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Spindle alignment is achieved without rotation after the first cell cycle in *Drosophila* embryonic neuroblasts

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Spindle alignment along the apicobasal polarity axis is mandatory for proper self-renewing asymmetric division in *Drosophila* neuroblasts (NBs). In embryonic NBs, spindles have been reported to assemble orthogonally to the polarity axis and later rotate to align with it. In larval NBs, spindles assemble directly aligned with the axis owing to the differential spatiotemporal control of the microtubule organising activity of their centrosomes. We have recorded embryonic NBs that express centrosome and microtubule reporters, from delamination up to the fourth cell cycle, by two-photon confocal microscopy, and have found that the switch between these two spindle orientation modes occurs in the second cell cycle of the NB, the first that follows delamination. Therefore, predetermined spindle orientation is not restricted to larval NBs. On the contrary, it actually applies to all but the first cell cycle of embryonic NBs.

KEY WORDS: *Drosophila*, Spindle alignment, Neuroblast, Asymmetric division

INTRODUCTION

Asymmetric division of *Drosophila* neuroblasts (NBs) results in a differentiating ganglion mother cell (GMC) and a self-renewed NB. This process involves the differential sorting of several protein complexes to the apical and basal sides of the cell cortex and the controlled orientation of the spindle (Chia et al., 2008; Gonczy, 2008; Gonzalez, 2007; Knoblich, 2008).

Pioneering live microscopy studies carried out on embryos demonstrated the first reported mechanism of spindle alignment in *Drosophila* NBs: spindles assemble at an angle that is nearly perpendicular to the apicobasal axis of the cell and later rotate to align with it (Kaltschmidt et al., 2000). More recent studies carried out on larval NBs revealed a different mechanism by which spindles assemble already closely aligned along the cortical polarity axis of the NB and only minor rotations refine their alignment before division occurs (Rebollo et al., 2007; Rusan and Peifer, 2007). This second mechanism relies on the differential spatiotemporal control of the microtubule-organising center (MTOC) activity of the NB centrosomes (Rebollo et al., 2007; Rusan and Peifer, 2007). Because larval NBs originate from quiescent embryonic NBs, these observations raise the question of when during development the switch from the rotational to the predetermined spindle alignment mode occurs.

MATERIALS AND METHODS

Fly stocks

Flies expressing GFP–α-Tub84B and YFP-Asl were used for live-cell imaging (Rebollo et al., 2007).

Embryo immunostaining

W^{1118} embryos were collected and immunostained as described (Gonzalez and Glover, 1993), using anti-Cnn (Heuer et al., 1995; Vaizel-Ohayon and Schejter, 1999), DM1a (Sigma, Aldrich), and Alexa antibodies (Invitrogen).

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Live-embryo preparations

Embryos were processed as described (Kaltschmidt et al., 2000). NBcc1 was followed in a total of 22 NBs. NBcc2 was followed in 12 NBs in which NBcc1 was also recorded. NBcc3 was followed in 12 NBs of which four were also recorded during NBcc1 and NBcc2. NBcc4 was followed in four NBs in which NBcc1, NBcc2 and NBcc3 were also recorded. For the recording of centriole motility by spinning disk confocal microscopy, embryos were gently squashed by pressing on the Teflon membrane.

Microscopy

Immunostained embryos were recorded on a Leica TCS-SP2 microscope (Leica Microsystems, Heidelberg, Germany). Stacks of 20 to 30 sections every 1 μm were acquired. Live embryos were recorded with a Leica TCS-SP5 multiphoton microscope equipped with a Ti:sapphire laser (Spectra Physics, USA). Stacks of 20 to 30 sections every 1 μm were captured at intervals of 30 seconds to 1 minute. Isolated NBs were recorded in an Andor Spinning Disk Confocal System (Andor Technology). All microscopes were equipped with a 60×/1.42 or a 63×/1.40 (oil PL APO) objective. Images were processed using the Leica Confocal Software, Image J 1.37n (http://rsb.info.nih.gov/ij/) and Adobe After Effects 7.0.

RESULTS AND DISCUSSION

The onset of the predetermined spindle alignment mode takes place after the first NB cell cycle

During stages 8 to 11 of embryogenesis, about one third of the epithelial cells from the ventral neuroectoderm delaminate becoming NBs (Campos-Ortega and Hartenstein, 1997). Once delaminated, NBs can be found at different levels towards the interior of the embryo. To monitor MTOC activity and spindle assembly in NBs as they progress from delamination to later cell cycles, we resorted to two-photon confocal microscopy in embryos expressing YFP-Asl and GFP–α-Tub84B. Two-photon confocal microscopy produces high-contrast optical sections that span from the surface to several cell diameters deep into the embryo, showing delaminating NBs (Fig. 1A, stage 9, boxed area), as well as NBs that have already undergone one or more cell cycles, as revealed by the number of GMCs associated with them (Fig. 1A, stage 10, asterisks).

A time-lapse series including the most representative time points of the delaminating NB framed in Fig. 1A is shown in Fig. 1B (see also Movie 1 in the supplementary material). It covers the first and second cell cycle of this NB (NBcc1 and NBcc2, respectively).

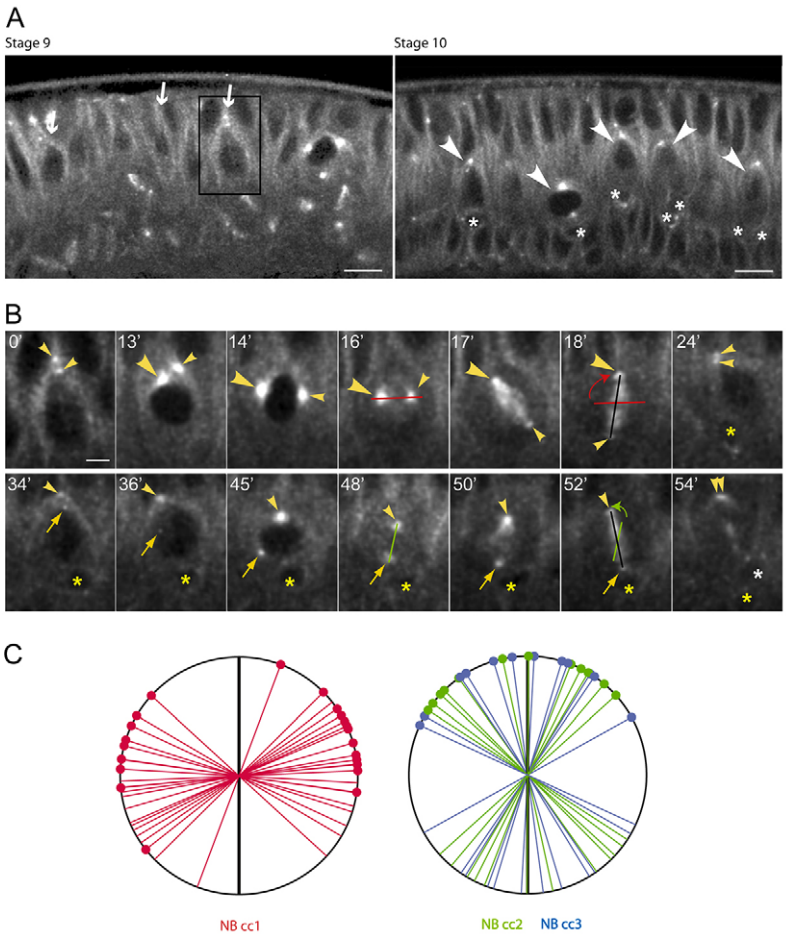

Fig. 1. Switch between spindle orientation modes in embryonic neuroblasts. (**A**) Single frames from two time-lapse series obtained with a two-photon microscope taken from embryos expressing GFP–α-Tub84B and YFP-Asl. At stage 9, several delaminating NBs can be observed (arrows). At stage 10, delaminated NBs (arrowheads) containing one or two GMCs (asterisks) can be seen deeper inside the embryo. Scale bar: 10 μm. (**B**) Time-lapse series of the delaminating NB boxed in Fig. 1A, showing the most representative time points over the first two cell cycles (NBcc1 and NBcc2). During NBcc1, arrowheads label the centrosomes, initial and final spindle orientations are highlighted in red and black, respectively, and the first delivered GMC is labelled with a yellow asterisk. During NBcc2, the large apical and the small wandering centrosomes are labelled by an arrowhead and an arrow, respectively; the initial and final spindle orientations are highlighted in green and black, respectively, and the second GMC is labelled with a white asterisk. Scale bar: 5 μm. (**C**) Plots of spindle orientation at the time of assembly in NBcc1 (red), and NBcc2 (green) and NBcc3 (blue). Dots indicate the position of the larger MTOC.

Early in NBcc1, when delamination is taking place, two fairly weak MTOCs are already visible, located apically within the stalk (Fig. 1B, NBcc1, 0′, arrowheads). At prophase, as the stalk retracts, microtubule nucleation increases synchronously in both centrosomes. In this cell, as in all other NBcc1s recorded (n=22), one of the MTOCs is slightly larger than the other (Fig. 1B, NBcc1, 13′, arrowheads) and remains so throughout mitosis. The two centrosomes migrate, first down towards the nucleus, and then in opposite directions over the nuclear envelope until they reach opposite positions across the nucleus (Fig. 1B, NBcc1, 13′ to 14′). By nuclear envelope breakdown, the axis of the incipient spindle is closely parallel to the epithelia (Fig. 1B, NBcc1, 16′, red line). At metaphase, the spindle rotates to orient itself approximately orthogonally to the epithelium (Fig. 1B, NBcc1, 17′-18′, black line). Mitosis proceeds with the spindle aligned along the apicobasal axis and the first GMC is delivered basally (Fig. 1B, NBcc1, 24′, yellow asterisk). In most cases (20/22), rotation resulted in the apical localisation of the slightly larger MTOC, possibly reflecting a higher efficiency in interacting with the apical cortex. These observations confirm the spindle rotation model previously reported (Kaltschmidt et al., 2000). In addition, our results show that in NBcc1 centrosomes duplicate early, in interphase, always remain apically located, and have very feeble MTOC activity until mitosis onset, when they mature. Finally, our results suggest that the proposed difference between the two centrosomes that would prime one of them to move apically after the metaphase spindle is set up (Kaltschmidt et al., 2000) in NBcc1 might be the slight difference in microtubule organising activity between the two centrosomes.

MTOC activity and spindle orientation are markedly different in this same NB in the following cell cycle, NBcc2, the first one that takes place entirely after delamination. Shortly after cytokinesis, one main MTOC organises a microtubule aster that stays through interphase at the apical cortex (Fig. 1B, NBcc2, 34′ to 45′, arrowhead), which already at this early stage acquires the slightly pointed shape previously described in larval NBs (Rebollo et al., 2007; Rusan and Peifer, 2007). The other focus of centriolar signal has little, if any, MTOC activity and moves extensively through the cytoplasm (Fig. 1B, NBcc2, 34′ to 45′, arrow). At prophase, this centrosome, which is located close to the basal side of the cell starts to acquire significant microtubule nucleation activity whereas the apical centrosome approaches the nucleus, always on the apical side (Fig. 1B, NBcc2, 45′). As a result, the spindle is directly assembled apicobasally (Fig. 1B, NBcc2, 48′, green line). Little spindle rotation takes place before mitosis in this cell, which, once more, divides along the apicobasal axis delivering its second GMC basally (Fig. 1B, NBcc2, 50′ to 54′, white asterisk). The same process of aligned spindle assembly was observed in NBs in the third and fourth cell cycle.

A plot of the actual rotation angles observed during NBcc1, NBcc2 and NBcc3 is shown in Fig. 1C. In NBcc1, spindle orientation at the time of assembly very rarely falls within the apical quadrant centered over the axis of cortical polarity and significant rotation occurs to achieve alignment (Fig. 1C, NBcc1). By contrast, spindle orientation at the time of assembly in the following cycles is close to the final orientation (Fig. 1C; NBcc2, NBcc3). From these observations we conclude that the onset of the predetermined spindle orientation mode

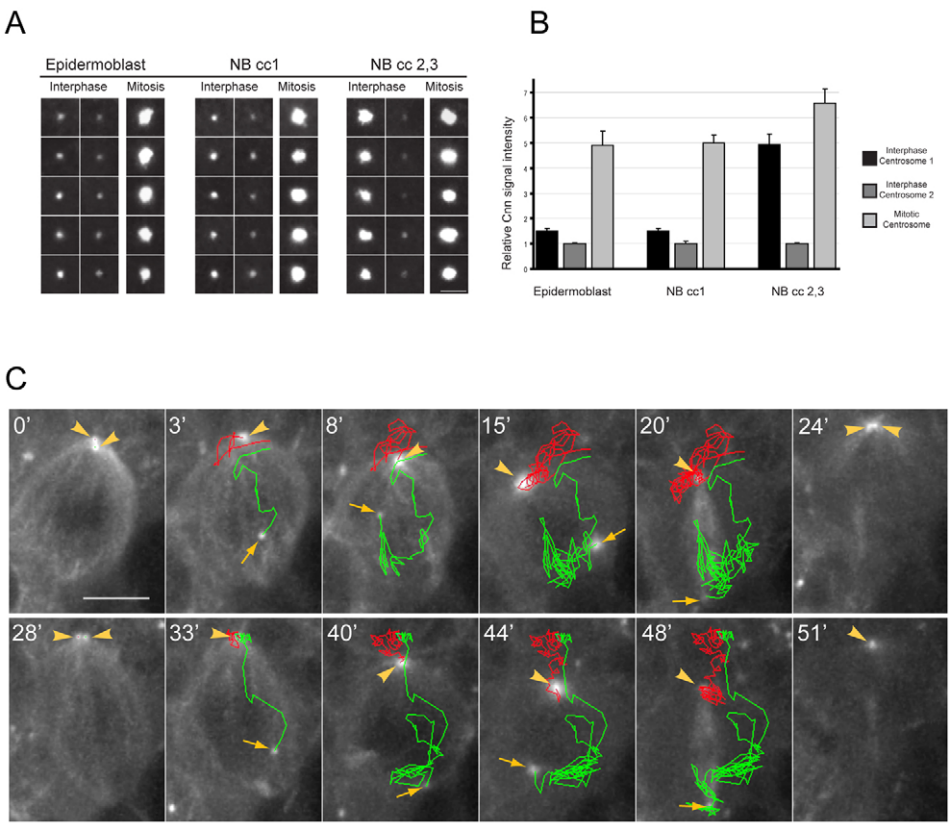

Fig. 2. Asymmetry of PCM content and centriole motility during the second and later cell cycles in embryonic NBs. (**A**) Comparative series of five panels showing the intensity of the Cnn immunofluorescence signal for each centrosome pair, in interphase and mitosis, in epidermoblasts, NBcc1 and NBcc2/NBcc3. (**B**) Quantitated plot of the data shown in A. The values of each pair are normalised to a relative value of one for the weakest signal. (**C**) Centrosome movement during two consecutive cell cycles in a delaminated embryonic NB expressing YFP-Asl and GFP–α-TUB84B. Movements of the active apical centrosome (arrowhead) and the motile centrosome (arrow) are traced in red and green, respectively. Scale bar: 5 μm.

takes place in NBcc2, the first cell cycle that takes place entirely after delamination. Therefore, rather than being limited to larval NBs as initially suspected, predetermined spindle orientation appears to be a distinct feature of NBs throughout most of their lifespan.

Predetermined spindle alignment mode in embryonic NBs relies on the same features described for larval NBs

The three main features that characterise the predetermined mechanism of spindle orientation discovered in larval NBs are (1) the permanent apical residence of the main MTOC of the cell, (2) the unequal recruitment of pericentriolar material (PCM), and (3) the high motility of the centriole fated to the GMC. Having demonstrated that the first applies in embryonic NBs from NBcc2 onwards, we decided to test whether the other two features also apply.

To this end, we first determined the amount of PCM in the centrosomes during cycles NBcc1, NBcc2 and NBcc3, by immunostaining with an antibody against Centrosomin (Cnn) (Heuer et al., 1995; Vaizel-Ohayon and Schejter, 1999). The amount of PCM in epidermoblast centrosomes was also recorded. We found that epidermoblasts and delaminating NBs were indistinguishable in this regard (Fig. 2A). During interphase, both cell types contain two rather small, but distinct foci of PCM. Interestingly, consistent with the slight differences in MTOC activity that we had observed, the two foci are also reproducibly different, with a size ratio close to 1.5 (Fig. 2B). At mitosis, PCM sizes increase significantly in both centrosomes in epidermoblasts and delaminating NBs, up to about 5 times the size of the smaller interphase centrosome (Fig. 2A,B). This pattern changes dramatically in the following cell cycles (NBcc2 and NBcc3) when, in interphase, the two PCM foci are quite different in size: one is small, similar or even smaller than the interphase foci of epidermoblasts and delaminating NBs; the other is much larger, similar to the mitotic foci of epidermoblasts and delaminating NBs. At mitosis, the large PCM foci are roughly as large as in the previous mitoses (Fig. 2A,B). Remarkably, in interphase cells, the large PCM focus was always localised at the apical side of the NB. These results strongly suggest that the differences in MTOC activity, both the mild ones observed in NBcc1 and the more dramatic ones seen in the following cell cycles, are accounted for by differential PCM accumulation. Moreover, because PCM size during interphase is rather similar in all but the much larger apical MTOC of NBcc2 and later, it appears that the asymmetric MTOCs characteristic of the predetermined spindle orientation mode result from a strong upregulation of the apical centrosome rather than a downregulation of the other.

We then monitored centriole motility in embryonic NBs. To achieve the required time resolution, we resorted to spinning disk confocal microscopy. Fig. 2C shows a series of time points summarising two consecutive cycles in a delaminated embryonic NB (see also Movies 2, 3 in the supplementary material). Centriole behaviour is identical in both cycles. One of the dots of centriolar signal is associated with the main MTOC and remains apical throughout most of the cell cycle, moving only marginally as the cell cortex expands or retracts (Fig. 2C, arrowhead, red tracing); the other centriolar signal moves extensively through the cell until, once basal, it acquires MTOC activity shortly before mitosis (Fig. 2C, arrow, green tracing). Such differential centriole behaviour is almost identical to that previously observed in larval NBs (Rebollo et al., 2007). However, the initial phase of centriole movement that is charateristic of larval NBs and during which the centrosome stays mostly on the apical half of the cell, migrating back and forth to the apical MTOC, is absent in these early cycles of embryonic NBs. Instead, in these cells, the motile centrosome goes almost directly to

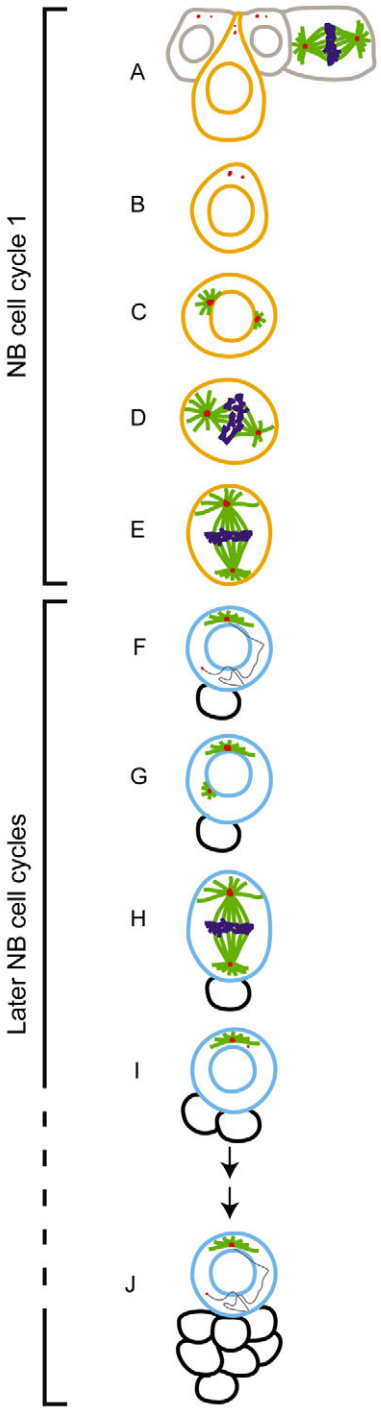

Fig. 3. Model of centrosome behaviour and spindle assembly orientation in embryonic NBs. Epithelial cells are delineated in grey, GMCs in black, NBcc1 in orange, and NBcc2 and later cycles in blue. Centrosomes are depicted in red and microtubules in green. (**A**) As in epithelial cells, duplicated centrosomes are located apically in the delaminating NB, within the protruding stalk. (**B**) When mitosis approaches, the NB body rounds up and the two centrosomes, which start to gain MTOC activity, move towards the nucleus and migrate to opposite sides around the nuclear envelope, one aster remaining bigger than the other (**C**). During mitosis of this first NBcc, the spindle assembles at an axis almost parallel to the epithelium (**D**) and, by metaphase, the spindle rotates to align with the apicobasal axis, always keeping the larger aster at the apical side (**E**). Right after the first asymmetric division, the two newly duplicated NB centrosomes acquire the differential MTOC behavior observed in larval NBs (**F**) that leads to the assembly of the spindle directly along the apicobasal axis (**H**), thus determining the orientation of the asymmetric division (**I**). All subsequent divisions occur in this mode (**J**).

the basal half of the cell (Fig. 2C, 3′ and 33′, green tracing). Remarkably, although the cell cycle length is significantly shorter in embryonic NBs (31.2±2.4 minutes, n=15) than in larval NBs (94.7±27.2 minutes, n=10), the approximate time of arrival at the basal side, expressed as a fraction of the cell cycle length, is not so different between embryonic (0.34±5) and larval (0.41±11) NBs. These results demonstrate that, like in larvae, the predetermined spindle alignment mode observed in embryonic NBs relies on the apical residency of the permanently upregulated centrosome and on the controlled upregulation of the MTOC activity of the other centrosome, right before spindle assembly, and only once it is located on the basal side of the cell.

The revised model of spindle alignment in embryonic NBs highlights new perspectives on NB polarisation

A schematic summary of our results is shown in Fig. 3. In terms of centrosome behaviour and MTOC activity, NBs in their first cell cycle are somewhat between the neuroectodermal cells from which they derive and older NBs. In epidermoblasts and delaminating NBs, centrosomes duplicate long before mitosis and both centrosomes have feeble MTOC activity (Fig. 3A,B). MTOC activity has been shown to be very weak during interphase in many *Drosophila* cell types (Rogers et al., 2008). At mitosis onset, the centrosomes of epidermoblasts and delaminating NBs start to gain MTOC activity and to migrate to opposite sides of the nucleus defining a line that is nearly orthogonal to the apicobasal axis along which the spindle assembles (Fig. 3C,D). However, while spindle orientation remains unchanged through mitosis in epidermoblast (Fig. 3A), it changes in the newly differentiated NB at metaphase (Fig. 3E) to an apicobasal orientation. In most cases, rotation occurs in the direction that positions the slightly larger aster on the apical side. Completion of the first cytokinesis results in the basal delivery of the first GMC and, as previously hypothesised (Kaltschmidt et al., 2000), leaves the NB centrosome on the apical side of the cell (Fig. 3F). This is the landmark of the switch to the predetermined alignment mode in which differential centrosome behaviour leads to spindle assembly directly along the apicobasal axis, and in which no major spindle rotation occurs (Fig. 3F-I). The apical centrosome contains a considerable amount of PCM, organises a large microtubule aster, and stays at the apical cortex of the cell (Fig. 3F-I). The other centrosome, almost totally devoid of PCM and microtubules, moves away from the apical cortex and remains motile, mostly around the basal half of the cell, until mitosis onset, when it starts to accumulate PCM and to nucleate microtubules near to the basal cortex (Fig. 3G). As a result, the spindle assembles directly along the apicobasal axis (Fig. 3H), and once more, but this time without significant spindle rotation, asymmetric division delivers a basal GMC (Fig. 3I). This process of asymmetric centrosome behaviour and aligned spindle assembly is repeated in the following cycles in the embryo (Fig. 3J) and indeed in larval NBs (Rebollo et al., 2007; Rusan and Peifer, 2007). We have observed this mode in all the post-delamination NB cell cycles that we were able to unequivocally identify in the embryo

by two-photon microscopy (NBcc2, NBcc3 and NBcc4). It is therefore likely that such a mode operates in all but the first cell cycle in *Drosophila* NBs.

When a NB delaminates from the epithelium, the apical stalk carries the Par complex from the apical cortex of the corresponding epidermoblast, which triggers the recruitment cascade that establishes apicobasal polarity during the first round of NB asymmetric cell division (Yu et al., 2006). However, because the Par complex and other known polarity markers fade away from the cortex after mitosis, it is unclear how cortical polarity orientation is passed on in the following cell cycles. Our results strongly suggest that, as previously proposed (Rebollo et al., 2007; Januschke and Gonzalez, 2008), the aster that stays anchored to the cortex during interphase might convey such information.

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Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/20/3393/DC1

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