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The role of centrosomes and astral microtubules during asymmetric division of *Drosophila* neuroblasts

Maria Grazia Giansanti, Maurizio Gatti and Silvia Bonaccorsi*

Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Genetica e Biologia Molecolare, Universita' di Roma 'La Sapienza', P.le Aldo Moro 5, 00185 Rome, Italy
*Author for correspondence (e-mail: bonaccorsi@axcasp.caspur.it)

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SUMMARY

Drosophila **neuroblasts are stem cells that divide asymmetrically to produce another large neuroblast and a smaller ganglion mother cell (GMC). During neuroblast division, several cell fate determinants, such as Miranda, Prospero and Numb, are preferentially segregated into the GMC, ensuring its correct developmental fate. The accurate segregation of these determinants relies on proper orientation of the mitotic spindle within the dividing neuroblast, and on the correct positioning of the cleavage plane. In this study we have analyzed the role of centrosomes and astral microtubules in neuroblast spindle orientation and cytokinesis. We examined neuroblast division in *asterless* (*asl*) mutants, which, although devoid of functional centrosomes and astral microtubules, form well-focused anastral spindles that undergo anaphase and telophase. We show that *asl* neuroblasts assemble a normal cytokinetic ring around the central spindle midzone and undergo unequal cytokinesis. Thus, astral microtubules are not required for either signaling or positioning cytokinesis in *Drosophila* neuroblasts. Our results indicate that the cleavage plane is dictated by the positioning of the central spindle midzone within the cell, and suggest a model on how the central spindle attains an asymmetric position during neuroblast mitosis. We have also analyzed the localization of Miranda during mitotic division of *asl* neuroblasts. This protein accumulates in morphologically regular cortical crescents but these crescents are mislocalized with respect to the spindle orientation. This suggests that astral microtubules mediate proper spindle rotation during neuroblast division.**

Key words: *Drosophila*, Neuroblasts, Unequal cytokinesis, Miranda, Astral microtubules

INTRODUCTION

Asymmetric cell division is one of the main developmental mechanisms that generate cell diversity in multicellular organisms. Generation of daughter cells that adopt different fates is usually achieved through unequal partition of cell fate determinants, and is often accompanied by unequal cytokinesis (reviewed by Horwitz and Herszkowitz, 1992; Strome, 1993; Knoblich, 1997; Jan and Jan, 1998; Lu et al., 1998a). *Drosophila* neuroblasts (NBs) are one of the best characterized examples of asymmetrically dividing cells (Knoblich, 1997; Jan and Jan, 1998). During *Drosophila* embryogenesis NBs delaminate basally from the neuroectodermal epithelium. They then divide in a stem cell-like fashion along the apical basal axis, producing another large NB and a smaller ganglion mother cell (GMC). The newly generated NB remains adjacent to the neuroectoderm and retains the potential to divide, while the GMC divides only once into a pair of equally sized cells that eventually differentiate into neurons or glia (Goodman and Doe, 1993). During NB division, the spindle is initially oriented parallel to the surface of the embryo and to the spindle axis of the neuroectodermal epidermoblasts. It then rotates by 90°, so that at metaphase its axis is perpendicular to the epidermoblast spindle axis (Kaltshmidt et al., 2000).

Most embryonic NBs divide from stage nine to 14 of embryogenesis, undergoing five to nine mitotic divisions. They then become dormant for the rest of embryogenesis but are reactivated in first instar larvae (Truman et al., 1993). The brain NBs actively divide throughout larval life (Truman and Bate, 1988). Some of their earlier divisions are symmetric and are thought to produce two daughter NBs (Hofbauer and Campos-Ortega, 1990). However, their later divisions are asymmetric and generate a larger NB and a smaller GMC (Hofbauer and Campos-Ortega, 1990). In third instar larval brains, NB divisions do not display an obvious apical-basal orientation with respect to the surface of the brain, and exhibit differently oriented spindle axes (Parmentier et al., 2000; M. G. G., M. G. and S. B., unpublished observations). However, the GMC (basal) side of the dividing brain NBs is often clearly identified as it faces a discrete cluster of daughter GMCs and neurons (Truman and Bate, 1988; see Fig. 4A and below).

During embryonic NB division, several proteins are unequally partitioned between the two daughter cells. The proteins Miranda (Mira), Numb, Partner of Numb (Pon), Prospero (Pros) and Staufen (Stau) accumulate in cortical

crescents at the basal NB side, and are thus preferentially segregated into the GMC (reviewed by Knoblich, 1997; Jan and Jan, 1998; see also Lu et al., 1998b). Mira plays key role in the asymmetric location of Pon, Pros and Stau: it binds each of these proteins and is thought to be a multidomain adapter that brings Pon, Pros and Stau to the cell cortex to form crescents (Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Shen et al., 1998; Lu et al., 1998a; Lu et al., 1998b; Schuldt et al., 1998). The proper localization of Mira, Numb, Pon, Pros and Stau is controlled by a multi-protein complex that forms a crescent at the apical side of the NB. This complex includes Bazooka (Baz), Inscuteable (Insc) and Partner of Inscuteable (Pins; Raps – FlyBase). Together, these proteins play at least two functions: they cooperate to ensure the cortical localization of Mira, Numb, Pon, Pros and Stau, and mediate proper spindle rotation within the dividing NB (Kraut et al., 1996; Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999; Kaltshmidt et al., 2000; Schaefer et al., 2000; Yu et al., 2000).

Although the hierarchical relationships between the various proteins showing asymmetrical distribution during NB division are well understood, much less is known about the mechanisms underlying their cortical accumulation. It has been shown that microfilament inhibitors delocalize Mira, Numb, Pros, Stau and Insc, suggesting that proper asymmetric accumulation of these proteins requires the integrity of the actin-based cortical cytoskeleton (Kraut et al., 1996; Broadus and Doe, 1997; Knoblich et al., 1997; Shen et al., 1998). However, disruption of microtubules by colchicine treatment has no effect on cortical accumulation of Numb, Pros, Stau and Insc, indicating that this process occurs independently of microtubules (Knoblich et al., 1995; Kraut et al., 1996; Broadus and Doe, 1997). However, microtubules, in addition to spindle formation, are likely to be involved in other aspects of asymmetric NB division. For example, microtubules must be involved in the phenomenon of spindle rotation that occurs during embryonic NB divisions (Kaltshmidt et al., 2000). In addition, either the astral or the central spindle microtubules are likely to play an important role in determination of the cleavage plane during NB unequal cytokinesis (Strome, 1993; Gatti et al., 2000).

In this study, we have addressed the role of astral microtubules in NB spindle rotation and unequal cytokinesis. We have analyzed these processes in larval NBs of *asterless (asl)* mutants. We have previously shown that *asl* NBs, despite the absence of functional centrosomes and astral microtubules, assemble anastral spindles and undergo anaphase and telophase (Bonaccorsi et al., 2000). We show that *asl* NBs normally accumulate Mira in cortical crescents. These crescents, however, are often misplaced with respect to the spindle, suggesting that astral microtubules mediate spindle rotation. In addition, we show that *asl* NBs undergo unequal cytokinesis as their wild-type counterparts. Our results indicate that the positioning of the cleavage plane solely depends on the location of the central spindle within the dividing NB. We show that aster-independent interactions between the central spindle and the nascent GMC nucleus, and between this nucleus and the polar cortex, shift the central spindle towards the GMC pole, causing unequal cytokinesis.

MATERIALS AND METHODS

Drosophila stocks

Larval brain preparations were made using the wild-type Oregon-R stock, and the asl^2/asl^2 and asl^2/asl^3 mutants. The asl^2 and asl^3 lethal mutations were maintained over the *TM6C* balancer that carries the body shape marker *Tubby*. asl^2/asl^2 homozygous larvae and asl^2/asl^3 larvae obtained from $asl^2/TM6C \times asl^3/TM6C$ crosses, were identified on the basis of their non-*Tubby* phenotype.

Orcein-stained chromosome preparations

Mutant and control brains were dissected, fixed and squashed in aceto-orcein, according to our previously described procedures (Gatti and Goldberg, 1991). The mitotic index was estimated by determining the average number of mitotic figures per optic field in aceto-orcein squashes of non-colchicine-treated, non-hypotonically swollen brains dissected from late third instar larvae (Gatti and Baker, 1989). Mitotic figures were scored every fourth field in each brain examined. The optic field chosen for this analysis is the circular area defined by a phase-contrast Neofluar 100× Zeiss objective, using 10× oculars and the Optovar set at 1.25.

Immunostaining of larval brains

To obtain cytological preparations for immunofluorescence, brains from late third instar larvae were dissected and fixed as described previously (Bonaccorsi et al., 2000). Immunostaining with anti-tubulin, anti-centrosomin and anti-anillin antibodies, and Hoechst 33258 staining were performed as described for larval testes (Bonaccorsi et al., 1998). Myosin II was detected by overnight incubation at 4°C with a rabbit antibody obtained by C. Field (Harvard University), diluted 1:250 in PBS. Staining for Miranda was carried out as previously described (Shen et al., 1997). Multiple stainings were performed in the following orders: centrosomin, tubulin, Hoechst 33258; anillin or Myosin II, tubulin, Hoechst 33258; Miranda, tubulin, Hoechst 33258.

Immunostained preparations were examined with an Axioplan Zeiss microscope equipped with a cooled charge-coupled device (CCD, Photometrics) as described (Bonaccorsi et al., 1998; Bonaccorsi et al., 2000; Giansanti et al., 1999). Gray-scale digital images were collected separately using the IP Lab Spectrum software, converted into Photoshop 3.0 format (Adobe) and merged in pseudocolors.

RESULTS

Mitotic parameters and chromosome segregation in *asl* larval brains

We have previously shown that NBs and GMCs of *asl* larval brains assemble bipolar spindles that undergo anaphase and telophase (Bonaccorsi et al., 2000). To determine whether these spindles can mediate proper progression through the mitotic process and ensure the fidelity of chromosome segregation, we examined aceto-orcein squashes of asl^2/asl^2 and asl^2/asl^3 larval brains. asl^2 and asl^3 are both lethal mutations; asl^2/asl^2 and asl^2/asl^3 individuals die at the larval/pupal boundary, while asl^3/asl^3 individuals have an earlier lethal phase (Bonaccorsi et al., 1998).

We initially examined brain squashes in aceto-orcein without pretreatment with colchicine and hypotonic solution. These preparations allow observation of all phases of mitosis and permit evaluation of the mitotic index (the fraction of cells undergoing mitosis; Gatti and Baker, 1989; Gatti and Goldberg, 1991). However, the analysis of orcein-stained

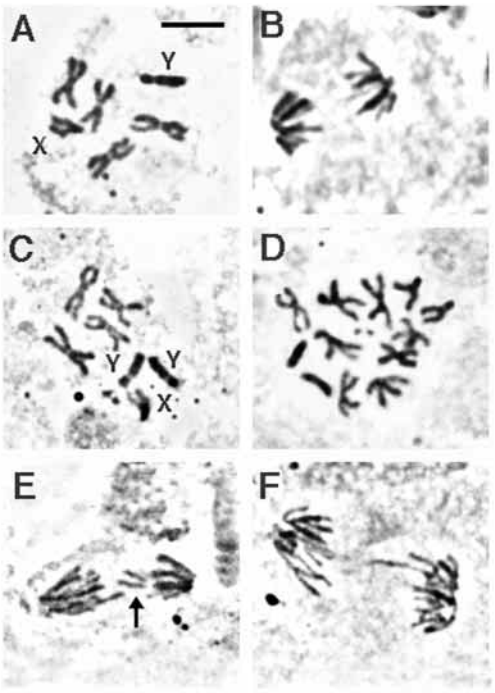

Fig. 1. Abnormal mitotic figures observed in *asl* mutant brains. (A,B) Wild-type controls (Oregon R); (C-F) *asl*. Anaphase figures are from brains immediately squashed in aceto-orcein after dissection. Metaphases are from brains treated with colchicine and hypotonic solution before squashing. (A) Normal male metaphase; (B) normal male anaphase; (C) hyperploid male metaphase with two Y chromosomes; (D) tetraploid male metaphase; (E) anaphase showing a lagging chromosome (arrow); (F) tetraploid anaphase. Scale bar: 5 μm.

chromosomes does not allow distinction between NBs and GMCs. As shown in Fig. 1 (and see Table 2) the mitotic indexes and the frequencies of metaphase and anaphase figures observed in asl^2/asl^2 and asl^2/asl^3 brains are comparable with those seen in Oregon-R control cells. This indicates that *asl* NBs and GMCs, despite the absence of functional centrosomes and astral microtubules, proceed through mitosis at the same pace as wild-type cells. However, an examination of Table 1 reveals that in *asl* mutants the fidelity of chromosome segregation is slightly compromised: 4-5% of *asl* anaphases exhibit lagging chromosomes and a small fraction of *asl* metaphases appears to be polyploid (Fig. 1).

In brain squashes without hypotonic pretreatment, chromosome morphology is rather poor and chromosomes are often overlapped, preventing reliable identification of aneuploid metaphases. Thus, to better evaluate the frequency of mitotic errors in *asl* mutant cells, we made aceto-orcein squashes of hypotonically swollen brains that had been treated for 1 hour with colchicine. In the analysis of these preparations we only scored hyperploid and polyploid metaphases (Fig. 1C,D); hypoploid metaphases were not recorded as they can result from squashing artifacts (Gatti and Goldberg, 1991). As shown in Table 2, *asl* brains exhibit 5-6% hyperploid cells and 2.6-2.8% tetraploid metaphases. Because GMCs divide only once, giving rise to daughter cells that differentiate into neurons, these abnormal metaphases must have been originated by events occurred during NB mitosis. We thus conclude that in *asl* mutants about 5% of the NBs suffer of errors in chromosome segregation and 3% of the NBs of complete failures in chromosome segregation.

Cytokinesis in *asl* mutants

Polyploid cells are an expected outcome of failures in NB cytokinesis, and are observed in frequencies ranging from 18 to 89% in mutants defective in cytokinesis, such as *twinstar*,

Table 1. Mitotic parameters in *asl* brains*

Genotype	Number of mitotic figures	Number of metaphases‡		Number of anaphases			% Polyploid	% Anaphases	Mitotic index§
		Diploid	Polyploid	Diploid	Polyploid	Irregular¶			
asl2/asl2	634	491	7	125	4	7	1.7	21.4	0.96
asl2/asl3	746	587	8	140	6	5	1.9	20.2	1.06
Oregon R	502	399	0	103	0	0	0	20.5	0.99

*Brains were fixed immediately after dissection without colchicine and hypotonic pretreatments.
‡This class also includes late prophases and prometaphases.
§The mitotic index is the average number of mitotic figures per optic field (see Materials and Methods).
¶All irregular anaphases display lagging chromosomes.

Table 2. Frequency of hyperploid and polyploid metaphases in *asl* brains treated with colchicine and hypotonic solution

Genotype	Number of metaphases	Types of metaphases*					Hyperploid (%)	Tetraploid (%)
		2n	2n+1	2n+2	2n+3‡	Tetraploid		
asl2/asl2	755	697	23	9	6	20	5.0	2.6
asl2/asl3	1498	1363	69	8	16	42	6.2	2.8
Oregon R	447	444	3	0	0	0	0.7	0

*In counting the number of chromosomes per cell, the fourth chromosomes were not taken into account.
‡This class also includes the 2n+4 cells.

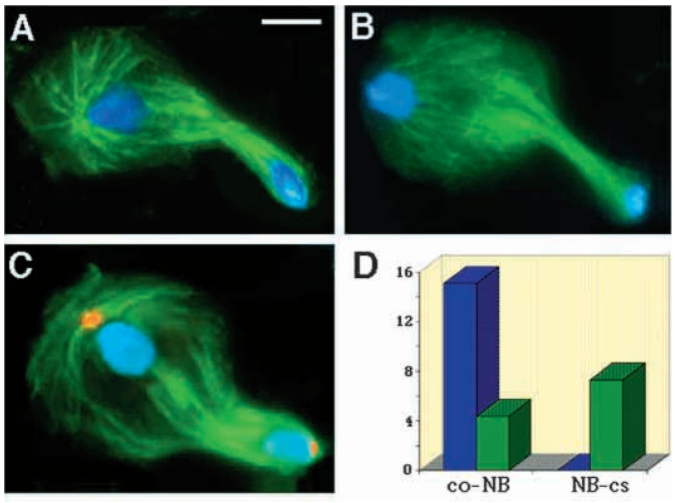

Fig. 2. Central spindle positioning in wild-type and *asl* NB telophases. (A,C) Wild type; (B) *asl*. (A,B) Stained for tubulin (green) and DNA (by Hoechst 33258, blue); (C) stained for tubulin, DNA and centrosomin (red). Note that in the wild-type telophases (A,C) the NB nucleus (at the left of each panel) lies close to the central spindle (the prominent bundle of microtubules between the two daughter nuclei). In contrast, in the *asl* telophase (B), this nucleus is disconnected from the central spindle and is located near the polar cortex. (D) Relative distances between the cortex and the NB nucleus (co-NB) and between the NB nucleus and the central spindle (NB-cs) in wild-type and *asl* telophases. These distances have been determined by measuring the co-NB and NB-cs distances in each telophase, and dividing these values by the pole-to-pole length of the cell. Blue bars: wild-type telophases (n=44); green bars: *asl* telophases (n=45). Scale bar: 5 μm.

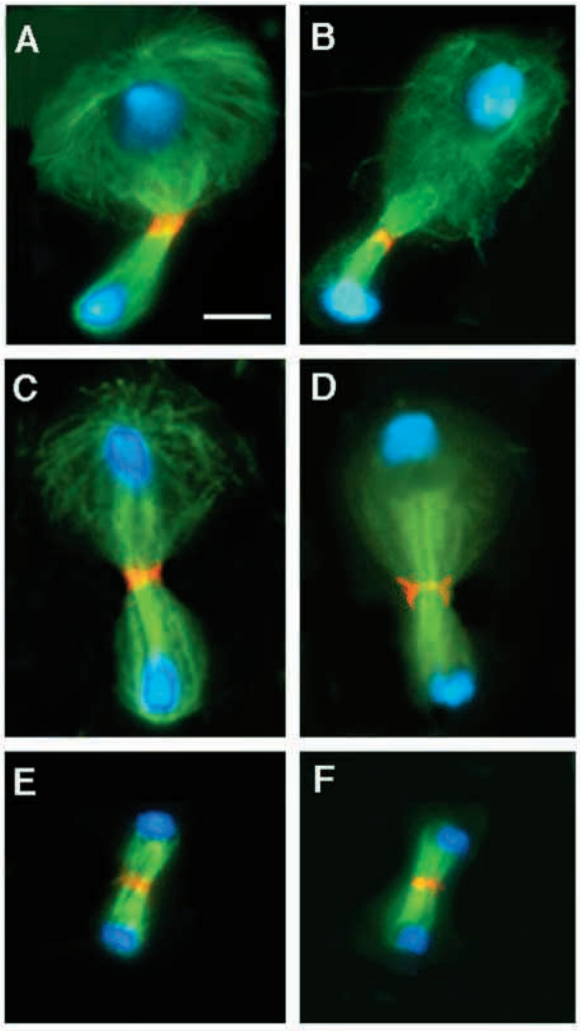

Fig. 3. Cytokinesis in wild type (Oregon-R) and *asl* mutants. (A,C) Wild-type NB telophases; (B,D) *asl* NB telophases; (E) a wild-type GMC telophase; (F) an *asl* GMC telophase. Cells were stained for tubulin (green), DNA (by Hoechst 33258, blue) and either anillin (A,B,E,F; orange) or myosin II (C,D; orange). (A-D) NB nucleus is at the top. The microtubules surrounding the NB nucleus of the *asl* telophase of B are not astral microtubules, as they are randomly oriented and do not emanate from a single, discrete microtubule organizing center. Note that *asl* mutations do not disrupt the accumulation of components of the cytokinetic apparatus to the midzone of the central spindle. Scale bar: 5 μm.

diaphanous and *l(3)7m62* (Gatti and Baker, 1989; Castrillon and Wasserman, 1994; Gunsalus et al., 1995). Thus, the finding that *asl* mutant brains display only 2-3% polyploid cells indicates that most *asl* NBs, despite the absence of asters, successfully complete cytokinesis.

Wild-type NBs undergo unequal cytokinesis, giving rise to a large NB and a small GMC. To determine whether the asters play a role in NB unequal cleavage, we compared mitotic divisions of wild-type and *asl* NBs. We made fixed preparations of Oregon-R, asl^2/asl^2 and asl^2/asl^3 larval brains and stained them with Hoechst 33258 and anti-tubulin antibodies for simultaneous visualization of chromatin and microtubules. Third instar larval brains from asl^2/asl^2 and asl^2/asl^3 animals exhibit comparable cytological phenotypes, so we will not distinguish subsequently between these two genotypes.

As previously described, NB prophases of wild-type larval brains exhibit two prominent asters nucleated by centrosomes of similar sizes. Later in mitosis, the centrosome associated with the nascent GMC nucleus becomes smaller than the one associated with the nascent NB nucleus at the opposite cell side. Concomitantly, the microtubules emanating from the aster at the GMC side shorten dramatically, while those nucleated by the centrosome at the NB side elongate slightly (Bonaccorsi et al., 2000; Kaltshmidt et al., 2000). These differences between the two asters are particularly evident during late anaphase and telophase (Figs 2A,C, 3A,C). In addition, the two ana-telophase nuclei differ for the degree of compaction, with the GMC nucleus being more condensed than the other (Figs 2A,C, 3A,C). During ana-telophase NBs develop a prominent central spindle that becomes pinched in the middle at the site of cleavage furrow (Figs 2A,C, 3A,C).

asl mutant larval NBs lack functional centrosomes and are completely devoid of astral microtubules. Nevertheless, they develop well focused anastral spindles that undergo anaphase and telophase (Bonaccorsi et al., 2000), and they form central spindles indistinguishable from their wild-type counterparts (Figs 2B, 3B,D). Moreover, both in wild-type and in *asl* NB telophases, the central spindle is tightly associated with the nascent GMC nucleus, which is in turn closely apposed to the polar cortex (Figs 2A-C, 3A-D). However, wild-type and *asl*

Table 3. Localization of Miranda in *asl2/asl2* larval neuroblasts

	Metaphases		Anaphases		Telophases	
Genotype	Number scored	% Irregular*	Number scored	% Irregular*	Number scored	% Irregular*
asl2/asl2	103	52	37	46	108	11
Oregon R	30	0	15	0	35	0

*In all the irregular mitotic figures Miranda is accumulated in a cortical crescent which, however, is incorrectly localized with respect to the spindle axis.

telophases differ in the positioning of the NB nucleus with respect to both the central spindle and the polar cortex. In wild type, the NB nucleus lies very close to the central spindle but is separated from the polar cortex by a large astral array of microtubules (Figs 2A,C,D, 3A,C). The situation is reversed in *asl* telophases: the NB nucleus is usually disconnected from the central spindle and located much closer to the polar cortex than its wild type counterpart (Figs 2B,D, 3B,D; see Fig. 5D).

Our findings on the dynamic transformations of the mitotic spindle, and more specifically on central spindle positioning in ana-telophases of wild-type larval NBs, are in full agreement with those of Kaltshmidt et al. on embryonic NBs (Kaltshmidt et al., 2000). By examining GFP-labeled spindles of embryonic NBs, they observed that the central spindle is asymmetrically placed with respect to the centrosomes. They reported that the distance between the NB centrosome and the middle of the central spindle is 30% longer than that between the GMC centrosome and the middle of the central spindle. We performed the same measurements on 38 fixed wild-type larval ana-telophases immunostained for both tubulin and centrosomin (Bonaccorsi et al., 2000; see also Fig. 2C), and found that the NB half spindle is indeed about 30% longer than the GMC half spindle (Fig. 2D). However, because the NB nucleus is larger than the GMC nucleus, the distances between the middle of the central spindle and the inner edges of either the NB or the GMC nucleus are identical. Thus, while the wild-type central spindle is asymmetrically positioned between the centrosomes, it is symmetrically positioned between the two daughter nuclei.

To determine precisely the site of the cleavage furrow, we immunostained larval brain preparations of both wild type and *asl* mutants with either anti-myosin II or anti-anillin antibodies. Myosin II is a well-known component of the acto-myosin contractile ring that mediates cytokinesis in animal cells (Goldberg et al., 1998; Field et al., 1999). Anillin is a 190 kDa actin-binding protein that concentrates in the cleavage furrow of a variety of *Drosophila* cells (Field and Alberts, 1995; Giansanti et al., 1999), where it is thought to anchor the contractile ring to the equatorial plasma membrane (Giansanti et al., 1999). In wild-type NB ana-telophases, both myosin II and anillin concentrate in circumferential bands around the central spindle midzone (Fig. 3A,C). In *asl* NBs, despite the absence of asters, both proteins exhibit the same patterns of accumulation seen in wild type, forming morphologically normal cytokinetic structures across the middle of central spindle (we observed normal cytokinesis rings in over 100 *asl* NB telophases; Fig. 3B,D). Thus, in both wild-type and *asl* NBs, the cytokinetic apparatus is displaced towards the GMC distal cortex. This would result in the formation of two unequally sized daughter cells upon execution of cytokinesis.

The importance of the central spindle in organizing the cleavage furrow is underlined by the examination of subsequent symmetrical divisions of the GMCs. GMC ana-telophases of both wild type and *asl* mutants always exhibit myosin II and anillin accumulations in the middle of the central spindle (Fig. 3E,F). Because in both types of cells the central spindle is symmetrically positioned between the cell poles, cytokinesis would result into two equally sized products.

Taken together, our results show that, despite the absence of asters, most, if not all, *asl* NBs assemble a normal contractile apparatus and undergo unequal cleavage. This indicates that asters are not required for either the assembly or the localization of the cytokinetic ring during wild-type NB mitosis.

Localization of Miranda in dividing *asl* NBs

To assess the role of astral microtubules in the distribution of cell fate determinants, we compared the localization of Mira in wild-type and *asl* larval NBs. We focused on Mira because this protein directs proper localization of other cell fate determinants such as Pros, Numb and Pon (see Introduction), and because the anti-Mira antibody results in a very clear immunostaining signal that can be seen in all dividing larval NBs.

Studies on embryonic NBs have shown that Mira accumulates at the apical side of the NB during late interphase. Then, at prophase, Mira moves and concentrates at the basal NB side, where it remains throughout cell division (Shen et al., 1998; Schuldt et al., 1998). In wild-type larval brains, we were not able to see any clear cortical concentration of Mira in any interphase cell. However, in all NB prophases, prometaphases and metaphases, Mira forms a crescent at one of the spindle poles (Fig. 4; Table 3). We were able to identify this pole as the GMC (basal) side of the dividing NB because, in many cases, the Mira crescent faces a cluster of daughter GMCs (Fig. 4A; Truman and Bate, 1988). In late anaphases and telophases of wild-type NBs, Mira is always concentrated at the GMC pole. This pole can be unambiguously identified because it contains an aster smaller than that located at the opposite cell side (Fig. 4C; Bonaccorsi et al., 2000; Kaltshmidt et al., 2000).

We also failed to observe Mira cortical signals in interphase cells of *asl* mutant brains. Mira accumulates in cortical crescents only in dividing *asl* NBs. These crescents are comparable with their wild-type counterparts, but are often incorrectly localized with respect to the spindle (Fig. 5; Table 3). In 52% of *asl* NB metaphases the Mira signal does not overlay one of the spindle poles but is localized laterally (Fig. 5B; Table 3). This abnormal localization is also seen in 46% of *asl* NB anaphases (Fig. 5C; Table 3). However, as *asl* NBs proceed through mitosis, Mira progressively attains a normal localization (Fig. 5D), so that only 11% of the *asl* telophases exhibit mislocalization of Mira.

Together, these data indicate that asters play an important role in determining the correct localization of the Mira crescent during the early stages of mitosis. However, Mira localization

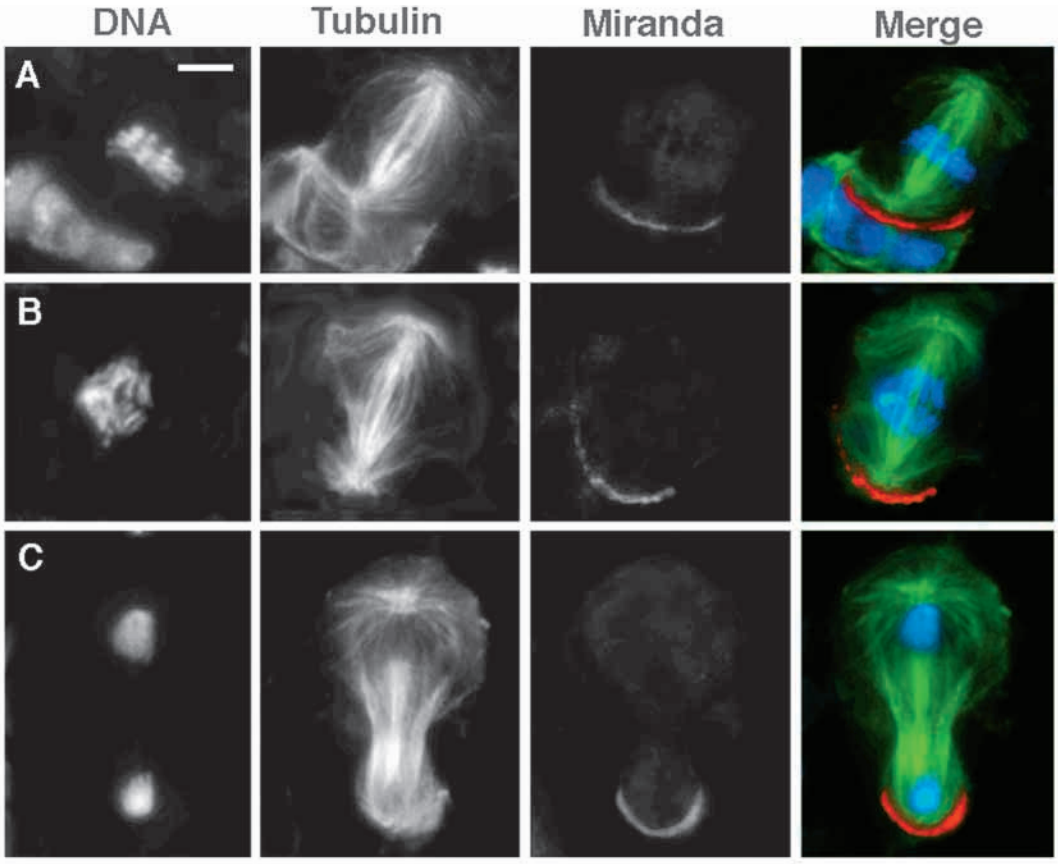

Fig. 4. Localization of Mira during mitotic division of wild-type NBs. Cells were stained for DNA (blue), tubulin (green) and Mira (red). (A) Metaphase; (B) early anaphase; (C) telophase. Note that Mira forms a cortical crescent at one of the cell poles. (A) This crescent faces a cluster of daughter GMCs, suggesting its localization at the cell pole that buds off GMCs. (C) The Mira crescent overlays the GMC pole which is unambiguously identified for its smaller aster. Scale bar: 5 μm.

at telophase, and thus its segregation into the GMC, appears to be, at least in part, independent of asters.

DISCUSSION

The role of asters in the fidelity of chromosome segregation during NB mitosis

Our results show that anastral spindles of *asl* NBs can mediate chromosome segregation as their wild-type centrosome-containing counterparts. The fact that *asl* brain cells display a mitotic index comparable with control cells indicates that in the absence of centrosomes and astral microtubules, there is no increase in the duration of mitosis. Moreover, the finding that *asl* and wild-type cells exhibit comparable frequencies of the various types of mitotic figures further indicates that *asl* anastral spindles proceed through mitosis at the same pace as centrosome-containing spindles. Thus, NB spindle assembly and dynamics appear to be substantially similar when microtubules are nucleated around the chromatin, as occurs in *asl* mutants (Bonaccorsi et al., 2000), and when microtubules are nucleated by the centrosomes.

However, *asl* mutant brains exhibit low frequencies of hyperploid and tetraploid metaphases, suggesting that *asl* slightly affects the fidelity of mitotic chromosome segregation. Most likely, the hyperploid metaphases are the consequence of errors in chromosome segregation that occurred during the anaphase of the previous cell cycle. This is suggested by the observation that about 5% of *asl* anaphases exhibit lagging chromosomes (Fig. 1E). However, the mechanisms underlying the delayed poleward migration of these chromosomes are unclear. One possibility is that the kinetochore of these lagging chromosomes is simultaneously attached to microtubules derived from the opposing spindle poles. This event, that has been previously described in vertebrate cells (Khodjakov et al., 1997), could be a consequence of the mode of spindle formation in *asl* mutants. The massive microtubule nucleation that occurs near the chromosomes of these mutants might favor the attachment of a single kinetochore to microtubules emanating from opposite poles. This would prevent the anaphase movement of the bipolarly attached chromosome, leading to its random inclusion in one of the two daughter cells upon execution of cytokinesis.

Also unclear is the origin of the few tetraploid cells observed in *asl* mutant brains. Given that a normal contractile apparatus is found in all *asl* NB telophases (see Results), we suspect that the tetraploid cells observed in *asl* mutants are not the consequence of failures in cytokinesis. We believe that these cells derive from cells that failed to assemble a functional mitotic spindle and reverted to interphase to give rise to a tetraploid nucleus.

The role of asters in NB cytokinesis

Our results provide insight into an important unanswered question about cell cleavage in animal cells: are the signals that stimulate cytokinesis provided by the asters or instead by the central spindle (reviewed in Goldberg et al., 1998; Field et al., 1999; Gatti et al., 2000)? We have already shown that the meiotic cells of *asl* males, which are completely devoid of asters, assemble a functional contractile apparatus around the

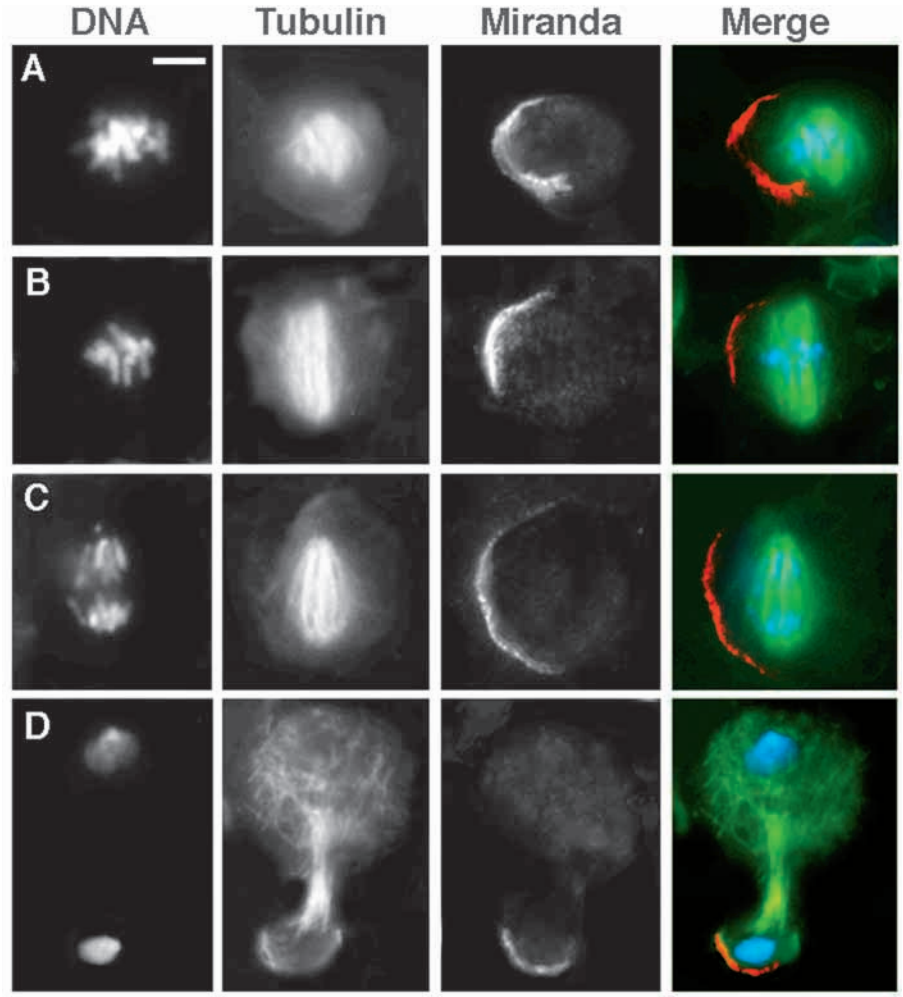

Fig. 5. Localization of Mira during mitotic division of *asl* NBs. Cells were stained for DNA (blue), tubulin (green) and Mira (red). (A) Prometaphase; (B) metaphase; (C) anaphase; (D) telophase. Note that Mira accumulates in cortical crescents that are comparable with those seen in wild-type NBs (see Fig. 4). In the cells shown in A-C, these crescents do not overlay one of the spindle poles as occurs in wild type but are parallel to the spindle axis. In the telophase shown in D, the Mira crescent is regularly positioned at the GMC pole. Scale bar: 5 μm.

central spindle midzone (Bonaccorsi et al., 1998). Here we demonstrate that *asl* NBs and GMCs also form functional cytokinetic structures even though their spindles are anastral. Moreover, the cytokinetic apparatus is always located in the middle of the central spindle, whether the cells divide symmetrically or asymmetrically. Our findings indicate that in several different *Drosophila* cell types (spermatocytes, NBs and GMCs), the molecular signals for cytokinesis are specifically provided by the central spindle midzone; these signals are properly generated, even in the absence of asters.

Because the central spindle midzone is the only source of the cytokinetic stimulus, our findings on central spindle positioning in *asl* NBs suggest a model for unequal cytokinesis in *Drosophila* NBs (Fig. 6). We propose that the presumptive GMC cell possesses a specialized cortical site that interacts with the GMC nucleus, anchoring it to the polar cortex. The GMC nucleus would in turn interact with the central spindle, shifting its position within the dividing NB cell.

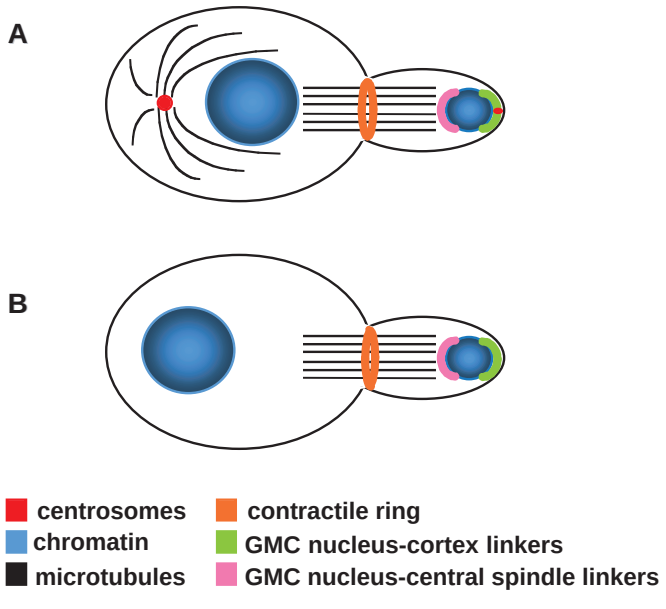

Fig. 6. A model for the mechanisms underlying unequal cytokinesis in *Drosophila* NBs. (A) Wild-type NB telophase; (B) *asl* NB telophase. We propose that molecular links between the central spindle and the GMC nucleus and between this nucleus and the polar cortex result in an eccentric positioning of the central spindle within the dividing cell. The shifting of the central spindle towards the GMC side is particularly evident in *asl* telophases, where, in the absence of the large aster on the NB side, the NB nucleus can be seen to be disconnected from the central spindle. Because the central spindle midzone emanates the cytokinetic signal(s), its position within the cell sets the cleavage plane.

At present we do not know what kind of molecular linkers anchor the GMC nucleus to the polar cortex, nor do we have many clues about the molecular mechanisms that underlie the close relationship between the central spindle and the GMC nucleus. One interesting possibility is that minus end-directed molecular motors associated with the GMC nuclear envelope might link the minus ends of central spindle microtubules to the envelope of the GMC nucleus. Precedents for this model can be found in reports that microtubule-dependent nuclear motility in several cell types is mediated by cytoplasmic dynein at the nuclear envelope (reviewed in Reinsch and Gonczy, 1998) In the asymmetric NB divisions, this hypothesis would require that the motors themselves, or molecules that target these motors to the nuclear envelope, must segregate preferentially to the presumptive GMC. Alternatively, the smaller size of the GMC nucleus might ensure that the concentration of these motors or targeting molecules is higher on the surface of this nucleus than on that of the NB daughter nucleus.

Unequal divisions characterized by the presence of differently sized asters have been observed in a variety of systems (reviewed in Strome, 1993). In some of these systems (e.g. the unequally dividing blastomeres during the fourth cleavage of sea urchin embryos (Schroeder, 1987)), the nucleus of the smaller daughter cell reaches the polar cortex, while in others (e.g. the one-cell embryo of *Caenorhabditis elegans* (Albertson, 1984)) this nucleus remains detached from the polar cortex but is connected to it by astral microtubules. Thus, while our model's assumption of a cortical anchor for the nucleus of the smaller daughter cell could explain unequal cleavage in sea urchins, it does not apply to *C. elegans* unequal cytokinesis. In *C. elegans*, the shifting of the mitotic apparatus towards the small-cell polar cortex is thought to be mediated by interactions between astral microtubules and localized cortical anchors (Hyman, 1989). However, it is quite possible that all types of unequal cleavage involve in common an association between the central spindle with either the small-cell nucleus or both daughter nuclei. Such associations may be particularly important in maintaining the integrity of the mitotic apparatus during the processes that lead to its eccentric positioning within the dividing cell. Because in ana-telophase spindles astral microtubules no longer overlap and because most central spindle microtubules are detached from the centrosomes (Mastronarde et al., 1993), connections between the central spindle and one or both daughter nuclei might ensure the stability of the mitotic apparatus during its shift towards one of the cell poles.

The role of centrosomes and astral microtubules in Miranda localization

We have shown that in wild-type larval brains Mira is concentrated in a cortical crescent overlaying the GMC side of the NB throughout cell division. In *asl* NB metaphases and anaphases, Mira accumulates in cortical crescents that are comparable with those observed in wild type, but are often incorrectly localized with respect to the spindle. However, as *asl* NBs proceed through mitosis, Mira progressively attains a normal localization, so that in nearly 90% of telophases it is enriched in the GMC polar cortex.

The localization pattern of Mira in *asl* larval NBs has some interesting similarities with the distribution patterns of Mira, Numb and Pros in embryonic NBs of *inscuteable* (*insc*) and *bazooka* (*baz*) mutants. In a large fraction of *insc* and *baz* NBs, Mira, Numb and Pros are evenly distributed. However, in some mutant NBs these proteins accumulate into cortical crescents that are randomly oriented with respect to the spindle (Kraut et al., 1996; Kuchinke et al., 1998; Shen et al., 1997; Schober et al., 1999; Wodarz et al., 1999). In addition, in both *insc* and *baz* mutants the Mira, Numb and Pros proteins, although irregularly distributed during prophase and metaphase, concentrate over the basal spindle pole at telophase (Schober et al., 1999; Wodarz et al., 1999). These results, together with recent *in vivo* observations of spindle rotation in *insc* NBs (Kaldshmidt et al., 2000), have led to the conclusion that *insc* and *baz* play two main roles: proper spindle rotation within the dividing NB, and asymmetric localization of cell fate determinants (such as Mira, Numb and Pros) during prophase and metaphase. However, during late anaphase and telophase there is a second mechanism, independent of *baz* and *insc*, that concentrates these determinants at the NB basal side (Kraut et al., 1996; Schober et al., 1999; Wodarz et al., 1999).

Our results on Mira localization in *asl* larval NBs indicate that centrosomes and astral microtubules are not required for the *insc*- and *baz*-dependent asymmetric accumulation of this protein during early mitotic stages. This conclusion is in good agreement with previous studies showing that disruption of microtubules by colchicine treatment has no effect on cortical accumulation of several cell fate determinants, including Numb, Pros and Insc (Knoblich et al., 1995; Kraut et al., 1996; Broadus and Doe, 1997). In addition, our finding that Mira attains a normal telophase localization in most *asl* NBs, shows that centrosomes and astral microtubules are not involved in the second, *insc*- and *baz*-independent mechanism that concentrates Mira, Pros and Numb at the basal cortex during telophase. However, the observation that *asl* mutations disrupt the positioning of the Mira crescents with respect to the spindle orientation, as do the *insc* and *baz* mutations, strongly suggests that in *asl* NBs the spindle fails to rotate properly. This implies that centrosomes and astral microtubules play an essential role in spindle rotation, and that this process is not a specific feature of embryonic NBs but also occurs in larval NBs. Our finding that simultaneous staining for Mira and tubulin can detect defects in spindle orientation within larval NBs, opens the way for a mutational dissection of the mechanisms underlying spindle rotation. For example, this analysis could be performed by using extant mitotic mutations that disrupt diverse aspects of cell division. The effects of these mutations, however, cannot be studied in embryonic NBs but only in larval NBs. Indeed, owing to the perdurance of their maternal products, most of the *Drosophila* genes with essential mitotic functions do not affect embryonic divisions but display mitotic defects only in late larval stages (Gatti and Baker, 1989; Gatti and Goldberg, 1991).

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