The centrosome is a dynamic structure that ejects PCM flares

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Summary

The *Drosophila* Centrosomin (Cnn) protein is an essential core component of centrosomes in the early embryo. We have expressed a Cnn-GFP fusion construct in cleavage stage embryos, which rescues the maternal effect lethality of *cnn* mutant animals. The localization patterns seen with GFP-Cnn are identical to the patterns we see by immunofluorescent staining with anti-Cnn antibodies. Live imaging of centrosomes with Cnn-GFP reveals surprisingly dynamic features of the centrosome. Extracentrosomal particles of Cnn move radially from the centrosome and frequently change their direction. D-TACC colocalized with Cnn at these particles. We have named these extrusions 'flares'. Flares are dependent on microtubules, since disruption of the microtubule array severs the movement of these particles. Movement of flare particles is cleavage-cycle-dependent and appears to be attributed mostly to their association with dynamic astral microtubules. Flare activity decreases at metaphase, then increases at telophase and remains at this higher level of activity until the next metaphase. Flares appear to be similar to vertebrate PCM-1-containing 'centriolar satellites' in their behavior. By injecting rhodamine-actin, we observed that flares extend no farther than the actin cage. Additionally, disruption of the microfilament array increased the extent of flare movement. These observations indicate that centrosomes eject particles of Cnn-containing pericentriolar material that move on dynamic astral microtubules at a rate that varies with the cell cycle. We propose that flare particles play a role in organizing the actin cytoskeleton during syncytial cleavage.

Movies available on-line

Key words: *Drosophila*, Centrosomes, Centrosomin (Cnn), PCM, Flares

Introduction

The centrosome is the major microtubule-organizing center (MTOC) in most animal cells, but its functions are still relatively obscure (Palazzo and Schatten, 2000). At mitosis, a pair of centrosomes nucleates and organizes microtubules into the bipolar spindle apparatus, the accurate assembly of which is necessary for the proper segregation of chromosomes into the daughter cells. If the spindle apparatus is not assembled into a bipolar array, the chromosomes may not be distributed equally at cell division, and the resulting aneuploidy can lead to apoptosis or oncogenesis (Brinkley, 2001; Lingle and Salisbury, 2000; Doxsey, 1998).

In the absence of centrosomes, the bipolar spindle assembles by an alternate, anastral, pathway where the microtubules are nucleated on the chromosomes and subsequently organized into the bipolar spindle with the minus ends of the microtubules focused at the poles (Hyman, 2000; Compton, 2000). This process involves the coordinate activities of motor proteins (Wittmann et al., 2001; Walczak, 2000; Heald, 2000; Sharp et al., 2000; Compton, 2000). Since centrioles are degraded during oogenesis in many species, this alternate spindle assembly pathway appears to act exclusively during female meiosis (Theurkauf and Hawley, 1992; Rieder et al., 1993; McKim and Hawley, 1995; Megraw and Kaufman, 2000; Compton, 2000).

Found throughout the animal kingdom, the centrosome consists of over 100 proteins (Kalt and Schliwa, 1993; Kellogg et al., 1994). At the heart of the centrosome lies a pair of centrioles. These are surrounded by the pericentriolar material (PCM), which has a filamentous structure (Dictenberg et al., 1998; Schnackenberg et al., 1998; Schnackenberg and Palazzo, 1999). The centromatrix, a substructure of the PCM, is a filamentous basal component of the centrosome that remains after many PCM proteins are removed by high salt (Schnackenberg et al., 1998; Schnackenberg and Palazzo, 1999; Moritz et al., 1998; Dictenberg et al., 1998; Palazzo et al., 2000). Additionally, γ-tubulin ring complexes (γTURCs) are bound to the PCM and are the sites of microtubule nucleation at the centrosome (Gunawardane et al., 2000).

Cnn is a core centrosome constituent, and a major protein component of purified *Drosophila* centrosomes (Lange et al., 2000), but it is not part of the centromatrix (Moritz et al., 1998). In the hierarchical assembly of the centrosome (Palazzo et al., 2000), Cnn lies between the centromatrix and the incorporation of other PCM components including CP60, CP190 and γ-tubulin (Megraw et al., 1999; Megraw et al., 2001). Changes in the centrosome composition with the cell cycle are reflected in the discovery that Cnn is required for γ-tubulin accumulation at mitotic, but not interphase, centrosomes (Megraw et al., 2001).

Currently, the role of the centrosome in cell division is being re-evaluated (Rieder et al., 2001; Marshall, 2001; Raff, 2001; Hyman, 2000; de Saint Phalle and Sullivan, 1998; Bonaccorsi et al., 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Bonaccorsi et al., 2000; Khodjakov et al., 2000; Megraw et al., 2001; Khodjakov and Rieder, 2001; Hinchcliffe et al., 2001; Piel et al., 2001; Giansanti et al., 2001). In the absence of centrosomes, or in cells where functional centrosomes are not assembled, such as in *asterless* or *centrosomin* (*cnn*) mutants, the bipolar spindle apparatus is assembled by the anastral pathway described above (de Saint Phalle and Sullivan, 1998; Bonaccorsi et al., 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Bonaccorsi et al., 2000; Khodjakov et al., 2000; Megraw et al., 2001; Khodjakov and Rieder, 2001; Hinchcliffe et al., 2001). When centrosomes are completely removed from cultured cells by laser ablation (Khodjakov et al., 2000; Khodjakov and Rieder, 2001) or by microsurgery (Hinchcliffe et al., 2001), the cells enter mitosis and divide, but then arrest at G1. However, a *Drosophila* cell line that lacks centrosomes can be perpetually maintained, although a delay at cytokinesis is observed (Debec and Abbadie, 1989; Piel et al., 2001). These results demonstrate that bipolar spindle assembly can occur efficiently without centrosomes in vivo and in vitro. Furthermore, much of *Drosophila* development can occur without fully functional mitotic centrosomes (Megraw et al., 2001). These data also indicate that although the centrosome plays an essential role in the cell cycle, it is not required at mitosis but for progression through G1 (Khodjakov and Rieder, 2001; Hinchcliffe et al., 2001; Piel et al., 2001). The mother centriole may mediate a checkpoint at G1 by migrating to the midbody and signaling the completion of cytokinesis (Piel et al., 2001). Whatever its total functional repertoire, it is evident that the centrosome has functions that change with the cell cycle.

In *Drosophila cnn* mutants, the mitotic centrosome is not fully functional, but the interphase centrosome may be normal (Megraw et al., 2001). *cnn* mutant animals develop into adults, with their mitotic divisions occurring by the anastral pathway for bipolar spindle assembly (Megraw et al., 2001). *cnn* mutant females produce no offspring because the eggs they lay arrest in early embryogenesis (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999) due, at least in part, to the fusion of nuclei at telophase (T.L.M., S.K., F.R.T. and T.C.K., unpublished).

In the early *Drosophila* embryo, where the first 13 cleavage divisions occur synchronously in a syncytium, the centrosome has an indispensable role. Here, spindle assembly and nuclear divisions can occur in the absence of centrosomes, but the actin cytoskeleton does not organize properly and cellularization of the blastoderm does not occur (de Saint Phalle and Sullivan, 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). Although the centrosome has long been viewed as an MTOC, it also organizes the actin cytoskeleton and appears to function in this capacity independently of microtubules (Stevenson et al., 2001).

Fluorophore-conjugated and GFP-fused centrosomal proteins have been used to understand the assembly and dynamics of the centrosome in several studies (Oegema et al., 1995; Khodjakov and Rieder, 1999; Kubo et al., 1999; Young et al., 2000; Piel et al., 2000; White et al., 2000; Strome et al., 2001; Piel et al., 2001). Pericentrin is pre-assembled into particles with γ-tubulin prior to import/assembly into the centrosome (Dictenberg et al., 1998). GFP-Pericentrin revealed the presence of Pericentrin particles in the cell that move in a microtubule- and dynein-dependent manner into the centrosome (Young et al., 2000). GFP-γ-tubulin was used to show that γ-tubulin dynamically exchanged at the centrosome and is accumulated at the centrosome in higher levels at mitosis in a microtubule-independent manner (Khodjakov and Rieder, 1999). Using GFP-centrin, the dynamics of the centrioles and their genesis were examined (Piel et al., 2000; White et al., 2000). With GFP-Centrin it was shown that the mother and daughter centrioles have a far less static relationship with one another than was previously thought, especially following telophase, and the movements of mother and daughter centrioles depend upon the microtubule and actin cytoskeletons (Piel et al., 2000; Piel et al., 2001). GFP-PCM-1 was used to show the dynamics of satellites, which are subcentrosomal structures of the PCM. In this study, it was shown that satellites are very dynamic and move in a microtubule-dependent manner back-and-forth from the centrosome (Kubo et al., 1999). Taken together, live imaging of centrosomes and its components has revealed some surprisingly dynamic features of this organelle. Here, we show that during the syncytial cleavage divisions of the early *Drosophila* embryo, the centrosome has a dynamic structure, from which PCM material is ejected. Ejected particles of PCM material move upon microtubules, have properties in common with vertebrate satellites and may have a relationship with actin cytoskeleton organization.

Materials and Methods

Plasmids

We constructed a UAS-GFP cloning vector (pUASpEGFPc1) to accommodate the insertion of various coding sequences as fusions to GFP for expression in *Drosophila*. A 0.8 kb DNA fragment containing the EGFP coding sequence and multiple cloning sites was isolated from pEGFPc1 (Clontech) by cleavage with *Nhe*I, filling in with Klenow, followed by cleavage with *Xba*I. This DNA fragment was ligated to the vector pUASp (Rorth, 1998), which had been cleaved with *Not*I, filled in with Klenow, and then cleaved with *Xba*I. The GFP-Cnn fusion construct (pUASpGFP-Cnn) was made by inserting into pUASpEGFPc1 a 3.44 kb DNA fragment of *cnn* coding sequence that was amplified by PCR from a cDNA clone (Heuer et al., 1995). The *cnn* coding sequence was amplified with the following primers: Cnn4: CCT GGA TCC GAC CAG TCT AAA CAGT GTT and Cnn5: TAT AGT CGA CGT CGG CGC GAT CGT TAT A. The PCR fragment was cleaved with *Bam*HI at its 5′ end and left blunt at the 3′ end. This DNA fragment was ligated to pUASpEGFPc1, which was cleaved with *Bam*HI, filled in with Klenow, and digested with *Bgl*II.

Drosophila stocks

Transgenic lines were recovered following microinjection of plasmids directly into early embryos collected over a 30-40 minute period as described (Robertson et al., 1988). We injected plasmids at a concentration of 1 mg/ml in water directly through the chorion and vitelline membrane of early embryos of the genotype: w*;TMS, P{ry$^{+t7.2}$=Delta2-3}99B/TM6B, Tb^{1}. Embryos were collected, rinsed with water, arranged onto coverslips in a parallel register with a spacing of approximately two embryos' width and then blotted dry with a paintbrush. After drying briefly, the embryos stick to the coverslip. Embryos were injected at their posterior end. We recovered transgenic lines of the pUASpGFP-Cnn plasmid with inserts on chromosomes I, II and III (w*; P{w^{+mC}=UASp-GFP-Cnn1}). Two

stocks were used in this study, lines 26-1 and 29-3. To express pUASpGFP-Cnn and GFP-α-tubulin in the embryo a stock that expresses Gal4 in the ovary from the *nanos* promoter was used (P{GAL4::VP16-nos.UTR}^{MVD1}) (Van Doren et al., 1998). A fly stock expressing a GFP-Histone 2A variant (w^{1118}; P{w^{+mC}=His2Av$^{T:Avic\GFP–S65T}$}62A) (Clarkson and Saint, 1999) was a gift from Rob Saint. The GFP-α-tubulin (P{w^{+},UASp-GFPS65C-a1-tub84B}) stock, an insertion on chromosome III, (Grieder et al., 2000) was a gift from Allan Spradling. The *cnn* allele used in these studies was the null *cnn^{hk21}*(Megraw et al., 1999). All mutant animals analyzed were hemizygous *cnn^{hk21}*/Df(2R)cnn.

Immunostaining

Embryos were fixed using either MeOH or formaldehyde fixation according to Theurkauf (Theurkauf, 1994). Fixed embryos were incubated with Guinea pig antibodies raised against Cnn at a 1:1000 dilution, anti-α-tubulin monoclonal antibody DM1A (Sigma) at 1:500, and the fluorescent DNA dye TOTO-3 (Molecular Probes) at 1:400. Rabbit antibodies to D-TACC, a gift from Bill Theurkauf, were used at a 1:500 dilution. Fluorescent secondary antibodies (Jackson ImmunoResearch Labs) were used at a 1:200 dilution. In order to detect Cnn colocalization with membrane bearing particles or Golgi, embryos were stained for Cnn and Discontinuous actin hexagon (Dah), or Lava lamp (Lva) or beta-Coatomer protein (β-Cop). Rabbit Dah (1:120) (Zhang et al., 1996) and rabbit Lva (1:3000) (Sisson et al., 2000) antibodies were generously provided by Bill Sullivan and John Sisson. Rabbit anti-β-Cop (1:200) (Ripoche et al., 1994) was a gift from Vivek Malhotra. Embryos were incubated with primary antibodies and RNase A (50 μg/ml) overnight at room temperature with rocking in 300 μl PBT [phosphate-buffered saline (Theurkauf, 1994) with 0.1% Triton X-100]. Embryos were then rinsed three times with PBT, and then washed three times for 20 minutes each with PBT. Secondary antibodies were diluted in PBT and incubated with embryos with rocking for 90 minutes. Embryos were rinsed and washed as above, then mounted on glass slides in PBS containing 90% glycerol. Images were captured with a Leica TCS NT scanning confocal microscope and saved as a maximal projection through several optical sections totaling approximately 10 μm, then imported into Adobe Photoshop 5.0. For deconvolution microscopy, images were captured on a Nikon TE 300 inverted microscope and processed with Applied Precision deconvolution software. For Fig. 4, images were captured using an Olympus FV500 Confocal microscope equipped with an IX81 inverted microscope.

Electron microscopy

Embryos were fixed in 1% glutaraldehyde in PBS, then stage 14 specimens were selected and hand dechorionated. Fixed embryos were embedded, stained and prepared for electron microscopy as described previously for testis (Li et al., 1998). Guinea pig anti-Cnn was diluted 1:50 in PBS and incubated with the sample at room temperature. 12 nm colloidal gold-Donkey anti-Guinea pig secondary antibodies (Jackson ImmunoResearch Labs) were diluted 1:10 in PBS.

Microinjection and live cell imaging

Embryos were collected for 1-2 hours, dechorionated by rolling on double-stick tape, placed onto a coverslip that was coated with double-stick tape extract and, for injections, dehydrated mildly (Schubiger and Edgar, 1994). The coverslip was mounted onto an upright microscope using a coverslip holder with a gas permeable Teflon membrane (Kiehart et al., 1994). For both microinjection and viewing of live material the embryos were mounted in Halocarbon Oil 700. Colchicine (Sigma) and Cytochalasin-D (Sigma) were dissolved in water and injected at a concentration of 0.1 mg/ml. Rhodamine-tubulin (Molecular Probes, Inc) was injected at a concentration of 10 mg/ml. Rhodamine-actin (Cytoskeleton, Inc) was injected at a concentration of 1 mg/ml. Embryos were treated with 10 μM Paclitaxel (Taxol, Sigma) in Grace's Insect Medium (Invitrogen) while being permeated with high grade octane (Sigma) for 30 seconds as described (Raff et al., 1993). Taxol mock control embryos were similarly treated, except their medium contained only 0.4% DMSO and no Taxol. Time-lapse images were collected every3 seconds with a Leica TCS NT scanning confocal microscope using a 63×/1.2NA water immersion lens. Images were processed for animation using NIH Image software (http://rsb.info.nih.gov/nih-image/) and, for the two-color images, Final Cut Pro software.

Results

Cnn is a core component of the centrosome PCM

Centrosomin (Cnn) is localized to the centrosome throughout the division cycles of the *Drosophila* early embryo. *cnn* mutant embryos fail to assemble centrosomes properly and arrest division before cellularization of the blastoderm can occur (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). Using antibodies directed toward the Cnn protein, we observed that Cnn is localized at the perimeter of the centrosome, as is characteristic of components of the centrosome PCM, producing an image with a doughnut-shaped structure (Fig. 1A). When we viewed early embryos using a higher gain on the fluorescence signal, we observed punctate particles of Cnn in the cytoplasm in addition to the accumulation at the centrosomes (Fig. 1B). When embryos or *Drosophila* S2 cells were treated with colchicine to inhibit microtubule polymerization, Cnn remained localized to the centrosome (Li et al., 1996) (T.L.M., S.K., E.R.T. and T.C.K.), indicating that it is a core component of the centrosome (Whitfield et al., 1995; Rothwell and Sullivan, 2000). Electron microscopic imaging of embryonic centrosomes showed that the pericentriolar material (PCM) is arranged in a tentacle-like configuration (Fig. 1C), which was also observed by immunostaining (Fig. 1B). Immunogold labeling shows that Cnn is a component of the centrosome PCM (Fig. 1C).

Cnn has a dynamic relationship with the centrosome

To examine Cnn and centrosome dynamics in living embryos, we constructed a green fluorescent protein (GFP)-Cnn fusion construct that contains Gal4 UAS transcription control elements 5′ of the ORF and ovary permissive control elements 5′ and 3′ of the ORF (Rorth, 1998). This construct, contained in a P element transformation vector, was integrated into the fly genome. A *nanos*-Gal4 'driver' construct, in which *nanos* transcription regulatory elements control the expression of the yeast Gal4 ORF (Van Doren et al., 1998), was used to express GFP-Cnn during oogenesis. Embryos from mothers containing both transgenes express GFP-Cnn in laid eggs. When this combination of ovarian Gal4 and GFP-Cnn constructs are present in a *cnn* mutant female, she produces viable embryos, thereby rescuing the maternal effect lethal phenotype associated with the *cnn* mutation (Megraw et al., 1999).

In the early embryo, GFP-Cnn is localized to the centrosome and more weakly to the mitotic spindle (Fig. 2). When live GFP-Cnn embryos were viewed by time-lapse imaging, the dynamics of centrosomes during the early embryonic cleavage cycles were apparent (Fig. 2; Movie 1, available at

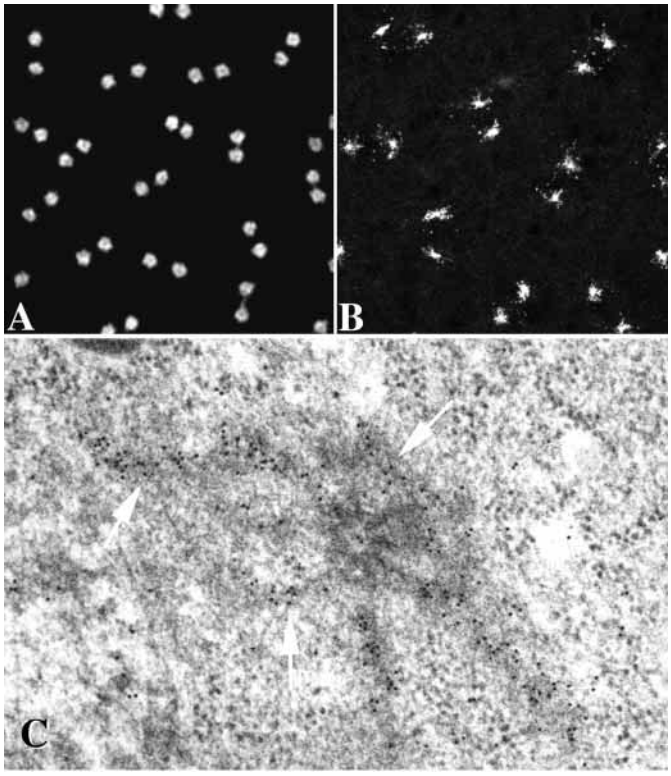

Fig. 1. Centrosomin localizes to centrosomes and to extracentrosomal particles. The localization of Cnn in early syncytial embryos was determined by immunostaining with antibodies directed against Cnn. The image of the specimen in A was acquired with a low signal gain, showing Cnn localization at the centrosome to have a doughnut-like appearance. In contrast to A, the specimen in B was acquired at a high signal gain, showing the presence of Cnn-containing extracentrosomal particles around the centrosomes. (C) An electron micrograph of an embryonic centrosome at interphase of cycle 14 that has been immunogold labeled for Cnn. The image shows that Cnn is a component of the electron-dense pericentriolar material, the tentacle-like projections of which are enriched in Cnn. The Cnn signal appears as black dots, some of which are indicated with white arrows, that are more concentrated within the PCM. See also Movie 1, available at jcs.biologists.org/supplemental.

Table 1. Average flare particle number per centrosome at different stages of the cleavage cycle

Interphase	Prophase	Metaphase	Anaphase	Telophase
6.83 (*n*=30)	5.74 (*n*=23)	1.29 (*n*=28)	0.76 (*n*=21)	6.07 (*n*=28)

jcs.biologists.org/supplemental). Changes in Cnn intensity at the centrosome with the cell cycle are also apparent. The GFP-Cnn signal increases at the centrosome as the cleavage cycles progress from interphase into mitosis, beginning at prophase.

Closer inspection revealed that the centrosome and the punctate Cnn-containing particles seen by immunostaining (Fig. 1B) share an intimate and dynamic relationship (Fig. 3; Movie 2, available at jcs.biologists.org/supplemental). The particles emerge from the centrosome and move back and forth radially. We will refer to this process as 'flaring' and will refer to the moving particles of PCM material as 'flare particles'. Flare particles move in a saltatory fashion. They jump 0.2 to 1.0 μm in less than 3 seconds and then may continue in the same or in the reverse direction. The rate of movement of the flare particles could not be precisely measured because each step occurs more quickly than the time it takes to capture each individual image. From these observations, however, it is clear that flare movements are in the range of 0.06-0.3 μm/second.

The number of flares associated with centrosomes varies with the cell cycle. We compared the average number of flare particles associated with centrosomes at different stages of the cleavage cycle, and these data are presented in Table 1. The intensity of flares is highest at cleavage telophase/interphase centrosomes and lowest at mitotic centrosomes, especially during metaphase and anaphase. In addition, Cnn appears to be associated with metaphase/anaphase centrosomes more tightly, giving the centrosome a more rounded appearance, with fewer of the projections that spawn flare particles seen on centrosomes at other phases of the cleavage cycle (see Fig. 1B). Furthermore, at telophase when the centrosome is dividing, projections of Cnn material transiently span the divide between the separating centrosomes.

To observe the dynamics of both Cnn and microtubules in living embryos, fluorescent-rhodamine-labeled tubulin protein was injected into GFP-Cnn embryos (Fig. 4). In a recorded time-lapse series the formation of astral microtubules around centrosomes and the dynamics of the spindle through

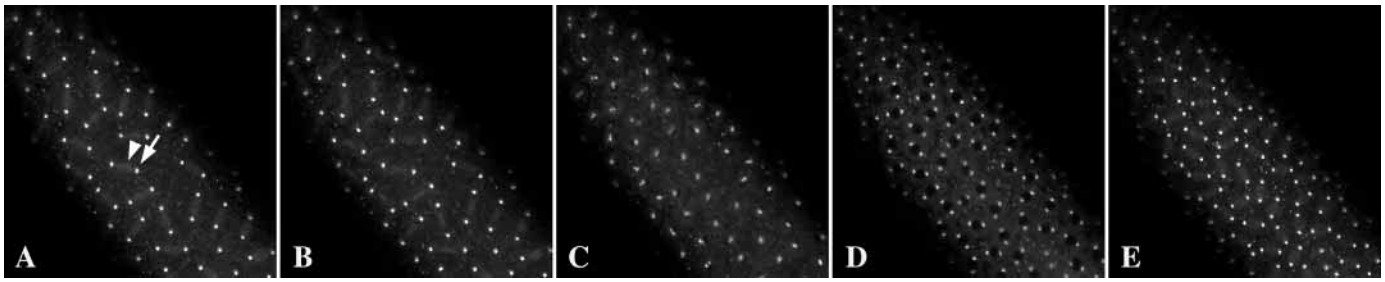

Fig. 2. GFP-Centrosomin localizes to centrosomes. GFP-Centrosomin fusion protein (GFP-Cnn) was expressed in the early embryo. This expression rescues the maternal effect lethal phenotype associated with *cnn* mutants and is therefore biologically active. These representative still images show GFP-Cnn localized to centrosomes (arrow in A), weakly to the spindle microtubules (arrowhead in A) and to punctate particles throughout the embryo. The stills show the first cycle in anaphase (A), late anaphase (B), telophase, when the centrosomes duplicate (C), prophase (D) and metaphase of the next cycle (E). The nuclei can be seen as the dark areas in D that are devoid of GFP-Cnn signal. See also Movie 1 at jcs.biologists.org/supplemental.

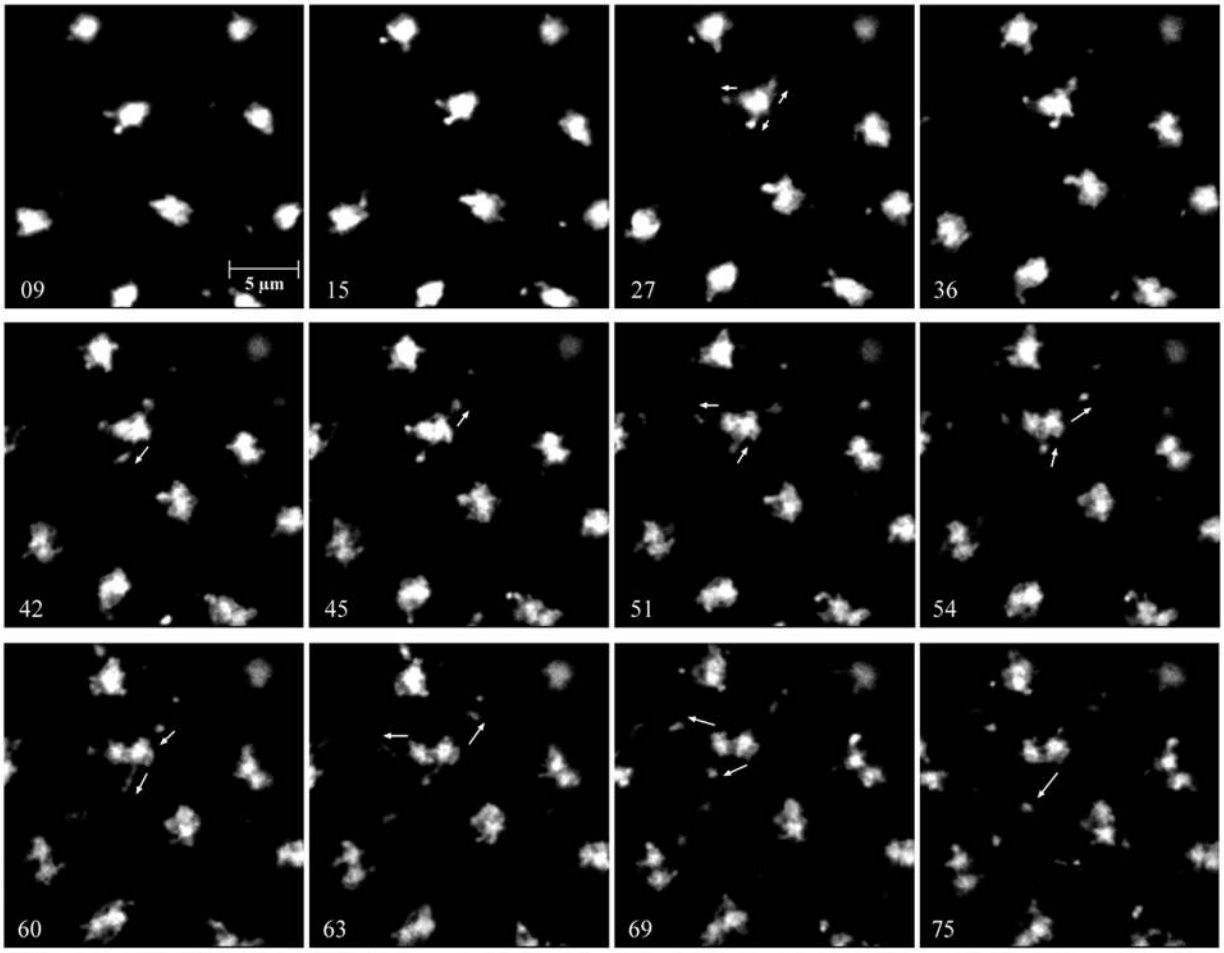

Fig. 3. Cnn has a dynamic relationship with the centrosome. Emerging as 'flares', GFP-Cnn particles move back and forth from the centrosome. Shown are 12 time-lapse images of a centrosome dividing at telophase. The time code in seconds is shown at the lower left corner of each frame. The arrows indicate the direction of flare particle movement. See also Movie 2 at jcs.biologists.org/supplemental.

the cleavage cycle can be seen (Movie 3, available at jcs.biologists.org/supplemental).

Flares contain a subset of known centrosomal proteins

We examined the colocalization of several centrosomal proteins with Cnn in wild-type syncytial stage embryos. We found that the core centrosomal protein D-TACC colocalized with Cnn at centrosomes and flare particles (Fig. 5). However, not all Cnn-containing particles contained a detectable signal of D-TACC, and conversely some D-TACC particles did not have a detectable amount of Cnn. Immunostaining against the centrosomal proteins γ-tubulin and CP190 showed no signal at flare particles when co-stained with anti-Cnn antibodies (data not shown).

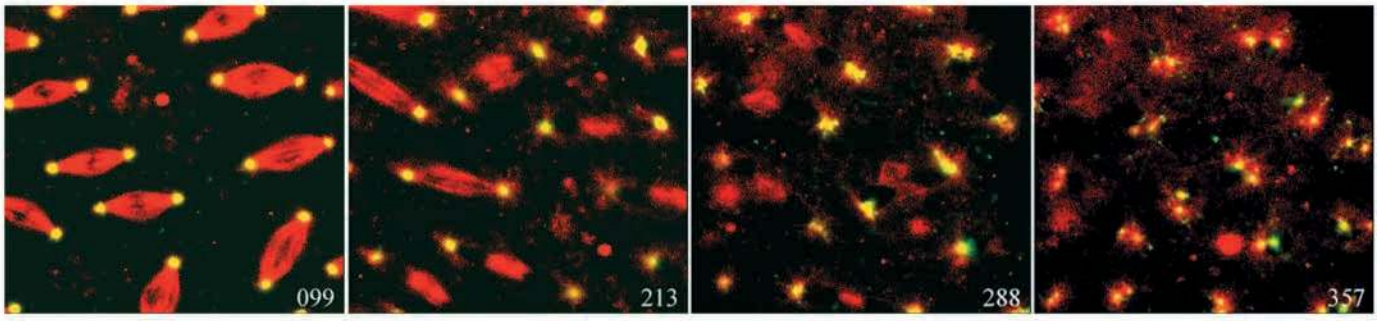

Fig. 4. Flare activity changes through the cleavage cycle. A GFP-Cnn embryo was injected with Rhodamine-tubulin to reveal the dynamics of centrosomes and microtubules simultaneously in living embryos. This analysis shows that flares appear throughout the division cycle in the syncytial embryo, with flaring occurring most actively at telophase and interphase (Table 1). The time code in seconds appears in the lower right corner of each frame. See also Movie 3 at jcs.biologists.org/supplemental.

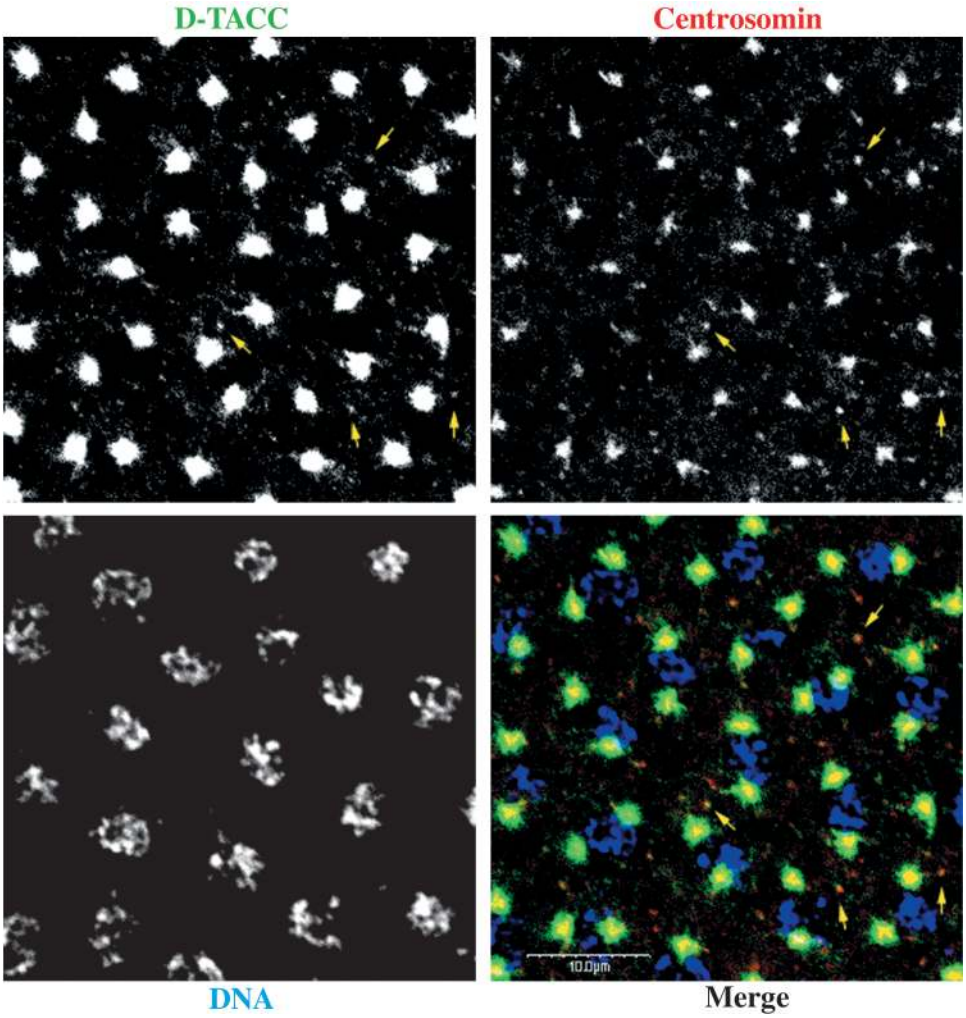

Fig. 5. Cnn and D-TACC colocalize at flare particles. Shown is a wild-type (OreR) interphase syncytial stage embryo double immunostained for D-TACC (green) and Cnn (red). DNA, stained with TOTO-3, is shown in blue. Yellow arrows indicate several flare particles in the D-TACC, Cnn and the merged images. Not all Cnn-immunoreactive particles contained D-TACC and vice versa. In the merged image the D-TACC signal at the centrosome appears to extend further from the center than does Cnn, but this is an inaccurate representation of centrosome structure and is due to an higher gain placed on the D-TACC signal at data collection, which was necessary to bring the flare particle signal to a similar level to that achieved with Cnn. See also Movie 4 at jcs.biologists.org/supplemental.

Flares are dependent on microtubules for their activity

To investigate the involvement of microtubules in flare activity, we injected the microtubule-destabilizing drug colchicine into GFP-Cnn embryos. The inhibition of microtubule polymerization caused the cleavage cycle to arrest and the normal movement of flare particles to cease (Fig. 6A). Specifically, the centrosomes in colchicine-treated embryos oscillate and appear to attempt to release flares but fail to do so (see Fig. 6A; Movie 4, available at jcs.biologists.org/supplemental). Thus although flare particles are clearly seen in colchicine-treated embryos, their normal movement is disrupted and is therefore dependent on microtubules. Although we injected rhodamine-labeled tubulin into GFP-Cnn embryos to observe the microtubules and Cnn simultaneously (Fig. 4), we were unable to resolve the astral microtubules that the flare particles are apparently moving upon using our imaging conditions. To see the microtubules more clearly, we immunostained for both Cnn and α-tubulin in fixed embryos and deconvoluted the images. From this, we observed that flare particles appear associated laterally and with the plus (+) ends of astral microtubules (Fig. 6B).

Flare movement was also inhibited by the drug Taxol (Fig. 7). Treatment of embryos with Taxol stabilized the microtubules and their cleavage was arrested (Schiff et al., 1979; Wani et al., 1971; Callaini and Riparbelli, 1997). Taxol caused the spindle to arrest in metaphase, which could be readily seen in GFP-tubulin embryos (compare Fig. 7A to Fig. 7B; Movie 5A-D, available at jcs.biologist.org/supplemental). In GFP-Cnn embryos we found that Taxol inhibited flare movement significantly (compare Movie 5C with Movie 7D), although some small movements can still be detected in these embryos (Fig. 7D; Movie 5, available at jcs.biologists.org/supplemental). In control animals that were permeabilized with octane but not exposed to Taxol, cleavage defects were observed. The nuclei did not reassemble properly following cleavage and often collided with each other (Fig. 7A; Movie 5, available at jcs.biologists.org/supplemental) (data not shown). Nonetheless, flaring was not inhibited under these control conditions (Fig. 7C; Movie 5, available at jcs.biologists.org/supplemental).

We investigated the relationship between the actin cytoskeleton and flares using GFP-Cnn and Rhodamine-labeled actin together in living embryos. At anaphase B and telophase, actin assembles densely along the outer edge of the centrosome on the face opposite to the spindle (Fig. 8, 297 seconds). These dense patches of actin juxtapose at division, preventing neighboring centrosomes from colliding. The characteristic formation of actin caps over the centrosomes in

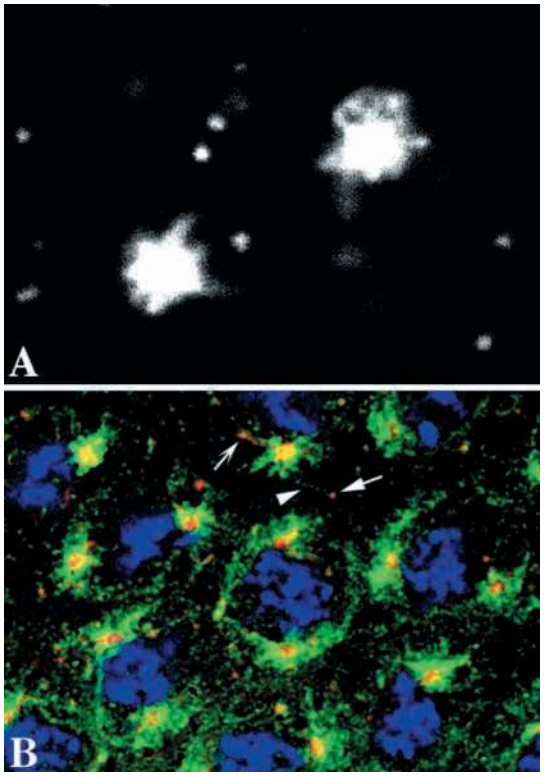

Fig. 6. Flares are dependent on microtubules. (A) GFP-Cnn embryos were injected with the microtubule-destabilizing drug colchicine to inhibit the formation of microtubules. Shown is a single still of the movie. Flare particles surround the centrosome but no longer move back and forth as they do in the untreated embryos. Incipient flares appear to emerge from the centrosome but do not bud off as flare particles. (B) Flares are associated with microtubules. Shown are images of embryos stained for Cnn (red), microtubules (green) and DNA (blue). Flare particles are often associated with microtubules (arrows in B), appearing at the end (solid arrow) of a microtubule (arrowhead) or projecting out like a tentacle along microtubules (arrow). The image in B was deconvoluted to resolve the Cnn and microtubule signals better. See also Movie 4 at jcs.biologists.org/supplemental.

interphase and the assembly of actin cages at mitosis around the mitotic spindle occurred (Fig. 8; Movie 6, available at jcs.biologists.org.supplemental). At mitosis, flare particles moved to the boundary of the actin cage but did not go beyond it.

To determine whether there was any dependence of flares on the actin cytoskeleton, actin polymerization was inhibited by the injection of cytochalasin-D into GFP-Cnn embryos. Cytochalasin-D did not inhibit flare activity. However, flare particles appeared to move farther from the centrosome than they did in untreated embryos (Fig. 9; Movie 7, available at jcs.biologists.org/supplemental). Flare particles also jumped to neighboring centrosomes in the cytochalasin-treated embryos (Fig. 9; Movie 7, available at jsc.biologists.org/supplemental), which rarely occurred in untreated embryos. Notably, multiple (more than two) centrosomes appear with each nucleus in the cytochalasin-treated embryos. This is due to the fusion of nuclei at division (Zalokar and Erk, 1976; Callaini et al., 1992; Sullivan et al., 1993). Thus, the nuclei become larger as the chromosomes and centrosomes become hyperploid at each division.

Discussion

Here we show that Cnn, a major component of *Drosophila* centrosomes (Lange et al., 2000), is a core component of the PCM that participates in some apparently novel centrosome dynamics. Time-lapse imaging of GFP-Cnn revealed that particles ('flares') emerge from the centrosome and move back and forth in a microtubule-dependent and actin-independent manner. D-TACC, a core component of centrosomes, colocalizes with Cnn at centrosomes and at flare particles. The relative intensity of flare activity changes with the cell cycle. Most of the movement of flare particles appears to be attributed to the dynamics of astral microtubules. Our observations with GFP-Cnn probably reflect the dynamics of native Cnn as GFP-Cnn expression in the early embryo rescued a *cnn*-null mutant.

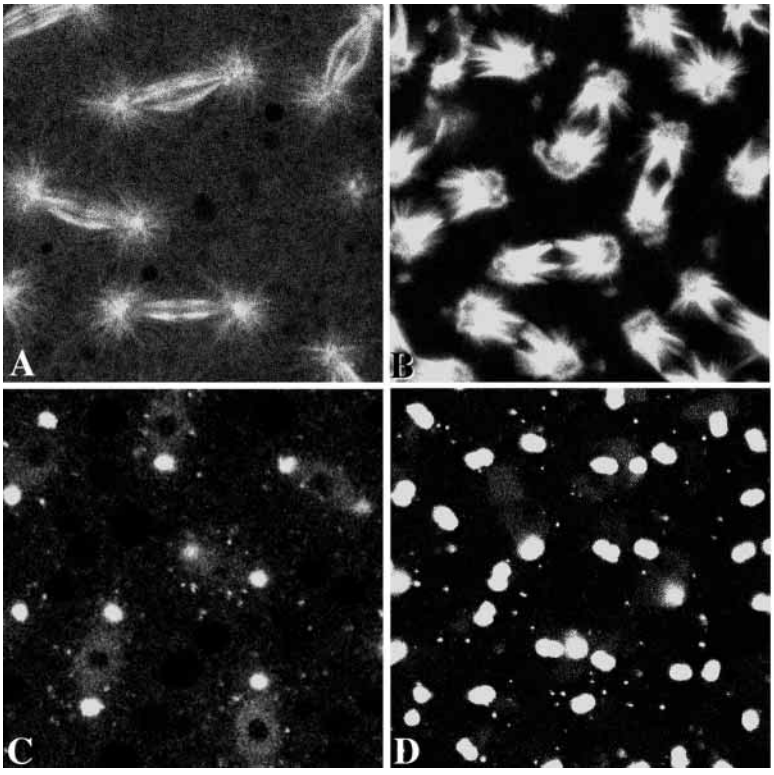

Fig. 7. Flare movement is dependent on microtubule dynamics rather than on microtubule-based motors. Live embryos were treated with paclitaxel (Taxol) to stabilize microtubules. GFP-tubulin embryos treated with Taxol (B) exhibit intense spindle microtubules, bright asters and have an arrested cycle as compared to mock control GFP-tubulin embryos (A). Flare activity was minimal in Taxol-treated GFP-Cnn embryos (D) as compared with the mock control animals (C). See also Movie 5 at jcs.biologists.org/supplemental.

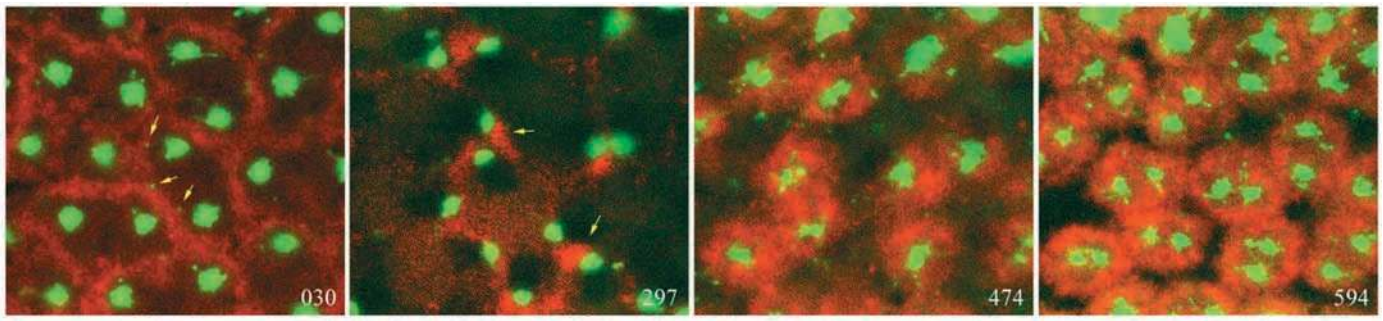

Fig. 8. Flare particles are restricted by the actin cage. Rhodamine-actin was injected into GFP-Cnn embryos to view the dynamics of the centrosomes and the actin cytoskeleton live. In metaphase (030), the flare particles, indicated by yellow arrows, moved to the actin cage boundary and no further. At telophase, when the centrosomes approach each other in close proximity (297), actin assembled densely at the centrosomes (see arrows) on the sides opposite to the nucleus. Following division, actin caps formed over the centrosomes (474) and in interphase the actin cage bound the flares (594). See also Movie 6 at jcs.biologist.org/supplemental.

Cnn is localized at the periphery of the centrosome, with the center appearing devoid of the protein. This was observed by both immunostaining (Fig. 1A) and by live imaging with GFP-Cnn. Imaging gives the appearance of a 'doughnut-like' structure, but it is more likely that the structure being viewed is actually a sphere devoid of Cnn at its center. With GFP-Cnn, as with immunostaining, the hole is visible only at low gain, where flare particles are difficult to detect (images of GFP-Cnn at low gain are not shown). The center of the centrosome is where the centrioles lie, and these structures may preclude the accumulation of Cnn. A 'hollow sphere' structure has also been observed in *C. elegans* centrosomes during mitosis when viewed by live imaging of GFP-γ- or GFP-β-tubulin (Strome et al., 2001). Additionally, EM studies on interphase cleavage stage *Drosophila* centrosomes reported 'clouds' of PCM material (Callaini and Riparbelli, 1990) and structures with 'spider-like' extensions of electron dense PCM material (Debec et al., 1999), that are similar to those in Fig. 1C. Moreover, similar projections of PCM material from centrosomes like those seen here with anti-Cnn antibodies (Fig. 1B, Fig. 5 and Fig. 6B), with GFP-Cnn (Fig. 4, Fig. 7C and Fig. 9) and by EM (Fig. 1C) were also observed on mouse

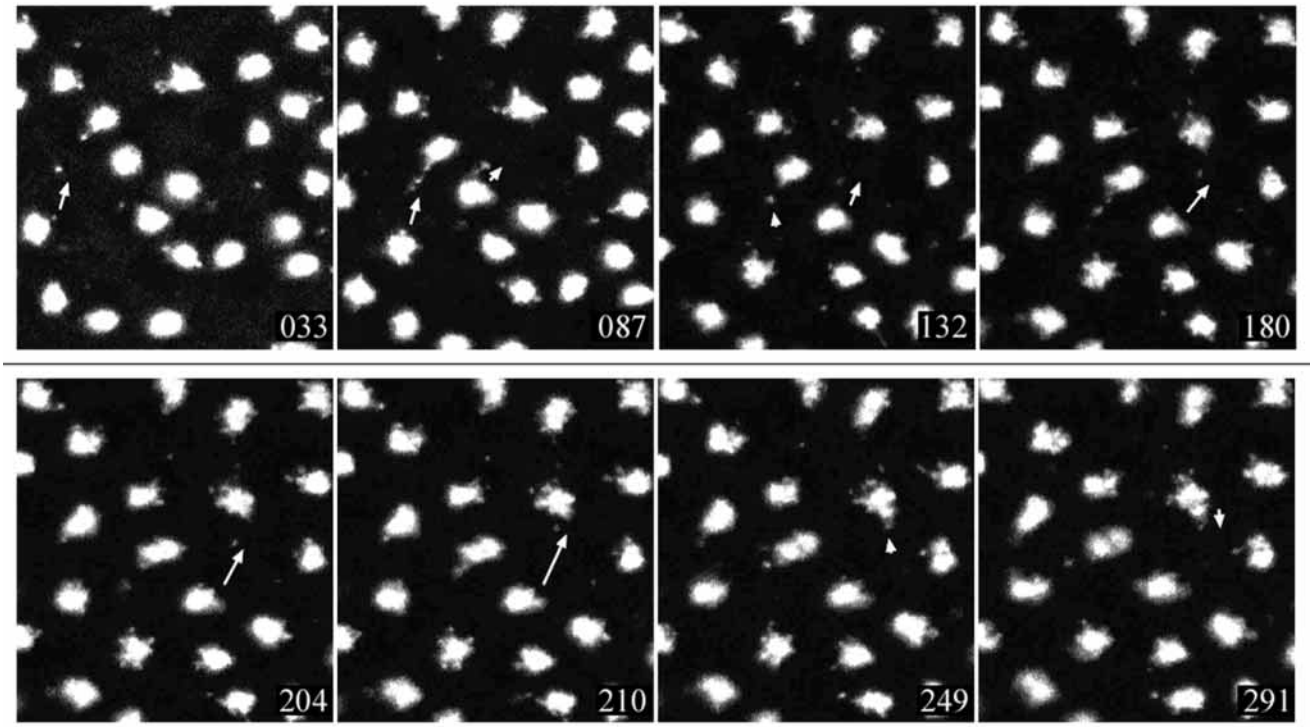

Fig. 9. Flare activity is not wholly dependent on actin assembly. GFP-Cnn embryos were injected with the actin destabilizing drug cytochalasin-D. In the absence of the actin cytoskeleton, the nuclear and centrosome divisions were not inhibited. The drug apparently does not affect flare activity. The centrosomes flared under these conditions and appeared to transfer flare particles more readily from one centrosome to another (see arrowheads in 132 and 249). The arrows in the figures indicate the direction of movement of the flare particles located closest to the tips of the arrows. movies available at jcs.biologists.org/supplemental.

centrosomes with anti-Pericentrin antibodies (Mogensen et al., 1997).

The localization of GFP-Cnn to the centrosome and to particles (flares) was consistent with immunostaining seen previously (Megraw et al., 1999; Li and Kaufman, 1996). However, live imaging revealed a strikingly dynamic relationship between Cnn and the centrosome. Live imaging revealed that flare particles emerged from centrosomes and moved back and forth in a microtubule-dependent manner to the boundary of the actin cage. Flares appear to be extrusions of Cnn-containing PCM material that contain only a subset of known PCM components (see below). Flares are probably not unique to cleavage stage centrosomes and may exist in other cells in addition to the early embryo. We have observed particles of Cnn near centrosomes in neuroblasts and spermatocytes by immunostaining (data not shown). Live imaging will be required to demonstrate whether these particles have the dynamic flaring relationship with the centrosome that we observed in the cleavage-stage embryo.

Flare activity oscillates with the cleavage division cycle. Specifically there are few flare particles associated with metaphase centrosomes, and this persists through mitosis until telophase, when flare activity increases again (see Table 1). When flare numbers are high, especially at interphase, the centrosomes have a more tentacled appearance when viewed with GFP-Cnn. Flare particles are born from these extrusions that emanate from the PCM. At metaphase Cnn has a relatively more intense and 'tight' association with the centrosome, producing a rounded appearance for the centrosome as compared with interphase.

Flares appear to be associated primarily with astral microtubules. Spindle microtubules had few flare particles associated with them. These observations are consistent with the changes in astral microtubule length that occur during the cleavage cycle (Karr and Alberts, 1986). Thus, flare numbers per centrosome approximate to the changes in astral microtubule length during the cleavage cycle. That is, in metaphase, when astral microtubules are short, flaring is less intense, whereas in interphase, when astral microtubules are long, flares are more numerous. It should be noted however that flare numbers do not absolutely mirror astral microtubule length changes. Asters grow longer during anaphase (Karr and Alberts, 1986), which precedes the observed increase in flaring that occurs at the ensuing telophase.

It is unclear what, if any, relationship flares have with Cnn assembly into centrosomes. To examine assembly, we attempted to use FRAP (fluorescence recovery after photobleaching) analysis, but were unable to photobleach centrosomes sufficiently without causing damage to the nuclei. These attempts at photobleaching caused the associated nucleus to arrest at interphase and fall away from the cortex toward the center of the embryo.

Flares are dependent upon microtubules for their movements. Colchicine injection stops all movement of flares, but does not affect the localization of Cnn to the centrosome or to the immobilized flare particles. The movie of GFP-Cnn with colchicine (Fig. 6A; Movie 4, available at jcs.biologists.org/supplemental) shows that the centrosome forms projections that appear to attempt budding of new flare particles, but these fail to exit the PCM, probably because the astral microtubules have been destabilized. Cnn may not bind flare particles directly to microtubules, since little microtubule-binding activity is detected with Cnn in vitro (Li and Kaufman, 1996). However, if Cnn is associated with Msps (see below), as D-TACC is (Lee et al., 2001; Cullen and Ohkura, 2001), then Cnn may be bound to microtubules indirectly through the microtubule-binding activity of Msps.

Flare particles contain a subset of centrosomal proteins. While we were unable to detect γ-tubulin or CP190 at flare particles, D-TACC protein does colocalize with Cnn at centrosomes and flare particles. In a recent report, D-TACC-GFP was examined in the early embryo where it localized to centrosomes and to punctate particles among astral microtubules at interphase (Gergely et al., 2000). Additionally, D-TACC was found to be in a complex with Msps (Lee et al., 2001; Cullen and Ohkura, 2001), and the two proteins interact directly with one another (Lee et al., 2001). When D-TACC-GFP and Msps-GFP fusion proteins were both expressed in *Drosophila* embryos, both were localized in particles that move to and fro from the centrosome (Lee et al., 2001). It is therefore likely that Cnn, D-TACC and Msps are all components of flares. However, colocalization will need to be examined to verify this for Msps.

We favor a model that posits that flare particles are passive passengers on dynamic microtubules, as opposed to a model where flare particles are transported by microtubule-based motor proteins. Taxol stabilizes microtubules but does not inhibit motor proteins as taxol is used routinely in vitro to stabilize microtubules for motor movement assays (Saxton, 1994). Thus since Taxol inhibited the activity of flares, flare movement is probably attributed to the dynamic instability inherent to microtubules and not to microtubule-based motor proteins. This suggests that flare particles are mostly static passengers upon dynamic microtubules. Thus, flare particles emerge from the centrosome, are associated with astral microtubules and move back and forth upon them as they grow and shrink. There may be a minor component of motor protein contribution to flare movement since flare particles moved to a small degree when microtubules were stabilized with taxol.

Consistent with the above model is the apparent rate at which flare particles move. We estimate that flares move at a speed of 4-20 μm/minute, a rate faster than that of spindle microtubule flux, estimated at 0.3-2 μm/minute (Mitchison, 1989; Mitchison and Salmon, 1992; Zhai et al., 1995; Desai et al., 1998), and more similar to the rate of interphase microtubule treadmilling measured at 12 μm/minute (Rodionov et al., 1999). Our estimate of flare velocity is also consistent with the rates of plus-end dynamics of centrosomal microtubules (Rodionov et al., 1999). Thus, the movement of flare particles on astral microtubules is probably a direct consequence of dynamic instability at the plus ends of these centrosome-anchored microtubules.

Injection of cytochalasin to disrupt the actin cytoskeleton does not affect flare movement but does cause flare particles to transfer to neighboring centrosomes. Such transfers rarely occur in embryos not treated with the drug. So actin is required to limit the extent of flare particle movement. During the cortical cleavage divisions, actin is organized into caps at interphase and furrows at metaphase (Warn et al., 1984; Karr and Alberts, 1986; Kellogg et al., 1988). Centrosomes orchestrate these cytoskeletal rearrangements (Raff and Glover, 1989) but can do so independently of microtubules

during cleavage in *Drosophila* (Stevenson et al., 2001). Live observations from GFP-Cnn embryos injected with Rhodamine actin showed that flares transit to the edge of the actin cage at mitosis. From this observation, it is tempting to speculate that flares act with the centrosome to organize the actin cage boundary. If flares, which clearly are microtubule dependent, participate in promoting actin organization by the centrosome during cleavage, their role in this process may not be absolutely required.

During the cortical cleavage divisions, membrane-bearing particles are recruited to the cleavage furrows for deposition of membrane, actin and membrane proteins such as Discontinuous actin hexagon (Dah) (Zhang et al., 2000; Rothwell et al., 1999). Membrane-bearing particle recruitment requires the activity of Nuclear fallout (Nuf), a protein found at the centrosome (Rothwell et al., 1999). Flare particles are probably not these membrane-bearing particles since Dah and Cnn did not colocalize when we immunostained for the two proteins (data not shown). In addition, immunostaining for Golgi bodies using anti-β-Cop (β-coatomer protein) (Ripoche et al., 1994) or anti-Lva (Lava lamp) (Sisson et al., 2000) antibodies also showed no colocalization to Cnn flare particles (data not shown).

To our knowledge, the ejection of PCM particles from centrosomes has not been described previously. The appearance of 'ejected asters' from frog oocyte spindle poles in vitro (Murray et al., 1996) may be related to the flaring phenomenon. However, unlike ejected asters, flare particles do not appear to emanate microtubules. Additionally, particles that contain Pericentrin, a centrosomal protein that is required for microtubule nucleation at centrosomes (Doxsey et al., 1994), were reported recently (Young et al., 2000). Pericentrin particles also contain γ-tubulin and move toward centrosomes where they dock and facilitate proper centrosome assembly (Young et al., 2000). However, Cnn-containing flare particles behave differently, because they emerge from the centrosome and move bi-directionally, both retreating and advancing toward the centrosome. In addition, although Pericentrin particles are carried by the minus-end-directed motor cytoplasmic dynein (Young et al., 2000), movement of Cnn-containing flare particles appears to be largely dependent upon intrinsic microtubule dynamics.

In vertebrate cells, early electron micrograph studies of centrosomes revealed the presence of electron-dense spherical granules approximately 70-100 nm in diameter that localized around centrosomes and within the PCM and have been called satellites or centriolar satellites (Rattner, 1992; Kalt and Schliwa, 1993; Kubo et al., 1999). PCM-1, a component of the centrosome PCM that localizes to the centrosome during interphase, but dissociates from it at mitosis, is required for mouse zygotes to pass through interphase (Balczon et al., 1994; Balczon et al., 2002). PCM-1 is a component of satellites, and live imaging of GFP-PCM-1 particles showed that satellites move bi-directionally in a microtubule- but not actin-dependent manner (Kubo et al., 1999). Thus the behavior of satellites in vertebrate cells is similar to that of the flares we describe here in *Drosophila* embryos except that the reported rate of satellite movement is 0.7-0.8 μm/second (Kubo et al., 1999), which is approximately four-fold faster than the maximum rates we measured for flare movement. Since TACC, and probably Msps, appear to be components of flares – it will be interesting to investigate whether the homologs of these proteins in vertebrates are localized at satellites.

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