

# miR-430 regulates oriented cell division during neural tube development in zebrafish

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## ABSTRACT

MicroRNAs have emerged as critical regulators of gene expression. Originally shown to regulate developmental timing, microRNAs have since been implicated in a wide range of cellular functions including cell identity, migration and signaling. miRNA-430, the earliest expressed microRNA during zebrafish embryogenesis, is required to undergo morphogenesis and has previously been shown to regulate maternal mRNA clearance, Nodal signaling, and germ cell migration. The functions of miR-430 in brain morphogenesis, however, remain unclear. Herein we find that miR-430 instructs oriented cell divisions in the neural rod required for neural midline formation. Loss of miR-430 function results in mitotic spindle misorientation in the neural rod, failed neuroepithelial integration after cell division, and ectopic cell accumulation in the dorsal neural tube. We propose that miR-430, independently of canonical apicobasal and planar cell polarity (PCP) pathways, coordinates the stereotypical cell divisions that instruct neural tube morphogenesis.

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## 1. Introduction

MicroRNAs are evolutionarily conserved small noncoding RNAs that induce the translational repression and degradation of target mRNAs (Fabian et al., 2010; Huntzinger and Izaurralde, 2011). Animal genomes contain hundreds of microRNA genes, and each microRNA, in turn, can directly regulate hundreds of target mRNAs (Alvarez-Garcia and Miska, 2005; Ambros, 2004; Bartel, 2009; Takacs and Giraldez, 2010). Given this widespread reach, an individual microRNA has the potential to both regulate multiple components within particular cellular pathways as well as impact several distinct processes simultaneously within the cell (Flynt and Lai, 2008). The global impact of microRNA function can be assessed through removal of *dicer*, a RNase III ribonuclease required for microRNA biogenesis (Bernstein et al., 2001; Denli et al., 2004; Harfe et al., 2005; Ketting et al., 2001; Lee et al., 2004; Murchison et al., 2007; Wienholds et al., 2003). Zebrafish deficient for *dicer* activity develop normal A-P and D-V body axes, as well as undergo cell fate specification and tissue differentiation (Giraldez et al., 2005). *MZdicer* mutant embryos display morphogenetic defects during gastrulation and brain ventricle development.

Reintroduction of a single microRNA, miR-430, rescues these defects, indicating that loss of miR-430 alone is primarily responsible for these early *dicer* phenotypes (Giraldez et al., 2005). Endogenous miR-430 is expressed ubiquitously in the early embryo and regulates the expression and stability of hundreds of maternal mRNAs (Chen et al., 2005; Giraldez et al., 2006; Lund et al., 2009). Distinct phenotypes can be attributed to the misregulation of specific cellular pathways and target mRNAs. For instance, miR-430 directly represses *squint* and *lefty* mRNA expression to dampen and balance Nodal signaling during mesendoderm induction (Choi et al., 2007; Rosa et al., 2009), buffers chemokine signaling effectors *sdf1a* and *cxc7* to ensure robust germ cell migration (Staton et al., 2011) and regulates the expression of germline-specific genes in the soma (Mishima et al., 2006). Perhaps the most striking *MZdicer* phenotype is severely reduced brain ventricle formation and subsequent morphogenesis defects (Giraldez et al., 2005). However, the cellular basis underlying neural defects remains unclear.

Brain morphogenesis in zebrafish initiates with the convergence of neural progenitors to the dorsal midline (Hong and Brewster, 2006; Papan and Camposortega, 1994). Mutants in the planar cell polarity (PCP) pathway display delayed convergence movements and neurulation defects (Quesada-Hernandez et al., 2010; Tawk et al., 2007). As convergence proceeds, neural progenitors ingress ventrally to generate a solid neural rod primordium (Lowery and Sive, 2004; Papan and Camposortega, 1994). Teleost neurulation is distinctive in that neural progenitors

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do not undergo apicobasal polarization or organize into distinct neuroepithelia until after the neural rod forms (Geldmacher-Voss et al., 2003; Hong and Brewster, 2006). Neural midline formation is mediated by a stereotypic series of oriented cell divisions in which daughter pairs segregate across the presumptive midline and establish mirror-symmetric apicobasal polarity (Ciruna et al., 2006; Clarke, 2009; Quesada-Hernandez et al., 2010; Tawk et al., 2007). The localization, timing, and mitotic orientation of these divisions are critical to ensure the faithful distribution of daughter cells and the establishment of a coherent neural midline. Defects in any of these steps leads to ectopic neural tube formation and/or disorganization of the midline (Ciruna et al., 2006; Quesada-Hernandez et al., 2010; Tawk et al., 2007; Zigman et al., 2014; Zigman et al., 2011).

In this study, we investigate how microRNAs regulate neural tube formation in zebrafish. We find that loss of miR-430 activity causes the dorsal accumulation of ectopic neural progenitors that fail to incorporate into the nascent neuroepithelium. In the absence of miR-430 function, apicobasal and planar cell polarity are established. In contrast, we show that miR-430 is required for proper mitotic spindle orientation of neural rod cell divisions during neural midline formation. Finally, we identify a set of neuroectodermal miR-430 target genes, providing an entry point for understanding how miR-430 coordinates oriented cell divisions during neural tube development and tissue morphogenesis.

## 2. Methods

### 2.1. Zebrafish lines

MZ*dicer* fish were generated as previously described (Giraldez et al., 2005). Embryos used were *dicer*<sup>hu896/hu896</sup>, *dicer*<sup>hu896/hu715</sup> or *dicer*<sup>hu715/hu715</sup>.

### 2.2. mRNA reporter constructs

Capped mRNA was transcribed from reporter constructs using mMessage mMachine kit (Ambion). 1 nl of mRNA was injected at one-cell stage at following concentrations: pCS2+GFP (50 pg), GFP-Pk ([10]; 75 ng/ul), histone2B-mCherry (100 ng/ul) and pCS2-Kaede (100 ng/ul). Scatter labeling was performed by injecting a subset of blastomeres in 16-cell stage embryos. For chimaeric analysis of miR-430 function, wildtype and MZ*dicer* embryos were injected with GFP mRNA at one-cell stage. Equivalently staged donor and host embryos were grown under similar environmental conditions until mid-blastula stages, at which point donor cells were transferred to wildtype host embryos (20–50 cells/host).

### 2.3. Embryo sectioning and labeling

Embryos were fixed in 4% paraformaldehyde O/N at 4°C, mounted in 2% low-melting point agarose and sectioned at 200–250 μm intervals using a vibratome (1500 System, Vibratome). Transverse sections through the hindbrain were identified based on the presence of otic vesicles and size/presence of notochord. Immunohistochemistry was performed on floating sections with: rabbit-aPKC (C-20; Santa Cruz Biotechnology 1:200) and rabbit-GFP (Molecular Probes; 1:1000). Secondary antibodies conjugated to Alexa-488 (Invitrogen) were used at a 1:200 dilution. Nuclei and F-actin were stained with Topro-3 (Life Technologies; 1:2000) and rhodamine-phalloidin (Molecular probes; 1:75), respectively. Imaging was performed on a Zeiss 510 Meta confocal microscope.

In situ hybridization using *dlx3* riboprobe (plasmid obtained from Rachel Brewster, University of Maryland) was performed as described (Thisse and Thisse, 2008)

### 2.4. Time-lapse confocal microscopy

Live embryos were mounted in 1.2% low-melting point agarose prior to imaging. For lineage tracing, embryos injected with *kaede* mRNA were mounted at approximately 3-somite stage, photo-converted by scanning ROI with a 405uM-diode laser on a Leica TCS SP5 Spectral Confocal Microscope, then imaged immediately and approximately 10 h later.

### 2.5. Cell division inhibition

To block cell divisions at neural keel/rod stages, embryos were placed in medium containing 100 μM aphidicolin (Sigma) and 20 mM hydroxyurea (Sigma) in 4% dimethylsulfoxide (DMSO) at 90% epiboly stage. Control embryos were incubated for same time period in media containing 4% DMSO.

### 2.6. Target site analysis

Fold enrichment for 135 microRNA target sites (7mers) in the UTRs of upregulated genes (> 1.5 fold) was determined:

Fold enrichment for single miRNAs

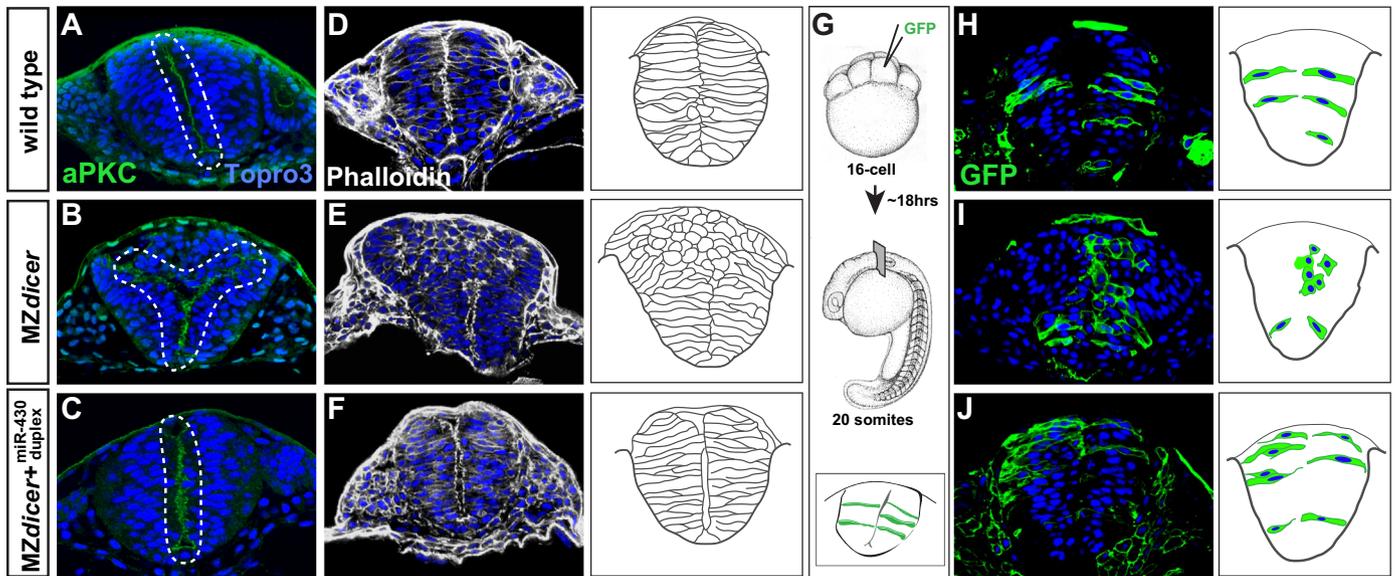
$$= \frac{\frac{\# \text{Target sites for miR-X}}{\# \text{Target sites for all miRNAs}} (\text{experimental})}{\frac{\# \text{Target sites for miR-X}}{\# \text{Target sites for all miRNAs}} (\text{control})}$$

Gene ontology clusters were defined using DAVID (<http://david.abcc.ncifcrf.gov>) Gene Functional Annotation Clustering on GO 'FAT' annotations and 'high' stringency. Clusters are annotated with representative GO terms and corresponding Benjamini-Hochberg FDR corrected *P* values.

## 3. Results

### 3.1. Loss of miR-430 results in neural midline defects

Neurocoel formation is severely impaired in MZ*dicer* embryos (Giraldez et al., 2005). MiR-430 is the earliest expressed miRNA in the early embryo (Chen et al., 2005; Giraldez et al., 2005) and, reintroduction of the miR-430 duplex (which does not require Dicer processing) into MZ*dicer* embryos rescues neurulation defects (Giraldez et al., 2005), indicating that early neurulation defects in MZ*dicer* embryos are exclusively due to loss of miR-430 activity. To further investigate neural tube defects in the absence of miR-430, we first compared neural rod morphology in the hindbrain of wildtype and MZ*dicer* embryos at the ~20 somite stage (Fig. 1). At this stage, neural plate ingression has occurred and neural progenitors are organized within coherent epithelia that display mirror-symmetric apicobasal polarity. Localized enrichment of cell polarity and junctional proteins within apical membranes defines the nascent neural midline from which lumen inflation soon follows. We analyzed apicobasal polarity (aPKC; Fig. 1A–C; (Tabuse et al., 1998)), cell morphology (scatter labeling; Fig. 1G–J) and cytoskeletal organization (F-actin; Fig. 1D–F). In contrast to wildtype progenitors, MZ*dicer* neural cells display defects in epithelial organization. Specifically, we observe the accumulation of multiple rounded cells within medial regions of the dorsal neural rod. Scatter labeling with membrane GFP reveals that these cells often fail to elongate and establish contact with the basal membrane (Fig. 1I), and display abnormal F-actin organization (Fig. 1E), reminiscent of neural rod defects associated with PCP/Wnt pathway impairment (Ciruna et al., 2006). The presence of ectopic cells prevents the formation of a continuous apical membrane along the neural midline as assessed by the apical marker aPKC, precluding proper lumen inflation and neurocoel formation (Fig. 1B). In most cases (77%, *n*=30 embryos), we



**Fig. 1.** miR-430 is required for neural tube morphogenesis. Transverse sections through the hindbrain region of 20-somite stage embryos. Topro3, nuclear stain. Wildtype neural progenitors (A, D and H) display apical localization of aPKC and bilateral organization along the presumptive neural midline (A), display normal subcellular localization of F-actin (D), and acquire elongated cell morphology (H). In contrast, *MZdicer* neural progenitors (B, E and I) form ectopic apical membranes (B), display abnormal F-actin organization (E), and fail to establish contact with lateral membrane and acquire epithelial character in dorsal regions (I). (C, F and J) Neural progenitors from *MZdicer* embryos injected at the 1-cell stage with miR-430 RNA duplex display rescued cell morphology and organization. (G) Scatter labeling strategy. 1-2 blastomeres of 16-cell embryos were injected with GFP mRNA.

observed ectopic neural midline formation within dorsal regions and/or a branching or bifurcation of the neural midline. Variability in phenotypic severity was observed both among different embryos as well as within different hindbrain regions of the same embryo (Fig. S1). Importantly, reintroduction of mature miR-430 rescued neural midline defects in *MZdicer* embryos displaying a single coherent midline (Fig. 1C, F and J). Taken together, these data indicate that miR-430 function is required for proper neuroepithelial organization in the zebrafish dorsal neural rod.

### 3.2. Neural daughter cells fail to segregate across nascent neural midline

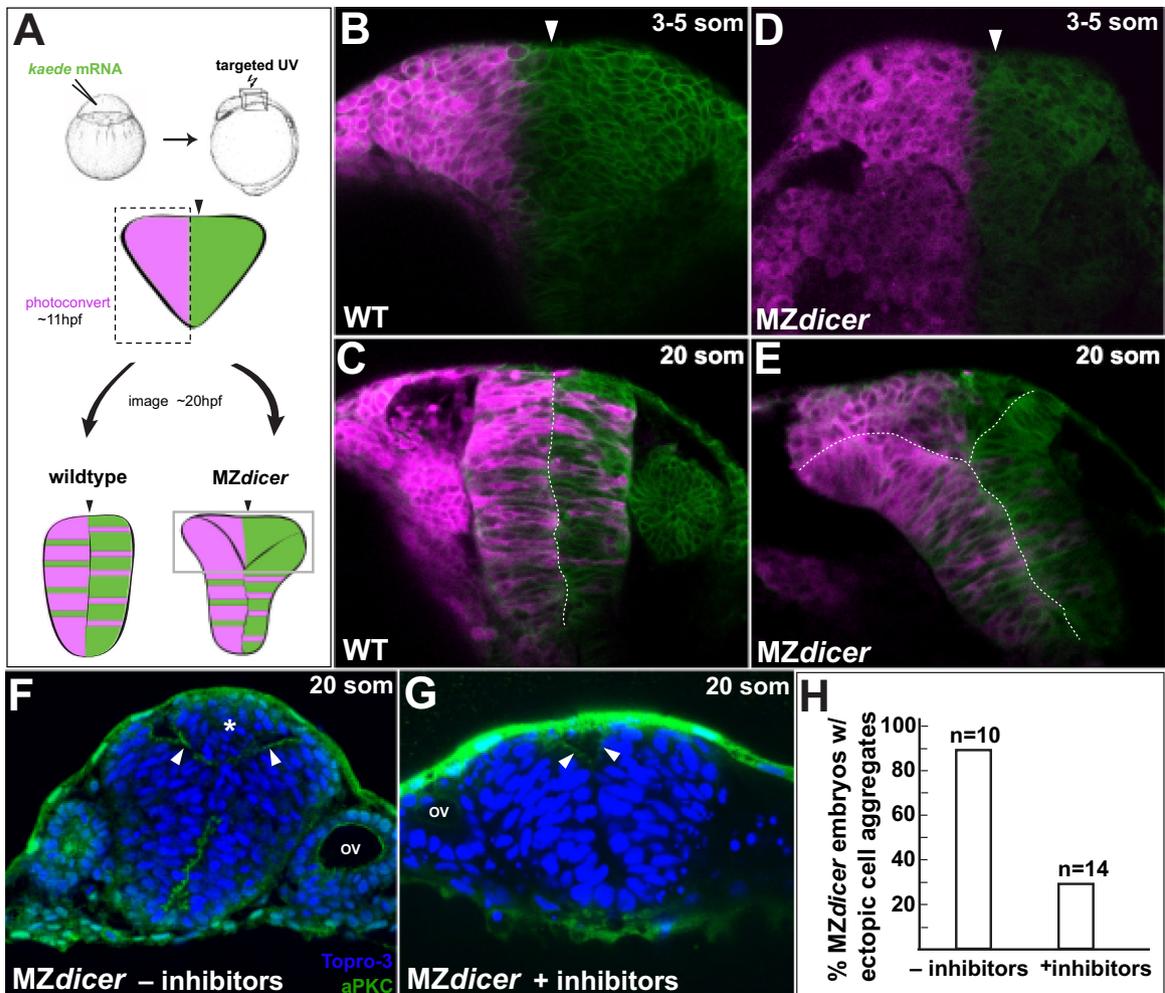
Neural midline formation is instructed by a series of crossing divisions that result in the bilateral segregation of daughter cells across the axial midline with mirror-symmetric apicobasal polarity (Clarke, 2009). To determine whether midline defects in the *MZdicer* mutant arise from aberrant crossing divisions, we employed the photoconvertible fluorophore Kaede (Hatta et al., 2006) to follow the fate of neural progenitors and their progeny during the neural keel/rod transition (Fig. 2A). We assessed the degree of midline crossing by photoconverting progenitors exclusively on one side of the midline before the onset of crossing divisions and then recording the position of daughter cells ~10 h later after midline formation. A successful crossing division requires that a daughter cell originating from the ipsilateral side contacts, and integrates into, the contralateral epithelium (Ciruna et al., 2006; Tawk et al., 2007). The majority, if not all, of cell divisions during the neural keel/rod stage result in midline crossing (Concha and Adams, 1998; Geldmacher-Voss et al., 2003). In wildtype embryos the progeny of converted cells become bilaterally distributed between opposing epithelia (Fig. 2C). Consistent with proper midline formation in the ventral neural rod of *MZdicer* embryos, *MZdicer* progenitors in more ventral regions displayed a heterogeneous bilateral distribution. In contrast *MZdicer* progenitors remained segregated within dorsal regions (Fig. 2E). Failure to cross was associated with an accumulation of cells at the presumptive midline. Previous studies have shown that midline defects arising

from ectopic or misoriented crossing divisions can be rescued through chemical inhibition of mitosis (Ciruna et al., 2006; Quesada-Hernandez et al., 2010; Tawk et al., 2007; Zigman et al., 2014; Zigman et al., 2011). To determine whether crossing divisions contribute to *MZdicer* neural midline defects, we incubated *MZdicer* embryos with cell cycle inhibitors before the onset of crossing divisions. We find that cell cycle inhibition rescues ectopic cell accumulation in the *MZdicer* mutant (Fig. 2F–H). Taken together, these results suggest that ectopic *MZdicer* neural progenitors arise from defective crossing division mitoses in which daughter cells fail to successfully navigate midline crossing and contralateral integration.

### 3.3. *MZdicer* neural progenitors establish apicobasal and PCP polarity

Both apicobasal and planar cell polarity (PCP) pathways instruct the correct partitioning daughter cells within the neural rod (Ciruna et al., 2006; Geldmacher-Voss et al., 2003; Quesada-Hernandez et al., 2010; Tawk et al., 2007; Zigman et al., 2011). We next tested whether miR-430 function is required for proper establishment of cell polarity in the neural rod. To address whether ectopic *MZdicer* cells retain an ability to adopt apicobasal polarity, we assessed the localization of the polarity marker aPKC (Tabuse et al., 1998) later in development (~27 hpf; Fig. 3). aPKC normally localizes to apical membrane within the neuroepithelium (Hong and Brewster, 2006). We find that, at later stages, ectopic progenitors, despite failed integration, adopt epithelial character and establish apical polarity as assessed by apical enrichment of aPKC (Fig. 3E). Moreover, we find that ectopic cells often generate bifurcated lumens indicating that they retain the ability to self-organize into epithelial structures.

Loss-of-function mutations in the PCP pathway result in delayed neural convergence, failed planar polarization within neural progenitors, and disruption of C-division positioning and midline crossing (Ciruna et al., 2006; Quesada-Hernandez et al., 2010; Tawk et al., 2007; Zigman et al., 2011). To determine whether planar polarity is affected by loss of miR-430 function, we assessed



**Fig. 2.** *MZdicer* neural progenitors in dorsal regions fail to cross midline. (A) Lineage tracing after unilateral Kaede photoconversion at 11 hpf. (B–E) Kaede lineage tracing after photoconversion (3–5 somites; B and D) and ~8 h later (20 somites; C and E). (B–C) Wildtype neural progenitors undergo bilateral distribution. (D–E) In contrast, *MZdicer* neural progenitors retain unilateral distribution in dorsal regions of the neural anlage. Arrowhead demarcates the presumptive neural midline. (F–H) Cell cycle inhibition reduces *MZdicer* ectopic neural cell accumulation. Asterisk indicates presence of ectopic cell aggregate. Arrowheads indicate apical membrane. OV, otic vesicle.

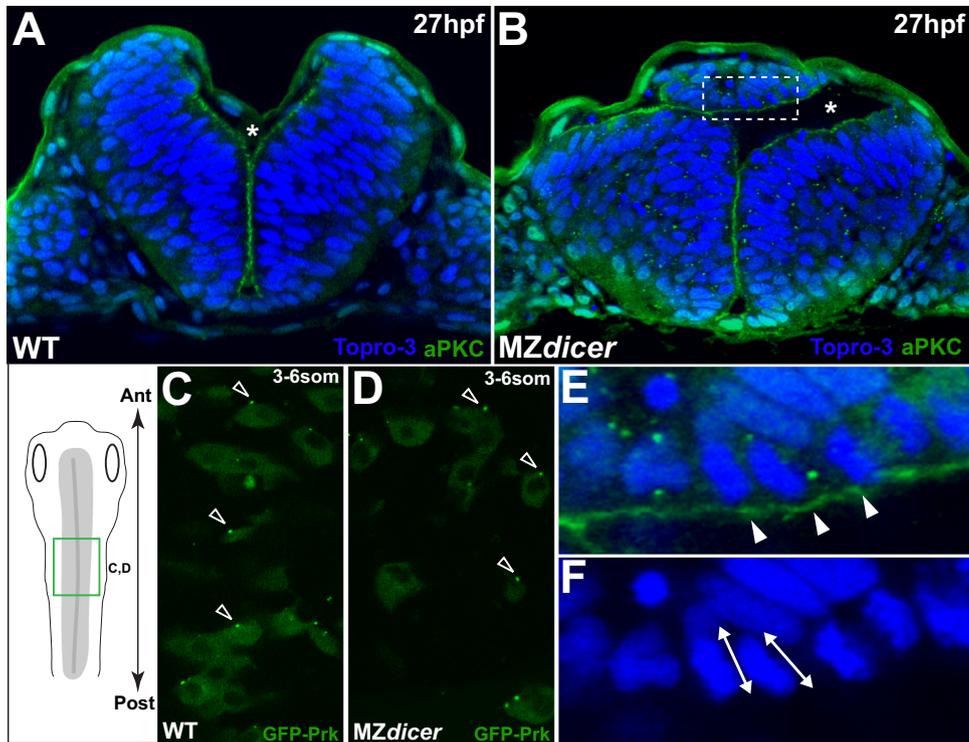
the localization of the PCP component Prickle (Jenny et al., 2003). In wildtype embryos, the PCP reporter GFP-Prickle (GFP-Prk) is the earliest known marker of planar polarity during neurulation and localizes to discrete foci at the anterior edge (71/94; 76%) of neural keel stage progenitors. (Fig. 3C and D; (Ciruna et al., 2006)). We find that GFP-Prk correctly displays anterior localization in *MZdicer* neural cells (39/49; 80%), suggesting that the PCP pathway is not disrupted upstream of Prickle localization in *MZdicer* neural progenitors. Taken together, these data suggest that *MZdicer* cells maintain an ability to polarize in both planar and apicobasal orientations, and are both capable of, and predisposed toward, epithelial organization.

#### 3.4. Neural midline defects are not due to mislocalized crossing divisions

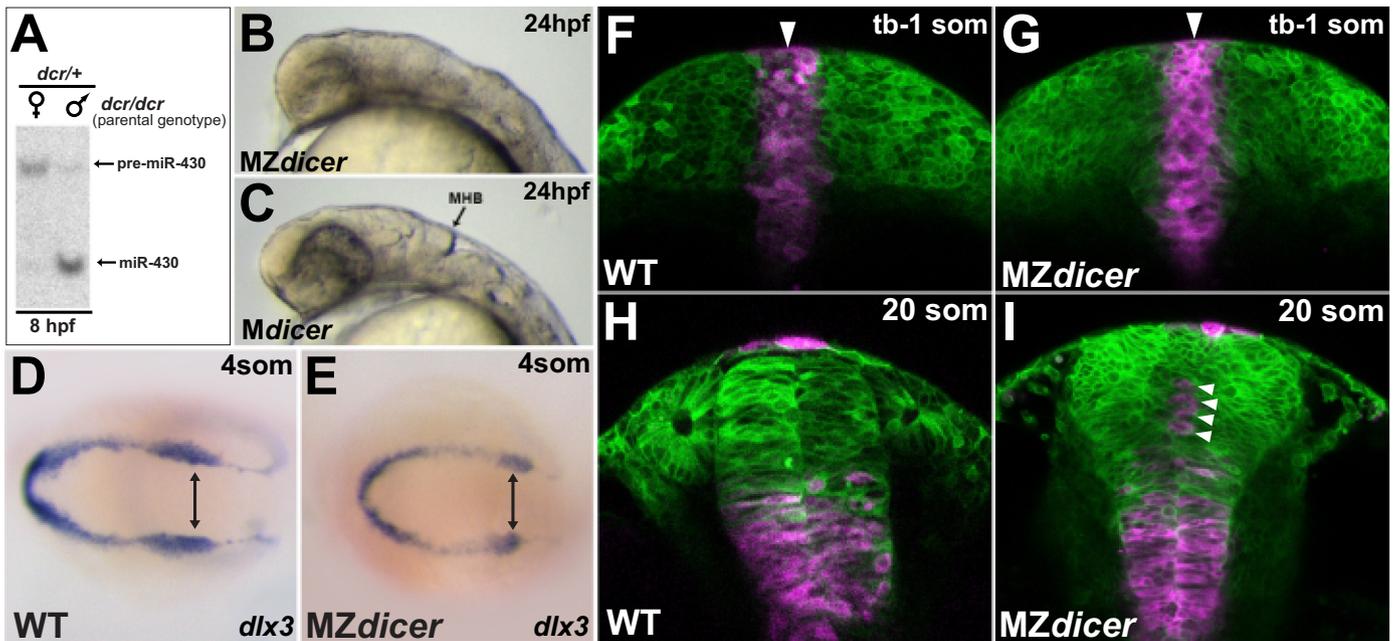
The failure of neural progenitors to cross the midline could be a function of their distance from the midline at the time of crossing division. For example, in the PCP mutant *vang2*, convergent extension defects result in delayed medial convergence of neural progenitors to the axial midline (Quesada-Hernandez et al., 2010; Tawk et al., 2007). Mislocalized crossing divisions on either side of the axial midline give rise to ectopic neural midlines in the *vang2* mutant. To address whether *MZdicer* neural defects arise from convergence defects, we first assessed the temporal requirements

for miR-430 in neural tube development. Embryos that are maternally deficient for *dicer* but express Dicer zygotically (*Mdicer*) do not generate significant levels of mature miR-430 until 8hpf (vs. ~4–5 hpf in wildtype embryos) (Fig. 4A). However, in contrast to *MZdicer* embryos, *Mdicer* embryos undergo brain morphogenesis and form a single contiguous lumen through the hindbrain region (Fig. 4C). This observation suggests that *MZdicer* neurulation defects are due to relatively late function(s) of miR-430 in neurulation as opposed to an earlier role in convergent extension. Indeed, analysis of *dlx3*, a marker of the lateral edges of the neural anlage, does not reveal differences in the width of the neural keel, indicating that early defects in epiboly and convergence do not explain the observed neural midline defects in *MZdicer* embryos. (Fig. 4D and E).

We reasoned that if the distance from the axial midline is not a determining factor in whether a neural progenitor undergoes a defective crossing division, then neural progenitors positioned close to the midline in the neural keel should also give rise to ectopic daughter cells that fail to cross and integrate into the contralateral epithelium. To follow the fate of medial progenitors, we photoconverted cells located close to the midline in the early neural keel (Fig. 4F–G). In a wildtype embryo, medial neural cells adopt ventral positions in the neural rod due to ingression as neurulation proceeds (Fig. 4H; Fig. S2); (Lowery and Sive, 2004; Papan and Camposortega, 1994). Accordingly, neural progenitors at



**Fig. 3.** Ectopic MZdicer neural progenitors retain apicobasal and PCP polarity. (A and B) Transverse sections at 27hpf. Topro3, nuclear stain. At this stage, lumen inflation has initiated. Asterisk indicates luminal space. (B) MZdicer embryo with a dorsal ectopic cell aggregate. (E) Inset of panel B; arrowheads indicate aPKC (green) localization to apical membrane. (F) Inset of panel B; double arrows indicate elongated nuclear shape characteristic of polarized epithelial cells. (C and D) Dorsal view (green box in schematic) of GFP-Prickle scatter labeling. GFP-Prk localizes to the anterior edge (arrowheads) of (C) wildtype neural cells (71/94; 76%) and (D) MZdicer mutant cells (39/49; 80%). Anterior to top. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** miR-430 activity is required post-gastrulation within proximal neural progenitors (A) Northern blot analysis of miR-430 expression: lane 1, embryos derived from *dcr/dcr* female germline (maternally deficient) X *dcr/+* male; lane 2, embryos derived from *dcr/dcr* male germline X *dcr/+* female (maternally sufficient). Embryos deficient for maternal *dicer* function do not generate significant levels of mature miR-430 until after 8hpf. (B) MZdicer embryo (maternally and zygotically deficient) fails to generate contiguous neural lumen and ventricular organization. (C) Mdicer embryo (maternally deficient) undergoes ventricle formation, suggesting that zygotic Dicer contribution (and delayed miR-430 production) is sufficient for neural tube generation. (D and E) *In situ* analysis of *dlx3* expression, marking the lateral edges of neural keel. (F–I) Kaede lineage tracing after photoconversion at dorsal midline (tailbud-1somite stage; F, G) and ~10 hours later (20 somite stage; H, I). Arrowheads demarcate dorsal midline. As ingress proceeds, proximal progenitors adopt ventral positions in neural rod. In MZdicer embryos, a subset of proximal progenitors fails to ingress ventrally (arrowheads in panel I).

the lateral edges of the neural keel are destined to the dorsal neural rod. This dorsoventral profile is maintained through the crossing division phase since opposing daughter pairs maintain similar dorsoventral positions as ingress proceeds. In contrast to wildtype progenitors, we observed labeled midline *MZdicer* cells that failed to co-migrate ventrally during epithelial ingression. Labeled cells separated from their ventral neighbors and remained “left behind” within *MZdicer* dorsal cell aggregates (6/6 embryos displayed > 1 labeled cell within dorsal aggregate; Fig. 4I). These results provide multiple insights. First, a subset of progenitors proximal to the midline give rise to ectopic cells, indicating that distance from the midline is not a principal determining factor in failed *MZdicer* epithelial integration. Second, *MZdicer* ectopic cells fail to migrate ventrally with the surrounding neuroepithelium and, as a result, are relegated to the dorsal midline. Taken together, these results suggest that miR-430 activity facilitates neuroepithelial integration after crossing division within progenitors positioned at the axial midline

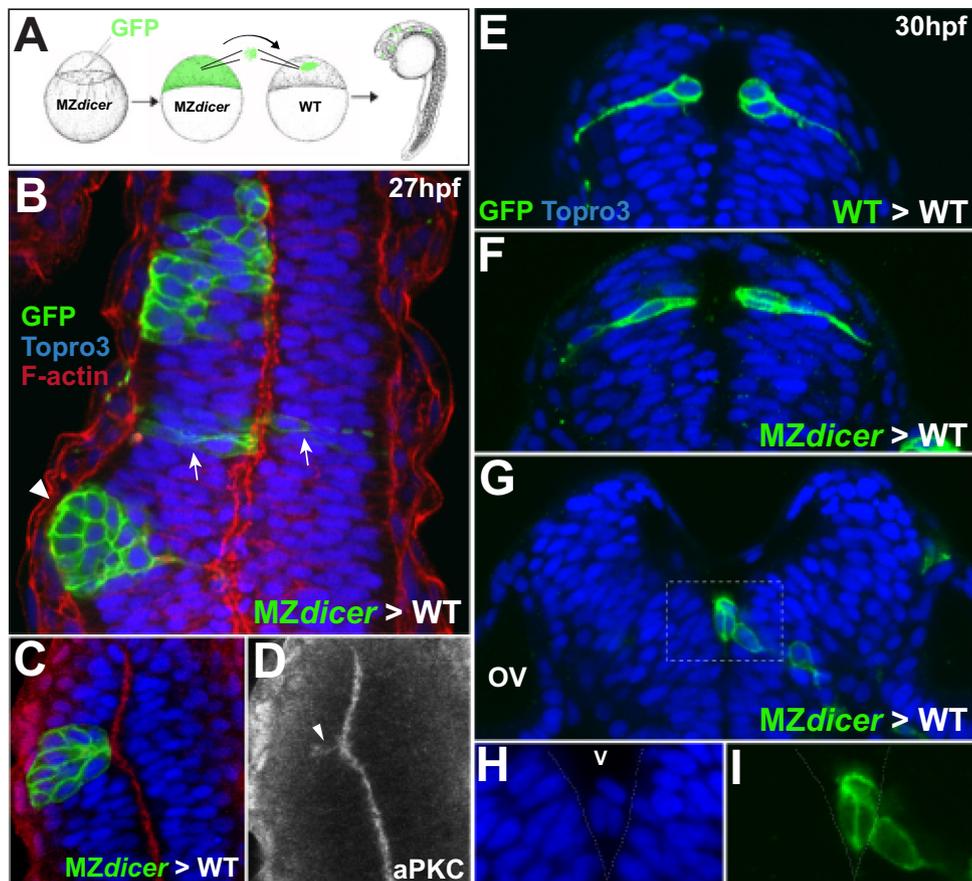
### 3.5. Transplanted *MZdicer* neural progenitors display defects in neuroepithelial integration

The above results suggest that defects underlying *MZdicer* neurulation originate during the neural keel-to-rod transition. To assess whether Dicer function is required within individual neural progenitors, we transplanted labeled *dicer* cells into wildtype hosts (Fig. 5A). A failure of mutant daughter cells to cross after division would suggest that Dicer function is required cell-autonomously.

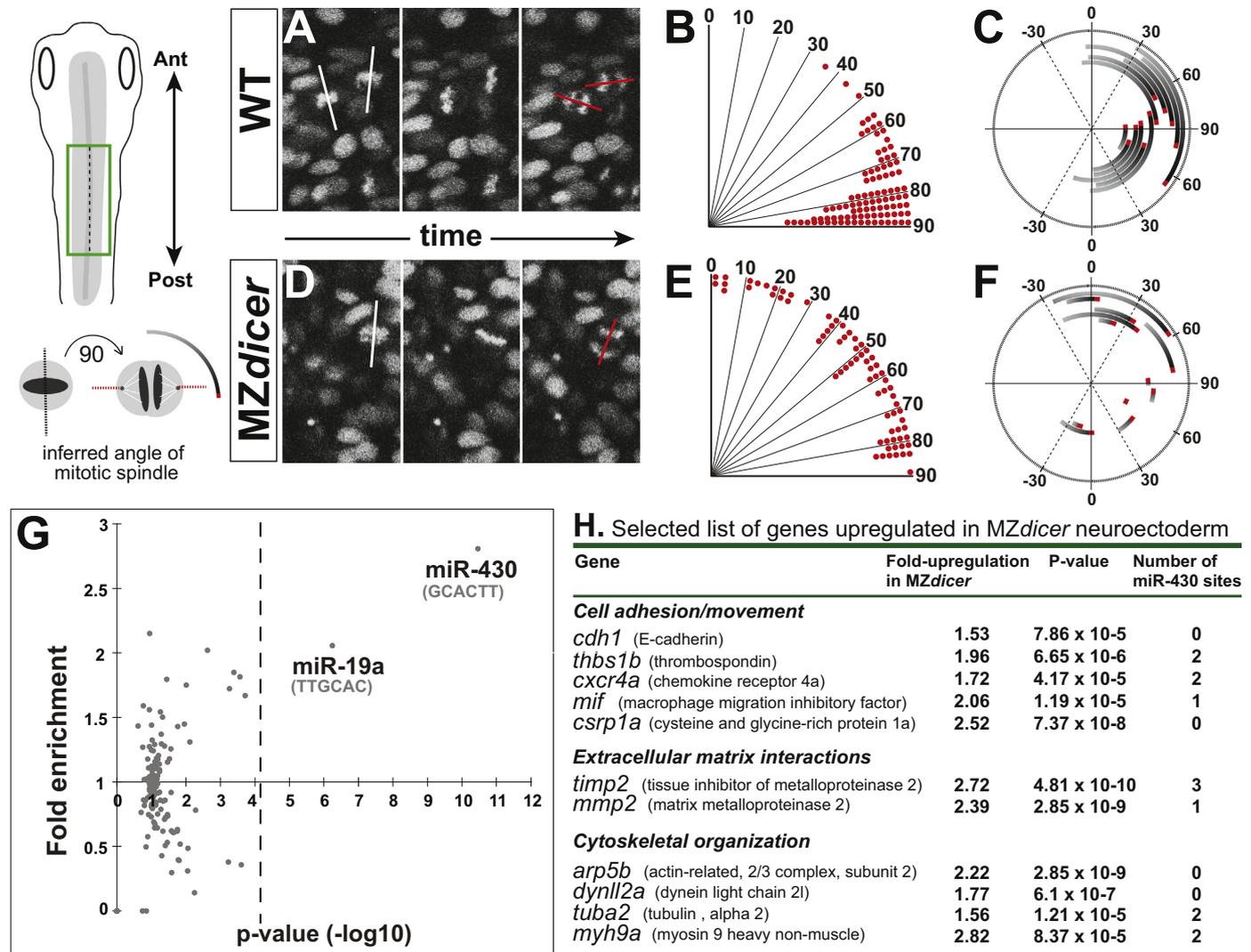
This could result in either the deposition of a daughter cell outside of the nascent epithelium or, alternatively, its reintegration within the same side leading to a predominantly unilateral distribution. Labeled wildtype cells transplanted into wildtype hosts generated bilaterally organized clones suggestive of successful crossing events (15/17 (88%) clones). Transplanted mutant *MZdicer* clones (or miR430 KD cells; Fig. S3A) of similar size (< 6 cells) into wildtype hosts also displayed bilateral organization in wildtype hosts (Fig. 5B and F) albeit to a lesser extent (20/28 (71%) clones;  $p$ -val=0.2759). However, in a subset of clones that failed to establish bilateral organization (2/8 clones), we observed the exclusion of mutant cells from the neuroepithelium (Fig. 5G–I; Fig. S4). Larger clones (> 6 cells) revealed additional qualitative differences between wildtype and mutant *MZdicer* clones. Specifically, larger mutant clones formed unilateral clusters that remained segregated from the neuroepithelium (Fig. 5B). In one instance, we observed the formation of ectopic apical membrane through a larger clone (Fig. 5C). These behaviors were never observed for wildtype cell clones of similar size. Taken together, these results indicate that *MZdicer* cell morphology and organization can be largely rescued by neighboring wildtype cells and is consistent with cell nonautonomous functions.

### 3.6. Abnormal mitotic spindle orientation during neural midline divisions

In contrast to prior divisions that occur longitudinal to the anteroposterior axis, crossing divisions (usually the 16th cell



**Fig. 5.** Transplanted *MZdicer* cells display reduced ability to integrate into wildtype neuroepithelium. (A) At midblastula stage, ~50 cells (WT or *MZdicer*) were transplanted into equivalently staged WT embryos. (B) *MZdicer* clones that display unilateral distribution (asterisk) and/or segregation away from wildtype epithelium (arrowhead). Arrows demarcate a *MZdicer* clone that displays normal cell shape and bilateral distribution. (C and D) Unilateral *MZdicer* clone that is organized around ectopic apical membrane (localized aPKC signal; arrowhead in panel D). (E and F) Transverse views of wildtype clone (E) and *MZdicer* clone (F) displaying epithelial integration and bilateral distribution. (G) Transverse view of *MZdicer* progeny cells excluded from the neuroepithelium. (H and I) Inset of panel F.



**Fig. 6.** MZdicer neural progenitors undergo misoriented neural rod cell divisions. (A and D) Time-lapse recording from dorsal view (green box in schematic). Histone2A-Cherry localizes to chromosomes and enables measurement of mitotic orientation (red lines) at anaphase. White line indicates orientation at initiation of mitosis. (B and E) Quantification of mitotic orientation at anaphase relative to anteroposterior axis (0 degrees) in (B) wildtype (96 cells; 4 embryos) and (E) MZdicer embryos (72 cells; 5 embryos). (C and F) Representation of mitotic spindle rotation for randomly chosen (C) wildtype ( $n=12$ ) and (F) MZdicer ( $n=12$ ) progenitors. Inferred spindle orientation (relative to anteroposterior axis) was measured at prophase (start of line) and then again at anaphase (red). (G) miRNA target site (7-mer sites) enrichment in 3'UTRs of upregulated genes ( $> 1.5$  fold) in MZdicer neuroectodermal cells. The enrichment of 135 target sites compared to a control set is plotted. The p-value cutoff after multiple test correction is shown as a dashed line. (H) Selected candidate list of upregulated transcripts in MZdicer mutant. Fold-change upregulation and p-value derived from published microarray data (Shkumatava et al., 2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

division) orient perpendicularly to the anteroposterior axis through a 90° rotation of the mitotic spindle (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Kimmel et al., 1994). It has been reported that proper mitotic spindle orientation during the crossing division phase is critical for both the establishment of an organized neural midline as well as for the faithful bilateral segregation of daughter cells (Quesada-Hernandez et al., 2010; Zigman et al., 2014; Zigman et al., 2011). To assess cell division orientation, we conducted time-lapse recording of neural keel/rod stages (8som–14som stages) in wildtype and MZdicer embryos injected with an mRNA encoding the fusion protein histone2A-Cherry. We limited our analysis to cell mitoses within 30  $\mu$ m on either side of the axial midline. Consistent with previous reports, cell mitoses during this stage predominantly oriented orthogonal to the anteroposterior axis in wildtype embryos (median  $81.6^\circ \pm 11.4^\circ$ ; Fig. 6A and B). In contrast, cell divisions in MZdicer embryos displayed a heterogeneous distribution without final orientation preference (median  $52.7^\circ \pm 25.0^\circ$ ; Mann–Whitney Test ( $p < 0.0001$ ); Fig. 6D and E). Misoriented cell divisions could be

due to abnormal cell orientation at start of mitosis and/or due to abnormal spindle rotation as mitosis proceeds (Zigman et al., 2011). To distinguish between these two possibilities, we measured the initial orientation of neural progenitors during prophase and compared to orientation during anaphase (Fig. 6C and F). Consistent with previous work, wildtype cells predominantly oriented along the anteroposterior axis upon entry into mitosis (median  $7^\circ \pm 17^\circ$ ;  $n=12$  cells, 2 embryos) and rotated an average of  $80^\circ \pm 28^\circ$  (Fig. 6C). In contrast, MZdicer progenitors were less consistently oriented in planar orientation (median  $30^\circ \pm 27^\circ$ ;  $n=12$  cells, 2 embryos) and additionally, displayed reduced rotation (average of  $29^\circ \pm 24^\circ$ ; Fig. 6F). Taken together, these results suggest that miR-430 function is required to both establish initial spindle orientation and facilitate spindle rotation as mitosis proceeds during neural rod cell divisions.

### 3.7. Upregulated neural mRNAs are enriched for miR-430 target sites

Our phenotypic analysis has uncovered abnormal cellular

behaviors that underlie the *MZdicer* neural phenotype. We reasoned that this phenotype arises from the derepression of miR-430 targets in neuroectoderm. To identify misregulated miR-430 target genes involved in neural tube morphogenesis, we analyzed RNA-seq datasets derived from whole embryos (shield stage) (Lee et al., 2013), as well as microarray datasets derived exclusively from neuroectoderm (18hpf; (Shkumatava et al., 2009)). In both cases, we compared wildtype and *MZdicer* mutant expression profiles to identify transcripts upregulated in the absence of microRNA function. This analysis revealed 166 transcripts that were significantly upregulated ( $> 1.5$  fold;  $p$ val  $< .05$ ) in *MZdicer* neuroectoderm. Examination of 3'UTRs for the presence of microRNA target sites (pairing to seed region) revealed significant enrichment of miR-430 target sequences within 3'UTRs of upregulated genes (Fig. 6G), with 85 (51%) transcripts possessing at least one miR-430 target site (8mer sites,  $n=22$ ; 7mer-m8 sites,  $n=34$ ; 7mer-A1,  $n=11$ ; 6mer,  $n=53$ ), suggesting that miR-430 activity directly regulates mRNA transcript stability within neuroectoderm. To identify affected cellular processes, we conducted gene ontology enrichment analysis for misregulated genes. Representative GO terms for the top five enriched functional clusters were lysosome (GO:0005764), cell migration (GO:0016477), peptidase inhibitor activity (GO:0030414), extracellular matrix (GO:0031012), and cytoskeletal organization (GO:0007010). Closer inspection revealed genes involved in cell–cell adhesion (e.g. *cdh1*, *mif1*), ECM-cell interactions (e.g. *mmp2*, *timp2*), and cytoskeletal dynamics (e.g. *tuba2*, *arpc5b*, *dynll2a*) (Fig. 6H). Taken together, these results suggest that miR-430 provides an essential and pervasive layer of regulation across a range of cellular processes to coordinate mitotic spindle orientation during neural tube morphogenesis.

#### 4. Discussion

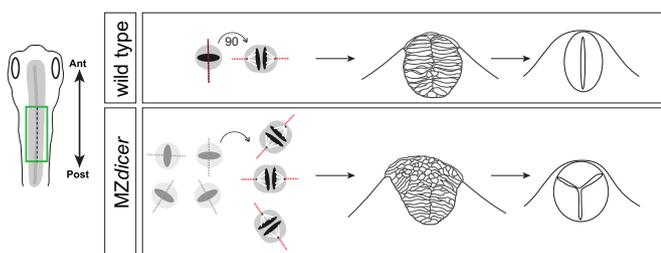
A fundamental challenge in developmental biology is to understand how molecular information influences cellular behaviors that shape tissue morphogenesis. We find that miR-430 coordinates a specialized cell division that directly instructs neural tube development. Photolabeling experiments indicate that a subset of neural progenitors that lack miR-430 activity fail to incorporate into the nascent neural epithelium. We propose that spindle misorientation results in the failed integration and dorsal accumulation of 'orphan' daughter cells that self-organize to form ectopic neural structures (Fig. 7). Our data suggest that *MZdicer* neural progenitors are in the right location to engage in effective crossing divisions. However, we cannot rule out the possibility that the timing of crossing divisions is askew in the *MZdicer* mutant. MicroRNAs have been shown to regulate developmental timing in multiple contexts (Ambros, 2011; Decembrini et al., 2009; La Torre et al., 2013), and in turn, miR-430 might function to ensure that crossing divisions are temporally coordinated within a defined

developmental window. Such coordination would be especially important given that crossing divisions deviate dramatically in their orientation compared to both precedent and subsequent cell divisions (which orient parallel to the anteroposterior axis). Recently published transplantation experiments suggest that crossing division timing is regulated by the neural progenitor's intrinsic schedule and not from surrounding environmental cues (Girdler et al., 2013). Interestingly, larger clones of transplanted *MZdicer* cells resemble heterochronically-displaced cell transplants in that they tend to cluster rather than integrate into the host neuroepithelium, suggesting that *MZdicer* cells might possess incompatible cellular properties (e.g. cell adhesive properties) due to an asynchrony in developmental time (Girdler et al., 2013).

Alternatively, the crossing division *per se* is defective. Spindle positioning is determined by forces exerted on astral microtubules associated with the cell cortex. The localization of cortical protein complexes that pull on astral microtubules (i.e. Gi-LGN-NuMA/Dynein/Dynactin) is determined, in turn, by an interactive network of intrinsic mechanisms that sense and respond to external cues. Among these, we first assessed cell polarity cues. The apical par-3/par-6/aPKC complex coordinates spindle geometry in early *C. elegans* embryos (Colombo et al., 2003) and *Drosophila* neuroblasts (Cai et al., 2003), as well as centrosome positioning during zebrafish neurulation (Hong et al., 2010). A recent study by Buckley et al. demonstrates that broad apical localization of Pard3-GFP is initiated immediately before and independently of crossing divisions. Therefore, localized Pard3 could potentially influence the mitotic orientation of crossing divisions as apical polarization proceeds. Planar cell polarity has also been shown to instruct spindle orientation in multiple contexts (Gong et al., 2004; Segalen and Bellaiche, 2009), and loss of the PCP component *frizzled7* results in misoriented crossing divisions in the zebrafish neural keel (Quesada-Hernandez et al., 2010). In contrast, we find that *MZdicer* neural progenitors establish apical and planar polarity and therefore, do not interpret spindle orientation defects as due to the loss of these polarizing cues.

In addition to cell polarity cues, defects in cell adhesion might also contribute to *MZdicer* neural tube misorganization. *N-cadherin* mutant embryos display neural tube defects that are similar to those observed in *MZdicer* embryos (Hong and Brewster, 2006; Lele et al., 2002). Specifically, dorsal cells become excluded from the neuroepithelium and often form aggregates that retain the ability to self-organize. Knockdown of zebrafish *N-cadherin* (*cdh2*) function results in spindle misorientation in the ventral neural tube (Zigman et al., 2011). Further implicating cell adhesion and spindle orientation, reduced alpha-catenin accumulation at nascent adhesive junctions is associated with misoriented neural keel divisions in *MZscribble* neural progenitors (Zigman et al., 2011). Interestingly, *MZscribble* neural progenitors display both initial misorientation and reduced spindle rotation, suggesting that similar defects observed in *MZdicer* embryos may be rooted in altered cell adhesion. Consistent with this hypothesis, several misregulated molecules in *MZdicer* neuroectoderm are involved in cell adhesion, as well as cell-extracellular matrix interactions, suggesting that miR-430 loss affects the adhesive interactions between neighboring cells or with the basal lamina

MicroRNAs can interact with hundreds of mRNAs in the cell and provide a versatile mechanism whereby multiple target mRNAs within a common cellular process can be coordinately regulated. Given this complexity, it is likely that the majority of microRNA phenotypes arise as a composite outcome of derepression across a range of targets affecting multiple pathways. We have been able to show that microRNA activity provides an essential regulatory layer to ensure the precise and coordinated elaboration of a series of oriented cell divisions involved in neural tube morphogenesis. Future work will entail identifying those



**Fig. 7.** Model of neurulation defects in *MZdicer* embryos. Loss of miR-430 results in abnormal spindle orientation of neural keel cell mitoses and failed neuroepithelial integration. Displaced neural progenitor daughter cells within dorsal regions retain ability to organize into ectopic neural structures.

target mRNAs whose functions in oriented division are most sensitive to perturbations in gene expression.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2015.11.016>.

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