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Report

Cortical Actin Dynamics Facilitate Early-Stage Centrosome Separation

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Summary

Proper centrosome separation is a prerequisite for positioning the bipolar spindle. Although studies demonstrate that microtubules (MTs) and their associated motors drive centrosome separation [1], the role of actin in centrosome separation remains less clear. Studies in tissue culture cells indicate that actin- and myosin-based cortical flow is primarily responsible for driving late centrosome separation [2], whereas other studies suggest that actin plays a more passive role by serving as an attachment site for astral MTs to pull centrosomes apart [3–6]. Here we demonstrate that prior to nuclear envelope breakdown (NEB) in *Drosophila* embryos, proper centrosome separation does not require myosin II but requires dynamic actin rearrangements at the growing edge of the interphase cap. Both Arp2/3- and Formin-mediated actin remodeling are required for separating the centrosome pairs before NEB. The Apc2-Armadillo complex appears to link cap expansion to centrosome separation. In contrast, the mechanisms driving centrosome separation after NEB are independent of the actin cytoskeleton and compensate for earlier separation defects. Our studies show that the dynamics of actin polymerization drive centrosome separation, and this has important implications for centrosome positioning during processes such as cell migration [7, 8], cell polarity maintenance [9, 10], and asymmetric cell division [11, 12].

Results and Discussion

Centrosome Separation Is Concomitant with Actin Cap Expansion

To define the role of the actin cytoskeleton in centrosome separation, we examined centrosome separation in early *Drosophila* embryos. During the rapid synchronous divisions in the syncytial *Drosophila* embryo, the nuclei divide on a plane just beneath the plasma membrane, providing a means to simultaneously follow centrosomes, microtubules (MTs), and actin dynamics (Figure 1A). During these divisions, centrosomes duplicate during telophase when actin caps form directly above each centrosome pair. Centrosome pairs migrate along the nuclear envelope at nuclear envelope formation (NEF) and move to the opposite poles (close to 180°) before nuclear envelope breakdown (NEB) (Figure 1A, arrows; see also Figures S1A–S1E and Movie S1 available online). During this time, lateral expansion of the actin caps occurs (Figures 1A and 1B). Centrosome separation is concomitant with actin cap expansion (Figure 1A).

Disruption of F-Actin Cytoskeleton Prevents Centrosome Separation before, but Not after, NEB

To investigate the roles of the cortical actin cytoskeleton in centrosome separation and spindle assembly, we injected embryos expressing GFP-tubulin with latrunculin A (LatA) just prior to NEF. Because F-actin is constantly turning over [13], LatA injection resulted in a rapid loss of F-actin and prevented furrow invagination in the following cell cycle (Figure S2). In wild-type uninjected cycle 12 embryos, the distance between centrosome pairs at cycle 12 NEB was about 8 μm (Figures 2A and 2K). Dimethyl sulfoxide (DMSO) injection had very little effect on centrosome separation (Figures 2B, 2K, and 2L). In LatA-injected embryos, approximately a quarter of the nuclei clustered during early interphase, which resulted in failed centrosome separation and multipolar spindles (Figure S3 and Movie S2). To avoid secondary effects on centrosome separation because of LatA-induced clustering of nuclei, we only quantified centrosome separation in nuclei that did not cluster (the same criteria also applies to the other genetic or drug manipulations). For the unclustered nuclei, LatA did not appear to affect centrosome splitting, because the centrosome pairs were clearly distinguishable and detached from each other after NEF (Figure 2C; Movie S2). However, during the interval between centrosome splitting and NEB, centrosomes failed to separate normally (Figures 2C, 2K, and 2L; Movie S2). The distance between centrosomes (4.0 ± 0.5 μm) was significantly shorter, and the separation angle (65° ± 11°) was also significantly smaller at NEB of cycle 12 than in control embryos injected with DMSO (7.4 ± 0.5 μm and 158° ± 5°), indicating a role for actin in early separation of centrosomes (Figure 2C; Movie S2). Defects in early centrosome separation were also observed in embryos derived from females homozygous for the *sponge* (*spg*) maternal-effect mutation (Figures 2D, 2K, and 2L). These embryos also fail to form actin caps and furrows [14]. However, following NEB in LatA-treated embryos and embryos laid by *sponge* mutant females, sister centrosomes separated fully to ultimately establish a bipolar spindle during prometaphase-metaphase (Figures 2C, 2D, and 2M; Movie S2), indicating that a nuclear envelope and actin-independent pathway compensate for the earlier actin-based separation defects.

Actin Turnover Is Required for Centrosome Separation before NEB

To determine whether actin dynamics are required for centrosome separation, we injected the actin-stabilizing drug Jasplakinolide (Jasp) into embryos immediately prior to NEF. Similar to LatA-mediated inhibition of actin polymerization, Jasp-mediated actin stabilization strongly inhibited centrosome migration before NEB (Figures 2E, 2K, and 2L; Movie S3). The respective pole-pole distance and separation angle were 5.5 ± 0.4 μm and 98° ± 25° in Jasp-treated embryos, compared to 7.4 ± 0.5 μm and 158° ± 5° in DMSO-treated control embryos. Because both disruption and stabilization of F-actin inhibit early centrosome separation, these data suggest that actin turnover is important for proper centrosome separation before NEB.

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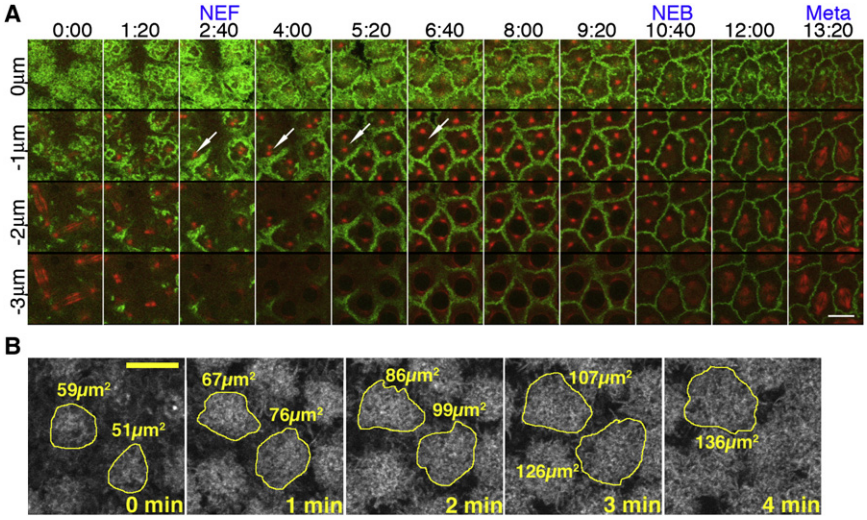

Figure 1. Centrosome Separation Is Concomitant with Actin Cap Expansion

(A) Cycle 12 syncytial blastoderm. Green depicts GFP-moesin; red depicts Rhodamine-tubulin; x axis represents time; y axis represents depth. Z series are shown, starting from the cortical surface (z = 0) to 3 μm below the surface (z = −3 μm) at 1 μm increments. At telophase, actin caps form at the cortical surface and initiate lateral expansion. This cap expansion is shown in the top panel, where gaps between caps are seen initially from time 0:00 to 6:40, after which GFP-moesin-marked actin has filled the entire frame. Concurrently, centrosome pairs separate from each other (arrows). See also Figure S1.

(B) Panels depict the method of measuring actin cap area in wild-type embryos expressing GFP-moesin. The freehand tool in ImageJ was used to outline individual caps at the beginning of cycle 12. Each cap is measured every minute until it can no longer be distinguished from its neighbors. Scale bars represent 10 μm.

Inhibition of Actin Turnover during Interphase Prevents Actin Cap Expansion

Injecting Jasp before NEF resulted in strong actin accumulation at the cap (Figure S2). In control embryos, actin caps expanded laterally from NEF through early interphase and eventually made contact with one another (Figures 3A and 3B; Movie S4). In contrast, actin caps failed to expand and actually shrunk over time after Jasp treatment (Figures 3A and 3B; Movie S5). Fluorescence recovery after photobleaching (FRAP) analysis indicated that the Jasp-induced defects in actin cap expansion are likely due to failed actin turnover at the cap. Actin turnover rates ($t_{1/2}$) at the interphase caps in untreated (n = 10 embryos) or DMSO-injected (n = 10 embryos) embryos were 18.9 ± 1.7 s and 17.9 ± 2.2 s, with most of the actin (87.3% ± 2.4% and 85.0% ± 4.3%, respectively) recovered in the photobleached region after 80 s (Figures 3C and 3D). However, in Jasp-treated embryos, only 25.5% ± 3.0% of the total actin recovered after 80 s (n = 10 embryos), with a very slow turnover half-time of 50.5 ± 5.3 s (Figure 3C and 3D).

Thus, inhibition of actin turnover results in failed actin cap expansion and failed centrosome separation. Previous studies have demonstrated that centrosome separation is not required for actin cap expansion: centrosome separation fails in colchicine-treated embryo, but there is very little effect on actin cap expansion [6]. Our experiments confirm this finding (Figure 2F; Figure 3A).

Disruption of Arp2/3, an Actin Branching Complex, Strongly Inhibits Actin Cap Expansion and Centrosome Separation

To test the converse relationship, whether actin cap expansion is required for centrosome separation, we analyzed mutants in Arpc1, a key component of the Arp2/3 complex. The Arp2/3 complex has been shown to localize to the margins of actin caps and promote cap expansion, presumably through its actin branching activity [15]. In control embryos, actin caps expanded to their maximum size (117.4 ± 4.1 μm^2) 5 min after NEF. However, the cap size in *Arpc1* embryos (the progeny of *Arpc1* mutant maternal germline clones) had only increased slightly at 5 min after NEF and from then on maintained an almost constant size until NEB (63.7 ± 7.8 μm^2, Figures 3A and 3B). This is about half of the maximum cap size observed in wild-type controls. These data are consistent with the published *Arpc1* phenotype [15]. Concomitantly, *Arpc1* mutant embryos displayed a significant reduction in the distance and angle of separation between centrosome pairs at NEB (6.1 ± 0.2 μm and 136° ± 7°, compared to 8.0 ± 0.2 μm and 169° ± 1° in wild-type controls, Figures 2G, 2K, and 2L).

Blocking Formin/Diaphanous and RhoA Activities Also Interferes with Cap Expansion and Centrosome Separation before NEB

Cap expansion driven by formin-mediated actin bundling is also required for centrosome separation before NEB. Diaphanous (Dia), the *Drosophila* formin homolog, is required for actin bundling and metaphase furrow formation [16]. Initial cap size was normal in *dia* embryos (the progeny of *dia* mutant maternal germlines), but cap expansion was strongly inhibited, with the cap size at NEB (76.6 ± 4.4 μm^2) reaching only 65% of the normal cap size (Figures 3A and 3B). Inhibition of RhoA, the upstream regulator of Diaphanous [17], through C3 exotransferase (C3), resulted in stronger defects in cap expansion (Figures 3A and 3B). Corresponding decreases in centrosome separation before NEB were observed in *dia* and C3-treated embryos (Figures 2H, 2I, 2K, and 2L). Together, these studies demonstrate that cap expansion is required for centrosome separation.

Apc2 Mutant Embryos Exhibit Relatively Normal Cap Expansion

The Apc2-Armadillo complex in the fly early embryo has been shown to regulate centrosome separation [3]. Based on its localization to cortical sites where actin and MTs interact [18], it was proposed that the Apc2-Armadillo complex facilitates centrosome separation through stabilizing the interaction between the actin cortex and astral MTs [3]. Alternatively, because we have shown that actin cap expansion is required for centrosome separation before NEB, it is possible that the Apc2-Armadillo complex facilitates centrosome separation by directly promoting actin cap expansion. To differentiate these two possibilities, we examined cap expansion in *Apc2* mutant embryos. The initial cap size and the cap expansion rate in *Apc2* embryos were very similar to those observed in uninjected control embryos (Figures 3A and 3B). To confirm that these *Apc2* embryos were defective in centrosome

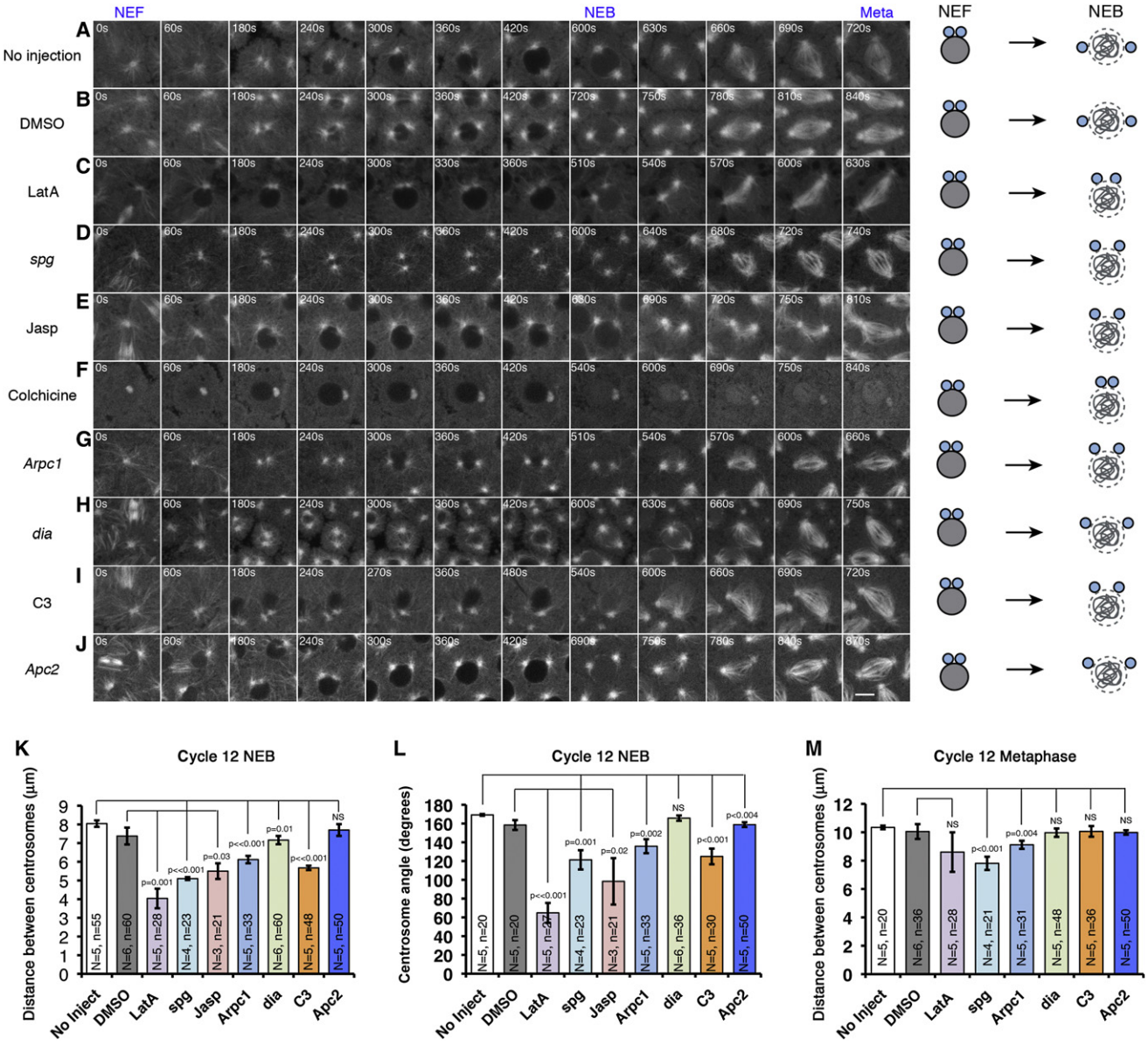

Figure 2. Cortical Actin Reorganization Facilitates Centrosome Separation before Nuclear Envelope Breakdown

Time-lapse images of:

(A) Uninjected GFP-Tub embryo. See Movie S1.

(B) Dimethyl sulfoxide (DMSO)-injected GFP-Tub embryo.

(C) 10 mM latrunculin A (LatA)-injected GFP-Tub embryo. See also Figure S3 and Movie S2.

(D) Rhodamine-tubulin-injected *spg* embryo.

(E) 1 mM Jasp-injected GFP-Tub embryo. See Movie S3.

(F) 0.5 mM colchicine-injected GFP-Tub embryo.

(G) Rhodamine-tubulin-injected *Arpc1* embryo.

(H) Rhodamine-tubulin-injected *dia* embryo. Centrosomes in *dia* and *Arpc1* embryos were imaged more apically than controls, indicating a slowed migration toward the midline of the nucleus.

(I) 1 mg/mL C3 exotransferase-injected GFP-Tub embryo.

(J) Rhodamine-tubulin-injected *Apc2* embryo.

Schematic drawings on the right side of each image series (A–J) illustrate the degree of centrosome separation from nuclear envelope formation (NEF) to nuclear envelope breakdown (NEB).

(K) Mean distance between centrosome pairs at NEB.

(L) Mean angle between centrosome pairs at NEB with respect to the nuclear center. The centrosome pairs of *dia* embryos failed to migrate basally but were able to separate from each other normally at a focal plane above the equator of each nucleus.

(M) Mean centrosome pair distances at metaphase. The following abbreviations are used: N, total number of embryos counted; n, total number of nuclei counted; NS, not statistically significant. Error bars represent the standard error of the mean (SEM) from at least three different embryos.

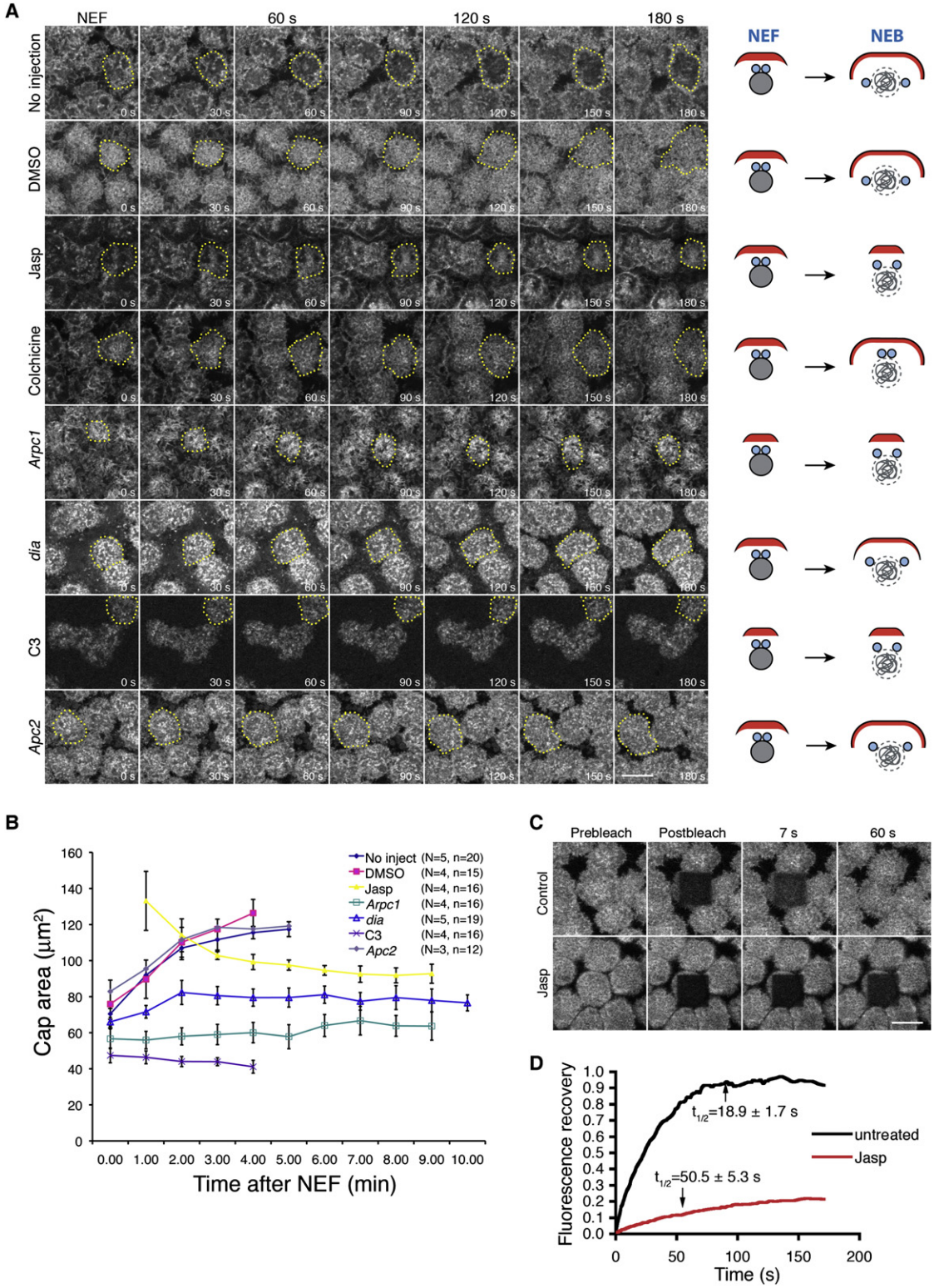

Figure 3. Actin Cap Expansion Is Driven by Arp2/3- and RhoA-Diaphanous-Mediated Actin Remodeling

(A) Actin-based cap expansion was imaged after NEF in wild-type untreated, DMSO-treated, Jasp-treated, colchicine-treated, C3-treated, *Arpc1*, *dia*, and *Apc2* embryos. In each row, cap expansion is illustrated by dotted lines. Schematic drawings on the right side of each image series illustrate actin cap expansion (actin is in red) relative to centrosome separation from NEF to NEB. See also Figure S2, Movie S4, and Movie S5.

(B) The rate of actin cap area expansion after NEF in the drug-treated and mutant embryos imaged in (A). The following abbreviations are used: N, total number of embryos; n, total number of caps counted.

(C and D) Fluorescence recovery after photobleaching analysis of actin turnover at the interphase cap in untreated and Jasp-treated embryos, showing relative fluorescence intensities of Rhodamine-actin at the caps after photobleaching. The prebleach intensities were arbitrarily set to 1. Scale bars represent 10 μm. Error bars represent the SEM from at least three different embryos.

separation, we injected Rhodamine-labeled tubulin into these embryos to follow centrosome movements. Our data showed very similar centrosome separation defects before NEB (159° ± 2° compared to 169° ± 1° in wild-type controls, Figures 2J and 2L), as did previously published results [3]. Because *Apc2* embryos do not disrupt actin cap organization [18, 19], the effects on centrosome separation were not as dramatic as those observed for the actin inhibitors, and thus we did not observe significant differences in the distance in which the centrosomes separated (Figure 2K). In addition, after NEB, the incomplete separated centrosome pairs were able to correct the earlier defects and achieve full separation by metaphase (Figure 2M). Taken together, these suggest that the Apc2-Armadillo complex functions downstream of actin cap expansion to regulate centrosome separation before NEB.

Myosin II-Driven Cortical Flow Is Not Required for Centrosome Separation before NEB

Actin-myosin II-driven cortical flow has been proposed to separate centrosomes after NEB in mammalian cultured cells [2]. To determine whether myosin II, similar to actin, facilitates centrosome separation during the cortical divisions in *Drosophila* embryos, we relied on the small molecule inhibitor Y-27632, a drug that inhibits Rho kinase, which in turn blocks myosin II light-chain kinase and thus myosin II activity. We used a drug concentration (50 mM) that has been proven to effectively block myosin II activity in our system [20]. A significant delay into mitosis was often observed after Y-27632 injection (from cycle 12 NEF to NEB, 1455 ± 296 s versus 588 ± 15 s in control embryos). Unlike LatA injection, myosin II inhibition by Y-27632 had only a very mild effect on centrosome separation before NEB, and the metaphase spindle length was about the same as that in control embryos (Figures 4A–4C). The relatively normal centrosome separation and spindle formation after inhibiting myosin II is consistent with previously published results [20]. Thus, it appears that centrosome separation during the cortical divisions in the *Drosophila* embryo does not rely on myosin II activity.

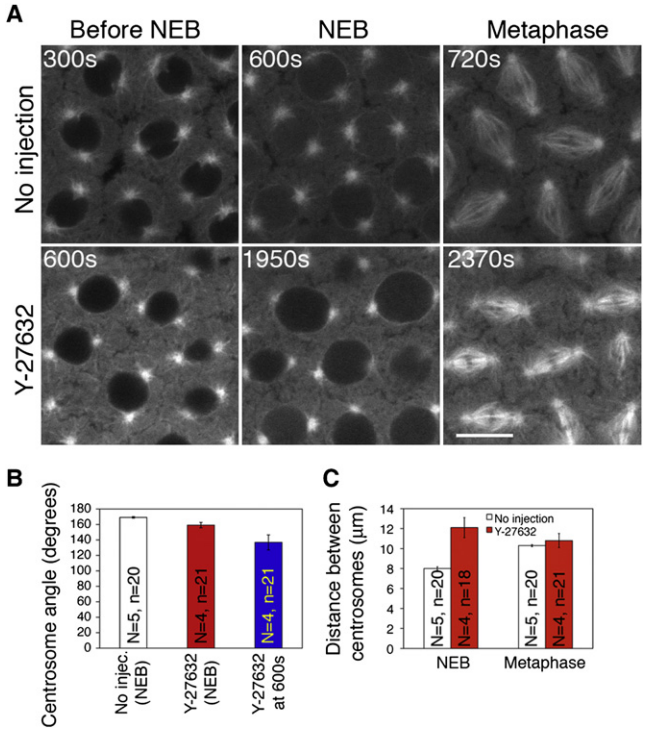

Figure 4. Disruption of Myosin II by Y-27632 Has a Very Mild Effect on Centrosome Separation before NEB

(A) 50 mM Y-27632 was injected into GFP-tubulin embryos. Prolonged interphase, nuclear swelling, and centrosome detachment from the nuclear envelope were observed. A lower drug concentration (2 mM) failed to produce any of these phenotypes (data not shown). Scale bar represents 10 μm.

(B) Mean angle of centrosome pairs at NEB of uninjected embryos and Y-27632-treated embryos. Because the 50 mM Y-27632 injection induced prolonged interphase, the centrosome angle of Y-27632-treated embryos was also measured at 600 s after NEF, the equivalent timing of NEB in control embryos.

(C) Centrosome distance at NEB and metaphase of uninjected embryos and Y-27632-treated embryos. The increase in centrosome distance in Y-27632-treated embryos at NEB was due to the nuclear swelling phenotype induced by this drug injected at 50 mM. The following abbreviations are used: N, total number of embryos; n, total number of nuclei counted. Error bars represent the SEM from at least three different embryos.

Conclusions

We have described an essential role for actin turnover in centrosome separation prior to NEB. During this stage, centrosome separation occurs along the nuclear envelope and requires dynein, a minus end directed motor protein [4, 21–23]. However, additional mechanisms must be required, because some separation of centrosomes still occurs in *dynein* mutants [4]. Work by Rosenblatt and colleagues demonstrated that actin-myosin II-based cortical flow drives the late stages of centrosome separation after NEB [2], raising the possibility that similar cortical-based mechanisms are driving the initial stages of centrosome separation. In fact, previous studies in both mammalian and *Drosophila* systems indicated that actin is necessary for centrosome separation before NEB [6, 24], but it remained unclear where and how actin functions to separate centrosomes and whether this is myosin based. Our results demonstrate that in the *Drosophila* embryo, the initial stages of centrosome separation prior to NEB rely on dynamic actin turnover but are independent of myosin.

Specifically, our results demonstrate a role for both Arp2/3-mediated actin branching and formin/Diaphanous-mediated actin bundling in cortical actin cap expansion, which in turn drives centrosome separation prior to NEB. There was also a strong positive correlation between the severity of the centrosome separation defects and the cap expansion defects. Among the drugs and mutations that didn't remove actin completely, Jasp and C3 produce the greatest disruption in cap expansion and also the greatest disruption in centrosome separation. *Arpc1* and *dia* produced progressively less severe defects in cap expansion and centrosome separation. Recent models propose that centrosome separation is driven by the dynein-dynactin complex associated with cortical actin [3–5]. Cortical dynein localization at dynamic actin caps may function to pull astral MTs and their associated centrosomes toward the cortex. This force is opposed by action of the Ncd motor protein on interpolar MT bundles [22]. In addition, the MT depolymerase Klp10A also plays a role in the initial phase of centrosome separation [25]. Our results suggest that either the astral MT plus ends are constantly seeking some unknown cues at the growing edge of the cortical expanding caps or the actin-MT plus-end interaction is stabilized preferentially at the growing edge of the expanding caps. Based on our results and previous studies [3], the Apc2-Armadillo complex may provide a link between the

expanding actin cap and astral microtubules. It will be of interest to determine whether actin turnover at the cortex also plays a role in centrosome and spindle positioning in other cell types [12, 26, 27]. Our finding that the actin-based centrosome separation defects are fully corrected after NEB is particularly intriguing and may provide insight into mechanisms regulating spindle size.

Experimental Procedures

Fly Strains and Genetics

Germline clones of *diaphanous*^{5} or *Arpc1*^{R337st} were generated via the flipase-dominant female sterile technique [15, 16, 28]. These two mutants and *Apc2*$^{\Delta S}$ were acquired from the Bloomington Drosophila Stock Center. *sponge*^{335} was a gift from E. Wieschaus. For live embryo imaging, we used the following stocks: GFP-alpha tubulin (a gift from T. Kaufman; [29]), GFP-moesin (a gift from D. Kiehart; [30]), and GFP-Dlg (Discs Large) (FlyTrap Project; [31]). All stocks were raised at 25°C on standard corn meal and molasses media.

Live Embryo Analysis

Embryos were prepared for microinjection and time-lapse scanning confocal microscopy as previously described [32]. All of the reagents were injected at 50% egg length and were diluted approximately 100-fold in the embryos [33]. For *sponge*, *diaphanous*, and *Arpc1* mutant embryos, Rhodamine-conjugated tubulin (10 mg/ml, Cytoskeleton) or Rhodamine-conjugated actin (10 mg/ml, Cytoskeleton) was injected into the embryos at late cycle 10 or early cycle 11. The following drugs were injected at cycle 11 anaphase: DMSO alone (Sigma-Aldrich), LatA (10 mM in DMSO, Sigma-Aldrich), Y-27632 (50 mM and 2 mM, Tocris), Jasp (1 mM in DMSO, Calbiochem), C3 exotransferase (1 mg/ml, Cytoskeleton), and colchicine (0.5 mM, Sigma-Aldrich). GFP-Dlg was used to mark the furrow membrane [13].

Confocal Microscopy and FRAP Analysis

Confocal microscope images were captured on an inverted photoscope (DMIRB; Leitz) equipped with a laser confocal imaging system (TCS SP2; Leica) with an HCX PL APO 1.4 NA 63× oil objective (Leica). ImageJ software (National Institutes of Health) was used to quantify the confocal images.

FRAP analysis was performed as previously described [13]. Imaging was controlled by the Leica Confocal Software Microlab. After five prebleach scans of an entire image, 10 bleaching scans (0.7 s each) with 100% intensity of 488 nm and 543 nm over the region of interest in the actin caps (10 μm × 10 μm) were performed. After photobleaching, fluorescence recovery was monitored 10 times every 0.7 s, 60 times every 2 s, and 10 times every 5 s. The recovery of fluorescence intensities was measured with Microlab. The intensity of the bleached cap area was normalized to the background nonbleached area. Recovery percentage was calculated as the final plateau intensity (I_F) minus the first intensity after photobleaching (I_0), all divided by the difference between prebleach (I_I) and postbleach (I_0) intensities $([I_F - I_0]/[I_I - I_0])$. The fluorescence intensity of each time point (I_t) was transformed into a 0–1 scale calculated by $(I_t - I_0)/(I_I - I_0)$. The values of relative intensities versus time were plotted with Excel (2004; Microsoft), and the recovery $t_{1/2}$ was measured from the plots.

Image Quantifications and Statistics

Centrosome pair distances were quantified in the optical section with the strongest centrosome signal (GFP-tubulin or injected Rhodamine-tubulin). Centrosome pair angles were measured with the angle tool in ImageJ by placing the vertex at the approximate center of each nucleus. In Jasp-injected embryos, although centrosomes can separate further during subsequent prometaphase and metaphase, spindles tend to fuse with each other. To avoid secondary defects because of spindle fusion, we analyzed centrosomes only prior to NEB in Jasp-treated embryos.

For cap expansion analysis, time-lapse confocal images were taken of either GFP-moesin-expressing or Rhodamine-actin-injected embryos from NEF to NEB of cycle 12. A Z series was taken every 30 s during this time period, with Z steps of 0.75 μm starting at the very surface of the embryo. Cap expansion was measured with ImageJ. Confocal sections depicting just the actin cap were used in all experiments. The freehand tool in ImageJ was used to encircle each cap, which allowed an area measurement. Four individual caps were measured in each embryo from the beginning of cycle 12. These four were followed every minute until the boundaries of each cap could no longer be differentiated from that of neighboring caps (Figure 1B). In C3-treated embryos, caps were tracked only until 4 min after NEF, when the cap boundaries became indiscernible. At least three embryos were analyzed per genotype or drug treatment.

Student's t tests (two-tailed, equal variance) were performed to analyze the data. For each embryo, multiple mitotic apparati were quantified and averaged. These averaged values were then used for statistics to estimate the variance between embryos under the same treatment. Error bars represent the standard error of the mean from at least three independent experiments. For videos, image series collected over time were cropped in ImageReady (version 9.0; Adobe) and converted to QuickTime (Apple) videos via portable network graphics lossless compression.

Supplemental Information

Supplemental Information includes three figures and five movies and can be found with this article online at doi:10.1016/j.cub.2010.02.060.

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