In vivo imaging reveals a role for Cdc42 in spindle positioning and planar orientation of cell divisions during vertebrate neural tube closure

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Summary
Specialization of the cell-division process is a common feature of developing embryos, but most studies on vertebrate cell division have focused on cells dividing in culture. Here, we used in vivo four-dimensional confocal microscopy to explore the role of Cdc42 in governing cell division in the developing neural epithelium of Xenopus laevis. We find that Cdc42 is crucial for stable positioning of the metaphase spindle in these cells, but was not required for spindle positioning in epidermal epithelial cells. We also find that divisions in the Xenopus neural plate are planar oriented, and that rotations of mitotic spindles are essential for establishing this orientation. When Cdc42 is disrupted, spindles over-rotate and the final orientation of divisions is changed. Finally, the planar orientation of cell divisions in this tissue seems to be independent of planar cell polarity (PCP) signaling and does not require normal neural morphogenesis. Our data provide new insights into the coordination of cell division and morphogenesis in epithelial cell sheets and reveal novel, cell-type-specific roles for Cdc42 in spindle positioning and spindle orientation.

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Key words: Cdc42, Metaphase plate, Neural tube closure, Oriented cell division, Spindle

Introduction
Developmentally regulated cell division is a central facet of embryogenesis, and how cells establish orientation and asymmetries during cell division is a fundamental issue at the interface of cell and developmental biology. Precisely oriented divisions are important for controlling determination of the embryonic axes in Caenorhabditis elegans (Gonczy and Rose, 2005; Gotta et al., 2001), for axis elongation and epithelial-tube elongation in vertebrates (Concha and Adams, 1998; Fischer et al., 2006; Gong et al., 2004; Saburi et al., 2008) and for the diversification of cell types in the embryonic nervous systems of Drosophila and Xenopus (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Chalmers et al., 2003; David et al., 2005).

Oriented cell divisions have been thoroughly described in more mature vertebrate nervous systems as well. In the closed neural tube, divisions that occur parallel to the plane of the neural epithelium are generally proliferative, whereas divisions that are perpendicular to the apical membrane tend to be neurogenic (Gotz and Huttner, 2005; Haydar et al., 2003; Wilcock et al., 2007). Moreover, proliferative divisions occurring within the plane of the neural epithelium can display stereotypical orientations with respect to the anteroposterior axis. Earlier in development, the hollow neural tube forms by the intricate folding of an initially flat neural plate during a process termed neural tube closure (Wallingford, 2005). During neural tube closure in chick embryos, the axis of cell division is preferentially parallel to the long axis of the embryo (Sausedo et al., 1997), whereas, in zebrafish, divisions are initially parallel to the long axis and shift to a perpendicular orientation as neurulation proceeds (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Gong et al., 2004). The Xenopus embryo represents a special situation, however. The neural plate of Xenopus has two cell layers, a deep layer giving rise to the primary neurons and a superficial layer that only differentiates much later and continues to proliferate dramatically during neural tube closure (Chalmers et al., 2002; Hartenstein, 1989).

Many oriented cell divisions have a few controlling factors in common. In particular, the planar cell polarity (PCP) pathway has been found to be a major player. PCP signaling was first shown to regulate spindle rotations that orient cell divisions in Drosophila neuroblasts, facilitating the asymmetric distribution of determination factors (Bellaiche et al., 2001a; Bellaiche et al., 2001b; David et al., 2005). PCP signaling in the zebrafish governs orientation of cell divisions along the anteroposterior axis that facilitate elongation of the embryo (Gong et al., 2004). Most recently, PCP signaling has been found to control oriented cell division in the developing mammalian kidney (Saburi et al., 2008). It should be noted, however, that this role for PCP signaling in controlling oriented cell division is not universal. For example, extension of the germ band in Drosophila also relies on oriented cell divisions, but this orientation is independent of PCP signaling (da Silva and Vincent, 2007).

Another common player in developmental regulation of oriented cell divisions is the small GTPase Cdc42. In C. elegans, Cdc42 controls spindle orientation during the first cell cycle and thereby is involved in defining the anteroposterior axis (Gonczy and Rose, 2005; Gotta et al., 2001). A clear role for Cdc42 in controlling oriented cell division in vertebrate somatic cells has not been reported as yet, but Cdc42 is essential for proper localization and
organization of the meiotic spindle in mouse oocytes (Na and Zernicka-Goetz, 2006). Moreover, Cdc42 is activated asymmetrically on meiotic spindles in Xenopus oocytes, where it links spindle position to the position of the cytokinetic ring (Ma et al., 2006). These functions might reflect the role Cdc42 plays in bud-site selection in yeast (Kozminski et al., 2003).

The involvement of Cdc42 in oriented cell division in developing embryos is particularly intriguing in light of recent findings that suggest that Cdc42 plays cell-type-specific roles in many fundamental aspects of cell division. In some cell lines, Cdc42 has been reported to control microtubule attachment to kinetochore (Yasuda et al., 2004), whereas, in others, it governs assembly of the cytokinetic septime cytoskeleton, as it does in yeast (Caviston et al., 2003; Garcia et al., 2006; Joberty et al., 2001). Moreover, biosensors in live cells have revealed differing patterns of Cdc42 activity during division of different cell types (Garcia et al., 2006; Yoshizaki et al., 2003). These differences might reflect the fact that even fundamental aspects of cell division are under cell-type-specific developmental control.

By contrast, manipulation of Cdc42 in epidermal epithelial cells demonstrates a key role for Cdc42 in spindle stability in the cell center during metaphase. For these experiments, embryos were injected into both dorsal blastomeres at the four-cell stage with histone-2B–GFP mRNA to allow for monitoring of division orientation throughout the neural plate (Fig. 1C, 1). Embryos were then grown to the eight-cell stage and injected again, this time into only one of the dorsal, animal blastomeres (Fig. 1C, 2). This blastomere was injected with a combination of mRNA encoding our experimental construct and mRNA encoding membrane-RFP, to distinguish the experimental cells from normal cells at neurula stages (Fig. 1C, 2 and 3). Wild-type clones and experimental clones can then be compared directly within a single embryo.

Cdc42 is required to stably position metaphase spindles in neural-plate cells during neural tube closure

Cdc42 has been shown to control spindle position and orientation during cell divisions in early C. elegans embryos, and in mouse and Xenopus oocytes (Bement et al., 2005; Gotta et al., 2001; Ma et al., 2006; Na and Zernicka-Goetz, 2006). We therefore used mosaics (see above) to examine what role Cdc42 might play in controlling spindle dynamics in the Xenopus neural plate. During cell divisions in neural epithelial cells, the metaphase plate normally remains positioned stably in the cell center (Fig. 2A,A’). (Kieserman et al., 2008). In these mosaics, cells expressing the dominant-negative Cdc42-N17 displayed chaotic movements of the condensed chromosomes during metaphase (supplementary material Movie 2).

In control cells within these mosaics, metaphase plates remained in place, centered in the middle of the cell, as was observed in unmanipulated embryos (Fig. 2A,A’).

Cdc42-N17 disrupts Cdc42 function by blocking the ability of the GTPase to exchange GDP for GTP. Therefore, by binding and sequestering guanine-nucleotide exchange factors, Cdc42-N17 might disrupt the function of other GTPases that share such exchange factors (Karnoub et al., 2004; Zhang et al., 1995). To confirm the role of Cdc42 in spindle stability in the Xenopus neural plate, we repeated our experiment with another Cdc42 dominant-negative, Cdc42-F37A (Lamarche et al., 1996; Richman et al., 2002; Zhang et al., 1995). This construct is a more-specific disruptor of Cdc42 function, blocking the interaction between Cdc42 and its downstream effector proteins (Lamarche et al., 1996). In mosaics, cells expressing Cdc42-F37A displayed the aberrant movement of condensed chromosomes within the cell during metaphase. The violent movements in Cdc42-F37A-expressing cells can be observed in Fig. 2A,A’ and supplementary material Movie 3. Immediately after forming, the metaphase plate moves quickly from the upper-left-hand portion of the cell to the lower-left-hand portion. The plate...
then moves back, past the center of the cell, to the cortex in the upper left, and finally returns to the approximate center of the cell as anaphase begins (Fig. 2A,A’; supplementary material Movie 3).

We next sought to quantify the excessive spindle movement following manipulation of Cdc42. During neural tube closure, the entire neural plate is deforming dramatically, so it was necessary to develop a measurement scheme that isolates intracellular movement of the spindle from the overall movement of the cells in the neural plate. We therefore quantified the intracellular distance of metaphase-plate movement as a function of the length of the long axis of that cell, per unit of time (Fig. 3A). We performed these measurements over the 14 minutes preceding anaphase onset.

In control cells, metaphase plates moved on average only 5% of the cell length per minute (Fig. 3B), and metaphase plates almost never moved more than 20% of cell length in a given minute of time, with the maximum movement being 26% (Fig. 3C). By contrast, cells in embryos expressing either of the dominant-negative forms of Cdc42 frequently moved greater than 20% of cell length per minute, and the maximum movement for both manipulations was over 46% (Fig. 3D,E). Likewise, these nuclei on average moved greater than 8% of the cell length per minute (Fig. 3B). This difference in the average distance that was moved between control and experimental groups was extremely significant ($P<0.001$, Kruskal-Wallis multiple-comparisons test).

Cdc42 does not control spindle positioning in epithelial cells of the developing *Xenopus* epidermis

Given that core cytokinesis mechanisms differ surprisingly between neural epithelial cells and epidermal epithelial cells examined in vivo (Kieserman et al., 2008), we next investigated whether epidermal cell division was affected by disruption of Cdc42. We made in vivo 4D movies of *Xenopus* tail epidermis. In control epidermal cells, chromosomes separated to the geometric center of the new daughter cells and cytokinesis initiated at about 4 minutes after anaphase onset (supplementary material Movie 4) (Kieserman et al., 2008). In contrast to the effect on neural cells, metaphase, anaphase and cytokinesis in epidermal cells expressing Cdc42-N17 were indistinguishable from that in control cells (supplementary material Movie 5).

We quantified the intracellular movement of metaphase plates in epidermal cells and found that they moved on average only 3.9% of the cell length per minute, and movements of 20% or more of the cell length were never observed (Fig. 3F-H). Thus, intracellular movement of metaphase plates in non-manipulated neural cells was significantly greater than that of non-manipulated epidermal cells ($P<0.001$). The jostling of metaphase plates of neural cells might reflect the fact that the neural plate is actively moving, or it might be related to the spindle rotations that establish the planar orientation of these cells divisions (see below). Regardless, expression of

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**Fig. 1.** Analysis of cell divisions during neural tube closure. (A) Method to analyze cell divisions during neural tube closure in *Xenopus laevis*. Eggs are collected and undergo in vitro fertilization as described in the Materials and Methods. Solutions of mRNA, previously made by an in vitro transcription kit (Sigma), are created to label appropriate portions of cells (i.e. chromosomes via H2B-GFP or membranes via mem-RFP). Mixes of mRNA are put in small glass needles and are injected directly into the embryo at the four-cell stage by use of a picospritzer (1). For analysis of the early neural plate, the two dorsal cells are injected and, for analysis of the late tail epidermis, all four cells are injected. Embryos are then grown to an appropriate stage: stage 13 for early neural plate and stage 30 for late tail epidermis (2). Embryos are then placed on a microscope and imaged. Images of the early neural plate are obtained from horizontal confocal slices of the dorsal surface of the spinal-chord region prior to neural tube closure (3, see red box). Stacks are obtained every 30 seconds or 1 minute for 150 iterations (4). Movies are then projected into an x confocal projection in the Pascal program (5). Confocal projections are then exported to tiff files and analyzed by use of ImagePro Plus (6). (B) Use of this method allows for easy analysis of morphogenesis of the neural plate. Movies are started at early neural plate stage ($t=0$ minutes). As time progresses, neural folds come into view ($t=80$ minutes). These neural folds continue to move laterally until the neural plate is no longer well seen and the future epidermis of the embryo is easily seen ($t=80$ minutes and $t=110$ minutes) (supplementary material Movie 1). Scale bar: 50 m. (C) Method to assess division-polarity effectors. Embryos are injected so that they have different clones for analysis of polarity effectors. Embryos are injected on their entire dorsal side with a histone-2B-GFP marker at the four-cell stage (1). Embryos are grown up one stage, to the eight-cell stage, and injected with a membrane-RFP marker and an mRNA of interest (2). Embryos are grown to the late neurula stage and cells injected with the mRNA of interest can be distinguished by the expression of GFP and RFP, whereas control cells only express the GFP marker (3). DN, dominant negative. Scale bar: 100 m.
Cdc42-N17 had no effect whatsoever on the stable position of metaphase plates in dividing epidermal cells in vivo (Fig. 3F; compare Fig. 3H with 3G). These data demonstrate that Cdc42 plays a cell-type-specific role in governing cell division in vertebrate embryonic epithelia, revealing a neural-specific role for Cdc42 in the stable positioning of the metaphase spindle.

Planar orientation of cell divisions in the closing neural tube of *Xenopus*

In *C. elegans*, Cdc42 governs not only spindle positioning but also the orientation of cell divisions (Gotta et al., 2001). Because the orientation of cell divisions is developmentally regulated in the closing neural tube of other vertebrates, we investigated whether the same might be true for *Xenopus* and whether Cdc42 has any role in controlling the process. Previous results from fish and chick suggest that the planar cell divisions during neural tube closure would be aligned along the anteroposterior or mediolateral axes (see Introduction, above). We quantified the orientation of neural-plate cell divisions by plotting the position of the mitotic spindles on a rose diagram, and this analysis clearly demonstrates that the vast majority of cell divisions in the neural plate during neural tube closure are aligned roughly perpendicular to the anteroposterior axis.

However, the division angles of *Xenopus* neural-plate cells varied more widely than those observed in zebrafish (Concha and Adams, 1998); many cells in the *Xenopus* superficial neural plate divided at an angle that was oblique with respect to the mediolateral axis of the embryo (supplementary material Movie 6; Fig. 4A-B). Indeed, in our movies, more cells were observed dividing obliquely with respect to the mediolateral axis than were observed dividing directly parallel to the mediolateral axis (Fig. 4C). Curiously, the ‘diagonal’ or ‘slanted’ orientation of a cell-division angle was irrespective of the mediolateral position of a cell in the neural plate. Fig. 4A provides an example: within a small area on the right side of a
neural plate (Fig. 4A,A') some cells were observed to divide with their anterior-most daughter cells facing towards the midline (Fig. 4A', pink arrows), whereas other cells in the same region divided with their anterior-most daughter cells away from the midline (Fig. 4A', blue arrows). In many cases, we observed adjacent cells in the neural plate dividing simultaneously, one oriented obliquely towards the midline and the other oriented obliquely away from the midline (supplementary material Movie 7; Fig. 4B).

To quantify the orientation of cell divisions, each division was assigned an angle on the basis of the position of the anterior-most daughter cell relative to the midline. To simplify this quantification, all division angles were converted to represent divisions on the right side of the embryo. Thus, a positive value indicates an orientation obliquely towards the midline (Fig. 4, pink arrows) and a negative value indicates a division obliquely away from the midline (Fig. 4, blue arrows). A cell dividing parallel to the mediolateral axis is given an angle of 0°. Representative division angles are provided in Fig. 4A',B. This data can also be gleaned from a color-coded rose diagram (Fig. 4C). The circles in the rose diagram refer to the number of data points for each bin (the outer ring in Fig. 4C represents n=40).

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We next sought to characterize the cellular basis of oriented cell division in the closing *Xenopus* neural tube (supplementary material Movies 8, 9). Because our in vivo imaging approach allows for in-depth analysis of the dynamics of cell division, we were able to observe that metaphase plates often rotated just prior to anaphase (supplementary material Movie 9). Such rotation was reminiscent of mitotic-spindle rotations observed in a variety of developmentally controlled cell divisions (Bellaiche et al., 2001a; Geldmacher-Voss et al., 2003; Haydar et al., 2003; Kaltenschmidt et al., 2000). We therefore examined microtubule dynamics in the neural plate by expression of tau-GFP (Kwan and Kirschner, 2005), and we observed dramatic rotations of the mitotic spindle (supplementary material Movie 8; Fig. 5B).

We quantified the orientation of the mitotic spindles upon their initial formation during metaphase and found that spindles were initially oriented more or less randomly with respect to the midline (Fig. 5A,B, white lines; Fig. 5C). However, when the angle of the spindle in these cells was assessed again at anaphase, the pattern reflected the mediolateral orientation of telophase cell divisions described above (Fig. 5A,B, yellow lines; Fig. 5D).

Disruption of Cdc42 function results in over-rotation of mitotic spindles and shifts the angle of cell division

Because Cdc42 controls spindle positioning, we investigated whether Cdc42 also controlled spindle rotation. Indeed, we found

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Mitotic spindles are easily seen and a rotation of 15° is observed before anaphase onset. White line represents the orientation of spindle-pole separation (supplementary material Movie 8). Scale bars: 10 μm. (C) Rose diagram of the angle of 144 cells in six embryos expressing membrane-RFP and tau-GFP. Angles of spindle-pole separation are shown from 0° to 360° in bins of 10°. Yellow line represents the orientation of spindle-pole separation. (D) Rose diagram of the angle of cell division in the same 144 cells shown in C. Angles of spindle set up are shown from 0° to 360° in bins of 10°.

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that disruption of Cdc42 function resulted in spindles rotating to a far greater extent than those in control cells (compare supplementary material Movie 10 with Movie 9 and Fig. 6C with Fig. 6A). Control cells in the early neural plate had a restricted field of movement: the average rotation was 45° (Fig. 6E), the majority of control cells rotated between 0° and 60°, and we rarely observed control cells that rotated beyond 120° (Fig. 6B). By contrast, metaphase plates in cells expressing Cdc42-F37A rotated farther, with an average of 78° (Fig. 6E). Cdc42-F37A-expressing cells were frequently observed to rotate more than 90° (compare Fig. 6D with 6B) and, in many cases, chromosome rotation exceeded 200° (Fig. 6D). The mean degree of rotation in Cdc42-F37A-expressing cells was significantly higher than that of controls (P = 0.0005 using the Mann-Whitney U-test).

We next examined the final orientation of cell divisions following manipulation of Cdc42. Expression of Cdc42-N17 resulted in a shift in the final angle of cell divisions such that the number of cells with anterior daughters facing away from the midline increased at the expense of the number of cells with anterior daughters facing towards the midline (Fig. 7C,C'). Thus, the average angle of cell division shifted roughly 18° following expression of Cdc42-N17.

Injection of mRNA encoding Cdc42-F37A elicited the same specific alteration to the orientation of neural-cell division. In Cdc42-F37A-expressing clones, the number of cells dividing with their anterior-most daughter cell oriented laterally increased at the expense of those oriented medially, resulting in a 12° shift in the average orientation of division (Fig. 7B,B'). The Kolmogorov-Smirnov (K-S) test confirmed a significant shift in the distribution of cell-division orientations in these cells, as compared with controls (P = 0.001 and 0.039, respectively). These data suggest that Cdc42 contributes to the distribution of oriented cell divisions in the Xenopus neural plate.

Spindle rotations align divisions with the cellular long axis, but cellular axis alignment is not affected by disruption of Cdc42. "Hertwig’s rule" states that cells generally divide along their cellular long axis and, in mammalian cells in culture, variable rotations align the mitotic spindle with the long axis of the cell (Hertwig, 1893;...
O’Connell and Wang, 2000; Thery and Bornens, 2006). We therefore examined cell shape in the Xenopus neural plate and found that the long axes of cells in the neural plate were oriented in an angle comparable to the angle of observed divisions (Fig. 8A, compare to Fig. 3C). Thus, cell shape is initially polarized in these neural-plate cells, although spindles form in random orientations; spindle rotations then align the spindles such that division occurs parallel to the long axis of the cell (supplementary material Movie 8). When we assessed the alignment of cellular long axes in embryos expressing Cdc42-F37A or Cdc42-N17, we found no difference from controls (Fig. 8B,C) (K-S test, P = 0.431 and 0.083, respectively). We also found that manipulation of Cdc42 did not affect the length-to-width ratio of these cells (not shown). These data suggest that Cdc42 does not govern cell polarization in this tissue, but rather acts directly to govern the positioning of the spindle within the cells.

Cell-division orientation is not controlled by PCP signaling and does not require normal neural tube morphogenesis

Given the very specific role for Cdc42 in governing the distribution of oriented neural-cell divisions (above), we next examined PCP signaling, which might play a broader role than Cdc42 in orienting these divisions in Xenopus, because it controls oriented divisions in the fish and the fly (Bellaiche et al., 2001a; Gho and Schweisguth, 1998; Gong et al., 2004; Kaltschmidt et al., 2000). To disrupt PCP signaling, we expressed Xdd1, a dominant-negative deletion of the PCP protein Dishevelled that effectively disrupts planar polarization of cell behavior in several contexts, including polarized cell division in zebrafish (Gong et al., 2004; Park et al., 2008; Wallingford et al., 2000). Surprisingly, overexpression of Xdd1 did not alter the predominant mediolateral orientation of these divisions and did not affect the distribution of oblique cell divisions in the neural tube (Fig. 9B, compare to 9A). The K-S test for comparison of histograms was used to confirm that there was no difference in the distribution
of cell-division orientations in control and Xdd1-expressing cells \((P=0.892)\).

To confirm this result, we also tested the effect of Nxfz-8, a dominant-negative version of Frizzled-8 that disrupts PCP-mediated convergent extension (Deardorff et al., 1998). Similar to Xdd1, Nxfz-8 did not alter the orientation of neural-cell divisions (Fig. 9C) \((K-S\ test, \ P=0.49)\). Finally, we tested overexpression of wild-type Frizzled-8, which also disrupts PCP-mediated convergent extension \((\text{Deardorff et al., 1998; Wallingford et al., 2001})\). Overexpression of wild-type Frizzled-8 also failed to alter cell-division orientation \((\text{not shown, K-S test}, \ P=0.296)\). Our data thus suggest that the PCP signaling system is dispensable for oriented cell divisions in the \textit{Xenopus} neural plate, similar to PCP-independent oriented cell divisions in the extending \textit{Drosophila} germ band \((\text{da Silva and Vincent, 2007})\).

Although dispensable for oriented cell divisions, PCP signaling is required for normal neural tube closure \((\text{Wallingford and Harland, 2002})\), and we noted that expression of either Xdd1 or Nxfz-8 resulted in severely disrupted neural morphogenesis \((\text{data not shown})\) \((\text{Wallingford and Harland, 2001; Wallingford and Harland, 2002})\). This phenotype serves as a useful indicator for the efficacy of our reagents, and this result also suggests that cell-division orientation is independent of neural morphogenesis. To further test the independence of cell-division orientation and neural morphogenesis, we examined cell divisions in clones of cells expressing Shroom3-ASD1, a dominant-negative form of the Shroom3 protein that potently disrupts neural tube closure \((\text{Haigo et al., 2003})\). Expression of Shroom3-ASD1 in clones disrupted neural tube closure \((\text{not shown})\) but did not change the pattern of oriented cell divisions \((\text{Fig. 9D})\) \((K-S\ test, \ P=0.127)\).

**Discussion**

The specialization of cell-division mechanisms is a hallmark of developing embryos. Perhaps the most notable specialization is the absence of G1 and G0 phases of the cell cycle in the first several divisions of most embryos \((\text{Etkin, 1988; Masui and Wang, 1998; Philpott and Yew, 2008})\). Orientation of cell-division angle is another important specialization that is essential for normal development in many animals, and a small number of conserved mechanisms are typically associated with setting up these division planes. Here, we describe a novel mode of oriented cell division that occurs in the closing neural tube of \textit{Xenopus} embryos.

Previous studies have shown that cells tend to divide in one particular orientation within a given tissue and rotations of the mitotic spindle are a general feature of oriented cell divisions. Typically, spindles rotate to a precisely defined degree to achieve this orientation for a given tissue \((\text{Bellaiche et al., 2001a; Bellaiche et al., 2001b; Concha and Adams, 1998; Gong et al., 2004})\). For example, during oriented cell divisions in \textit{Drosophila} neuroblasts and in the neural plate of zebrafish embryos, mitotic spindles consistently rotate precisely 90° \((\text{Geldmacher-Yoss et al., 2003; Kalt schmidt et al., 2000})\). In \textit{C. elegans} embryos, some cells undergo rotations of 90° whereas others rotate 45° \((\text{Hyman and White, 1987})\). In all of these cases, however, spindles in a given cell type consistently rotate to a precisely set degree.

We found that these themes are not universal, and we describe here a system of oriented cell divisions in which variable rotations align spindles in the \textit{Xenopus} neural plate. Some spindles divided in approximately the orientation that they were set up in, whereas others underwent large, rapid rotations to divide nearly perpendicular to the orientation in which they set up \((\text{Fig. 5})\). We also found that, although the extent of spindle rotation varied from cell to cell, all spindles rotated to the extent necessary to align them with the cellular long axis \((\text{Fig. 8})\). Similar orienting rotations have been observed in cultured cells when cell shape is experimentally altered \((\text{O’Connell and Wang, 2000})\).

It is noteworthy that the orientations of cells on opposite sides of the midline are mirror symmetric \((\text{Fig. 7, diagrams})\). Signals emanating from the midline have been shown to direct the cellular behavior of deep neural cells engaged in convergent extension \((\text{Ezin et al., 2003})\), and it is possible that similar signals guide the orientation of cellular long axes in the superficial neural plate, which appear to impose the orientation of divisions. Regardless, the orientation of cell division is independent of normal neural morphogenesis, as disruption of neural tube closure by a variety of different molecular manipulations failed to alter cell-division orientation \((\text{Fig. 9B-D})\). This result might be important because the neural plate is subject to defined mechanical strains, and such strains are likely to influence cell shape and cell division in epithelial sheets \((\text{Benko and Brodland, 2007; Brodland and Veldhuis, 2002; Thery and Bornens, 2006})\).

The oriented cell divisions characterized here are also notable for their independence from PCP signaling, which plays a major role in controlling oriented cell divisions in a wide variety of vertebrate and invertebrate animals. The divisions reported here thus bear some resemblance to the PCP-independent oriented cell divisions in the elongating \textit{Drosophila} germ band \((\text{da Silva and Vincent, 2007})\). Instead of PCP signaling, we found that Cdc42, which has not previously been shown to influence oriented cell division in vertebrate embryos, plays a role in establishing division orientation in the closing \textit{Xenopus} neural tube. Cells expressing two different dominant negatives to Cdc42, Cdc42-N17 or Cdc42-F37A shifted their average angle of these divisions by roughly 15° \((\text{Fig. 7B,C})\). This shift was modest, but very consistent. This modest role for Cdc42 in orienting these divisions suggests that there are multiple signaling inputs controlling spindle orientation in the \textit{Xenopus} neural plate. Such a mechanism is known to act in \textit{Drosophila} neuroblasts, in which disruption of Ric-8 results in consistent misalignment of spindle by only fewer than 10° \((\text{David et al., 2005})\).

We also found that alteration of Cdc42 function has a dramatic effect on the extent of mitotic-spindle rotation in the \textit{Xenopus} neural plate \((\text{Fig. 6C-F})\). Whereas control cells have a limited range of rotation, with very few spindles rotating more than 120°, cells with altered Cdc42 function had spindles that rotated in excess of 200° \((\text{Fig. 6B,D})\). These data suggest a model for the orientation of cell divisions in the neural plate. Spindle positioning in general involves interactions between astral microtubules and the cell cortex \((\text{Gundersen et al., 2004; O’Connell and Wang, 2000; Siller and Doe, 2008})\), and we propose that spindle rotations are initiated essentially at random, and that astral microtubules identify ‘catch points’ on the cell cortex that stop rotation prior to anaphase and in accordance with the long axis of a cell. One such catch point that is necessary for stopping spindle rotation so that the final division angle is aligned with the cellular long axis is Cdc42-dependent. In this model, if the Cdc42-dependent catch point is disrupted, spindles rotate excessively, and eventually are able to stop by responding to other, Cdc42-independent mechanisms. This general mechanism, by which multiple inputs ‘compete’ to control spindle orientation, has been demonstrated in \textit{Drosophila} neuroblasts \((\text{Bellaiche et al., 2001a; Bellaiche et al., 2001b; David et al., 2005})\).

We also found that, during cell divisions in the \textit{Xenopus} neural plate, observed in vivo, spindles in cells expressing Cdc42-N17 or Cdc42-F37A were not stably positioned during metaphase.
Chromosomes ‘bounce’ from one side of the cell to the other during and after spindle rotation (Fig. 2A and Fig. 6C). This phenotype might be related to the chaotic interactions between meiotic spindles and the cell cortex of mouse oocytes expressing mutants of Cdc42 (Na and Zernicka-Goetz, 2006). Moreover, such hyperactive nuclear movement is linked to spindle positioning in C. elegans (Couwenbergs et al., 2004). Cdc42 has been shown to control the reorientation of the microtubule-organizing center (MTOC) in migrating cells by governing association of the microtubules with the cell cortex (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001). Our results suggest that a similar mechanism functions to position the spindle in Xenopus neural-plate cells. Curiously, Cdc42 had no such effect on spindle positioning in Xenopus epidermal cells observed in vivo.

Finally, this work provides a rare glimpse into the process of cell division in an intact epithelial cell sheet that is simultaneously engaged in both massive proliferation and a massive morphogenetic event. Our data reveal cell-type-specific roles for Cdc42 in spindle positioning and spindle orientation, suggesting that additional roles will be important.

Materials and Methods
Collection and manipulation of embryos
Female Xenopus laevis were injected with 700 ml of human chorionic gonadotropin hormone (HCG) and stored at 18°C overnight. The next day, eggs were isolated and fertilized. Embryos were de-jellied using a 3% cysteine solution in 1/3× Marc’s modified Ringers solution (MMR).

Time-lapse analysis of cell divisions in the neural plate and tail epidermis
Embryos were injected dorsally, at the four-cell stage, with 200 pg histone-eGFP, 200 pg histone-eRGFP, 200 pg histone-REP or any combination of these to examine cell divisions in the neural plate. To examine the epiphris, all cells were injected at the four-cell stage (Fig. 1). To examine the neural plate, embryos were grown to stage 13 and then placed upside down in a culture dish. This dish contained 2% agarose with wells made from a hair comb. To image the tail epiphris, embryos were grown to stage 30 and covered in agarose in a culture dish. Both of these sets of embryos were imaged on a Zeiss Pascal LSM5, at 10× (NA, 0.3), 20× (NA, 0.5), 40× (oil, NA, 1.3) or 63× (oil, NA, 1.4) and cell divisions were analyzed using Image-Pro Plus.

Determination of division polarity
Stacks were projected and exported from the Zeiss LSM5 Pascal program and analyzed using Image-Pro Plus. The polarity of cell division was assessed at the onset of telophase for all cells. Lines were drawn bisecting the center of each set of daughter chromosomes in a posterior-to-anterior manner. The features of these lines were then exported to Microsoft Excel. The angles of divisions were converted and placed into a Stacks were exported to Microsoft Excel. The angles of divisions were converted and placed into a

Effect of molecular effectors on cell-division polarity
To examine the effect of PCP signaling and Cdc42 function, embryos were first injected with 200 pg of histone-eGFP along the entire dorsal side at the four-cell stage. These embryos were grown to the eight-cell stage and 200 pg of membrane-RFP along with either 500 pg Xdd1, 500 pg Xfz8N, 1 ng Shroom3-ASD1, 750 pg of Cdc42-N17 or 750 pg of Cdc42-F37A. Embryos were then imaged and analyzed as above.

Quantifying spindle rotations
This longer axis of the metaphase spindle was assessed upon initial formation, and again 1 minute before anaphase onset. This was done for control cells and cells expressing Cdc42-F37A. The difference of these angles were determined and plotted in Delta-Graph. The Mann-Whitney comparison test was conducted on the two sets of data using GraphPad Instat software.

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References


