Title:

Morphogenetic forces planar polarize LGN/Pins in the embryonic head during *Drosophila* gastrulation

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Abstract:

Spindle orientation is often achieved by a complex of Pins/LGN, Mud/NuMa, Gai, and Dynein, which interacts with astral microtubules to rotate the spindle. Cortical Pins/LGN recruitment serves as a critical step in this process. Here, we identify Pins-mediated planar cell polarized divisions in several of the mitotic domains of the early *Drosophila* embryo. We found that neither planar cell polarity pathways nor planar polarized myosin localization determined division orientation; instead, our findings strongly suggest that Pins planar polarity and force generated from mesoderm invagination are important. Disrupting Pins polarity via overexpression of a myristoylated version of Pins caused randomized division angles. We found that disrupting forces through chemical inhibitors, laser ablation, and depletion of an adherens junction protein disrupted Pins planar polarity and spindle orientation. Furthermore, *snail* depletion, which abrogates ventral furrow forces, disrupted Pins polarization and spindle orientation, suggesting that morphogenetic movements and resulting forces transmitted through the tissue can polarize Pins and orient division. Thus, morphogenetic forces associated with mesoderm invagination result in planar polarized Pins to mediate division orientation at a distant region of the embryo during morphogenesis. To our knowledge, this is the first *in vivo* example where mechanical force has been shown to polarize Pins to mediate division orientation.

Introduction:

Epithelial morphogenesis and homeostasis are influenced by cell division orientation, which can occur along specific axes relative to both the epithelial plane and the polarity of an organ/organism (Baena–Lopez et al., 2005; Kulukian and Fuchs, 2013; Lancaster and Knoblich, 2012; Morin and Bellaiche, 2011). Epithelia are tissues composed of cells that are organized in a sheet and held together by junctions, which maintain the integrity of the tissue as well as the apical-basal polarity of cells (Choi et al., 2013; Classen et al., 2005; Cox et al., 1996; Gumbiner, 2005; Harris and Peifer 2005; Tepass, 1997; Ulrich et al., 2005; Wang and Margolis, 2007). In epithelia, asymmetric cell divisions, in which cellular components are distributed unequally to daughter cells, result in one daughter cells leaving the epithelium. In contrast, symmetric divisions in epithelia divide cellular components equally, and always result in both daughter cells remaining in the tissue. Orientation of the mitotic spindle is what determines whether a division is symmetric or not, as spindle orientation determines division orientation. (Cabernard and Doe, 2009; Gonczy and Rose, 2005; Lechler and Fuchs, 2005; Mao et al., 2011; Nakajima et al., 2013). In addition, the spindle orientation with respect to organ/organism axis can promote directional growth and/or stress dissipation in tissues (Campinho et al., 2013; Legoff et al., 2013; Mao et al., 2011; Mao et al., 2013; Wyatt et al., 2015).

Spindle orientation is often achieved by a complex of Partner of Inscuteable (Pins)/LGN, Mushroom Body Defect (Mud)/Nuclear Mitotic Apparatus (NuMa), G*α*i, and Dynein. In this complex, Pins/LGN acts as a linker between G*α*i and Mud, which is also bound to a Dynein-Dynactin complex. Dynein's motor activity provides the force to rotate the spindle (Bergstrahl et al., 2013; Busson et al., 1998; Fielmich et al., 2018; Grill and Hyman, 2005; Johnston et al., 2009; Nguyen-Ngoc et al., 2007; Siller and Doe, 2008). In *Drosophila* neuroblasts, asymmetric division and spindle orientation is dependent on Pins/LGN recruitment to the apical cortex. Apical proteins

recruit Inscuteable (Insc), which in turn recruits Pins/LGN and the spindle rotation complex. Pins null mutants exhibit defects in spindle orientation in neuroblasts (Cabernard and Doe, 2009; Schaefer et al., 2000; Siller et al., 2006; Yu et al., 2006). In symmetric epithelial cell divisions, spindle orientation also depends on Pins, which is recruited to the lateral cortex by the lateral domain protein, Discs large (Dlg). Loss of Pins, Dlg, or disrupted recruitment of Pins by Dlg causes aberrant asymmetric cell divisions in epithelia and cell loss (Bell et al., 2015; Bergstrahl et al., 2013; Carvalho et al., 2015). Furthermore, in planar cell polarized divisions of the sensory organ precursor cells in the *Drosophila* dorsal thorax, asymmetric determinant segregation and spindle orientation depend on planar polarized Pins and E-cadherin localization, respectively (Bellaïche et al., 2001; Le Borgne et al., 2002).

In addition to molecular cues, mechanical cues also orient cell division. For example, in isolated cell cultures, cell division orientation has been shown to directly respond to stress by aligning with applied forces (Fink et al., 2011; Matsumura et al., 2016; They et al., 2007; They et al., 2005). Cells also respond to force through its influence on cell shape. The connection between cell shape and division axis is known as the long axis rule or Hertwig's rule and was first observed over 100 years ago (Hertwig, 1884). However, the mechanisms through which force, cell shape, and molecular cues are interconnected to influence cell division remain an active area of research. In epithelial tissues, cell division/spindle orientation occurs along the tension axis, which can dissipate tension and influence cell packing (Campinho et al., 2013; Gibson et al., 2011; Godard et al., 2020; Mao et al., 2011; Scarpa et al., 2018; Wyatt et al., 2015). In the *Drosophila* pupal notum and *Xenopus*, cell shape has been shown to affect the spindle angle through the influence of molecular cues localized at tri-cellular junctions, which provide a 'memory' for interphase cell shape (Bosveld et al., 2016; Nestor-Bergmann et al., 2019). Whereas in the *Drosophila* oocyte, follicular epithelium, and parasegmental boundaries and cultured MDCK cells, molecular cues do not localize to tri-cellular junctions, implying other mechanisms are responsible for force-oriented spindle rotation (Finegan et al., 2019; Gloerich et al., 2017; Scarpa et al., 2018;

Yu et al., 2006). For example, mechanical cues have been shown to recruit the Pins/LGN machinery to adherens junctions in MDCK cells (Hart et al., 2017). Mitotic rounding forces have also been shown to be important for proper spindle orientation and morphology (Chanet et al., 2017; Kunda et al., 2012). The combinatorial action of mechanical and molecular cues used to orient spindles is incompletely understood. Here, we use the *Drosophila* embryo as a powerful model organism that is uniquely tractable, and exhibits mirror symmetric domains that undergo highly stereotyped divisions to uncover the relationship between mechanical and molecular cues to coordinate division orientation in a developing embryo.

During development the early *Drosophila* embryo undergoes thirteen cycles of synchronous nuclear divisions with no intervening gap phases. After these thirteen cycles of nuclear division, embryonic cells arrest in G2. Cycle 14 divisions occur in a highly stereotyped pattern defined by *string* (*Drosophila* Cdc25 homologue) expression in pockets of cells across the embryo. These pockets of cells that divide together are termed mitotic domains (Edgar and O'Farrell, 1989; Foe, 1989). Some of these domains have been observed to have oriented divisions, but the mechanism underlying division orientation is unknown (da Silva and Vincent, 2007; Hartenstein and Campos-Ortega, 1985; Hartenstein et al., 1987; Stern et al., 2021; Wang et al., 2017). We found that spindle orientation is a highly dynamic process in which spindles are not assembled oriented, but rather have to rotate to achieve orientation in mitotic domains 1, 3, and 5 (MDs 1, 3, and 5) in the dorsal 'head' and are oriented in the direction of tissue movement/elongation. We discovered that these oriented symmetric divisions and those of MD 14, which had previously been shown to exhibit force dependent oriented symmetric divisions, require planar polarized Pins (da Silva and Vincent, 2007; Wang et al., 2017).

Results:

Early mitotic domains in the Drosophila head exhibit spindle rotation and planar polarized division.

Located on the dorsal side of the *Drosophila* embryo 'head', MD 1, 3, and 5 undergo division during gastrulation, 200 minutes after egg deposition (AED). MD3 is the anterior most mitotic domain and comprises around thirty cells, MD1 is the next most anterior domain and consists of two mirrored clusters of around 40 cells, and MD5 is located just anterior to the cephalic furrow and consists of two mirrored clusters containing around 30 cells (Figure 1A, B).

We initially visualized division axis in embryos expressing fluorescently-tagged non-muscle myosin II to mark cytokinetic rings and fluorescently tagged myristoylation sequence of Gap43 to mark cell membranes. Cytokinetic rings form perpendicular to the division axis. In MDs 1, 3, and 5, cytokinetic rings are oriented roughly perpendicular to the AP axis. (Figure 1C). To further visualize the division process and assess dynamics during division, we used embryos expressing fluorescently-tagged CLIP170 to mark the mitotic spindle (Figure 1D, movie 1). To quantify the alignment of the divisions with the AP axis, we measured the angle between the spindle and the AP axis, where an angle of 0 indicates perfect alignment with the AP axis and an angle of 90 indicates alignment with the dorsal-ventral (DV) axis (Figure 1E). Spindles were measured after anaphase, when spindle position becomes fixed. MDs 1, 3, and 5 divisions occur almost entirely within 30° of the AP axis (Figure 1E-H).

Notably, we found that mitotic spindles are not initially oriented with the AP axis and form with no preferential orientation. Spindles then rotate up to 90° to achieve a final orientation (Figure 1D). Together these data show that MDs 1, 3, and 5 exhibit oriented divisions and this orientation is achieved by spindle rotation toward the AP axis. We next asked what cue(s) could coordinate the spindle rotation and division orientation.

Planar polarized divisions require polarized Pins localization

Because spindles exhibited a pronounced rotation after assembly, we examined components of the spindle rotation machinery, Pins and Mud. We discovered that MDs 1, 3, and 5 all exhibit planar cell polarity of Pins. The Pins planar cell polarity is dependent on cell cycle state: mitotic cells exhibit planar polarized Pins, whereas Pins is uniformly distributed along lateral membranes of interphase cells (Figure 2A, movie 2). We quantified Pins polarization by modifying previously described polarization quantifications of mitotic cells (Besson et al., 2015). We used embryos expressing fluorescently labelled Pins and fluorescent membrane marker Gap43::mCherry, and measured the intensity of Pins::YFP and Gap43::mCherry around the membrane. We found that in MD1, the Pins crescent is on the posterior side of the cells and Pins intensity peaks just past the posterior-most part of the cell (Figure 2C and C'). The ratio of the point of greatest Pins intensity to the average of the two adjacent troughs was greater than the same ratio calculated from the Gap43::mCherry signal (peak-to-trough ratio Pins, 1.39 ± 0.1017 and Gap43::mCherry, 1.04 ± 0.0406 , mean and S.D.), demonstrating that this polarity was not caused by membrane inhomogeneity. Gap43::mCherry also had a more uniform labeling along the membrane in MD cells (Figure 2B, D, and D'). Though our imaging resolution is not sufficient to determine whether Pins is localized solely on the posterior side of MD1 cells or both the posterior and anterior sides when groups of cells round up together, we find that cells on the boundary of MD1 display Pins crescents only on the posterior side. Interestingly, from observation of boundary cells, we find Pins is localized on the posterior side of MDs 1 and 3, but the anterior side of MD5 (Figure S1).

In addition to MDs 1, 3, and 5 we decided to look at MD14, which has previously been shown to exhibit force dependent planar polarized divisions (Wang et al., 2017). Mitotic cells within MD14 exhibited planar polarized Pins, similar to what we saw for MD 1 (Figure 2H). Posterior crescents of Pins form in the mitotic cells of MD 14, though the crescent is broader than

in MDs 1, 3, and 5. The peak to trough ratio for Pins in MD14 was different from control Gap43::mCherry (1.22 ± 0.0914 and 1.04 ± 0.1115 , respectively, mean and S.D.) (Figure 2H-K').

To ensure Pins polarization was not a consequence of expressing non-endogenous levels of Pins, we fixed and stained embryos to localize endogenous Pins. We found that similar to Pins::YFP, endogenous Pins was planar polarized in mitotic MD 1 cells (Figure 2E). We also looked at components of the spindle rotation machinery complex downstream of Pins, namely Mud. It appears that Mud follows a similar localization pattern as Pins and is polarized in crescents in mitotic cells of MD1, although Mud also strongly labels spindle poles (Figure 2F-G). Based on our live and fixed imaging, we conclude that MDs 1, 3, 5, and 14 cells exhibit planar polarized Pins localization during mitosis.

We next sought to establish whether there was a connection between Pins polarity and spindle orientation. Using embryos expressing fluorescently-tagged tubulin and fluorescently-tagged Pins we monitored the timing of Pins polarization and spindle rotation. We found that Pins polarization preceded spindle rotation and is abolished shortly after anaphase, when the spindle position is finalized (Figure 3A-B, movie 3). The correlated timing between Pins polarization and spindle rotation suggests Pins is responsible for the spindle achieving its polarized orientation. To determine the relationship between Pins polarity and spindle rotation we compared the angle of maximum Pins intensity to the division angle. We found that there was a strong correlation between angular position of maximum Pins intensity and the anaphase spindle angle in MD 1 (Figure 3C). Previous studies have shown division orientation often follows the long axis of the interphase cell shape (Hertwig, 1884; Minc et al., 2011; Tsou et al., 2003). To determine whether division angle in the MD1 could also be related to cell shape, we compared the interphase cell long-axis angle to the division angle. In contrast to Pins polarity, there was no correlation between interphase cell shape and division angle, indicating the divisions of MD1 do not follow the long axis rule (Figure S2). Together these data show that division orientation correlates with Pins localization, but not interphase cell shape. We also analyzed Pins and tubulin within MD14, which

is located at the ventral midline (Figure 3D). Similar to MD 1, Pins polarization preceded spindle rotation. (Figure 3E-F).

To test the functional requirement of Pins polarity, we analyzed spindle orientation in embryos overexpressing a myristoylated Pins. Myristoylation covalently attaches a myristoyl group to a protein so the protein associates with the plasma membrane in an unregulated manner. Overexpression of myristoylated Pins, but not wild-type Pins, caused Pins to be uniformly distributed along the membrane during mitosis and abolished Pins planar polarity (Figure S3). We overexpressed the myristoylated version of Pins instead of using a Pins null mutant because we and others have previously shown that the Pins null mutant causes aberrant asymmetric divisions and our goal was to disrupt Pins polarity while keeping the divisions in-plane. We have previously shown myristoylated Pins is functional and able to rescue off-axis divisions that occur after Dlg-RNAi (Chanet et al., 2017). Despite occurring in-plane, spindles in embryos expressing myristoylated Pins fail to orient along the AP axis. Instead, division angles are evenly distributed, occurring with seemingly no preference between the DV and AP axes for cells within MD1 (Figure 3I-J, movie 4). In comparison, spindles in embryos expressing control Pins orient along the AP axis, with most divisions occurring within 30° of the AP axis (Figure 3G-H). Myr-Pins expressing embryos had an average division angle within MD1 that was significantly different from controls (42.7 and 20.1, respectively, $p=0.000103$). Results for MDs 3 and 5 are consistent with MD1 (Figure S4A - D). Myristoylated Pins expression also disrupted division orientation in MD14. Myr-Pins expressing embryos had an average division angle within MD14 that was significantly different from controls (41.2 and 18.1, respectively, $p=0.0079$) (Figure 3K-N). These data suggest that Pins polarity is necessary for mitotic spindle orientation and planar polarity of divisions within MDs 1, 3, 5, and 14. Next we investigated what mechanisms establish Pins planar polarity.

Adherens junctions, but not Toll-like receptors or other PCP pathways, are required for division orientation.

We hypothesized that Pins polarity could require an upstream planar cell polarity pathway and/or a mechanical cue. We first tested the requirement of planar cell polarity pathways. Some well-known planar polarity pathways include the Fat-Dachsous, the core PCP, and the PAR pathways (Hale and Strutt, 2015; Strutt and Strutt, 2009). The Fat-Dachsous and core PCP pathways were not good candidates from orienting divisions within MDs 1, 3, and, 5 because previous work has shown the components of these pathways are not expressed early enough and/or in the MDs to be involved (Clark et al., 1995; Tomancak et al., 2002; Tomancak et al., 2007). The PAR pathway was also not a good candidate because the *Drosophila* homologue of Par 3, Bazooka (Baz), did not exhibit planar cell polarity in the MDs, despite retaining apical localization during mitosis (Ko et al., 2020). Therefore, we explored the possible involvement of Toll-like receptors (TLRs), which act downstream of even-skipped (Eve) and have been shown to be necessary for the planar polarity of non-muscle myosin II (Myo II) and Baz in the *Drosophila* trunk (Lavalou et al., 2021; Pare et al., 2014). We investigated whether TLRs were involved in division orientation by injecting live embryos with dsRNA of TLRs 2, 6, and 8 and control 0.1x TE buffer. Further we analyzed fixed embryos that were triple mutants for these TLRs. These perturbations did not disrupt division orientation to the extent of expressing Myr-Pins, and distributions of division angles and average division angles were similar to controls (Figure S5) further, Myo II and Baz were not planar polarized in the *Drosophila* head (Chanet et al., 2017; Ko et al., 2020), leading us to conclude that TLRs are not solely responsible for division orientation in MDs 1, 3, 5.

We next tested the function of AJs. AJs mechanically couple epithelia cells and are composed of cadherin-catenin complexes (Hinck et al., 1994; Kovacs and Yap, 2008; Leckband and Prakasham, 2006; Oda et al., 1994; Rimm et al., 1995; Takeichi, 1991; Yap et al., 1997). AJs are connected to F-actin, which is required for force transmission (Nelson et al., 2005; Rauskolb et al., 2014). Junctional proteins, such as Canoe/Afadin (cno), have been implicated in spindle positioning (Carminati et al., 2016; Speicher et al., 2008; Wee et al., 2011). Additional work has

shown that E-Cadherin is able to recruit Pins in a tension dependent manner (Hart et al., 2017). While AJs are not required for in-plane divisions in certain tissues (Bergstrahl et al., 2013), they could serve as platforms for Pins to cue division orientation. To perturb AJs, we maternally depleted α -catenin (α cat) using RNAi. We have shown that α cat depletion is the most severe way to disrupt adherens junctions, totally uncoupling mesoderm cell actomyosin from the junctions, preventing their apical constriction, and lowering adherens junction levels throughout the entire embryo (Denk-Lobnig et al., 2021; Yevick et al., 2019). We crossed the RNAi (hairpin) stock to flies expressing a maternal GAL4 and fluorescently labelled tubulin to visualize spindles. When compared to a control RNAi (Rh3), embryos expressing the α cat RNAi had an even distribution of division angles in MD1, occurring with seemingly no preference between the DV and AP axes. α cat-depleted embryos exhibited an average division angle that was significantly different from controls (36.7 and 26.4, respectively; $p=0.000583$) (Figure 4A-D, movie 4). α cat RNAi also disrupted spindle orientations in MD3 and MD5 (Figure S4E - H).

Because Cno has been shown to interact with Pins (Speicher et al., 2008; Wee et al., 2011), we tested Cno function in MDs 1, 3, and 5. We used two RNAi lines to maternally deplete Cno and crossed each to flies expressing fluorescently labelled tubulin. Measuring division angles, as previously described, we found that Cno depletion does not significantly disrupt division orientation within MDs 1, 3, and 5 compared to controls, Cno-depleted embryos exhibited an average division angle that was not significantly different from controls (27.9 and 26.4, respectively; $p=0.2698$) (Figure 4E - F). Note that Cno-RNAi with these lines disrupted myosin localization, similarly to genetic mutants (Sawyer et al., 2011; Sawyer et al., 2009), but did not fully abrogate midline convergence during ventral furrow formation (Jodoin et al., 2015), unlike α cat RNAi (Denk-Lobnig et al., 2021). Together these data suggest a role for AJs and possibly force transmission in spindle orientation. Given our results, we next tested whether AJs are involved in polarization of Pins within MDs 1, 3, and 5.

269

270 *Disruption of adherens junction proteins disrupts Pins polarization*

271 To determine whether AJs are involved in Pins planar polarized localization, we examined
 272 fluorescently labelled Pins in α -catenin-depleted embryos. In contrast to control embryos, we
 273 found that Pins planar polarity is abolished when α -cat was depleted (Figure 5A-D, movie 5). Pins
 274 appeared to have no preferential localization, though given that cells divide in the epithelial plane,
 275 a process that depends on Pins, Pins must still localize laterally. Pins peak to trough ratio at the
 276 posterior was different in controls compared to α -catenin-depleted embryos (1.33 ± 0.0751 and
 277 1.06 ± 0.0173 , respectively, mean and S.D.). These data demonstrate a role for AJs in Pins planar
 278 polarization in MD 1.

279

280 *Mechanical cues affect Pins polarization and division orientation*

281 Given our results that AJs are involved in division orientation and Pins polarity and our finding
 282 that Pins is planar polarized within the force dependent oriented divisions of MD14, we
 283 hypothesized that force transduction through AJs contributes to Pins polarization and division
 284 orientation in MDs 1, 3, and 5. To further test the role of force transmission, we injected embryos
 285 expressing fluorescently labelled Pins and tubulin with a low dose of cytochalasin D (CytoD) that
 286 we have shown to inhibit force transmission without globally depolymerizing F-actin (Jodoin et al.,
 287 2015; Mason et al., 2013). Actomyosin is also necessary for cell rounding, which is necessary for
 288 planar divisions for MD cells (Chanet et al., 2017). As we were interested in the orientation of
 289 planar divisions, we focused our analysis on divisions that occurred within the plane of the
 290 epithelium. We found that injections of CytoD disrupted planar polarized division orientation,
 291 CytoD injected embryos exhibited an average division angle that was significantly different from
 292 controls (53.7 and 21.9, respectively; $p=0.0043$) (Figure 6B, D, F, H). CytoD injection also
 293 disrupted spindle orientations in MD3 and MD5 (Figure S4I - L). In addition, Pins polarity was

disrupted in embryos injected with CytoD whereas Pins is polarized in posterior crescents in DMSO injected controls. The posterior Pins peak to trough ratio was different for DMSO injected controls and CytoD injected embryos (1.17 ± 0.0685 and 1.08 ± 0.0609 , respectively, mean and S.D.) (Figure 6A, C, E, G, movie 5). Interestingly, injection of DMSO seems to perturb the synchrony of divisions. In non-injected embryos, cells within the domain divide in a wave-like pattern, starting in the middle of the domain and propagating to the domain boundary. In DMSO injected embryos, nearly all cells round and divide simultaneously. This disruption in division timing within the domain does not disrupt division orientation or Pins polarization. As CytoD has many effects beyond disrupting forces, we next sought to test the role of tissue mechanics without chemical or genetic perturbation.

To that end, we performed laser ablation experiments to mechanically isolate MDs from the rest of the tissue. We focused on MD1 and performed a series of four cuts to isolate the domain (Figure 6I). Using embryos that expressed fluorescently labelled tubulin, we analyzed the effect of mechanical isolation on spindle orientation. Cells that divide within the mechanically isolated region fail to orient along the AP axis (Figure 6K, N). As an internal control, we utilized the mirrored spatial pattern of MD1 and cut one side of the domain and left the other side uncut (Figure 6I). In the same embryo, we were able to observe spindles within the cut region fail to orient and neighboring cells outside the cut region orient correctly (Figure 6J, M, movie 6). As a further control, and to compensate for the difficulty in acquiring images that permitted visualization of both sides of the domain, we performed sham ablations, where we irradiated the region with a level of light below the cutting threshold. In sham ablated control embryos, spindles oriented with preference towards the AP axis and had an average division angle that was significantly different from cells within the cut region (20.0 and 58.0, respectively; $p=0.0286$) (Figure 6J - M).

Mesoderm invagination polarizes Pins and division orientation in the embryonic head

The divisions in MDs 1, 3, and 5 occur during gastrulation. We hypothesized that forces from morphogenic events during gastrulation control Pins polarization and division orientation. We focused on mesoderm invagination, which precedes divisions in MDs 1, 3, and 5 by around 10 minutes and pulls ectoderm cells around circumference of the embryo (Lye et al., 2015; Rauzi et al., 2015). To perturb forces associated with mesoderm invagination, we decided to disrupt the transcription factor Snail because Snail-depletion most severely depletes apical actomyosin activity in the mesoderm (Martin et al., 2009; Mason et al., 2013). We injected Snail dsRNA into embryos expressing fluorescently labelled tubulin and Pins. We found that Snail depletion disrupted division orientation (Figure 7B, D, F, H, movie 7). Embryos injected with Snail dsRNA had a random distribution of division angles, occurring with an average division angle significantly different from controls (40.0 and 22.6, respectively; $p=0.0121$). In addition, Pins polarity was clearly disrupted in embryos injected with Snail dsRNA. For 0.1x TE buffer injected controls, the posterior Pins peak to trough ratio was different from Snail dsRNA injected embryos (1.27 ± 0.08401 and to 1.10 ± 0.10545 , respectively, mean and S.D.) (Figure 7A, C, E, G). Beyond division orientation and Pins polarity, injections of Snail dsRNA disrupted the patterning of MDs 1, 3, and 5. Instead of three distinct domains, a single group of anterior cells divide. Taken together, these data indicate morphogenic forces play a role in Pins polarity and division orientation within MDs 1, 3, and 5. A role for morphogenic forces is consistent with our observation that disruption of α cat or actomyosin (with CytoD) or laser ablation disrupt division orientation.

Discussion:

Here, we discovered that Pins exhibits planar cell polarity in mitotic domains of the dorsal head (MDs 1, 3, and 5) and along the ventral midline (MD14). We demonstrated that Pins polarity is required for spindle orientation by overexpressing a myristoylated version of Pins that exhibits unregulated cortical localization and disrupts spindle orientation. Furthermore, we found that Pins planar cell polarity and AP spindle orientation depend on adherens junctions. Finally, we showed

that disrupting mesoderm invagination, disrupting actomyosin, or disconnecting MD cells from surrounding tissue by laser ablation disrupted Pins polarization and spindle orientation, suggesting that morphogenetic movements and resulting forces transmitted through the tissue can polarize Pins.

Other mechanisms of division orientation

Several other mechanisms of planar polarized division orientation have been identified. Previous works have shown that planar cell polarity pathways, namely the Fat/Dachsous, the core PCP and the PAR pathways, coordinate divisions of the fly wing disc, during Zebrafish gastrulation, and in *Drosophila* sensory organ precursor (SOP) cells, amongst other contexts (Baena-Lopez et al., 2005; Besson et al., 2015; Concha and Adams, 1998; Gho and Schweisguth, 1998; Gong et al., 2004; Mao et al., 2006). We ruled out the Fat/Dachsous and core PCP pathways because previous studies indicated the components of these pathways are not expressed early enough in development to play a role in orienting the divisions of the dorsal head or ventral midline (Clark et al., 1995; Tomancak et al., 2002; Tomancak et al., 2007). We further confirmed this by examining fluorescently tagged versions of proteins within these pathways. As was reported, we found no evidence of appreciable expression of these proteins (data not shown). Further, we ruled out the PAR pathway because we saw no evidence of planar polarity in Bazooka/Par 3 (Ko et al., 2020). The Toll-like receptor planar polarity pathway identified in the *Drosophila* trunk was a candidate for involvement in the mitotic domains (Pare et al., 2014). We ruled out this pathway for two reasons, first the TLR pathway in the *Drosophila* trunk results in polarized Myosin and Bazooka/Par3. Observation of these proteins in the dorsal head showed no strong polarization of either protein (Chanet et al., 2017; Ko et al., 2020). Second, we generated a triple mutant of the TLRs identified and performed injections of dsRNA of these TLRs. In each case, spindle orientation was not affected to the extent of the myristoylated Pins perturbation. Indicating TLRs cannot be solely responsible for spindle orientation in MDs 1, 3, and 5.

Hertwig's rule or the long axis rule is perhaps the most established mechanism of division orientation. More recent studies have shown that tricellular junctions are a better predictor of division orientation than the long axis. Mud localizes to tricellular junctions which cues spindle orientation (Bosveld et al., 2016). We did not observe Mud localization to tricellular junctions within the dorsal head mitotic domains and along the ventral midline. Further, we find no correlation between cell long axis and division orientation within these domains.

Myosin-dependent tension and division orientation

Myosin-dependent tension has been shown to cue the orientation of the mitotic spindle. A growing body of work suggests a role for tension in division orientation, with notable examples in fly development. In the fly notum, it was shown that cells rely on isotropic myosin-dependent tension to sense cell shape and divide along the cell long axis (Lam et al., 2020). Unlike the cells of the fly notum, cells within MDs 1, 3, 5, and 14 do not follow the long axis rule. However, isotropic myosin-dependent tension is required for cell rounding and for symmetric cell divisions in MD cells (Chanet et al., 2017).

Tension has also been shown to overrule the long axis rule and orient divisions with the axis of tension rather than the cell long axis. In the follicular epithelium, cortical tension at the apical surface cues planar divisions in a Canoe dependent manner. Pins and Mud were not planar polarized but rather localized around the entire cell cortex and did not play a role in the planar divisions within the follicular epithelium (Finegan et al., 2019). Similarly, in parasegmental boundaries, local anisotropy in myosin-dependent tension overrides cell shape. In this context it was shown that tension is both necessary and sufficient to cue division orientation. Pins and Mud were shown to not be involved in division orientation but were necessary for planar divisions. (Scarpa et al., 2018). In the mitotic domains, we did not observe anisotropic myosin-dependent tension. Instead, we observe a uniform distribution of actin and myosin within the dorsal head.

While myosin-dependent tension plays an important role in division orientation in different context within fly development, we found no evidence of a role in the oriented divisions of the dorsal head.

Adherens junctions and division orientation

Prior work suggested that adherens junctions are involved in division orientation. Notably, within *Drosophila* male germline stem cells (Inaba et al., 2010) and sensory organ precursor cells (Le Borgne et al., 2002) the adherens junction component, E-cadherin is localized asymmetrically and coordinates the asymmetric divisions within these cells. Perturbation of E-cadherin results in misorientation of the mitotic spindle. Interestingly, in sensory organ precursor cells it was also shown that perturbation of E-cadherin disrupted the localization of Pins, although Pins still appears as a crescent (Le Borgne et al., 2002).

E-cadherin also has a demonstrated role in symmetric divisions. In MDCK cells E-cadherin recruits Pins in a tension-dependent manner. It was previously known that Pins/LGN localizes to junctions, but this study first showed a role for force in Pins' junctional localization (Hart et al., 2017). Our findings are consistent with these results. As seen in the MDCK cells, in cells within the dorsal head mitotic domains and ventral midline adherens junctions may sense anisotropic force within the tissue and recruit spindle rotation machinery in a polarized fashion to cue division orientation. To our knowledge, this is the first *in vivo* example where mechanical force has been shown to polarize Pins to mediate division orientation.

Role of divisions in morphogenesis

An important question that remains is the function of the oriented divisions of the dorsal head during morphogenesis. The oriented divisions of the ventral midline (MD14) have been shown to alleviate tissue strain and promote tissue elongation by promoting an increase in surface area of the mesectoderm (Wang et al., 2017). This agrees with other studies which suggest that oriented division facilitate tissue elongation during development (Aigouy et al., 2010; Baena–

Lopez et al., 2005; Gong et al., 2004; Mao et al., 2011). A recent study expanded on the role of divisions during gastrulation. By following all the cell intercalations, movements, and divisions during gastrulation, the authors show cell divisions compensate for the cell loss due to various invaginations (Stern et al., 2021). Indeed, past work has shown that cell division can lead to oriented pushing forces that could contribute to morphogenesis (Gupta et al., 2021). In the embryo, apical relaxation that results from mitotic entry and the following oriented division has been shown to give rise to ectopic tissue folds (Ko et al., 2020). Therefore, we speculate that the oriented division of the dorsal head facilitate tissue elongation and compensate for cell loss from mesoderm and cephalic furrow invagination.

Study limitations and future directions

While the evidence in this study supports the hypothesis that morphogenic forces polarize Pins which is required for division orientation, the interpretation of this evidence is limited by certain aspects of the study. First, in describing Pins crescents in mitotic cells, we argue that Pins is on the posterior side for MDs 1 and 3 and anterior side for MD5 (Figure 2A, C, movie 2, Figure S1). This result is based on observation of cells on the boundary of mitotic domains that are not surrounded by other mitotic cells. We do not have the resolution to determine whether Pins is on one or both sides of cells within the domains. One question that arises from this observation is: how does a junction that is under tension recruit Pins to just one side? It is of interest to understand the sidedness of Pins in the mitotic domains and what causes this sidedness.

More broadly, it is of interest to understand how Pins is specifically recruited along the AP axis. While we show that disruption of mesoderm invagination is necessary for division orientation, what provides the directionality of the divisions remains unknown. Previous work has shown tissue flows that correspond to the division orientations we observe (Streichan et al., 2018). More work is needed to determine how forces generated at the ventral midline orient divisions in the dorsal head.

Finally, we can only speculate on the significance of these oriented divisions during morphogenesis. Planar, oriented division have been shown to be involved in tissue elongation and folding (Baena-Lopez et al., 2005; Kulukian and Fuchs, 2013; Lancaster and Knoblich, 2012; Morin and Bellaiche, 2011). Given that the divisions of MDs 1, 3, 5, and 14 take place during gastrulation, we hypothesize that the divisions may aid in morphogenic movements such as germ-band extension and cephalic furrow invagination, but we have not directly tested this. These questions form the basis of future work into the relationship between mechanical and molecular cues in division orientation and the contribution of division orientation during development.

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Materials and Methods

Fly stocks and genetics

Fly stocks and crosses used in this study are listed in Supplemental Table S1. Crosses were maintained at 25°C. In the F1 generation, non-balancer females and males were used to set up cages that were incubated at 25°C. The F2 generation was used for imaging.

Live and fixed imaging

For live imaging, embryos were dechorionated in 50% bleach for 2 minutes, washed in water, and mounted onto a glass slide coated with embryo glue (double-sided tape dissolved in heptane). No. 1.5 coverslips coated in glue were attached to the slide to use as spacers and a No. 1 coverslip was attached on top to create a chamber. Halocarbon 27 oil was used to fill the chamber. All imaging took place at 23-25°C.

For imaging fixed embryos, embryos were dechorionated in 50 % bleach (Chlorox) for 2 minutes, washed in water, and then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1 M phosphate buffer at pH 7.4 with 50% heptane (Alfa Aesar) for 30 min and manually devitellinized (TLR stains) or devitellinized by removing fixative, adding 50% methanol (Sigma Aldrich), and vortexing (Pins and Mud). Manually devitellinized embryos were stored in 0.01% Tween 20 in PBS (PBS-T and embryos devitellinized with 50% methanol were stored in 100% methanol at -4°C and rehydrated in PBS-T.

Embryos were washed in PBS-T, blocked with 10% BSA in PBS-T, and incubated with antibodies diluted in PBS-T. Embryos were incubated with primary antibodies at room temperature for 2 h. Antibodies used in this study are listed in Supplemental Table S1. Secondary antibodies used were conjugated with Alexa Fluor 488, 568, or 647 (Invitrogen) diluted at 1:500 in 5% BSA in PBS-T incubated overnight at 4°C. After antibody incubation, embryos were mounted onto glass slides using AquaPolymount (Polysciences).

All images were taken on a Zeiss LSM 710 confocal microscope with a 40×/1.2 Apochromat water objective lens, an argon-ion, 561-nm diode, 594-nm HeNe, 633 HeNe laser, and Zen software. The pinhole was set to 1 airy unit (au) for fixed imaging and 2.5 au for live imaging. For two-color live imaging, band-pass filters were set at ~490–565 nm for GFP and ~590–690 nm for mCherry (mCh). For three-color imaging, band-pass filters were set at ~480–560 nm for Alexa Fluor 488, ~580–635 nm for Alexa Fluor 568, and ~660–750 nm for Alexa Fluor 647.

dsRNA injections

Primers included the sequence of the T7 promoter (TAATACGACTCACTATAGGGAGACCAC) followed by the following recognition sequences:

Toll 2 F: AGTTTGAATCGAAACGCGAG

Toll 2 R: GGACACTGCACCGGATGT

Toll 6 F: ATCGGCCAAAAAGAGCAGTA

Toll 6 R: AGCAGCGTGTGCAGATTATT

Toll 8 F: ATGCGTACCATTTTCCTACCA

Toll 8 R: TTTTACTCTTCGCTTTTTCGC

Snα-F: CGGAACCGAAACGTGACTAT

Snα-R: GCGGTAGTTTTTGGCATGAT

Primer pairs were used to amplify a PCR product from genomic DNA. PCR products were directly used in a transcription reaction with T7 polymerase using the MEGAscript transcription kit (Ambion). The reaction was placed in boiling water and allowed to cool to room temperature to

promote annealing. RNA was extracted with phenol:chloroform, washed with ethanol, and resuspended in 0.1M TE buffer.

Stage 2 embryos were dechorionated in 50% bleach for 2 minutes. The embryos were mounted onto glass slides and desiccated for 4 min using Drierite. Embryos were covered with a 3:1 mixture of halocarbon 700/halocarbon 27 oils and then injected laterally. Embryos were imaged 3 hours after injecting.

Drug injections

Embryos were dechorionated in 50% bleach for 2 minutes. The embryos were mounted onto glass slides and desiccated for 4 min using Drierite. Embryos were covered with a 3:1 mixture of halocarbon 700/halocarbon 27 oils and then injected laterally at the beginning of cephalic furrow formation. To disrupt F-actin we resuspended CtyoD (Enzo Life Sciences) at 0.125 mg/ml in DMSO. Embryos were imaged 3-5 minutes after injecting.

Laser ablation

Laser ablations were performed using a 2-photon Mai-Tai laser set to 800 nm on a LSM710 confocal microscope (Zeiss) through a 40x/1.1 objective, using the Zen software (Zeiss) (W. M. Keck Microscopy Facility, Cambridge, MA). For ablations, laser power was set at 20%, with a scan speed of 1.58 ms/px. Ablations were performed with four overlapping linear ROIs around one side of MD1.

Sham ablations were performed with the same setup as the laser ablations except the laser power was set at 10%. This caused the ROIs to become bleached, but not ablated.

Image processing and analysis

All figure images were processed using MATLAB (MathWorks) and FIJI (<http://fiji.sc/wiki/index.php/Fiji>). A Gaussian smoothing filter (kernel = 0.75 pixel) was applied to all figure images. Brightness and contrast were adjusted to optimally visualize the mitotic spindle or Pins crescents in figure images. Control and experimental images were adjusted the same.

Division angle analysis

Division angle was taken using the angle tool in FIJI and measured as the angle between spindle poles axis and the AP axis in mitotic cells at anaphase or later. The angle values range from 0 degrees, indicating alignment along AP, to 90 degrees, indicating alignment along DV. Unless otherwise stated, 10 embryos and between 100-250 spindles were counted of each genotype. Resulting angles were plotted in rose plots, with percentage of spindles on the r axis and angle on the theta axis.

Intensity profile analysis

Intensity profiles were taken using the segmented line tool in Fiji with a line width of 2 and measured using the Plot Profile feature in Fiji. The perimeter of the cell was outlined starting at the apical most point of mitotic cells in anaphase. The direction of the lines was toward the dorsal midline or ventral midline (clockwise for the left half of the domains and counterclockwise for the right half of the domains). Intensity was normalized to cytoplasmic intensity, which was measured as the mean grey value of a line drawn through the cytoplasm. Unless otherwise stated, 10 cells per embryo and 3 embryos per genotype were analyzed, averaged, and graphed as line plots with normalized intensity on the y axis and distance from start on the x axis.

Anisotropy analysis

Aspect ratios of interphase cells were taken using the polygon tool in Fiji and the Fit Ellipse measurement, which gives the major axis length, the minor axis, and the angle of the major axis.

Aspect ratio is ratio between the major axis and the minor axis. The average aspect ratio was recorded and the angle of the major axis was compared to the axis of division. Unless otherwise stated, 10 cells per embryo and 3 embryos per genotype were analyzed.

Quantification and statistical analysis

Statistical analyses were performed using the MATLAB statistics toolbox. p values were calculated using a two-sample Mann-Whitney test, the reference sample is the angle distribution in wild-type. Correlation coefficients r are Spearman correlation coefficient. p values are calculated against the null hypothesis $r = 0$.

Figure Legends

Figure 1. Early mitotic domains in the *Drosophila* head exhibit spindle rotation and oriented division (A) Schematic of the dorsal head of a *Drosophila* embryo with mitotic domain 1 (MD1) in red, mitotic domain 3 (MD3) in blue, and mitotic domain 5 (MD5) in orange. Anterior-posterior (AP) axis is indicated by arrow, the top of images will consistently be anterior, as in the schematic. (B) Image of the dorsal head of a live *Drosophila* embryo expressing Jupiter::GFP to label microtubules/spindle (greyscale). Jupiter::GFP signal is detected in image. MD1 is outlined in red, MD3 in blue, and MD5 in orange, scalebar is 50 μ m. (C) MD1 cells exhibit planar polarized cell division. Left, Image of MD1 in live embryo showing labeled myosin (greyscale, Sqh::mCherry), to show cytokinetic rings, and labeled F-actin (green, UtrABD::GFP) to show cell cortex, scalebar is 10 μ m. Right, Same image of MD1 annotated with black lines marking the cytokinetic ring of each dividing cell and magenta dashed lines marking division angle. (D) MD1 cells exhibit spindle rotation and alignment. Images are time series of MD1 with Jupiter::GFP marking the mitotic spindle (greyscale), blue lines indicate spindles still rotating and magenta lines represent spindle that have reached their final orientation, scalebar is 10 μ m. (E) Schematic of division angle analysis. Division angle is taken with respect to the AP axis such that angle of 0 indicates alignment with the AP axis. Note only spindles during or after anaphase, when division angle is fixed, are measured. (F-H) Rose plots indicating division angles of MD1 (F), MD3 (G), and MD5 (H). Red lines indicate mean division angle. Mean division angle for MD=21.6°, MD3=20.4°, and MD5=19.0°. For MD1, n=10 embryos and 200 spindles, MD3, n=5, embryos and 123 spindles and MD5, n=5 embryos and 140 spindles.

Figure 2. Pins is planar polarized in mitotic domain cells. (A-B) Time series of MD1 of embryo expressing Pins::YFP, to label spindle rotation machinery, and Gap43::mCherry, to mark membranes. scalebar is 10 μ m. (C, J) Quantification of average cellular (7-9 cells) Pins::YFP signal intensity around the mitotic cell cortex for 3 separate embryos, lines indicate average of cells in each embryo for MD1 (C) or MD14 (J). Cortical intensity was measured at anaphase, indicated by cell elongation, and normalized to cytoplasmic Pins::YFP signal. (C', J') Average of embryos in C or J. (D, K) Quantification of Gap43::mCherry (membrane marker) intensity for same cells and 3 embryos as in C for MD1 or J for MD14. (D', K') Average of embryos in D, MD1, or K, MD14. Intensity was normalized to cytoplasmic Gap43::mCherry signal. (E) Fixed sample of metaphase cells in MD1 stained for Tubulin (Left), Ecadherin (center), and Pins (Right). (F) Fixed sample of anaphase cells in MD1 stained for Tubulin (Left), Pins (Right). (G) Fixed sample of anaphase cells in MD1 stained for Tubulin (Left) and Mud (Right). (H-I) Time series of MD14 cells expressing Pins::YFP and Gap43::mCherry, similar to A and B. scalebar is 10 μ m.

Figure 3. Planar polarized divisions depend on asymmetric Pins localization (A–B) Pins localizes in planar polarized crescents immediately prior to spindle orientation. Images are time series of MD1 of embryo with labeled Pins (Pins::YFP) and labeled tubulin (Tubulin::mCherry), to mark mitotic spindles, blue lines indicate spindles that are still rotating, magenta lines indicate spindles that have reached their final orientation. Colored boxes at tips of lines label spindles with the color marking the same spindle at each time point. Scalebar is 10 μ m. (C) Spindle orientation is correlated with Pins polarity. Plot of the correlation between the angle of maximum Pins::YFP intensity and division angle., $R^2=0.77$. (D) Schematic of MD 14, located on either side of the ventral midline. (E–F) Images are a time series of MD14 cells labeled with Pins::YFP and tubulin::mCherry, to mark mitotic spindles. (G, I) Myristoylated Pins disrupts division orientation. Left, image of MD1 of control embryo (G, UAS>PinsGFP) or MD1 of Myristoylated Pins expressing embryo (I, UAS>myr::Pins::GFP). Images show marker (CLIP170::GFP) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position. (H, J) Quantification of spindle angles for control embryos (H, UAS>PinsGFP) using rose plots or Myristoylated Pins expressing embryos (J, UAS>myr::Pins::GFP). For control embryos (H, UAS-PinsGFP) MD1, n=7 embryos and 175 spindles. For Myristoylated Pins expressing embryos, n=10 embryos and 284 spindles. Average orientation angles per embryo were statistically different between control and Myristoylated Pins expressing embryos, KS test, p=0.0326. (K, M) Pins asymmetry is required for MD14 spindle orientation. Left, image of MD14 of control embryo (G, UAS-PinsGFP) or MD14 of Myristoylated Pins expressing embryo (I, UAS>myr::Pins::GFP). Images show marker (CLIP170::GFP) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position. (L, N) Quantification of spindle angles for control embryos (UAS>PinsGFP) or Myristoylated Pins expressing embryos (N, UAS>myr::Pins::GFP) using rose plots. For control embryos (L, UAS>PinsGFP) MD14, n=3 embryos and 64 spindles. For Myristoylated Pins expressing embryos, n=3 embryos and 59 spindles. Average orientation angles per embryo were statistically different between control and Myristoylated Pins expressing embryos, KS test, p=0.0326.

Figure 4. Disruption of adherens junctions, but not Canoe, inhibits planar polarized division orientation (A, C, E) α -catenin depletion disrupts division orientation. Left, image of MD1 of control knock-down (A, Rh3 RNAi), MD1 of α -catenin depleted embryo (C, α -catenin RNAi), or MD1 of Canoe depleted embryo (E, Canoe RNAi). Images show marker (CLIP170::GFP) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position. (B, D, F) Quantification of spindle angles for control embryos (B, Rh3 RNAi), α -catenin depleted embryos (D, α -catenin RNAi), or Canoe depleted embryos (F, Canoe RNAi) using rose plots. For control embryos (Rh3 RNAi) MD1, n=10 embryos and 355 spindles. For α -catenin RNAi MD1, n=10 embryos and 248 spindles. For Canoe RNAi MD1, n=7 embryos and 166 spindles. Average orientation angles per embryo were statistically different between control and α -catenin RNAi, p=0.0012, but not for canoe RNAi, p=0.2698.

Figure 5. Adherens junction depletion disrupts Pins planar cell polarity. (A, C) α -catenin depletion disrupts Pins localization. Left, image of a MD1 cell of control embryo (A, Rh3 RNAi) or a MD1 cell of α -catenin depleted embryo (C, α -catenin RNAi). Images show labelled Pins (Pins::YFP), scalebar is 10 μ m. Right, same image annotated with dashed line to indicate cell outline used to plot intensity profile, * to indicate start, and arrow to indicate direction of line. (B, D) Quantification of Pins intensity for control embryos (Rh3 RNAi) or α -catenin depleted embryos (D, α -catenin RNAi). Intensity was normalized to cytoplasm. For control (Rh3) MD1, n=3 embryos and 30 cells. For α -catenin RNAi MD1, n=3 and 30 cells.

Figure 6. Mechanical force is necessary for Pins polarity and spindle orientation (A, E) Cytoskeletal disruption prevents Pins localization. Image of MD1 in control embryo (A, DMSO injection) or MD1 of cytochalasin D (CytoD) injected embryo (E, 0.25mg/mL CytoD injection). Images show labelled Pins (Pins::YFP), red arrows point to crescents of Pins in control MD1, scalebar is 10 μ m. (B, F) Cytoskeletal disruption interferes with division orientation. Image of MD1 of control embryo, same embryo as A, (B, DMSO injection) or MD1 of CytoD injected embryo (F). Images, from same embryo as E, show marker (Tubulin::mCherry) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position. (C, G) Quantification of Pins intensity for control embryos (C, DMSO injection) or CytoD injected embryo (G, 0.25mg/mL CytoD injection). Intensity was normalized to cytoplasm. For control (DMSO injection) MD1, n=3 embryos and 30 cells. For CytoD injection MD1, n=3 and 28 cells. (D, H) Quantification of spindle angles for control embryos (H, DMSO injection) or CytoD injected embryo (H) using rose plots. For control embryos (DMSO injection) MD1, n=5 embryos and 162 spindles. For CytoD injection MD1, n=6 embryos and 143 spindles. When compared to control (DMSO injection) using KS test, p=0.0023. (I) Schematic of laser ablation schema. The ROI for laser cutting was drawn around MD1 on one side of the embryo to mechanically uncouple MD1 from surrounding tissue. The other side of MD1 was not cut. (J-L) Separating MD1 from surrounding tissue disrupts spindle orientation. Left, image of MD1 on control side of embryo (J, non-cut), laser cut side of embryo (K), or sham ablation, control embryos (L). Images show marker (Tubulin::mCherry) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position, red box indicates laser cut ROI. (M-O) Quantification of spindle angles for control side (M, non-cut), laser cut side (N), or MD1 of sham ablated embryos (O) using rose plots. For control side (non-cut) MD1, n=2 embryos and 15 spindles. For laser cut side MD1, n=4 embryos and 56 spindles. For sham ablated embryos MD1, n=4 embryos and 58 spindles. Average orientation angles per embryo were statistically different between laser cut and non-cut sides of MD1, p=0.0469, and between laser cut embryos and sham ablation embryos, p=0.0111.

Figure 7. Snail depletion disrupts Pins polarization and spindle orientation (A, E) Disrupting ventral furrow formation disrupts Pins polarity. Image of MD1 of control embryo (A, 0.1x TE buffer injection) or MD1 of Snail dsRNA injected embryo (E). Images show labelled Pins (Pins::YFP), red arrows point to crescents of Pins in control, scalebar is 10 μ m. (B, F) Snail depletion disrupts division orientation. Left, image of MD1 of control embryo (B, 0.1x TE buffer injection) or MD1 of Snail depleted embryo (F). Images show marker (Tubulin::mCherry) to mark mitotic spindles (greyscale), scalebar is 10 μ m. Right, same image annotated with dashed lines to indicate spindle position. (C, G) Quantification of Pins intensity for control embryos (C, 0.1x TE buffer injection) or Snail dsRNA injected embryo (G). Intensity was normalized to cytoplasm. For control (0.1x TE buffer injection) MD1, n=3 embryos and 30 cells. For Snail depletion MD1, n=2 and 20 cells. (D, H) Quantification of spindle angles for control embryos (H, 0.1x TE buffer injection) or Snail dsRNA injected embryo (H) using rose plots. For control embryos (0.1x TE buffer injection) MD1, n=6 embryos and 166 spindles. For Snail dsRNA injection MD1, n=2 embryos and 23 spindles. Average orientation angles per embryo were statistically different between control and Snail dsRNA injected embryos, KS test, p=0.0326.