Poleward Microtubule Flux Is a Major Component of Spindle Dynamics and Anaphase A in Mitotic Drosophila Embryos

Paul Maddox,1,2,5,6 Arshad Desai,1,3,6 Karen Oegema,1,3 Timothy J. Mitchison,1,4 and E.D. Salmon1,2

1The Cell Division Group
Marine Biological Laboratory
Woods Hole, Massachusetts 02543
2Department of Biology
UNC-Chapel Hill
Chapel Hill, North Carolina 27599
3Max Planck Institute for Cell Biology and Genetics (2–3)
Dresden, Germany
4Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

Summary

During cell division, eukaryotic cells assemble dynamic microtubule-based spindles to segregate replicated chromosomes [1, 2]. Rapid spindle microtubule turnover, likely derived from dynamic instability, has been documented in yeasts [3, 4], plants [5] and vertebrates [6]. Less studied is concert spindle microtubule poleward translocation (flux) coupled to depolymerization at spindle poles [7]. Microtubule flux has been observed only in vertebrates [7], although there is indirect evidence for it in insect spermatocytes [8, 9] and higher plants [10]. Here we use fluorescent speckle microscopy (FSM) to demonstrate that mitotic spindles of syncytial Drosophila embryos exhibit poleward microtubule flux, indicating that flux is a widely conserved property of spindles. By simultaneously imaging chromosomes (or kinetochores) and flux, we provide evidence that flux is the dominant mechanism driving chromosome-to-pole movement (anaphase A) in these spindles. At 18°C and 24°C, separated sister chromatids moved poleward at average rates (3.6 and 6.8 μm/min, respectively) slightly greater than the mean rates of poleward flux (3.2 and 5.2 μm/min, respectively). However, at 24°C the rate of kinetochore-to-pole movement varied from slower than to twice the mean rate of flux, suggesting that although flux is the dominant mechanism, kinetochore-associated microtubule depolymerization contributes to anaphase A.

Results and Discussion

Investigation of the biological role of microtubule flux in spindles has focused on defining the extent to which it contributes to chromosome segregation. In vertebrate somatic cells, photoactivation analysis revealed that kinetochores microtubules connecting kinetochores regions of chromosomes to spindle poles continuously flux poleward at about 0.5 μm/min during metaphase and slow to 0.3 μm/min during anaphase [11–14]. The major mechanism for anaphase A in vertebrate somatic cells is kinetochore movement coupled to microtubule depolymerization near the kinetochore, with poleward flux making a relatively minor (~30%) contribution [11–14]. In contrast, photoactivation analysis in Xenopus egg extracts revealed that the bulk microtubule population in extract spindles moves poleward at much higher rates (2–3 μm/min) [15, 16] and that flux is potentially a major contributor to anaphase A [15]. Because Xenopus extracts exhibit microtubule assembly in meiotic cytoplasm, these studies raised the possibility that flux is a major component of anaphase A during meiosis but not mitosis.

To determine if poleward microtubule flux exists in invertebrate mitosis, we analyzed spindle dynamics in Drosophila embryos. During the syncytial blastoderm divisions (nuclear cycles 10–13), close apposition of the spindles to the embryo surface makes them uniquely accessible for high-resolution fluorescence imaging. However, analyzing flux by photoactivation in the 10–14 μm-long spindles of these rapidly dividing embryos (anaphase A lasts about 1 min) was not feasible. Therefore, we adapted fluorescent speckle microscopy (FSM; [17]), a recently developed alternate technique for monitoring cytoskeletal polymer movements, to the Drosophila embryo.

To analyze Drosophila embryonic spindles with FSM, we injected dechorionated embryos [18] with a mixture of DAPI, Alexa 488-tubulin to completely label spindle microtubules, and Alexa 594 or X-rhodamine-labeled tubulin at an empirically determined low concentration to generate fluorescent speckles (speckles) within microtubules (Figure 1) [17, 19]. Wide-field time-lapse movies obtained at 18°C revealed the presence of robust poleward speckle movement throughout metaphase and anaphase in these embryonic spindles (Figures 2A and 2B). These observations provide the first direct evidence for poleward microtubule flux in an invertebrate as well as in a mitotic embryo. They suggest that flux is a widely conserved property of spindles and should be considered for any analysis of the mechanisms of spindle morphogenesis.

To determine if flux can make a significant contribution to the segregation of sister chromatids during embryonic mitosis, we simultaneously followed speckle and chromosome movement. During metaphase, chromosomes remained in the middle of the spindle while speckles could be seen originating near them and moving poleward in a linear fashion (Figure 2A, arrows). After anaphase onset, separated sister chromatids moved poleward while speckles continued moving poleward. Point tracking of the leading edges of chromosomes and of fluorescent speckles in close proximity to these leading edges showed that poleward flux occurred at a mean velocity of 3.2 ± 0.7 μm/min (n = 110 speckles from eight embryos) while sister chromatids moved poleward at a mean velocity of 3.6 ± 1.0 μm/min (n =
Poleward Microtubule Flux in Drosophila Embryos

1671

Both kinetochore and non-kinetochore, flux poleward. These results demonstrate that flux is a major mechanism for sister chromatid segregation in Drosophila embryos at 18°C.

Recent antibody and dominant-negative inhibitor injection experiments suggest that poleward chromosome movement in Drosophila embryos at 24°C is driven in large part by the kinetochore-associated minus-directed motor protein cytoplasmic dynein [20]. The results described in Figure 2 are inconsistent with this conclusion because there is little net movement of kinetochores relative to spindle microtubules during anaphase A. Because our analysis was performed at 18°C, one possible source of discrepancy is the difference in temperature between the two studies. Therefore, we wanted to test whether embryonic divisions observed at higher temperatures (23°C–25°C) exhibited significant kinetochore motility in addition to microtubule flux during anaphase. We also wanted to directly visualize the microtubules that connect kinetochores to spindle poles because it is the dynamic properties of these microtubules that are most important for understanding the mechanism of anaphase A. To achieve these two goals, we modified our experimental design in two ways. First, we used embryos expressing GFP fused to MEI-S332, a protein that localizes to the kinetochore region of the chromosomes [21]. Second, we used spinning-disk confocal imaging to more precisely follow moving kineto-

Figure 1. Injection of Low Levels of Labeled Tubulin Generates Speckled Spindle Microtubules in Mitotic Drosophila Embryos

We injected embryos with DAPI to label chromosomes ([A], red in [D]), low levels of Alexa-594-labeled tubulin to speckle the microtubules ([B], green in [D]), and high levels of Alexa-488-labeled tubulin to visualize the entire spindle ([C], blue in [D]). The three fluorescent channels were imaged sequentially with wide-field microscopy. Note that the speckles overlay with spindle fibers in the merged image (D). The three channels are illustrated schematically in (E). To eliminate error due to variable separation of centrosomes from the spindle poles, we measured chromosome (red arrow)-to-pole (cp) and speckle (green arrow)-to-pole (sp) distances to the end of the spindle fibers (gray arrow) and not the centrosome. The scale bar represents 2 µm.

75 chromosomes from eight embryos). Kymograph analysis directly revealed the correlation between chromosome movement and poleward flux (compare white and black lines in Figure 2B; Figure 2C). We did not detect static speckles, suggesting that all spindle microtubules, both kinetochore and non-kinetochore, flux poleward. These results demonstrate that flux is a major mechanism for sister chromatid segregation in Drosophila embryos at 18°C.

Figure 2. Wide-Field FSM of Speckled Drosophila Embryo Mitotic Spindles Reveals Robust Poleward Microtubule Flux and Shows that Flux Is a Major Mechanism for Sister Chromatid Segregation at 18°C–19°C

Drosophila embryos were followed by time-lapse digital imaging [26] during nuclear division cycles 10–13.

(A) Five frames from a single time-lapse recording show chromosomes (red) and speckles (green). During metaphase, fluorescent speckles (white arrow and arrowhead) appeared near the chromosomes and then moved poleward. During anaphase, the leading edges of chromosomes also moved poleward (black arrow and arrowhead). The black arrow marks a chromosome leading edge moving at the same rate as a neighboring speckle (white arrow). The black arrowhead marks a chromosome leading edge moving at a slightly faster pace than a neighboring speckle (white arrowhead).

(B) Kymograph analysis shows movements of chromosomes (red) relative to movements of speckles (green) during mitosis. Selected speckle trajectories are marked by white dotted lines, while the leading edges of the separating chromosomes are marked by black dotted lines. Note that the slopes of the lines, which reflect the velocities, are similar.

(C) Positions of the spindle poles, several speckles, and two separating chromosomes with time. Again note the similarity between poleward movement of speckles and chromosomes.

(D) Traces showing the chromosome-to-pole distance versus time for three different chromosomes.
Figure 3. Real-Time Spinning-Disk Confocal FSM of Microtubules and GFP-Labeled Kinetochores Shows that Poleward Flux Makes the Major Contribution to Anaphase A at 24°C

Embryos obtained from a strain expressing GFP-MEI-S332, a fluorescent fusion protein that labels kinetochores, were injected with X-rhodamine-labeled tubulin and imaged in both GFP and X-rhodamine channels by spinning-disk confocal microscopy [22]. (A) Six frames from a time-lapse recording showing the poleward movement of kinetochores (red) and speckles (green) during anaphase. The arrow marks a kinetochore that appears to move more quickly than an adjacent speckle (marked by the arrowhead). Note that the speckle disappears as the kinetochore moves past it. The spindle shown in (B) was analyzed by construction of kymographs for three sections of the spindle (delineated by boxes C, D, and E). The resulting kymographs are shown in panels (C), (D) and (E), respectively. Note that all parts of the spindle exhibit poleward flux (angled green streaks in [C], [D], and [E]) and that the rates are similar in each area. In (E), one pair of sister kinetochores (red) can be seen moving poleward during anaphase. The left sister exhibits three distinct phases of poleward motility. The phase labeled “1” occurs at 2.4 μm/min. This velocity of kinetochore movement increases to 6.6 μm/min in phase 2 before slowing to 3.6 μm/min in phase 3. The average rate of anaphase A was 4.0 μm/min; faster than the average rate of poleward flux (3.5 μm/min; compare slopes of the green and red streaks). Speckle trajectories on the right side of the spindle are marked by white dotted lines. Note that the poleward flux (3.9 μm/min) for the entire length of anaphase A. The double arrow in (E) indicates the region of reduced fluorescence between separated sisters that increases in width as the sisters move poleward. (F) A histogram showing the distribution of rates of poleward flux and kinetochore-to-pole motility. The average values are marked by arrows (n = 112 and 99 measurements for speckles and chromosomes, respectively, from ten embryos; an average of five spindles were analyzed per embryo). (G) Three representative traces of kinetochore-to-pole distance versus time. The kinetochores moved erratically and exhibited periods of rapid, as well as slower, poleward movement. In contrast, speckles (green streaks in [C], [D], [E]) moved poleward at a constant velocity. The scale in (C) is the same for all panels.

Figure 3 and supplementary Figure S1 show examples of speckled Drosophila embryonic spindles observed by spinning-disk confocal microscopy at room temperature (23°C–25°C). As in the previous experiment, kinetochores aligned at the metaphase plate, and poleward movement of speckles was observed throughout mitosis. Confocal images revealed microtubule bundles, some of which were in close apposition to aligned kinetochores. In addition, these spindles also contain non-
kinetochore polar microtubules that overlap to form inter-polar spindle fibers [23]. In meiosis, the number of sister kinetochore microtubules is reported to be about five, similar to the number in inter-polar spindle fibers [23, 24]. Because of their similar fluorescence intensity, it was difficult to discriminate kinetochore bundles from inter-polar bundles throughout anaphase A (Figure 3A). To most closely approximate the behavior of the kinetochore microtubules, we focused our analysis on speckles moving with the same trajectory and in close vicinity to segregating kinetochores. We performed quantification by two methods, point tracking and kymography (Figure 3 and Supplementary Experimental Procedures). To exclude artifacts associated with focal shifts, we only analyzed spindles that remained in focus for the duration of mitosis. At 24°C, poleward flux occurred at an average of 5.2 ± 1.9 μm/min (n = 112 measurements from 10 embryos), nearly twice the speed observed at 18°C. Before anaphase onset, sister kinetochores on aligned chromosomes moved little while microtubules fluxed poleward (Figures 3D and 3E and supplementary Figure S1B). As in the wide-field analysis, we did not observe static speckles. Speckles were seen to originate near kinetochores and move poleward at rates similar to speckles in other regions of the spindle (Figures 3C–F). Thus, as with Xenopus extract spindles (P.M., T.J.M., and E.D.S., unpublished data), kinetochore microtubules appeared to be fluxing at similar rates to those of the bulk spindle microtubule population and were not static. At anaphase onset, kinetochores separated and moved poleward at an average rate of 6.6 ± 2.4 μm/min (n = 99 measurements from ten embryos, Figure 3F). As seen in the kymographs in Figure 3E and supplementary Figure S1B, most kinetochores were seen to move near the rate of poleward flux, in accordance with the results described above. However, unlike kinetochores at 18°C, those at 24°C underwent measurable periods of increased velocity relative to microtubule flux (Figures 3E and 3G). During these periods, speckles could be seen to disappear as the kinetochores moved past them (arrowheads in Figure 3A), confirming that at least some of these speckles are on kinetochore microtubules. As kinetochores moved poleward, the fluorescence between separating sisters decreased substantially, as expected for the shortening of kinetochore microtubules during anaphase A (double arrows in Figure 3E and Supplementary Figures S1B and S1C). Occasionally kinetochores passed each other while moving poleward. Virtually all kinetochores examined exhibited variable rates of poleward movement; some changed rates up to three times during anaphase A. These rates were anywhere from slower than to nearly twice that of poleward flux (Figures 3E and 3G; supplementary Figure S1).

These results indicate that, although flux is the dominant mechanism for anaphase A, kinetochore motility associated with microtubule depolymerization near the kinetochore adds on average 1.4 μm/min to the flux velocity (Figures 3F and 4). Kinetochore-associated dynein may contribute to the faster-than-speckle kinetochore movement observed at 23°C–25°C. However, our results are inconsistent with the assertion that dynein-driven kinetochore movement is the major mechanism for anaphase A in mitotic Drosophila embryos [20]. Instead, our results indicate that both kinetochore and flux-based mechanisms contribute, with poleward flux being the dominant mechanism (Figure 4). Given the well-documented role of dynein in spindle pole formation and maintenance [25], one possibility is that chromosome movement defects in dynein-inhibited embryos are an indirect consequence of disrupting anchorage of kinetochore fibers at spindle poles. A second possibility is that dynein maintains kinetochore-microtubule attachment during anaphase such that flux-generated forces are able to move the separated chromatids poleward.

Our study in Drosophila embryos provides the first example for a mitotic division in which microtubule flux is the dominant mechanism for poleward chromosome movement. Thus, flux-dominated chromosome segregation is not meiosis specific. Viewed in the context of previous work in mitotic vertebrate somatic cells and meiotic Xenopus egg extracts, these results strongly suggest that both microtubule flux and kinetochore motility are widely conserved, concurrently acting mechanisms that contribute to anaphase A. However, there is a dramatic difference among systems in the extent to which each mechanism is utilized.

Our results illustrate the power of FSM as a technique for studying mitotic spindles in the Drosophila embryo. The existence of a simple and robust assay for spindle dynamics in a classical genetic model organism with a sequenced genome should help analysis of spindle morphogenesis and function.

Supplementary Material
Supplementary Experimental Procedures, Figure S1 and time-lapse movies of Figures 1, 2, 3, and S1 are available with this article online at http://images.cellpress.com/supmat/supmatin.htm.

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Supplementary Experimental Procedures
Labeled Tubulins

We labeled tubulin with X-rhodamine or Alexa NHS esters as described at http://mitchison.med.harvard.edu/protocols/label.html. All labeled tubulins were stored at –80°C in 1B (50 mM K-glutamate, 0.5 mM MgCl₂, pH 7). For assaying flux via FSM, it is essential to extensively dilute the labeled tubulin prior to injecting it into embryos. To avoid tubulin inactivation, we performed all dilutions serially into unlabeled tubulin or tubulin labeled with a different fluorophore.

Microscopy

FSM was performed with diffraction-limited wide-field microscopy [S1] or spinning-disc real-time confocal microscopy [S2]. We injected dechorionated embryos with 50 μg/ml DAPI to label chromosomes (Figure 1A in the main text, red in 1D), low levels (750 nM) of Alexa-594-labeled tubulin to speckle the microtubules (Figure 1B in the main text, green in 1D), and high levels (40 μM) of Alexa-488-labeled tubulin to visualize the entire spindle (Figure 1C in the main text, blue in 1D), as described in [S3] and Figure 1 in the main text. For wide-field microscopy, images were acquired at 5–15 s intervals with a Nikon E600 upright microscope equipped with a 60×, 1.4 N.A. Plan Apo bright-field objective lens with no intermediate magnification. A multiple bandpass dichromatic filter allowed sequential acquisition of blue (DAPI-stained chromosomes), green (Alexa 488-labeled tubulin, GFP), and red (Alexa 594- or X-rhodamine-labeled tubulin) fluorescent images in register on the detector of the camera. We used MetaMorph software (Universal Imaging Corporation) to control illumination through a shuttered dual filter wheel (excitation wavelength and intensity, Ludl), a stepping motor (Nikon) to focus on specimens, and a CCD detector (Hamamatsu Orca 100, Hamamatsu Photonics) to acquire images. Multi-mode fluorescence images were collected every 5–15 s depending on the experiment, with exposure times of 750–1250 ms. Confocal microscopy differs from the above described system in its use of the Yokogawa Spinning Disk Confocal unit [S2] and a TE300 Nikon inverted microscope. For this application, a Krypton/Argon air-cooled laser was used for excitation, and an Orca ER cooled CCD (Hamamatsu Photonics) was used for detection.

Data Analysis

We mainly performed measurements with MetaMorph software to hand track positions of the leading edges of DAPI-labeled chromosomes (Figure 1 in the main text) or GFP-labeled kinetochores (Figures 2 and 3 in the main text) and Alexa 594- or X-rhodamine-labeled tubulin speckles relative to the spindle poles. Measurements were made from the margin of the structure being analyzed to the end of the spindle fibers (Figure 1E in the main text). Speckles were chosen for analysis based on the following criteria: the speckle was in front of and near a chromosome (or kinetochore) and the speckle could be followed for at least three consecutive time points before disappearing from view.

We performed kymograph analysis on select samples as a second method to verify hand-tracking results. We used two kymograph methods to compare trajectories of chromosomes/kinetochores and speckle movements. In one method, we drew a narrow box cursor in the direction that a chromosome moved poleward to include images of both the leading edge of a chromosome (or kinetochore) and proximal speckles. Alternatively, we created kymographs by first drawing a line on the image in the direction of movement and then using the “Kymograph” function in MetaMorph [S4]. For both methods, we then montaged the line or narrow box to produce an image with one axis being time and the other being the data from the pixels along the line or box [S1, S5]. We used color overlays to compare movements of speckles to chromosomes/kinetochores. Velocities were obtained from the slopes of the speckle or leading-edge trajectories in the kymograph images.

Supplementary References

Figure S1. Another Example of Spinning-Disk Confocal FSM of Microtubules and GFP-Labeled Kinetochores during Anaphase at 24°C
See also the legend of Figure 3 in the main text. A reduced concentration of tubulin gave higher contrast to the fluorescent speckles [S6]. (A) Seven frames from a time-lapse recording showing the poleward movements of a pair of sister kinetochores (red) and speckles (green) during anaphase. The arrow marks one kinetochore that moves more quickly than an adjacent speckle (arrowhead). Note that the speckle disappears as the kinetochore moves past it, most likely because of depolymerization of the speckled microtubule(s) at the kinetochore. (B) Color kymograph through the sister kinetochore pair along the trajectory of their poleward movements. (C) The speckle channel and (D) the fluorescent kinetochore channel of the color kymograph in (B). The left-hand pole is the left-most bright spot, whereas the right-hand pole is barely visible at the right margin of the kymograph. In each case, time increases downward, and the times for the frames shown in (A) are indicated. Note that all parts of the spindle exhibit poleward flux at similar rates (angled green streaks in [B] and white streaks in [C]). Both sister kinetochores exhibit variable velocities of poleward movement that are independent of each other. The sister on the right begins moving at a rate (4.6 μm/min) slower than flux (6.1 μm/min), then briefly moves at a rate (9.9 μm/min) faster than flux and persists in moving slightly more quickly (7.1 μm/min) than flux to the pole. On average, this kinetochore moved poleward at 7.4 μm/min, 1.3 μm/min faster than flux. The sister on the left begins moving poleward at a rate (7.0 μm/min) faster than flux (5.1 μm/min), then slows down (3.9 μm/min) before moving much more rapidly (11.0 μm/min) than flux to the pole. The average value for anaphase A of the left sister kinetochore was 7.6 μm/min. The double arrow in (B) and (C) indicates the region of reduced fluorescence between separated sisters. This region increases in width as the sisters move poleward and their kinetochore fibers shorten. The scale bar in (A) represents 5 μm and is the same for all panels.