

The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the *Drosophila* S2 cell line

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Kinesins and dyneins play important roles during cell division. Using RNA interference (RNAi) to deplete individual (or combinations of) motors followed by immunofluorescence and time-lapse microscopy, we have examined the mitotic functions of cytoplasmic dynein and all 25 kinesins in *Drosophila* S2 cells. We show that four kinesins are involved in bipolar spindle assembly, four kinesins are involved in metaphase chromosome alignment, dynein plays a role in the metaphase-to-anaphase transition, and one kinesin is needed for cytokinesis. Functional

redundancy and alternative pathways for completing mitosis were observed for many single RNAi knockdowns, and failure to complete mitosis was observed for only three kinesins. As an example, inhibition of two microtubule-depolymerizing kinesins initially produced monopolar spindles with abnormally long microtubules, but cells eventually formed bipolar spindles by an acentrosomal pole-focusing mechanism. From our phenotypic data, we construct a model for the distinct roles of molecular motors during mitosis in a single metazoan cell type.

Introduction

Accurate chromosome segregation is essential for genome inheritance in eukaryotes. Microtubules, the polymers that compose the mitotic spindle, and microtubule-based motor proteins have vital roles in mitosis. Several members of the kinesin motor superfamily have been implicated in spindle formation, chromosome movement, and cytokinesis. Kinesin motors share in common a conserved ~340-aa enzymatic domain, but have different “tail” domains that mediate oligomerization, regulation of motor activity, and interactions with specific cargo (for review see Hirokawa, 1998; Goldstein, 2001; Vale, 2003). Cytoplasmic dynein, also implicated in mitosis in several organisms, is a dimer of two large (~530 kD) motor-containing polypeptides (the dynein heavy chains [DHCs]) and several associated smaller subunits (Hirokawa, 1998; Vale 2003).

The roles of microtubule motors in mitosis have been comprehensively analyzed in the budding yeast *Saccharomyces cerevisiae*, which has six kinesin genes belonging to five subfamilies and one cytoplasmic DHC gene. Gene disruption

experiments show that five yeast kinesins have important roles in spindle assembly and orientation, chromosome positioning and segregation, and spindle elongation during anaphase (for review see Hildebrandt and Hoyt, 2000). Dynein is not needed for spindle formation or chromosome movements, but plays an important role in orienting the mitotic spindle at the mother-bud neck before cytokinesis (for review see Bloom, 2001). Double or triple deletions of motor protein genes have revealed functional redundancy or antagonistic functions of different motors (Saunders and Hoyt, 1992).

In contrast to *S. cerevisiae*, the roles of microtubule-based motors in a single higher eukaryotic cell type have not been comprehensively examined. Moreover, it is precarious to extrapolate results from yeast to other eukaryotes that have very different mitotic spindle structures and chromosome behaviors. Furthermore, yeast has lost many kinesin genes that are present in other unicellular organisms, and some mitotic kinesins appear to be metazoan inventions (Vale, 2003).

Although a systematic investigation of motor proteins has not been undertaken in metazoan cells, insights into the functions of kinesins and cytoplasmic dynein in higher eukaryotes

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Abbreviations used in this paper: DHC, dynein heavy chain; dsRNA, double-stranded RNA; MTOC, microtubule-organizing center; NEB, nuclear envelope breakdown; Pav, Pavarotti; RNAi, RNA interference.

Table I. Kinesin superfamily genes in *Drosophila melanogaster*

Kinesin	Subfamily	S2 RNAi mitotic phenotypes	Protein reduction	Orthologues in			
				<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>C. elegans</i>	Mammals
Klp61F	BimC/Eg5	Monopolar	+	Cin8, Kip1	Cut7	KLP-14	Eg5
Klp10A	Kin I	Monopolar	+ ^a	-	-	KLP-7	KIF2
Klp59C	Kin I	None	+ ^a	-	-	KLP-7	KIF2
Klp59D	Kin I	None	+ ^a	-	-	KLP-7	KIF2
Klp67A	Kip3	Monopolar	-	Kip3	Klp5, Klp6	-	KIF18
Ncd	Kin C	Multipolar	+	Kar3	Pkl1, Klp2	KLP-15	KIFC1
CENP-meta	CENP-E	Chr. misalign	-	-	-	-	CENP-E
CENP-ana	CENP-E	None	-	-	-	-	CENP-E
Klp3A	Chromo-K	Chr. misalign	+	-	-	KLP-19	KIF4
Klp31E	Chromo-K	None	-	-	-	KLP-12	KIF21
Nod	Kid	Chr. misalign	-	-	-	-	Kid
Pavarotti	MKLP1	Cytokinesis	+	-	-	ZEN-4	MKLP1
KHC	KHC	None	+	-	Klp3	UNC-116	KIF5
Klp64D	Kin-II	None	-	-	-	KLP-20	KIF3A
Klp68D	Kin-II	None	-	-	-	KLP-11	KIF3B
CG17461	Homo Kin-II	None	- ^b	-	-	OSM-3	KIF17
Klp53D	Unc104	None	-	-	-	UNC-104	KIF1
Klp98A	Unc104	None	-	-	-	-	KIAA1590
Kinesin-73	Unc104	None	- ^b	-	-	KLP-4	KIF13
Klp38B	Unc104	None	-	-	-	KLP-6	KIF14
Klp54D	KIF12	None	- ^b	-	-	-	KIF12
CG9913	?	None	-	-	-	-	-
Subito	?	None	- ^b	-	-	-	-
Costal2	?	None	+	-	-	-	-
CG10845	?	None	- ^b	-	-	-	-
Dhc64C	Cytoplasmic DHC	Ana. delay	+	Dyn1	Dhc1	Dhc-1	DHC1

+, protein reduction after RNAi confirmed using specific antibodies (see Fig. 2); -, not tested because antibodies were unavailable.

^aImmunoblot will be described elsewhere (Rogers, G.C., and D. Sharp, personal communication).

^bExpression of these genes was not detected above background in S2 cell line by Affymetrix DNA microarray analysis (Hollien, J., and J. Weissman, personal communication).

have been obtained by a number of different approaches. Spindles can be formed de novo in *Xenopus* egg extracts, and the roles of motors has been explored by immunodepletion and re-addition in this in vitro system (Walczak et al., 1998). Function-blocking motor antibodies have been microinjected into fly embryo or mammalian tissue culture cells as another means of inhibiting kinesin function (Sharp et al., 2000c; Levesque and Compton, 2001). Small molecule inhibitors also have been developed against mammalian Eg5, a tetrameric kinesin (Mayer et al., 1999). RNAi of a few mitotic kinesins and cytoplasmic DHC have also been performed in *C. elegans* (Powers et al., 1998; Raich et al., 1998; Gonczy et al., 1999).

The most extensive genetic analyses have been performed in the fruit fly *Drosophila melanogaster* (in this paper, we use the *Drosophila* kinesin nomenclature followed by the most commonly used kinesin subfamily name; see Table I for closely related motors in other organisms). Mutations of several kinesins and cytoplasmic dynein cause mitotic defects, which include spindle formation defects (Klp61F [BimC/Eg5], Heck et al., 1993; Ncd [Kin C], Endow et al., 1994; Dhc64C [cytoplasmic DHC], Robinson et al., 1999), chromosome missegregation (Klp38B [Unc104], Alphey et al., 1997; Molina et al., 1997; Ruden et al., 1997; CENP-meta [CENP-E], Yucel et al., 2000), or cytokinesis failure

(Klp38B [Unc104], Ohkura et al., 1997; Pav [MKLP1], Adams et al., 1998). Some kinesin mutants affect specifically meiotic cell divisions (e.g., Subito [ungrouped], Giunta et al., 2002; Nod [Kid], Theurkauf and Hawley, 1992; and Klp3A [chromokinesin], Williams et al., 1995). However, functional analyses have not been reported for 12 kinesin genes, and redundancies of different kinesin genes have not been extensively tested because mutant isolation and genetic crossing are not as easy to perform as in yeast. Furthermore, the effect of loss-of-function has been investigated in different tissues for each kinesin mutant (early stage embryo, larval neuroblast, etc.). Therefore, it is difficult to build a complete picture of the involvement of kinesins and dynein in mitosis in higher eukaryotes.

The *Drosophila* S2 cell system is excellent for functional analysis of mitotic genes because they are very sensitive to double-stranded RNA (dsRNA)-mediated gene silencing (Clemens et al., 2000). We have reported previously that S2 cells spread on Con A-coated surfaces and execute normal mitosis (Rogers et al., 2002). This preparation provides outstanding imaging of the mitotic spindle and enables real-time observation of mitotic events by light microscopy.

In this work, we have screened all 25 *Drosophila* kinesins and cytoplasmic dynein for mitotic phenotypes in S2 cells using RNAi methods and microscopic observation, and have

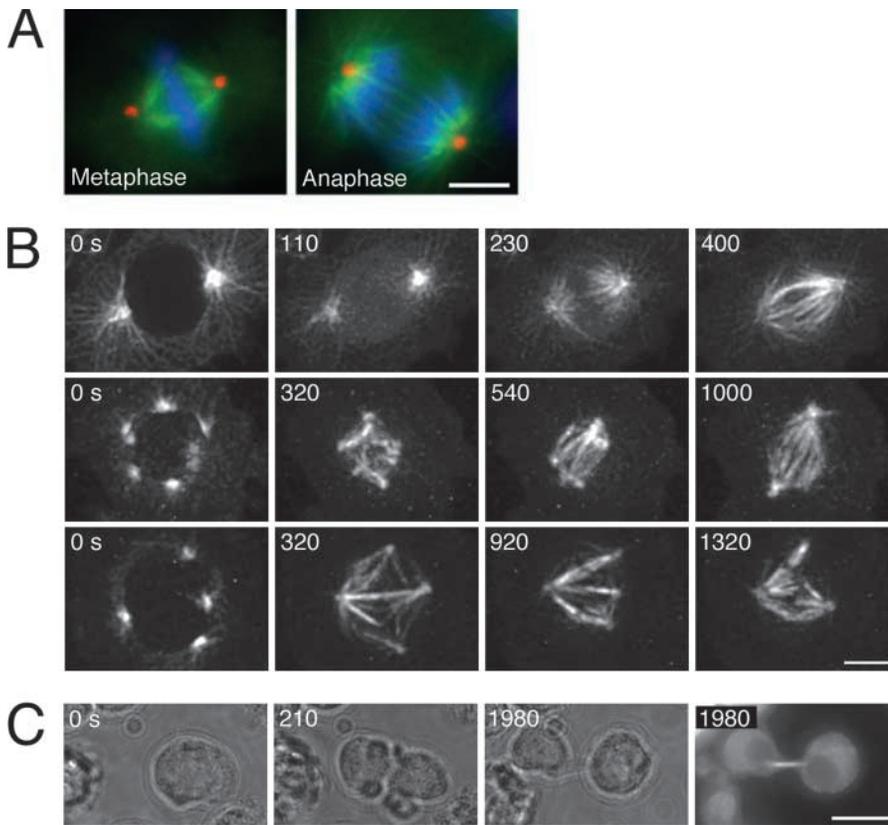


Figure 1. Mitosis of untreated S2.

(A) Untreated S2 cells expressing GFP-tubulin (green) were fixed and stained with γ -tubulin antibody (red) and Hoechst 33342 (blue). Bar, 5 μ m. (B) Time-lapse observation of GFP-tubulin in untreated cells, which have variable numbers of prophase MTOCs (Table II). Bipolar spindle was formed directly from two MTOCs (top) or in an extreme case, from eight MTOCs joined through fusion process (middle). The bottom cells failed to complete MTOC fusion, and a tripolar spindle was formed. Images were taken every 10 s (top; single optical section) or 20 s (middle and bottom; Z-projection, 0.66 μ m \times 10 and 0.58 μ m \times 10, respectively) using a spinning-disk confocal microscopy. See also Videos 1–3 (available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). Bar, 5 μ m. (C) Time-lapse phase-contrast images of a GFP-tubulin cell from anaphase to cytokinesis. Phase images were taken every 30 s using wide-field microscopy. The image of GFP-tubulin was obtained at the last frame (1980 s). See also Video 4. Bar, 10 μ m.

also performed simultaneous RNAi of multiple kinesins to investigate functional redundancy or coordination between different kinesin genes. We find that RNAi of eight kinesins and cytoplasmic dynein causes mitotic defects, including monopolar spindle formation, chromosome misalignment, anaphase delay, and cytokinesis failure. Some of the phenotypes are unexpected, and we also report the first live-cell imaging of several mitotic kinesin defects. This paper represents the first comprehensive analysis of microtubule-based motor function during mitosis in a single metazoan cell type.

Results

Kinesin superfamily genes in *Drosophila*

Before beginning functional analysis, we first identified and analyzed the sequences of all *Drosophila* kinesin superfamily proteins. A BLAST search was performed on the fly database using the conserved motor domain of fly conventional kinesin (1–340 aa). 25 genes emerged as exhibiting significant (E-value < 1e-15) sequence homology, one more than a previous search for kinesins in the *Drosophila* genome (Goldstein and Gunawardena, 2000). Sequence alignments of the motor and nonmotor domains with kinesins from other organisms (unpublished data) were used to assign the *Drosophila* kinesins to different subfamilies. This analysis identified clear subfamilies and mammalian homologues for 21 of the 25 genes (Table I). The remaining four are divergent kinesins that have no homology in their tail domains to kinesins in other organisms. Five kinesins may not be present or are expressed at very low levels in S2 cells (Table I). Nevertheless, we performed RNAi for all 25 kinesins so

as not to miss a potential mitotic involvement of a low copy number kinesin.

Characterization of mitosis in untreated S2 cells

Before investigating RNAi-induced mitotic phenotypes, we first characterized the process of cell division in untreated S2 cells. For clear imaging of mitosis, cells were adhered onto a Con A-coated dish (2 h) and then fixed and stained with anti-tubulin antibody and Hoechst 33342 (DNA staining dye). Untreated S2 cells proceed through mitosis in a characteristic progression of nuclear envelope breakdown (NEB), bipolar mitotic spindle formation, chromosome alignment at the metaphase plate, anaphase movement of chromosomes to the pole and spindle elongation, and cytokinesis (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). The mitotic index of untreated cells was usually \sim 3%, and it increased up to 15% when spindle formation was blocked by addition of a high concentration (50 μ M for 24 h) of colchicine, a microtubule polymerization inhibitor.

To visualize poles of the mitotic spindle, we stained with γ -tubulin antibody in cells stably expressing GFP-tagged tubulin (Rogers et al., 2002). Mitotic γ -tubulin foci always colocalized with microtubule-organizing centers (termed here γ -tubulin–MTOCs) from which astral microtubules emanate and may reflect the presence of centriole-containing centrosomes. In prophase before NEB, we found that the number of γ -tubulin–MTOCs was quite variable (Fig. 1; Fig. S1 B). Half of the cells contained three or more foci of γ -tubulin staining (Table II). Such abnormal γ -tubulin–MTOC numbers in prophase have been observed in many mammalian tu-

Table II. Quantitation of γ -tubulin–MTOC number determined by immunostaining

Prophase before NEB		Percentage of cells					
γ -Tubulin no.		1	2	3	4	>5	<i>n</i>
Untreated		2	46	12	12	27	41
Klp61F		0	33	6	28	33	18
Klp10A		10	45	10	20	15	20
Klp67A		3	31	3	25	38	32
Ncd		8	52	8	8	32	25
Monopolar spindle phase		Percentage of cells					
γ -Tubulin no.		1	2	3	4	>5	<i>n</i>
Untreated		-	-	-	-	-	-
Klp61F		97	3	0	0	0	30
Klp10A		85	15	0	0	0	20
Klp67A		100	0	0	0	0	30
Ncd		-	-	-	-	-	-
Bipolar or multipolar spindle phase		Percentage of cells					
γ -Tubulin no.		1	2	3	4	>5	<i>n</i>
Untreated		10	62	19	5	4	105
Klp61F		-	-	-	-	-	-
Klp10A		31	54	7	6	3	127
Klp67A		43	55	7	6	3	127
Ncd		12	41	9	15	24	34

Frequency percentage of the number of γ -tubulin punctate signals observed for indicated RNAi cells. Signals were detected only during mitosis by our staining method. Prophase was characterized by partially condensed chromosomes and γ -tubulin foci near nuclear envelopes, whereas monopolar or bipolar spindles were recognized by GFP-tubulin signals. *n*, number of cells scored.

mor cell lines (Marx, 2001), and may reflect the long-term propagation and immortalization of the S2 cell line. After NEB, the proportion of cells with three or more γ -tubulin–MTOCs decreased to 30%, suggesting that the MTOC fusion took place during spindle formation. To gain more insight on this abnormal spindle formation process, we performed time-lapse observation of GFP-tubulin in living cells using spinning-disk confocal microscopy. Cells that had two MTOCs in prophase (Fig. 1 B, top row; Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>) successfully formed bipolar spindle within 5 min after NEB. Cells with more than three MTOCs in prophase formed either bipolar spindles through the fusion of MTOCs (Fig. 1 B, middle row; Video 2) or multipolar spindles (Fig. 1 B, bottom row; Video 3). Even in the latter case, MTOC fusion partially took place, as four MTOCs initially observed in prophase fused into three. Although phototoxicity prevented continuous live observation from prophase through to late mitotic stages, fixed-cell images suggest that chromosomes can be segregated equally to daughter cells by multipolar spindles (Fig. S1 B, bottom right cell).

We also performed time-lapse imaging of S2 cell divisions by phase microscopy on glass without Con A. These observations revealed that cells proceed rapidly through anaphase (~4 min), but proceed slowly through cytokinesis; daughters can remain connected by a cytoplasmic bridge for >30 min (Fig. 1 C; Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). On Con A–coated surfaces, cells did not complete cytokinesis,

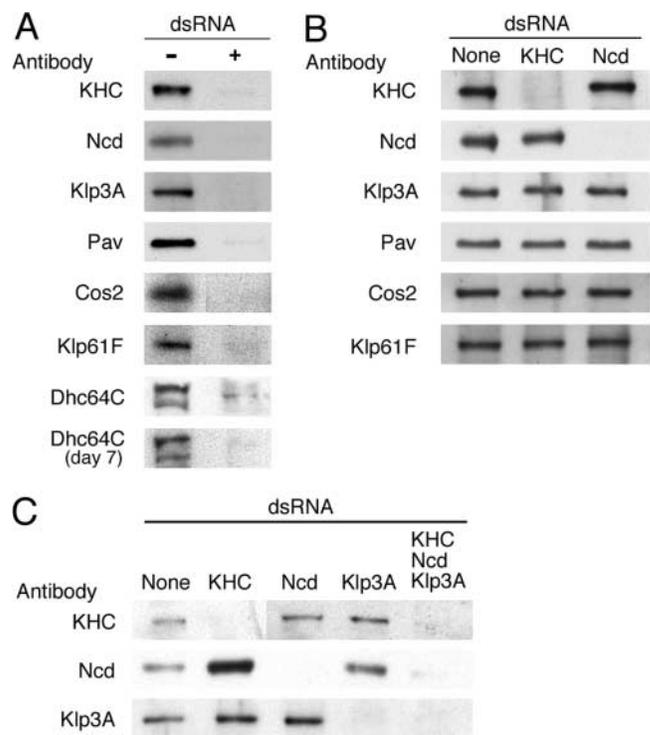


Figure 2. Reduction of kinesins and dynein after RNAi. (A) Reduction of seven microtubule motor proteins after dsRNA addition. Immunoblotting of KHC, Ncd, Klp3A, Pav, Cos2, Klp61F, and Dhc64C for cultures subjected to RNAi for 3 (Pav and Klp61F), 4 (others), or 7 (Dhc64C) days. All lanes contained an equal load of total proteins (not depicted). Quantitative analyses (not depicted) indicated >90% of reduction of each protein. (B) Specificity of RNAi. dsRNA against KHC or Ncd did not affect the levels of the other five kinesins. (C) Triple RNAi (KHC/Ncd/Klp3A, right lane) yielded efficient protein reduction of all three kinesins.

probably because the tight adherence to the dish interfered with complete invagination.

RNAi of microtubule motor proteins in the S2 cell line

To investigate the roles of motors in mitosis, we treated S2 cells with unique motor protein dsRNA and cultured them for 3–4 d without Con A, then adhered them to Con A–coated coverslips, and examined the mitotic spindle and chromosomes by anti-tubulin and Hoechst DNA staining, respectively. To confirm the decrease of protein levels, immunoblotting was performed with nine specific anti-kinesin antibodies and an anti-dynein antibody. Levels of these motors decreased dramatically (90–99%) 4 d after dsRNA treatment (Fig. 2 A; Table I). We confirmed that RNAi directed at one kinesin did not affect the levels of other kinesins (Fig. 2 B). We also confirmed that simultaneous knock-down of two or three different kinesin proteins by mixing dsRNAs worked almost as efficiently as single RNAi (Fig. 2 C). Furthermore, DNA microarray analysis revealed that expression of five kinesins are not detected above background in untreated S2 cells (Table I; Hollien, J., and J. Weissman, personal communication).

As an overview of our results, RNAi of eight kinesins and the cytoplasmic DHC caused mitotic defects, but did not produce discernible changes in the morphology or microtubu-

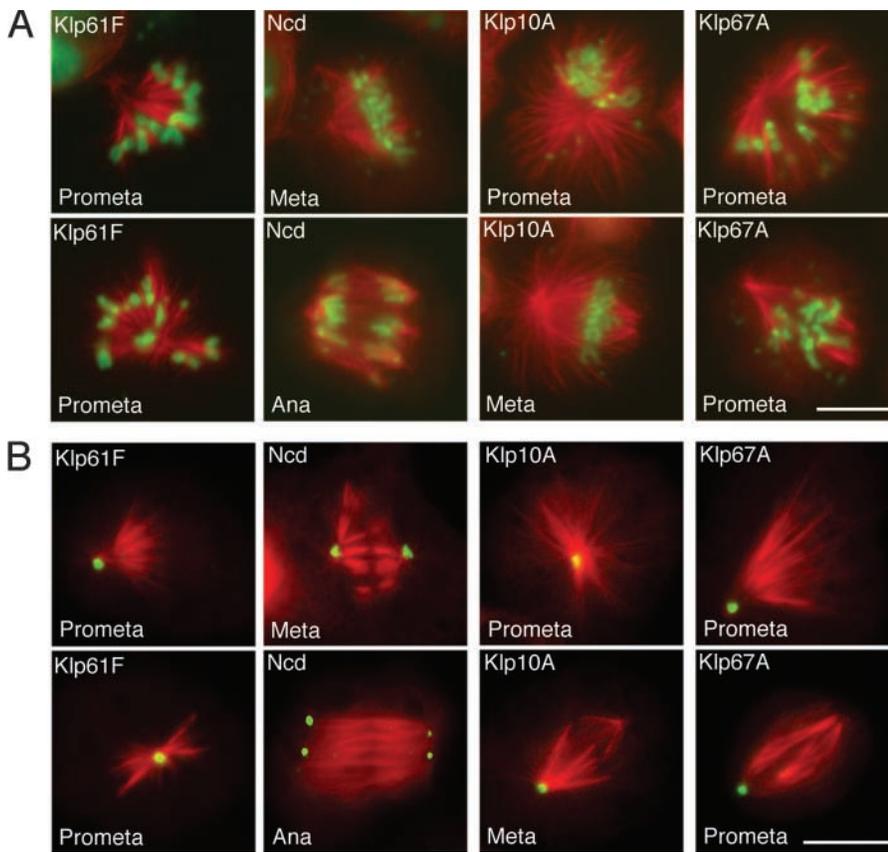


Figure 3. Bipolar spindle formation defects caused by Klp61F [BimC/Eg5], Ncd [Kin C], Klp10A [Kin I], and Klp67A [Kip3] RNAi. (A) Abnormal spindle formation after RNAi of indicated four kinesins. Cells were fixed and stained by anti-tubulin antibodies (red) and Hoechst 33342 (DNA; green) at d 3 (Klp61F) or d 4 (Ncd, Klp10A and Klp67A). The majority of the mitotic cells had monopolar spindles after Klp61, Klp10A, or Klp67A RNAi, whereas reduction of Ncd induced multipolar spindle formation. In the case of Klp10A or Klp67A RNAi, long bipolar spindles were also observed. (B) γ -Tubulin staining (green) after RNAi. Spindle (red) was visualized by GFP-tubulin expression. Bipolar spindle formed in Klp10A and Klp67A RNAi cells often had only one of the two poles stained for γ -tubulin. Quantitative data and additional cell images are presented in Table II, Table III, and Figs. S2–S8. Prometa, prometaphase; Meta, metaphase; Ana, anaphase. Bars, 10 μ m.

bule pattern in interphase cells (Table I). No mitotic phenotype was detected for the other 17 kinesins, including the five whose expression was not detected by DNA microarray analysis. In the following sections, we describe the mitotic defects that arose from the RNAi treatments.

Klp61F [BimC/Eg5], Klp10A [Kin I], Klp67A [Kip3], and Ncd [Kin C] are required for proper spindle formation during prometaphase

RNAi of four kinesins (Klp61F [BimC/Eg5], Klp10A [Kin I], Klp67A [Kip3], and Ncd [Kin C]) produced distinct types of abnormal spindles (Fig. 3; Figs. S2–S6, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). In Klp61F [BimC/Eg5] RNAi cells, the mitotic index was fourfold higher than control cells, and virtually all mitotic cells (97%; $n = 102$) had monopolar spindles with a single γ -tubulin staining foci at the MTOC, and either a single chromosome mass (72%; Fig. 3 A, top) or scattered chromosomes (25%, Fig. 3 A, bottom; also see Table II and Table III, and Fig. S2). This phenotype is consistent with the fly Klp61F [BimC/Eg5] mutant (Heck et al., 1993). From time-lapse imaging of GFP-tubulin in Klp61F [BimC/Eg5] RNAi cells, it was apparent that the two or more MTOCs in prophase fused into one monopolar spindle after NEB (Fig. 4 A; Video 5). The monopolar spindle was stable for >30 min (Video 6).

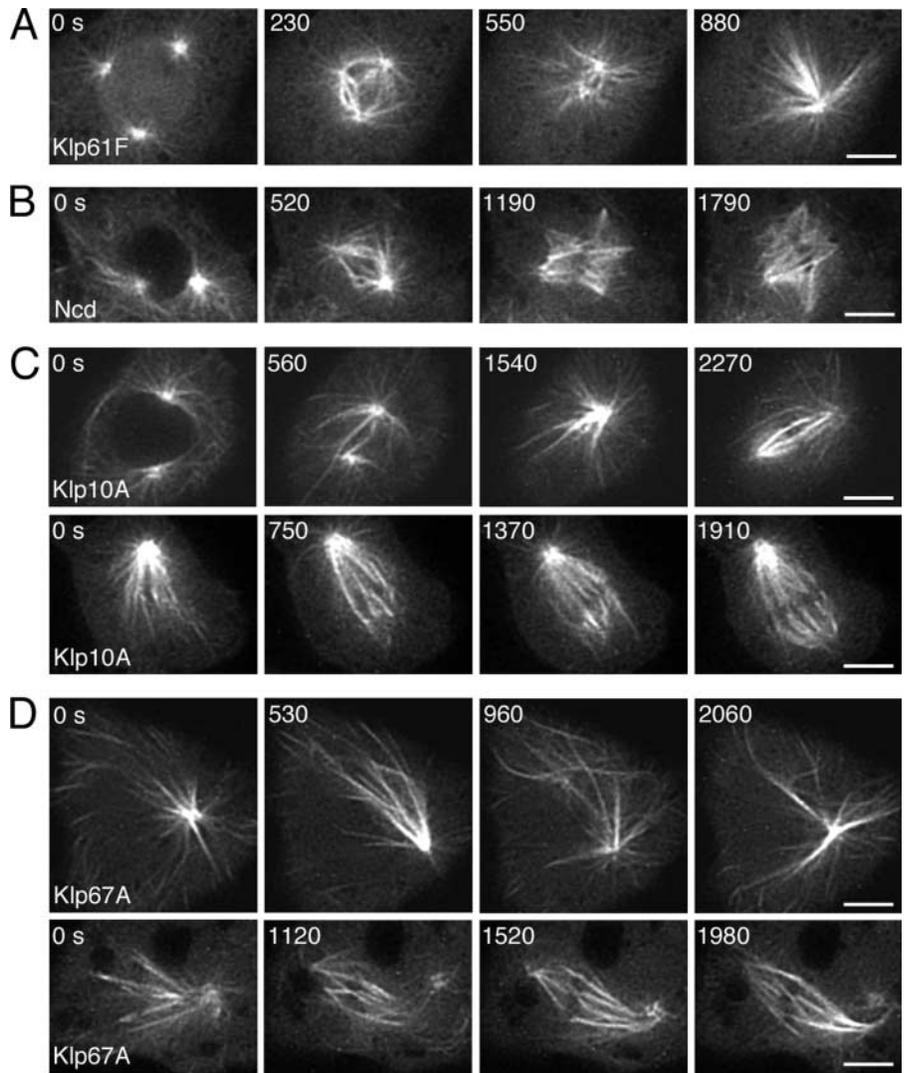
Depletion of Ncd, the sole Kin C-type kinesin in *Drosophila*, led to the formation of a spindle with unfused poles (72% of mitotic cells [$n = 105$]; Fig. 3 A; Fig. S3 E) and multiple γ -tubulin foci (Fig. 3 B and Fig. S3 F). In contrast

to untreated cells, the distribution of γ -tubulin–MTOC number after NEB was similar to that in prophase, suggesting that MTOC fusion rarely took place in the absence of Ncd [Kin C] (Table II). Microtubules also were focused to poles that had no γ -tubulin staining (Fig. S3 F). Chromosomes generally were aligned in the middle of each spindle, anaphase cells were clearly observed, and mitotic progression was not severely delayed (Table III). In some anaphase cells, more than three chromosome masses were visible, which may have been independently captured and segregated by more than two bipolar-like spindles. The frequency of interphase cells with more than three small nuclei (15%) was higher than control (0.3%), which is likely to be the consequence of nuclear envelope reformation around each separate chromosome mass (Fig. S3 E).

Time-lapse imaging of Ncd [Kin C]–depleted cells showed that the MTOCs in prophase frayed to form more than four independent bipolar spindle-like structures in metaphase (Fig. 4 B; Video 7, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). The newly appeared poles did not have astral microtubules, suggesting that they are not γ -tubulin–MTOCs. When mitosis began with more than three MTOCs, they did not fuse together, and spindles with multiple MTOCs were formed in metaphase (Video 8). Thus, live-cell imaging confirms that MTOC fusion does not occur in Ncd [Kin C] RNAi cells, and that additional acentrosomal poles are formed.

Simultaneous inhibition of BimC/Eg5 and minus end-directed motors (either Kin C or dynein) has been shown to rescue bipolar spindle formation (Saunders and Hoyt,

Figure 4. Real-time imaging of GFP-tubulin during the abnormal spindle formation in RNAi cells. Images were taken every 10 s at single optical section using spinning-disk confocal microscopy. (A) A Klp61F [BimC/Eg5] RNAi cell. A monopolar spindle is formed through MTOC fusion. (B) A Ncd [Kin C] RNAi cell. Multiple spindles are formed during prometaphase. (C) Klp10A [Kin I] RNAi cells. Monopolar spindle is initially formed, but is converted to bipolar spindle through acentrosomal pole fusion. (D) Klp67A [Kip3] RNAi cells. A monopolar spindle is maintained for 34 min (top panels), or is eventually converted to a monastral bipolar spindle (bottom panels). See also Videos 5–12 (available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). Bars, 5 μ m.



1992; Sharp et al., 2000b). To investigate whether this holds true for S2 cells, we performed double RNAi of Klp61F [BimC/Eg5] and Ncd [Kin C], or Klp61F [BimC/Eg5] and Dhc64C [DHC] (Fig. S4). After such double RNAi, normal bipolar spindles were observed in only 2% ($n = 50$) and 5% ($n = 58$) of the mitotic cells, respectively. In the case of Klp61F [BimC/Eg5]/Ncd [Kin C] double RNAi, monopolar spindles (unpublished data) or nearly monopolar spindles, where γ -tubulin foci came close together but not completely fused (Fig. S4 G b), were observed in 12 and 40% of the mitotic cells, respectively. The other 43% of the mitotic spindles were similar to those seen after Ncd [Kin C] single RNAi, including ones with congressed chromosomes (Fig. S4 G a). Strikingly, 20% of the spindles were segregating chromosomes in anaphase (bottom three cells). As metaphase-like or anaphase cells were rarely, if ever, seen after Klp61F [BimC/Eg5] single RNAi (Table III), this finding suggests that Kin C inhibition rescues chromosome segregation in BimC/Eg5-depleted cells. In contrast, double RNAi of Klp61F [BimC/Eg5]/Dhc64C [DHC] yielded only monopolar spindles (98%; Fig. S4 H). The phenotype of Dhc64C [DHC] single RNAi will be described later in this paper.

In Klp10A [Kin I] RNAi cells, spindle microtubules were longer and their density was higher than in control cells (Fig. 3 A; Fig. S5 I), which is consistent with other reports showing microtubule-destabilizing activity of Kin I motors (Desai et al., 1999; Rogers, G.C., and D. Sharp, personal communication). The mitotic index (5.0%) was higher than controls, and monopolar spindles with a single chromosomal mass were prevalent (57% of mitotic cells; $n = 102$). However, in contrast with Klp61F [BimC/Eg5] RNAi-treated cells, bipolar spindles with congressed or separating chromosomes were observed in many (37%) mitotic, Klp10A [Kin I] RNAi-treated cells (Table III). These bipolar spindles were often >50% longer than those in control cells. Moreover, the morphology of bipolar spindle was often asymmetric, and one pole of the spindle did not have astral microtubules.

Cells treated with dsRNA of Klp67A [Kip3] exhibited similar, but not identical, phenotypes to Klp10A [Kin I]. Like Klp10A [Kin I] RNAi treatment, the spindle microtubules were abnormally long, often reaching the cortex, and monopolar spindles were common (74%, $n = 119$; mitotic index = 9.6%; Fig. 3 A; Table III; Fig. S5 J). However, unlike the findings for Klp10A [Kin I], chromosomes were often dispersed and did not form a single com-

Table III. Quantitation of prometaphase/metaphase and anaphase cells in mitosis after RNAi

dsRNA	Mitotic index	Monopolar	Prometaphase and metaphase	Anaphase	<i>n</i>
	%	%	%	%	
None	3.0	<5	61	14	168
Klp61F	12.0	97	3	0	102
Ncd	3.4	<5	63	18	59
Klp10A	5.0	57	37	12	102
Klp67A	9.6	74	18	1	119

Frequency percentage of each mitotic phase after RNAi is shown. Prometaphase and metaphase cells are defined as any pre-anaphase cell with a bipolar or multipolar spindle. *n*, number of mitotic cells observed for each RNAi sample.

pact mass. Furthermore, the longer astral microtubules observed after Klp10A [Kin I] RNAi were not observed after Klp67A [Kip3] RNAi; instead, the abnormally long microtubules were only observed on the chromosomal side from the spindle pole. Abnormally long bipolar spindles also were observed in 18% (*n* = 119) of the mitotic cells, but the chromosomes were rarely fully congressed to a metaphase plate, and few (<1%) anaphase-like cells were detected. These results strongly suggest that Klp67A [Kip3] acts to destabilize microtubules during mitosis like Klp10A [Kin I], but its cellular role differs from Kin I-type motors (see Discussion).

A mechanism for monopolar to bipolar spindle conversion in Klp10A [Kin I] and Klp67A [Kip3] RNAi-treated cells

We wished to further understand the basis of the two populations of monopolar and bipolar spindles in Klp10A [Kin I] and Klp67A [Kip3] RNAi-treated cells. First, we examined γ -tubulin–MTOC distributions in these cells by staining with anti- γ -tubulin antibody in cells stably expressing GFP-tagged tubulin. Interestingly, 31% (*n* = 127) and 43% (*n* = 80) of the bipolar spindles after Klp10A [Kin I] and Klp67A [Kip3] RNAi, respectively, had a single γ -tubulin foci at one pole. Such bipolar spindles were also observed in a minor population of untreated metaphase cells (10%; *n* = 105; Fig. 3 B; Fig. S1 B; Fig. S6; Table II). The pole without γ -tubulin did not develop astral microtubules. This indicates that Klp10A [Kin I] or Klp67A [Kip3] RNAi cells, and also some of untreated cells, have monastral (one γ -tubulin-staining MTOC) bipolar spindles.

Time-lapse observations of GFP-tubulin in Klp10A [Kin I] RNAi cells shows that monopolar spindles with elongated microtubules were formed by the fusion of MTOCs after NEB (Fig. 4 C; Video 9 and Video 10, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). However, in all seven cells observed, the monopolar spindle converted to bipolar spindles with a dark mass in the middle, which appears to be congressed chromosomes. The newly formed pole lacked astral microtubules. The origin of the newly focused microtubules is not clear. Microtubules released from the centrosome may be captured and stabilized by chromosomes. Alternatively, chromosomes may nucleate these microtubules, as observed during meiotic spindle as-

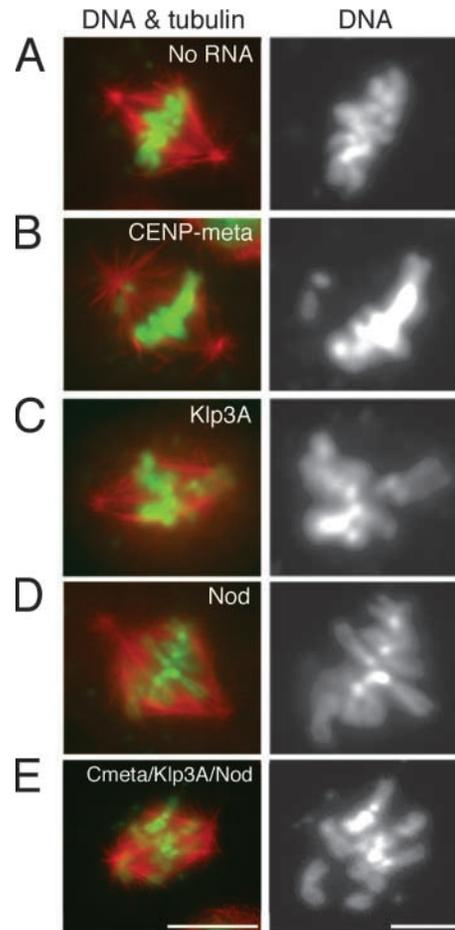


Figure 5. Chromosome congression defects after CENP-meta [CENP-E], Klp3A [chromokinesin], and Nod [Kid] RNAi. Cells were treated with dsRNA targeting for indicated genes and were fixed and stained by anti-tubulin antibodies (red) and Hoechst 33342 (green) at d 4. Bar in the left column represents 10 μ m. Magnified images of the chromosomes are shown in the right column (Bar, 5 μ m). Control nontreated cell is shown in A. Misaligned chromosomes were frequently detected after CENP-meta (B) and Klp3A (C) RNAi, whereas stretched chromatin was observed after Nod RNAi (D). More severe misalignment phenotypes appeared when RNAi of a kinetochore kinesin and two chromokinesins were combined in E (see also Table IV). Additional images are presented in Fig. S9 and Fig. S10.

sembly (for review see Compton, 2000). From these results, together with images of γ -tubulin staining, we conclude that chromosome-directed, acentrosomal pole focusing is efficiently induced after monopolar spindle formation in Klp10A [Kin I] knockdown cells.

Similar observations were made in Klp67A [Kip3]-depleted cells. Monopolar spindles were initially formed, chromatin directed new microtubule growth and/or stabilization, and then microtubules became reorganized into an acentrosomal pole (Fig. 4 D; Video 12, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). However, some monopolar spindles remained stable for >30 min (Fig. 4 D, top; Video 11). The spindle microtubules occasionally dissociated from the poles (Fig. 4 D, bottom; Fig. S6 L); the mechanism of this detachment is not clear.

Table IV. Quantitation of prometaphase and metaphase cells in mitosis after RNAi

dsRNA	Mitotic index	Prometaphase ^a	Metaphase ^b	Anaphase	n
	%	%	%	%	
None	3.0	23	46	14	306
CENP-meta	3.5	37	28	11	100
Klp3A	3.5	36	25	16	107
Nod	3.6	34	33	12	105
CENP-meta/Klp3A	3.2	60	10	12	109
CENP-meta/Nod	3.2	50	18	17	100
Klp3A/Nod	3.4	67	15	4	114
CENP-meta/Klp3A/Nod	3.2	72	3	13	67

Frequency percentage of prometaphase, metaphase and anaphase for mitotic cells after RNAi shown. *n*, number of mitotic cells observed for each RNAi sample.

^aPrometaphase, at least one misaligned chromosome observed in the bipolar spindle.

^bMetaphase, chromosomes are all aligned (congressed) at the metaphase plate in the bipolar spindle. In the case of Nod RNAi, fully congressed chromosomes still display abnormal orientation of the chromosome arms away from the metaphase plate.

Then, we investigated whether BimC/Eg5 and minus end-directed motors (Kin C and dynein) are involved in the monastral bipolar spindle formation by double RNAi analyses. The acentrosomal recovery of a bipolar spindle observed in Klp10A [Kin I] and Klp67A [Kip3] RNAi cells was completely dependent on Klp61F [BimC/Eg5] (>98% monopolar spindles with long microtubules observed after such double RNAi treatment; Fig. S7, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). Cytoplasmic dynein has been shown to be required for acentrosomal pole formation in *Xenopus* egg extract system by antibody inhibition (Heald et al., 1996). However, RNAi reduction of Dhc64C [DHC] in conjunction with RNAi of Klp10A [Kin I] or Klp67A [Kip3] did not affect the frequency or morphology of monastral bipolar spindles in S2 cells (50% [*n* = 38] and 25% [*n* = 53]) of all bipolar spindles, respectively; Fig. S8 O). On the other hand, spindle structure with variable numbers of unfocused poles were seen after Klp10A [Kin I]/Ncd [Kin C] and Klp67A [Kip3]/Ncd [Kin C] RNAi, as observed for Ncd [Kin C] single RNAi (except for the appearance of longer microtubules as well; Fig. S8, P and Q). Monopolar spindles were not observed (*n* > 50), most likely because reduction of Ncd [Kin C] dominantly inhibited MTOC fusion at the beginning of prometaphase. Therefore, it could not be directly tested whether Ncd [Kin C] is required for the monopolar to bipolar conversion step.

RNAi of a kinetochore kinesin and two chromokinesins additively affects metaphase chromosome alignment

In addition to Klp67A [Kip3] RNAi, depletion of three kinesins, CENP-meta [CENP-E], Klp3A [chromokinesin], and Nod [Kid], caused chromosome misalignment at the metaphase plate. RNAi of Klp3A [chromokinesin] (Williams et al., 1995) and the kinetochore-localized CENP-meta [CENP-E] kinesin (Yucel et al., 2000) exhibited indistinguishable phenotypes: an increased frequency of prometaphase-like cells with bipolar spindles and misaligned chromosomes that were detached from the metaphase plate (Fig. 5, B and C; Table IV; Fig. S9, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). The mitotic indices (3.5%) were only slightly higher than the control, and spindle morphology looked normal. Similarly, RNAi of Nod, a DNA-binding kinesin reported to be essential for chromosome alignment in meiosis I (Theurkauf and Hawley, 1992), had no effect on spindle morphogenesis but showed higher frequencies of prometaphase-like cells (Fig. 5 D and Fig. S9 T). In this case, complete separation of chromosomes from the metaphase plate was rare, but most chromosome arms were extended along the spindle axis toward the poles.

Depletion of the aforementioned three kinesins individually did not completely inhibit congression to the metaphase plate, as only one or two unaligned chromosomes were visible in most cells and >25% of the mitotic cells had normal metaphase-like configurations (Table IV). However, reduc-

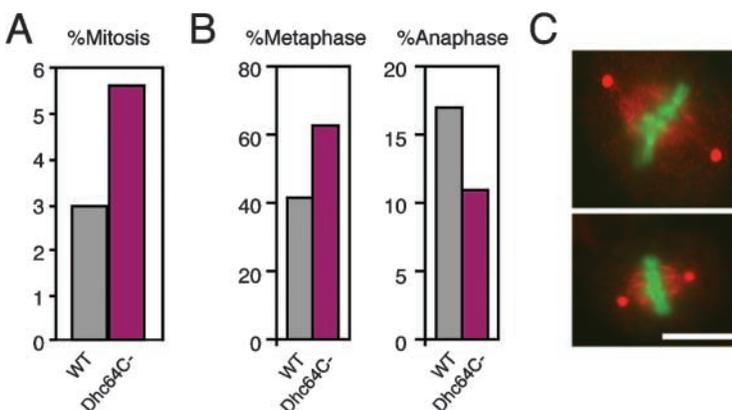


Figure 6. Accumulation of metaphase by cytoplasmic dynein knockdown. (A) Mitotic index of Dhc64C [DHC] RNAi at d 4 was 1.9-fold higher than controls. (B) Percentage of metaphase and anaphase in mitotic cells. Metaphase cells were accumulated, whereas anaphase was less frequently observed in RNAi samples. (C) Metaphase cells with congressed chromosomes. Cells treated with dsRNA for Dhc64C [DHC] were fixed and stained by γ -tubulin (red) and Hoechst 33342 (green) at d 4. Spindle-like structure was visualized by the overexposure of anti- γ -tubulin signals. Majority of the metaphase cells (64%; *n* = 22) had two punctate signals of γ -tubulin like untreated cells, and no apparent morphological defects in the spindle were detected (not depicted). Bar, 10 μ m.

tion of one kinesin might be compensated for by the two other chromosome-associated kinesins. To test this possibility, we performed simultaneous RNAi of two or three of these kinesins. Each double RNAi pair had more severe chromosome misalignment phenotypes than single RNAi treatments (Table IV; Fig. S10, U–W, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>), although 10–18% of the metaphase-like cells appeared normal. However, simultaneous RNAi of all three genes greatly reduced the number of cells with normal metaphase chromosomal alignments (3% of mitotic cells; Fig. 5 E, Table IV, and Fig. S10 X), and triple RNAi cells that underwent anaphase often had lagging chromosomes (unpublished data). These results indicate that chromosome congression is ensured by the independent actions of several chromosomal kinesins in the S2 cell line.

CENP-ana (Yucel et al., 2000) and Klp31E potentially affect chromosome behavior, as they have sequence similarity to CENP-meta [CENP-E] and chromokinesin, respectively (Table I). However, RNAi of these kinesins did not yield mitotic phenotypes, and double RNAi of CENP-ana and CENP-meta yielded an identical phenotype and mitotic index to CENP-meta single RNAi (unpublished data). Thus, S2 cells appear to use CENP-meta as their primary kinetochore kinesin for congression.

RNAi of cytoplasmic dynein induces metaphase arrest, but does not affect spindle morphology, centrosome positioning, or chromosome congression

Genetic analyses and antibody inhibition experiments in *Drosophila* suggested that cytoplasmic DHC (Dhc64C) is involved in spindle formation (Robinson et al., 1999; Sharp et al., 2000b), chromosome movement in prometaphase and anaphase A (Sharp et al., 2000a), and transport of Rod, a spindle checkpoint protein (Wojcik et al., 2001). After Dhc64C [DHC] RNAi, we did not observe defects in spindle morphology, γ -tubulin localization, or chromosome segregation, but detected a 1.5-fold accumulation of cells with fully congressed metaphase chromosomes and a higher mitotic index (5.6%; Fig. 6). The congressed chromosome mass was more compact than normal metaphase, most likely reflecting the prolonged metaphase state. This phenotype indicates that the metaphase-to-anaphase transition is significantly delayed by the absence of Dhc64C [DHC], but that chromosome movements and spindle integrity are not affected. Identical anaphase delay phenotypes were observed for both 4 and 7 d of dsRNA treatment (unpublished data).

Formation and maintenance of the central spindle during cytokinesis requires the kinesin Pavarotti [MKLP1]

As expected from the previous RNAi analysis in S2 cells (Somma et al., 2002) and mutant fly analysis (Adams et al., 1998), RNAi of Pavarotti (Pav; the orthologue of MKLP1/ZEN-4/CHO1) caused cytokinesis defects and showed multiple nuclei in interphase cell at very high frequencies (75%; Fig. 7 A). RNAi of the other kinesins, including Klp3A [chromokinesin] and Klp38B [Unc104], mutants of which have been shown to exhibit cytokinesis defects in testes and follicle cells, respectively (Williams et al., 1995; Ohkura et al., 1997), did not produce cytokinesis defects in S2 cells.

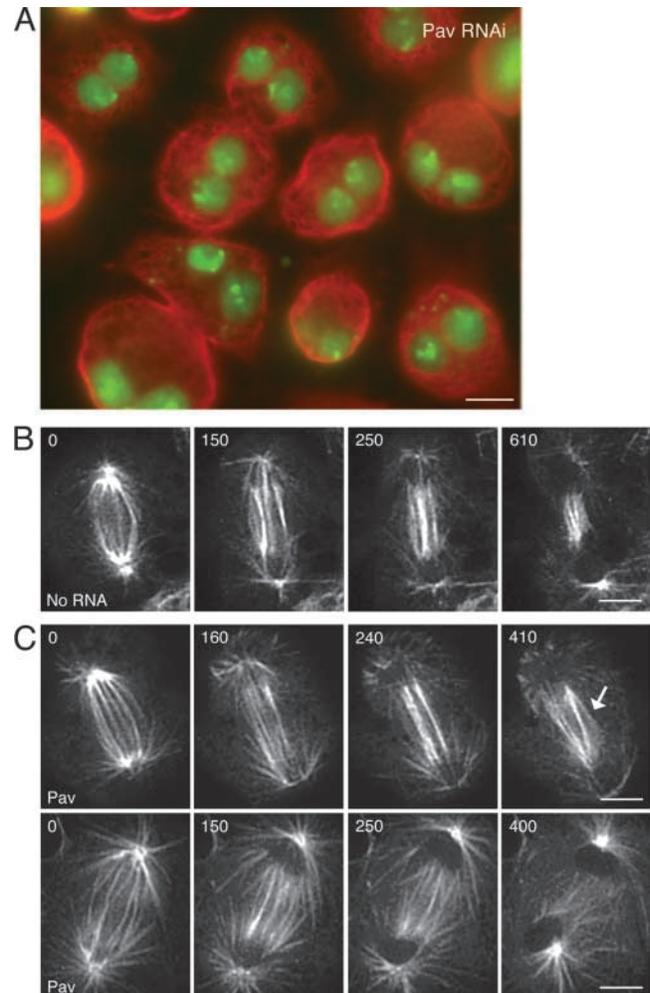


Figure 7. Pav [MKLP1] functions in the formation and maintenance of the central spindle bundling during cytokinesis. (A) Cells with binuclei are abundant after Pav RNAi. Cells were fixed and stained by anti-tubulin antibodies (red) and Hoechst 33342 (green) at d 3. Bar, 10 μ m. (B and C) Real-time imaging of GFP-tubulin during cytokinesis in an untreated cell (B) and Pav RNAi cells (C) on Con A-coated dish. Arrow indicates the microtubules splayed apart from the bundle. See also Videos 13–15 (available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). Bars, 5 μ m.

Because time-lapse observations of GFP-tubulin from anaphase through interphase have not been made in Pav and its orthologue mutants, we undertook such analysis. The untreated S2 cells on Con A-coated dish did not complete cytokinesis, but they maintained a central microtubule bundle for >20 min and partially invaginated (Fig. 7 B; Video 13, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>). In sharp contrast, time-lapse observations of Pav [MKLP1] RNAi cells showed clear defects in central spindle bundling and invagination. Seven out of 10 cells treated with dsRNA against Pav transiently made central spindle-like structures, but then the microtubules splayed apart (Fig. 7 C, arrow in 410 s), and membrane invagination was not initiated (Fig. 7 C; Video 14). The other three recorded cells never showed central spindle bundling (Fig. 7 C, bottom panels; Video 15). These results indicate that Pav [MKLP1] functions both in the formation and maintenance of the central spindle.

Discussion

In this work, we investigated the mitotic roles of all 25 *Drosophila* kinesins and cytoplasmic dynein in S2 cells using RNAi methods and microscopic observation. We found that RNAi of eight kinesins and cytoplasmic DHC caused a variety of mitotic defects, including monopolar spindle formation, chromosome misalignment, anaphase delay, and cytokinesis failure. We cannot absolutely rule out the involvement of other motors in mitosis due to several experimental caveats. First, due to unavailability of antibodies, we could not confirm the reduction of 16 motors by RNAi. However, for the 10 motors that were examined, we confirmed drastic (>90%) protein reductions by RNAi (Fig. 2; Table I). Moreover, our laboratory has confirmed similar reductions of 24 other cytoskeleton-related proteins by immunoblotting and/or immunostaining after dsRNA treatment, regardless of the appearance of phenotypes, and we have not encountered a case where protein levels were not dramatically reduced. Based on such results, we feel that it is highly likely that all motors were reduced to very low levels by RNAi. A second caveat is that even the small amount of residual protein that remains after RNAi treatment may be sufficient for cellular function. A third caveat is that functional redundancy may obscure a mitotic phenotype for some motors. This could be potentially examined by exhaustive double or triple RNAi for many combinations of motor proteins.

Our results provide several general insights into the roles of molecular motors in mitosis. First, cells appear to have redundant or alternate mechanisms for completing mitosis, despite the absence of a kinesin motor. Our experiments, for example, uncovered a novel rescue mechanism for converting monopolar to bipolar spindles by an acentrosomal pole-focusing mechanism (discussed below). By performing multiple RNAi, we also could show that several kinesins act in a partially redundant manner to ensure congression of chromosomes to the metaphase plate. Only three kinesins appear to be absolutely essential for completing mitosis; Klp61F [BimC/Eg5] and Klp67A [Kip3] RNAi cells could not proceed into anaphase, and Pav [MKLP1]-depleted cells could not execute cytokinesis. The phenotypes from our comprehensive RNAi analysis enable us to derive a model for the roles of microtubule-based motors in the sequential steps of mitosis in S2 cells (Fig. 8), the details of which will be described in the following paragraphs.

Functions of mitotic motor proteins: mitotic spindle assembly

The establishment of spindle bipolarity, the first step of mitosis, requires Klp61F [BimC/Eg5], a bipolar homotetrameric kinesin. Our time-lapse imaging results are consistent with previous proposals that Klp61F [BimC/Eg5] cross-links and slides apart antiparallel spindle microtubules (for review see Sharp et al., 2000c).

Ncd [Kin C] depletion causes defects in spindle pole integrity. MTOC fusion is inhibited, and multiple poles, frequently acentrosomal, are produced. Time-lapse observation of GFP-tubulin in Ncd [Kin C] RNAi cells suggests that microtubules are frequently released from the existing pole in prometaphase, and these released microtubules can build ad-

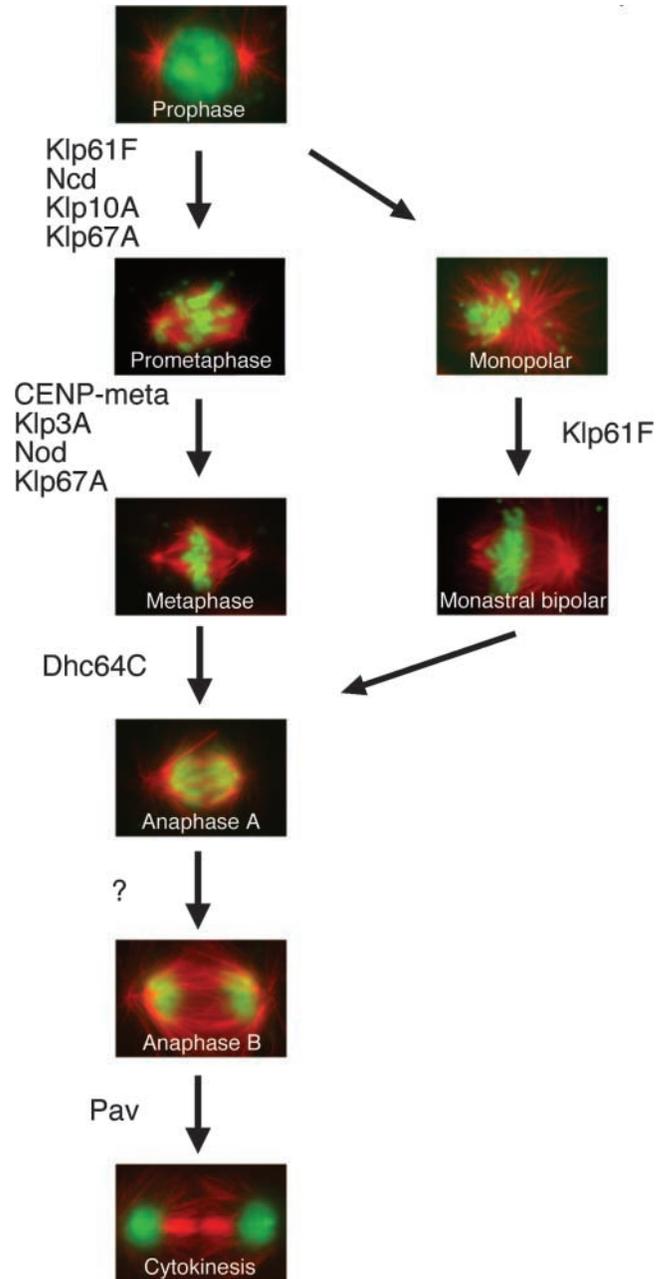


Figure 8. Roles of microtubule-based motors in the sequential steps of mitosis in S2 cells. Proper bipolar spindle assembly requires Klp61F [BimC/Eg5], Ncd [Kin C], Klp10A [Kin II], and Klp67A [Kip3] (left pathway). Once a bipolar spindle is formed, chromosomes congress to the metaphase plate by the redundant actions of four chromosomal kinesins, CENP-meta [CENP-E], Klp3A [chromokinesin], Nod [Kid], and Klp67A [Kip3]. If a cell fails to assemble a bipolar spindle initially and a monopolar spindle is formed, it can be eventually converted to monastral bipolar spindle by chromatid-directed, acentrosomal pole-focusing mechanism that requires Klp61F [BimC/Eg5] and possibly Ncd [Kin C] functions (right pathway). After establishment of metaphase, Dhc64C [DHC] (cytoplasmic dynein) controls the timing of anaphase onset. Anaphase B spindle elongation is caused by sliding of antiparallel microtubules, which may require the functions of certain motor proteins that were not identified in our screening. Finally, central spindle formation and cytokinesis requires Pav [MKLP1].

ditional acentrosomal spindles. Ncd [Kin C] may move toward the microtubule minus ends and physically tether the microtubules to the poles. The S2 Ncd [Kin C] RNAi phenotype is quite different from the *Drosophila* Ncd [Kin C] null mutant, in which meiotic spindle defects are observed, but spindle formation defects in mitotic cells are not severe or not observed (Endow et al., 1994). Variations in phenotype after inhibition of Kin C kinesins are also evident in comparing results from different experimental systems. For example, antibody inhibition of XCTK2 induced accumulation of asters and half spindle in *Xenopus* egg extract (Walczak et al., 1997). We speculate that Ncd [Kin C] may serve as a primary motor for spindle pole coalescence in S2 cells, whereas in most of the fly tissues, Ncd [Kin C]'s function may be secondary and/or compensated by cytoplasmic dynein.

The regulation of microtubule dynamics by motor proteins is essential for efficient spindle assembly. RNAi of Klp10A [Kin I], a member of the microtubule-destabilizing Kin I kinesins, resulted in excessively long microtubules emanating radially from the MTOC, suggesting that Klp10A [Kin I] destabilizes most, if not all, spindle microtubules. Monopolar spindles were frequently formed, as found in fixed images after protein inhibition of the vertebrate homologue KCM1/MCAK (Kline-Smith and Walczak, 2002). Our live-cell imaging clearly shows that separated MTOCs fuse together after NEB, despite the presence of Klp61F [BimC/Eg5]. Longer than normal microtubules may be physically difficult for Klp61F [BimC/Eg5] to cross-link into antiparallel bundles. Alternatively, pushing forces against the cell cortex exerted by long astral microtubules might overcome the forces generated by Klp61F [BimC/Eg5]. However, Klp10A [Kin I] RNAi cells eventually succeed in forming monastral bipolar spindles, which segregate sister chromatids in anaphase (Fig. S5 I), strongly suggesting that stable kinetochore–spindle interaction can be achieved in the absence of Klp10A [Kin I]. We did not identify a mitotic function for the two other Kin I motor proteins (Klp59C and Klp59D), although another analysis involving antibody microinjection found anaphase A defects for Klp59C in fly embryos (Rogers, G.C., and D. Sharp, personal communication).

Klp67A [Kip3] was originally localized at mitochondria (Pereira et al., 1997). However, we did not find a mitochondria transport defect after Klp67A [Kip3] RNAi in S2 cells (unpublished data). Instead, RNAi of Klp67A [Kip3] produces long mitotic microtubules, indicating that it destabilizes microtubules. This result is consistent with sequence analysis showing that Klp67A [Kip3] is related to *S. cerevisiae* Kip3 and *Schizosaccharomyces pombe* Klp5/Klp6, null mutants of which have longer than normal spindle microtubules (Garcia et al., 2002b). Excessively long microtubules may give rise to monopolar spindle formation in Klp67A [Kip3] RNAi cells by the mechanism described above for Klp10A [Kin I]. However, unlike Klp10A [Kin I], which destabilizes all spindle microtubules, Klp67A [Kip3] appears to act selectively on microtubules between the poles and chromosomes; astral microtubules have normal length in Klp67A [Kip3] RNAi cells. Thus, Klp67A [Kip3] and Klp10A [Kin I] have distinct roles as microtubule-destabilizing proteins in the spindle.

Functions of mitotic motor proteins: chromosome congression to the metaphase plate

Three and possibly four chromosomal kinesins are important for chromosome movement. Previous reports have shown that the kinetochore-localized CENP-meta [CENP-E] is essential for chromosome congression at early developmental stages (Yucel et al., 2000). The genetic phenotypes of the two chromosome-associated kinesins, Nod [Kid] and Klp3A [chromokinesin], for mitotic chromosome alignment are less evident. Nod [Kid] is important for chromosome positioning in meiosis, but is nonessential for fly viability (Theurkauf and Hawley, 1992). Klp3A [chromokinesin] is also nonessential for fly viability, and null mutation causes cytokinesis failure in meiosis (Williams et al., 1995). However, our data suggest that these two chromokinesins act redundantly in mitotic cells for prometaphase chromosome movement, as double RNAi of Klp3A [chromokinesin] and Nod [Kid] causes more severe chromosome misalignment phenotypes than single RNAi treatments. The survival of adult fly without either Nod [Kid] or Klp3A [chromokinesin] might be due to this redundancy. However, our phenotypic analyses show that the actions of these two chromokinesins are distinct. The chromosome arms were abnormally extended along the direction of spindle axis during prometaphase and metaphase after Nod [Kid] RNAi, suggesting that Nod [Kid] functions to transport chromosomal arms away from the pole, as previously proposed for vertebrate homologues (Antonio et al., 2000; Funabiki and Murray, 2000; Levesque and Compton, 2001). In contrast, Klp3A [chromokinesin] may be needed for both kinetochore and arm-directed chromosome motility. The redundant function of chromatin- and kinetochore-localized kinesins for chromosome congression may be a general feature of eukaryotes.

The Klp67A [Kip3] RNAi phenotype also suggests an additional role for this motor in proper chromosome–spindle interaction, most probably at the kinetochore. After depletion of Klp67A [Kip3], chromosomes are scattered in the spindle (in contrast to Klp10A [Kin I] RNAi), possibly reflecting unbalanced spindle tension on chromosomes. Additionally, very few cells enter anaphase, presumably because of activation of the spindle checkpoint that monitors spindle–kinetochore interaction. In *S. pombe*, two Klp67A [Kip3]-like proteins, Klp5 and Klp6, are localized at mitotic kinetochores, and have been proposed to generate stable kinetochore–spindle interactions and tension at kinetochores (Garcia et al., 2002a). Because RNAi phenotypes of Klp67A [Kip3] are similar in many aspects to Klp5/Klp6 mutants, Klp67A [Kip3] also might act at kinetochores.

Functions of mitotic motor proteins: anaphase and cytokinesis

In addition to Klp67A [Kip3], cytoplasmic dynein plays a role in the metaphase-to-anaphase transition. Dhc64C (cytoplasmic dynein) may control the timing of anaphase onset, possibly by transporting Rod or other checkpoint proteins away from kinetochores as proposed for the fly embryo (Wojcik et al., 2001). A similar checkpoint inactivation model was proposed for mammalian dynein/dynactin based on inhibition analyses (Howell et al., 2001).

The roles of motors in anaphase A (chromosome to pole motion) and anaphase B (spindle elongation) are less clear from this paper, as we did not detect defects by fixed cell images after RNAi. This may be due to the fact that RNAi depletion of a single motor protein may cause kinetic defects in anaphase, which may be difficult to detect in fixed population images. For example, recent live-cell imaging of anaphase in living *Drosophila* embryos shows that antibodies against Kin I motors slow down rather than completely block anaphase (Rogers, G.C., and D. Sharp, personal communication). It is also possible that Klp61F [BimC/Eg5] or Klp67A [Kip3] play roles in anaphase; however, RNAi of these motors arrests cells earlier in mitosis.

Finally, central spindle formation and cytokinesis in S2 cells require Pav [MKLP1], as described previously (Somma et al., 2002). However, it has not been clear whether Pav is needed for the formation or maintenance of the central spindle. Our live-cell observations of Pav [MKLP1] RNAi cells shows that some cells formed central spindles, but could not maintain them, whereas others showed a complete failure in formation.

Monastral bipolar spindle formation as a rescue of monopolar spindle

An interesting phenomenon observed in this paper is the conversion of a monopolar spindle to a monastral bipolar spindle, in which one of the poles lacks a centrosome. Anastral bipolar spindles, in which both poles lack centrosomes, are commonly observed in higher plant mitosis and animal female meiosis (for review see Compton, 2000), but the formation and relevance of acentrosomal poles in animal mitotic cells have been less clear. Khodjakov et al. (2000) found that bipolar spindles containing one centrosomal and one acentrosomal pole can be formed if one centrosome is destroyed by laser ablation. In addition, there has been a previous report of asymmetrical bipolar spindles in nonmeiotic cells (Wilson et al., 1997).

Our live-cell analysis provides a mechanism for monastral bipolar spindle formation. First, microtubules emerge from the chromosomal region in the monopolar spindle. Similar observations of chromosome-directed microtubule formation have been made for acentrosomal spindle formation in meiosis (Theurkauf and Hawley, 1992; for review see Compton, 2000). Our simultaneous RNAi experiments show that Klp61F [BimC/Eg5], but not cytoplasmic dynein, is required for monastral bipolar spindle formation. Bipolar kinesins may be necessary for bundling the chromosome-generated microtubules (Walczak et al., 1998). The role of Ncd [Kin C] during monopolar to bipolar conversion is ambiguous by our double RNAi method, as Ncd [Kin C] depletion dominantly inhibited monopolar spindle formation. However, considering Ncd [Kin C]'s role in MTOC fusion in early prometaphase and pole maintenance, it also may be needed for acentrosomal pole-focusing in the final step of the conversion. Klp67A [Kip3] may also play a minor role in this process because monopolar spindles generated after Klp67A [Kip3] RNAi do not always form an acentrosomal pole, perhaps due to problems in chromosome–microtubule interaction as discussed above.

Monopolar to monastral bipolar spindle conversion also appears to occur in untreated cells as well, because we observed both monopolar (<5% of mitotic cells) and monastral bipolar spindles (10% of bipolar spindles) in untreated S2. Moreover, Wilson et al. (1997) reported that 0.9% of mitotic spindles of the wild-type *Drosophila* larval neuroblasts display monastral bipolar spindles. In the monastral bipolar spindle of Klp10A [Kin I] RNAi and untreated cells, anaphase chromosome movement can occur (Fig. S1 B; Fig. S5 I). Thus, the mitotic checkpoint is activated during the monopolar spindle phase, but is properly down-regulated once metaphase is achieved in monastral bipolar spindle. Thus, in the presence of a functional spindle checkpoint system, monastral bipolar spindle formation enables completion of mitosis in rare cases when the spindle gets trapped in a monopolar state after NEB. Although originally believed to be a property of plant and meiotic animal cell, we propose that acentrosomal pole formation constitutes a general backup mechanism for mitosis in somatic animal cells.

S2 cell as a model system for animal mitosis

Our work indicates that the *Drosophila* S2 cell provides a powerful model system for investigating the mechanisms of mitosis through a combination of gene inactivation and high resolution fixed- and live-cell imaging. Moreover, the use of combination RNAi to explore protein redundancy constitutes a powerful approach to dissect complex biological processes. These methods have allowed us to systematically define and dissect the roles of molecular motors in mitosis in these cells. In general, the results are likely to be broadly applicable, as evidenced by the similarity between many of the RNAi phenotypes described here with those obtained through genetics in various organisms. However, it should be noted that S2 cells display abnormal features that are characteristic of many immortalized cell culture systems, such as variable γ -tubulin–MTOC numbers. Moreover, certain motor functions that we define here may not translate precisely to other organisms or even other cell types in *Drosophila*. As an example, inhibition of Dhc64C [DHC] produces numerous mitotic defects, including spindle disintegration during fly development (Robinson et al., 1999), whereas RNAi of Dhc64C [DHC] in S2 cells only elicited anaphase delay. This difference could be explained by residual dynein after RNAi treatment. However, another likely possibility is that the two minus end–directed motors, dynein and Ncd [Kin C], perform similar functions in pole formation; in some cells, dynein may constitute the dominant activity for this aspect of spindle formation, whereas in other cells (e.g., S2), Ncd [Kin C] may play the dominant role. A similar situation may exist for the two kinetochore motors CENP-meta and CENP-ana; the latter does not produce a phenotype in S2 cells by RNAi, but elicits a mitotic phenotype by genetic inactivation in embryos (Yucel et al., 2000). Such variability is consistent with notion that certain biological activities can be driven by different types of molecular motors (Vale, 2003). This idea also raises the possibility that some tumors might be more susceptible than normal tissues to inhibition of cell division by inactivation of particular mitotic kinesin motors.

Materials and methods

Cell culture and RNA interference

Drosophila Schneider cell line (S2) was cultured and RNAi was performed according to the methods of Clemens et al. (2000) and Rogers et al. (2002). Templates for in vitro transcription were generated by PCR using the primers listed in Table S1. Purified PCR products were used as templates for in vitro transcription using the MEGAscript[®] T7 kit (Ambion). The synthesized dsRNA was added to cell culture in 24- or 96-well plates in amounts of 5 and 1 μ g, respectively. If no phenotype was found in the d 4 sample, the culturing was prolonged until d 7 with an additional treatment with dsRNA at d 4. In the case of Klp61F [BimC/Eg5] and Pav [MKLP1], d 3 samples were observed, as cells were sick and detached from the plate after d 4. At the end of the RNAi, cells were resuspended and replated on Con A-coated coverslips for 2 h before imaging (Rogers et al., 2002).

Immunofluorescence microscopy

Immunofluorescence of tubulin DM1A (anti-tubulin; 1:500) and γ -tubulin (GTU-88, 1:500; Sigma-Aldrich) were performed according to the methods described previously (Rogers et al., 2002), and 0.5 μ g/ml Hoechst 33342 was added for DNA staining. The signal of GFP-tubulin was well maintained during this procedure. Specimens were imaged by a cooled CCD camera mounted on either an inverted microscope (TE300; Nikon) or an Axioplan (Carl Zeiss MicroImaging, Inc.) microscope. The mitotic index was calculated by counting the number of cells with condensed chromosomes (>1,000 cells scored). The mitotic index of untreated S2 was generally ~3%, but was sometimes higher (~5%) or lower (~2%) in different sets of RNAi experiments. Therefore, we prepared a control untreated sample for all the RNAi experiments. The mitotic indices of some RNAi samples described in Table II were normalized by proportionally adjusting the control index to 3%.

Time-lapse imaging of GFP-tubulin

Cells expressing GFP-tubulin were treated with dsRNA and put onto Con A at d 3 or 4. Images were collected at 5–20-s intervals at RT (20–22°C) by an intensified CCD camera (MEGA10; Stanford Photonics) attached to a spinning-disk confocal scanhead (Yokogawa) that was mounted on an inverted microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.). Images were acquired using software package QED in vivo (QED Imaging, Inc.). Images were deconvoluted using the maximum likelihood estimate-time algorithm as implemented in the software package Huygens Pro (Scientific Volume Imaging). A theoretical point spread function calculated for a confocal microscope with a pinhole of 1,000 nm was used. Maximum intensity projection of Z-stacks was done for four-dimensional images using ImageJ. Phase-contrast images were collected at 30-s intervals by cooled CCD camera (Princeton Instruments, Inc.) mounted on an inverted microscope (Axiovert 200M).

Antibodies for immunoblotting

Specific antibodies were used for immunoblotting as follows: anti-KHC (SUK4 [COVANCE]; 1:300), anti-Dhc64C (Sharp et al., 2000a; mouse, 1:50), anti-Klp3A (Williams et al., 1995; rabbit, 1:1,000), anti-Pav (Adams et al., 1998; rabbit, 1:100), anti-Costal2 (Sisson et al., 1997; rat, 1:50), anti-Klp61F (Sharp et al., 1999; rabbit, 1:100) and anti-Ncd (a gift of Dr. Jon Scholey, rabbit, 1:200).

Online supplemental material

The supplemental material (Figs. S1–S10 and Videos 1–15, available at <http://www.jcb.org/cgi/content/full/jcb.200303022/DC1>) contains additional immunofluorescence and live-cell imaging.

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