LETTERS

A mirror-symmetric cell division that orchestrates neuroepithelial morphogenesis

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The development of cell polarity is an essential prerequisite for tissue morphogenesis during embryogenesis, particularly in the development of epithelia[1,2]. In addition, oriented cell division can have a powerful influence on tissue morphogenesis[3]. Here we identify a novel mode of polarized cell division that generates pairs of neural progenitors with mirror-symmetric polarity in the developing zebrafish neural tube and has dramatic consequences for the organization of embryonic tissue. We show that during neural rod formation the polarity protein Pard3 is localized to the cleavage furrow of dividing progenitors, and then mirror-symmetrically inherited by the two daughter cells. This allows the daughter cells to integrate into opposite sides of the developing neural tube. Furthermore, these mirror-symmetric divisions have powerful morphogenetic influence: when forced to occur in ectopic locations during neurulation, they orchestrate the development of mirror-image pattern formation and the consequent generation of ectopic neural tubes.

The movements of neurulation in zebrafish embryos involve convergence of left and right sides of the neural plate towards the dorsal midline, followed by invagination of the neural plate to form a neural keel that then condenses to form a solid neural rod[4–7]. At the midline of the neural rod, an epithelial seam forms that divides left from right and initiates formation of the ventricular system of the brain and spinal cord[8]. The neural keel and rod stages are uniquely characterized by neural progenitor divisions that deposit one daughter cell on either side of the midline, so that each side of the neural tube receives contributions from both left and right sides of the neural plate[4–7,9] (Supplementary Fig. 1). Once deposited on either side of the neural rod, both daughter cells elongate across the apico-basal extent of the left and right neuroepithelium (Fig. 1a). More than 90% of neural plate cells undergo this midline-crossing division (C-division)[10]. When cell division is blocked during this period, very few cells are able to cross the midline[7] (Fig. 1b–e), thus demonstrating that division itself is required for crossing. The daughter cells that cross the midline integrate into a neuroepithelium that develops mirror-image apico-basal polarity with respect to their original side. To integrate into the contra-lateral neuroepithelium, a mechanism must exist to generate mirror-image polarity in the crossing daughter cells.

C-divisions are most prevalent at neural keel and rod stages (14 to 18 hours post fertilization (h.p.f.))[6,9], which coincides with the first midline expression of markers of apical epithelial character[9]. Before these stages, apical markers are either not expressed (aPKC) or are expressed diffusely in neural plate cell membranes (β-catenin and ZO-1)[9]. Because Par3 has a fundamental role in the establishment of cell polarity in a variety of invertebrate and vertebrate species[11], we chose to follow the dynamics of tissue and cell polarization using time-lapse confocal microscopy of a fusion protein of Pard3 with green fluorescent protein, Pard3–GFP (Pard3, initially described as ASIP/PAR-3, is a zebrafish orthologue of the *Caenorhabditis elegans* Par3 protein[9,12,13]). A few evenly distributed puncta of Pard3–GFP are observed in the neural plate[9], but the first coordinated localization appears towards the end of the neural keel phase when Pard3–GFP is expressed in bright puncta within a zone approximately 40 μm wide at the midline (Fig. 2b). These puncta are mobile and gradually coalesce towards the midline over the next few hours. By 18 h.p.f., a distinct single midline expression domain that extends throughout the dorso-ventral extent of the neural rod is evident (Fig. 2c; and Supplementary Fig. 2a, b).

When expression in individual cells is examined, the development of bright Pard3–GFP puncta is particularly striking in cells undergoing the C-division. When cell bodies round up at the start of prophase, Pard3–GFP has no consistent subcellular localization, however, by telophase, Pard3–GFP is specifically enriched at the cleavage furrow (Fig. 2d; and Supplementary Fig. 2). In most cells, bright expression remains symmetrically localized across the cleavage

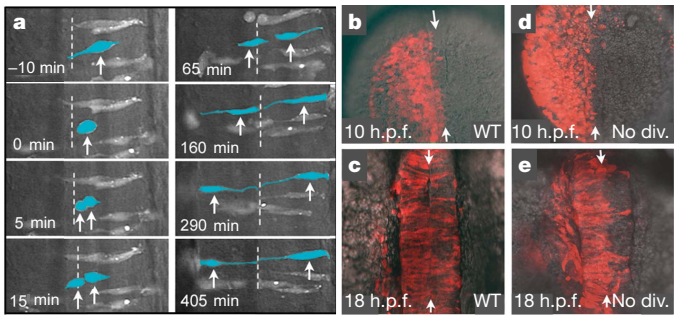

Figure 1 | Cell division separates daughter cells across the midline of the neural keel and rod. **a**, Time-lapse sequence of a C-division (crossing-division) viewed from the dorsal surface of a neural rod. The cell divides on the right side of the neural rod and is followed by the medial daughter crossing the midline (dotted line) and integrating into the contra-lateral side. Mother and daughter cells are highlighted by arrows and blue overlay. Time points are in minutes relative to the beginning of mitosis. **b**, Dorsal view of a normal neural plate with cells labelled by red fluorescence on only the left side. Arrows point to the midline. **c**, Dorsal view of the same embryo as **b** but 8 h later, showing bilateral distribution of red cells. **d**, Dorsal view of a neural plate in an embryo treated with inhibitors of cell division (No div.) during the period of C-divisions. Red fluorescent cells are predominantly on the left side of the neural plate. **e**, Dorsal view of the neural keel 8 h later than **d**, indicating that very few cells crossed the midline (arrows) in the absence of cell division. WT, wild type.

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furrow throughout cytokinesis (40 out of 47 monitored divisions; Fig. 2e). In a few cells, Pard3–GFP was only enriched at the cleavage plane towards the end of cytokinesis (7/47). Intense expression of Pard3–GFP remains at the medial ends of the two daughter cells following all C-divisions (Fig. 2e). During this period, the most medial daughters move across the midline and elongate to stretch across the full prospective apico-basal extent of the contra-lateral epithelium, while their sister cells elongate across the full extent of the ipsi-lateral epithelium. Thus, mirror-image polarity of daughter cells is established during cytokinesis by a novel mechanism that localizes apical information to the cleavage furrow.

To test whether Pard3 is important for establishing mirror-image polarity during C-divisions and for initiating midline crossing of the medial daughters, we first reduced Pard3 levels by morpholino-mediated antisense knockdown (Supplementary Fig. 3). Reduction of Pard3 had no effect on the frequency, location or completeness of cell divisions (Supplementary Fig. 4), but did dramatically decrease midline crossing in 7/10 embryos (Fig. 2f). We next tested whether a mutant version of Pard3 that does not preferentially localize to the cleavage furrow during the C-division (97/102 cells monitored; Fig. 2g) would inhibit midline crossing. We mosaically expressed the mutant Pard3-Δ6–GFP, which lacks amino acids 688–1127 including the aPKC binding domain[13]. In neuroepithelial cells, this protein is expressed widely in the cell membrane rather than being restricted to apical poles and also associates with intracellular filaments (probably microtubules)[13]. Time-lapse analyses revealed that for Pard3–GFP-expressing cells 80% (32/40) of cell divisions led to midline crossing less than 10 min after prophase, and 100% cross by 35 min. In contrast, only 49% (39/80) of daughter cells expressing Pard3-Δ6–GFP cross the midline within 10 min, and in the remaining 51% (41/80) of divisions, both daughters stay on the same side until lost from focus (Fig. 2g and Supplementary Table 1). Together, these results demonstrate that Pard3 function is required for midline crossing and indicate that this role for Pard3 is probably mediated by specific localization to the cleavage plane and mirror-symmetric inheritance at the prospective apical poles of the daughter cells.

To investigate the regulation of the C-division, we looked for mirror-symmetric divisions in embryos in which convergence of the neural plate and formation of the neural keel was delayed by reducing the function of the non-canonical Wnt signalling component Vangl2 (refs 14–16; Fig. 3a, b). We reasoned that C-divisions would occur 'on time' but in ectopic lateral locations, if regulated by a mechanism intrinsic to the neuroepithelial precursors, but would occur 'late' in the delayed neural keel, if regulated by the process of neurulation or the keel environment. We find the former is true. Time-lapse analyses in the region of the caudal hindbrain and anterior spinal cord reveal that ectopic C-divisions occur in neural plate cells before keel formation in *trilobite/vangl2* mutants (Fig. 3c, d; and Supplementary Movies 1 and 2). These divisions occur across the deep-to-superficial axis of the convergence-delayed neural plates and generate pairs of daughter cells that remain in register as they

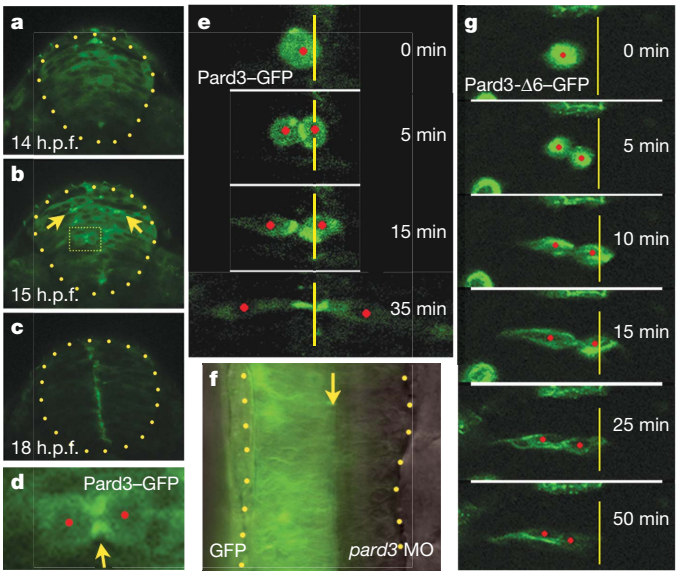

Figure 2 | Subcellular distribution of Pard3–GFP reveals that C-division is a mirror-symmetric division. **a**–**c**, Three frames from a time-lapse sequence of Pard3–GFP expression in neural keel and rod seen in transverse section. In early keel, Pard3–GFP is diffusely expressed throughout the cytoplasm of cells and shows no polarization. At approximately 15 h.p.f., bright puncta appear within a territory 20 μm either side of the midline and by 18 h.p.f. these puncta accumulate at the midline of the rod and the diffuse cytoplasmic distribution has disappeared. **d**, High magnification of a telophase cell outlined in **b** shows distinct Pard3–GFP accumulation around the cleavage furrow (arrow). **e**, A time-lapse sequence showing Pard3–GFP distribution throughout a C-division. Pard3–GFP is distributed across the cleavage furrow and inherited in medial poles of the two daughter cells. The medial daughter rapidly crosses the midline (yellow line). Time points are in minutes, starting at the beginning of mitosis. **f**, Dorsal view of a neural tube in which the level of Pard3 was reduced by injection of 0.35 pmoles of morpholino (MO). The unilateral distribution of GFP-labelled cells indicates that few cells crossed the midline (arrow), compared to the normal embryo in Fig. 1c. **g**, Time-lapse sequence of a mutant Pard3-Δ6–GFP-expressing cell dividing close to the neural keel midline (yellow line). Pard3-Δ6–GFP expression is not localized to the cleavage furrow or to prospective apical poles of the daughter cells. Both daughter cells remain on the same side of the keel for at least 50 min before drifting out of focus. Time points are in minutes, starting at the beginning of mitosis.

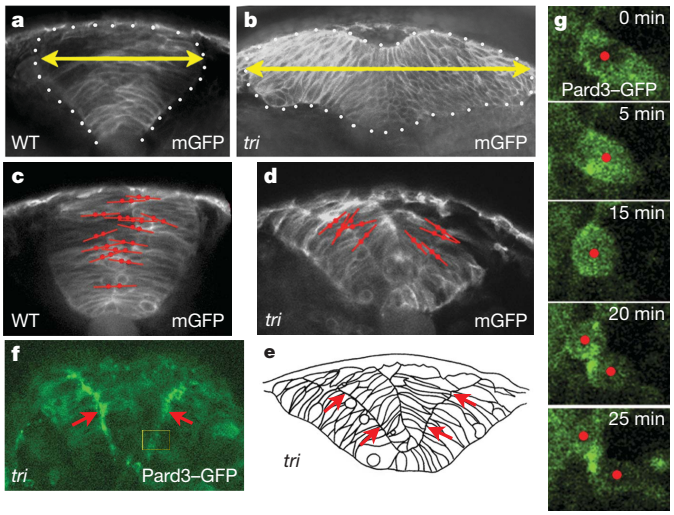

Figure 3 | Delayed convergence of the neural plate leads to ectopic Pard3-mediated C-divisions. **a**, Arrowheads indicate the width of wild-type neural keel in live confocal transverse sections at 14 h.p.f. Cells are labelled with membrane-bound GFP (mGFP). **b**, The *trilobite/vangl2* mutant neural plate, also at 14 h.p.f., has a much wider profile owing to delayed convergence. **c**, The orientations of cell divisions are superimposed on wild-type neural rod. Red lines and dots indicate direction of separation of daughter cells, leading to midline crossing. **d**, Positions and orientations of cell divisions towards the end of the invagination of *trilobite/vangl2* (*tri*) neural plate. Daughters are separated across what was the superficial-deep axis of the plate. **e**, Line drawing of cell-outlines traced from the *trilobite/vangl2* specimen illustrated in **d** to show ectopic interface (arrows) between developing bilayers in mutant neural primordium. **f**, A section of *trilobite/vangl2* neural primordium at 18 h.p.f. reveals two ectopic, bilateral lines of Pard3–GFP expression (arrows) coincident with the ectopic bilateral interface between cells illustrated in **e**. **g**, Time-lapse sequence showing Pard3–GFP expression concentrated at the cleavage furrow of a cell dividing across the neural plate in a *trilobite/vangl2* embryo. Location of the division is shown by the boxed area in **f**. Time points are in minutes, starting at the beginning of mitosis. Red dots indicate the centre of mother and daughter cells.

elongate across the neural plate (Supplementary Fig. 5). Because the daughter cells remain in register, the neural plate becomes a bilayer of pseudostratified epithelia (Fig. 3e). In contrast to wild-type embryos, in which a single domain of Pard3–GFP expression develops only in the midline of the neural keel (Fig. 2c), Pard3–GFP in *trilobite/vangl2* mutants is expressed in two domains on either side of the embryo's midline, sandwiched midway between the superficial and deep surfaces of the neural plate (Fig. 3f). Examination of individual cells reveals that Pard3–GFP is mirror-symmetrically expressed across the cleavage plane of cells that divide across the mutant bilayered neural plate (30/37 cells; Fig. 3g; and Supplementary Movies 3 and 4). These observations all suggest that ectopic neural 'midlines' are being generated by ectopic C-divisions on either side of the actual embryonic midline, and that Pard3-mediated C-divisions are independent of non-canonical Wnt function (see also Supplementary Fig. 6).

Despite the abnormal development of the neural plate, neural tissue continues to converge and invaginate in *trilobite/vangl2* mutant embryos. Thus, by 24 h.p.f., the ectopically bilayered neural plates are re-oriented and appear to fuse at the midline, to generate a four-layered neural primordium. Molecular and structural analyses show this four-layered structure comprises two neural tubes side-by-side. The apical marker aPKC, which normally delineates the single neural midline (Fig. 4a), is expressed along the two ectopic neural midlines in *trilobite/vangl2* mutants (Fig. 4d). Remarkably, GFAP

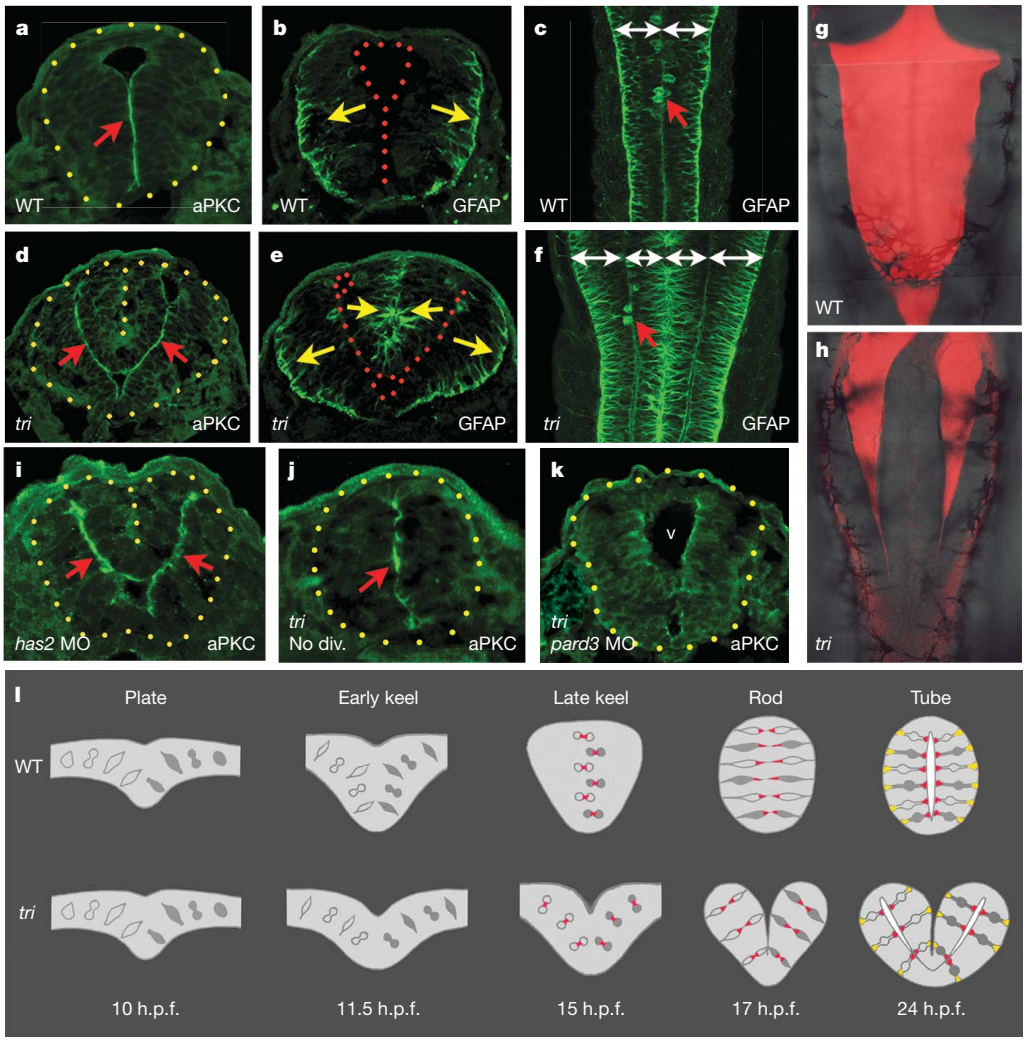

Figure 4 | Delayed neural plate convergence generates duplicate neural tubes complete with mirror-image apico-basal polarity and ventricles. **a**, A transverse section of wild-type neural tube at the level of the caudal hindbrain at 24 h.p.f. reveals aPKC expression at the apical surface (red arrow) of the epithelium. Yellow dots indicate the basal surface of the tube. **b**, A transverse section of neural tube reveals GFAP expression at the basal edge (yellow arrows) of epithelia. Red dots indicate the apical surfaces of the tube. **c**, A horizontal confocal section of a wild-type neural tube whole-mount stained for GFAP expression. As well as marking basal ends of neuroepithelial cells this antigen is highly expressed in mitotic cells (red arrow) at the apical surface. **d**, **e**, A transverse section of *trilobite/vangl2* neural primordium at 24 h.p.f. reveals bilateral aPKC expression (two red arrows) and mirror-image expression of GFAP (four yellow arrows) on either side of apical surfaces. **f**, A horizontal confocal section of *trilobite/vangl2* neural tube stained as a whole-mount for GFAP expression reveals duplicate mirror-image epithelial organization (compare to **c**). **g**, Fluorescent dextran injected into a ventricle of a wild-type embryo reveals a single, large ventricle in the hindbrain. **h**, Fluorescent dextran injected into a ventricle of a *trilobite/vangl2* embryo reveals twinned ventricles in the caudal hindbrain. **i**, A transverse section of neural primordium in a *has2* morpholino embryo at 24 h.p.f. reveals bilateral aPKC expression that phenocopies the *trilobite/vangl2* phenotype. **j**, A transverse section of neural tube from a *trilobite/vangl2* embryo treated with inhibitors of cell division. Epithelial duplications are significantly reduced in 76% (32/42) of mutant embryos as revealed by single midline expression of the apical marker aPKC (compare to **d**). **k**, A transverse section of neural tube from a *trilobite/vangl2* embryo injected with 0.35 pmoles of *pard3* morpholino oligonucleotides. Epithelial duplications are significantly reduced in 46% of mutant embryos as revealed by the single midline ventricle (v) and expression of the apical marker aPKC at the ventricular surface. **l**, A schematic of transverse sections to show mirror-symmetric C-divisions during the morphogenesis of normal and mirror-duplicate neural tubes. Pard3 localization is shown in red and the basal marker GFAP is shown in yellow.

which is a marker of basal (outer) surface of the neuroepithelium (Fig. 4b, c) is expressed with mirror-image symmetry on either side of each of the two apical aPKC domains (Fig. 4e, f). By 30 h.p.f., ectopic ventricles with appropriate dorso-ventral morphology are generated on either side of the midline (Fig. 4g, h). Identical phenotypes are obtained by abrogation of other components of the non-canonical Wnt pathway such as Pk1 (ref. 17) and Dvl (refs 18, 19) (Supplementary Fig. 7).

To confirm that generation of mirror-image duplicates is a consequence of delayed convergence rather than other potential defects downstream of non-canonical Wnt signalling (see ref. 7 and Supplementary Fig. 6), we analysed embryos with convergence defects caused either by reduced function of the Hyaluron synthesizing enzyme, Has2, which is in the mesoderm[20], or by surgical separation of the left from right side of the neural plate in wild-type embryos (Supplementary Fig. 8). We find both interventions lead to mirror-image duplication of the neural tubes, identical to embryos with non-canonical Wnt signalling deficits (Fig. 4i; and Supplementary Fig. 8 and Supplementary Movie 5). We conclude that when neural keel formation is delayed or prevented, ectopic mirror-symmetric divisions occur across the neural plate, and we propose that these specialized divisions initiate mirror-image apico-basal organization across the neuroepithelium.

If the ectopic mirror-image apico-basal organization is really a downstream consequence of the ectopic mirror-symmetric cell divisions then blocking division should eliminate this phenotype. In fact a previous study has demonstrated that blocking division during keel formation rescues normal development of the neural tube in *trilobite/vangl2* embryos[7], and we confirm this here (Fig. 4j). In addition, because Pard3 function is crucial for the mirror-symmetric division, we tested whether it is also required for mirror-image duplications by reducing Pard3 levels in *trilobite/vangl2* mutants. We find that a partial reduction of Pard3 levels by morpholino knockdown reduces mirror-image duplication in 46% of the mutants (7/39 were fully rescued, whereas 11/39 showed reduced duplication; Fig. 4k; and Supplementary Fig. 9). More severe reduction of Pard3 function disrupts morphogenesis such that it is impossible to score for duplications. These results demonstrate that ectopic mirror-symmetric divisions and Pard3 function are required to generate duplicated mirror-image neural tubes in embryos with delayed convergence.

A prerequisite for lumen formation in the solid neural rod is the development of apical specializations at the tissue midline and mirror-image cell polarization on either side of this midline. Our results (summarized in Fig. 4l) demonstrate that this tissue organization is orchestrated by mirror-symmetric cell division, which localizes the apical polarity protein Pard3 to the mitotic cleavage furrow. It will be interesting in the future to see if mirror-symmetric divisions are a more general mechanism for initiating mirror-image pattern or lumen formation in other embryonic organ primordia.

METHODS

Embryo care. Embryos were staged and cared for according to standard protocols. The *trilobite/vangl2* mutant allele used was tri^{m209} (ref. 14).

Time-lapse imaging. Confocal time-lapse imaging was carried out by embedding embryos in low melting point agarose (Sigma) and viewing the neural keel in the transverse or dorsal planes using a ×40 or a ×63 long-working-distance water immersion objective. Embryos were maintained at 28.5 °C in an environmental chamber and *z*-stacks collected at 5-min intervals, usually starting at 10 or 11 h.p.f. and continuing through to 18 h.p.f.

Immunocytochemistry. For immunostaining, embryos were fixed in 4% paraformaldehyde and sectioned at 10 μm on a cryostat, or stained as wholemounts. Antibodies and dilutions used were aPKCζ (Santa Cruz C20, 1:500), GFAP (Dako Z0344, 1:200) and phospho-histone H3 (Sigma H0412, 1:200).

Cell division inhibitors. To block cell division at neural keel and rod stages, embryos were cultured in embryo medium containing 100 μM aphidicolin (Sigma) and 20 mM hydroxyurea (Sigma) dissolved in 4% dimethylsulphoxide[7,8,21], from approximately 90% epiboly onwards.

Dextran injection into ventricles. To reveal ventricular organization in live embryos, a small volume of 4% rhodamine dextran was pressure-injected into the midbrain ventricle of wild-type and experimental embryos at 30 h.p.f.

Additional details of methods, including information about the morpholinos and RNA constructs used can be found in Supplementary Information.

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