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The Concentration of Nuf, a Rab11 Effector, at the Microtubule-organizing Center Is Cell Cycle–regulated, Dynein-dependent, and Coincides with Furrow Formation[D] [V]

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Animal cytokinesis relies on membrane addition as well as acto-myosin–based constriction. Recycling endosome (RE)-derived vesicles are a key source of this membrane. Rab11, a small GTPase associated with the RE and involved in vesicle targeting, is required for elongation of the cytokinetic furrow. In the early *Drosophila* embryo, Nuclear-fallout (Nuf), a Rab11 effector, promotes vesicle-mediated membrane delivery and actin organization at the invaginating furrow. Although Rab11 maintains a relatively constant localization at the microtubule-organizing center (MTOC), Nuf is present at the MTOC only during the phases of the cell cycle in which furrow invagination occurs. We demonstrate that Nuf protein levels remain relatively constant throughout the cell cycle, suggesting that Nuf is undergoing cycles of concentration and dispersion from the MTOC. Microtubules, but not microfilaments, are required for proper MTOC localization of Nuf and Rab11. The MTOC localization of Nuf also relies on Dynein. Immunoprecipitation experiments demonstrate that Nuf and Dynein physically interact. In accord with these findings, and in contrast to previous reports, we demonstrate that microtubules are required for proper metaphase furrow formation. We propose that the cell cycle–regulated, Dynein-dependent recruitment of Nuf to the MTOC influences the timing of RE-based vesicle delivery to the invaginating furrows.

INTRODUCTION

Cytokinesis, the final event in the cell cycle, relies on assembly of an actomyosin contractile ring that drives furrow invagination. The contractile ring forms perpendicular and midway to the anaphase spindle, and its location is determined by the position of the centrosomes and their associated microtubule arrays (Rappaport, 1996; D'Avino *et al.,* 2005). An early step in cytokinesis is the recruitment of furrow components such as actin, Myosin II, Anillin, Formins, and Septins to the site of contractile ring formation (Eggert *et al.,* 2006). Immediately after contractile ring formation furrow invagination occurs.

A major advance in our understanding of animal cytokinesis has been the finding that furrow invagination relies on vesicle-mediated membrane addition as well as acto-myosin based contraction (for recent reviews, see Albertson *et al.,* 2005; Burgess and Chang, 2005; Otegui *et al.,* 2005). The most visually striking evidence comes from electron microscopy (EM) and live confocal studies revealing concentrations of exocytic fusion pores at the base of the invaginating furrows in *Xenopus laevis* embryos (Danilchik *et al.,* 2003). Studies in *Caenorhabditis elegans* and *Drosophila*, using a variety of functional approaches, demonstrate that Golgi-derived vesicles provide a key source of membrane required for furrow invagination (Lecuit and Wieschaus, 2000; Sisson *et al.,* 2000; Skop *et al.,* 2001; Xu *et al.,* 2002; Farkas *et al.,* 2003). Genomic studies support this conclusion as one quarter of the proteins associated with the midbody are Golgi-derived and RNA interference (RNAi) studies demonstrate that many of these proteins are essential for cytokinesis (Skop *et al.,* 2004). Functional studies in *C. elegans*, *Drosophila*, and mammalian cells demonstrate that the recycling endosome (RE) is also required for invagination of the cytokinetic furrow (Skop *et al.,* 2001; Riggs *et al.,* 2003; Wilson *et al.,* 2005). The RE is responsible for trafficking vesicles to the plasma membrane and this organelle is often closely associated with the microtubule-organizing center (MTOC). These properties make it well suited for delivering membrane to the cytokinetic furrow. Rab11, a small GTPase localized at the RE, is required for proper RE organization and function (Ullrich *et al.,* 1996; Horgan *et al.,* 2007). RNAi studies in *C. elegans* demonstrate that inhibition of Rab11 disrupts the early and late stages of

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Abbreviations used: RE, recycling endosome; Nuf, nuclear-fallout; DHC, dynein heavy chain; DIC, dynein intermediate chain; MTOC, microtubule-organizing center.

cytokinesis (Skop *et al.*, 2001). Functional studies in mammalian cell culture demonstrate a role for the RE in the late stages of cytokinesis (Wilson *et al.*, 2005).

The furrows that form during the cortical divisions of *Drosophila* embryogenesis, known as the metaphase and cellularization furrows, are particularly well suited for studying the role of membrane addition during furrow formation. During the cortical divisions of early *Drosophila* embryogenesis thousands of furrows form simultaneously (Sisson *et al.*, 1999). These furrows are structurally and compositionally equivalent to the furrows formed during conventional cytokinesis and include actin, Myosin, Spectrin, Cofilin, ARP, Anillin, Septin, and Formin (Miller and Kiehart, 1995; Stevenson *et al.*, 2002). As with conventional cytokinesis, these components are closely associated with the invaginating furrow. A number of studies demonstrate that these furrows rely extensively on membrane addition rather than acto-myosin–based contraction. Recycling-endosome and Golgi-based vesicle delivery play a key role in furrow formation and elongation during the cortical divisions and cellularization (Sisson *et al.*, 2000; Riggs *et al.*, 2003; Papoulas *et al.*, 2005). In contrast, disrupting Myosin function has relatively little effect on metaphase and cellular furrow elongation (Royou *et al.*, 2004).

The furrows formed in the early *Drosophila* embryo and those formed during conventional cytokinesis differ in their timing and position. Conventional cytokinesis furrows form during anaphase and telophase and the furrow forms perpendicular to and bisects the elongating spindle. In contrast metaphase furrows are initially formed during prophase and are dismantled during anaphase and telophase. These furrows encompass rather than bisect the spindle. Cellular furrows form during the prolonged interphase of nuclear cycle 14 and encompass the inverted baskets of microtubules that are formed around each nucleus. Although it is clear that microtubules play a key role in furrow formation during conventional cytokinesis, the role of microtubules in metaphase furrow formation remains unresolved. Given the structural and compositional conversation between metaphase and conventional cytokinesis furrows, the regulatory role of microtubules in furrow formation would be conserved as well (D'Avino *et al.*, 2005). In addition, The fact that the pattern of overlapping astral microtubule arrays from neighboring centrosomes is well correlated with pattern of newly formed metaphase furrows suggests that microtubules play an important role in metaphase furrow formation (Sisson *et al.*, 1999). However, a recent study exploring this issue examined the effects of microtubule inhibitors on furrow formation and concluded that microtubules do not play a role in metaphase furrow formation (Stevenson *et al.*, 2001).

The studies presented here focus on the role of microtubules in regulating the subcellular localization of Nuclear-fallout (Nuf), a Rab11 effector required for proper metaphase and cellular furrow formation (Rothwell *et al.*, 1998, 1999). Nuf is a homolog of mammalian FIP4/Arfophilin 2 (Arfo2), an ADP ribosylation factor (Arf) effector that binds and colocalizes with the small GTPase Rab11 at the RE (Hickson *et al.*, 2003; Riggs *et al.*, 2003). Rab11 is required for RE organization and the transport of vesicles to and from the plasma membrane (Mellman, 1996). Nuf and FIP4 share a conserved 20 amino acid C-terminal Rab11-binding domain (Hickson *et al.*, 2003; Riggs *et al.*, 2003). Nuf exhibits a cell cycle–regulated colocalization with Rab11 at the MTOC. Nuf and Rab11 physically associate and are mutually required for their localization at the RE (Riggs *et al.*, 2003). Embryos with reduced levels of Rab11 produce membrane recruitment and actin-remodeling defects strikingly similar to *nuf*-derived embryos. These studies support a common role for Nuf and Rab11 in membrane trafficking and actin remodeling during the initial stages of furrow formation.

Rab11 maintains a constant localization at the MTOC throughout the cell cycle, whereas Nuf localization at the MTOC is cell cycle regulated. Significantly Nuf localization at the MTOC is maximal during furrow formation. During the cortical divisions (nuclear cycles 10–13) Nuf concentration at the MTOC is highest during prophase at the time of metaphase furrow elongation. Significantly during cellularization at nuclear cycle 14, Nuf concentration is highest during interphase. In contrast to the metaphase furrows, cellular furrow invagination occurs during the prolonged interphase of nuclear cycle 14.

These observations raise the possibility that the cell cycle–regulated concentration of Nuf at the MTOC activates RE-mediated vesicle delivery to the invaginating furrows. These findings prompted us to explore the mechanisms by which Nuf concentrates at the MTOC during prophase of the cortical nuclear cycles in the early *Drosophila* embryo. We demonstrate that Nuf protein levels remain constant throughout the cell cycle and Nuf localization at the MTOC occurs through Dynein-based recruitment and maintenance at the MTOC. We also demonstrate a physical association between Nuf and Dynein. In accord with these findings, and in contrast to a previous report (Stevenson *et al.*, 2001), we demonstrate that microtubules are required during the initial stages of furrow formation for proper actin recruitment of the metaphase furrow consistent with well-established findings in conventional cytokinetic furrow formation.

METHODS AND MATERIALS

Drosophila Stocks

The initial characterization of the GFP-Nuf stock has been previously described (Riggs *et al.*, 2003). The GFP-Moesin stock was kindly provided by Daniel Kiehart (Biology, Duke University) (Edwards *et al.*, 1997). The Krupple-GFP and the *sponge* stocks were obtained from the Bloomington *Drosophila* Stock Center (Indiana). Experiments were performed using the *sponge*^{16} allele (Postner *et al.*, 1992). Stocks were maintained on standard corn meal/molasses medium.

Live Embryo Analysis

GFP-Nuf and GFP-Moesin embryos were prepared for microinjection and time-lapse scanning confocal microscopy as previously described (Yu *et al.*, 2000; Tram *et al.*, 2001). The following reagents were injected at 50% egg length: cytochalasin D, 100 μg/ml (Sigma-Aldrich, St. Louis, MO); colchicine, 100 μg/ml (Sigma-Aldrich); rhodamine-conjugated tubulin (Invitrogen-Molecular Probes); monoclonal anti-mouse Dynein heavy-chain (DHC) antibody (McGrail and Hays, 1997; Papoulas *et al.*, 2005); and monoclonal anti-mouse (74-1) Dynein intermediate-chain (DIC), 200 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). Colchicine and cytochalasin D were diluted in 20% DMSO to their final concentration. The DHC and DIC antibodies are well characterized and have been successfully used in disruption studies examining the functional role of Dynein during *Drosophila* development (McGrail and Hays, 1997; Boylan *et al.*, 2000; Wojcik *et al.*, 2001). All antibodies were affinity-purified and dialyzed against phosphate-buffered saline (PBS)/40% glycerol solution (pH 7.5) before injection. Control injections were performed by injecting water, PBS/40% glycerol, or a 20% DMSO solution.

Fixation and Immunofluorescence

Immunofluorescence analysis was performed as described by Rothwell and Sullivan (2000) and Sisson *et al.* (2000). Embryos were stained using anti-rat Rab11 polyclonal antibodies generously supplied by Robert Cohen (Molecular Biosciences, University of Kansas) (Dollar *et al.*, 2002) at a concentration of 1:500 in PBTA (1× PBS, 0.1% Triton X-100, 1% bovine serum albumin). Secondary Alexa Fluor 488 anti-rat antibodies (Invitrogen-Molecular Probes, Eugene, OR) were applied to the embryos as described previously (Karr and Alberts, 1986). Injected embryos were fixed by physically removing as much of the halocarbon oil as possible and using heptane to wash the embryos from the coverslip into a glass Petri dish. The embryos were immediately transferred into a solution of heptane saturated with 37% formaldehyde and fixed as previously described (Rothwell and Sullivan, 2000).

Microscopy and Quantification of Fluorescence

Microscopy was performed using a Leitz DMIRB inverted photoscope (Rockleigh, NJ), equipped with a Leica TCS NT laser confocal imaging system (Deerfield, NJ). To measure the variation of Nuf protein in the embryo before and after injections, the intensity of brightness of the GFP-Nuf signal was quantified. The Leica TCS NT software analysis system was used for this purpose. The mean value of brightness of an area of 70×70 pixels was measured for all frames of each movie. The background brightness value was subtracted from both the control and experimental injections. This value was obtained at telophase in the untreated GFP-Nuf embryo, when the intensity of Nuf is at its lowest. The variability of brightness as a function of time was reported in all the graphs. Control embryos were either injected with water, PBS/40% glycerol, or a 20% DMSO solution, and experimental embryos were injected with inhibitor (Colchicine or anti-Dynein antibody) dissolved in 20% DMSO and PBS/40% glycerol, respectively. All measurements were taken at a site ~30 μm from the site of injection. All injections were performed 3–6 min after entry into interphase of nuclear cycle 14. Cellularization occurs from 10 min after entry into interphase of nuclear cycle 14 and lasts 70 min (Foe, 1989).

Protein Preparation

Immunoprecipitation experiments were carried out on extracts of *Drosophila* embryos aged 0–4 h. Homogenization, incubation, and wash steps were in 50 mM HEPES, pH 7.4, 150 mM KCl, 0.9 M glycerol, 0.5 mM dithiothreitol (DTT), and 0.1% Triton X-100 supplemented with protease inhibitors, plus 2 mM phenylmethylsulfonyl fluoride (PMSF). Antibodies to the rat cytoplasmic DIC (MAB 1618, Chemicon, Temecula, CA), the *Drosophila* DHC P1H4; (McGrail and Hays, 1997), or the mouse anti-GFP (Invitrogen-Molecular Probes) were allowed to bind to protein A-Sepharose (Sigma-Aldrich) and then incubated with equal amounts of embryo extract (0.6 mg of total protein in 400 μl) for 3 h at 4°C. Beads were washed three times, the last two times in buffer lacking Triton X-100. Each pellet was eluted into 20 μl of SDS-PAGE sample buffer, and the entire volume was loaded onto a gel for blot analysis. The blot was probed with monoclonal anti-GFP antibody (Clontech, Palo Alto, CA) at a dilution of 1:1000. Equal volumes of supernatants were analyzed by blot analysis, 25 μg total protein.

Single Embryo Western Immunoblots

Immunoblots of individually staged embryos were prepared as previously described (Edgar *et al.*, 1994; Su, 2000). Embryos were collected and dechorionated in 50% bleach for 2 min, extensively rinsed, immersed in heptane, and rapidly transferred into a mixture with equal volume of heptane and methanol (containing 1 mM Na_3VO_4) for fixation. These embryos were rinsed three times in ice-cold 99% methanol, 1 mM Na_3VO_4 and rehydrated with embryo buffer (EB) containing 10 mM of NaF. The embryos were then stained with EB containing 4 μg/ml Hoechst 33258 for 3–4 min, rinsed twice in EB, and transferred to 40%EB/60% glycerol. Embryos were staged visually using the DAPI channel of a fluorescent microscope with 20× objective. Handpicked cycle 12 embryos (4 per sample) were dissolved in 2× SDS sample buffer containing 50 mM NaF and 100 mM sodium β-glycerophosphate and proceeded with SDS-PAGE and immunoblotting using standard procedures. Na_3VO_4, 1 mM, was also added to polyacrylamide gels to stabilize phosphoisoforms of Nuf.

RESULTS

Nuf Protein Levels Remain Relatively Constant throughout the Cell Cycle

Previous studies demonstrated that Nuf concentration at the MTOC steadily increases from telophase through prophase, abruptly decreases during prometaphase, and remains at low levels from prometaphase through anaphase (Rothwell *et al.*, 1998; Figure 1A). This cell cycle–dependent pattern of Nuf localization at the MTOC can be explained either by overall cell cycle–dependent changes in Nuf abundance or cell cycle changes in Nuf concentration at and dispersion from the MTOC. To distinguish between these alternatives, we performed immunoblots on embryos at defined stages of the cell cycle (Figure 1B). Fixed DAPI-stained embryos were individually scored for cell cycle stage based on their chromosome morphology. Embryos from specific stages of the cell cycle were selected and extracts prepared for immunoblot analysis. Probing the blots with anti-Nuf antibody reveals Nuf protein levels do not exhibit dramatic alterations in abundance and remain relatively constant throughout the cell cycle (Figure 1B). From this analysis, we conclude that protein degradation is unlikely to be a major cause in the dramatic cell cycle–dependent changes in Nuf concentration at the MTOC. Instead, these results favor a model in which cell cycle–regulated cycles of recruitment and dispersion dictate Nuf levels at the MTOC. As previously described, the less abundant higher molecular weight bands are phosphoisoforms of Nuf (Rothwell *et al.*, 1998). It is interesting to note that the pattern of phosphorylation appears to be cell cycle regulated with the highest amounts occurring during prophase when Nuf is concentrated at the MTOC and furrow invagination is occurring.

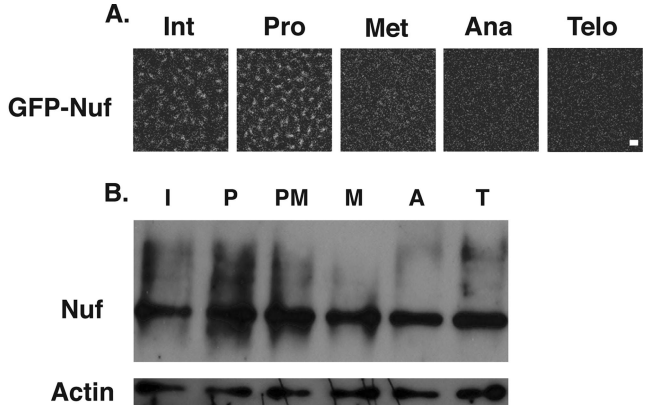

Figure 1. The abundance of Nuf protein does not change dramatically throughout the cell cycle. (A) GFP-Nuf localization throughout syncytial cortical nuclear cycle 12. From interphase through prophase, Nuf concentration at the MTOC increases. At metaphase, Nuf concentration at the MTOC dramatically decreases and remains low during anaphase and telophase. Scale bar, 5 μm. (B) Western analysis of Nuf protein levels throughout the syncytial nuclear cycle 12. Nuf protein levels remain relatively constant at each stage of the cell cycle, suggesting that protein degradation is not responsible for changes in Nuf concentration at the MTOC. I, interphase; P, prophase; PM, prometaphase; A, anaphase; T, telophase. Actin was used as a loading control (bottom row).

Microtubules Are Continuously Required To Recruit and Maintain Nuf at the MTOC

With the above model in mind, we tested the role of microtubules in localizing and concentrating Nuf at the MTOC. We injected the microtubule depolymerizing agent, colchicine, into living GFP-Nuf embryos (see *Materials and Methods*). These embryos were also injected with rhodamine-labeled tubulin to simultaneously follow microtubule dynamics throughout the cell cycle. As shown in Figure 2, we followed Nuf from interphase to metaphase of nuclear cycle 12. Colchicine was injected precisely at anaphase of nuclear cycle 12. Images taken immediately after the injection reveal that colchicine induces extensive microtubule depolymerization resulting in failed centrosome separation and monopolar spindles at prophase (Figure 2, second column). Recording images every 30 s for a total of 15 min, we found a significant reduction in Nuf localization at the MTOC in the injected embryos. This is demonstrated by comparing Nuf localization at the MTOC during the prophase before and after colchicine injection. This is also demonstrated by comparing Nuf localization at the MTOC during prophase in injected and uninjected control embryos (Figure 2, bottom row). These studies demonstrate that Nuf localization to the MTOC requires normal microtubule arrays.

We also performed precisely timed colchicine injections at telophase and interphase and monitored Nuf localization in the following prophase as described above. As with the anaphase injections, injecting colchicine at telophase and

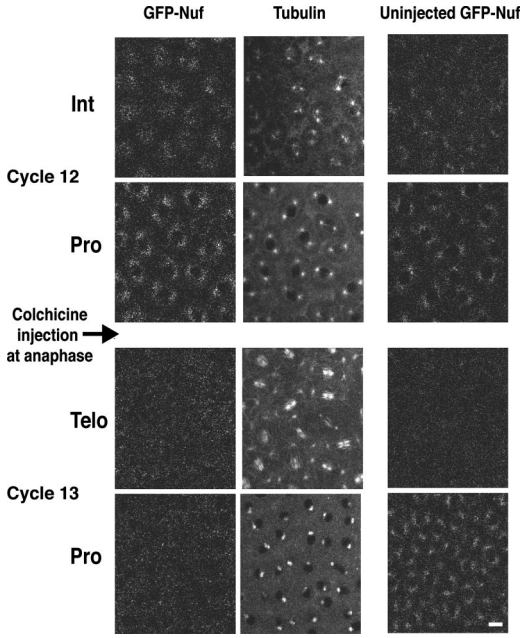

Figure 2. Nuf localization at the MTOC requires microtubules. Colchicine, a microtubule-depolymerizing agent, was injected in early anaphase into an embryo containing GFP-Nuf and rhodamine-labeled tubulin. Nuf fails to reaccumulate at the centrosomes in the next cell cycle (compare Nuf interphase localization before and after injection with uninjected controls, rightmost column). Scale bar, 5 μm.

interphase in the cell cycle disrupts Nuf localization at the MTOC in the following prophase (data not shown). We conclude that microtubules are required from anaphase through interphase for proper recruitment of Nuf to the MTOC in the following prophase.

To determine if microtubule arrays are required to maintain Nuf at the MTOC, we took advantage of the fact that Nuf is stably maintained at the MTOC during the prolonged interphase of nuclear cycle 14 (Rothwell *et al.*, 1998). Using the approach described above, we monitored microtubule and Nuf dynamics as the embryos entered nuclear cycle 14 and initiated cellularization (Figure 3A, Supplementary Movie M1). Injecting colchicine 5 min after the start of cellularization produces a rapid dispersal of Nuf from the MTOC.

Quantifying total GFP fluorescence intensity at the MTOC in untreated embryos, reveals Nuf levels remain relatively constant at the MTOC from 5 through 25 min after the injection (Figure 3B). Over the same time span in colchicine injected embryos, Nuf levels decrease 76% (from a mean brightness value of 17 to 4). We conclude that microtubules are required to maintain as well as recruit Nuf to the MTOC.

Microtubules Are Required for Proper Rab11 Localization

To determine the role of microtubules on the localization and maintenance of Rab11 at the MTOC, cellularizing embryos were injected with the microtubule-depolymerizing agent colchicine, fixed, and prepared for Rab 11 immunofluorescent analysis (Figure 4). Like Nuf, Rab11 is stably maintained at the MTOC during cellularization. Colchicine injection results in a dramatic reduction in Rab11 at the MTOC, whereas control injections (20% DMSO) do not disrupt Rab11 localization. Thus maintenance of both Rab11 and Nuf at the MTOC during cellularization requires microtubules.

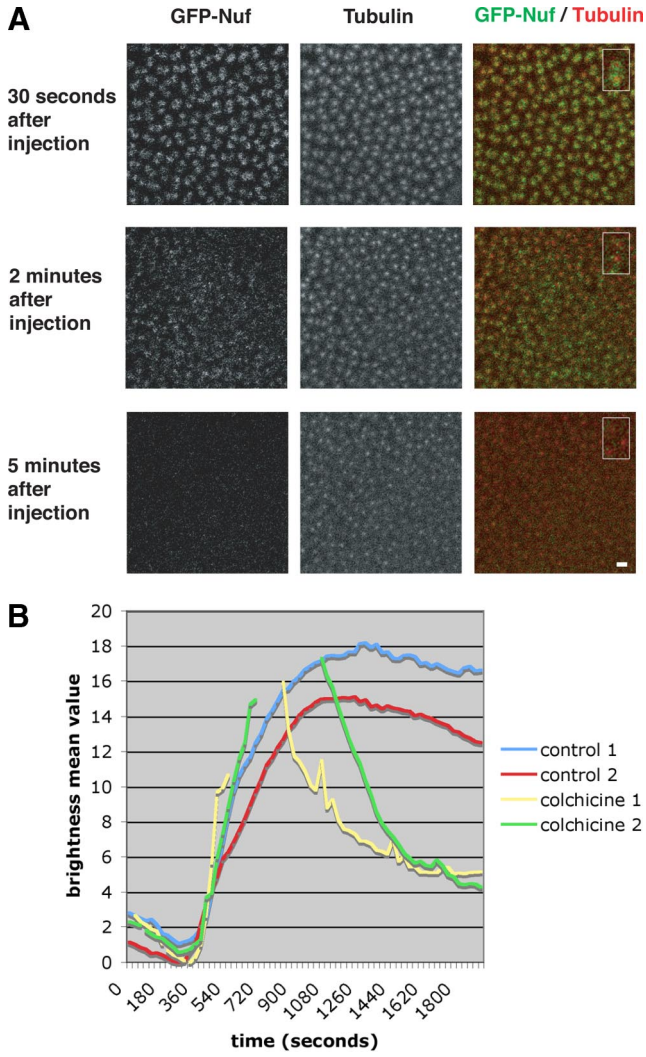

Figure 3. (A) Microtubules are necessary for maintaining Nuf at the MTOC during cellularization. Nuf is stably maintained at the MTOC from the beginning of interphase of nuclear cycle 14 until the completion of cellularization. The microtubule inhibitor, colchicine, was injected during early cellularization in an embryo containing GFP-Nuf (green) and rhodamine-labeled tubulin (red). Within 5 min of colchicine injection, Nuf localization at the MTOC is greatly diminished. Scale bar, 5 μm. (B) Quantification of Nuf concentration at the MTOC during cellularization. The graph depicts the concentration of GFP-Nuf at the MTOC in two uninjected control embryos and two embryos injected with the microtubule inhibitor colchicine, 0.5 mM, at the beginning of cellularization (cycle 14). Metaphase of syncytial cycle 13 occurs at the 90-s time point, anaphase of syncytial cycle 13 occurs at the 210-s time point, and telophase of syncytial cycle 13 occurs at the 330-s time point. Breaks in the curve indicate the time of injection.

Nuf Localization Is Independent of Microfilaments

To determine if cortical actin plays a role in Nuf localization, we examined Nuf localization in embryos derived from homozygous *sponge* females (Postner *et al.*, 1992). In this mutant, reorganization of the actin into interphase caps and metaphase furrows fails. Shown in Figure 5A are GFP-Nuf–bearing *sponge*-derived embryos injected with rhodamine-labeled tubulin. The disrupted cortical actin organization results in nuclei receding from the cortex (arrow), irregularly spaced nuclei (arrowhead), and spindle fusions (see

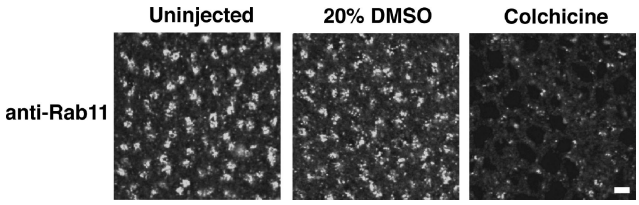

Figure 4. Proper Rab11 localization requires microtubules. Cellularizing embryos were injected with a 20% DMSO solution with and without 100 μg/ml colchicine. Immediately after injection, the embryos were fixed and processed for immunofluorescent analysis using the anti-Rab11 antibody. Injection with the 20% DMSO had no effect on Rab11 localization at the MTOC, whereas colchicine injections produced a severe reduction of Rab11 at the MTOC. Scale bar, 5 μm.

bottom row, Met panel). In spite of these defects, Nuf dynamics and accumulation at the MTOC during prophase are normal in *sponge*-derived embryos.

We confirmed these results by injecting the microfilament inhibitor cytochalasin D in GFP-Nuf–bearing embryos (Figure 5B). The injected embryos displayed irregular nuclear spacing and spindle fusions similar to the phenotypes observed in *sponge*-derived embryos (Figure 5B, bottom row). In spite of disrupting the organization of the cortical microfilaments, Nuf is normally recruited and maintained at the MTOC.

Dynein Is Required for Proper Nuf Centrosomal Localization

The rapid microtubule-dependent accumulation of Nuf at the MTOC suggests the action of a minus-end microtubule motor protein. To test this hypothesis, we injected affinity purified anti-DHC antibodies (McGrail and Hays, 1997) into GFP-Nuf–bearing embryos during interphase of nuclear cycle 12 (Figure 6) and observed Nuf dynamics through prophase of nuclear cycle 13. The top panels depict images immediately after injection (the embryos were also injected with rhodamine-labeled tubulin to directly follow other aspects of the nuclear cycle). These images indicate that Nuf accumulation at the MTOC is slightly reduced in prophase relative to uninjected controls (left insets). When the embryos enter the next prophase, having been exposed to the antibody for a full cycle, Nuf localization at the MTOC is greatly reduced (in the bottom row compare the anti-DHC injected embryo to the uninjected control embryo). As previously described, we find anti-DHC injection does not disrupt progression through metaphase (middle rows), but does inhibit separation and attachment of centrosomes to the nuclear envelope (Robinson *et al.*, 1999).

To determine whether Dynein is required to maintain Nuf at the MTOC, we injected anti-DHC antibodies into GFP-Nuf–bearing embryos during the prolonged interphase of nuclear cycle 14 (Figure 7, Supplementary Movie M2). This stage provides an excellent opportunity to address this issue because Nuf is stably maintained at the MTOC for the ~70 min it takes to complete cellularization. The top panels show images immediately after antibody injection (the embryos were also injected with rhodamine-labeled tubulin to highlight the MTOC). An observable decrease in Nuf concentration occurs ~5 min after injection and Nuf levels are dramatically decreased by 10 min after injection. At 20 min after injection, Nuf is almost completely absent from the MTOC.

Injecting an antibody directed against the DIC provides additional support for these results. This antibody has been used to disrupt Dynein function in the *Drosophila* oocyte (Serbus *et al.*, 2005). Injection of this antibody during cellularization results in significant reduction in Nuf concentration at the MTOC similar to that observed for the anti-DHC injections described above (Supplementary Figure S1). Quantifying total GFP fluorescence intensity at the MTOC in cellularizing embryos injected with anti-DIC antibodies reveals that Nuf levels decrease 80% (from mean brightness value 25 to 5) in contrast to PBS-injected controls (Figure 8).

We also examined the role of Dynein in actin recruitment and furrow elongation in the early embryo. Injection of anti-DHC antibodies into GFP-Moesin–bearing embryos produced

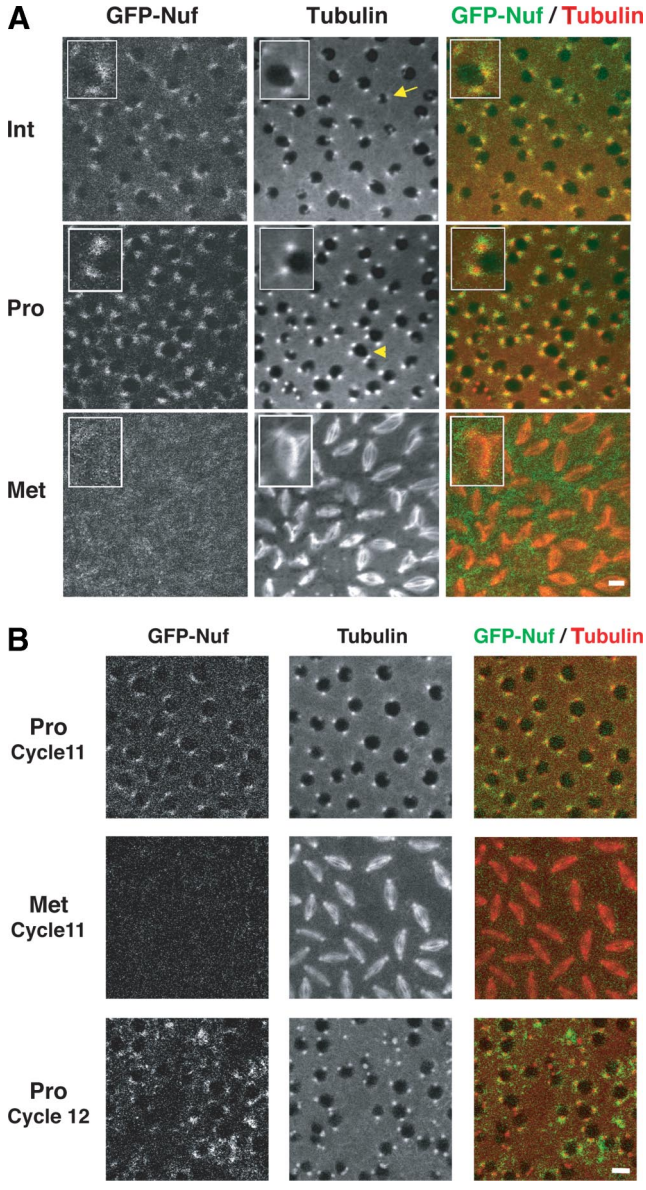

Figure 5. Nuf localization is independent of microfilaments. (A) *sponge*-derived embryos severely disrupt cortical microfilament organization resulting in lost (arrows) and fused nuclei (arrowheads), as well as multipolar spindles (see insets). In spite of these defects, live analysis of GFP-Nuf (green) and rhodamine tubulin (red) reveals that Nuf cell cycle–dependent concentration at the MTOC occurs normally. (B) Injection of embryos during interphase of nuclear cycle 11 with cytochalasin D disrupts cortical microfilaments. As with the *sponge*-derived embryos, live analysis of GFP-Nuf (green) and rhodamine-labeled tubulin (red) reveals Nuf cell cycle–dependent localization at the MTOC is unaffected. Scale bar, 5 μm.

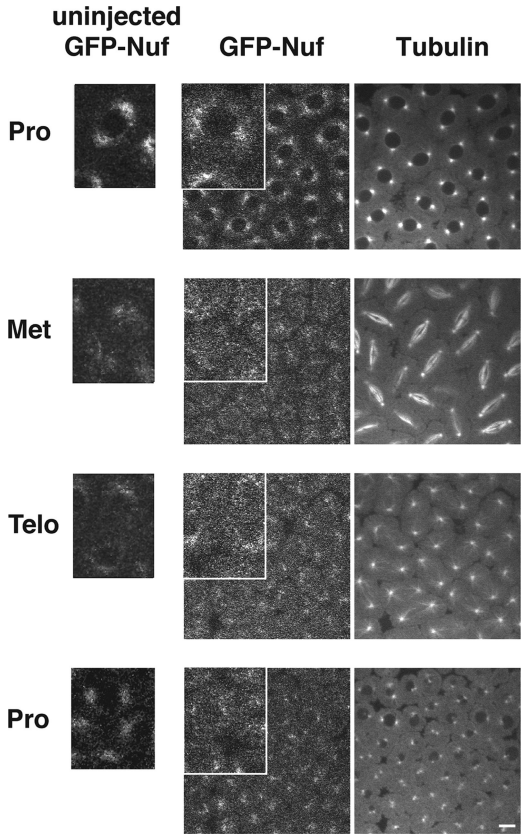

Figure 6. Dynein is required for proper Nuf localization during the cortical syncytial divisions. Affinity-purified anti-Dynein heavy-chain antibodies (DHC) were injected into an embryo containing GFP-Nuf and rhodamine-labeled tubulin during interphase of nuclear cycle 11 and immediately visualized as the embryo enters prophase (top row). Relative to uninjected controls (left), there is a slight reduction of Nuf at the centrosomes. As the embryos progress through mitosis and into prophase of nuclear cycle 12, there is a dramatic reduction of Nuf at the MTOC (compare uninjected and DHC-injected in the bottom panels). The defects in centrosome separation (previously reported consequences of disrupting Dynein function) verified antibody efficacy (compare tubulin column top and bottom panels). Scale bar, 5 μm.

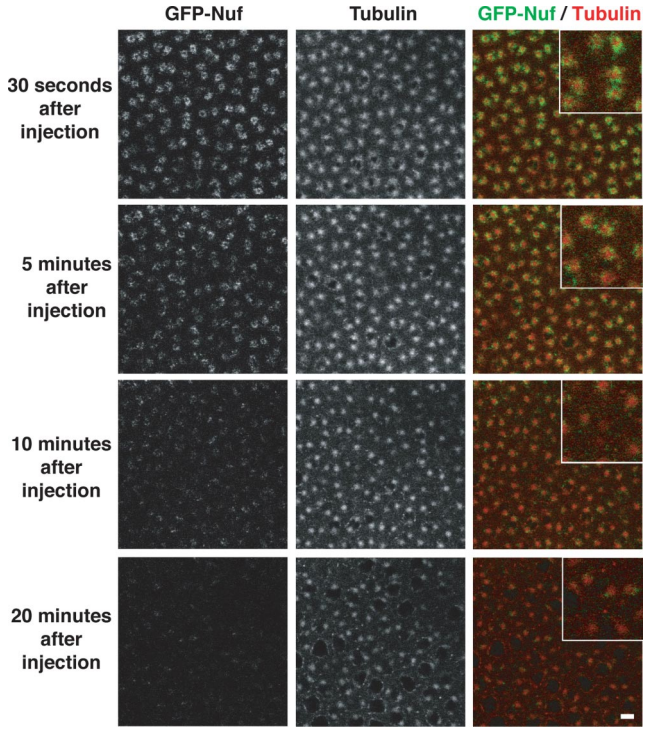

Figure 7. Dynein is required for the maintenance of Nuf at the MTOC. Affinity-purified anti-DHC antibodies were injected into an embryo containing GFP-Nuf (green) and rhodamine-labeled tubulin (red) 5 min after the start of cellularization (nuclear cycle 14). Gradually, Nuf is depleted from the MTOC in the absence of cytoplasmic Dynein. At 20 min after injection there is no Nuf present at the MTOC (bottom row) Insets, 2×. Scale bar, 5 μm.

defects in the recruitment of actin at the site of furrow formation (Supplementary Figure S6, Supplementary Movie M5). The Dynein complex is involved in many processes in the cell, and the actin defects observed could be due to a number of different Dynein-mediated functions. However, these data are consistent with the notion that the localization of Nuf at the MTOC is required for actin recruitment.

Dynein Physically Associates with Nuf

To determine whether Nuf and cytoplasmic Dynein physically associate, we performed immunoprecipitation reactions using extracts derived from GFP-Nuf embryos. An antibody against green fluorescent protein (GFP) pulled down the GFP-Nuf gene product and significant amounts of the DHC (Figure 9, lane 3). As a negative control, immunoprecipitation reactions were also performed using embryo extract derived from a GFP-tagged version of the transcription factor Kruppel (Kr; Casso *et al.*, 2000). Dynein does not coprecipitate with Kr-GFP (Figure 9, lane 5), indicating that the interaction of Dynein with Nuf is not due to the GFP tag itself. The reciprocal experiment using antibodies against Dynein subunits failed to pellet GFP-Nuf. This result is not unexpected, given that Nuf is one of many cargoes that associates and is transported by Dynein. These results, together with the cellular studies in the previous section, are consistent with the hypothesis that Dynein physically associates and transports Nuf along microtubules to the MTOC.

Microtubules Are Required during the Anaphase/Telophase Transition for Proper Actin Recruitment to the Furrow at Prophase

Nuf exhibits a microtubule-dependent concentration at the MTOC during the time of metaphase furrow formation and is required for proper membrane recruitment and actin organization at the metaphase furrow. These observations raise the issue of whether Nuf localization at the MTOC is necessary for its proper function. Arguing against this possibility are studies demonstrating that depolymerizing microtubules from interphase through metaphase do not disrupt actin recruitment during metaphase furrow formation (Stevenson *et al.*, 2001).

We experimentally revisited this issue through live analysis using a GFP-Moesin transgenic stock, which serves as an excellent marker of metaphase furrow formation (Edwards *et al.*, 1997). GFP-Moesin bearing embryos were injected with colchicine at either anaphase, telophase of nuclear cycle 12, or interphase of nuclear cycle 13 and assayed for effect on actin recruitment to the furrow during prophase of nuclear cycle 13 (Figure 10). Before the colchicine injection, the embryos were injected with rhodamine-labeled tubulin to directly follow the effect of the colchicine on microtubule dynamics and distribution. Shown in Figure 10A, colchicine was injected precisely at

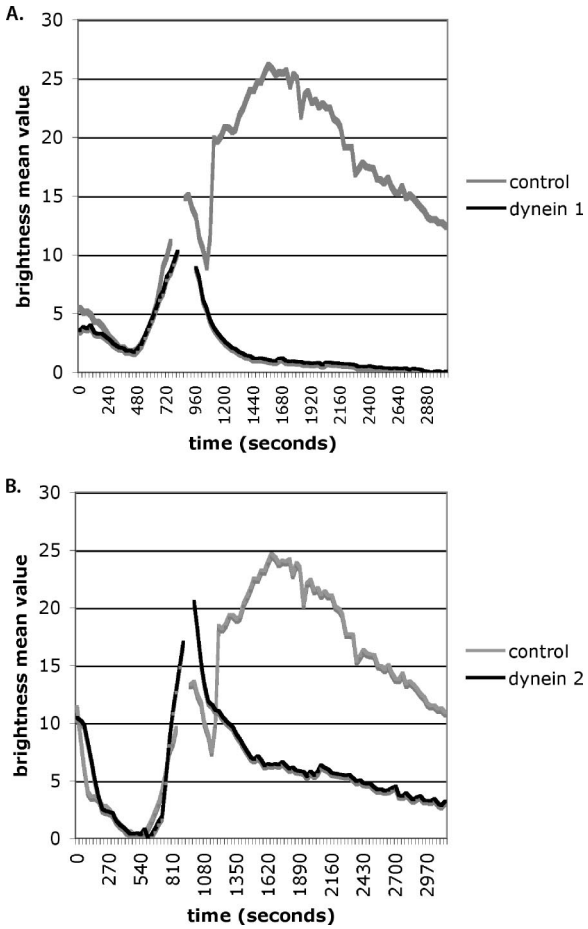

Figure 8. Dynein preserves Nuf intensity at centrosomes. The two graphs (A and B), quantify the decrease of GFP-Nuf signal in two different embryos after the injection of anti-Dynein IC (DIC) antibodies 5 min after the start of cellularization (cycle 14). PBS was injected as a control. The time of injection is represented by the broken line in the graphs. In graph A, metaphase of syncytial cycle 13 occurs at the 60-s time point, anaphase of syncytial cycle 13 occurs at the 210-s time point, and telophase of syncytial cycle 13 occurs at the 420-s time point. Compared with the control injection, the GFP-Nuf signal is significantly decreased upon injection with the anti-DIC antibody.

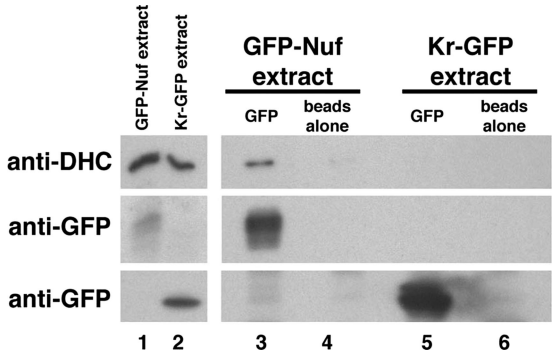

Figure 9. Dynein coprecipitates with Nuf. immunoprecioitation (IP) experiments were carried out using extracts from GFP-Nuf embryos. As a negative control, IP experiments were run in parallel on embryo extracts expressing GFP, driven by the Kruppel (Kr) promoter (Casso *et al.*, 2000). A Western blot of extracts from embryos expressing GFP-Nuf and Kr-GFP is shown in the right-hand patterns. This blot was probed with anti-Dynein heavy-chain (DHC) antibodies and anti-GFP antibodies and demonstrates the antibodies and extracts behave as expected (lanes 1 and 2). The left-hand panels show IP reactions using the same extracts and antibodies as described above. This figure shows that in GFP-Nuf extracts, but not Kr-GFP extracts, anti-GFP precipitates Dynein. This indicates that Dynein does not coprecipitate with the GFP tag itself (lane 5), and coprecipitation requires Nuf. Control reactions using protein A beads in the absence of antibody (lanes 4 and 6) do not precipitate Dynein, GFP-Nuf, or the Kr-driven GFP tag. Lanes 1 and 2 show samples of the starting extracts. Note that in the middle panel, the image of lanes 1 and 2 represent a longer exposure than the rest of the figure; this was required to visualize the low levels of expressed GFP-Nuf.

anaphase and assayed for proper furrow formation in the following prophase (Supplementary Movie M3). This experiment reveals that actin recruitment to the metaphase furrows is severely disrupted (compare the actin localization panel at prophase 12 with the actin localization panel at prophase 13). In contrast, injecting colchicine at telophase does not affect the recruitment of actin in the following prophase. Shown in Figure 10B, upon injection of colchicine at telophase, actin is able to form a proper hexagonal array at the corresponding prophase (bottom row, Supplementary Movie M4). Injecting colchicine at interphase also has no effect on actin recruitment (data not shown). These experiments demonstrate that microtubules are required during the anaphase for proper actin recruitment to the furrows in the following prophase. Although these studies certainly do not prove the Nuf localization at the MTOC is necessary for furrow formation, they are consistent with this possibility.

Interestingly, disruption of the microtubule network does not affect actin cap formation (Supplementary Figure S5). This suggests that formation of the actin caps is independent of microtubules.

DISCUSSION

Microtubule and Dynein-dependent Transport of Nuf to the MTOC

Microtubule-based motility has been implicated in many steps in endocytosis, and there is increasing evidence that it influences the distribution and activity of endocytic organelles (Burkhardt *et al.*, 1997; Apodaca, 2001; Allan *et al.*, 2002; Hoepfner *et al.*, 2005; Papoulas *et al.*, 2005; Driskell *et al.*, 2007). The work presented here suggests that motor-based movement of Rab effectors may be another means of regulating endosomal activity. Previous studies have shown that the *Drosophila* Rab11 effector, Nuf, is required for stable Rab11 localization at the RE and thus RE activity (Riggs *et al.*, 2003). Nuf concentrates at the MTOC during interphase through prophase and disperses into the cytoplasm at metaphase (Rothwell *et al.*, 1998). Here we demonstrate that Nuf relies on microtubules and minus-end microtubule motor Dynein both for its accumulation and maintenance at the MTOC. This raises the possibility that the Dynein-dependent delivery of Nuf to the RE may play a role in regulating Rab11 activity at the RE. Significantly maximal localization of Nuf at the MTOC-associated RE occurs during late interphase and prophase. This is the time of the establishment and formation of the metaphase furrows, which rely on RE-based vesicle delivery (Riggs *et al.*, 2003).

Our immunoprecipitation data demonstrates a physical interaction between Nuf and Dynein. This raises the possibility that the cell cycle–regulated localization of Nuf at the

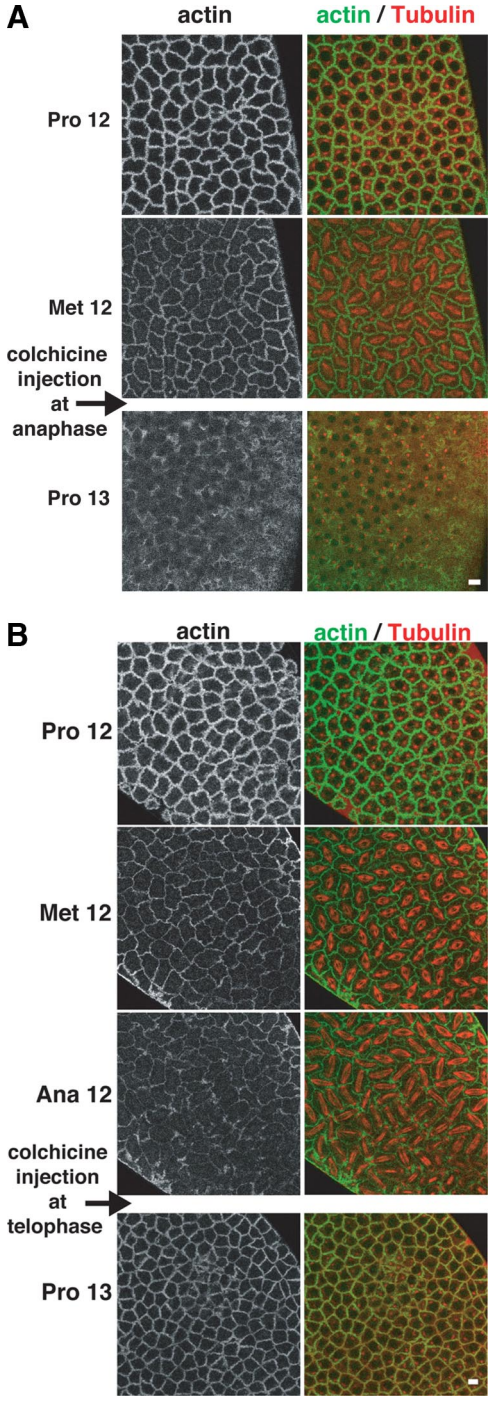

Figure 10. Microtubules are required at anaphase, but not telophase for proper furrow formation. (A) Microtubules are necessary at anaphase for actin recruitment and furrow elongation. Colchicine was injected into a live embryo containing GFP-Moesin (green) and rhodamine-labeled tubulin (red) at anaphase of nuclear cycle 12 and assayed for proper furrow formation in the following prophase. Depolymerization of the microtubules at anaphase of nuclear cycle 12 affects actin recruitment and furrow formation at prophase of nuclear cycle 13 (bottom row). (B) Disruption of microtubules at telophase does not affect actin recruitment and furrow formation. Colchicine was injected into an embryo containing GFP-Moesin (green) and rhodamine-labeled tubulin (red) at telophase of nuclear cycle 12 and assayed for proper furrow formation at prophase of nuclear cycle 13. Even in the absence of microtubules, actin recruitment and furrow elongation occurs normally (bottom row). Scale bar, 5 μm.

MTOC is mediated by a corresponding cell cycle–regulated interaction between Nuf and Dynein. Support for this idea comes from a study in vertebrate cells, demonstrating that Polo-like kinase (Plk) mediated phosphorylation of Nineinlike protein (Nlp), a microtubule-nucleating protein, directly determines its cell cycle–regulated localization at the centrosome (Casenghi *et al.*, 2003, 2005). Like Nuf, Nlp localizes to the centrosome by associating with the minus-end–directed motor protein Dynein. As cells progress into metaphase, Plk is activated and phosphorylates Nlp on sites that are required for its association with Dynein. This disrupts Nlp ability to associate with Dynein and results in loss of Nlp from the centrosome.

Microtubules and Dynein, But Not Actin, Are Continuously Required for Maintenance of Nuf at the MTOC

There is a strong correlation between maximal Nuf localization at the MTOC and furrow invagination. During the cortical divisions, furrow invagination and maximal Nuf concentration at the MTOC occurs during prophase. During cellularization, furrow invagination and maximal Nuf concentration at the MTOC occurs during interphase. Stable localization of Nuf and Rab11 at the MTOC during cellularization enabled us to demonstrate that microtubules are continuously required for maintaining Nuf and Rab11 at the MTOC. Colchicine-induced disruption of the interphase microtubules results in the rapid loss of Nuf from the MTOC. One interpretation of this result is that colchicine disrupts MTOC organization, which is required for maintaining Nuf at the MTOC. In contrast to the colchicine injections, injecting anti-Dynein antibody does not alter microtubule organization and results in a slow steady decrease of Nuf at the MTOC. This result suggests that the steady-state level of Nuf at the MTOC is maintained by continuous Dynein-dependent recruitment of Nuf to the MTOC (Figure 9). This also implies that Nuf is continuously released from the MTOC as well. The mechanism driving the release is unclear. Previous live analysis revealed vectorial movement of Nuf away from the centrosome, suggesting that it may rely on a kinesin, a plus-end–directed microtubule motor (Riggs *et al.*, 2003). If kinesin is involved, this implies that the balance between plus- and minus-end motor activities dictates whether Nuf is concentrated at the MTOC or dispersed in the cytoplasm. Recent work by Hoepfner *et al.* (2005) indicates that the positioning and activity of the early endosome is mediated through a balance of plus- and minus-end motor activities. In addition, investigations into cellular furrow elongation demonstrated that Lava lamp, a Golgi-associated protein, is complexed with Dynein and is responsible for Golgi-based movements necessary for latter half of furrow elongation (Papoulas *et al.*, 2005).

Microtubules Are Required during Anaphase for Proper Metaphase Furrow Formation in the Following Prophase

The above studies demonstrate that microtubules are continuously required for proper Nuf localization at the MTOC. This raises the possibility that microtubule-based localization of Nuf at the MTOC is necessary for its association with the Rab11 and proper RE function. Because RE function is necessary for metaphase furrow formation, this predicts that microtubules are required for proper metaphase furrow formation. However previous studies did not observe defects in furrow formation when embryos were treated with microtubule inhibitors (Stevenson *et al.*, 2001). The authors concluded that microtubules were dispensable for proper metaphase furrow formation in the early embryo. We reexamined this issue by injecting microtubule inhibitors at precise times throughout the cell cycle during the syncytial divisions. Because disrupting the

microtubules at metaphase activates the spindle assembly checkpoint, we injected the embryos immediately after entry into anaphase. In these experiments, the nuclear cycle progressed normally but formation of the metaphase furrows were profoundly disrupted. Incorporation of GFP-tagged Moesin into the furrows that form at the next prophase completely fails. Thus these experiments define anaphase as a key time in which microtubules are required for recruiting actin to the furrows that form in the following prophase. The previous study failed to appreciate the role of microtubules in metaphase furrow formation because they were not able to produce disruptions in the microtubule network at defined stages of the cell cycle (Stevenson *et al.*, 2001).

These studies also revealed that injecting colchicine at telophase produced no defects in actin recruitment. Similar injections at interphase through prophase also produced no defects in actin recruitment to the metaphase furrows. One interpretation of these results is that microtubules are specifically required during anaphase but not telophase or later for furrow formation in the next prophase. However it must be pointed the different classes of microtubules are differentially sensitive to microtubule inhibitors (Wheatley and Wang, 1996; Downing and Nogales, 1998). Thus this differential sensitivity may contribute to the observed cell phase sensitivity of metaphase furrow formation to colchicine.

That microtubules are required during anaphase for metaphase furrow formation in the following prophase is significant for a number of reasons. First, these studies support, although certainly do not prove, a model in which microtubule-based transport of Nuf to the MTOC is necessary for normal metaphase furrow formation. Second, anaphase/telophase is the point at which the metaphase furrows begin to regress. Thus the timing of furrow regression corresponds to the time at which microtubules are involved in establishing the next round of furrow formation. This indicates that the speed of the cortical divisions is not only achieved by an accelerated nuclear cycle but also by overlapping furrow regression with furrow formation. During anaphase, the replicated centrosomes possess robust astral arrays and the midbody has not yet fully formed. We hypothesize that the plus ends of these overlapping arrays from neighboring centrosomes define the position of the metaphase furrow in the next cell cycle. This readily explains why furrows encompass the spindle and do not form at the midzone microtubules. Finally, although the furrows form at prophase, these studies identify anaphase as a critical time in which furrow is established. This also corresponds to the time at which microtubules are required during conventional furrow formation (Hamaguchi, 1975; Rappaport, 1996).

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