Analysis of kinesin motor function at budding yeast kinetochores

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Accurate chromosome segregation during mitosis requires biorientation of sister chromatids on the microtubules (MT) of the mitotic spindle. Chromosome–MT binding is mediated by kinetochores, which are multiprotein structures that assemble on centromeric (CEN) DNA. The simple CENs of budding yeast are among the best understood, but the roles of kinesin motor proteins at yeast kinetochores have yet to be determined, despite evidence of their importance in higher eukaryotes. We show that all four nuclear kinesins in Saccharomyces cerevisiae localize to kinetochores and function in three distinct processes. Kip1p and Cin8p, which are kinesin-5/BimC family members, cluster kinetochores into their characteristic bilobed metaphase configuration. Kip3p, a kinesin-8/13/KinI kinesin, synchronizes poleward kinetochore movement during anaphase A. The kinesin-14 motor Kar3p appears to function at the subset of kinetochores that become detached from spindle MTs. These data demonstrate roles for structurally diverse motors in the complex processes of chromosome segregation and reveal important similarities and intriguing differences between higher and lower eukaryotes.

Introduction

Kinetochores are multiprotein complexes that assemble on centromeric (CEN) DNA and attach chromosomes to spindle microtubules (MTs; Mitchison and Salmon, 2001). Kinetochore–MT attachments generate the forces required for sister chromatid biorientation during metaphase and poleward movement during anaphase (Maiato et al., 2004). Evidence from a variety of organisms suggests that regulation of MT dynamics by kinetochores is critical to both of these processes and that multiple motor and nonmotor MT-associated proteins (MAPs) are involved (Kline-Smith et al., 2005). The comparative simplicity of budding yeast CENs makes Saccharomyces cerevisiae an attractive organism in which to undertake a thorough study of this aspect of kinetochore biology (McAinsh et al., 2003).

S. cerevisiae has six kinesins and a single dynein heavy chain (for review see Hildebrandt and Hoyt, 2000), but only the four nuclear kinesins—Cin8p, Kip1p, Kip3p, and Kar3p—are potential kinetochore subunits. Yeast nuclear kinesins belong to different subfamilies with distinct directionalities, structures, and functions. Cin8p and Kip1p are members of the kinesin-5 family of plus end–directed motors (BimC motors; Dagenbach and Endow, 2004) that form homotetramers that are active in cross-linking parallel and antiparallel MTs (Gordon and Roof, 1999; Kapitein et al., 2005). Cin8p and Kip1p function in spindle assembly and in other MT-based processes (Hildebrandt and Hoyt, 2000). cin8Δ mutants are viable at 25°C, but have high rates of chromosome loss and undergo frequent spindle collapse (Hoyt et al., 1992); at 37°C, cin8Δ cells are dead. cin8Δ and kip1Δ are synthetically lethal, and KIP1 overexpression suppresses the spindle collapse phenotype of cin8Δ, though kip1Δ does not cause elevated chromosome loss. cin8Δ, but not kip1Δ, is synthetically lethal with mad2Δ (Geiser et al., 1997), presumably because checkpoint-mediated cell cycle delay is required for cin8Δ cells to complete mitosis successfully. Overall, these data show that Kip1p and Cin8p are functionally redundant (Hoyt et al., 1992; Roof et al., 1992), but that Cin8p plays the larger role under normal circumstances.

Kip3p belongs to either the kinesin-8 or -13 families (formerly Kip3 and KinI kinesins; Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200509101/DC1). These families include the kinetochore motors MCAK in mammals; XKCM1 in Xenopus laevis; KLP10A, KLP59C, and KLP59D in Drosophila melanogaster; and Klsp5 and Klsp6 in Schizosaccharomyces pombe (Table S1). Kinesin-13 motors destabilize MT protofilaments, causing MT depolymerization primarily at plus ends (Niederstrasser et al., 2002). D. melanogaster KLP10A and KLP59C mediate the disassembly of MTs from the plus and minus ends, respectively (Rogers et al., 2004).
S. cerevisiae kip3Δ cells are resistant to the MT-depolymerizing drug benomyl, which is consistent with a role for Kip3p in MT destabilization in yeast (Cottingham and Hoyt, 1997). Kinesin-8 and -13 motors are also thought to function during metaphase to correct improper kinetochore–MT attachment and to align chromatid pairs at the metaphase plate (for review see Moore and Wordeman, 2004). Thus, functions for kinesin-8 and -13 motors in vivo include kinetochore–MT attachment during metaphase and kinetochore MT (kMT) depolymerization during anaphase.

Kar3p, the fourth nuclear motor in budding yeast, is a minus end–directed kinesin-14 family member that localizes to spindle pole bodies (SPBs) and the tips of cortical MTs (Meluh and Rose, 1990). Kar3p destabilizes MT minus ends in vitro and cytoplasmic MTs in vivo (Endow et al., 1994; Sproul et al., 2005) and has been found at low levels in biochemical preparations of the CBF3 CEN-binding complex (Hyman et al., 1992; Middleton and Carbon, 1994). Like Cin8p (He et al., 2001), Kar3p associates with CEN DNA when assayed by chromatin immunoprecipitation (ChIP; Tanaka et al., 2005). Kar3p is involved in the sliding of mini-chromosomes laterally along MTs when newly formed kinetochores are captured by MTs. Endogenous S. cerevisiae chromosomes are bound to MTs throughout the cell cycle, however, making it unclear whether Kar3p functions at kinetochores during normal cell division.

Figure 1. Localization of GFP-tagged kinesins in wild-type cells. (A) Typical 2D projections of 3D images showing metaphase (top) and anaphase (bottom) cells coexpressing the SPB marker Spc42p-CFP (red) and Cin8p-GFP, Kip1p-GFP, Kip3p-GFP, Kar3p-GFP, or Ndc80p-GFP (green). Surface plots beneath each image depict fluorescence signal intensity distribution in arbitrary units for CFP (red) and GFP (green). The intensity distributions were generated from 2D summed projections of 3D image stacks. Intensities are not comparable between images. (B) Representative 2D projections of 3D images of G1 cells coexpressing Kip3p-GFP, Kar3p-GFP, or Ndc80p-GFP (green) with Spc42p-CFP (red). (C) Images of cells expressing Cin8p-GFP, Kip1p-GFP, and Kip3p-GFP (green) with Spc42p-CFP (red) in ndc10-1 cells. Strains were grown to midlog phase at 25°C and shifted to 37°C for 3 h before analysis. Spindles elongate abnormally in many ndc10-1 cells, creating metaphase cells with spindle lengths that are typical of anaphase (~2.0-μm long). Bars, 1 μm.
Functional analysis of nuclear kinesins in budding yeast is complicated by their involvement in multiple mitotic processes either individually or in combination. This multiplicity of function creates complex loss-of-function phenotypes. To begin to understand kinesin functions specifically at kinetochores, we have applied a series of fixed and live-cell assays that focus on kinetochore biology. We find that all four *S. cerevisiae* nuclear kinesins localize to kinetochores and perform the following three distinct functions: Cin8p and Kip1p are required for correct alignment and clustering of kinetochores on the metaphase spindle; Kip3p is required for coordinated movement of sister chromatids to spindle poles at anaphase; and Kar3p appears to function specifically at a subset of kinetochores on which MT attachments are slow to form. Thus, although nuclear kinesins in budding yeast are best known as essential players in spindle assembly, they also have important roles in ensuring the accurate attachment of kinetochores to MTs.

**Results**

To determine whether Cin8p, Kip1p, Kip3p, and Kar3p localize to kinetochores, we applied three criteria previously used in the analysis of other kinetochore proteins (He et al., 2001). First, GFP-tagged kinesins were examined in fixed cells and localization patterns were compared with patterns for known kinetochore proteins; second, tagged kinesins were tested for *CEN* association by ChIP; and third, the role of CBF3 in *CEN* binding of kinesins was examined by using a temperature-sensitive mutation (*ndc10-1*) in a subunit of CBF3. CBF3 is an essential four-protein complex that is required for initiating kinetochore assembly and for the recruitment of all known kinetochore proteins to *CEN* DNA (Lechner and Carbon, 1991; Goh and Kilmartin, 1993; He et al., 2001). Ndc10p-dependent localization to kinetochores and association with *CEN* DNA are diagnostic of kinetochore proteins.

To image motor proteins, kinesins were fused at their COOH termini to GFP and integrated into endogenous loci in a strain containing Spc42p-CFP–labeled SPBs. Genetic tests established that the tagged motors were biologically active (see Materials and methods). In early mitosis, kinetochore proteins localize to a single focus spanning the short (<1-μm-long) distance between the spindle poles. Subsequently, at spindle lengths of 1.0–1.2 μm, kinetochores resolve into two distinct foci lying between the SPBs. This bilobed pattern is analogous.
to the metaphase plate in metazoans and is maintained until anaphase, at which time chromatids move poleward and become tightly associated with SPBs (Fig. 1 A; He et al., 2000). When metaphase cells were examined by three-dimensional (3D) deconvolution microscopy, Cin8p-GFP, Kar3p-GFP, and Kip3p-GFP were found to have bilobed localization patterns similar to that of Ndc80p-GFP, a well characterized kinetochore protein (Fig. 1 A, top). In addition, these kinesins also decorated interpolar MTs during metaphase (unpublished data). In anaphase, Cin8p-GFP and Kip3p-GFP were found at the spindle midzone and Kar3p-GFP localized to faint puncta along pole-to-pole MTs (pMTs), whereas Ndc80p-GFP was visible only near kinetochores (Fig. 1 A, bottom; He et al., 2001). Biochemical experiments have shown that the majority of Kip1p is degraded at the metaphase–anaphase transition (Gordon and Roof, 2001), implying that the Kar3p-GFP visible in anaphase cells represents a small fraction of undegraded MT-bound protein. Overall, imaging data suggest that Cin8p, Kip1p, and Kip3p localize to kinetochores, as well as to other MT-based structures. To further demonstrate this point, we established that the bilobed localization of Cin8p, Kip1p, and Kip3p was lost in ndc10-1 cells, but that GFP fluorescence along spindle MTs was maintained (Fig. 1 C). Cin8p-GFP localized close to the poles in ndc10-1 cells, whereas Kar3p-GFP was found along the spindle (Fig. 1 C) and Kip3p-GFP was concentrated at the spindle midzone. We interpret these data to mean that in the absence of CBF3 the association of Cin8p, Kip1p, and Kip3p with kinetochores was disrupted, whereas localization to other MT-based structures was retained.

In contrast to Cin8p-GFP, Kip1p-GFP, and Kip3p-GFP, Kar3p-GFP was found primarily along the nuclear face of SPBs and did not appreciably colocalize with Ndc80p-CFP (Fig. 1 A and Fig. 2, A and B). Localization of Kar3p-GFP to SPBs in living cells confirms previous immuno-EM data (Zeng et al., 1999). However, faint Kar3p-GFP foci were also visible along the spindle in ∼30% of early (<1.5-μm-long spindles) and 20% of late (1.5–2.5-μm-long spindles) metaphase cells (Fig. 2, C and E). These Kar3p-GFP foci were rarely, if ever, seen during anaphase (Fig. 2 E) and did not adopt the bilobed pattern typical of core kinetochore proteins. To test the idea that Kar3p might associate specifically with detached or partially attached kinetochores (which are more abundant early in mitosis), cells were arrested in α-factor and released into the MT poison nocodazole. Most kinetochores in nocodazole-treated cells migrate to SPBs, associated by following shrinking MTs (Gillett et al., 2004), but in 75% of cells one or more faint foci were visible distal to the SPBs (Fig. 2 D). These fainter, more distant Kar3p-GFP foci colocalized with Ndc80p-GFP (Fig. 2 D, bottom). Based on our previous analysis (Gillett et al., 2004), Kar3p-GFP signals distant from SPBs almost certainly represent kinetochores that have detached from MTs (Fig. 2, D and E). SPB distal Kar3p-GFP represented 8 ± 2.5% of the total punctate GFP signal; implying efficient recruitment of Kar3p to detached kinetochores. We conclude that in vegetatively growing cells Kar3p becomes bound to detached or improperly attached kinetochores, but that most Kar3p is associated with SPBs.

**Association of Cin8p, Kip1p, and Kip3p with kinetochores by ChIP**

Cin8p and Kar3p have previously been shown to associate with CEN DNA by ChIP (He et al., 2001; Tanaka et al., 2005), and we found that Kip1p- and Kip3p-GFP also cross-link efficiently by ChIP to a 200-bp region centered on CEN IV, but not to equal length fragments lying 400 bp upstream and downstream (Fig. 3 A). As specificity controls, we showed that GFP-tagged kinesins did not cross-link appreciably with the URA3 locus, that immunoprecipitation of untagged kinesins yielded a negative ChIP signal at CEN IV, and that shifting ndc10-1 cells carrying Kip1p-GFP, Kip3p-GFP, or Cin8p-GFP to 37°C for 3 h lowered ChIP signals by 10-fold or more (Fig. 3 B; He et al., 2001). Overall, these data show that Cin8p, Kip1p, and Kip3p associate specifically with CEN DNA in a CBF3-dependent manner.

**Recruitment of kinesins to kinetochores**

To investigate how Cin8p, Kip1p, and Kip3p are recruited to kinetochores, ChIP was performed in ndc80-1 and spc25-7 cells. Ndc80p and Spc25p are components of the Ndc80 complex,
a multiprotein “linker” that bridges the DNA and MT-binding components of kinetochores (McAinsh et al., 2003). Kinetochores partially disassemble in ndc80-1 and spc25-7 mutants, and chromosomes dissociate from spindle MTs (He et al., 2001; Janke et al., 2002). We observed an approximately fourfold drop in the CEN-specific ChIP signal for Cin8p-GFP and Kip1p-GFP in ndc80-1 and spc25-7 cells (Fig. 3, C and D). In contrast, Kip3p-GFP showed wild-type levels of CEN-binding in ndc80-1 and spc25-7 cells (Fig. 3, C and D). Because a functional Ndc80 complex is required for kinetochores to bind to MTs, we conclude that Kip3p is CEN–bound in the absence of MTs, implying that Kip3p is a core kinetochore protein, as are kinisin-8 and -13 proteins in higher eukaryotes (Wordeman and Mitchison, 1995; Garcia et al., 2002; West et al., 2002). Data are more ambiguous for Cin8p and Kip1p; the motors could either require MTs for CEN association or the Ndc80 complex could be directly involved in recruiting Cin8p and Kip1p to kinetochores.

Cin8p and Kip1p organize kinetochores during metaphase

To probe the functions of kinetochore-bound motors, we asked whether mutations in kinesins would alter the localization of the Ndc80p and Mtw1p core kinetochore proteins. The bilobed distribution of these proteins represents the mean position of kinetochores during metaphase and is therefore a sensitive readout of MT attachment and chromosome congression (He et al., 2000; Pearson et al., 2004). We examined the localization of Ndc80p-GFP or Mtw1p-GFP in cells carrying Spc42p-CFP–tagged SPBs and cin8Δ or kip1Δ mutations. Because cin8Δ kip1Δ double deletions are inviable (Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992; Gheber et al., 1999) and it was necessary to focus on the subset of cells with metaphase spindles that were >1.2-μm long (for cin8Δ cells, 20–30% could be scored at t = 60–105 min). When these cells were examined, >60% contained supernumerary Ndc80p-GFP foci along the spindle. Error bars represent the SEM. n > 100.
spindle axis or had abnormally diffuse GFP lobes (“declustering”; Fig. 4, B and H). The same phenotype was observed in cells in which kinetochores were labeled with Mtw1p-GFP (unpublished data). In cin8Δkipp1Δ cells, Ndc80p-GFP and Mtw1p-GFP localization patterns were altered to an even greater extent, although only a subset of cells could be scored because of spindle collapse. In the 5–10% of cells with >1.2-μm-long bipolar spindles, kinetochores were distributed throughout the spindle, and 10–15 partially resolved foci were visible (Fig. 4 E). In contrast, kinetochore distribution was only slightly altered in kipp1Δ and kipp3Δ single mutants (Fig. 4, C and D and H). Collectively, these data suggest that Cin8p is involved in establishing or maintaining the normal metaphase configuration of yeast chromosomes, perhaps by bundling kMTs. kipp1Δ alone does not significantly alter kinetochore localization, but the severity of the cin8Δkipp1Δ phenotype suggests that Cin8p and Kip1p work together to cluster kinetochores, as they do to assemble spindles.

Because cin8Δ mutants are known to have unstable mitotic spindles, one concern with the aforementioned localization data is that declustering might be a simple consequence of the failure to form a spindle. To explore this possibility, spindle morphology was compared in wild-type and mutant cells using GFP-Tub1p (α-tubulin; Straight et al., 1997). In wild-type cells, MTs were visible as a thick bar with a slight increase in intensity near the SPBs, reflecting the termination of many kMTs near the spindle poles (Fig. 4 F; Maddox et al., 2000). kMTs are particularly prominent in budding yeast because they outnumber pMTs (Winey et al., 1995). GFP-Tub1p morphology was similar in cin8Δ cells, indicating that bipolar spindles had formed, although the GFP-Tub1p signal was less highly concentrated near SPBs (Fig. 4 G). This is precisely what one would expect if pMTs were correctly assembled, but kMTs mislocalized because of defects in congression. We conclude from these data that gross defects in spindle morphology are not responsible for the disruption of kinetochore clustering in cin8Δ cells.

We were also concerned that kinetochore declustering might be a consequence of spindle collapse and regrowth. Were this the case, we would expect the mutant phenotype to be more severe as mitosis progressed and spindles had time to undergo multiple cycles of collapse and regrowth. However, when metaphase declustering was measured in cin8Δ cells as a function of time after α-factor release, the fraction of cells with Ndc80p-GFP declustering early (t = 60–75 min) and late (t = 90–105 min) was similar (Fig. 4 I). Therefore, we concluded that declustering was not a consequence of altered mitotic timing in cin8Δ cells or of rounds of spindle collapse and regrowth.

Unattached or improperly attached chromosomes were a third possible explanation for kinetochore declustering. To investigate this possibility, we assayed the degree of transient separation in α-factor–synchronized cells carrying a CENIV-proximal TetO/TetR-GFP tag and Spc42p-GFP–labeled SPBs (Straight et al., 1996; Ciosk et al., 1998; He et al., 2000). Transient separation arises when kMTs pull sister kinetochores in opposite directions (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000) and represents an in vivo measure of force generation. No significant decrease in transient separation was observed in cin8Δ and kipp1Δ single mutants relative to wild-type cells (Fig. 5 A and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509101/DC1). Quantitation was difficult in cin8Δkipp1Δ cells because of their very short spindles, but live-cell imaging revealed transient separations qualitatively similar to those seen in wild type (Fig. 5, B and C). Although kipp3Δ cells and kipp1Δkipp3Δ cells both exhibited wild-type levels of transient separation, cin8Δkipp3Δ cells had a statistically significant 30% decrease (Fig. 5 A). Overall, these data show that chromosome–MT attachment is not severely disrupted by the deletion of individual kinesins, but that CIN8 and KIP3 might work together in force generation during metaphase. We also conclude that kinetochore declustering in cin8Δ cells is not simply a consequence of defective kinetochore–MT attachment. Instead, Kip1p and Cin8p appear to have a specific role in maintaining the metaphase configuration of budding yeast kinetochores in a process analogous to congression.

Kip3p regulates anaphase A movement

Because many kinesin-13 motors play a role in the poleward movement of chromatids, we asked whether KIP3 deletion...
would alter the anaphase movement of budding yeast kinetochores. We observed sister chromatid disjunction to be complete in wild-type cells with 2.0–4.0-μm-long spindles spanning the bud neck (a morphological marker of anaphase), as indicated by the appearance of two bright puncta of Ndc80p-GFP or Mtw1p-GFP immediately adjacent to the SPB. The puncta represent clusters of disjoined kinetochores (Fig. 6 A). In ∼20–25% of these cells, particularly those that are very early in anaphase, an extra focus of Ndc80p-GFP or Mtw1p-GFP was visible away from the SPBs (Fig. 6 A and not depicted). In contrast, kip3Δ cells with 2.0–4.0-μm-long spindles had multiple supernumerary kinetochore foci, typically two to five (Fig. 6, B and C), and these foci persisted for longer. The number of supernumerary GFP foci in kip3Δ cells fell as anaphase progressed, and none were visible when spindles had reached their maximum anaphase length of 7–10 μm, indicating that all chromatids had eventually moved to the poles (Fig. 6, C–E).

We propose that supernumerary Ndc80p-GFP and Mtw1p-GFP foci represent lagging chromosomes. An alternative explanation for supernumerary kinetochore foci is that kip3Δ cells with abnormal metaphase spindle morphology are transiently delayed in metaphase by the spindle checkpoint. However, when checkpoint-dependent cell cycle arrest was abolished in kip3Δ cells by deleting MAD2 (Li and Murray, 1991), the number of lagging chromosomes was unaltered (Fig. 6 F). Thus, supernumerary Ndc80p-GFP or Mtw1p-GFP foci in kip3Δ cells are unlikely to arise from malorientation of chromatid pairs during an extended metaphase. The ability of chromatids to disjoin in kip3Δ cells before cytokinesis supports previous data showing that chromosome loss rates are normal (DeZwaan et al., 1997). Collectively, our findings argue that in the absence of Kip3p the synchronous movement of chromatids toward the spindle poles is disrupted.
To obtain further evidence for lagging chromatids in *kip3Δ* mutants, we filmed anaphase in live cells carrying TetO/TetR-GFP–tagged *CENIV* and Spc42p-GFP–tagged SPBs. In wild-type cells, *CEN*-proximal GFP tags were briefly stretched into a line along the spindle axis. Such “hyperstretching” presumably reflects increased pulling forces on *CEN* DNA before the dissolution of sister chromatid cohesion, leading to the unraveling of chromatin ultrastructure (Thrower and Bloom, 2001).

**Figure 7. Live-cell analysis of hyperstretched chromatids in *kip3Δ* cells.** (A and B) Consecutive 2D projections of 3D images from representative videos of wild-type or *kip3Δ* cells were collected every 5 s. Numbers on bottom left of each image indicate seconds. Abnormally prolonged stretching of the TetO/TetR-GFP tag is visible in *kip3Δ* cells. (C) Percentage of anaphase cells with hyperstretching before anaphase A. Error bars are SEM. *n* = 22. (D) Mean duration of hyperstretching. Error bars are SEM. *n* = 12. (E) Mean MT length after anaphase A completion. Error bars are SEM. *n* > 300.
After 15–45 s (mean of 25 s) of hyperstretched, individual TetO/TetR tags resolved in two compact dots (Fig. 7, A and C–E) and moved rapidly to within 0.4 μm of the SPBs, where they remained for the duration of anaphase (Fig. 7 E). In 15% of kip3Δ cells (n = 13), one chromatid made a swift movement poleward, but then paused for several minutes before finally moving all the way to the pole (Fig. 8, A and D, with pause highlighted in yellow). In other kip3Δ cells, chromosomes remained hyperstretched for significantly longer than in wild type (30–100 s; mean of 70 s; Fig. 7, B and D), implying imposition of pulling forces before the dissolution of sister cohesion. The accumulation of lagging chromatids demonstrates the failure of kip3Δ kinetochores to complete poleward movement in a timely fashion, and the existence of hyperstetching suggests abnormally early imposition of anaphase A forces. Thus, Kip3p may function both to mediate kMT depolymerization and to coordinate pulling forces with the metaphase–anaphase transition.

Kip3p regulates MT length and dynamics in G1 and α-factor-arrested cells

The involvement of Kip3p in chromosome movement during anaphase suggested that it might regulate MT dynamics. To directly measure the effects of kinesin mutations on chromosome movement, we used fast-acquisition live-cell imaging coupled...
with Machine Vision software. The software makes it possible to track Tet/TetR-tagged CENs with precision and to extract rates of MT growth, shrinkage, rescue, and catastrophe (Dorn et al., 2005). Currently, accurate tracking is possible only within the simple monopolar spindle geometry of G1 cells. Cin8p and Kip1p are not present in G1; therefore, we focused on analyzing Kip3p and Kar3p (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Kip3p deletion led to a statistically significant decrease in mean MT growth ($P < 10^{-3}$) and shrinkage speeds ($P < 10^{-3}$), implying a role for Kip3p in the regulation of G1 MT dynamics (Fig. 9 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200509101/DC1). In contrast, kar3Δ did not affect either of these key parameters to a significant degree. As a second means to show that Kip3p alters kMT behavior, we examined the distribution of Ndc80p-GFP or Mtw1p-GFP in α-factor–arrested cells. After 2 h in α-factor, Ndc80p-GFP or Mtw1p-GFP foci in wild-type cells averaged a distance of 0.8 μm from SPBs and were rarely >1.5 μm away (Fig. 9, B–D); in kip3Δ cells under identical conditions, kinetochore foci averaged a distance of 1.2 μm from SPBs and were often as far as 2.5 μm away (Fig. 9, B–D). Collectively, these data show that Kip3p is involved in regulating the dynamics and mean lengths of kMTs in G1 and α-factor–arrested cells.

**Discussion**

Cin8p, Kip1p, and Kip3p localize to kinetochores

In this paper, we determine which kinesin motor proteins in *S. cerevisiae* localize to kinetochores and analyze the functions of kinetochore kinesins in metaphase and anaphase. Because *S. cerevisiae* has a closed mitosis, only the four nuclear kinesins Cin8p and Kip1p (kinesin-5 family members), Kip3p (a kinesin-8,-13/KinI motor), and Kar3p (a minus-end directed kinesin-14) have the potential to bind to kinetochores. ChIP has previously established that Cin8p and Kar3p associate with CEN, and we show that this is also true of Kip1p and Kip3p, the two remaining nuclear motors. Live- and fixed cell imaging shows that kinetochores are one of the primary structures to which Kip1p-, Cin8p-, and Kip3p-GFP are localized during mitosis in normally growing cells. However, association with other structures is observed in cells lacking active kinetochores (as a consequence of disrupting the CEN-binding complex CBF3), which is consistent with previous data showing that kinesins play important roles in spindle assembly.

How are kinesins recruited to kinetochores? In the case of Kip3p, it appears that the motor binds directly to core kinetochore components; Kip3p remains CEN-bound in ndc80-1 and spc25-7 mutants, despite the dissociation of chromosomes from MTs. In this respect, Kip3p is similar to the human kinesin-13/KinI motor MCAK, which is a component of the inner kinetochore (Wordeman and Mitchison, 1995). The finding that CEN binding by Cin8p and Kip1p is partially, but not entirely, dependent on NDC80 and SPC25 is ambiguous in respect to the role of MT attachment, but it seems likely that both motors require MTs to associate with kinetochores. Other yeast kinetochore proteins, including members of the MT-binding Dam1–DASH complex, require MTs for kinetochore association (McAinsh et al., 2003; Miranda et al., 2005; Westermann et al., 2005), as do plus end MAPs, such as CLIP-170, in higher eukaryotes (Maiato et al., 2004). Therefore, it seems that MT-binding kinetochore
proteins in *S. cerevisiae* fall into two classes: those that are recruited directly by core kinetochore proteins and those that bind to, or are transported to, MT plus ends and then associate with kinetochores. These two classes of kinetochore MAPs must then interact to form a fully functional kinetochore–MT attachment site.

In contrast to Kip1p, Kip3p, and Cin8p, which mainly localize to kinetochores, Kar3p-GFP is found primarily on the nuclear face of SPBs. It has been suggested that Kar3p might be a kinesin motor, based on its copurification with CBF3 (Hyman et al., 1992; Middleton and Carbon, 1994) and genetic interaction with other motors (Hildebrandt and Hoyt, 2000). However, previous immuno-EM data (Zeng et al., 1999) are consistent with our live-cell imaging in showing Kar3p to be primarily SPB bound. Low levels of Kar3p can be detected at a subset of kinetochores early in mitosis, and higher levels can be detected on kinetochores that are detached from MTs by nocodazole-treatment. Kar3p has recently been implicated in lateral MT sliding of newly captured ectopic kinetochores (Tanaka et al., 2005). However, kinetochores normally remain MT-bound throughout the cell cycle (Dorn et al., 2005), and MT capture is probably important only during a brief period in S phase. This may explain both the low levels of Kar3p on kinetochores under normal conditions and the absence of elevated chromosome loss in kar3Δ cells. Moreover, whereas Kar3p may function in de novo kinetochore–MT attachment, we have not been able to detect a kinetochore function for Kar3p in cells under normal growth conditions.
Organization of metaphase chromosomes by Cin8p and Kip1p

Our data show that Cin8p and, to a lesser extent, Kip1p are involved in the generation or stabilization of the distinctive bilobed kinetochore clusters found in budding yeast from mid- to late metaphase. The disruption of bilobed clustering in cin8Δ mutants does not appear to reflect gross disorganization of the spindle, dramatic increases in the number of detached chromosomes, or changes in the fraction of transiently separated sister CENs. Instead, we speculate that Cin8p and Kip1p, like other kinesin-5 motors that can cross-link parallel and antiparallel MTs (Gordon and Roof, 1999), are involved in cross-linking MTs. Because S. cerevisiae CENs associate with a single MT, kinetochore-bound Cin8p and Kip1p must cross-link MTs from different kinetochores. Metaphase sister kinetochores can transiently separate by 0.5 μm or more; therefore, it seems unlikely that Cin8p and Kip1p are able to cross-link sisters; instead, we propose that cross-linking involves kMTs from different chromatids, though not necessarily kMTs emanating from the same pole (Fig. 10 B). If Cin8p and Kip1p, like human Eg5, can translocate to MT plus ends and remain attached (Kapitein et al., 2005), the motors may actively bundle and link kinetochores together. Kinetochore–MT cross-linking could couple the polymerization of multiple MTs, perhaps explaining the requirement for Cin8p/Kip1p in forming bilobed kinetochore clusters. The significance of clustering is suggested by the appearance of detached kinetochores in cin8Δ mutants and an elevated rate of chromosome loss. Higher eukaryotes contain kinetochore fibers made up of 20 or more MTs. Bundles of yeast kMTs created by Cin8p and Kip1p may therefore resemble metazoan multi-stranded kinetochore fibers, except that multiple chromatids would be involved in the yeast MT bundles. Further analysis of Cin8p and Kip1p function during metaphase will require a deeper understanding of the forces that generate the bilobed configuration of yeast kinetochores, an effort that is currently underway in several laboratories.

Kip3p and anaphase chromosome movement

Live- and fixed cell imaging of kip3Δ cells reveals abnormally asynchronous sister chromatid separation during anaphase. A subset of chromatids in kip3Δ cells lags behind the majority and is found arrayed along spindle MTs at a point when the bulk of disjoined sisters have already arrived at the spindle poles. Surprisingly, a second subset of kip3Δ chromatids exhibits the opposite behavior—prolonged CEN hyperstretching. Transient sister separation and chromosome stretching are observed in wild-type cells, but coordinated dissolution of sister cohesion and poleward movement generate only a brief period of CEN hyperstretching at anaphase A onset. In kip3Δ cells, stretching is greater in magnitude and duration. Hyperstretching presumably reflects the initiation of poleward movement before the complete degradation of cohesion. Despite these problems early in anaphase, chromatids in kip3Δ cells are disjoined correctly by the end of anaphase B, consistent with a normal rate of chromosome loss in kip3Δ mutants (DeZwaan et al., 1997). The simultaneous generation of lagging and hyperstretched chromatids in kip3Δ cells implies a role for Kip3p in ensuring the synchronicity of poleward movement, presumably by coupling plus end MT depolymerization to the release of tension on sisters after cohesion degradation. In D. melanogaster, a similar function has been proposed for kinesin-13 motors in Rogers et al. (2004). Kip3p function in yeast does not appear to be restricted to anaphase, however, because kinetochore dynamics during G1 and MT length in α-factor are altered in kip3Δ cells. Moreover, Cin8p and Kip3p function together during metaphase to generate pulling forces on kinetochores, as indicated by the 30% decrease in transient sister separation observed in cin8Δkip3Δ double mutants. Overall, we conclude that budding yeast Kip3p, like kinesin-13/KinI motors in higher eukaryotes, plays an important role in the timely and efficient depolymerization of kMTs during anaphase and probably also during other phases of the cell cycle.

Conclusions

We have established that all four nuclear kinesins localize to mitotic kinetochores in S. cerevisiae, implying considerable complexity in kinetochore–MT interaction. During normal cell division, Cin8p, Kip1p, and Kip3p are found at high levels on most, if not all, kinetochores, whereas Kar3p is found transiently on only a subset of maloriented or unattached kinetochores. The absence of Kar3p from the majority of metaphase chromatids suggests that kinetochores do not normally move poleward along the sides of MTs, though such motion may be observed during MT capture by newly assembled ectopic kinetochores (Tanaka et al., 2005). Instead, it appears that in yeast, as in other organisms, the primary way that kinetochores move is by binding to MT plus ends and then altering their dynamics. Our data suggest that Kip3p is one protein involved in this regulation. Among our most striking observations is that Cin8p and Kip1p are important in organizing the bilobed metaphase configuration of yeast kinetochores. No precedent exists for this in higher cells, but we speculate that kinetochores with a single bound MT, such as those in S. cerevisiae, present mechanical problems not found in complex kinetochores that bind multiple MTs. Perhaps by bundling ~16 kMTs (the number bound to one pole in a haploid) in S. cerevisiae cells creates a structure similar to a kinetochore fiber in higher cells, thereby strengthening MT attachment.

Materials and methods

Yeast strains and manipulations

Strains were derived from W303 or S288C. GFP-tagged proteins were constructed as previously described (Gillett et al., 2004) and integrated into the genome to replace the endogenous wild-type copy. Because loss-of-function phenotypes for individual motor deletions are subtle, GFP-tagged kinesins were tested in strains carrying deletions exhibiting synthetic lethality, and the resulting compound mutants were tested for viability and growth. Kip1p-GFP was examined in cin8Δ cells; Cin8p-GFP in kip1Δ cells; Kar3p-GFP in kip3Δ cells; and Kip3p-GFP in kar3Δ cells. In all cases, compound mutants were viable, with growth rates that were indistinguishable from wild type. Kar3p-GFP has previously been shown to localize to the plus ends of cortical MTs in cells treated with α-factor (Maddox et al., 2003). In α-factor–arrested cells, Kar3p-GFP bound MT plus ends, furthering the belief that the GFP fusion was active. Kar3Δ deletion strains were constructed by amplifying the deleted gene of interest
from American Type Culture Collection deletion strains via PCR, using primers that were located 500 bp upstream and downstream of the deleted gene. PCR products were transformed into fresh cells, and correct integrants were confirmed by PCR. pFA6A-HisMX6 deletion strains were made as described in Longtine et al. (1998), using primers with at least 50 bp of homologous sequence.

**Microscopy analysis**

Image acquisition and processing were performed as previously described (Gillett et al., 2004), using a microscope with 100×, 1.4 NA, optics (Deltavision RT; Applied Precision) and a CoolSnap camera (Photometrics). Probes were localized in fixed cells. Figs. 6 and 7 show consecutive frames from live-cell videos. All images are two-dimensional (2D) projections of 3D data. Fixed cells were prepared by treatment with 2% formaldehyde for 2–5 min, followed by 0.1 M phosphate buffer, pH 6.6, for at least 10 min before microscopy analysis. Live cells were grown in SD media for several hours and then resuspended in fresh media before imaging at either room temperature or at 30°C.

**ChIP**

Temperature-sensitive strains and wild-type controls were grown at 37°C for 3 h before cross-linking (Megee et al., 1999), with minor modifications, as described in Gillett et al. (2004). Untagged strains served as a control. To establish the linearity of the ChIP assay, serial dilutions of immunoprecipitated or total DNA were used as substrates for PCR amplification of 200-bp CENIV or flanking fragments. ChIP signals were determined as a ratio of CENIV DNA in the immunoprecipitation to CENIV in the total DNA preparation. ChIP data is presented as a ratio of signals for mutant versus wild-type strains or CENIV versus flanking DNA.

**Online supplemental material**

Table S1 lists the known kinesin-like function of kinesin family members in yeast and other organisms. We have also included two figures further describing our live-cell imaging data. Fig. S1 shows graphs depicting live-cell movements of sister chromatids in metaphase in relation to a reference SPB in wild type, as compared with kinesin mutant depicting live-cell movements of sister chromatids in metaphase in relation to this manuscript. Further describing our live-cell imaging data. Fig. S1 shows graphs depicting live-cell movements of sister chromatids in metaphase in relation to a reference SPB in wild type, as compared with kinesin mutant

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