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A default mechanism of spindle orientation based on cell shape is sufficient to generate cell fate diversity in polarised *Xenopus* blastomeres

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The process of oriented divisions of polarised cells is a recurrent mechanism of cell fate diversification in development. It is commonly assumed that a specialised mechanism of spindle alignment into the axis of polarity is a prerequisite for such systems to generate cell fate diversity. Oriented divisions also take place in the frog blastula, where orientation of the spindle into the apicobasal axis of polarised blastomeres generates inner and outer cells with different fates. Here, we show that, in this system, the spindle orients according to the shape of the cells, a mechanism often thought to be a default. We show that in the embryo, fate-differentiative, perpendicular divisions correlate with a perpendicular long axis and a small apical surface, but the long axis rather then the size of the apical domain defines the division orientation. Mitotic spindles in rounded, yet polarised, isolated *Xenopus* blastula cells orient randomly, but align into an experimentally introduced long axis when cells are deformed early in the cell cycle. Unlike other systems of oriented divisions, the spindle aligns at prophase, rotation behaviour is rare and restricted to small angle adjustments. Disruption of astral microtubules leads to misalignment of the spindle. These results show that a mechanism of spindle orientation that depends on cell shape rather than cortical polarity can nevertheless generate cell fate diversity from a population of polarised cells.

KEY WORDS: Spindle, Cell shape, *Xenopus*, Blastula, Asymmetric division, Microtubules

INTRODUCTION

Asymmetric cell division has been shown in a variety of organisms to be an important mechanism through which to generate cell fate diversity (Wodarz and Huttner, 2003; Roegiers and Jan, 2004; Betschinger and Knoblich, 2004; Knoblich, 2001; Lu et al., 2000; Doe and Bowerman, 2001). The basic requirements for this mechanism are that an axis of polarity is established within the cell, that the mitotic spindle aligns with this axis of polarity, and that cell fate determinants become distributed asymmetrically along the axis of polarity so that they are differentially inherited by the daughter cells. As a result of this differential inheritance of determinants, the daughter cells attain different fates in development (reviewed by Betschinger and Knoblich, 2004; Knoblich, 2001).

Orientation of the spindle is key in these systems and most of what is currently known about the molecular control of spindle orientation in metazoans is based on results from asymmetric divisions in invertebrate systems such as the *Drosophila* neuroblasts and sensory organ precursor cells (SOPs) or the *C. elegans* zygote. In these systems, spindle orientation is tightly linked to the molecules that establish cell polarity (Cowan and Hyman, 2004; Betschinger and Knoblich, 2004; Doe and Bowerman, 2001). In the *Drosophila* neuroblast, for example, spindle rotation into the axis of polarity requires Inscuteable, which binds to the highly conserved apical Par3 (Bazooka)/Par6/aPKC complex via Par3 (Bazooka), and in turn interacts with the GoLoco domain protein Pins and its binding partner Gαi (reviewed by Betschinger and Knoblich, 2004). Both of these become localised to the apical domain upon Insc expression. Pins is a receptor-independent activator of heterotrimeric G proteins and mediates spindle rotation movements by modulating microtubule dynamics or the attachment of microtubules at the cortex and the activation of molecular motors that pull on them (reviewed by Betschinger and Knoblich, 2004; Hampoelz and Knoblich, 2004; Kusch et al., 2003). In addition, the mammalian Pins homologue (LGN) has been shown to interact simultaneously with Gαi and with the microtubule-binding protein NuMA [the homologues of which have recently been characterised in *Drosophila* (Siller et al., 2006; Izumi et al., 2006)], which can also bind to the dynein/dynactin complex (Du and Macara, 2004) (reviewed in Hampoelz and Knoblich, 2004). A similar mechanism has been shown for the *C. elegans* zygote that also involves Par protein-dependent localisation of the G-protein activators GPR1 and GPR2 (Betschinger and Knoblich, 2004; Colombo et al., 2003; Grill et al., 2003). From these results, a general model has emerged in which the polarity of the cells is causally linked to the molecular mechanisms that orient the spindle.

Spindle orientation has also been studied in the vertebrate nervous system, particularly in the developing brain and retina, but the causal relationship between spindle orientation and fate determination is less clear in vertebrates (Wodarz and Huttner, 2003; Roegiers and Jan, 2004; Betschinger and Knoblich, 2004; Gotz and Huttner, 2005; Huttner and Kosodo, 2005). However, vertebrate cells seem to employ the same conserved protein complexes to establish cell polarity (Wodarz, 2002; Suzuki and Ohno, 2006). Furthermore, the mammalian homologues of Inscuteable and Pins (AGS3) have been demonstrated to be necessary for spindle alignment into the axis of apicobasal polarity in the rat retina (Zigman et al., 2005) and the developing mouse brain via interactions with G proteins (Sanada and Tsai, 2005). Although it is not known how cells decide which way to divide, from these examples it seems likely that placing the control of spindle orientation downstream of cortical polarity factors

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is evolutionary conserved between vertebrates and invertebrates. However, it is not clear whether such a mechanism that links cell polarity with spindle orientation is a universal feature that applies to other systems of oriented, fate-asymmetric divisions.

Recently, we have described a new system of oriented divisions in vertebrates. As in other examples of fate-asymmetric divisions, the cells of the *Xenopus* blastula are morphologically and molecularly polarised along the apicobasal axis. Orientation of the mitotic spindle into this axis of polarity, perpendicular to the surface of the embryo, generates inner and outer cells, which are intrinsically programmed to follow different fates in development (Muller and Hausen, 1995; Chalmers et al., 2005; Chalmers et al., 2003; Chalmers et al., 2002). In the animal ectoderm region, outer cells give rise to neuronal progenitors, the ultimate fate of which is presently unknown, while inner cells give rise to early differentiating primary neurons (Chalmers et al., 2002; Hartenstein, 1989). The number of cells that align their spindles into the apical basal axis at each round of division is constant throughout the blastula stage (~25%), but their distribution pattern is apparently random, raising the possibility that the cells that divide perpendicularly are stochastically specified. However, we have observed a strong correlation between apicobasally elongated cells and perpendicular divisions, which led us to hypothesise that the parameter that determines spindle orientation might be cell shape (Chalmers et al., 2003).

Here, we test this hypothesis in several ways. We suggest that a simple default mechanism of spindle orientation that is based on monitoring the long axis rather than the polarity of the cell, is sufficient to generate cell fate diversity in this, and perhaps other, systems of oriented divisions.

MATERIALS AND METHODS

Embryos culture and blastomere dissociation

Xenopus embryos were obtained using standard procedures, cultured in 0.1× Marc's modified Ringer's solution (MMR) and staged according to cell number. Dissociated blastomeres were obtained by transferring embryos in Ca^{2+}/Mg^{2+}-free medium (CAMFM) as described (Muller and Hausen, 1995). Isolation of single blastomeres, filming and compression was carried out in 1×MMR. Isolated blastomeres and embryos were fixed in Dent's fixative, 80% methanol/20% DMSO.

Blastomere compression

To introduce an experimental long axis to round, isolated blastomeres a compression device was assembled from Superfrost glass slides (BDH), such that a single blastomere could be trapped in the gap between the narrow sides of a fixed and a moveable slide that was connected to a micromanipulator. By closing the gap via the micromanipulator while a cell sinks towards the bottom of the gap, it becomes suspended between the two vertical surfaces of the slides. The compression device was mounted within a container filled with 1×MMR. Cells were oriented with the pigmented surface to one side and filmed from the top. Statistical analysis for significance was performed using SPSS (v12.0).

Immunohistochemistry

Albino embryos were fixed in Dent's fixative and whole-mount antibody staining was carried out as described previously (Chalmers et al., 2003). After staining, embryos were dehydrated in methanol and cleared in 1:2 benzyl alkohol/benzylbenzoate. The following antibody combinations were used: anti α-tubulin (1/1000, Sigma, DM1A, T9026) with anti mouse TRITC (1/500, Sigma, T7782) or anti-mouse Alexa 488 (1/500, Molecular Probes, A:11001); rat anti α-tubulin (1/100, Abcam, YL1/2, ab6160) with anti-rat Alexa 488 (1/500, Molecular Probes, A:11006).

RNA microinjection

To visualise microtubules in vivo *tau-GFP* or *GFP-EB1* RNA was in vitro transcribed (Message Machine kit, Ambion) and injected at the two-cell stage into albino embryos (0.6-0.9 ng total RNA). The template for *tau-GFP* was generated by subcloning a 1958 bp *Sal*I-*Xho*I fragment derived from a construct used in *Drosophila* (A. Brand, unpublished) into the *Xho*I site of pCS2+ (E. Amaya, unpublished). The *GFP-EB1* expression plasmid pjMA2eGFP was a kind gift from Ewan Morrison (Morrison et al., 2002) and the 1587 bp *Apa*I-*Kpn*I *GFP-EB1* fragment was subcloned into pCS2+.

Time-lapse microscopy and confocal imaging

Time-lapse movies of uninjected embryos and isolated blastomeres were generated using a Leica MZFL111 microscope, a coolsnap camera (Photometrics) and Openlab software. Image time series of *tau-GFP* injected embryos were collected on a Radiance confocal microscope (BioRad); at each time point *z*-stacks were collected at a *z*-step size of 1 or 2 μm. Image time series of *EB1-GFP* injected embryos were taken between the 9th and 12th division on a spinning disc confocal microscope (Perkin Elmer), and movies were generated from projections of image stacks. Images of fixed, whole-mount stained embryos were collected either on an upright Radiance, or an inverted 1024 confocal microscope (BioRad). EB1-GFP plus ends were quantified for two cells in time-lapse movies of projections of image stacks by counting EB1-GFP signals frame by frame on the computer screen using a manual counter. One hundred and seventy frames were counted in each cell.

Image processing and angle measurements

For processing of single images, generating movie files and carrying out measurements, raw image files from the microscope software were imported into IMARIS 3D image analysis software (BITPLANE). For measuring the angle between spindle axis and long axis, cells with their spindles aligned parallel to the *x*-*y* plane of imaging (both ends of the spindle were always visible on all sections through the spindle) were selected in the animal pole region. For each selected cell first, its outline was probed manually by scrolling through the image stack and applying a measurement line tool to find one possible longest axis. With this tentative axis marked, the same process was repeated until confident that the longest axis was found. It was ensured that the cells did not have a longest axis in *z* using side projection views. Then, the spindle axis was marked by connecting the two spindle poles (or centrosomes) on the central section through the spindle. The angle between the two axes was measured on a projection of the long axis into *x*-*y*.

For a computational definition of the long axis and measurement of the angle between the long axis of a cell and the spindle axis, first 3D representations of cells and mitotic spindles were reconstructed from their outlines, which were traced on optical sections. Indices of shape were calculated by solving the eigenvalue problem for the reconstructed volumes, such that each cell and spindle could be represented by three orthogonal eigenvectors and their associated eigenvalues. The angle between the longest axis of each cell (axis with the largest eigenvalue) and the mitotic spindle was calculated as a measure of their alignment. Custom computer programs were written in IDL (ITT Industries, Boulder, CO).

Nocodazole treatment

Before fixation, embryos at the 256-cell stage were incubated in 60 μM nocodazole (Sigma, M1404) in 0.1×MMR, the solution was exchanged once during the 25 minutes of incubation. This concentration still allowed cytokinesis after one cell cycle, but caused a reduction of astral microtubules. Statistical analysis for significance by the Mann-Whitney test was performed using SPSS (v12.0).

Definitions

We refer to division types as follows: parallel (par), spindle parallel to the surface – cleavage plane divides the pigmented apical surface in approximately equal parts; perpendicular (per), spindle perpendicular to the surface – cleavage plane does not divide the pigmented apical surface; oblique (obl), oblique spindle – cleavage plane divides the pigmented surface in a ratio of 1:2 or less. Only perpendicular divisions give rise to inner cells and are fate differentiative; all other division types give rise to outer cells that remain part of the epithelium, although they differ in the amount of apical membrane they inherit.

RESULTS

Spindle orientation in the *Xenopus* blastula correlates with cell shape

The superficial cells of the *Xenopus* blastula have different shapes and often apicobasally elongated cells are found next to flatter cells with a long axis parallel to the surface of the embryo (Fig. 1A-C). In order to address whether alignment of the spindle with the long axis of a cell is a general feature of blastula cells in the animal pole, confocal image stacks of whole-mount stained embryos were analyzed. Side projection views confirmed that cells with a small apical surface have indeed a long axis pointing into the embryo (Chalmers et al., 2003). The spindles of these cells are perpendicular to the epithelium, aligned with the longest axis of the cell. Cells that appear to have their longest axis in the plane of the epithelium also have the spindle aligned with it (Fig. 1D,E).

To quantify spindle alignment with the long axis, first, we analysed parallel dividing cells, which are the most abundant type of spindle orientation during the blastula stage (about 55% at each division cycle) (Chalmers et al., 2003), using Tau-GFP live imaging. The long axis within the plane of the epithelium was manually defined (see

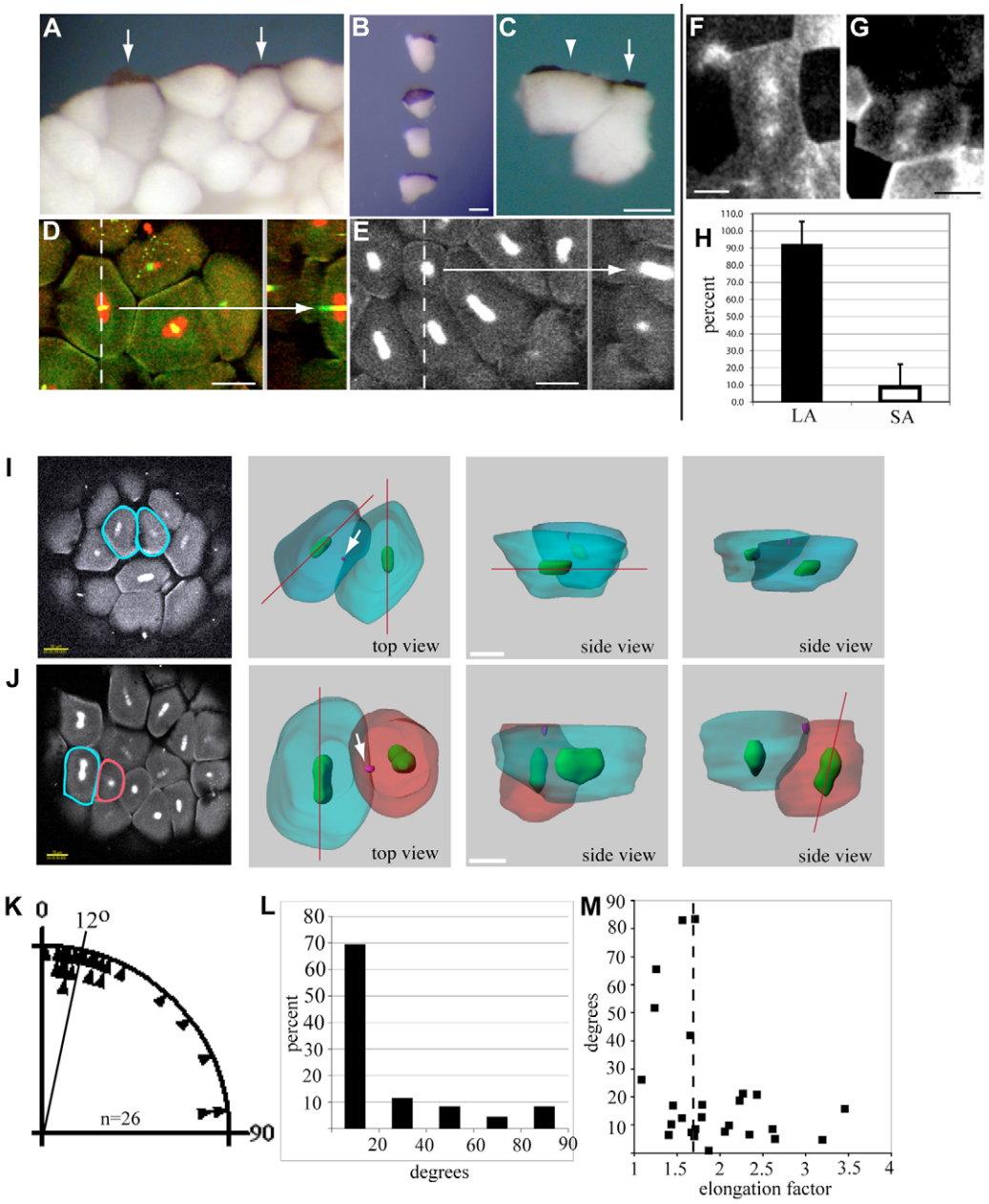

Fig. 1. Cells in the *Xenopus* blastula divide according to their shape. (**A**) *Xenopus* blastula cells have different shapes; arrows indicate cells with small apical surfaces and a long axis perpendicular to the surface. (**B**,**C**) Examples of single cells (B) and a pair of cells (C) dissected out (arrowhead indicates flat cell with a large apical surface; arrow indicates apicobasally elongated cell with a small apical surface). (**D**,**E**) Confocal image stacks of embryos stained for α-tubulin (red in D, white in E) and DNA (yellow in D). A top view section and the corresponding side projection of the stack are shown. In D, the spindle is oriented parallel to the surface; in E it is perpendicular to the surface. The cell in E has a small apical surface and a spindle aligned with the long axis that is perpendicular to the surface. Such cells will undergo a fate-asymmetric division. Spindle alignment with the long axis of the cell is also observed in cells with parallel spindles (D). (**F-H**) Spindle orientation was visualised in vivo using *tau-GFP*-injected embryos and alignment was analysed at late anaphase between the 8th and the 10th division. Spindles aligned within 25° of the long axis were scored as aligned with the long axis (LA) (F,H). Alignment within 25° of the short axis was scored as aligned with the short axis (SA) (G,H). *n*=216 divisions in 11 embryos. Scale bars: 50 μm. (**I**,**J**) 3D reconstructions of sister cells that were identified by the remnant of the midbody (white arrow). (I) A pair of flat cells that are the product of a parallel division and (J) a flat cell and an apicobasally elongated cell, the product of an oblique division. The previous cleavage plane can be deduced from the position of the midbody remnant. In all these cells, the axis of the spindle (red line) is aligned close to the long axis of the cell. Movies 1 and 2 in the supplementary material correspond to these images. (**K**,**L**) Computational analysis of spindle alignment with the long axis in 3D reconstructions shows that the median deviation is 12° at metaphase and most spindles are within 20° of the long axis. The distribution of angles was non-random, as determined by Watson's U^2_n test (P<0.005). (**M**) Correlation of the elongation factor with spindle alignment to the long axis. (A value of 1 corresponds to a sphere.) Elongated cells show better alignment with the long axis.

material and methods). At late anaphase, 91% of spindles were aligned within 25° of the long axis of the cell, and only 9% within 25° of the short axis (Fig. 1F-H). Second, to measure the angle between the spindle axis and the longest axis of a cell more accurately, we have written software that defines the longest axis of a 3D reconstructed volume, as well as calculates angles between axes. A sample of 26 cells containing all division types was analysed. The angle measurements found (Fig. 1K,L) confirmed that in the majority of cases the spindle is indeed aligned with the long axis.

The shape of the superficial cells of the *Xenopus* blastula is determined by two factors: spatial constraints (defined by fixed apical tight junctions and the blastocoel cavity that exerts osmotic pressure towards the exterior); and the previous cell division. For example, parallel divisions generate cells that are elongated within the plane of the epithelium, but with an axis orientation different from the mother cell (Fig. 1I; see Movie 1 in the supplementary material). Oblique divisions tend to generate one daughter cell that is apicobasally elongated and one cell with a long axis within the epithelial plane (Fig. 1J; see Movie 2 in the supplementary material). In both cases, spindle orientation can differ between the two daughter cells as they align with their respective long axes.

As the orientation of division according to cell shape is often thought to be a default mechanism, we considered whether another 'default' rule might apply, i.e. that spindles might undergo alternating 90° rotations from one division to the next (reviewed by Wilson, 1987). Two observations suggest that this is not always the case. First, the spindles of each of the two daughter cells may not be oriented in the same direction (see, for example, Fig. 1I,J). Second, apicobasally elongated cells, which are recognised by their small apical surface, can undergo two or more sequential divisions with the spindle oriented perpendicular to the apical surface. Out of 122 perpendicularly dividing cells (n=5 embryos), scored in time-lapse movies from the 256-cell to the 512-cell stage, 35 (29%) showed two sequential perpendicular divisions. Of those 35 cells, 60% had an apical surface that was smaller than one-third of that of its sister-cell, indicating that they were elongated in the apicobasal axis. An example of a cell dividing perpendicularly in two consecutive divisions is shown in Movie 3 (see supplementary material). Thus, spindles do not always rotate 90° to the previous cell division, but presumably may do so if the cell shape dictates it.

Finally, if cell shape was a determining factor in spindle orientation, one might predict that round cells would align less accurately with the long axis than elongated ones. To test this, we defined the ratio of the lengths of the longest axis to the next longest one as an elongation factor using vectorial data from our computational analysis. As shown in Fig. 1M, as the elongation factor increases, the angle of the spindle becomes more constrained. In particular, for elongation factors above 1.7, the deviation of the spindle becomes constrained within 25°.

Rounding of cells alters the proportions of divisions

If cell shape plays a role in controlling spindle orientation, then rounded cells should divide with different proportions than cells in the embryo. In Ca^{2+}/Mg^{2+}-free medium, blastomeres dissociate from each other and attain a spherical shape, but continue to divide (Fig. 2B). They also maintain apicobasal polarity: pigment markers, molecular markers and tight junction components at the boundary of the apical and basolateral membrane domains remain localised correctly (Fesenko et al., 2000; Muller and Hausen, 1995; Chalmers et al., 2003).

Spindle orientation in isolated cells was deduced as being 90° to the cytokinesis furrow and was classified as in the intact embryo with respect to the pigmented apical surface (Fig. 2B; see

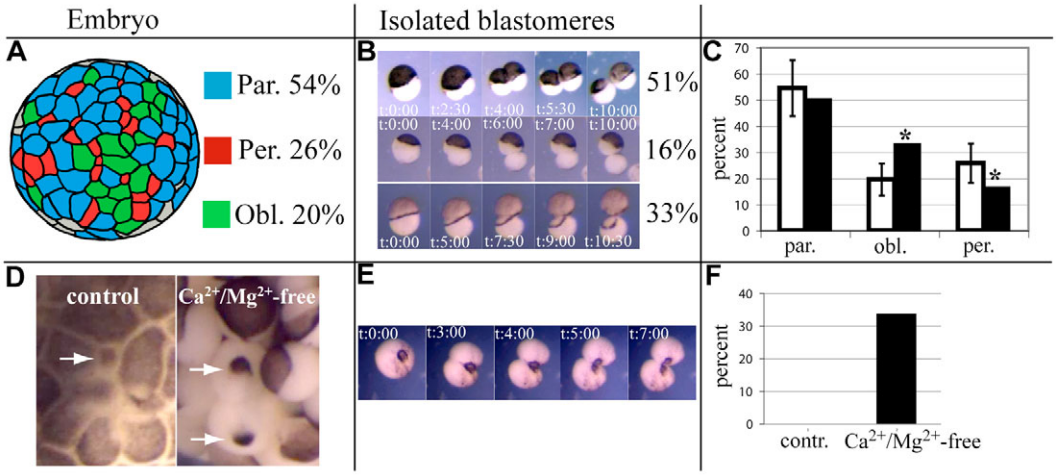

Fig. 2. Isolated spherical blastomeres divide differently from cells in the embryo and often bisect a small apical surface. (**A**) Example of a colour-coded frame from a time-lapse movie that shows the three possible spindle orientations in the embryo, quantified for the 128- to 256-cell division in 10 embryos (n=447 divisions). Par., parallel; Per., perpendicular; Obl., oblique, as defined in the Materials and methods. (**B**) These three types of spindle orientation occur also in isolated blastomeres at 128-cell stage, as example frames from Movies 1-7 in the supplementary material show; however, the proportion of each type of division differs. (**C**) Comparison of the proportions of the three division types between the embryo (white bars) and isolated blastomeres, n=151 (black bars). Oblique and perpendicular divisions have significantly different proportions in isolated cells when compared with the embryo (χ-square goodness of fit, P=0.001). (**D**) In control embryos, cells with a very small apical surface (less than or equal to one quarter of its sister) will always divide perpendicularly and the apical pigment is not divided. Arrows in D indicate cells with a small apical domain. (**E**,**F**) In isolated blastomeres (E) and in embryos that were raised in Ca^{2+}/Mg^{2+}-free medium (D), such small apical surfaces do become divided. (F) Comparison of frequencies with which the cleavage plane divides a small apical surface. In control embryos: 0% (n=42 divisions in time-lapse movies of 24 embryos). In embryos raised in Ca^{2+}/Mg^{2+}-free medium, with rounded cells: 32% (n=60 cells with small apical surface were analysed in 12 different embryos).

definitions in the Materials and methods). For comparison, division types in the intact embryo were scored in time-lapse movies (Fig. 2A,C).

Three types of division were observed, as is the case also in the embryo (Fig. 2A,B). However, in the intact embryo, there are a number of columnar cells, and these tend to divide perpendicularly. In isolation, cells round up and therefore the number of perpendicular divisions should decrease. Indeed, in isolated, spherical cells, the number of perpendicular divisions decreased to 16% from 26% (Fig. 2C). We note that the numbers for each division type in round cells are not evenly distributed between the three categories (51% parallel, 16% perpendicular, 33% oblique), which could be interpreted as some sort of regulation of division orientation. However, these numbers are very close to what one would expect from random divisions, if one takes into account the geometrical definition of each division type in a sphere by its difference in surface area, which corresponds to the probability for each division type (see Fig. S1 in the supplementary material).

The size of the apical domain does not determine spindle orientation in round cells

In the embryo, superficial cells that are elongated towards the interior also have a small apical domain and cleave perpendicularly in a very high number of cases (90%) (Chalmers et al., 2003), or in other words, they have a very low probability of bisecting the apical surface upon division (Fig. 2D,F). Either of these parameters, the long axis or the size of the apical domain could influence spindle orientation. For example, in *C. elegans* the Par3/Par6 complex inhibits spindle rotation in the anterior (AB) cell of the zygote (reviewed by Doe and Bowerman, 2001; Knoblich, 2001). If some apical complex inhibited rotation into the apicobasal axis in *Xenopus*, one could imagine that in cells with 'less' apical complex, the spindles would rotate into the apicobasal axis.

To distinguish the influence of the long axis versus the size of the apical domain in orienting the spindle, we have analysed spindle orientation in embryos raised in Ca^{2+}/Mg^{2+}-free medium versus controls (Fig. 2D), as well as in isolated rounded blastomeres with a small apical surface (Fig. 2E). Daughter cells of an oblique division that had an apical surface of one-quarter of the other daughter, or less, were selected. In contrast to the situation in control embryos, where such cells never bisect their apical surface, in rounded isolated cells the apical membrane was often bisected (Fig. 2E; data not shown). In embryos raised in Ca^{2+}/Mg^{2+}-free medium, 32% of cells with such a small apical domain bisected it upon division (Fig. 2F). Thus, it seems that the size of the apical domain is unlikely to be the primary factor influencing the orientation of division.

Blastomeres align the spindle with an experimentally induced long axis

To test whether the long axis determines spindle orientation, we examined cleavage plane orientation in isolated round cells after a long axis was imposed experimentally. Single blastomeres from 128/256-cell stage embryos were compressed (see Materials and methods for details) and the orientation of the cleavage plane was classified with respect to the imposed long axis of the cell (Fig. 3A).

Cells divided at different times after compression, because they were at different time-points of the cell cycle when isolated (Fig. 3A,B). In cells that divided within 3 minutes of compression, all three types of division orientations were observed. When division occurred between 3 and 5 minutes after compression, almost 80% of divisions bisected the long axis. The frequency of such divisions increased to 100% for divisions that took place between 5 and 15 minutes (Fig. 3B). Divisions after more than 15 minutes did not take place.

The stage of mitosis five minutes before the onset of cytokinesis, corresponds to mid-metaphase, as determined by analysing confocal time-lapse movies of *tau-GFP*- and *Histone-GFP*-injected embryos (data not shown). We conclude that blastula cells are able to respond to external shape cues with spindle alignment into the longest axis of a cell, but only before mid-metaphase. This is in agreement with previous reports in other systems that the cleavage plane position becomes fixed around anaphase onset (Gonczy and Hyman, 1996; Reinsch and Karsenti, 1994; Zieba et al., 1986).

Spindle alignment with the long axis takes place in prophase; no large angle rotations are observed

To characterise the temporal dynamics of spindle orientation in the *Xenopus* blastula, we have analysed the movements of the centrosome/spindle axis in *tau-GFP* injected embryos from late prophase/prometaphase, until late anaphase (Fig. 4).

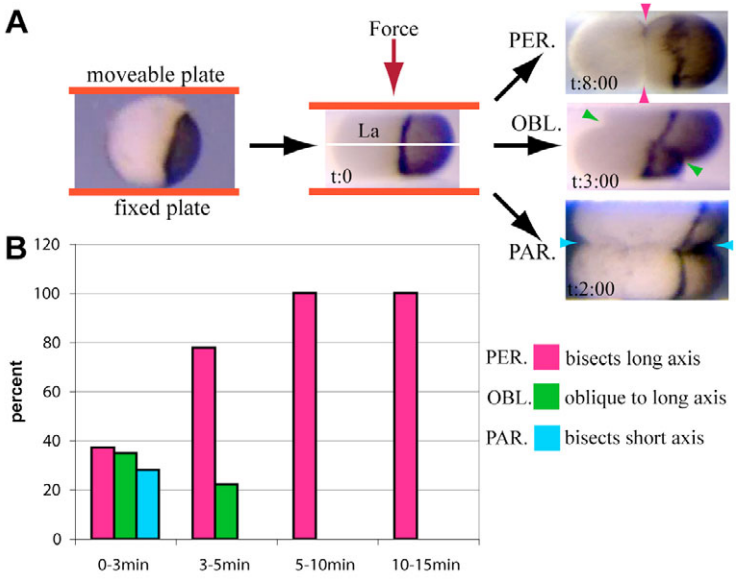

Fig. 3. Blastula cells align the spindle with an experimentally induced long axis. (**A**) Round isolated blastomeres were compressed and filmed to assess the orientation of the cleavage plane with respect to the introduced long axis: parallel (spindle perpendicular to the long axis), perpendicular (spindle parallel to long axis) and oblique (orientation between these two categories). Arrowheads indicate cleavage planes. (**B**) Cleavage plane orientations were analysed with respect to the time that has elapsed between mechanical deformation and cytokinesis (n=100 divisions). The shorter the elapsed time between deformation and cytokinesis (0-3 minutes, 3-5 minutes), the smaller the percentage of spindles that align with the long axis. When a long axis is imposed 5-15 minutes before cytokinesis, the spindle aligns with it in 100% of cases. The correlation between division type and time, measured to the nearest half minute, was highly statistically significant (Spearman's correlation –0.647, n=100).

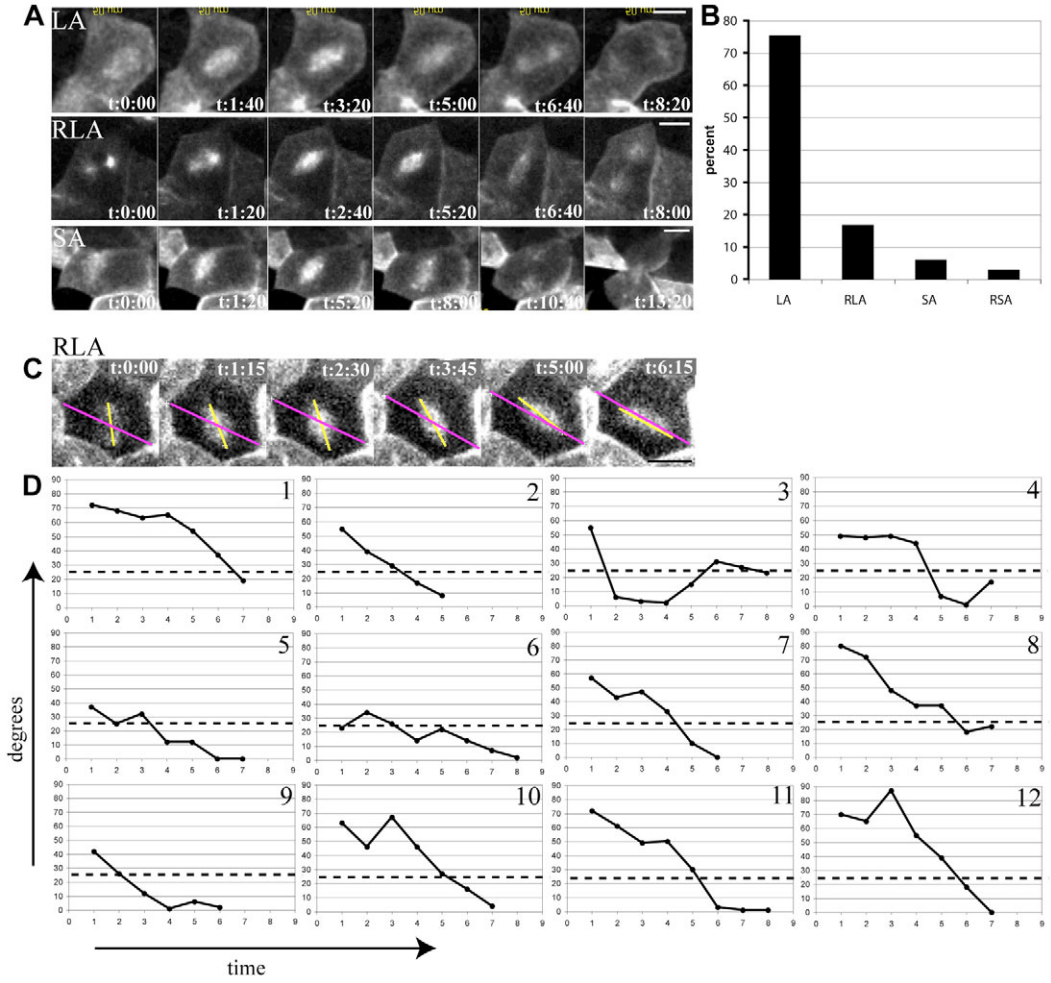

Fig. 4. Spindle alignment takes place early in the cell cycle; large angle oscillations do not occur. (**A**) Different spindle orientation behaviour is shown in frames from 4D series of *tau-GFP* injected embryos; LA, spindle is already set up within 25° of the long axis at prophase/prometaphase; RLA, spindle rotates into the long axis; SA, spindle is already set up within 25° of the short axis; RSA, spindle rotates into the short axis (see Movie 6 in the supplementary material). (**B**) Quantification of spindle orientation behaviour in 216 cells from 11 different embryos. The majority of spindles are already close to the long axis when the spindle is set up. (**C**) Example frames of a spindle rotating into the long axis, RLA. Scale bars in A,C: 20 μm. (**D**) Analysis of the temporal dynamics of such rotation movements in 12 cells from three different embryos. The angular changes (*y*-axis) with respect to the long axis are plotted against time (*x*-axis). Time intervals between frames are 92 seconds in cells 1-8 and 75 seconds in cells 9-12. The broken line indicates 25°. Overall, orientation movements into the long axis occur via small angle adjustments as for example in graphs 1 and 11 (number 11 corresponds to the cell shown in C).

We found that the majority of spindles were already within 25° of the long axis at prometaphase/metaphase (Fig. 4A,B: LA, 75%; see Movie 4 in the supplementary material). The second most common spindle behaviour was rotation into the long axis (Fig. 4A,B; RLA, 16%; see Movie 5 in the supplementary material). Spindles that were set up in the short axis (SA) or rotated into the short axis (RSA; see Movie 6 in the supplementary material) were found only occasionally in 6% and 3% of cases, respectively (Fig. 4A,B). Thus, in the great majority of cases, spindles are already close to the long axis at the onset of metaphase, which indicates that spindle orientation in the *Xenopus* blastula takes place early in mitosis.

In order to show the temporal dynamics of any rotation movements that may occur, angle values between the long axis and the spindle axis were plotted against time for 12 cells from the RLA category, from three different embryos (Fig. 4C,D). The first movie frame analysed was always the first time point when the two centrosomes became clearly visible on opposite sides of the nucleus, and the last frame was one time-point before the onset of midbody constriction (Fig. 4C). These angle/time graphs show that overall spindle alignment is a gradual movement towards the long axis (Fig. 4D). Although oscillating changes of orientation can be observed between time-points in some cells (e.g. cells 3,4,10,12) oscillations generally do not involve large degree changes of spindle orientation (Fig. 4D). In most cases, spindles adjust in steps smaller than 20° between time points (time interval 92 seconds in cells 1-8 and 75 seconds in cells 9-12). Therefore, systematic oscillations with large angle changes in directionality do not occur in the *Xenopus* blastula.

To exclude the possibility that *tau-GFP* expression might have influenced microtubule dynamics and consequently spindle behaviour, these results were confirmed in immunostained fixed embryos. Fig. 5 shows angle measurements as absolute deviation of the spindle from the long axis at prophase (Fig. 5A), metaphase (Fig. 5B) and anaphase (Fig. 5C). The median deviation angle at prophase is 18°, at metaphase 12° and at anaphase 9°. To confirm specifically

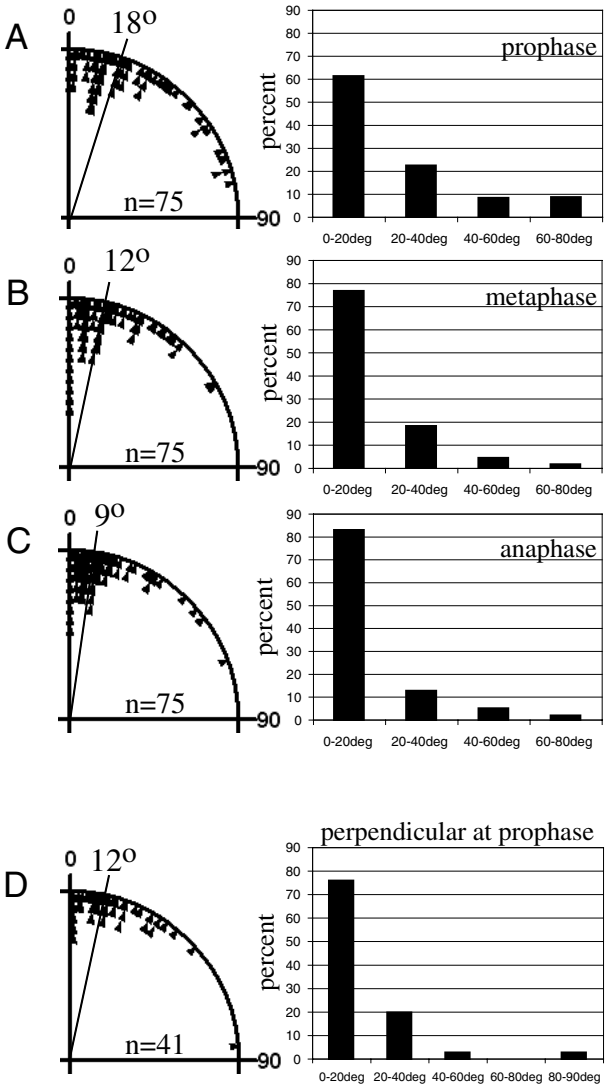

Fig. 5. Most spindles align with the long axis at prophase. The deviation of the spindle axis from the long axis was measured in parallel (symmetrically) dividing fixed cells at different time-points during mitosis (**A-C**). Charts on the left show the distribution of the raw data; *y*-axis=0° corresponds to long axis; each triangle represents one measurement. The radial line indicates the median angle deviation. Graphs on the right show the percentage of cells in angle classes of 20°. The majority of cells have their spindles aligned within 20° of the long axis throughout the cell cycle. (**D**) Cells with a spindle axis perpendicular to the surface were measured at prophase (*n*=41 cells).

that the spindle also aligns early with the long axis in cells that divide perpendicularly, measurements were taken in cells with a perpendicular centrosome orientation at prophase, in cross-section stacks. Fig. 5D shows that the median angle at prophase is 12° in these cells.

Astral microtubules are required for spindle orientation

To test the role of astral microtubules in spindle orientation, we have used the chemical microtubule polymerisation inhibitor nocodazole to reduce astral microtubules (Fig. 6A,D). Embryos at the 256-cell stage were incubated in nocodazole for 25 minutes (the average cell cycle time in the *Xenopus* blastula), and spindle orientation was analysed at anaphase onset in *z*-series of fixed embryos that were whole-mount stained for α-tubulin. Angle measurements between the spindle axis and the long axis of a cell were carried out as above. Reduction of astral microtubules leads to an increase of the observed median angle to 23°, indicating a trend towards randomisation of spindle orientation when compared with the controls (Fig. 6B,C,E,F). Thus, in the *Xenopus* blastula, astral microtubules are necessary for spindle alignment with the long axis of a cell.

Astral microtubules make contact with the cortex between anaphase and the end of the next prophase

To determine the time window in the cell cycle when cell shape is most likely to be monitored by astral microtubules, we looked at the quantitative dynamics of astral microtubules in vivo using *EB1-GFP* injected embryos. EB1 belongs to a highly conserved family of proteins that binds to microtubule plus-ends and centrosomes (Mimori-Kiyosue and Tsukita, 2003; Liakopoulos et al., 2003; Piehl and Cassimeris, 2003; Morrison et al., 1998; Rogers et al., 2002). Movies were generated from projections of image stacks that were collected on a spinning disk confocal microscope (Fig. 7A, see Movie 7 in the supplementary material). The mitotic stage was estimated by the signal from the spindle (Fig. 7A) or by co-injecting *Histone-GFP* to visualise DNA (data not shown) and the EB1 signals were counted (Fig. 7B). A period of EB1 reduction was observed during late prophase/prometaphase followed by complete absence of EB1 signal in metaphase, which could explain the observed lack of extensive spindle movements during this time period. Conversely, the time period of maximum astral microtubule density is between anaphase and prophase of the next cell cycle (see also Morrison and Askham, 2001). In particular, the high density of astral microtubules in prophase is consistent with our previous results that the main orientation event has already taken place by prophase.

To exclude the possibility that this observed reduction of EB1 plus end signals is due to a loss of binding activity of EB1 to microtubules rather than to a reduction of microtubules, we have examined the distribution of astral microtubules in fixed cells. We could confirm a reduction of astral microtubules during prometaphase and metaphase in fixed cells (Fig. 7C).

Interestingly we observed that the centrosomes begin to separate at late anaphase, and are already several micrometers apart before cytokinesis is completed (Fig. 7C, arrow in anaphase 2). This is also the time period of maximum astral microtubule density and it is therefore possible that already centrosome separation takes place according to the long axis of a cell. This would explain the observation that the majority of spindles are already set up in the correct orientation at prometaphase.

DISCUSSION

The *Xenopus* blastula exhibits many of the characteristics expected from a developmental system that uses oriented divisions to generate cell fate diversity. First, cells of the *Xenopus* blastula are polarised; they exhibit the hallmarks of epithelial cell polarity with conserved molecular markers distributed on the apical or basolateral membrane (Chalmers et al., 2003; Chalmers et al., 2005; Muller and Hausen, 1995; Gawantka et al., 1992). Second, conserved genes play a role in controlling polarity as they do in other systems; misexpression and/or loss of aPKC and Lgl2 function alters the proportion of apical versus basolateral membrane (Chalmers et al., 2005). Third, divisions where the spindle is oriented along the apicobasal axis are fate asymmetric (Chalmers et al., 2003). Such divisions generate cells with distinct fates throughout the blastula and in particular in

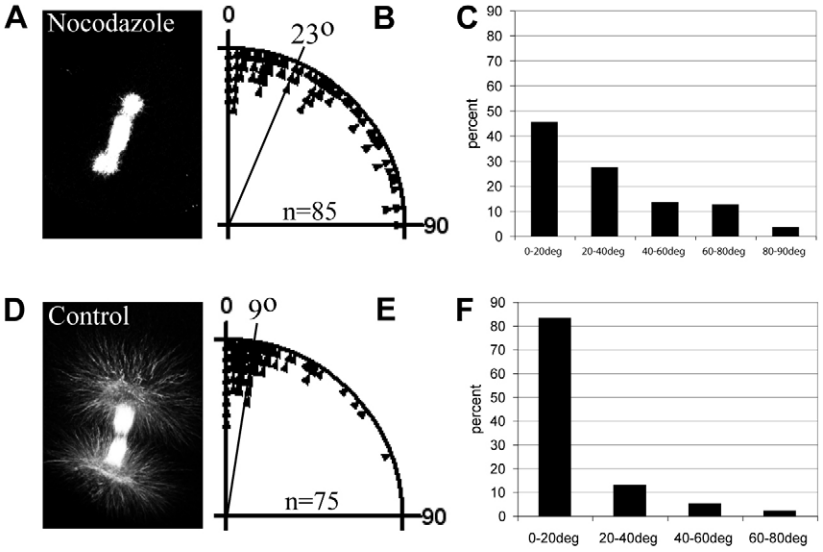

Fig. 6. Astral microtubules are important for spindle alignment into the long axis. The deviation of the spindle axis from the long axis was measured in cells treated with nocodazole. (**A**,**D**) Antibody staining for α-tubulin in treated (A) and control (D) cells. (**B**,**C**) A reduction of astral microtubules leads to an increase of the median deviation from the long axis compared with the controls (**E**,**F**). Eighty-five cells were analysed in ten nocodazole-treated embryos and 75 cells in 12 control embryos. The difference between the median angles is statistically significant by the Mann-Whitney test ($P<0.001$).

the animal region, they have an intrinsic difference in competence for neuronal differentiation (Chalmers et al., 2002). Although the molecules underlying these fate differences are only just beginning to be elucidated (Chalmers et al., 2006), it is clear that divisions at the blastula stage bear all the hallmarks of typical fate-asymmetric divisions of polarised cells.

However, as we show in this paper, the *Xenopus* blastula differs from other systems of oriented divisions in that it appears to lack a specialised mechanism to orient the spindle according to polarity. Instead, cells divide according to their shape, by aligning the spindle with the long axis and bisecting this axis during division. Thus, the cells that are elongated in the apicobasal direction have a high propensity of dividing perpendicularly (bisecting the axis of polarity), and consequently generate daughter cells with different fates. Although the apical membrane domain with its resident proteins/protein complexes may prove to be important for cell fate determination (Gotz and Huttner, 2005; Chalmers et al., 2003; Chalmers et al., 2005), it does not seem to be important for spindle orientation.

Spindle orientation: default versus specialised mechanisms

Cell division where the spindle is set up along the long axis of the cell, is often thought to be a default mechanism, taking place when other cues of spindle orientation are absent (Honda, 1983). Division along the long axis was first formulated in the 19th century as a general rule of division by Hofmeister and Sachs, based on observations in plant cells, and studied experimentally by Hertwig and others in amphibian eggs (reviewed by Wilson, 1987). Only a few examples where cells divide according to their long axis during development were studied experimentally, such as some cells in the zebrafish embryo (Concha and Adams, 1998), the mouse zygote (Gray et al., 2004) and *Xenopus* eggs, which align their spindles into the long axis when mechanically deformed (Black and Vincent, 1988). Finally, normal rat kidney (NRK) in culture divide and bisect their long axis; as described for the blastula cells here, they also correct their spindle orientation to adjust to an experimentally imposed new long axis (O'Connell and Wang, 2000).

However, in many cases the influence of cell-shape is over-ridden. For example, during zebrafish early development, cells that are located in the dorsal epiblast divide such that the cleavage plane bisects their short rather than their long axis, the opposite of what one would predict from geometric rules (Concha and Adams, 1998; Gong et al., 2004). In this case, 'ignoring' the long axis is thought to be mediated by the planar polarity pathway (PCP), (Gong et al., 2004), which has also been implicated in orienting cell divisions in *Drosophila* SOPs (Gho and Schweisguth, 1998) and *C. elegans* embryos (Schlesinger et al., 1999). Activation of this pathway presumably overrides the default influence of the long axis. In most examples of asymmetric divisions of polarised cells, the influence of cell shape has not been studied directly but it is believed not to be important as many specialised cues for spindle orientation have been described. It is presumed that these specialised cues are dominant over any influence of cell shape in defining the spindle orientation (Tsou et al., 2002; Tsou et al., 2003a) (reviewed in Cowan and Hyman, 2004). In cultured cells, the influence of cell shape can also be over-ridden by the spatial distribution of extracellular matrix components (Thery et al., 2005).

Spindle rotations and timing of spindle alignment

Many different cases have been described with regards to the timing of spindle alignment and the rotation behaviour of the spindle during mitosis. In *Drosophila* neuroblasts, the spindles are set up parallel to the plane of the epithelium and rotate by 90° in metaphase to align with the apicobasal axis (Kaltschmidt et al., 2000), although more recent reports place the alignment earlier in the cell cycle (Siegrist and Doe, 2006). In zebrafish keel/rod stage embryos, the majority of the spindles are set up parallel to the plane of the epithelium but undergo a rapid 90° rotation in the neural keel region (Geldmacher-Voss et al., 2003). Extensive metaphase rotations have also been described in the rat (Adams, 1996) and mouse (Haydar et al., 2003) cortex, including (in the mouse) 'flipping' between the plane of the epithelium and the apicobasal axis. However, in the rat cortex, most of the rotations are confined within the plane of the epithelium, either parallel or anti parallel to the final division axis, suggesting that a preference for alignment is set up early in the cell cycle (Adams, 1996).

We have reported here that in the *Xenopus* blastula, the final spindle orientation, which is aligned with the long axis, is already chosen at prophase, and that this is possibly achieved by the directed separation of duplicated centrosomes in the 'correct' direction. Spindles that are misaligned with respect to the long axis adjust with

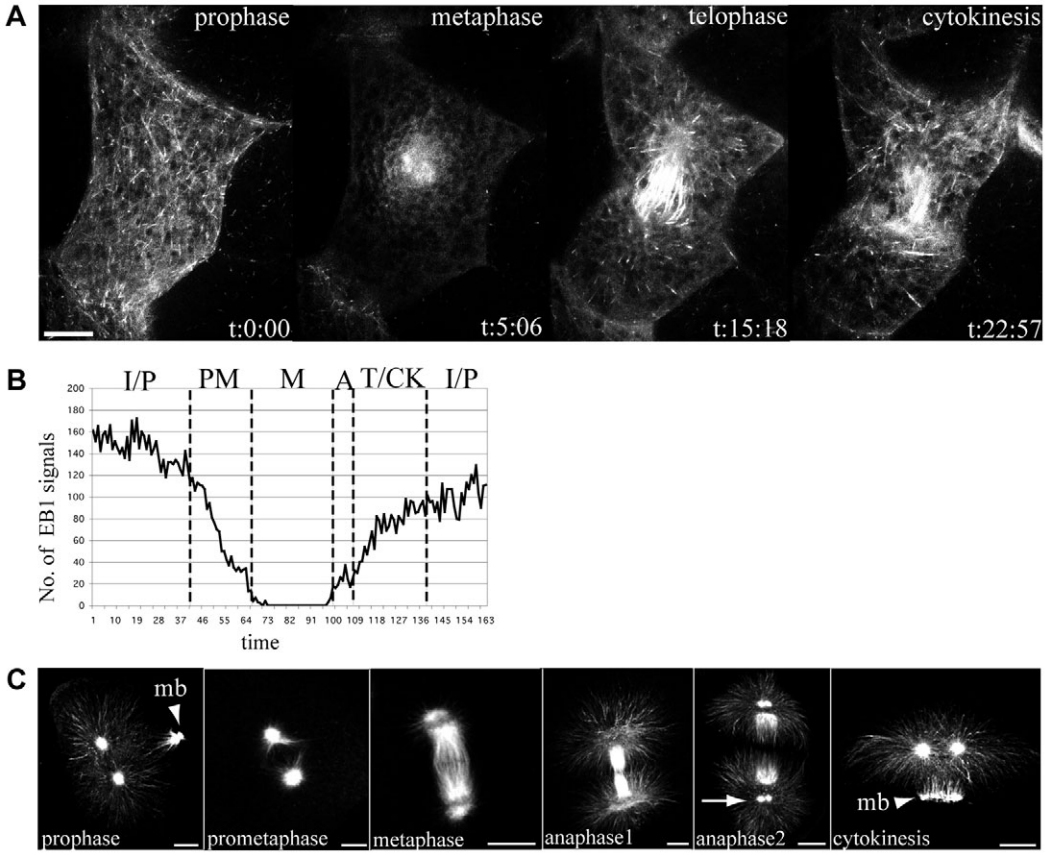

Fig. 7. Astral microtubules are most abundant in prophase and anaphase/telophase. (**A**) Example frames from a time-lapse movie showing a cell expressing *EB1-GFP*. Movies were generated from projections of *z*-stacks with an imaging depth of about 15 μm to visualise quantitative changes in cortical astral microtubules. Cortical EB1 signal disappears around metaphase. Seven cells were analysed throughout mitosis in three different embryos. (**B**) Quantification of EB1 signals throughout the cell cycle of one cell. I/P, interphase/prophase; PM, prometaphase; M, metaphase; A, anaphase; T/CK, telophase/cytokinesis; *y*-axis, number of EB1 signals; *x*-axis, time (15.3 seconds between frames). (**C**) Astral microtubules shown in fixed cells by antibody staining against α-tubulin. During prometaphase and metaphase, astral microtubules are strongly reduced; mb, midbody. Centrosomes are separated in late anaphase (arrow). Scale bars: 20 μm.

small angle steps towards it. In this sense, *Xenopus* blastomeres are more similar to the rat and chick retina, where spindles show only small rotations within a 30° band and the angle that the spindle assumes at metaphase entry tends to be the final angle at division (Tibber et al., 2004). Interestingly, similar to our case, but in contrast to the zebrafish retina (Das et al., 2003), in these systems the orientation of the spindle was found to be entirely random with respect to the anatomical landmarks of the retinal tissue (Tibber et al., 2004).

The reason for these differences in spindle behaviour across different systems is not known but it may ultimately be related to the mechanisms that align the spindle, such as the presence or absence of specialised orientation cues and the timing or mechanism by which astral microtubules interact with the cortex (e.g. Sanada and Tsai, 2005; Du and Macara, 2004).

Shape and mechanisms of spindle orientation

Spindle orientation based on specialised cues depends on the interaction of microtubules with specialised cortical domains, which can be defined either by polarity or cell contact (Doe and Bowerman, 2001; Gonczy, 2002; Thery et al., 2005). It is thought that these specialised cortical sites or cortical domains act as attachment sites or modulate forces on microtubules to 'pull' the spindle in a preferred orientation. By contrast, the mechanisms that orient the spindle according to cell shape are thought to rely on an overall balancing of forces acting on the spindle, which can only be achieved when the spindle is centered and aligned with the long axis (O'Connell and Wang, 2000). Different models, suggest either microtubule polymerisation – based pushing forces or motor-driven pulling forces (reviewed by Reinsch and Gonczy, 1998; Grill and Hyman, 2005; Vallee and Stehman, 2005). The common element is that forces should be proportional to microtubule length and that adjustment of spindle positioning takes place until a balance of forces on the spindle ends is achieved. Either of these models would be consistent with our observation that astral microtubules are important for spindle alignment in *Xenopus*.

Concluding remarks

One of the attractive features of the developmental system we described here is its simplicity. Interpreting the overall cell shape simultaneously defines the plane and the orientation of division within the plane. Furthermore, there is no need for a specific mechanism to 'select' cells that will divide perpendicularly. As long as the dividing cells are polarised and polarity is important for fate, perpendicular divisions that are randomly distributed with respect to anatomical landmarks in the blastula will generate inner and outer cells that will follow different fates.

In conclusion, a default mode of spindle orientation, featuring an element of spatial randomness, is sufficient for this system of polarised cells to generate different daughter cells. Thus, we propose that it is not obligatory to evoke a specialised mechanism for spindle orientation in all systems that generate cell fate asymmetry by division. Although well-studied examples from invertebrates have greatly influenced our thinking, perhaps we should expect more variety in the ways that different systems have adapted to control spindle orientation to generate cell fate diversity.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/19/3883/DC1

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