)CBF3 to *birΔ* extracts failed to restore CEN DNA-microtubule-binding activity (data not shown). Transformation with a plasmid encoding Bir1 under control of its endogenous promoter fully restored activity in the assay (Figure 3B) as well as the bands detected in the Bir1 molecular weight range in immunoblots (Figure 3A). We conclude that Bir1, a candidate identified by mass spectrometry of a fraction enriched for the complementing activity by conventional purification, is an essential component of the link between CBF3-bound CEN DNA and microtubules in vitro.

Sli15 and Bir1, but Not Ipl1, Are Required for the CBF3-Complementing Activity

Bir1, like its homolog Survivin in higher eukaryotes, is a subunit of the chromosomal passenger complex. In budding yeast, this complex includes Ipl1, the single Aurora

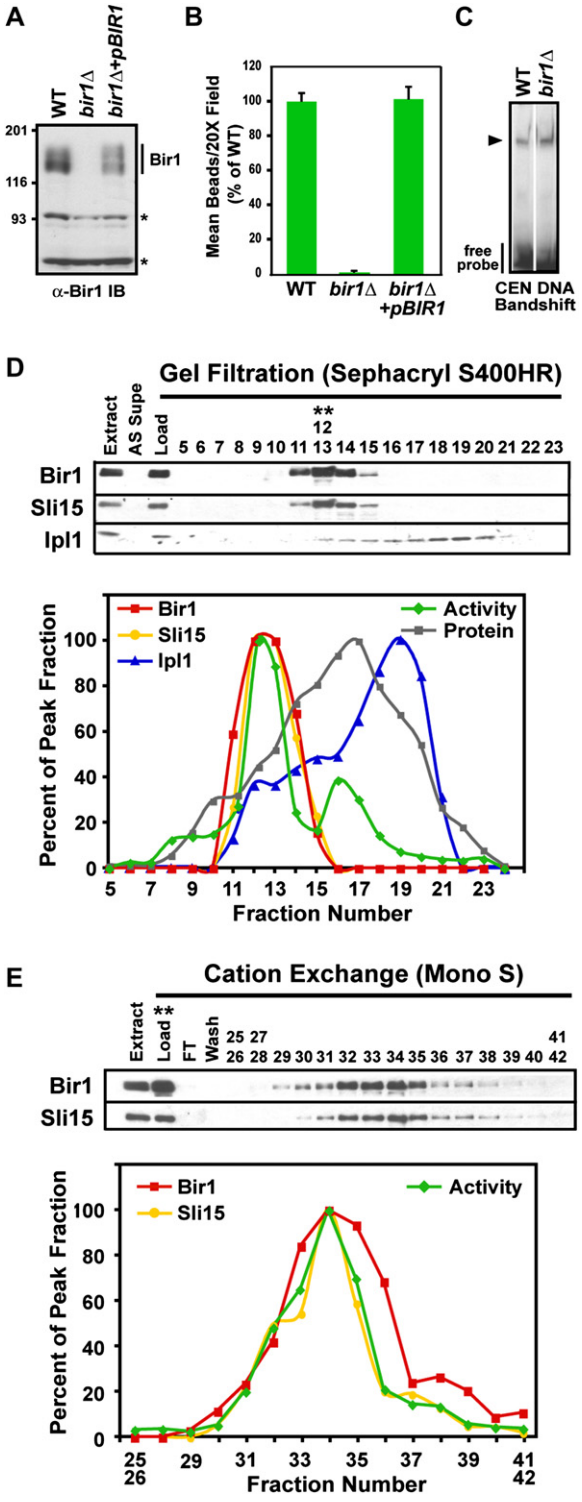

Figure 3. Bir1 Is Required for Linking CEN DNA to Microtubules In Vitro

(A) *bir1Δ* cells lack Bir1 protein. Western blot of extracts prepared from wild-type (ODY49), *bir1Δ* (ODY65), and *bir1Δ+pCEN-BIR1* (ODY114) strains probed with an anti-Bir1 antibody is shown. Asterisks indicate background bands that serve as loading controls.

family kinase in budding yeast, and Sli15, the homolog of the Aurora B activator and targeting subunit INCENP (Adams et al., 2000; Cheeseman et al., 2002; Kim et al., 1999). Since the complexity of the Mono S fraction could have prevented identification of the other two subunits by mass spectrometry, we repeated the purification and monitored the fate of each subunit. Bir1 and Sli15 showed a fractionation profile that was similar to the activity throughout the purification (Figures 3D and 3E). In contrast, the majority of Ipl1 fractionated away from Bir1-Sli15 into a distinct pool of smaller hydrodynamic radius during the gel filtration step (Figure 3D).

The fractionation analysis suggested that a complex containing Bir1 and Sli15 is responsible for the in vitro complementing activity, and that Ipl1 might be dispensable. Since Sli15 and Ipl1 are essential genes, and the available conditional alleles do not affect protein levels (see below), we tested this by immunodepleting >90% of Sli15 or Ipl1, each tagged with six copies of the hemagglutinin (HA) epitope (Figure 4A). Consistent with their fate during purification, depletion of Sli15 dramatically reduced activity whereas depletion of Ipl1 had no effect (Figure 4A). The assays were performed without ATP addition, and normal binding was observed at all tested dilutions, arguing against the catalytic activity of residual Ipl1 kinase accounting for the difference between extracts depleted of Sli15 and Ipl1.

Although Ipl1 does not appear to be required to connect CEN DNA to microtubules in vitro, its kinase activity regulates this linkage (Biggins et al., 1999). Addition of ATP to wild-type extracts severely inhibits binding of CEN DNA beads to microtubules (Figure 4B; Biggins et al., 1999). Sensitivity to ATP addition is lost in extracts prepared from *ipl1* mutants with compromised kinase activity but can be restored following addition of purified Ipl1 protein (Figure 4B). This result predicts that if Ipl1 is absent from fractions enriched for the complementing activity, they should be insensitive to ATP. We tested this prediction using the Mono S pool (Figures 2E and 2F). The binding observed in mixtures of the Mono S pool and (PP)CBF3 is insensitive to ATP but becomes sensitive following addition of purified Ipl1 protein (Figure 4B). These results suggest

(B) Bir1 is required for linking CEN DNA to microtubules. Extracts indicated in (A) were analyzed using the bead assay. Activity was normalized relative to the wild-type extract. Error bars show SD.

(C) Bir1 does not affect the ability of CBF3 to bind to CEN DNA. Arrowhead indicates position of the CBF3-CEN DNA complex.

(D) Bir1 and Sli15, but not Ipl1, cofractionate with the complementing activity. Gel filtration fractions of extracts prepared from *BIR1:6HA*; *SLI15:13Myc* (ODY97) were analyzed by western blotting using anti-HA, anti-Myc, and anti-Ipl1 antibodies. The blot signal intensity for all three proteins, as well as activity in the bead assay, is plotted as a percentage of the respective peak fractions (12/13 for Sli15, Bir1, and activity; 18 for Ipl1).

(E) Bir1 and Sli15 continue to cofractionate with the complementing activity during the cation-exchange step. The activity peak from gel filtration (fractions 12/13 in [D]) was further fractionated using a Mono S cation-exchange column and analyzed as in (D).

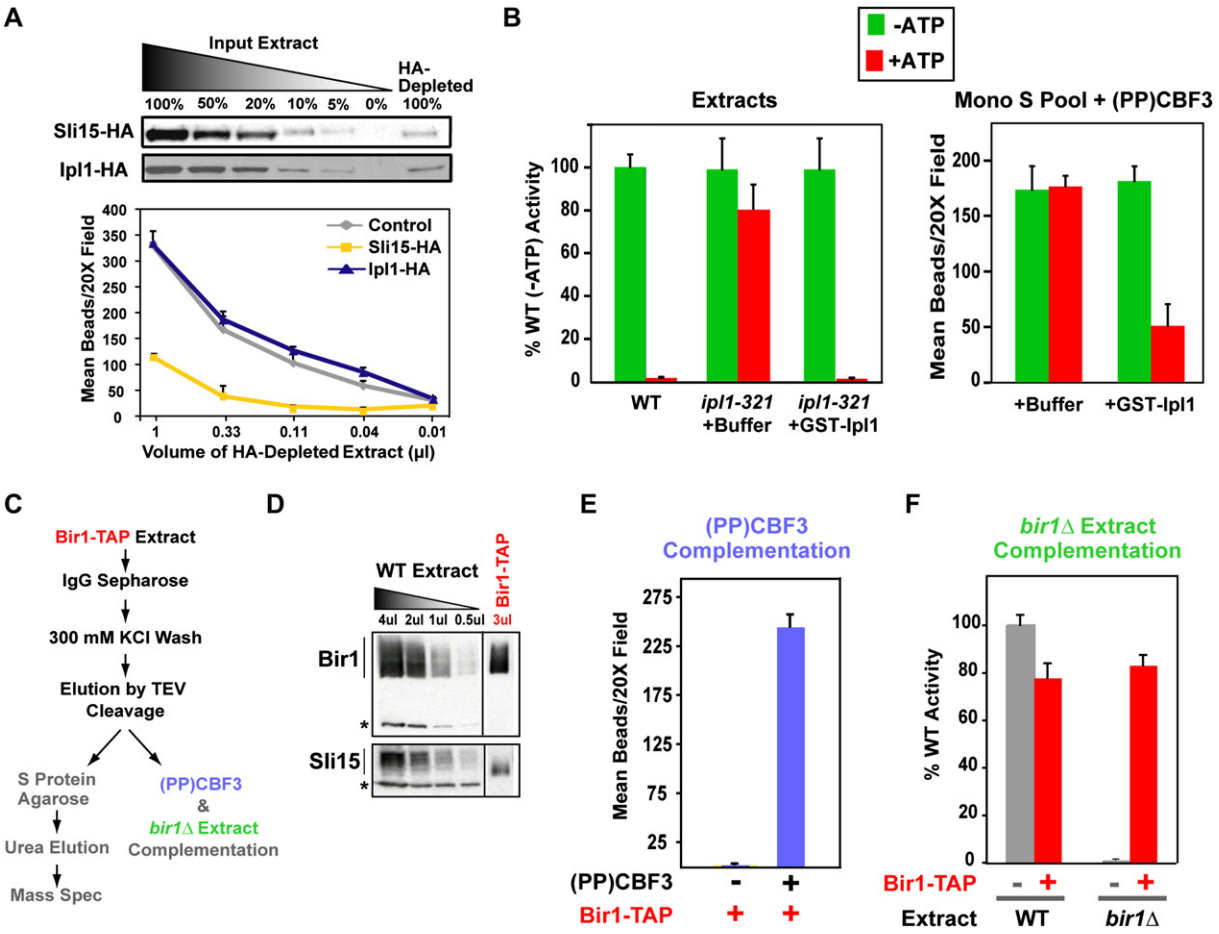

Figure 4. Sli15, but Not Ipl1, Is Required for the Complementing Activity

(A) Extracts prepared from *SLI15:6HA* (ODY54) and *IPL1:6HA* (ODY55) cells were immunodepleted using an anti-HA affinity resin. The depleted supernatants were serially diluted, and complementing activity was measured with constant (PP)CBF3. Comparing a standard curve of the input extract to the depleted extract using anti-HA immunoblotting assessed depletion efficiency. Both Sli15 and Ipl1 were successfully depleted by >90%, but only the Sli15 depletion resulted in a severe activity reduction. Error bars show SD.

(B) Ipl1 kinase activity regulates the binding observed in the in vitro assay. *ipl1-321* mutant extracts are insensitive to ATP addition, which is in contrast to wild-type extracts. Addition of purified GST-Ipl1 protein restores ATP sensitivity to the mutant extracts. The Mono S pool combined with (PP)CBF3 is ATP insensitive in the bead assay but becomes sensitive following addition of GST-Ipl1. Error bars show SD.

(C) Flow chart describing the purification and use of the Bir1-TAP complex.

(D) Immunoblot of Bir1-TAP after elution by TEV cleavage. Both Sli15 and Bir1 are present in the elution.

(E) Bir1-TAP complements (PP)CBF3. Error bars show SD.

(F) Bir1-TAP complements loss of activity in *bir1Δ* extracts. In both (E) and (F), the amount of Bir1-TAP added is similar to that present in 2 µl of extract, which is the standard amount analyzed in the bead assay (D). Error bars show SD.

that the CBF3-complementing activity is comprised of a complex containing Bir1-Sli15 that is independent of Ipl1 but subject to regulation by its kinase activity.

A Purified Endogenous Bir1-Sli15 Complex Complements CBF3 and *bir1Δ* Extracts

To determine whether a purified Bir1-Sli15 complex complements (PP)CBF3, we generated extracts from a strain expressing tandem affinity purification (TAP)-tagged Bir1. Following incubation with IgG resin, the Bir1-TAP complex was eluted by cleavage with TEV protease. A fraction of the elution was subjected to the second affinity purification step and analyzed by mass spectrometry; the rest of the elution was used for in vitro experiments (Figure 4C). Both immunoblotting and mass spectrometry indicated that the TAP prep of Bir1 contains Sli15 (Figure 4D), but not Ipl1, as expected from a previous study (Widlund et al., 2006). Neither protein was detectable in the (PP)CBF3 fraction (Table S3). The purified Bir1-Sli15 complex had no activity on its own but exhibited robust activity when mixed with (PP)CBF3 (Figure 4E). Mass spectrometry of (PP)CBF3 indicated that no other known

kinetochore proteins were present in this fraction (Table S3). Addition of Bir1-Sli15 also complemented the activity loss in *bir1Δ* extracts (Figure 4F). These findings strongly suggest that a complex containing Bir1 and Sli15 directly connects CBF3-bound CEN DNA to microtubules in vitro. This conclusion is supported by previous work documenting an interaction between Bir1 and the Ndc10 subunit of CBF3 (Bouck and Bloom, 2005; Gillis et al., 2005; Yoon and Carbon, 1999) and direct binding of Sli15 to microtubules (Kang et al., 2001).

Bir1-Sli15 and Ipl1 are chromosomal passenger proteins that localize prominently to the anaphase spindle. Dephosphorylation of Sli15 by the phosphatase Cdc14 is required for this localization (Pereira and Schiebel, 2003). Recent work has shown that the CBF3 complex also localizes to the anaphase spindle (Bouck and Bloom, 2005; Gillis et al., 2005). These observations raised the possibility that the in vitro CEN DNA-microtubule linkage assay is detecting microtubule binding activity of the pool of Bir1-Sli15 present in anaphase. To test this, we analyzed *cdc14-1* and *cdc15-2*, two mutants in which Sli15 anaphase spindle localization is not observed (Pereira and Schiebel, 2003; Stoepel et al., 2005). Extracts prepared from these mutants exhibited wild-type CEN DNA-microtubule linkage (Figure S2A). Linkage activity is also low in G1, when Sli15 is dephosphorylated, but high in mitosis, when Sli15 is hyperphosphorylated (Figure S2B). Together with the in vivo analysis described below, these results support the conclusion that the CEN DNA-microtubule linkage mediated by Bir1-Sli15 is of critical importance during chromosome segregation.

Shutting Off Sli15 or Deleting Its Microtubule-Binding Domain Eliminates the Linkage between CEN DNA and Microtubules In Vitro

To investigate the physiological role of the Bir1-Sli15-mediated linkage between CBF3-CEN DNA and microtubules, we focused our efforts on Sli15 because of the abnormal polyploidy observed in *bir1* mutants. Extracts prepared from strains harboring the only previously characterized mutant allele of *SLI15*, *sli15-3* (Kim et al., 1999), exhibited normal CEN DNA-microtubule binding but were insensitive to the addition of ATP (Figure 5A). *sli15-3* extracts are therefore similar to extracts prepared from *ipl1* mutants but distinct from extracts immunodepleted of Sli15 (see Figure 4A). This difference was explained by sequencing *sli15-3*, which revealed that the critical mutation is within the IN box domain that binds to and activates Ipl1 kinase (Figures S3A and S3B; Sessa et al., 2005). Thus, the mutant protein encoded by the *sli15-3* allele, while defective in Ipl1 activation, is present at normal levels (Figure S3C) and capable of linking CEN DNA to microtubules in vitro.

Since *sli15-3* encodes a protein that retains the ability to link CEN DNA to microtubules, a new mutant was needed to investigate the in vivo role of the Bir1-Sli15-mediated linkage. To obtain such a mutant, we generated a strain where the galactose promoter is integrated upstream of the *SLI15* coding region (*pGAL-SLI15*). Sli15 is overexpressed when this mutant is grown in galactose ($SLI15^{ON}$; Figure 5C and Figure S4A), but this does not result in a significant phenotype. Switching this strain to glucose medium ($SLI15^{OFF}$) causes a dramatic loss in Sli15 protein levels (Figure 5C and Figure S4A). Importantly, in agreement with results obtained from immunodepleting Sli15, no CEN DNA-microtubule linkage activity was detected in $SLI15^{OFF}$ extracts (Figure 5D).

The Sli15 shutoff mutant offered the opportunity to directly test the role of the Sli15 microtubule-binding domain in the in vitro linkage. Sli15 is a multidomain protein with a centrally located microtubule-binding domain (Kang et al., 2001). The C terminus of Sli15, like all INCENP family proteins, contains the Aurora-activating IN box which binds to and activates Aurora B (Kang et al., 2001; Sessa et al., 2005). To test the function of the microtubule-binding domain, we integrated a mutant (Sli15-MTBΔ; Figure 5B) into the URA3 locus of the strain harboring the *pGAL-SLI15* shutoff allele. Wild-type Sli15 coding sequence was integrated in parallel as a control. Both the mutant and wild-type coding regions were under control of the endogenous *SLI15* promoter.

Immunoblots confirmed expression of Sli15-MTBΔ before and after shutting off *pGAL-SLI15* (Figure 5C) and the mutant protein retained the ability to interact with Bir1 (Figure S5B), indicating that it is not misfolded. When expression of wild-type Sli15 was turned off, cells expressing the Sli15-MTBΔ mutant protein failed to survive, in contrast to controls expressing the wild-type protein, indicating that the microtubule-binding domain of Sli15 is essential for viability (Figure S5A). Analysis of nocodazole-arrested extracts prepared after shutting off *pGAL-SLI15* showed that the Sli15-MTBΔ mutant failed to support in vitro CEN DNA-microtubule linkage, in contrast to full-length Sli15, which supported normal binding (Figure 5D). Thus, the linkage detected in vitro requires the microtubule-binding domain of Sli15.

Both the Sli15 Shutoff and the Sli15 Microtubule-Binding Domain Deletion Mutant Phenocopy Ipl1 Kinase Activation Mutants In Vivo

The Sli15 shutoff and the Sli15-MTBΔ mutant both eliminate the linkage between CEN DNA and microtubules in vitro. Analyzing the phenotype of these mutants would therefore make it feasible to distinguish between two potential models for the physiological role of this linkage. First, as anticipated during the initial development of the bead assay, the Bir1-Sli15 linkage could play an Ipl1-independent role in the force-transducing connection of centromeres to spindle microtubules, which we will refer to as the "core" attachment. Alternatively, the role of Sli15 as an activator of Ipl1 raised the attractive possibility that Bir1-Sli15 mediate a "tension-sensing" attachment that ensures chromosome biorientation by locally controlling Ipl1 kinase activation.

To distinguish between these models, we used a GFP-marked chromosome to follow the segregation of sister

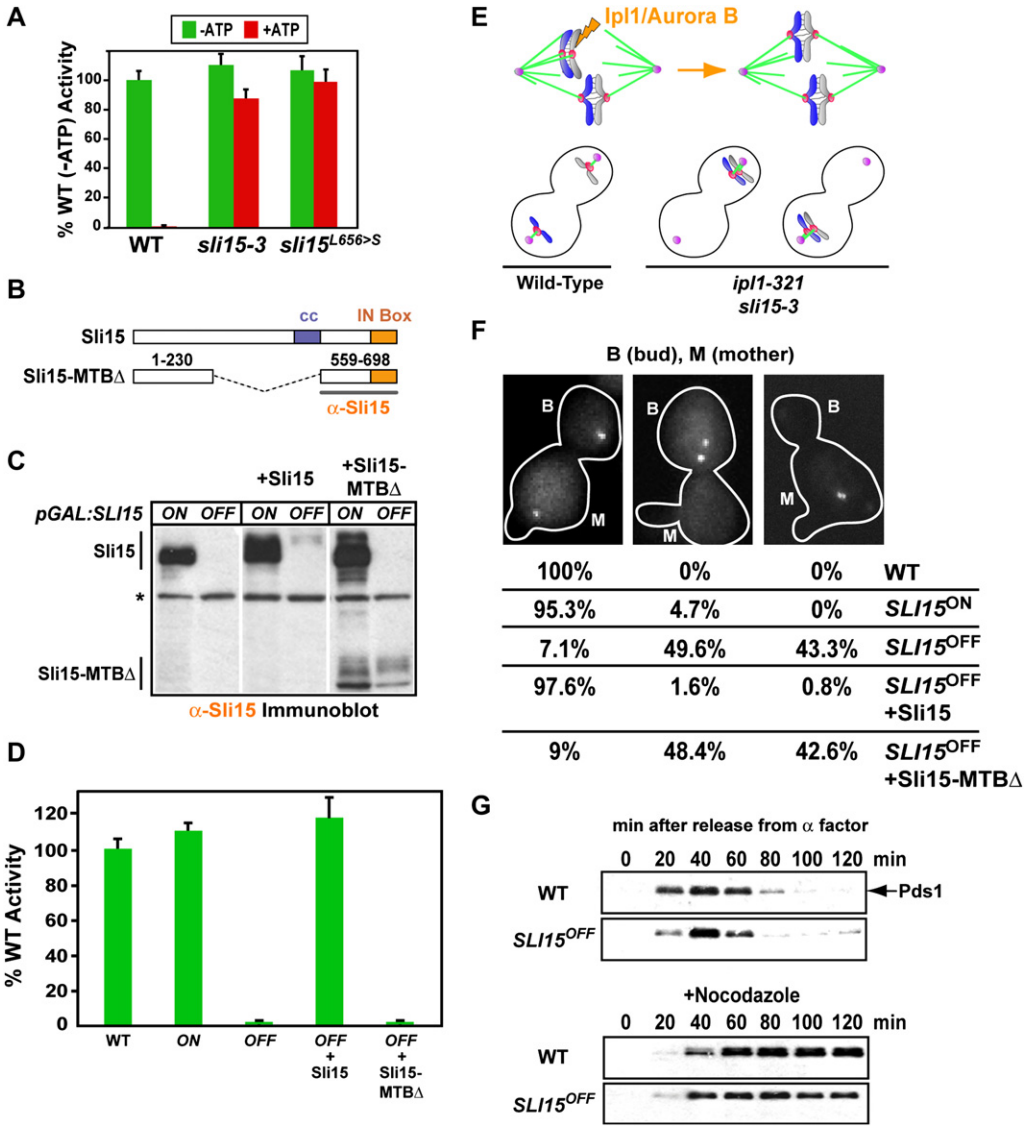

Figure 5. Sli15 Mutants that Eliminate CEN DNA-Microtubule Interactions In Vitro Phenocopy Ipl1 Kinase Activation Mutants In Vivo

(A) *sli15-3* extracts behave similarly to Ipl1 kinase mutant extracts. *sli15*$^{L656>S}$ is an engineered mutant with only the IN box amino acid change in *sli15-3* (see Figure S3). Error bars show SD.

(B) Schematic of Sli15 and the Sli15-MTBΔ mutant lacking the central microtubule-binding domain. The C-terminal region used to generate the anti-Sli15 antibody is underlined.

(C) Immunoblotting of ODY155 (p*GAL-SLI15*), ODY192 (*pGAL-SLI15*+Sli15), and ODY193 (*pGAL-SLI15*+Sli15-MTBΔ) in galactose (*ON*) or glucose (*OFF*) medium. All *SLI15*^{OFF} samples were prepared from cells arrested with nocodazole to maintain viability. Asterisk denotes the background band that serves as a loading control. See also Figures S4 and S5.

(D) Depletion of Sli15 or deletion of its microtubule-binding domain eliminates the CEN DNA-microtubule linkage in vitro. Error bars show SD.

(E) Ipl1/Aurora B is required for the correction of attachment errors where both sister chromatids are connected to the same spindle pole (syntelic attachment; upper panels). Mutants that perturb Ipl1 kinase activity (*ipl1-321*, *ipl1-2*, and *sli15-3*) fail to correct syntelic attachments, resulting in sister chromatid missegregation (lower panels).

(F) *SLI15*^{OFF} and *SLI15*^{OFF}+Sli15-MTBΔ both phenocopy Ipl1 kinase activity mutants in vivo. Segregation of a marked Chr IV was monitored 120 min after release from α factor arrest. Between 100 and 200 cells for each strain and growth condition were analyzed. The mother cell (M) was identified by residual α factor-induced shmoos (projections in cell outline).

(G) *SLI15*^{OFF} cells do not arrest in the cell cycle despite having a functional checkpoint. Wild-type (SBY818) and *pGAL:SLI15* cells expressing Pds1-Myc18 (ODY181) were grown as described above. Nocodazole was added to one set of cultures at 10 μg/ml upon release from α factor. Lysates were immunoblotted with an anti-Myc antibody.

chromatids. The failure to correct syntelic attachments in *ipl1* mutants results in sister chromatids cosegregating to the mother or the bud with roughly equal frequency (Figure 5E; Biggins and Murray, 2001; Biggins et al., 1999; Tanaka et al., 2002). In both the $SLI15^{OFF}$ and $SLI15^{OFF}$+ Sli15-MTBΔ cells, the marked chromosome exhibited a missegregation pattern similar to *ipl1* kinase activity mutants (Figure 5F). This missegregation pattern is distinct from different classes of kinetochore mutants required for the core attachment (reviewed in Biggins and Walczak [2003]). By contrast, the majority of sisters segregated properly in $SLI15^{ON}$ and $SLI15^{OFF}$+Sli15 cells (Figure 5F).

In *ipl1* mutants, despite a functional checkpoint and nearly 100% missegregation, the cell cycle is not delayed because core attachments, although incorrect, are present. This is in contrast to mutations that compromise the core attachment, which either delay in the cell cycle due to checkpoint activation or lack a functional checkpoint due to severe defects in kinetochore structure (Biggins and Walczak, 2003). To determine whether cells lacking Sli15 have a functional checkpoint and/or experience a cell-cycle delay, we monitored the levels of securin (Pds1) after releasing $SLI15^{OFF}$ cells from a G1 arrest in the presence or absence of nocodazole. $SLI15^{OFF}$ mutants arrested normally in nocodazole, indicating the presence of a functional checkpoint (Figure 5G). However, in the absence of nocodazole, $SLI15^{OFF}$ cells did not exhibit a significant cell-cycle delay (Figure 5G) despite extensive missegregation of sister chromatids (Figure 5F). This high rate of missegregation was associated with a rapid loss of cell viability (Figure S4C). These results indicate that cells lacking Sli15 behave similarly to *ipl1* kinase activity mutants (*ipl1-321*, *ipl1-2*, and *sli15-3*) but distinctly from mutants in kinetochore proteins important for the core attachment. We conclude that the Bir1-Sli15-mediated linkage between CEN DNA and microtubules is not part of the core attachment. Instead, our results indicate that the Bir1-Sli15-mediated linkage is essential for the tension-dependent regulation of microtubule attachments by Ipl1 that ensures biorientation.

DISCUSSION

A Complex of Bir1-Sli15 Links CBF3-CEN DNA to Microtubules In Vitro

The ability of budding yeast CEN DNA to interact with microtubules following incubation in a cell extract was first documented more than a decade ago. The importance of CBF3, the sequence-specific scaffold of the budding yeast kinetochore, was immediately apparent. However, the identity of an additional necessary component remained elusive. Here, we used a biochemical complementation assay to show that a complex of Bir1-Sli15 links CBF3-CEN DNA to microtubules in vitro. Bir1 directly interacts with the Ndc10 subunit of CBF3 (Bouck and Bloom, 2005; Gillis et al., 2005; Yoon and Carbon, 1999), and the central region of Sli15, required for the in vitro CEN DNA-microtubule linkage (this study), directly binds microtubules (Kang et al., 2001). Although a full reconstitution using recombinant proteins remains to be achieved, the evidence strongly suggests that the linkage observed in the in vitro assay can be accounted for by Bir1-Sli15 directly connecting CBF3-CEN DNA to microtubules.

It is interesting to note that the majority of proteins required for kinetochore assembly and function in vivo do not play a role in the in vitro linkage. This result was anticipated by the earlier finding that the CDEIII element of CEN DNA is sufficient in the in vitro assay but is not sufficient for centromere function in cells (Sorger et al., 1994). We suspect that the specialized chromatin domain on which kinetochores assemble, which includes nucleosomes containing the centromere-specific histone CENP-A, will be important for achieving in vitro reconstitution of the core attachment.

Ipl1 Kinase Regulates, but Is Not Required for, the CEN DNA-Microtubule Linkage In Vitro

Ipl1 kinase is dispensable for connecting CBF3-bound CEN DNA to microtubules but regulates this linkage (this study; Biggins et al., 1999). This observation raises the question as to what specific components are phosphorylated by Ipl1. In the original study describing Ipl1 regulation, the Ndc10 subunit of CBF3 was suggested to be the critical target (Biggins et al., 1999). Identification of Bir1-Sli15 as the linker between CBF3-CEN DNA and microtubules raises the possibility that phosphorylation of these components may also be important. In particular, Sli15 is a well-established Ipl1 substrate in addition to being its activator (Cheeseman et al., 2002; Kang et al., 2001). The binding of CBF3 to CEN DNA is unaffected in Ipl1 kinase mutant or *bir1Δ* extracts (this study; Biggins et al., 1999). An important future direction will be to determine whether Ipl1 phosphorylation regulates the interaction between CBF3 and Bir1-Sli15, between Bir1 and Sli15, or between Bir1-Sli15 and microtubules.

In Vivo Function of the Bir1-Sli15-Mediated Linkage Between CBF3-Bound CEN DNA and Microtubules

In considering the in vivo role of the Bir1-Sli15-mediated linkage in chromosome segregation, we focused on the connection to Ipl1, which is known to play a central role in biorientation. In the bioriented state, sister chromatids are connected to opposite spindle poles, placing centromere-spindle attachments under tension. Improper syntelic attachments are detected and eliminated in an Ipl1-dependent manner, facilitating new connection attempts until all chromosomes are bioriented. This paradigm for the mechanism of biorientation raises the question as to how changes in tension at centromere-microtubule connections are relayed to control Ipl1 kinase activity. The centromeric cohesion protector Sgo1 has been suggested to act as a tension sensor (Indjeian et al., 2005), but this function appears to be distinct from the Ipl1-dependent mechanism (Pinsky et al., 2006).

Our in vivo analysis indicates that the Bir1-Sli15-mediated linkage between CEN DNA and microtubules is

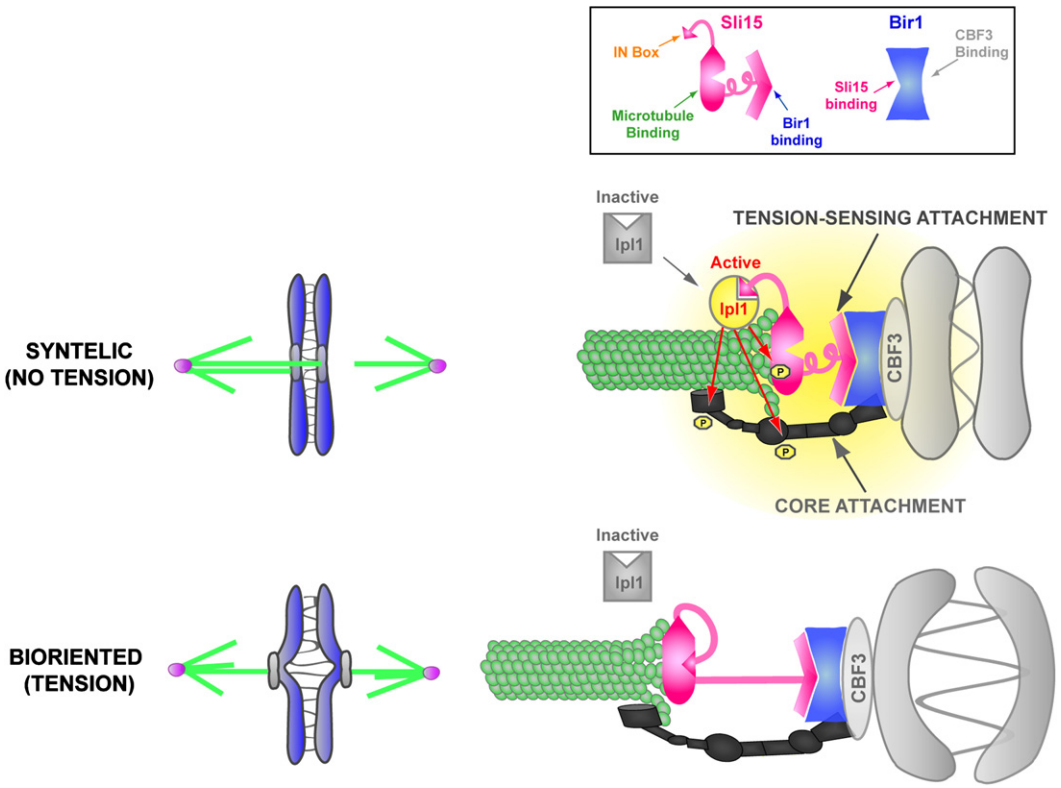

Figure 6. A Model for Tension-Regulated Ipl1 Activation by the Bir1-Sli15-Mediated Linkage between Centromeres and Microtubules

The central tenet of the model is based on the finding that the Bir1-Sli15-mediated linkage between CBF3-CEN DNA and microtubules is required for correction of syntely, but not for the core attachment between centromeres and microtubules in vivo. We propose that this linkage constitutes a tension-sensing attachment that is modulated by the primary force-generating (or force-transducing) core attachment such that syntely (no tension) promotes Ipl1 activation. Active Ipl1 phosphorylates multiple targets (red arrows) to dissociate the centromere from the microtubule. Biorientation (tension) silences Ipl1 activation, stabilizing the correct configuration.

required for Ipl1-dependent correction of core attachments lacking tension. One possibility to explain these findings is that the Bir1-Sli15 linkage facilitates recognition of Ipl1 substrates at syntelic attachments. Alternatively, this linkage, which includes the Aurora-activating IN box domain, may constitute the tension-sensitive activator of Ipl1. We favor the latter possibility and discuss it further below, although direct evidence for a tension-induced change in the Bir1-Sli15 linkage is necessary to distinguish between these alternatives.

A Speculative Model for Tension-Regulated Ipl1 Activation by Bir1-Sli15

The independent biorientation of multiple chromosomes on the same spindle poses a major challenge to understanding tension-regulated Ipl1 activation. We propose that Bir1-Sli15 links centromeres to microtubules in a manner that allows the IN box of Sli15 to locally activate Ipl1 when core attachments are not under tension (Figure 6). The finding that deletion of the microtubule-binding domain of Sli15 phenocopies *ipl1* kinase mutants suggests that formation of the ternary complex between Bir1-Sli15, CBF3-CEN DNA, and microtubules is necessary for robust Ipl1 activation. Such a mechanism could contribute to restricting Ipl1 kinase activity to the vicinity of attachments lacking tension. A potentially intriguing mechanistic parallel exists with Aurora A, the other major Aurora kinase present in metazoans but absent in fungi, that is activated by the TPX2 protein (Kufer et al., 2002; Tsai et al., 2003). Like INCENPs, TPX2 directly binds microtubules and the activation of Aurora A by TPX2 is strongly enhanced by microtubule binding (Tsai et al., 2003).

Ipl1 activated in the vicinity of syntelic attachments is predicted to phosphorylate components of both the core attachment and the tension-sensing attachment to release the centromere from the microtubule (Figure 6; Cheeseman et al., 2002; Kang et al., 2001). Recent work suggests that one of the critical core attachment targets is the Ndc80 complex, which binds directly to microtubules and whose microtubule-binding affinity is reduced by Ipl1/Aurora B-directed phosphorylation (Cheeseman et al., 2006). Phosphorylation-triggered detachment of the tension-sensing attachment, presumably recapitulated in the in vitro assay when ATP is present, may reduce

Ipl1 activation and promote dephosphorylation to allow new attachments to be formed.

Once a chromosome is bioriented and the core attachment is under tension, this in turn could modulate the tension-sensing attachment such that Sli15 is no longer able to activate Ipl1 (Figure 6). The modulation could occur by a tension-induced conformational change that buries the IN box, as depicted in Figure 6. Alternatively, tension may detach Bir1-Sli15 from the microtubule or from CBF3, preventing formation of the ternary complex that promotes activation (not depicted in Figure 6). Exploring potential tension regulation will be facilitated by the in vitro CEN DNA bead-microtubule interaction assay, which is amenable to direct biophysical analysis.

Significance to Metazoan Chromosome Segregation

The role of Aurora B in correction of syntelic attachments and the activation of Aurora B by INCENP family proteins are widely conserved (reviewed in Andrews et al. [2003], Meraldi et al. [2004], and Vagnarelli and Earnshaw [2004]). *Drosophila* and mammalian INCENPs directly bind to microtubules, indicating that this biochemical activity is also conserved (Adams et al., 2001; Wheatley et al., 2001). Although the CBF3-Bir1/Survivin interaction is restricted to budding yeast, Survivin and another accessory subunit Borealin/Dasra target Aurora B to centromeres in metazoans (reviewed in Vagnarelli and Earnshaw [2004]). Thus, we suspect that the general principle of utilizing a direct connection between centromeres and spindle microtubules that includes the activator of Aurora B will be widely relevant for understanding how tension helps ensure the correct distribution of chromosomes.

In summary, our work highlights the existence of a direct linkage between centromeres and microtubules that includes the activating scaffold protein for Ipl1/Aurora B. Such a linkage provides the potential for mechanically sensitive control of kinase activation. The concept of kinase-activating scaffolds acting as local mechanical sensors controlling phosphorylation cascades is likely to also be significant in other cellular contexts.

EXPERIMENTAL PROCEDURES

Strains and Extract Preparation

Strains are summarized in Table S4. For Pds1 blots, 1.5 ml culture was processed at each time point as described (Pinsky et al., 2006). To test candidate mutants in the bead assay, 200–500 ml cultures were grown to mid-log phase. Cells were washed in water, resuspended in a volume equal to the cell pellet of 2× breakage buffer (100 mM Bis-Tris Propane, 200 mM β-glycerophosphate, 400 mM KCl, 10 mM EDTA, 10 mM EGTA, 20% glycerol, and 1 mM PMSF [pH 7.6]), frozen as drops in liquid nitrogen, and ground in a liquid nitrogen-cooled mortar and pestle. The crude lysate was centrifuged at 15,000 × g for 2 × 15 min, and the supernatant was used for assays. Alpha factor was used at 1 μg/ml; nocodazole was used at 10 μg/ml.

Partial Purification of CBF3

CEN DNA band-shift assays were performed as described (Severin et al., 1997, 1999; Sorger et al., 1994). For the partial CBF3 purification, 50 ml extract was precipitated with 60% ammonium sulfate, resuspended in 40 ml BB150 (50 mM Bis-Tris Propane, 100 mM β-glycerophosphate, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 10% glycerol, and protease inhibitors [pH 7.0]), dialyzed into BB150, loaded onto a 20 ml Poros HQ/20 column, and eluted with a KCl gradient. Fractions with peak activity were pooled, flowed through a 5 ml HiTrap SP cation-exchange column, and concentrated on a 1.6 ml Poros HQ/20 column by elution with a KCl step gradient.

In Vitro CEN DNA-Microtubule Interaction Assay

The in vitro assay was performed as described (Severin et al., 1999), with the following modifications. Double-stick tape flow cells were constructed such that six samples could be analyzed on a single slide using a multichannel pipette. Taxol microtubules, polymerized using unlabeled, digoxigenin-labeled, and rhodamine-labeled tubulin (30:9:1), were adsorbed to anti-digoxigenin (10 μg/ml) antibody-coated flow cell surfaces for 5 min. Unbound microtubules were washed out, and the chamber was blocked for 5 min in BRB80 (80 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA [pH 6.8]) plus 10 μM taxol and 1.5 mg/ml casein. In parallel, 2 μl of ~20 mg/ml yeast extracts was incubated in 15 μl reactions in binding buffer (10 mM HEPES [pH 8.0], 6 mM $MgCl_2$, 10% glycerol, and ~120–140 mM KCl); ~3 μg sheared salmon sperm DNA was added as a nonspecific competitor. For complementation assays, (PP)CBF3 was added to ~3× the band-shift activity present in a typical extract assay, casein was added to 0.3 mg/ml, and the final reaction included 15 mM Bis-Tris Propane, 30 mM β-glycerophosphate, 0.6 mM EGTA, and 0.6 mM EDTA [pH 7.0], in addition to the binding buffer components. After 45 min, an equal volume of reaction buffer plus 10 μM taxol was added and reactions were introduced into the flow cell with adsorbed microtubules. Fifteen minutes later, unbound beads were washed out and ten fields of the coverslip surface per sample were photographed using a 20×, 0.7 NA objective. Bead binding was stable and did not change appreciably for 1 to 2 hr after the final wash. A custom macro was written to threshold and binarize each image and quantify bead number.

Enrichment of the CBF3-Complementing Activity and Mass Spectrometry

Conventional purification of the CBF3-complementing activity was performed using strain PS886. A fermenter was used to grow 100 liter cultures to mid-log phase. Cells were harvested using an ultrafiltration cassette followed by centrifugation. After a water wash, cell paste was directly extruded as fine vermicelli-like threads into liquid nitrogen using a compressor-driven caulk gun. Extracts were prepared by grinding the frozen cell paste threads in a liquid nitrogen-chilled stainless steel Waring blender. The fine cell powder was weighed and thawed by addition of an equivalent volume of 2× breakage buffer. Crude extract was clarified in two sequential 45,000 rpm spins (2 hr each, 4°C) and frozen in 50 ml aliquots. For a standard prep, 125–150 ml extract was precipitated with 40% ammonium sulfate and the pellet was resuspended in 0.5× extract volume of BB300 and loaded onto a 1 liter S400HR gel filtration column volume (in BB300). Peak activity fractions were dialyzed into SB150 (100 mM Bis-Tris Propane, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 10% glycerol, and protease inhibitors [pH 7.0]), loaded onto a 1 ml Mono S column, and eluted with a 20 column volume SB150 to SB1000 gradient. TCA was added to a final concentration of 20% to 50 μg of the Mono S pool, and the sample was left overnight on ice. The precipitate was washed in cold acetone and analyzed by MudPIT mass spectrometry (Washburn et al., 2001).

HA Immunodepletions, TAP Purifications, and Antibody Production

For immunodepletions, extracts prepared from 6HA-tagged strains were diluted with an equal volume of extract dilution buffer (50 mM Bis-Tris Propane, 1 mM EDTA, 1 mM EGTA, and 5% glycerol, plus protease inhibitors) clarified by centrifugation at 80,000 rpm for 10 min at 4°C in a TLA100.2 rotor and added to ~25 μl anti-HA resin (Roche). The

bead/extract mix was rotated at 4°C for 2 hr, and the supernatant was used for assays and quantifying depletions. TAP tagging and purifications were carried out as described (Cheeseman et al., 2002). GST-Ipl1 (full length), GST-Sli15 (aa 558–698), and GST-Bir1 (aa 700–945) were used as antigens for injection into rabbits. Affinity purifications were performed using standard methods.

Supplemental Data

Supplemental Data include five figures and four tables and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1179/DC1/.

ACKNOWLEDGMENTS

We are grateful to Sue Biggins and Iain Cheeseman for advice and encouragement, Paul Maddox for help with imaging, Scott Emr for use of his tetrad dissection scope, David Drechsel for help with the fermenter, and the yeast chromosome segregation community for its generosity, especially the Biggins, Drubin, Barnes, Kilmartin, Nasmyth, and Murray labs. For comments on the manuscript, we thank Don Cleveland, Ben Black, Iain Cheeseman, and Reto Gassmann. This work was supported by a training grant from the National Cancer Institute (to S.S.), by a grant from the National Center for Research Resources, NIH to Trisha N. Davis (P41 RR11823), by grants from the Human Frontier Science Program and the NIH to A.D., and by the Max Planck Institute (to F.S. and A.H.). K.O. is a Pew Scholar in the Biomedical Sciences; A.D. is the Connie and Bob Lurie Scholar of the Damon Runyon Cancer Research Foundation (DRS 38-04). K.O. and A.D. receive salary and additional support from the Ludwig Institute for Cancer Research.

Received: June 20, 2006
Revised: September 13, 2006
Accepted: September 30, 2006
Published: December 14, 2006

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