Zebrafish Neural Tube Morphogenesis Requires Scribble-Dependent Oriented Cell Divisions

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SUMMARY

How control of subcellular events in single cells determines morphogenesis on the scale of the tissue is largely unresolved. The stereotyped cross-midline mitoses of progenitors in the zebrafish neural keel [1–4] provide a unique experimental paradigm for defining the role and control of single cell orientation for tissue-level morphogenesis *in vivo*.

We show that the coordinated orientation of individual progenitor cell division in the neural keel is the cellular determinant required for morphogenesis into a neural tube epithelium with a single straight lumen. We find that Scribble is required for oriented cell division and that its function in this process is independent of canonical apico-basal and planar polarity pathways. We identify a role for Scribble in controlling clustering of α-Catenin foci in dividing progenitors. Loss of either Scrib or of N-cadherin results in abnormally oriented mitoses, reduced cross-midline cell divisions and similar neural tube defects. We propose that Scribble-dependent nascent cell-cell adhesion clusters between neuroepithelial progenitors contribute to define orientation of their cell division. Finally, our data demonstrate that while oriented mitoses of individual cells determine neural tube architecture, the tissue can in turn feed back on its constituent cells to define their polarization and cell division orientation to ensure robust tissue morphogenesis.

RESULTS

To identify mechanisms underlying the establishment of epithelial tissue form and shape, we focus on the immature epithelium of the zebrafish neural keel whose morphogenesis results in the formation of the lumenized neuroepithelial tube. Neural keel development is characterized by stereotyped oriented mitoses that generate two bilaterally distributed neural progenitors (Fig.1A,B) [1–4]. We focus on the *symmetric* oriented cell divisions of the

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neural keel in order to uncouple morphogenesis from cell fate. Here we set out to address whether oriented cell divisions are a determinant for morphogenesis of the zebrafish neural tube formation, and to define the underlying mechanisms that control the orientation of cell division *in vivo*.

We first analyzed *wt* mitotic spindle kinetics in keel stage progenitors and find that the mitotic spindle apparatus assembles with the spindle poles aligning with the anterior-posterior (planar) body axis and subsequently rotates about 90° into an apico-basal orientation (Fig.1A,B)[4]. Inferring the mitotic spindle orientation as orthogonal to the metaphase plate, we determined that in *wt* embryos the spindle orientation is on average 16.2 +/−14.6° from the planar axis at the onset of mitosis and 70 +/−21° after rotation (Fig. 1F).

Mitotic spindle positioning can be regulated by the interaction of astral microtubules with the cell cortex and with the cortical actin-based network [5, 6]. Using a potent actin dynamics inhibitor, cytochalasin-D [7, 8], we find that perturbing actin dynamics influences the orientation of mitosis in the neural keel, resulting in a distribution of anaphase angles that is statistically similar to random (Fig.1C).

The molecular machinery that mediates the interactions between the cortical cues and actin to locally stabilize spindle position is not well understood. Scribble functions in other systems in apico-basal epithelial polarity and junction formation [9–11], in planar cell polarity (PCP)-dependent processes [12–14] and in mitotic spindle asymmetry in *Drosophila* neuroblasts [15]. Scrib has also been indirectly implicated in actin organization [16]. Therefore we tested whether the single zebrafish scribble gene [13], *scrib*, plays a role in orienting mitoses in the neural keel. We analyzed spindle orientation in live embryos lacking maternal and zygotic *scrib* (*mzscrib*) and in *Tg(h2a.f/z-GFP)* transgenics injected with *scrib* morpholinos. We find that the cell division axes of *mzscrib* mutants and *scrib* morphants display abnormally homogeneous distributions, very different from *wt* (Fig.1C).

This finding allowed us to ask whether the oriented mitosis in the neural keel is required for normal neural tube morphogenesis. We find that in *mzscrib* the apical cell surfaces that constitute the epithelial neural tube lumen are not aligned along the neural axis (Fig.1D, Suppl.Fig.1A,B). However the general apical cortical organization remains intact in *mzscrib* (Suppl.Fig.1A,B). Consistent with this, apical Pard3, visualized as a GFP fusion protein [17], is correctly localized near the cleavage plane of misoriented *mzscrib* mutant cells (Fig. 1E). Subsequent sub-apical Pard3 localization and junction formation between neighbours generated by such misoriented divisions would be expected to result in the disorganized neural tube lumen such as that observed in *mzscrib* mutants. We observe the strongest morphogenetic perturbations in *mzscrib* mutants in more dorsal regions of the neural tube (Suppl.Fig.1C) and at posterior hindbrain and anterior spinal cord levels, resulting in disorganized neuronal architecture there (Suppl.Fig.1D).

The morphogenesis defects we observe in mzscrib mutants is not due to abnormalities in the neural plate before the onset of cross-midline divisions or in convergent extension movements that would result in abnormally positioned mitoses in the lateral neural keel

(Suppl. Fig.2A-D). However the *mzscrib* neural tube phenotype is rooted in a defect in cell division, since blocking cell proliferation rescues it. Applying inhibitors of cell proliferation [17, 18] decreases morphogenetic defects from 87% in untreated *mzscrib* embryos to 30.5% in treated *mzscrib* embryos (Fig.1H). Since it is the orientation of cell division, not its timing or position, that is affected in *mzscrib* mutants, we conclude that correct spindle orientation is essential for the morphogeneis of a straight neural tube and lumen.

Abnormally oriented mitoses in *mzscrib* could be due to abnormal planar spindle orientation at the entry into mitosis and/or due to abnormal subsequent spindle rotation. While 90% of *wt* progenitors enter mitosis in the planar orientation (mitotic spindle angle of less than 30° from the midline), only 25% of *mzscrib* progenitors do so. Furthermore *wt* spindles rotate on average for 72° (±15°) into the apicobasal orientation at a rate of 24°/minute while in *mzscrib* embryos the spindle rotates less (44°±34°) and slower (15°/minute)(Fig.1F,G). We observe that the 25% of *mzscrib* mutant cells that are correctly oriented at the onset of mitosis do not enter anaphase correctly with any higher probability than *mzscrib* mutant cells that are incorrectly oriented at the onset of mitosis (Fig.1F,G and data not shown). Thus Scribble is required to control both initial spindle positioning and rotation into the final correct apico-basal orientation.

In the *wt* zebrafish neural keel, only daughters of dividing cells cross the midline [3, 17–19]. To address whether correct orientation of mitoses in the neural keel is required for the bilateral daughter cell distribution in the neural tube, we labeled neural progenitors on one side of gastrulating embryos and assessed the subsequent distribution of labeled cells in the neural tube. Whereas in *wt* embryos cells show a typical bilateral distribution, in the posterior hindbrain/anterior spinal cord region of *mzscrib* mutants where morphogenesis is most severely disrupted, labeled cells are predominantly unilateral (Fig.1I, n=30).

Vertebrate Scrib has been demonstrated to be functional in tissues that require PCP [13][12]. Furthermore, non-canonical Wnts and Vangl2, a core PCP component, are essential for normal neural tube morphogenesis in zebrafish [17, 18]. Scrib could thus potentially act in the PCP pathway, which could provide the cue for oriented cell divisions in the neural keel. The PCP reporter GFP-Prickle (GFP-Pk) [18] is localized in foci predominantly at the anterior sides of progenitors in *wt*. GFP-Pk puncta are lost in *vangl2*$^{-/-}$ mutants [18](Fig. 2A). In *mzscrib* embryos, while the ratio of anterior to posterior positioned foci is lower (1.7:1) than in *wt* (3.1:1), the GFP-Pk protein still localizes to distinct foci (Fig.2A). Furthermore, we note that the anterior localization of GFP-Pk is lost in *wt* neural keel progenitors during mitosis (Suppl.Fig.3A), further suggesting that PCP might not be directing the axis of neural keel mitoses. Consistent with this, the distribution of mitosis orientation in live *Tg(h2a.z/f-GFP)* Wnt11, Dsh2, Pk1a and Pk1b morphants or *vangl2−/−* mutants are all statistically similar to the *wt* distribution (Fig.2B). Comparing *mzscrib* and *vangl2* phenotypes more closely, there are multiple differences: *vangl2* morphants have slower neuroepithelial convergence movements resulting in ectopic laterally positioned mitoses and duplicated neural tube lumina, whereas mzscrib mutants have midline mitoses and a single, though branched, neural tube lumen (Suppl.Fig.2B-E) [17]. The phenotypic differences between *mzscrib* and PCP mutants/morphants thus suggest that Scrib does not function in the context of PCP to orient mitoses in the neural keel.

Scrib can restrict the Par complex to the apical cortical domain of polarized cells [9]. We asked whether the function of Scrib in oriented mitoses in the neural keel depends on apical Par components. Scrib is not polarized in mitotic progenitors, and Pard3-GFP localization is normal in *mzscrib* (Fig. 1E, Suppl.Fig.3B). Furthermore, *pard6gb*$^{-/-}$ mutant and *aPKCζ/aPKCλ* morphant progenitors divide with normal orientation (Fig.2C). Finally, *mzscrib;pard6gb*$^{-/-}$ double mutants have a branched, disorganized midline similar to *mzscrib* single mutants (Fig.2D). Thus, these results suggest that Scrib's function in orienting cell division in the neural keel is distinct from a role in establishing or maintaining apico-basal polarity.

Since Scribble polarizes epithelial non-dividing cells by regulating adhesion junctional complexes [10, 11, 20], Scrib might affect the organization of cell-cell adhesion complexes in the context of the neural keel. We analyzed the subcellular distribution of adhesion complexes with an α-Catenin (Ctnna) transgenic gene trap line in which citrine is expressed as a fusion protein with α-Catenin (Ctnna), *Gt(Ctnna-citrine)*^{ct3a}, reflecting endogenous Ctnna protein localization (Suppl.Fig.3C). In *dividing wt* progenitors the Ctnna-citrine is predominantly cortical. Importantly for this work, we noted that Ctnna-citrine is also enriched in cortical foci that lie at the presumptive cleavage plane and which, upon cytokinesis, segregate into subapical structures (Fig.3A, Suppl.Fig.3D). We discovered that overall cortical Ctnna-citrine, as well as enrichment at these cortical foci, was reduced in *scrib* morphants. Ctnna-citrine localization was similarly affected in *N-cadherin/cdh2* morphants, as would be predicted for loss of an essential component of the adherens junction (Fig.3B,B'). Whereas in *wt* progenitors the ratio of fluorescence intensity at the cortical foci compared to the adjacent cortex is 2.2, the ratio in *cdh2* and *scrib* morphants was on average 1.8 and 1.4, respectively (data not shown). This requirement for Scrib in the localization of Ctnna is restricted to the neural keel stage, since Ctnna-citrine is localized normally in the mature (but disorganized) neural tube epithelium of *scrib* morphants (Suppl.Fig.3D).

Since cadherin-mediated cell-cell contacts can be instructive in spindle orientation in other contexts [22, 23], we considered that Scrib might be regulating oriented mitoses in the neural keel via regulation of α-Catenin-based cell-cell adhesion. If this is the case, *cdh2*$^{-/-}$ mutants would be expected to have defects similar to *mzscrib* mutants. Focusing on the ventral neural keel of *cdh2* morphant and *cdh2*$^{-/-}$ mutants (since dorsal regions are severely disorganized [21]), we find that mitotic angles in *cdh2* morphants indeed show a more homogeneous distribution, different from the *wt* distribution (Fig.3C). Mitotic spindle kinetics in *cdh2* morphants were also similar to *scrib* morphants, with only 19% of cells oriented correctly at the onset of mitosis, and with prolonged, slow rotation thereafter (Fig. 3.E,F). The subsequent alignment of neuroepithelial apical surfaces in the ventral hindbrain is disorganized in the absence of Cdh2 (Fig.3D). The similar consequences of loss of Scrib and loss of Cdh2 on α-Catenin localization, oriented cell division and neural tube morphogenesis suggest that Scrib controls oriented mitosis, at least in part, through control of cell adhesion in the neural keel.

Finally, we used genetic mosaics to determine whether oriented cross-midline mitosis is the result of a genetically encoded program in a single cell or a property that emerges only in the

tissue. Whereas *wt* cells distribute daughters to both sides of the midline (Fig.4A), *cdh2$^{-/-}$* transplanted cells in a *wt* environment form tight unilateral clusters (Fig.4B). Interestingly, when four or fewer *cdh2$^{-/-}$* cells are surrounded by *wt* cells, they acquire a normal cell shape and are positioned as bilateral pairs (Fig.4C). Similar observations were made for *mzscrib* mutant cells (Fig. 4D). In both instances, isolated mutant cells that are rescued by surrounding *wt* cells acquire normal subcellular organization of F-actin visualized by phalloidin (Fig.4A-E) and 3D-live imaging of the Utr-CH-RFP reporter [24] (Fig.4F). Additionally, *in vivo* timelapse analyses of mosaic embryos confirm that single *cdh2$^{-/-}$* as well as single isolated *mzscrib* cells predominantly divide with correct orientation (Suppl.Fig.4 and data not shown). Our study thus reveals a previously unrecognized cellular community effect that ensures cytoskeletal polarization and orientation of mitosis in individual neural progenitor cells.

DISCUSSION

The goal of this work was to determine whether oriented cell division patterns per se could control tissue morphogenesis, and which cues regulate the highly stereotyped oriented mitoses in the zebrafish neural keel. Most oriented cell divisions studied to date occur in the context of mature epithelia (fly imaginal disc, chick neural tube, mammalian cortex, retina and kidney). In contrast, our work suggests that the well-established mechanisms that determine spindle orientation [6] in mature epithelia may not apply in epithelia undergoing junctional establishment. We have shown that Scrib is required for oriented mitoses in the zebrafish neural keel and that this results in abnormal morphogenesis of the neural tube.

How does Scrib control the orientation of cell division in the neural keel? In different contexts, Scrib has been implicated in PCP and apico-basal polarity, both of which can be instructive in oriented mitoses [25–29]. We find that Scrib functions in neither of these processes for mitosis orientation in the neural keel. Taking into account that Scrib functions as a tumor suppressor by promoting Cadherin-dependent cell-cell adhesion [30, 31] and is required for E-cadherin-mediated cell adhesion in cultured epithelial cells [20], we considered a function in regulating nascent adhesive structures in mitotic progenitors in the neural keel. We find that *scrib* knock-down prevents efficient recruitment of α-Catenin to equatorial cortical foci in mitotic cells that correspond to the future subapical junctional complexes of the mature epithelium. We argue that this function is responsible for the spindle orientation defects of *mzscrib* mutants because we find that *cdh2* mutants have similar defects in α-Catenin localization, spindle orientation and rotation in dividing progenitors.

How might Scrib control the establishment and localization of equatorial adherens complexes in dividing cells? Cadherin-mediated cell-cell contacts are instructive in spindle orientation in cultured epithelial cells and in the fly sensory organ lineage [22, 32]. Since the mitotic spindle is initially aligned with the equatorial α-Catenin foci, Scrib-dependent equatorial α-Catenin might tether a pool of astral microtubules at the onset of mitosis. Mechanistically, initial positioning could depend on Scrib engaging Cadherin and α-Catenin at the cortex [20] and functioning via regulation of the β-catenin-Dynein complex [33], or through the APC (Adenomatous Polyposis Coli) complex [34, 35]. Since the equatorial

adherens foci do not rotate, one possibility is that during the second phase Scrib prevents planar positioning of astral microtubules. Alternatively, the equatorially positioned α-Catenin foci could form a barrier restricting dynamic microtubule-mediated search-and-capture only to the apical- and basal-most cell hemispheres. Since cortical dynein anchor sites can be determined by actin organization [5] and we show that disruption of actin impairs the orientation of mitosis, Scrib might establish a barrier function via influencing the actin network. An intriguing possibility is that Scrib controls spindle orientation through Cdc42-dependent reorganization of the actin cytoskeleton [36, 37] as observed in the *Xenopus* [38]. However expressing a dominant negative form of Cdc42 does not disrupt the orientation of mitosis in the zebrafish neural keel (data not shown).

Unexpectedly, we find that cell shape, cytoskeletal organization and oriented cell division could be rescued if single *mzscrib* or *cdh2* mutant cells are surrounded by *wt* cells in mosaic embryos. One possibility is that external physical forces arising as community effect in the wildtype tissue could feed back into redirecting or reinforcing the cellular polarity in neighboring cells. The only similar feedback loop to our knowledge between cell shape and cytoskeletal organization for mitosis *in vivo* has been proposed for the fission yeast [39], and very recently for cell orientation by anisotropic tension in the *Drosophila* pupal wing epithelium [40]. It is therefore tempting to ask whether shape-dependent polarity control is an evolutionary ancient property that has been adapted in multicellular organisms for correct morphogenesis of complex forms.

We propose that the zebrafish neural keel represents an important model system for how single cell polarity sculpts developing tissue structure and the tissue structure in turn instructs individual cell polarization and cell division orientation *in vivo*. In the future, it will be essential to integrate cell-cell, cell-extracellular matrix interactions as well as biophysical properties as possible non-exclusive regulatory regimes that reinforce robust intracellular decisions in single cells to effect morphogenesis at the tissue level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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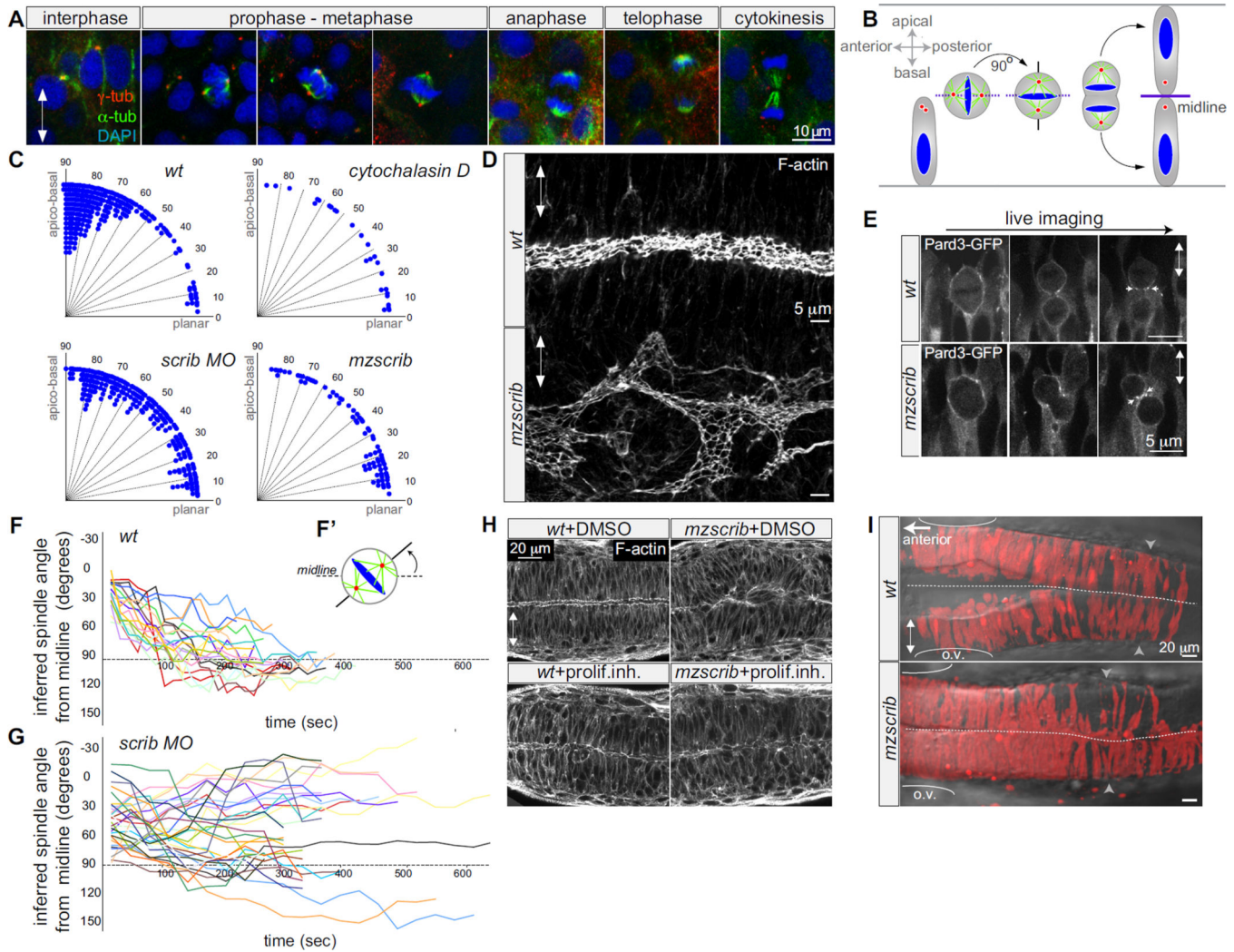
Figure 1. Scrib regulates mitotic spindle orientation and neural tube morphogenesis
A) Immunostainings of neural keel progenitors showing that the orientation of the mitotic spindle changes over the course of mitosis. γ-tubulin (centrosomes; red), α-tubulin (spindle; green), and DAPI (DNA, blue). This process results in the bilateral distribution of daughter cells as schematized in (**B**). (**C**) Quantification of mitosis orientation at anaphase. Chi-square analysis shows that the three distributions shown are highly significantly different from *wt* (n=269): 3 μg/ml Cytochalasin-D treated (n=28, X^2=51, 1 degree of freedom (df); p<0.001); *scrib* morphant (n=235, X^2=306, 8 df; p<<0.001); *mzscrib* mutant (n=76, X^2=201, 2 df; p<<0.001). While all three distributions are more homogeneous than *wt*, only Cytochalasin-D treatment results in a statistically randomized distribution (X^2=2.7, 3 df; p=0.44). (**D**) Posterior hindbrain lumen morphology, defined by apical F-actin, with an abnormal, branched organization in the *mzscrib* mutant compared to *wt*. (**E**) Pard3-GFP localization to subapical foci upon cytokinesis (arrows) occurs normally in *wt* and misoriented *mzscrib* mutant neural keel progenitors. (**F,G**) Representation of mitotic spindle rotation in live (**F**) *wt* (n=20) and (**G**) aberrantly rotating *scrib* morphant (n=32) progenitors. Plots present the angle between inferred mitotic spindle axis and the midline over time. (**F'**)

Scheme of spindle rotation in a mitotic progenitor. Mitotic spindle axis as solid line, midline as dashed line. **(H)** Inhibition of cell proliferation results in diminished neural tube morphogenesis defects in *mzscrib* embryos. **(I)** Requirement of Scrib for cross-midline cell divisions in the neural keel. Labeled cells in a 22 hpf *wt* embryo have a bilateral distribution, but have a predominantly unilateral distribution in the posterior hindbrain/anterior spinal cord region of an *mzscrib* mutant. Arrowheads indicate the position of the first somite (left is hindbrain, spinal cord is to the right), ovals mark the otic vesicle, dotted line indicates the midline. In all panels, anterior is to the left and double arrowheads indicate the apico-basal axis of neuroepithelial progenitors. See also Supp.Fig.1.
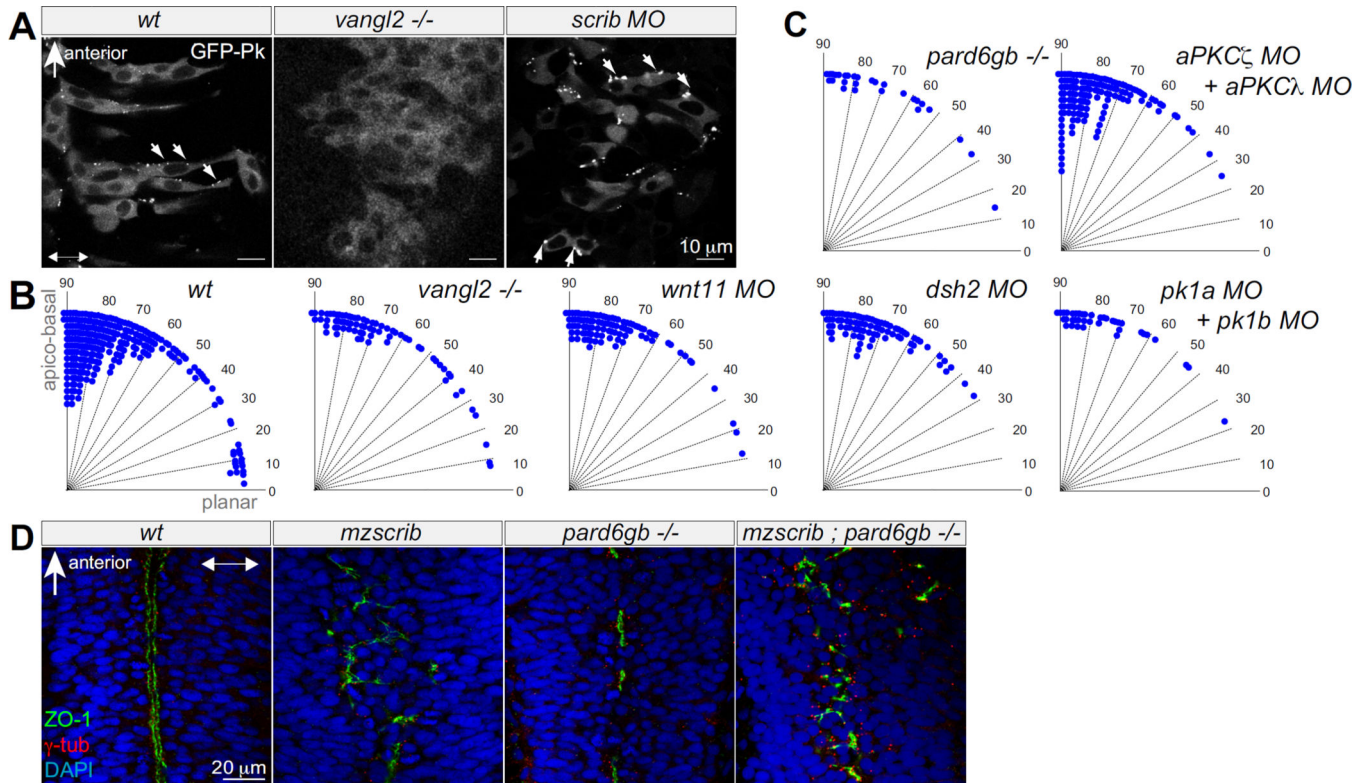
Figure 2. Neither PCP components nor Par complex are required for proper spindle orientation in the neural keel

(A) GFP-Prickle positive foci (arrows) in posterior hindbrain neural keel progenitors of *wt*, *vangl2*^{−/−} mutant and *scrib* morphant embryos. **(B,C)** Quantification of anaphase orientation without functional PCP and Par complex components. The distributions of the various mutant or morphant conditions shown are not significantly different from *wt* (n=269): *vangl2*$^{−/−}$ mutant (n=72; X^2=5.1; 4 df; p=0.28); *wnt11* morphant (n=74; X^2=2.6, 3 df; p=0.41); *dsh2* morphant (n=86, X^2=9.6, 4 df; p=0.05), *pk1a+pk1b* morphant (n=38, X^2=2.6, 3 df; p=0.46); *pard6gb*$^{−/−}$ mutant (n=28, X^2=2.7, 3 df; p=0.43); the distribution of mitotic angles in *aPKCζ+aPKCλ* double morphants is slightly more biased toward apicobasal than *wt* (n=139, X^2=14.4, 4 df; p=0.006). **(D)** The branched, disorganized neural tube lumen of *mzscrib* and *mzscrib;pard6gb*$^{−/−}$ double mutants. Dorsal optical sections at 18 hpf immunostained with γ-tubulin (red), ZO-1 (green), and DAPI (blue). In A and D, anterior is to the top, double arrowheads indicate the apico-basal axis. See also Suppl.Fig.2.
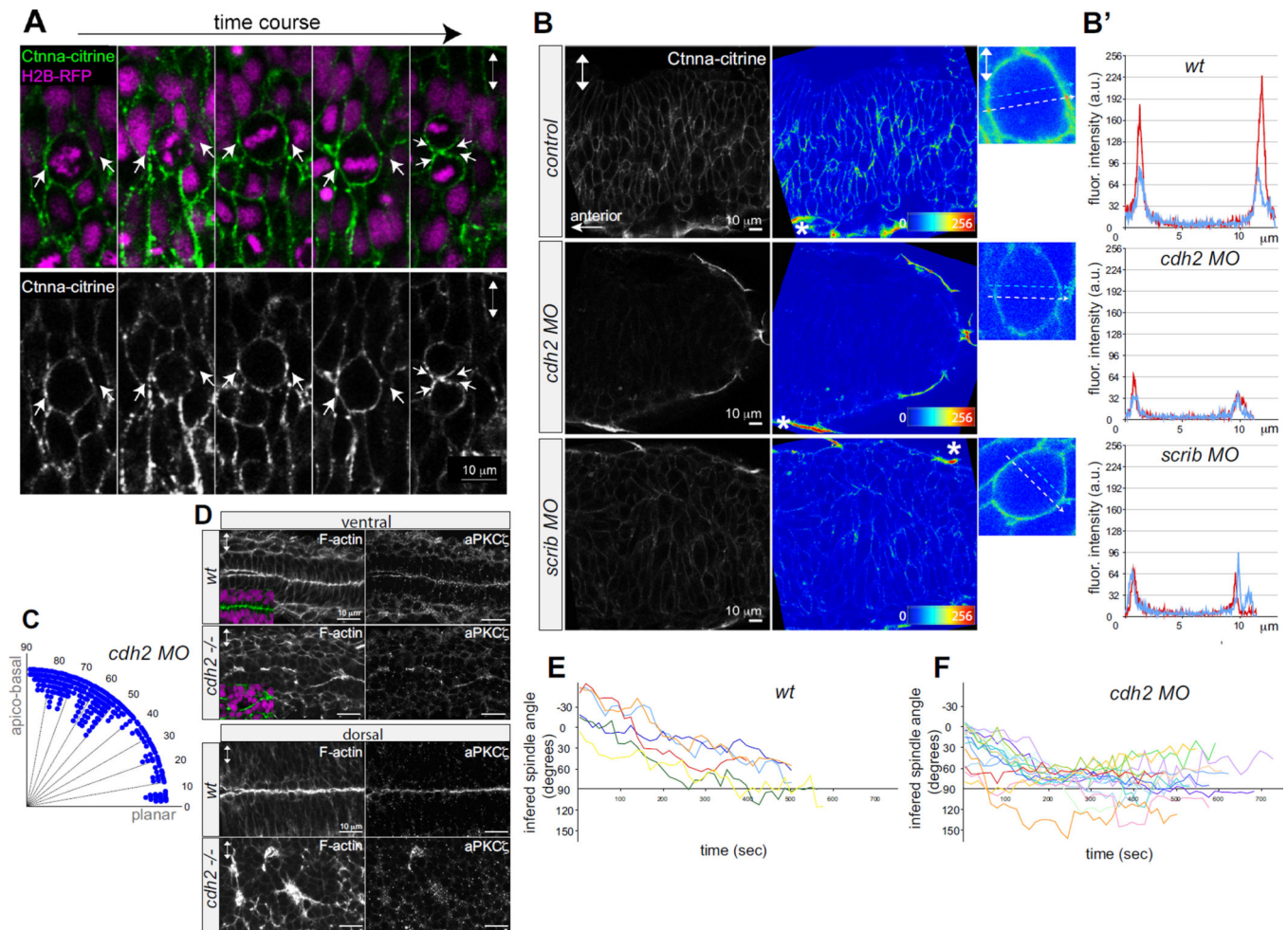
Figure 3. Reduction of α-Catenin foci in the neural keel correlates with aberrant mitotic orientation and neural tube architecture defects

(**A**) Equatorially positioned cortical Ctnna-citrine foci (α-Catenin, green, arrows) are stationary while chromosomes (H2B-RFP, purple) rotate in a timelapse of a dividing neural keel progenitor. Upper panel merged, lower panel showing Ctnna-citrine alone. (**B**) Scrib and Ncad/cdh2 are required for Ctnna-citrine abundance and the localization of equatorial cortical Ctnna-citrine foci in neural keel mitotic progenitors. Posterior hindbrain at 6–8 somites with Ctnna-citrine (left), fluorescence intensity in pseudocolors (middle), and in single mitotic progenitors (right). Localization of Ctnna-citrine in *wt* (upper row), *cdh2* morphants (middle row) and *scrib* morphants (lower row). The unchanged Ctnna-citrine signal in non-neural peridermal cells is marked by an asterisk. (**B'**) Fluorescence intensity plots of Ctnna-citrine levels in *wt*, *cdh2* MO, *scrib MO* mitotic cells (shown in **B**) with the *y*-axis displaying arbitrary gray values along a line across a mitotic cell at equatorial (white dashed arrow in pictures, red line in plots) and lateral (blue dashed arrow in pictures, blue line in plots) positions averaged over 8 pixels in width. (**C**) Quantification of mitosis orientation at anaphase in *cdh2* morphants. This distribution is highly significantly different from *wt* (n=267, X^2=224; 8 df; p<<0.001). For *wt* controls compare to Fig.1C. (**D**) Disorganized, branched neural tube midline in the $cdh2^{-/-}$ neural tube in horizontal cryo-

sections. F-actin/ phalloidin (green), aPKCζ and DAPI (purple). **(E,F)** Representation of mitotic spindle rotation in live *wt* (n=6) **(E)** and *cdh2* morphant (n=16) **(F)** neural keel progenitors. Spindle orientation is inferred from the orientation of the chromosomes marked with H2B-GFP. Images in all panels are in dorsal view. Double arrowheads indicate the apico-basal cell axis. See also Supp.Fig.3.
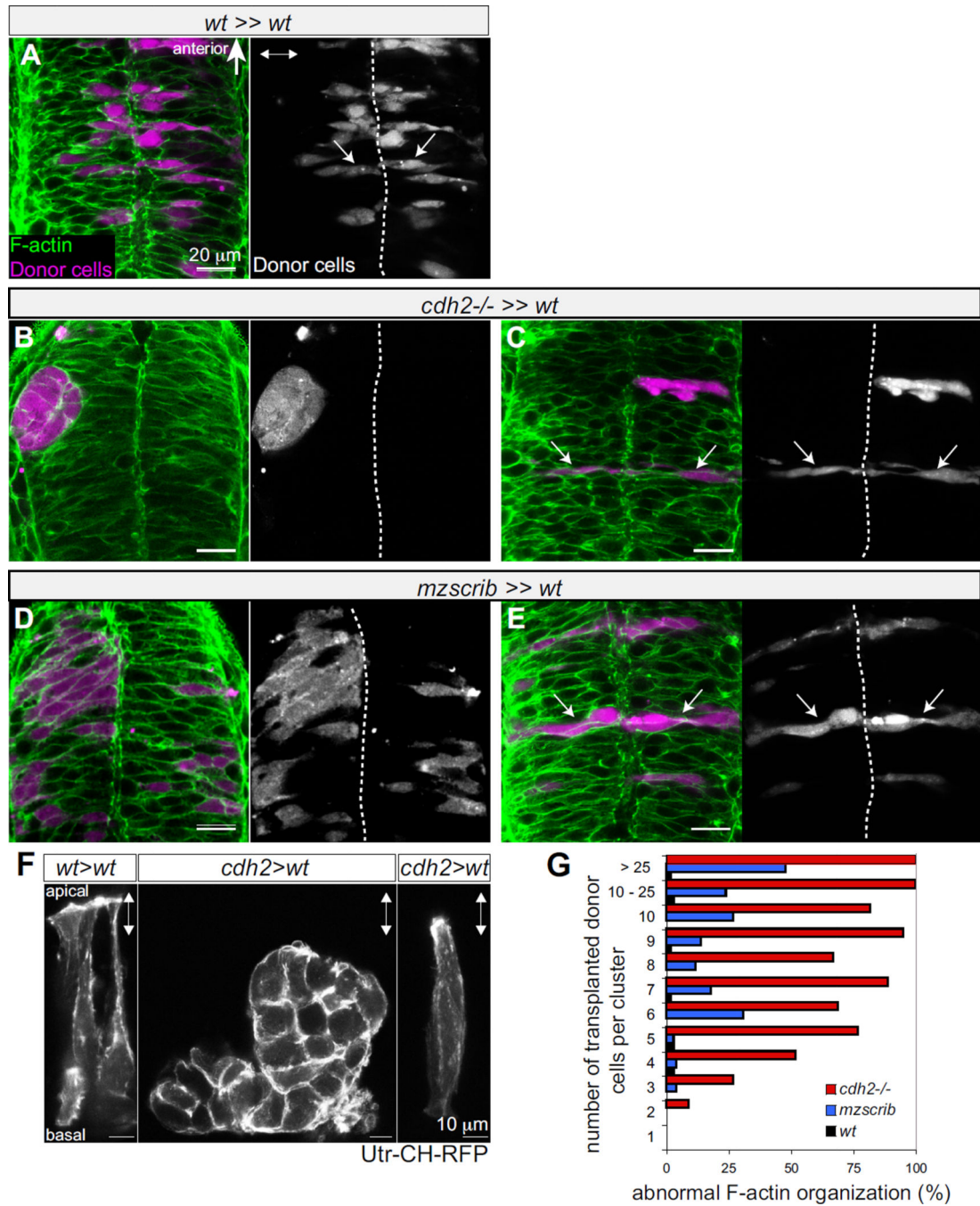
Figure 4. Non-autonomous rescue of oriented cross-midline cell division of single *cdh2*^{−/−} and *mzscrib* mutant cells

(A-E) Genetic mosaics at neural tube stage (21 hpf) in which donor derived cells (purple) were transplanted into the presumptive posterior hindbrain of *wt* host embryos (F-actin in green). (A) Bilateral distribution of *wt* cells (arrows) in a *wt* environment. (B) *cdh2*$^{-/-}$ cells form unilateral aggregates in a *wt* environment. (C) In contrast, isolated *cdh2*$^{-/-}$ progenitors show rescued bilateral distribution and normal cell shape. (D) A large group of *mzscrib* mutant cells in a *wt* environment with unilateral cell distribution. (E) Isolated *mzscrib* cells

in a wt environment have rescued shape and bilateral distribution. **(F)** Utr-CH-RFP reporter in mosaic embryos showing normal apical enrichment in *wt* control transplants, loss of apical enrichment in a cluster of transplanted $cdh2^{-/-}$ cells, and rescue in a single $cdh2^{-/-}$ cell that is surrounded by *wt* host cells. Images represent maximal intensity projections of optical sections. **(G)** Quantification of occurrence of abnormal F-actin organization in mosaics. *wt* black (n=5 experiments, 13 embryos), *mzscrib* blue (n=3 experiments, 21 embryos), $cdh2^{-/-}$ in red (n=2 experiments, 15 embryos). Embryos in dorsal view with anterior to the top. Dotted white line indicates the neural tube midline, double arrowheads indicate the apico-basal axis of the neuroepithelium. See also Supp.Fig.4.