

Maintaining the proper connection between the centrioles and the PCM requires *Drosophila* Centrosomin

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Abstract

Centrosomes consist of two centrioles surrounded by an amorphous pericentriolar matrix (PCM), but it is unknown how centrioles and PCM are connected. We show here that the centrioles in *Drosophila* embryos that lack the centrosomal protein Centrosomin (Cnn) can recruit PCM components, but cannot maintain a proper attachment to the PCM. As a result, the centrioles “rocket” around in the embryo and often lose their connection to the nucleus in interphase and to the spindle poles in mitosis. This leads to severe mitotic defects in embryos and to errors in centriole segregation in somatic cells. The Cnn-related protein CDK5RAP2 is linked to microcephaly in humans, but *cnn* mutant brains are of normal size, and we observe only subtle defects in asymmetric divisions in mutant neuroblasts. We conclude that Cnn maintains the proper connection between the centrioles and the PCM; this connection is required for accurate centriole segregation in somatic cells, but is not essential for the asymmetric division of neuroblasts.

Introduction

Centrosomes comprise a pair of centrioles and a surrounding pericentriolar matrix (PCM). They are the major microtubule organising centres (MTOCs) in animal cells and are thought to play an important part in organising many cell processes, including cell polarity, cell migration and cell division (Doxsey et al., 2005).

Centrioles are also required for the formation of cilia and flagella (Basto et al., 2006), and they have important roles in many developmental processes (Davis et al., 2006). Not surprisingly, centriole or centrosome dysfunction has been implicated in a wide variety of human genetic diseases (Badano et al., 2005).

While much is known about the protein composition of the centrosome, it remains unclear how centrosome structure and organisation are maintained. In flies, the centrosomal protein Centrosomin (Cnn) is required to recruit several proteins to the centrosome (Megraw et al., 2001; Megraw et al., 1999; Terada et al., 2003; Vaizel-Ohayon and Schejter, 1999). In *cnn* mutant embryos, and in somatic cells lacking Cnn, the centrosomes fail to function as MTOCs during mitosis and anastral spindles assemble through a centrosome-independent pathway. This leads to dramatic mitotic defects in embryos (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999) but only to subtle mitotic defects in somatic cells (Mahoney et al., 2006; Megraw et al., 2001), presumably because centrosomes are not essential for cell division in somatic cells (Basto et al., 2006; Bettencourt-Dias et al., 2005).

Cnn is a member of a family of structurally related proteins that have been implicated in organising microtubule (MT) arrays. In the yeast *S. pombe*, the Cnn-related protein Mto1 recruits the γ -tubulin complex to several types of MTOCs (Sawin et al., 2004; Venkatram et al., 2004). In human cells, the Cnn-related proteins CDK5RAP2 and Myomegalin/PDE4-DIP are concentrated at centrosomes, but their function is unknown (Bond et al., 2005; Verde et al., 2001). Mutations in the gene encoding CDK5RAP2, however, cause autosomal recessive primary microcephaly, in which the brain is small at birth and thereafter (Bond et al., 2005). The underlying cause of microcephaly is unknown, but it has been proposed that a failure of the centrosomes to function as efficient MTOCs in mitosis might lead to defects in asymmetric neuroblast (NB) divisions during foetal development (Basto et al., 2006; Bond and Woods, 2006; Fish et al., 2006).

Here we have used live confocal imaging to examine how Cnn functions to ensure the proper organisation of the centrosome in flies, and to test whether Cnn is required for asymmetric divisions in larval NBs.

Results and Discussion

Centrioles recruit PCM components and MTs, but cannot maintain their connection to them in the absence of Cnn

In fixed embryos and somatic cells that lack Cnn, PCM components are barely detectable at the poles of the mitotic spindles (Megraw et al., 2001; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). Centrioles are still present in *cnn* mutant cells (Megraw et al., 2001), but their function and positioning within the centrosome have not been analysed. To understand better how Cnn normally recruits PCM components to the centrioles, we generated transgenic *Drosophila* lines expressing a mRFP-centriolar marker (either mRFP-Fzr or mRFP-PACT), together with one of three PCM markers fused to GFP: Aurora A-GFP, Grip75-GFP (a component of the γ -tubulin ring complex — γ -TuRC), and GFP-D-TACC. Previous studies have suggested that Cnn interacts with both the γ -TuRC and Aurora A (Terada et al., 2003), but we found no evidence for an interaction between Cnn and D-TACC in co-immunoprecipitation experiments (not shown).

In wild-type (WT) syncytial embryos, centrioles recruited approximately equal amounts of PCM at all stages of the rapid mitotic cycles and they remained well centred within the PCM throughout the cell cycle (Figure 1, Supplementary Figure 1; Videos 1-2). During interphase, the centrioles were always closely associated with the nuclear envelope, whereas in mitosis, they were always closely associated with the spindle poles (Videos 1-2). In embryos laid by *cnn* homozygous females (hereafter, *cnn* embryos), we were surprised to observe that the centrioles were associated with appreciable amounts of PCM, but they were often not properly centred within it (Figure 1, Supplementary Figure 1; Videos 1-2). In video recordings of *cnn* embryos, the centrioles appeared to be constantly nucleating PCM, but seemed unable to maintain their connection to it. The centrioles often exhibited irregular, stochastic movements, leaving a trail of PCM behind them as they moved away. This PCM trail was most easily seen in *cnn* embryos expressing GFP-D-TACC (Video 1), as this protein was recruited in particularly large amounts to the centrioles, and large clusters of GFP-D-TACC often remained in the cytoplasm for some time after the centrioles had moved away. Smaller amounts of Aurora A-GFP and Grip75-GFP were recruited to the centrioles (Video 2), and so only small amounts of these proteins remained associated with the centrioles as they moved

around the embryo. As a result of this abnormal centriole behaviour, the centrioles in *cnn* embryos often lost their attachment to the nuclear envelope in interphase and to the spindle poles in mitosis. We refer to this behaviour of the centrioles as “centriole rocketing” (see below).

Previous studies suggested that centrosomes lacking Cnn fail to function as MTOCs during mitosis (Mahoney et al., 2006; Megraw et al., 2001; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). We therefore examined whether the PCM organised by the centrioles in *cnn* embryos was capable of nucleating MTs. As shown in Figure 2A-D and Videos 3-4, the centrosomes in *cnn* embryos organised astral MT arrays but seemed unable to maintain their connection with them. When the embryos entered mitosis, many nuclei were not associated with centrioles, and anastral spindles assembled around the mitotic chromatin (not shown). Many nuclei, however, were close enough to a centriole for the astral MTs to contribute to spindle assembly (Figure 2D, arrow). Often, however, these centrioles failed to maintain their position at the spindle pole and either wandered around within the spindle (Figure 2D, red arrowhead) or lost their connection to the spindle altogether (Figure 2D, yellow arrowhead). We conclude that the dramatic mitotic defects observed in *cnn* embryos do not result from a failure of the centrioles to recruit PCM, or of the centrosomes to nucleate astral MTs, but instead result from the failure of the centrioles to maintain a stable connection to the PCM and MTs that they organise.

Centriole rocketing in *cnn* embryos is MT-dependent

The centriole rocketing appeared to be driven by the asymmetric organisation of the PCM and MTs around the centrioles (Videos 1-3). To test whether the rocketing was MT-dependent, we injected the MT-depolymerising drug colchicine into embryos. In WT embryos in late interphase, the centrioles had already migrated around the nuclei and, when we plotted their movement over time, the centrioles moved regularly across the embryo cortex (Figure 2E; Video 5). This regular movement of the centrosomes and their associated nuclei across the cortex is driven by actin- and myosin-dependent cortical contractions, and it continued after colchicine injection (Video 5). In contrast, when we plotted centriole movement in *cnn* embryos in late interphase, we observed the rocketing behaviour described above (Figure 2F; Video 5). The rocketing ceased after colchicine injection, and the centrioles reverted to a

regular movement across the embryo cortex (Figure 2G; Video 5). Thus, centriole rocketing in *cnn* embryos depends on intact MTs.

The injection of colchicine into *cnn* embryos also enabled the centrioles to remain associated with the PCM (Figure 3), suggesting that it is the MT-dependent rocketing of the centrioles that ultimately breaks the link between the centrioles and the PCM in *cnn* embryos. Intriguingly, however, the injection of colchicine into *cnn* embryos did not correct the positioning defect of the centrioles within the PCM: whereas the centrioles were usually (>90%) well centered within the PCM in colchicine-injected WT embryos (between 50-100 centrioles observed with each of the three different PCM markers — Figure 3; Video 6), they were very rarely centered within the PCM in colchicine-injected *cnn* embryos (<10%) and were usually positioned at the very edge of the PCM (>100 centrioles observed with each of the three different PCM markers — Figure 3; Video 6).

This last observation was very unexpected, and we are unaware of any other perturbation to the centrosome that results in this very specific displacement of the centrioles from the centre of the PCM. This observation may have important implications for understanding how Cnn functions to maintain the link between the centrioles and the PCM. One interesting possibility is that the MT-dependent centriole rocketing we observe in *cnn* embryos may be mechanistically related to the actin-dependent rocketing of certain pathogenic bacteria (Borisy and Svitkina, 2000; Higgs and Pollard, 2001). These bacteria are coated with proteins that initially stimulate the polymerisation of an actin “cloud” symmetrically around the surface of the bacteria. If the actin surrounding the bacteria is structurally weak it can “fracture”, allowing the bacteria to move to the edge of the actin cloud and rocketing to begin (van der Gucht et al., 2005; van Oudenaarden and Theriot, 1999). Thus, we propose that the primary function of Cnn may be to mechanically strengthen the PCM: in the presence of Cnn, the PCM is structurally strong and the centrioles can maintain their position at the centre of the PCM; in the absence of Cnn, the PCM is weakened and the centrioles move to the edge of the PCM. This then initiates centriole rocketing, although the exact mechanism of this MT-dependent rocketing remains unclear.

Centriole segregation is defective in *cnn* mutant somatic cells

Maintaining the proper connection between the centrioles and the PCM is clearly crucial in syncytial embryos, as a lack of Cnn results in catastrophic failures in mitosis. In contrast, somatic cells that lack Cnn have few mitotic defects, and *cnn* mutant flies are viable (Mahoney et al., 2006; Megraw et al., 2001). To test whether Cnn was required to maintain the proper connection between the centrioles and the PCM in somatic cells, we treated 3rd instar larval brain cells with colchicine to depolymerise the MTs, and then fixed and stained them to examine the distribution of the centrioles and the PCM. We found that hardly any PCM was detectable around the centrioles in *cnn* brain cells that had not been treated with colchicine (not shown). In *cnn* cells treated with colchicine, however, significant amounts of PCM accumulated around the centrioles, but, as in *cnn* embryos, the centrioles were displaced from the centre of the PCM (Supplementary Figure 2).

To further investigate whether the centrioles in *cnn* somatic cells behaved in the same way as the centrioles in *cnn* embryos, we examined living 3rd instar larval NBs expressing the centriole marker DSas-4-mRFP and GFP- α -Tubulin. In WT NBs entering mitosis, the centrioles were always centred within astral MT arrays, and the centrioles remained tightly associated with the poles of the spindle throughout mitosis (Figure 4A). In contrast, the centrioles in *cnn* NBs were often not associated with prominent astral MTs, and exhibited irregular movements throughout the cell during mitosis. As a consequence, they were often abnormally displaced from the poles of the mitotic spindles (Figure 4B). Nevertheless, we could transiently detect astral MTs associated with some of the “rocketing” centrioles in some *cnn* NBs (Figure 4C, arrows; see below). In fixed larval *cnn* NBs, the centrioles were often randomly positioned around the cell (Figure 4E), and we noticed that 20-30% of brain cells had either too few or too many centrioles (Figure 4F). Taken together, these findings suggest that the centriole behaviour is similar in *cnn* embryos and somatic cells; while these defects do not lead to dramatic errors in somatic cell division, they do lead to errors in centriole segregation. These findings support the hypothesis that centrioles have evolved the ability to recruit PCM to ensure the equal partitioning of the centrioles during cell division, rather than to ensure the efficient assembly of the mitotic spindle (Pickett-Heaps, 1969; Rieder et al., 2001).

Asymmetric cell divisions are only mildly perturbed in *cnn* mutant neuroblasts

The Cnn-related protein CDK5RAP2 has been implicated in human microcephaly (Bond et al., 2005), and several recent studies have shown that centrosomes exhibit an asymmetric behaviour during the asymmetric divisions of male germline stem cells (GSCs) and larval neural stem cells (NBs) (Rebollo et al., 2007; Rusan and Peifer, 2007; Yamashita et al., 2007). During interphase in these cells, only one centrosome is initially associated with PCM and MTs, and this centrosome becomes anchored on one side of the cell (near the stem cell niche in GSCs, or near cortically localised cell polarity markers in NBs). When the second centrosome eventually associates with PCM and MTs, it localises to the opposite side of the cell, thus ensuring that the forming mitotic spindle is correctly oriented relative to these positional cues.

This asymmetric centrosome behaviour appears to be important in male GSCs as the asymmetric division of these cells is dramatically perturbed in *cnn* mutants (Yamashita et al., 2003). Although *cnn* mutant NBs have defects in aligning their spindles with cortical determinants early in mitosis (Megraw et al., 2001), it is not clear that this ultimately leads to failures in asymmetric division: early mitotic spindle alignment defects are often corrected in these cells by the time the cells divide (Cai et al., 2001). To determine whether *cnn* mutant NBs ultimately divide asymmetrically, we analysed living WT and *cnn* 3rd instar larval NBs expressing only GFP- α -Tubulin.

As reported previously (Rebollo et al., 2007; Rusan and Peifer, 2007), a single, anchored MTOC was usually visible in WT NBs prior to the entry into mitosis (not shown). After NEB, however, both centrosomes nucleated prominent arrays of MTs, and spindle assembly occurred primarily by a centrosomal pathway (Figure 5A; Video 7). As expected, the cells divided asymmetrically to produce a large NB and a small ganglion mother cell (GMC). In most *cnn* NBs no prominent MTOC was detectable prior to NEB, and spindle assembly occurred largely by an acentrosomal pathway (Figure 5B; Video 8). Nonetheless, ~95% of *cnn* NBs ultimately divided asymmetrically (n=81) (Figure 5B; Video 8), while ~4% divided symmetrically (Figure 5C; Video 9) and ~1% failed in cytokinesis (not shown). Although this failure rate is modest, we believe it is significant, as we observed only one symmetric division in more than 100 WT NBs examined (E. P. Lucas, R. Basto, and C. I. Dix, unpublished observations).

We previously showed that mutations in *DSas-4*, which encodes the *Drosophila* homologue of the human microcephaly protein CenpJ/CPAP (Bond et al., 2005), also lead to defects in the asymmetric divisions of larval NBs. The defects were much more severe in *DSas-4* mutants, which completely lack centrioles/centrosomes (~15% of NBs divided symmetrically, while ~15% failed in cytokinesis) (Basto et al., 2006). The much milder defects in asymmetric division that we observe in *cnn* NBs suggest that centrosomes are partially functional as MTOCs in *cnn* mutant somatic cells, consistent with the observations described above (Supplementary Figure 2; Figure 4C). Indeed, we frequently observed relatively well-focused astral MT arrays forming and disassembling in the cytoplasm, and these were often transiently associated with the spindle poles in *cnn* NBs (Figure 5D, arrow; Video 10).

Taken together, our observations on *cnn* and *DSas-4* mutant NBs reveal that, unlike the situation in male GSCs, the asymmetric behaviour of the centrosomes is not essential for the accurate asymmetric division of larval NBs. Nevertheless, mutations in the *Drosophila* homologues of two of the three human centrosomal proteins implicated in microcephaly do lead to relatively subtle defects in NB divisions in flies. *Drosophila cnn* and *DSas-4* mutants do not have small brains, suggesting that flies are able to compensate for defects in these divisions in a way that perhaps humans cannot.

Materials and Methods

Fly stocks

Oregon R or *yw* flies were used as wild-type, and *yw* flies as the parental stock for the generation of all transgenic lines. The *cnn*^{HK21} null allele has been described previously (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999), and the *cnn*^{f04547} allele, a *piggyBac* insertion in the middle of the *cnn* gene (Exelixis stock #f04547), was obtained from the Exelixis Stock Centre (Harvard Medical School, Boston, MA). In western blotting experiments, embryos laid by females homozygous for this allele had no detectable Cnn protein.

GFP- and mRFP-fusion proteins

GFP-fusions to the following proteins were used in this study: Aurora A-GFP (J. Lau, unpublished); GFP-D-TACC (Lee et al., 2001); Grip75-GFP (Schnorrer et al., 2002); GFP- α -Tubulin (Grieder et al., 2000); DSas-4-GFP (Peel et al., 2007). Fusions between mRFP- and the full length *fzr* cDNA, the PACT domain of D-PLP (Martinez-Campos et al., 2004) and DSas-4 (R. Basto, unpublished) were also generated (cloning details available upon request). Most of these fusions were subcloned into the pWR-Ubq transformation vector that drives the ubiquitous expression of the fusion protein at moderately high levels. For Grip75-GFP and GFP- α -Tubulin, we used previously established lines in which the expression of these proteins is under the control of the UASp promoter (Rorth, 1998); we drove their expression in embryos using the maternal 67C α -Tubulin-GAL4 promoter (Lee et al., 2001), and in brains using the 69B enhancer trap line (Brand and Perrimon, 1993).

Transgenic lines were generated using standard methods. Most studies were performed using the *cnn*^{HK21} allele, but we obtained similar results with either the *cnn*^{f04547} allele, or transheterozygous combinations of the two alleles.

Live imaging of syncytial embryos

Live embryos expressing fluorescent fusion proteins were examined as described previously (Huang and Raff, 1999). The embryos were observed on a Perkin Elmer ERS Spinning Disc confocal system, mounted on a Zeiss Axiovert 200M inverted microscope that was equipped with a CCD Orca ER camera (Hamamatsu), using a 63x/1.25NA objective. For each combination of GFP- and mRFP-fusions, 4 to 15 WT

and *cnn* mutant syncytial blastoderm stage embryos were examined. For drug injections, live embryos were injected at the desired stage with colchicine (5 mg/ml; Sigma-Aldrich). All images were captured and made into videos using the Ultraview ERS software (Perkin Elmer).

Live imaging of larval neuroblasts

Third-instar larval NBs were prepared as described (Basto et al., 2006), and followed by time-lapse confocal microscopy as described above with a 100x/1.3NA objective. Twelve to twenty focal planes spaced by 0.5 μ m were acquired every 25 or 45 s (0.5-1 s/frame, respectively). All images shown are maximum intensity projections of Z-stacks at selected time points.

Immunofluorescence of larval brain preparations

Third-instar larval brains were fixed and stained as described previously (Martinez-Campos et al., 2004). For drug treatment, dissected brains were incubated for 2h at 25°C in PBS containing colchicine (1 μ g/ml; Sigma-Aldrich) prior to fixation. The following primary antibodies were used: rabbit anti-D-PLP at 1-2 μ g/ml (Martinez-Campos et al., 2004); mouse anti- α -Tubulin at 1:1000 (DM1 α ; Sigma-Aldrich), mouse anti- γ -Tubulin at 1:1000 (GTU88; Sigma-Aldrich), and mouse anti-phospho-Histone H3 at 1:2000 (Abcam, UK). All secondary antibodies coupled to the appropriate fluorophore (Alexa 488 or 568; Molecular Probes) were used at 1:1000 in PBT. Fixed preparations were examined on a Zeiss Axioskop II widefield upright microscope, equipped with a CoolSnap HQ camera (Photometrics) and MetaMorph software (Molecular Devices), using a 100x/1.3NA objective. Images of fixed brain cells are all maximum intensity projections of optical sections acquired at 0.1-0.2 μ m intervals.

Preparation of Figures

Individual images were imported into Adobe Photoshop 7.0 and adjusted to use the full range of pixel intensities. In panels for some figures, pixel intensities were adjusted using the “curves” control panel, and an unsharp mask and despeckle filter were applied to the whole image. In all cases, the images from control and experimental embryos were adjusted in the same way.

Quantification of the PCM recruitment around the centrioles in syncytial embryos

To quantify the amount of PCM recruited to centrioles, individual images were

imported into MetaMorph (Molecular Devices). The centrosome was circled and the integrated intensity was calculated for independent centrosomes after subtraction of cytoplasmic background fluorescence. The integrated intensity per pixel area was determined from at least 3 different WT and *cnn* embryos per centrosomal marker. A total of 45 centrosomes were scored for each marker. Error bars represent the standard deviation (SD). The data was analysed for statistical significance using a two-tailed Student's *t*-test.

Quantification of centriole number in fixed larval brain cells

To quantify the number of centriole dots per mitotic cell, fixed preparations of 3rd instar larval brains were stained with anti-D-PLP and anti-phospho-Histone H3 antibodies. We examined a minimum of 70 mitotic (phospho-Histone H3 positive) cells per larval brain from at least 6 different brains. A total of 718 mitotic cells from WT and 976 mitotic cells from homozygous *cnn*^{HK21} mutants were scored. Error bars represent the SD.

Centriole tracking

Semi-automated tracking software (Imaris 4.5.2; Bitplane AG) was used to identify and track DSas-4-GFP trajectories over time (150 time frames, 1 s/frame). Image segmentation was performed to convert pixel intensities above a given threshold into computerised spots and this method was applied equally to all the images in the time series. Semi-automatic track building was based on autoregressive motion algorithms.

Online Supplemental Material

Fig. S1 shows the localisation of Aurora A-GFP, Grip75-GFP and centrioles in WT and *cnn* embryos. Fig. S2 shows the centriole positioning defects in *cnn* larval brain cells after colchicine treatment.

Ten additional videos are also included, showing the behaviour of: (1) centrioles and GFP-D-TACC in WT and *cnn* embryos; (2) centrioles and Aurora A-GFP, or Grip75-GFP in WT and *cnn* embryos; (3 and 4) centrioles and GFP- α -Tubulin in a *cnn* embryo; (5) centrioles in WT and *cnn* embryos prior to and after colchicine injection; (6) centrioles and PCM in WT and *cnn* embryos after colchicine injection; (7) a WT larval NB expressing GFP- α -Tubulin, dividing asymmetrically; (8) a *cnn* larval NB expressing GFP- α -Tubulin, dividing asymmetrically; (9) a *cnn* larval NB expressing GFP- α -Tubulin, dividing symmetrically; (10) a *cnn* larval NB expressing GFP- α -Tubulin with prominent astral MTs, and a small focus of MTs moving around the cell.

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Abbreviations list

Cnn, centrosomin; GSC, germline stem cell; MT, microtubule; MTOC, MT organising centre; NB, neuroblast; PCM, pericentriolar matrix; WT, wild-type.

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Figure Legends

Figure 1

The centrioles in *cnn* embryos cannot maintain a proper connection to the PCM.

(A-B) Still images from movies of WT and *cnn* syncytial embryos expressing the PCM marker GFP-D-TACC (pseudo-coloured *red*), and the centriole marker mRFP-Fzr (pseudo-coloured *green*; note that mRFP-Fzr is also concentrated in the nucleus in interphase). Time in min:sec. (A) In WT embryos, the centrioles are always well centered within the PCM. (B) In *cnn* embryos, the centrioles are associated with PCM, but they “rocket” around within the cytoplasm and no longer maintain their proper connection to the PCM (Video 1). (C) Quantification of the amount of PCM recruited to centrioles in WT and *cnn* embryos. Error bars represent SD (n=45 for each marker and genotype) and $p<0.001$ (Student’s *t*-test) are represented by asterisks. Scale bar, 10 μ m.

Figure 2

The centrioles associate with astral MTs in *cnn* embryos, and centriole rocketing is MT-dependent.

(A-D) The centrioles in a *cnn* embryo were visualized with mRFP-PACT (*red*) and the MTs with GFP- α -Tubulin (*green*). At the start of this time series (time in min:sec) (A), one centriole is associated with MTs (*arrowhead*), while another is not (*arrow*). The latter centriole starts to nucleate MTs from one side (B), and both centrioles exhibit “rocketing” movements as they recruit PCM and nucleate MTs asymmetrically around themselves (Video 3). As this embryo enters mitosis (C), the centrioles continue to rocket around in the cytoplasm (Video 4). In this panel, one centriole is located at the pole of a mitotic spindle (*arrow*), and this pole is associated with astral MTs. Another centriole loses its connection to the spindle pole (*red arrowhead*) and starts to move around within the spindle; another centriole (that is not in focus, but whose position can be inferred from its associated astral MTs — *yellow arrowhead*) starts to migrate away from the spindle into the cytoplasm. By the end of mitosis (D), the latter centriole has completely lost contact with the spindle (*yellow arrowhead*); the other centriole has migrated back to the pole (*red arrowhead*). (E-G) Traces of centriole movement over time are shown for a WT embryo (E), a *cnn* embryo (F), and the same *cnn* embryo after colchicine injection (G). The position of each centriole over time is indicated by the colour scale, which represents 150 seconds. See Video 5. Scale bars, 10 μ m.

Figure 3

The centrioles are displaced to the edge of the PCM in *cnn* embryos. The WT and *cnn* embryos shown here were injected with colchicine to depolymerise the MTs, and they express the centriole marker mRFP-Fzr (pseudo-coloured *green*) and the PCM markers GFP-D-TACC (A), Grip75-GFP (B) and Aurora A-GFP (C) (all pseudo-coloured *red*). Note how the centrioles are displaced towards the edge of the PCM in *cnn* embryos (right panels). See Video 6. Scale bar, 10 μ m.

Figure 4

Centriole segregation is abnormal in *cnn* mutant larval brain cells. (A-B)

Time series (min:sec) of living WT and *cnn* mutant NBs expressing the centriole marker DSas-4-mRFP (*arrows*, pseudo-coloured *green*) and GFP- α -Tubulin (pseudo-coloured *red*). (A) In WT cells, the centrioles are always located at the poles of the mitotic spindle. (B) In *cnn* NBs, the centrioles move erratically around the cell and no longer maintain their proper connection to the poles of the spindle. Note that one of the centrioles temporarily moves out of the focal plane (13:30), but reappears by the end of mitosis. (C) Another *cnn* NB expressing the same markers as above (colours are inverted). Both centrioles are able to nucleate astral MTs, but fail to maintain their connection to them. (D-E) The distribution of MTs (*red*), centrioles (D-PLP, *green*), and DNA (*blue*) in fixed WT and *cnn* mutant cells. In *cnn* mutant cells (E), the centrioles are more randomly distributed in the cell and are sometimes of unequal size (which may reflect the clustering of some centrioles). (F) Bar chart showing the number of centrioles present in WT and *cnn* mutant mitotic brain cells. Error bars represent SD. Scale bars, 5 μ m.

Figure 5

Asymmetric cell divisions are mildly perturbed in *cnn* mutant neuroblasts. Time series (min:sec) of MT behaviour in living 3rd instar larval NBs. (A) In a WT NB, the centrosomes nucleate prominent arrays of astral MTs before NEB (0:00), and the spindle is assembled primarily from these MTs. This cell divides asymmetrically (17:57) to produce a large NB and a small GMC (cell areas are outlined — Video 7). (B) In this *cnn* mutant NB, astral MTs are undetectable, and the spindle appears to assemble around the chromatin; nevertheless, the cell divides asymmetrically (Video 8). (C) In this *cnn* mutant NB, some astral MTs are visible, but the cell ultimately

divides symmetrically (Video 9). (D) In this *cnn* mutant NB, prominent astral MTs transiently form at one of the poles of the spindle (*arrow*), and a small, transient focus of MTs can be seen moving around in the cytoplasm (*arrowheads* — Video 10). Scale bar, 5 μm .

Online Supplemental Figure legends

Supplementary Figure 1

The centrioles in *cnn* embryos are associated with some PCM, but are not properly centered within it. The WT and *cnn* embryos shown here express the PCM markers Aurora A-GFP (A-B), or Grip75-GFP (C-D) (all pseudo-coloured *red* in the merged image), as well as the centriole marker mRFP-Fzr (pseudo-coloured *green* in the merged image; note that mRFP-Fzr is also concentrated in the nucleus in interphase). (A and C) In WT embryos, the centrioles are always well centered within the PCM. (B and D) In *cnn* embryos, the centrioles are associated with PCM, but they cannot maintain their proper connection to the PCM. Only relatively small amounts of Aurora A-GFP and Grip75-GFP are recruited to the centrioles compared to GFP-D-TACC, so only small amounts of these proteins remain associated with the centrioles as they move around the *cnn* embryo. See Video 2. Scale bar, 10 μm .

Supplementary Figure 2

The centrioles are not properly centered within the PCM in *cnn* mutant larval brain cells. (A-C) The distribution of centrioles (D-PLP, *green*), γ -Tubulin (*red*), and DNA (*blue*) in WT and *cnn* mutant cells after colchicine treatment. (A) In WT cells, the centrioles remain well centered within the PCM. (B and C) In *cnn* mutant cells, the centrioles are displaced to the side of the PCM, and often don't overlap with the PCM. This positioning defect was observed in 6/6 serially sectioned *cnn* mutant cells, and was not observed in 8/10 serially sectioned WT cells. Scale bar, 5 μm .

Online Supplemental Videos

Video #1

The behaviour of centrioles and GFP-D-TACC in WT and *cnn* embryos. This video relates to Fig. 1. Centrioles are visualised with mRFP-Fzr (pseudo-coloured *green*), and the PCM is visualised with GFP-D-TACC (pseudo-coloured *red*). Note that mRFP-Fzr also accumulates in the interphase nuclei. The PCM is highly dynamic, but in a WT embryo the centrioles remain well centred within it, and the centrosomes are closely associated with the nuclear envelope in interphase and with the spindle poles in mitosis. In a *cnn* embryo, not all of the centrioles are associated with nuclei. The arrow highlights the position of a centriole that has lost its connection to its nearby nucleus (and the nucleus is falling into the interior of the embryo). This centriole is constantly nucleating PCM, but cannot maintain a proper connection to the PCM as it rockets around within the embryo. Note how all of the centrioles move around erratically within their PCM and they often become partially or completely detached from their nuclei. Images were collected every 3 seconds and are displayed at 40 fps.

Video #2

The behaviour of centrioles, Aurora A and Grip75 in WT and *cnn* embryos. This video relates to Fig. S1. Centrioles are visualised with mRFP-Fzr (pseudo-coloured *green*), and the PCM is visualised with Aurora A-GFP, or Grip75-GFP (all pseudo-coloured *red*). Images were collected every 4 seconds and are displayed at 20 fps.

Video #3

The behaviour of centrioles and MTs in a *cnn* embryo. Images from this embryo are shown in Fig. 2 (A-B). Centrioles are visualised with mRFP-PACT (*red*), and MTs are visualised with GFP- α -Tubulin (*green*). Two centrioles are shown here, one of which starts out with no associated MTs. Both centrioles nucleate asters of MTs, but they cannot maintain their connection to the MTs, and they rocket about within the embryo. Images were collected every 3 seconds and are displayed at 20 fps.

Video #4

The behaviour of centrioles and MTs in a *cnn* embryo during mitosis. Images from this embryo are shown in Fig. 2 (A-D). Centrioles are visualised with mRFP-PACT

(red), and the MTs are visualised with GFP- α -Tubulin (green). This video shows an enlarged area of the same embryo shown in Video 3. The centrioles visualised with mRFP-PACT are very small, so most of the centrioles in this video are out of the plane of focus, although their position can be inferred from the position of the MT asters they nucleate. The centriole marked with the **large arrow** is closely associated with a spindle pole as the embryo proceeds through mitosis; this pole is therefore associated with astral MTs. The centriole marked with a **small arrow** is initially not in the focal plane of this video; it associates with a forming spindle pole but then migrates into the spindle and then back out to the spindle pole. The centriole marked with an **arrowhead** is out of the focal plane of this video; the astral MTs it organises are initially associated with the pole of a spindle but these then migrate off into the cytoplasm. Note how the spindle pole becomes anastral as the centriole moves away. Images were collected every 3 seconds and are displayed at 20 fps.

Video #5

Centriole behaviour in WT and *cnn* embryos prior to and after the injection of colchicine. This video relates to Fig. 2 (E-G). In all cases, centrioles are visualised with DSas-4-GFP. (Top left) This WT embryo is in late interphase and the centrioles have already migrated to opposite sides of the nuclei. Note how the centrioles exhibit very small stochastic movements, and they also gradually drift across the embryo cortex (from left to right). (Top right) This video shows the same WT embryo after a round of division, so there are twice as many nuclei and centrioles at the embryo cortex. Imaging was started approximately 30-60 seconds after colchicine had been injected into the embryo. Note how the centrioles continue to gradually drift across the embryo cortex. (Bottom left) This *cnn* embryo is in late interphase and many of the centrioles are no longer tightly associated with their nuclei. Note how many of the centrioles exhibit a rocketing behaviour, moving sharply in random directions. (Bottom right) This video shows the same *cnn* embryo, approximately 30-60 seconds after colchicine has been injected. Note how the centriole rocketing has largely ceased, and the centrioles now just gradually drift across the embryo cortex. Images were collected once every second and are displayed at 20 fps.

Video #6

The behaviour of centrioles and PCM in WT and *cnn* embryos after colchicine injection. Images from these embryos are shown in Fig. 3A. Centrioles are visualised

with mRFP-Fzr (pseudo-coloured *green*) and the PCM is visualised with GFP-D-TACC (pseudo-coloured *red*). Note how, in a WT embryo, the centrioles remain well centred within the PCM even after the MTs have been depolymerised. In a *cnn* embryo, most of the PCM (which was widely dispersed around the centrioles prior to the injection of colchicine — see Video 1) is now more tightly associated with the centrioles, although some aggregates remain dispersed in the cytoplasm. In contrast to the WT embryo, the centrioles are not positioned in the centre of the PCM but are located at the edge of the PCM. Images were collected every 3 seconds and are displayed at 20 fps.

Video #7

The behaviour of MTs in a WT 3rd instar larval neuroblast. This video relates to Fig. 5A. In this NB, both centrosomes are nucleating prominent astral arrays by the time of NEB. The spindle becomes positioned slightly asymmetrically in the cell during metaphase, and the cell ultimately divides asymmetrically. Note how the aster that remains in the large NB becomes greatly enlarged, while the aster in the smaller GMC rapidly disappears. Images are maximum intensity projections of Z-stacks that were acquired every 25 seconds and are displayed at 20 fps.

Video #8

The behaviour of MTs in a *cnn* mutant 3rd instar larval neuroblast. This video relates to Fig. 5B. In this NB, no astral arrays are visible at the time of NEB, and the spindle appears to assemble by the acentrosomal chromatin-mediated pathway. Note, however, that the cell still ultimately divides asymmetrically. Images are maximum intensity projections of Z-stacks that were acquired every 25 seconds and are displayed at 20 fps.

Video #9

The behaviour of MTs in a *cnn* mutant 3rd instar larval neuroblast. This video relates to Fig. 5C. This NB was already in mitosis at the start of the video. Although this spindle is relatively well organised, the cell ultimately divides symmetrically. Images are maximum intensity projections of Z-stacks that were acquired every 25 seconds and are displayed at 20 fps.

Video #10

The behaviour of MTs in a *cnm* mutant 3rd instar larval neuroblast. This video relates to Fig. 5D. This NB was already in mitosis at the start of the video. Note how the spindle pole on the left hand side of the cell has a prominent astral array that gets swept into the body of the spindle as mitosis proceeds. In addition, there is another, weaker, astral array that originates close to the right hand pole of the spindle, is swept away from the spindle, and then collapses into the bulk of the spindle. We presume that these astral arrays originate from centrioles that are rocketing within the cell. Images are maximum intensity projections of Z-stacks that were acquired every 25 seconds and are displayed at 20 fps.

Figure 1

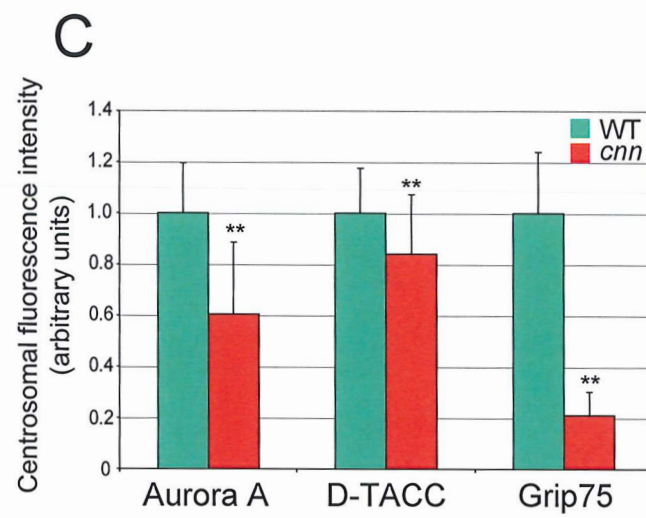
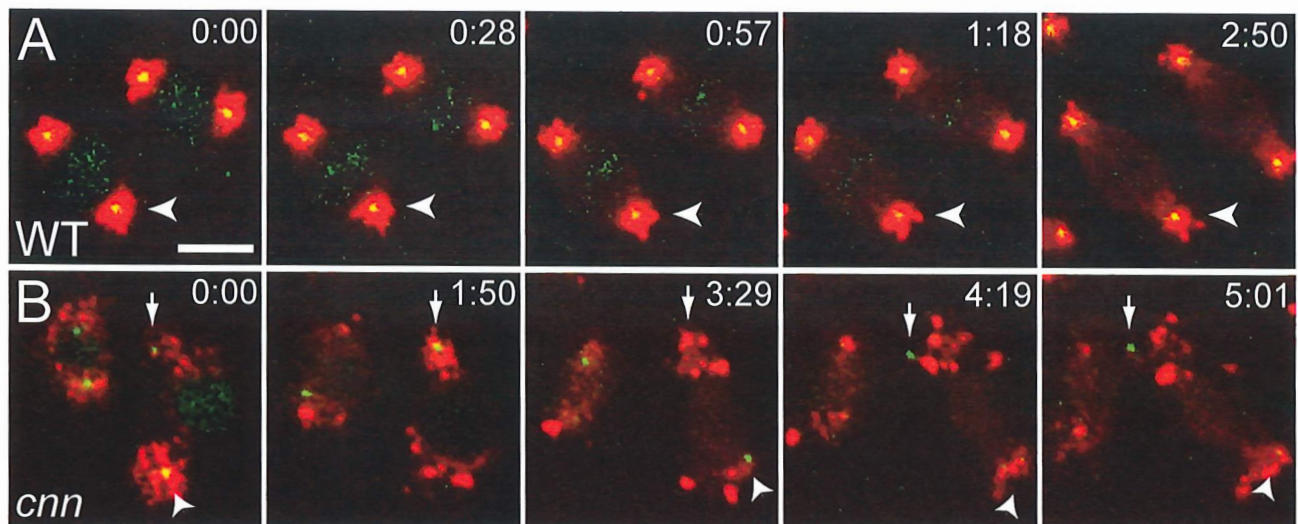


Figure 2

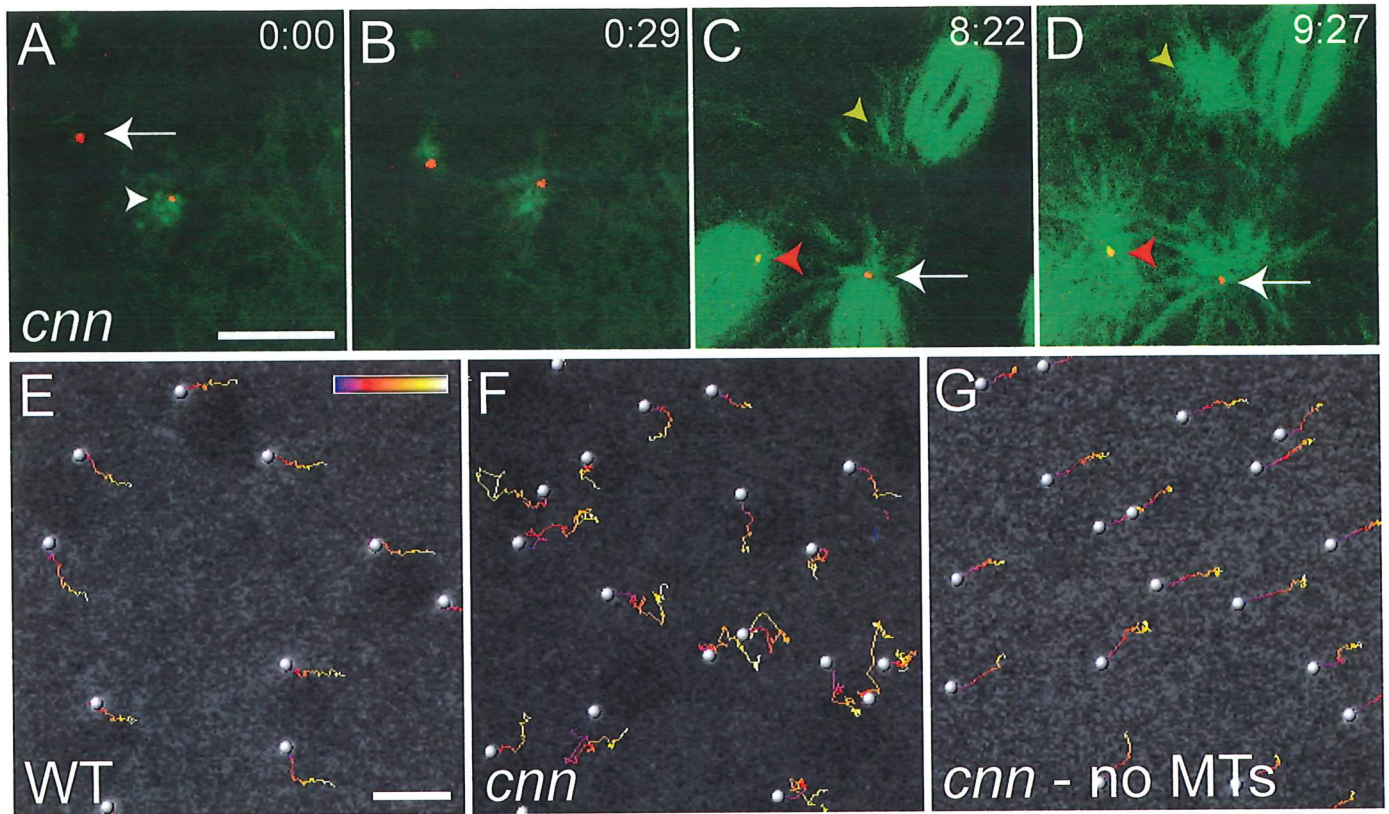


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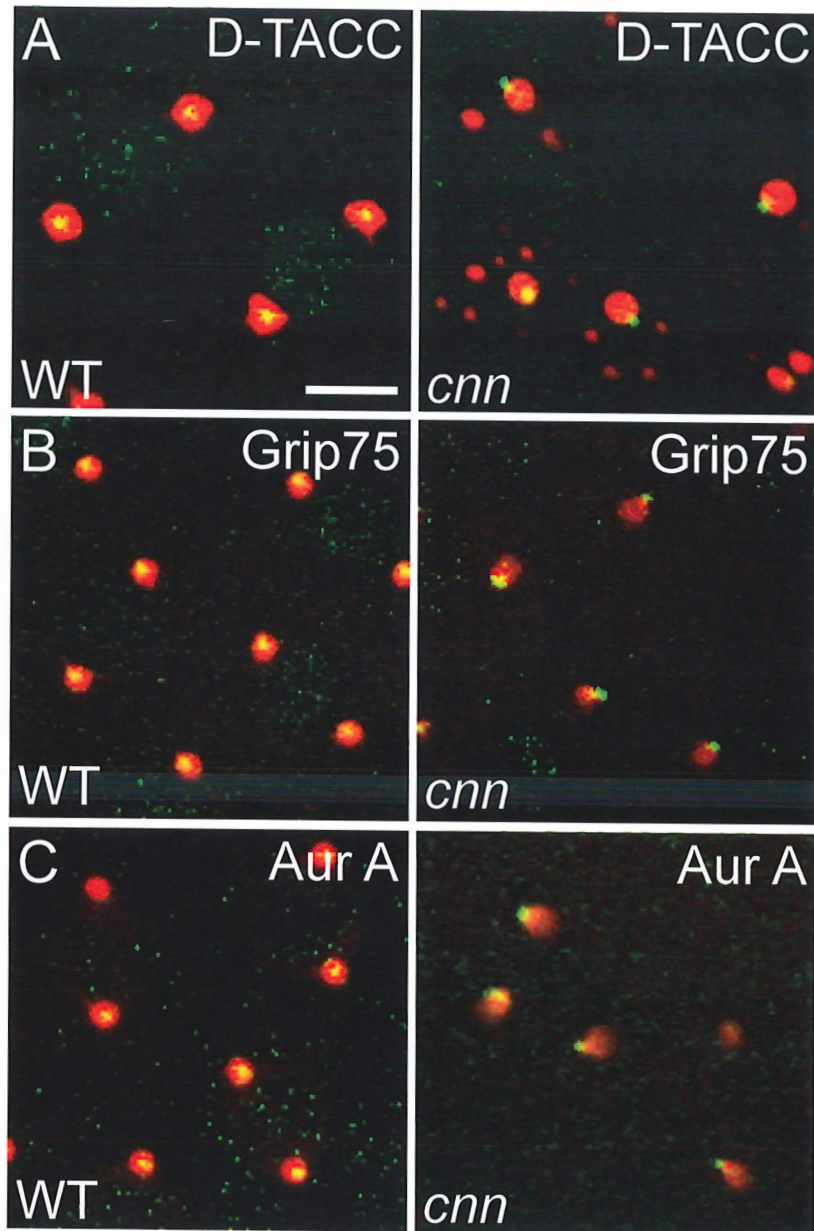


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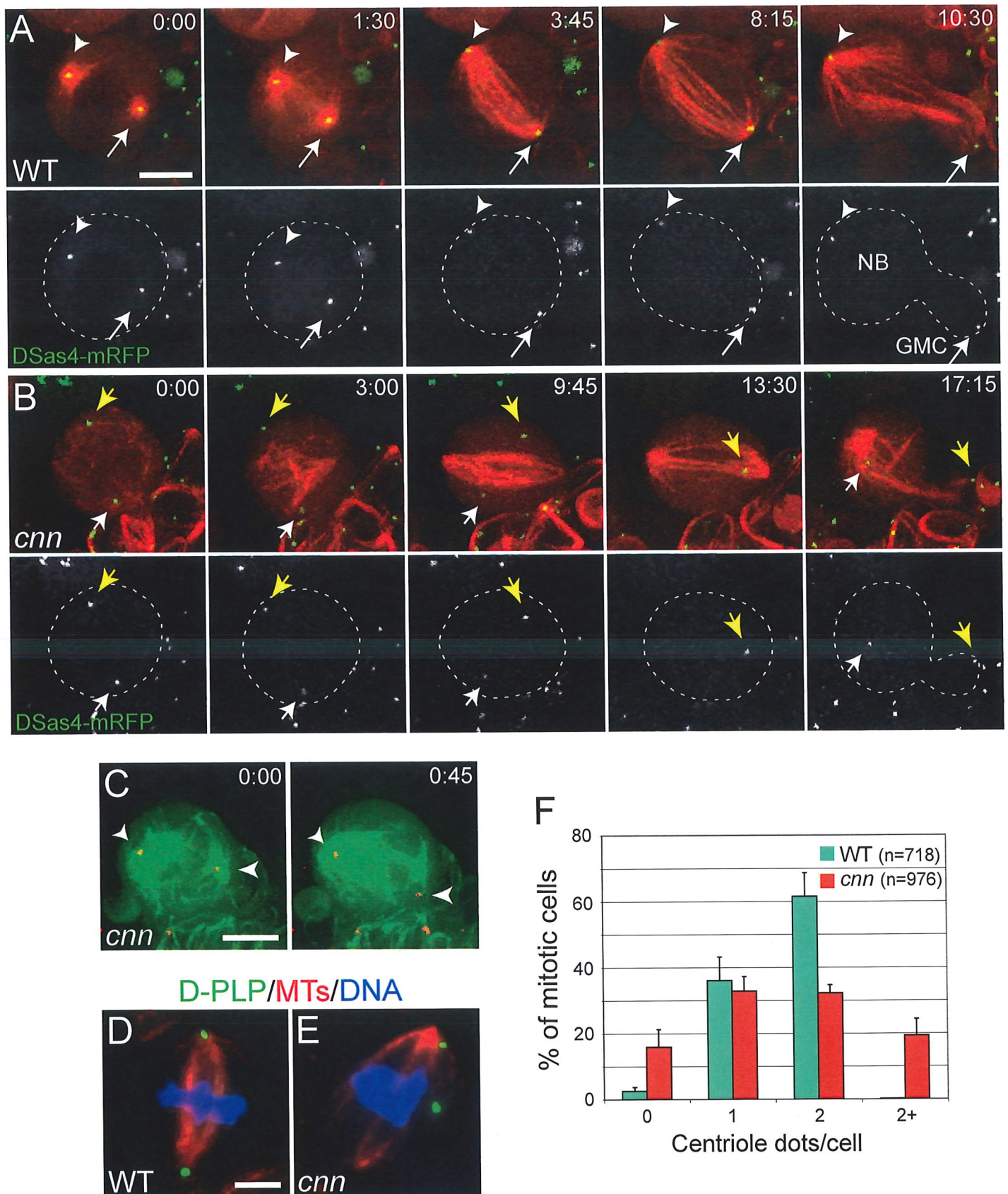
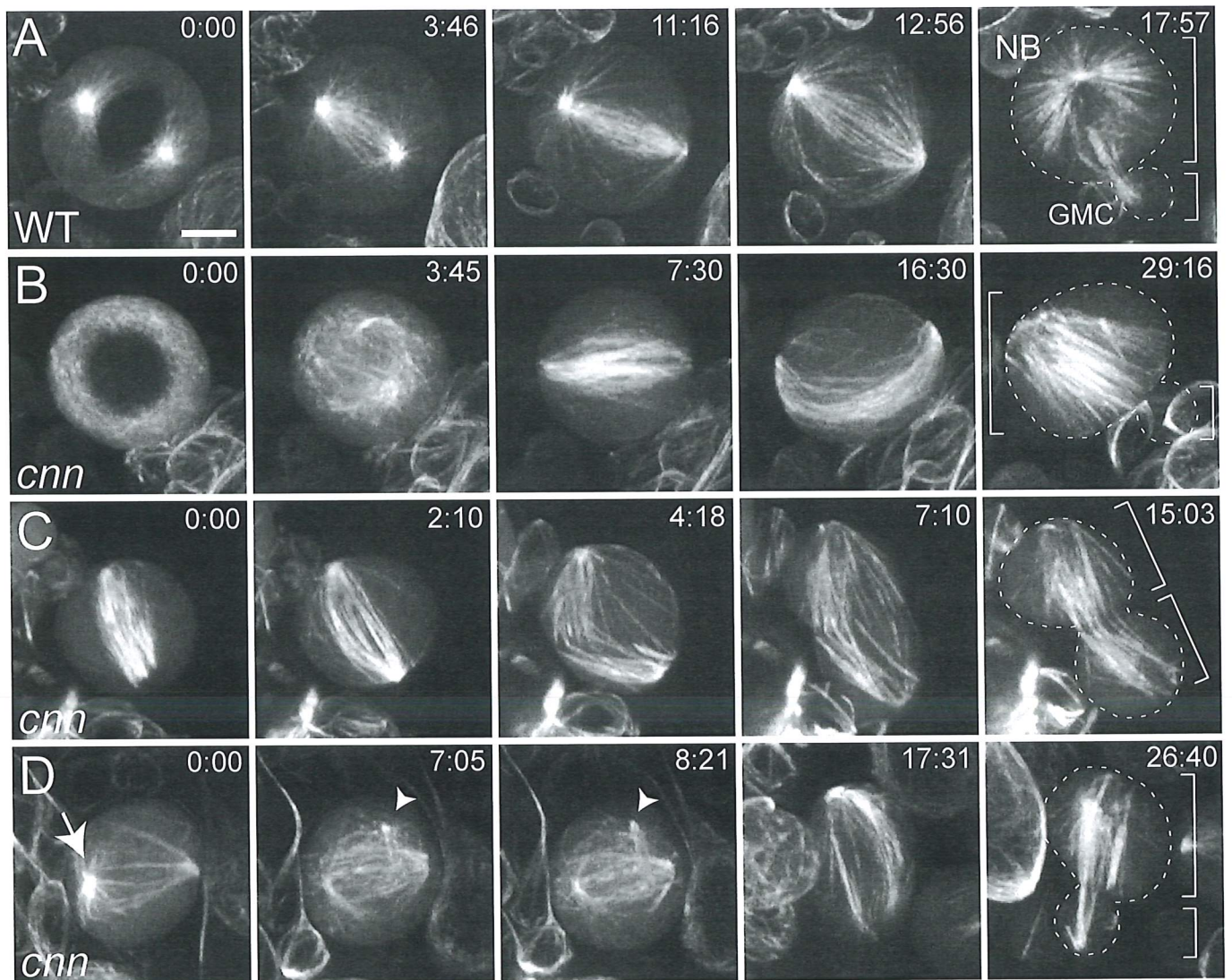
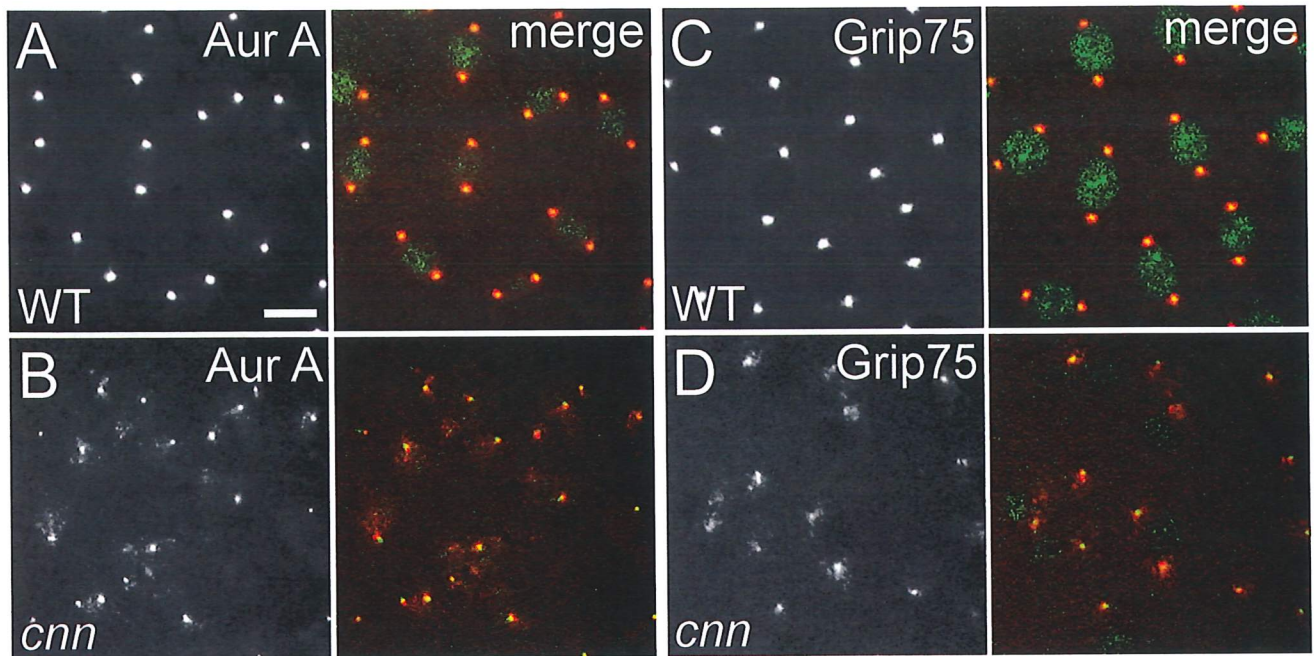


Figure 5



Supplementary Figure 1



Supplementary Figure 2

D-PLP/ γ -Tubulin/DNA

