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Report

Stereotypical Cell Division Orientation Controls Neural Rod Midline Formation in Zebrafish

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Summary

The development of multicellular organisms is dependent on the tight coordination between tissue growth and morphogenesis. The stereotypical orientation of cell divisions has been proposed to be a fundamental mechanism by which proliferating and growing tissues take shape. However, the actual contribution of stereotypical division orientation (SDO) to tissue morphogenesis is unclear. In zebrafish, cell divisions with stereotypical orientation have been implicated in both body-axis elongation and neural rod formation [1, 2], although there is little direct evidence for a critical function of SDO in either of these processes. Here we show that SDO is required for formation of the neural rod midline during neurulation but dispensable for elongation of the body axis during gastrulation. Our data indicate that SDO during both gastrulation and neurulation is dependent on the noncanonical Wnt receptor Frizzled 7 (Fz7) and that interfering with cell division orientation leads to severe defects in neural rod midline formation but not body-axis elongation. These findings suggest a novel function for Fz7-controlled cell division orientation in neural rod midline formation during neurulation.

Results

The orientation of cell division is thought to play a key role in various developmental processes, including cell-fate specification and tissue morphogenesis [3]. Previous studies have suggested that SDO contributes to body-axis elongation during both vertebrate and invertebrate gastrulation by positioning the daughter cells along the axis of elongation [1, 4, 5]. Cell divisions with stereotypical orientation have also been implicated in neural rod midline formation during zebrafish neurulation [2, 6–9]. The C-divisions are a special type of highly aligned neural progenitor cell division with a stereotypical orientation, and this type of division has been shown to be critical for neural rod midline formation [2, 6]. C-divisions are thought to occur along the forming midline and to give rise to pairs of neural progenitors with mirror-symmetric polarity [2]. Defective localization of C-divisions within the neural primordium, e.g., in embryos with defective neural progenitor cell convergence movements, leads to the formation of multiple ectopic midline structures [2, 6], suggesting that C-division localization determines the location of midline formation within the forming neural rod. In contrast, the role of C-division orientation in neural rod midline formation has not yet been analyzed.

Wnt signaling has previously been shown to control SDO in both vertebrates and invertebrates [1, 10, 11]. In zebrafish, the observation that Wnt/Planar Cell Polarity (Wnt/PCP) signaling is required for both SDO and axis elongation during gastrulation has led to the suggestion that Wnt/PCP signaling drives body-axis elongation by controlling SDO [1]. It has also been suggested that Wnt/PCP signaling is involved in neural rod midline formation through its control of neural progenitor cell polarization after division at the forming midline, which is required for daughter cell reintegration in opposing sides of the developing neural rod [6]. Furthermore, Wnt/PCP signaling has been shown to control neural progenitor cell convergence movements and thereby the proper localization of cells undergoing C-divisions that give rise to the neural rod midline [2]. However, it remains unclear whether Wnt signaling also controls neural progenitor cell division orientation during neurulation and whether the function of Wnt signaling in cell division orientation is linked to its activity in body-axis elongation and neural rod midline formation.

Here, we utilized mutants for *frizzled7* (*fz7*), a key component of noncanonical Wnt signaling in *Xenopus* and zebrafish [12] (Figure S1 in the Supplemental Information) to test the proposed coupling between Wnt signaling, SDO and the processes of body-axis elongation and neural rod midline formation. Maternal-zygotic double-mutant embryos for *fz7a* and *fz7b* (MZ*fz7a/b*) displayed impaired convergence and extension (CE) movements during gastrulation and diminished body-axis elongation and were thus similar to embryos with defective Wnt/PCP signaling (Figures 1A–1C and 1G–1I [13, 14]). MZ*fz7a/b* embryos overexpressing the Wnt/PCP ligand Wnt11 [15] exhibited disrupted CE movements indistinguishable from those of control-injected mutant embryos, suggesting that Fz7 functions downstream of Wnt11 during gastrulation. In contrast, overexpression of *fz7* in MZ*fz7a/b* embryos effectively rescued the mutant phenotype (Figure S2).

Analysis of cell division orientation in wild-type and MZ*fz7a/b* mutant embryos (for more details see Experimental Procedures and Figures 1M–1O) confirmed and extended previous findings [1] that wild-type epiblast and hypoblast cells display SDO that is parallel to the antero-posterior (A/P) axis and orthogonal to the dorso-ventral (D/V) axis (Figures 1D–1F). It further showed that SDO was reduced in MZ*fz7a/b* mutant cells (Figures 1J–1L), similar to the situation in other Wnt/PCP-signaling-defective embryos [1]. Taken together, these data suggest that Fz7 functions within the Wnt/PCP pathway to control both body-axis elongation and SDO during gastrulation.

The reduction in SDO in Wnt/PCP-signaling-defective embryos (Figure 1 [1]) suggests that Wnt/PCP directly controls SDO; alternatively, the alteration in SDO might be secondary to an effect of Wnt/PCP on body-axis elongation. To distinguish between these possibilities, we analyzed cell division

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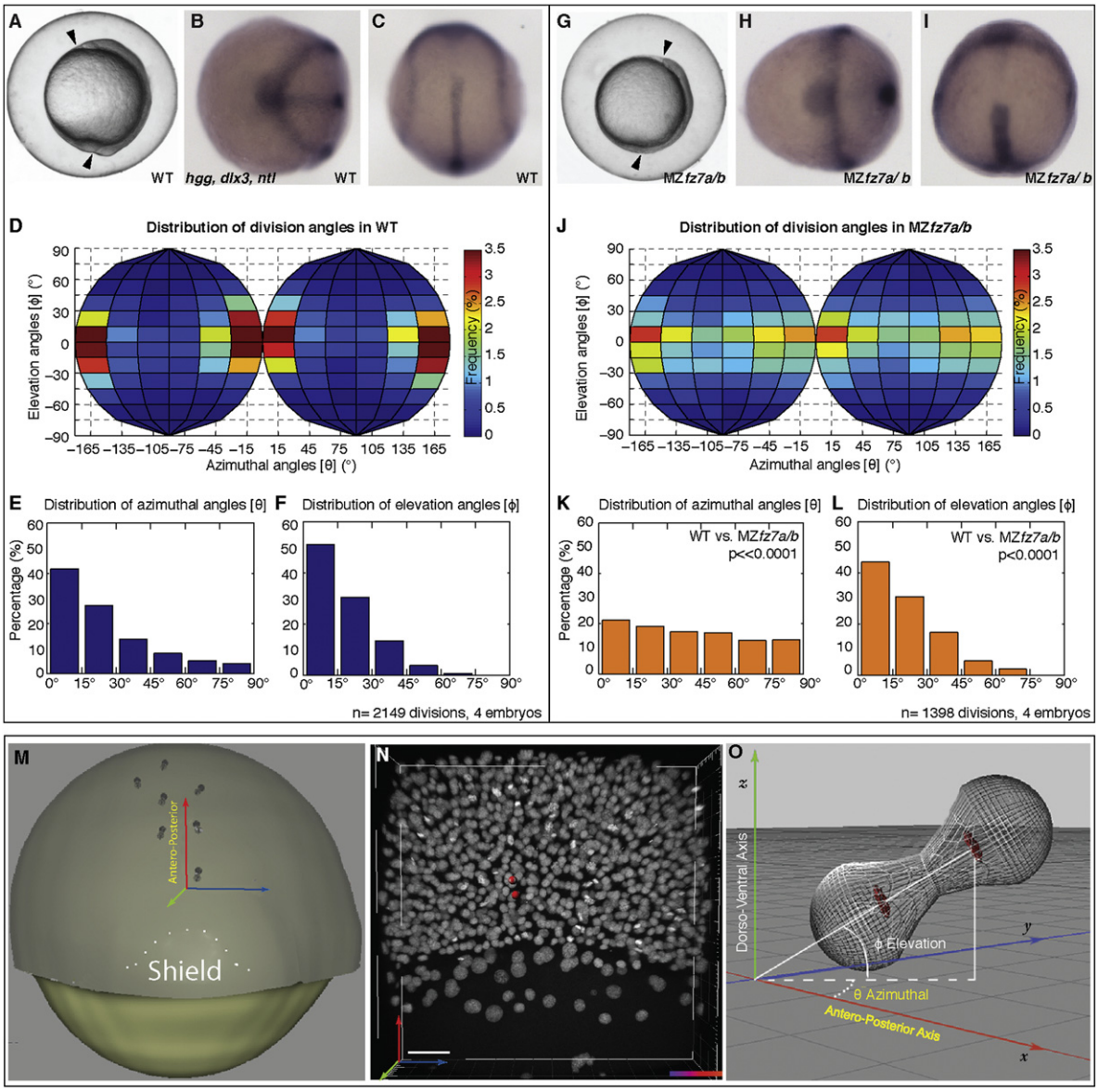

Figure 1. MZ*fz7a/b* Double Mutants Exhibit Reduced CE Movements and Reduced Stereotypical Germ-Layer Progenitor Cell Division Orientation

(A and G) Differential interference contrast (DIC) images of a wild-type (A) and MZ*fz7a/b* (G) embryo at bud stage (10 hpf). Arrowheads demarcate body axis.

(B, C, H, and I) Expression pattern of *no tail* (*ntl*), *hatching gland gene 1* (*hgg1*), and *distal-less homeobox gene 3* (*dlx3*) marking the notochord, prechordal plate, and anterior neural plate, respectively, in a wild-type (B and C) and a MZ*fz7a/b* (H and I) embryo at bud stage. Animal (B and H) and dorsal (C and I) views.

(D–F and J–L) Frequency distribution of pairs of division-orientation angles (θ, φ) in wild-type (D–F) and MZ*fz7a/b* (J–L) embryos during gastrulation (6–10 hpf). Chi-square test on the combined angles: wild-type versus MZ*fz7a/b* ($p < 0.05$).

(D and J) Distribution frequency of pairs of division-orientation angles in wild-type (D) and MZ*fz7a/b* (J).

(E and K) Distribution of azimuthal angles (θ) in wild-type (E) and MZ*fz7a/b* (K).

(F and L) Distribution of elevation angles (φ) in wild-type (F) and MZ*fz7a/b* (L).

(M) Schematic dorsal view of a zebrafish embryo at the early gastrula stage. The dotted line demarcates the shield. Pairs of daughter cells are shown as wired gray spheres. The reference coordinate system used to calculate the angles of the cell divisions is indicated by the red (antero-posterior), green (dorso-ventral), and blue (left-right) axes.

(N) Picture of the dorsal side of a wild-type embryo at 60% epiboly. Two daughter cells resulting from one representative division are marked by red spheres. The same coordinate system as in (M) is used. The scale bar represents 40 μm.

(O) Schematic diagram showing the azimuthal (θ) and the elevation (φ) angles for a single cell division represented by wired gray spheres and red nuclei. The embryonic axes are shown in respect to the reference system.

See also Figures S1 and S2 and Table S1.

orientation in *Prostaglandin E_2 synthase* (*ptges*) morphant embryos, which show body-axis elongation defects similar to those of Wnt/PCP mutants but are not defective in Wnt/PCP signaling (Figure S3; [16]). SDO was only mildly affected in *ptges* morphant embryos (Figure S3), suggesting that defective CE movements alone are not sufficient to explain the SDO defects in Wnt/PCP mutants.

To test whether Wnt/PCP-controlled SDO instructively functions in body-axis elongation during gastrulation, we analyzed *early mitotic inhibitor1* (*emi1*) mutants, in which cell divisions

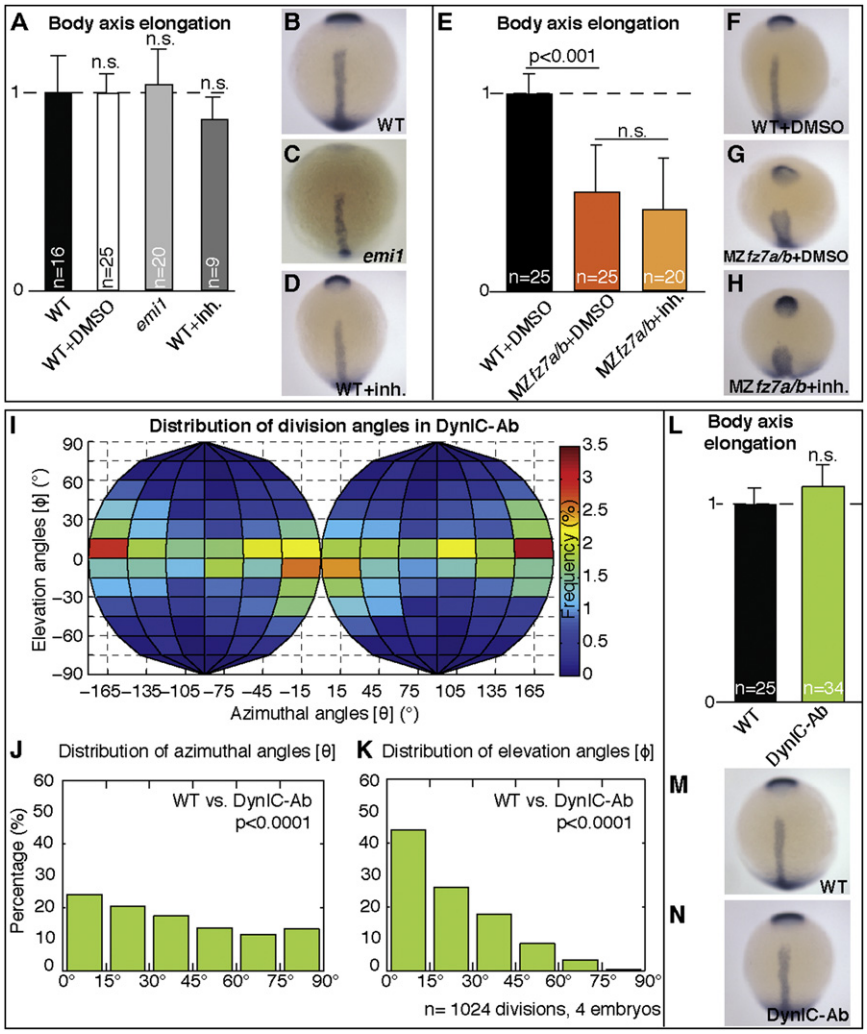

Figure 2. Stereotypical Germ-Layer Progenitor Cell Division Orientation Is Not Required for Body-Axis Elongation during Gastrulation

(A–D) Body-axis elongation (A) and *ntl* and *hgg1* expression in wild-type (B), *emi1* (C), and wild-type embryos treated with cell-division inhibitors (D) at bud stage (10 hpf).

(E–H) Body-axis elongation (E) and *ntl* and *hgg1* in wild-type (F), MZ*fz7a/b* embryos treated with DMSO (G) and MZ*fz7a/b* embryos treated with cell-division inhibitors (H) at bud stage (10 hpf).

(I–K) Frequency distribution of pairs of division-orientation angles (θ,φ) in DynIC-Ab-injected embryos during gastrulation (6–10 hpf). Distribution of azimuthal (θ) (J) and elevation (φ) angles (K). Chi-square test on the combined angles: wild-type versus DynIC-Ab ($p < 0.005$).

(L–N) Body-axis elongation (L) and *ntl* and *hgg1* expression in wild-type (M) and DynIC-injected embryos (N) at bud stage (10 hpf).

Error bars show the standard deviation.

See also Figure S3 and Table S1.

stop at the onset of gastrulation [17]. Body-axis length at the end of gastrulation in *emi1* mutants was indistinguishable from that of wild-type embryos (Figures 2A–2C), suggesting that Wnt/PCP-controlled SDO does not drive body-axis elongation during gastrulation. To confirm this conclusion, we treated wild-type embryos with cell-division inhibitors [18] during gastrulation (4–10 hpf). There was no significant change in body-axis length in treated embryos (Figures 2A–2D), supporting our conclusion that Wnt/PCP-controlled SDO has no instructive function in axis elongation during gastrulation.

Instead of instructively driving axis elongation, Wnt/PCP-controlled SDO might have a permissive function, allowing body-axis elongation to proceed normally when cell divisions are present. In this case, defective cell division orientation would obstruct axis elongation, and consequently the axis-elongation defect should be rescued when cell divisions are inhibited. To test this directly, we blocked cell divisions in MZ*fz7a/b* mutants during the course of gastrulation and determined whether this could partially rescue the elongation phenotype. However, this treatment did not rescue the body-axis-elongation phenotype (Figures 2E–2H), suggesting that Wnt/PCP-controlled SDO has no permissive function in body-axis elongation during gastrulation.

Although blocking cell division is a useful tool for addressing the role of cell divisions per se, it does not provide direct information about the role of cell division orientation. To address the specific function of cell division orientation in body-axis elongation, we injected embryos at the 1 cell stage with an antibody against Dynein-Intermediate-Chain (DynIC-Ab), which has previously been shown to randomize the orientation of the mitotic spindle in mammalian cells [19]. When compared to wild-type embryos, injected embryos exhibited reduced SDO along the A/P and D/V axes during gastrulation (Figures 2I–2K). In contrast, body-axis length at the end of gastrulation in DynIC-Ab-injected embryos was indistinguishable from that of uninjected control embryos (Figures 2L–2N), suggesting that Wnt/PCP-controlled SDO has no major function in body-axis elongation during gastrulation.

C-divisions within the neural rod have been implicated in the formation of the neural rod midline [2]. This prompted us to explore the role of Fz7 signaling in this process between 13 and 16 hpf. Consistent with previous studies [7, 20], we found that C-divisions during neural rod formation have stereotypical orientation: progenitor cells predominantly divided orthogonal to both the A/P and the D/V axes (Figures 3A–3C). Progenitor cells in the central axial region (~40 μm wide) showed the most pronounced C-divisions [2] and greater SDO than cells in paraxial regions (Figures 3A–3C; see also Figure S4). The polarization of the C-divisions was visualized in previous studies by expression of a fusion protein of Pard3 with green fluorescent protein [2, 7, 21] in neural rod cells. Our analysis of Pard3-GFP localization confirmed that C-divisions have a stereotypical orientation and are orthogonal to both the A/P and D/V axes (data not shown).

Analysis of neural progenitor cell division orientation in MZ*fz7a/b* mutants revealed that the SDO of C-divisions during neurulation depends upon Fz7 signaling. The SDO of neural progenitors was significantly reduced in the axial region of

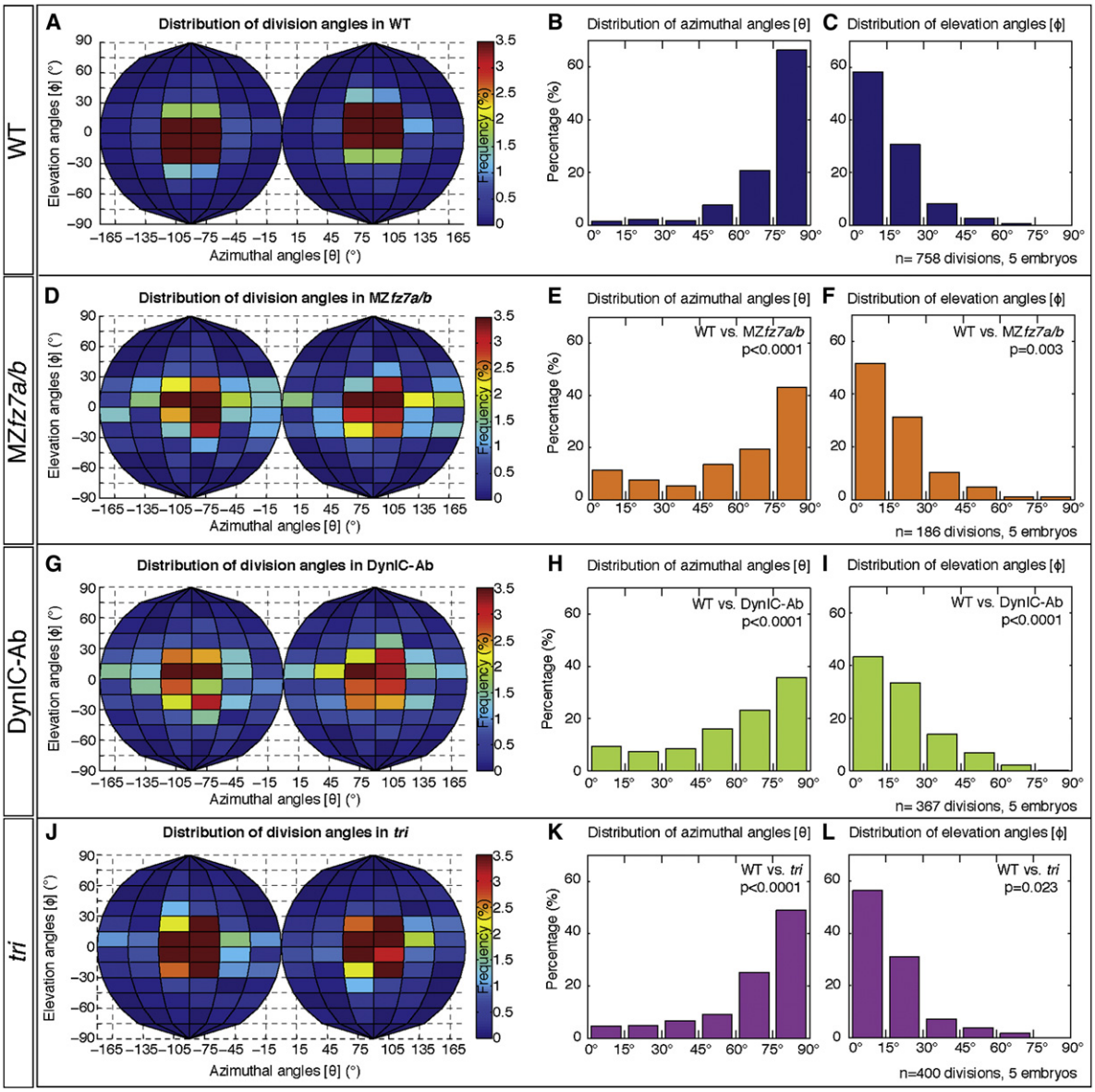

Figure 3. Wnt/PCP Signaling Controls Stereotypical Neural Progenitor Cell Division Orientation

(A–C) Frequency distribution of pairs of division-orientation angles (θ,φ) (A) in axial regions (~40 μm wide) of the forming neural rod in wild-type embryos during neurulation (13–16 hpf).

(D–F) Frequency distribution of pairs of division-orientation angles (θ, φ) in the axial regions of the forming neural rod in MZ*fz7a/b* embryos during neurulation (13–16 hpf). Chi-square test on the combined angles: wild-type versus MZ*fz7a/b* ($p < 0.005$).

(G–I) Frequency distribution of pairs of division-orientation angles (θ, φ) in the axial region of the forming neural rod in DynIC-Ab-injected embryos during neurulation (13–16 hpf). Chi-square test on the combined angles: wild-type versus DynIC-Ab ($p < 0.005$).

(J–L) Frequency distribution of pairs of division-orientation angles (θ, φ) in the axial region of the forming neural rod in *tri* mutants during neurulation (13–16 hpf). Chi-square test on the combined angles: wild-type versus *tri* ($p = 0.215$).

Distribution of azimuthal (θ) (B,E,H, and K) and elevation (φ) angles (C,F,I, and L).

See also Figure S4 and Table S1.

the forming neural rod in MZ*fz7a/b* mutants when these embryos were compared to wild-type embryos. Moreover, in contrast to the wild-type situation, SDO in MZ*fz7a/b* mutants was indistinguishable between axial and paraxial regions of the neural primordium (Figures 3D–3F; see also Figure S4).

A comparison of the midline morphology in wild-type and MZ*fz7a/b* mutant embryos showed that Fz7 signaling controls not only C-division orientation but also neural rod midline formation. At a stage when wild-type embryos showed a clearly recognizable neural rod midline (16 hpf; Figure 4A), no coherent midline structure was detectable in MZ*fz7a/b* mutant embryos (Figure 4C). To determine whether Fz7 controls neural rod midline formation by regulating neural progenitor cell division orientation, we first analyzed whether Fz7-controlled-SDO instructively drives neural rod midline formation during neurulation. Blocking cell divisions by either treating wild-type embryos with cell division inhibitors during neurulation (13–16 hpf) or using *emi1* mutants resulted in embryos that

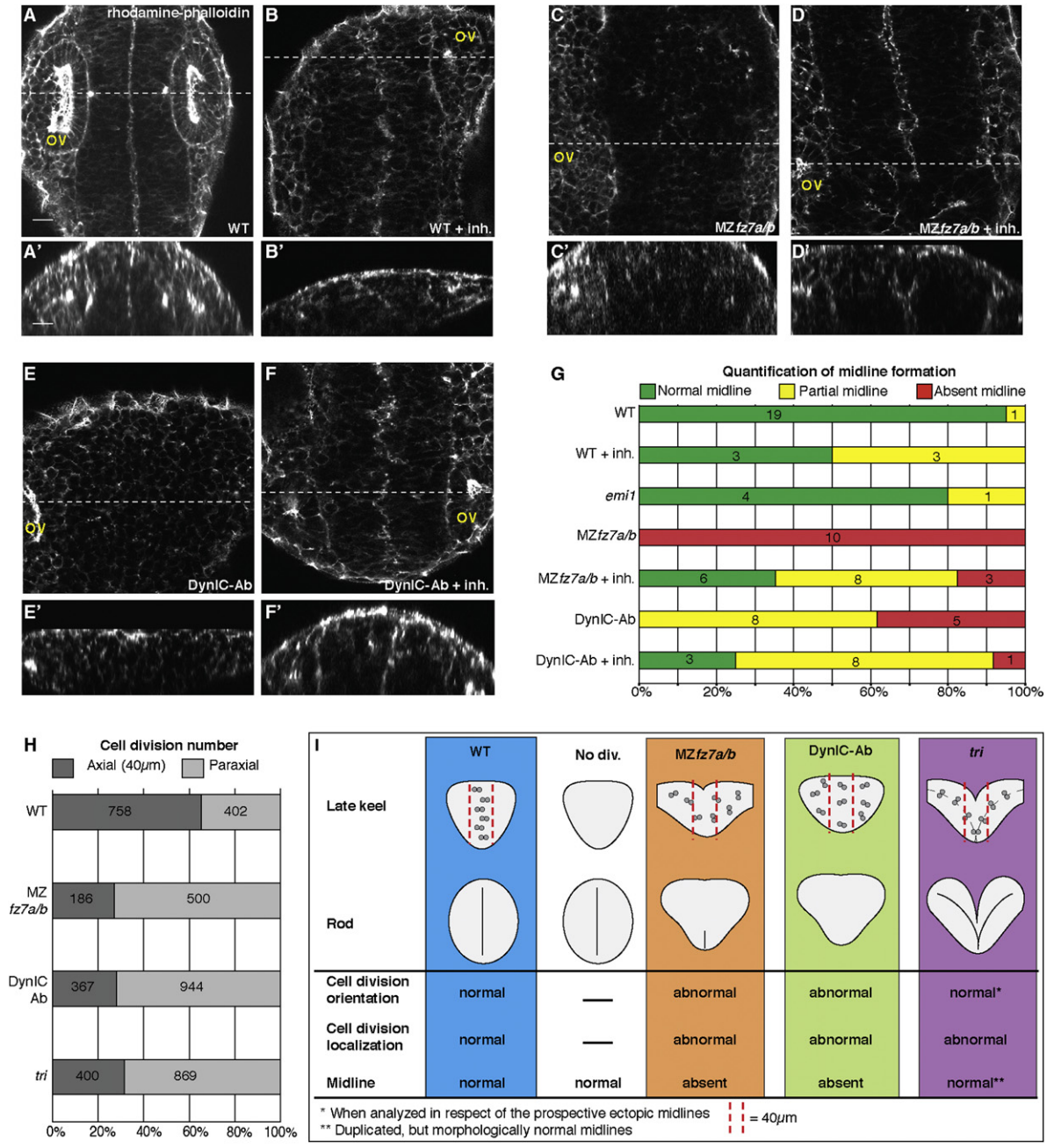

Figure 4. Stereotypical Neural Progenitor Cell Division Orientation Is Required for Neural Rod Midline Formation during Neurulation

(A–F′) Micrographs of F-actin distribution in dorsal (A–F) and transversal (A′–F′) confocal sections of Rhodamine-Phalloidin-stained neural rods in wild-type (A and A′), wild-type treated with cell-division inhibitors (B and B′), MZ*fz7a/b* mutants (C and C′), MZ*fz7a/b* mutants treated with cell-division inhibitors (D and D′), DynIC-Ab-injected embryos (E and E′), and DynIC-Ab-injected embryos treated with cell-division inhibitors (F and F′) at the 16-somite-stage (15 hpf). OV = otic vesicle; dashed lines in (A–F) demarcate the place of the transversal sections in (A′–F′). Scale bars represent 20 μm.

(G) Quantification of neural rod midline formation in wild-type embryos, wild-type embryos treated with cell-division inhibitors, *emi1* embryos, MZ*fz7a/b* mutants, MZ*fz7a/b* mutants treated with cell-division inhibitors, DynIC-Ab-injected embryos, and DynIC-Ab-injected embryos treated with cell-division inhibitors.

(H) Number of cell divisions in axial and paraxial regions of wild-type, MZ*fz7a/b*, DynIC-Ab injected, and *tri* embryos during neurulation (13–16 hpf)

(I) Schematic representations of the neural rod (transverse section), summarizing the effects on neural progenitor cell division localization and orientation and on neural rod midline formation in the different experimental conditions analyzed in this study.

See also Table S1.

formed a largely normal-appearing neural rod midline (Figure 4B and data not shown). This suggests that Fz7-controlled-SDO is not a key instructive factor driving midline formation. To test whether Fz7-controlled SDO is permissively required for neural rod midline formation to proceed normally, we blocked cell divisions in embryos with reduced SDO. If reduced SDO would indeed obstruct neural rod midline formation, then blocking cell divisions in embryos with

defective SDO should rescue the midline phenotype. We found that in MZ*fz7a/b* mutant embryos treated with cell division inhibitors during neurulation (13–16 hpf), midline formation was partially rescued (Figures 4D and 4G), suggesting that Fz7-controlled-SDO is permissively required for neural rod midline formation. To directly test this idea, we specifically interfered with cell division orientation by injecting DynIC-Ab into 1-cell-stage wild-type embryos and analyzed neural rod division orientation and midline formation in these embryos. Similar to the observations made at gastrula stages, neural rod SDO was reduced in DynIC-Ab-injected embryos as compared to wild-type embryos (Figures 3G–3I). However, in contrast to the situation during gastrulation, injected embryos failed to form a morphologically recognizable neural rod midline (Figure 4E), indicating that Fz7-controlled SDO is required for midline formation. Blocking cell divisions during neurulation (13–16 hpf) in DynIC-Ab-injected embryos partially rescued the midline defect (Figures 4F and 4G), confirming our hypothesis that Fz7-controlled-SDO permissively functions in midline formation.

Discussion

Cell divisions with stereotypical orientation have been observed in several morphogenetic processes during development [4, 5, 10, 20]. However, it remains unclear whether this stereotypical cell division orientation has a critical function in any of these processes. Here we show, by directly interfering with cell division orientation, that SDO plays a key role in neural rod midline formation during zebrafish neurulation.

Previous studies have suggested that the neural rod midline in zebrafish is formed by a special type of cell divisions with stereotypical orientation; these C-divisions occur along the A/P axis in medial portions of the neural primordium where the future midline will form [2]. When C-divisions occur in more lateral regions of the neural primordium, e.g., as a result of delayed convergence movements of neural progenitors, two bilateral midlines form, instead of one medial midline, suggesting that the localization of C-divisions within the neural primordium determines where the neural midline(s) will form [2]. In contrast, our finding that interfering with the stereotypical orientation of these divisions leads to defective midline formation indicates that the stereotypical orientation is a prerequisite for midline formation per se. It thus appears that the localization of neural progenitor cell divisions determines where the neural rod midline will form, whereas the stereotypical orientation is needed for midline formation per se (Figure 4I). Notably, we found that in both MZ*fz7a/b* mutant and DynIC-Ab-injected embryos, not only was the stereotypical orientation of progenitor cell divisions affected, but their localization was affected as well (Figure 4H). This suggests that the neural rod midline defect in these embryos is due to the combined defects in neural progenitor division orientation and localization. However, the finding that changes in the localization of cell divisions alone are not sufficient to affect midline formation per se [2] strongly suggests that the stereotypical orientation of neural progenitor cell divisions is a critical feature for midline formation.

Previous studies have shown that Wnt signaling controls cell division orientation in various morphogenetic processes, including body-axis elongation and primitive streak formation ([3]; Figure 1; see also Figure S2). We show that signaling through the Wnt receptor Fz7 controls SDO of neural progenitors during zebrafish neurulation and unravel a novel function for Fz7 signaling in neural rod midline formation by controlling stereotypical C-division orientation. It has recently been suggested that different noncanonical Wnt signaling components, such as Stbm/Vang, Wnt11, and Wnt5, contribute to neural rod midline formation by controlling neural progenitor cell polarization [6] and cell-division localization [2]. However, in contrast to Fz7, mutations in these genes have relatively little effect on the stereotypical orientation of neural progenitor cell divisions (Figures 3J–3L; see also Figure S4; data not shown), suggesting that Fz7 functions differently from Wnt11, Wnt5, and Stbm/Vang in neural rod midline formation and/or positioning. Whether Fz7 directly controls cell division orientation and whether it functions in this process by signaling through a Wnt signaling pathway(s) that is the same as that used by Wnt11, Wnt5, and Stbm/Vang are still unclear. However, our observations (Figure S2) and previous data [22] indicating that Fz7 can function as a receptor for the Wnt/PCP ligand Wnt11 suggest that both Fz7 and Wnt11-Wnt5-Stbm/Vang function in neural rod midline formation by signaling through the Wnt/PCP pathway.

Previous studies have suggested that Wnt/PCP signaling controls SDO [1] and that SDO drives body-axis elongation during vertebrate and invertebrate early development [4, 5]. SDO is expected to contribute to body-axis elongation by positioning daughter cells along the axis, so that each division lengthens the rank of cells. Here we carefully assessed the role of Wnt/PCP in both SDO and axis elongation during zebrafish gastrulation, and we found no major effect of diminished SDO on body-axis elongation (Figure 2). This clearly uncouples these developmental events and suggests that morphogenetic events other than SDO must explain the shortening of the axis in zebrafish embryos with altered Wnt/PCP signaling.

Our findings that epiblast and hypoblast cells indeed divide preferentially along the axis of elongation (Figure 1; [1, 20]) and that daughter cells do not show large changes in their relative positions after division (data not shown) are consistent with the notion that their divisions could in principle contribute to axis elongation. The extent to which SDO contributes to body-axis elongation critically depends on the total number of progenitor cell divisions and cell-shape changes and/or cellular rearrangements that accompany these divisions. Notably, the frequency of progenitor cell divisions strongly decreases at the onset of gastrulation [23], suggesting that SDO has no major function in body-axis elongation because the number of oriented cell divisions is simply not high enough.

SDO has been implicated in several morphogenetic processes [3]. However, many of these studies were based on experiments in which cell division orientation was correlated with a specific morphogenetic behavior, and/or in which blocking cell divisions led to morphogenetic defects consistent with a critical role for SDO in this process. Our study goes beyond those studies by directly analyzing the function of cell division orientation, instead of cell divisions per se, and systematically analyzing the different instructive and/or permissive functions of SDO in body-axis elongation and neural rod midline formation during zebrafish early development.

Supplemental Information

Supplemental Information include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at doi:10.1016/j.cub.2010.10.009.

Acknowledgments

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