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 *CEN*s 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 *CEN*s 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 Klp5 and Klp6 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).

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Abbreviations used in this paper: 2D, two-dimensional; 3D, three-dimensional; *CEN*, centromeric; ChIP, chromatin immunoprecipitation; kMT, kinetochore microtubule; MAP, MT-associated protein; MT, microtubules; SPB, spindle pole body; pMT, pole-to-pole MTs.

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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 minichromosomes 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.

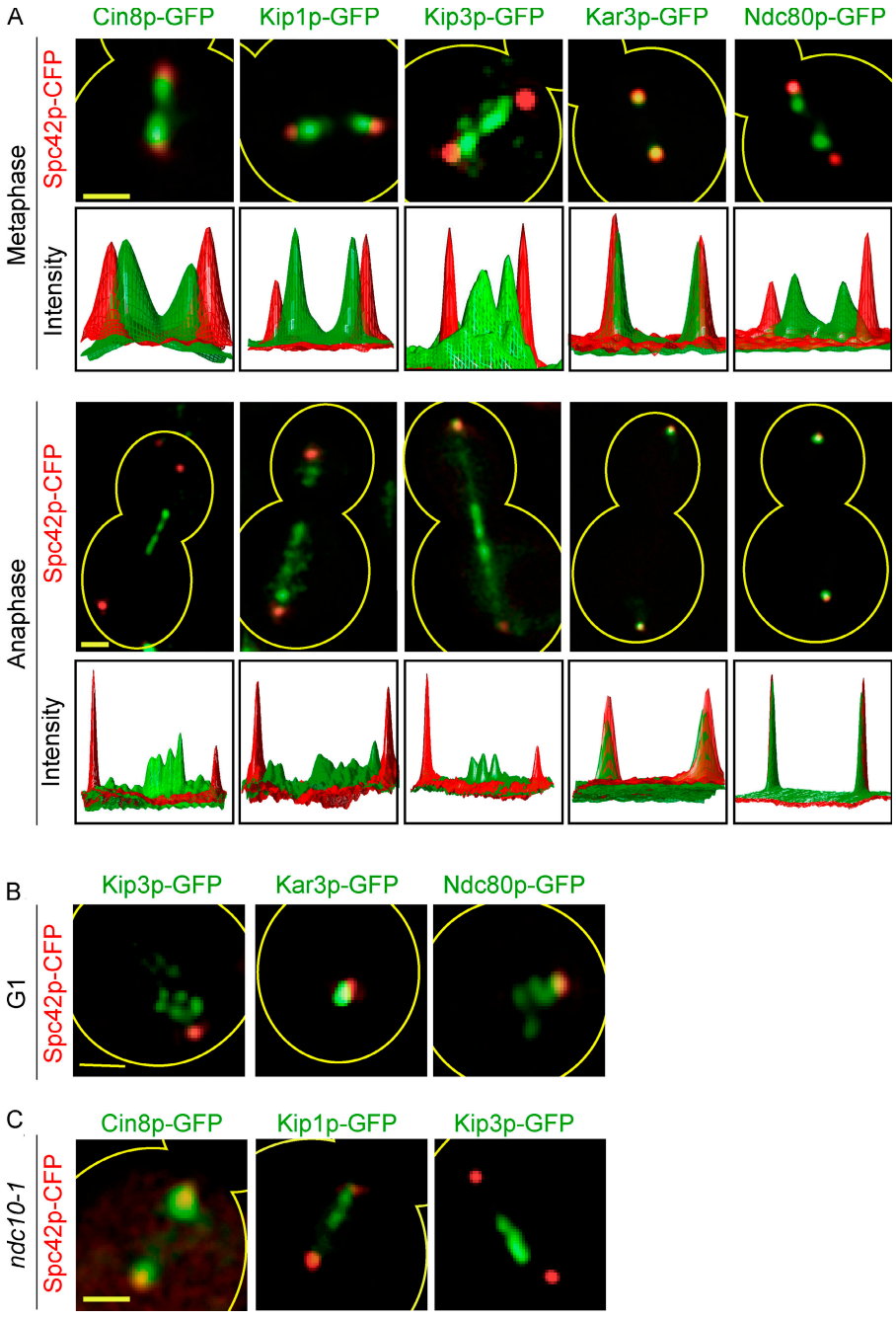

Figure 1. **Localization of GFP-tagged kinesins in wild-type cells.** (A) Typical 2D projections of 3D images showing metaphase (top) and anaphase (bottom) cells coexpressing the SPB marker Spc42p-CFP (red) and Cin8p-GFP, Kip1p-GFP, Kip3p-GFP, Kar3p-GFP, or Ndc80p-GFP (green). Surface plots beneath each 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 spindles lengths that are typical of anaphase (>2.0-μm long). Bars, 1 μm.

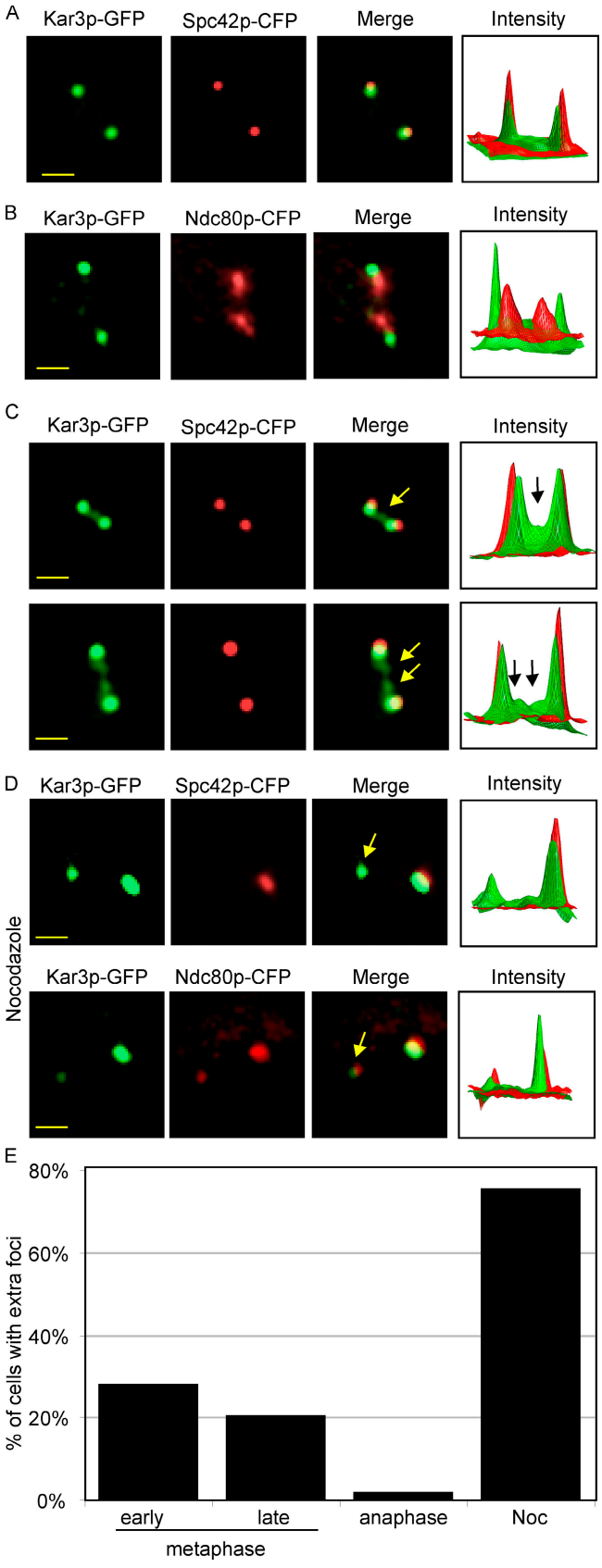

Figure 2. **Localization of Kar3p-GFP to SPBs and detached kinetochores.** 2D projections of 3D images and intensity plots were generated as described in Fig. 1. (A) Representative images of cells expressing Kar3p-GFP (green) and Spc42p-CFP (red) in which only two Kar3p-GFP foci were visible. (B) Images as in A, but with Kar3p-GFP (green) and the kinetochore protein Ndc80p-CFP (red). (C) Typical images of early metaphase (top; spindle length of <1.5 μm) or late metaphase (bottom; spindle length of 1.5–2.5 μm) cells in which Kar3p-GFP foci were visible along the spindle axis. Arrows show the positions of Kar3p-GFP foci away from SPBs. (D) Representative images of cells released from α-factor into nocodazole for 2 h to detach chromosomes from MTs. (top) Kar3p-GFP (green) and Spc42p-CFP (red) are coexpressed. (bottom) Kar3p-GFP (green) is coexpressed with Ndc80p-CFP (red). (E) Percentage of cells containing Kar3p-GFP foci along the spindle axis that are not coincident with the SPBs, as determined in early metaphase (spindle length of <1.5 μm), late metaphase (spindle length of 1.5–2.5 μm), anaphase (spindle length of <2.5 μm), or nocodazole-treated (as described in D) cells. 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,

Kip1p-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 Kip1p-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 Kip1p-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 Kip1p-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, apparently by following shrinking MTs (Gillett et al., 2004), but a subset becomes detached, moves farther from the SPBs, and recruits high levels of Bub1p, Mad1p, and Mad2p checkpoint proteins (Gillett et al., 2004). In nocodazole-treated cells, we observed that the majority of the Kar3p-GFP signal remained associated with SPBs (which were visualized with Spc42p-CFP), 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-CFP (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.

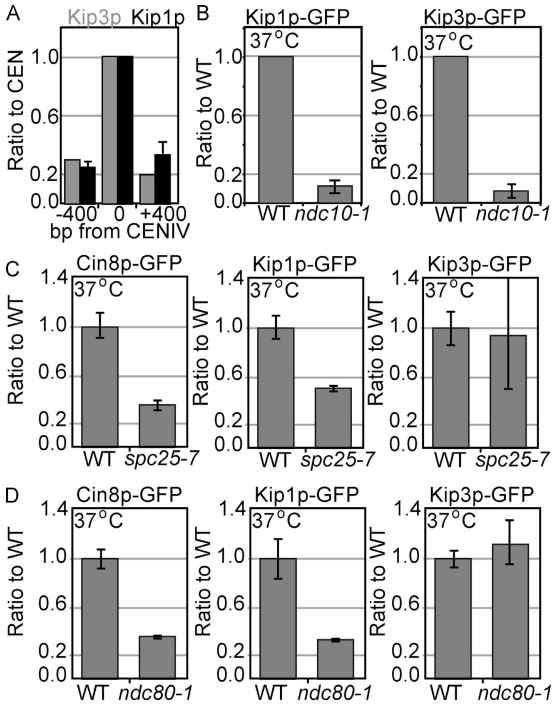

Figure 3. **Dependence of kinesin-*CEN* cross-linking by ChIP on core kinetochore components.** (A–D) ChIP of Kip1p-GFP, Kip3p-GFP, and Cin8p-GFP to *CENIV* or flanking DNA as assayed in wild-type (A), *ndc10-1* (B), *spc25-7* (C), or *ndc80-1* (D) cells. Asynchronous cultures were grown to midlog phase at 25°C and shifted to 37°C for 3 h (B–D) before analysis. (A) Results are expressed as the ratio of the percentage of immunoprecipitation from the arm regions to the percentage of immunoprecipitation of the *CEN*. (B–D) Results are expressed as a ratio of the percentage of immunoprecipitation in the mutant to the percentage of immunoprecipitation of the wild-type strain. Error bars represent the SEM. $n = 2$.

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 *CENIV*, 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 *CENIV,* 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,

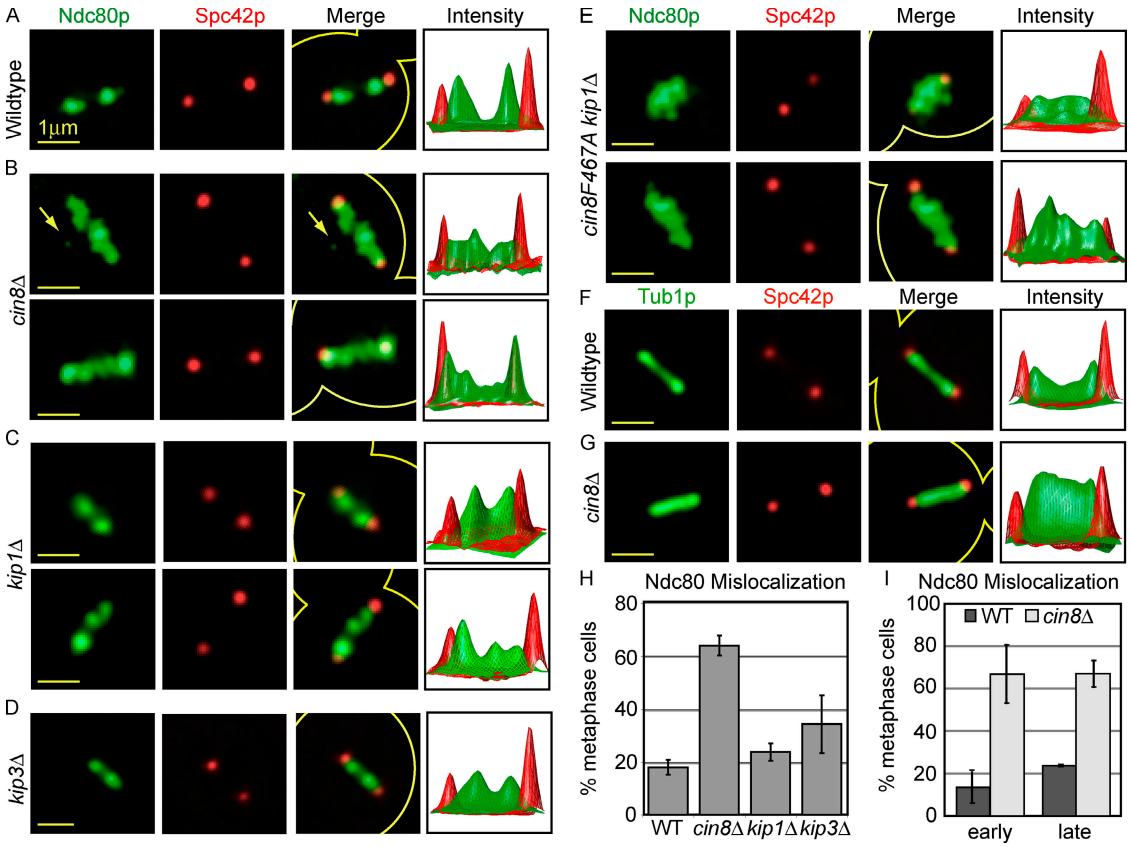

Figure 4. **Localization of Ndc80-GFP kinetochore protein in wild-type and kinesin mutants.** (A–E) Typical 2D projections of 3D images of Ndc80p-GFP (green) and Spc42p-CFP (red) in wild-type (A), *cin8Δ* (B), *kip1Δ* (C), *kip3Δ* (D), and *cin8F467Akip1Δ* (E) cells. (B, top) Arrow marks the location of a kinetochore that appears to have detached from spindle MTs. Spindles in *cin8F467Akip1Δ* cells rarely reach normal metaphase lengths. E (top) shows a metaphase cell with an abnormally short spindle (<1.0-μm long) and (bottom) a representative of the 10% of cells that reach wild-type spindle length (>1.5-μm long). (F and G) GFP-Tub1p (green) and Spc42p-CFP (red) in wild-type (F) and *cin8Δ* (G) cells. (H) Percentage of metaphase cells with atypical Ndc80p-GFP foci as scored in wild-type, *cin8Δ*, *kip1Δ*, and *kip3Δ* cells with spindle lengths of 1.2–2 μm. 100% of *cin8F467Akip1Δ* cells had gross defects in Ndc80p-GFP localization, but the interpretation of these images is complicated by the severity of the spindle assembly defect in these cells. Error bars represent the SEM. $n > 100$. (I) Percentage of atypical Ndc80p-GFP foci in metaphase *cin8Δ* cells as a function of time after α-factor release. 45–90 min after release is designated as early and 105–120 min as late. Error bars represent the SEM. $n > 100$.

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 kinesin-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), we examined the effects of impairing both kinesin-5 motors by combining *kip1Δ* with a temperature-sensitive *cin8F467A* mutation (which is defective in MT binding; Gheber et al., 1999). To obtain mitotic cells for imaging, cells were synchronized in α-factor and released into fresh medium. In wild-type and *kip1Δ* cells, a bilobed metaphase configuration was visible in cells 60–75 min after release (Fig. 4 A). However, in *cin8Δ* and *cin8F467Akip1Δ* cells, spindle assembly was delayed to a variable extent (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

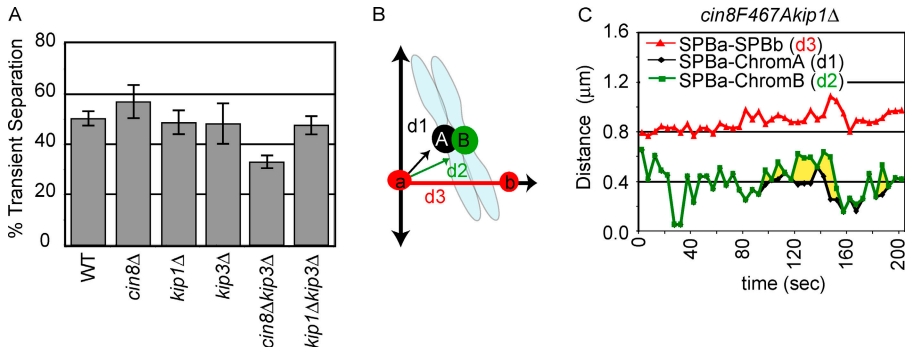

Figure 5. **Transient sister kinetochore separation in kinesin mutants.** (A) Percentage of metaphase cells undergoing transient sister separation in synchronized cultures of wild type, *cin8Δ*, *kip1Δ*, *kip3Δ*, *cin8Δ kip3Δ*, and *kip1Δ kip3Δ*, as judged in fixed cell assays. Transient separation was determined in α-factor–synchronized cells carrying a TetO/TetR-GFP tag near *CENIV* and Spc42p-CFP 75–90 min after release, at which point spindles averaged 1.5–2.5 μm in length. Error bars represent the SEM. $n > 100$. (B) Coordinates in live-cell tracking experiments. Distances d1 and d2 were measured between TetO/TetR tags on sister chromatids and a reference SPB, and d3 was measured between SPBs; differences between d1 and d2 during metaphase represent transient sister separation. (C) Plot of d1–d3 over time in a *cin8F467Akip1Δ*, with periods of transient separation denoted by yellow fill.

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 *cin8F467Akip1Δ* 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 *kip1Δ* and *kip3Δ* 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. *kip1Δ* alone does not significantly alter kinetochore localization, but the severity of the *cin8F467Akip1Δ* 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 *kip1Δ* 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 *cin8F467Akip1Δ* 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 *kip3Δ* cells and *kip1Δkip3Δ* cells both exhibited wild-type levels of transient separation, *cin8Δkip3Δ* 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

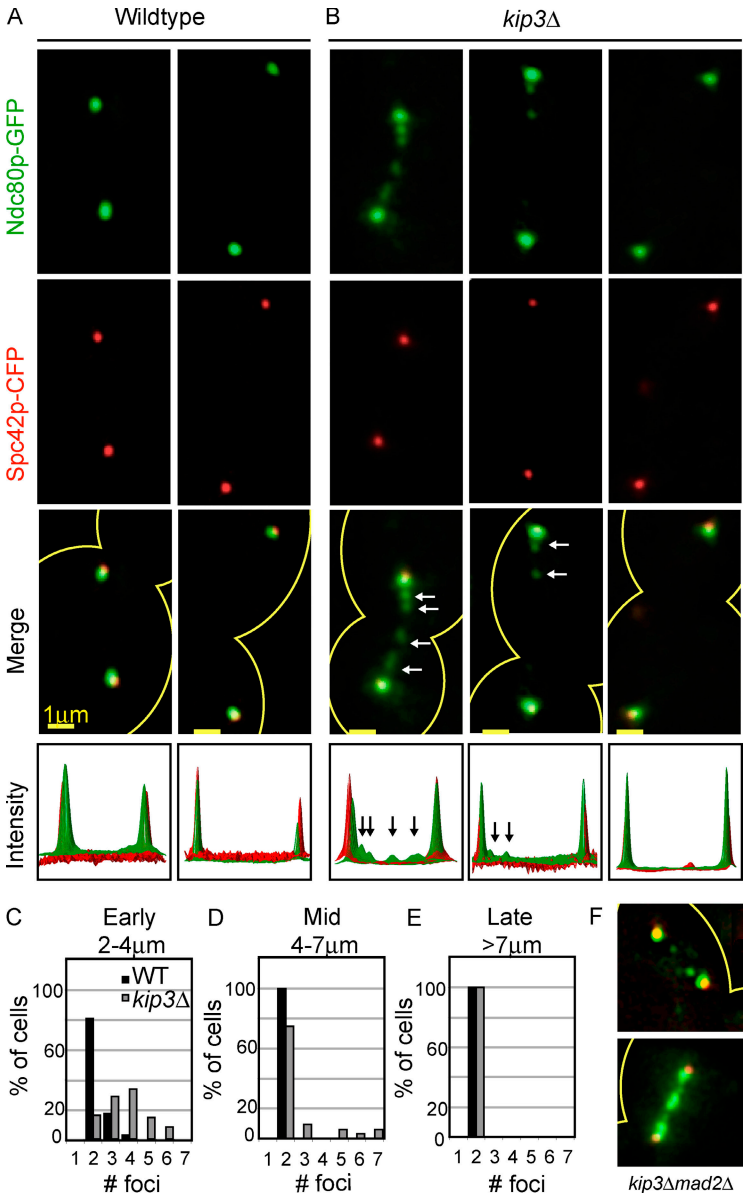

Figure 6. **Localization of kinetochore foci during anaphase.** (A and B) Representative 2D projections of 3D images of anaphase wild-type and *kip3Δ* cells expressing Ndc80p-GFP (green) and Spc42p-CFP (red). Arrows show the positions of the supernumerary Ndc80p-GFP foci, which represent lagging kinetochores. (C–E) Quantitation of supernumerary Ndc80-GFP foci during anaphase as sorted by spindle length, which is a marker of anaphase progression. (F) Anaphase localization of Ndc80p-GFP and Spc42p-CFP in *kip3Δmad2Δ* cells during anaphase.

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.

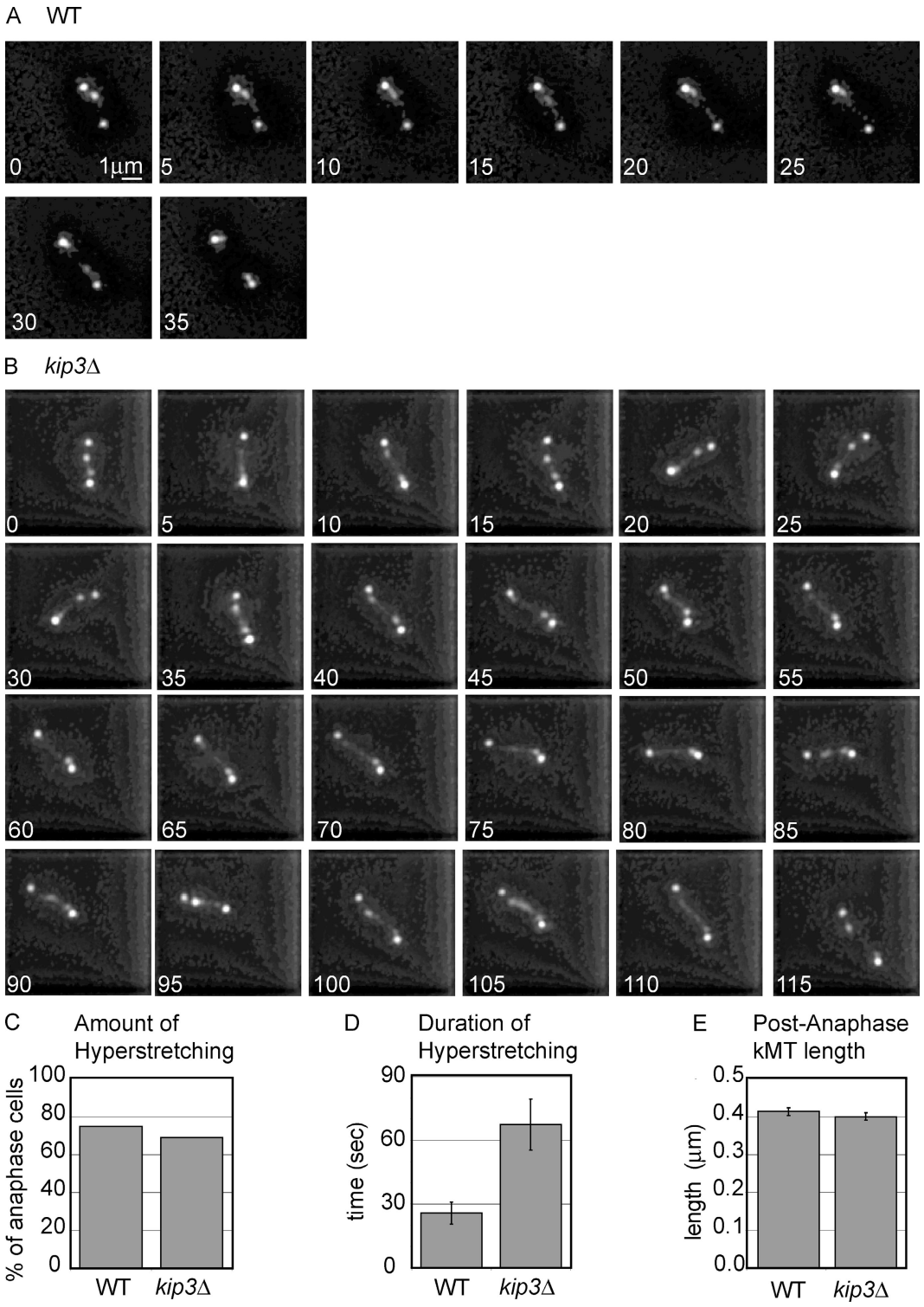

Figure 7. **Live-cell analysis of hyperstretched chromatids in *kip3Δ* cells.** (A and B) Consecutive 2D projections of 3D images from representative videos of wild-type or *kip3Δ* cells were collected every 5 s. Numbers on bottom left of each image indicate seconds. Abnormally prolonged stretching of 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. $n > 300$.

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).

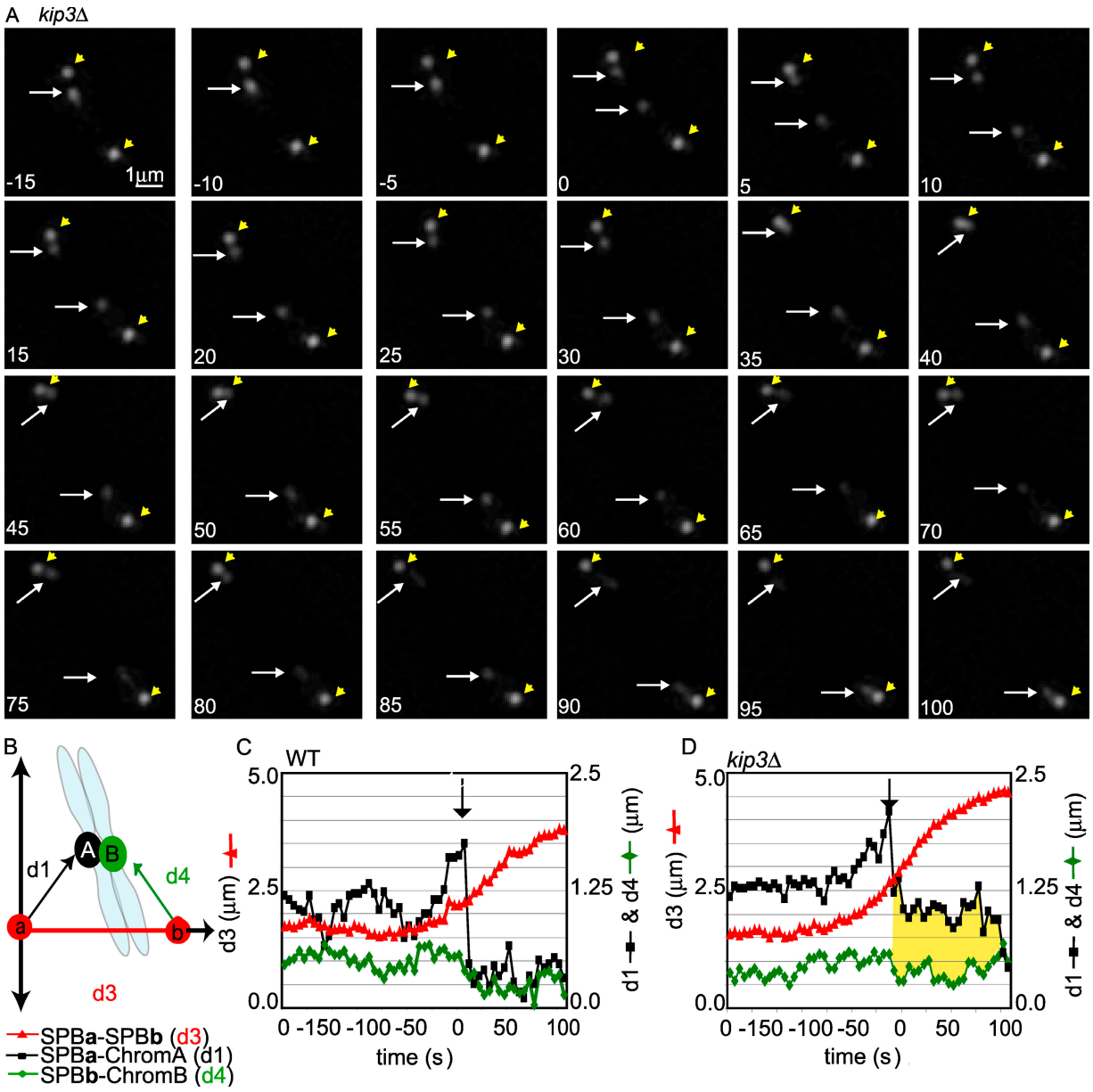

Figure 8. **Live-cell analysis of a lagging chromatid in *kip3Δ* cells.** (A) Consecutive frames from representative videos of *kip3Δ* cells containing a lagging chromosome. Arrows denote locations of TetO/TetR-GFP tags and yellow arrowheads denote SPBs. (B) Geometry of 3D live-cell tracking showing distances between labeled chromatids and the nearest SPB (d1and d4) and spindle length (d3). Chromatid trajectories in (C) wild-type and (D) *kip3Δ* cells; yellow fill denotes a pause in which one chromatid remained approximately 1.5 μm away from the nearest SPB for 100 s. Note the difference in scales for d1 and d4 versus d3. Times for A, C, and E are displayed relative to anaphase onset, which is set to t = 0.

After 15–45 s (mean of 25 s) of hyperstreching, 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 hyperstretching 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

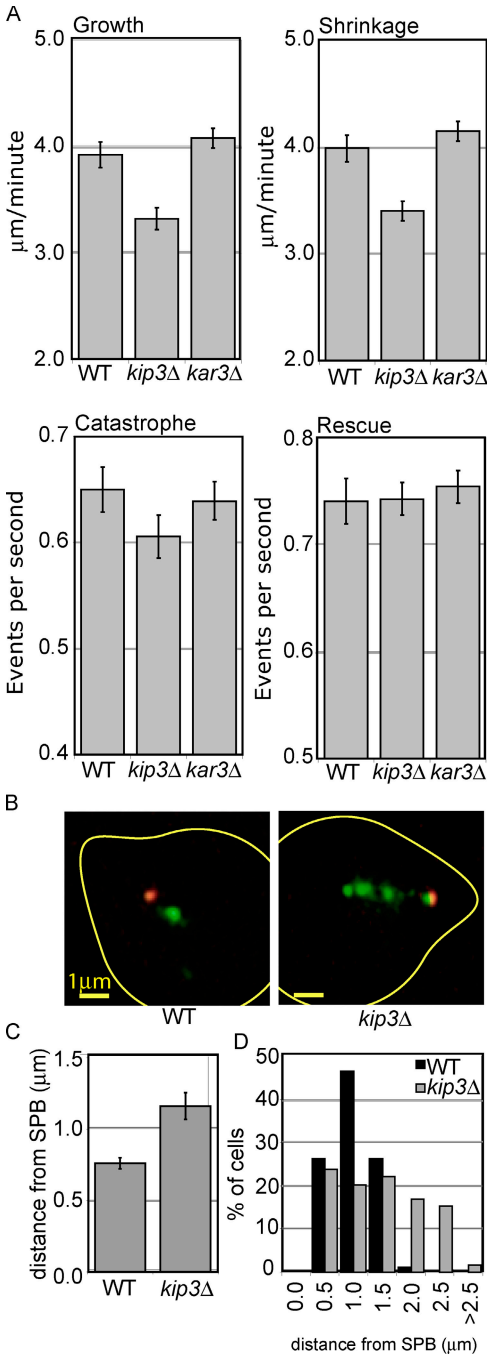

Figure 9. **Analysis of chromosome dynamics and MT length in G1 and α-factor–arrested cells.** (A) Comparison of MT growth, shrinkage, catastrophe, and rescue in wild-type, *kar3Δ*, and *kip3Δ* cells, as determined using automated tracking methods (Dorn et al., 2005). Error bars represent SD. $n > 2{,}000$. (B) Representative images in α-factor–arrested cells of Ndc80p-GFP localization (green) in wild-type or *kip3Δ* cells relative to Spc42p-CFP reference (red). (C) Quantitation of data in B that shows the mean distance of kinetochore foci to SPBs (in micrometers). Error bars are SEM from biological repeats. $n > 100$. (D) Distribution of the data shown in C.

with Machine Vision software. The software makes it possible to track TetO/TetR-tagged *CEN*s 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

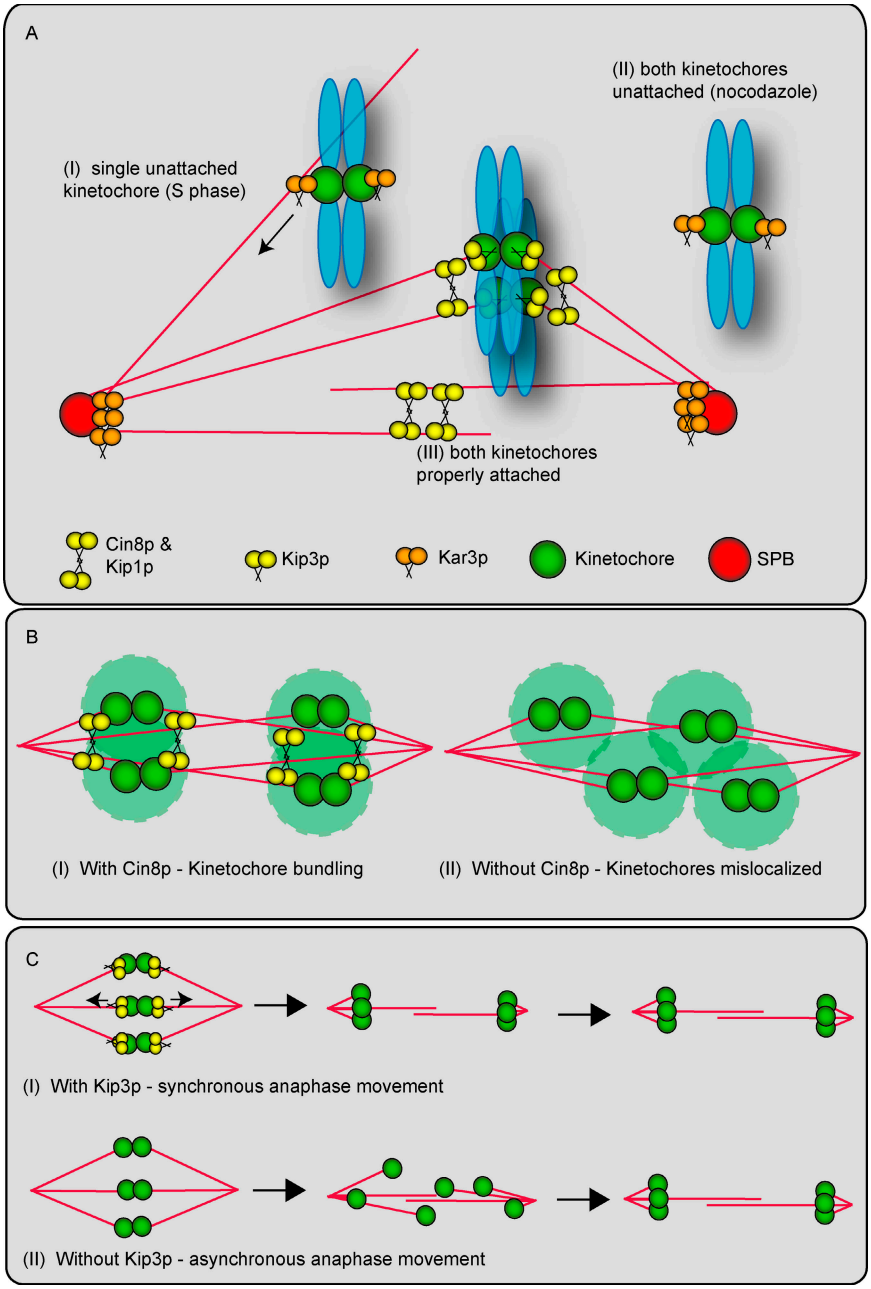

Figure 10. **Models of kinesin functions at budding yeast kinetochores.** (A) Kar3p is recruited to improperly attached (I) or unattached (II), but not bioriented (III), kinetochores, as well as to SPBs. In contrast, Cin8p, Kip1p, and Kip3p are present on bioriented kinetochores and on spindle MTs. (B) Model for Cin8p/Kip1p-mediated bundling of kinetochores. (C) Model depicting the role of Kip3p at MT plus ends in synchronizing the movement of chromatids toward spindle poles during anaphase A.

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 kinetochore 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 *CEN*s. 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 kMTs. 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 multistranded 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 cohesin. 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 by 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. KanMX 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). Proteins 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 kinetochore 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 cells. Fig. S2 shows a comparison of the probabilities of G1 dynamics data from wild-type and *kar3Δ* cells that are depicted graphically in Fig. 9. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200509101/DC1.

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