The Fz-Dsh Planar Cell Polarity Pathway Induces Oriented Cell Division via Mud/NuMA in Drosophila and Zebrafish

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DOI 10.1016/j.devcel.2010.10.004

SUMMARY

The Frizzled receptor and Dishevelled effector regulate mitotic spindle orientation in both vertebrates and invertebrates, but how Dishevelled orients the mitotic spindle is unknown. Using the Drosophila S2 cell “induced polarity” system, we find that Dishevelled cortical polarity is sufficient to orient the spindle and that Dishevelled's DEP domain mediates this function. This domain binds a C-terminal domain of Mud (the Drosophila NuMA ortholog), and Mud is required for Dishevelled-mediated spindle orientation. In Drosophila, Frizzled-Dishevelled planar cell polarity (PCP) orients the sensory organ precursor (pl) spindle along the anterior-posterior axis. We show that Dishevelled and Mud colocalize at the posterior cortex of pl, Mud localization at the posterior cortex requires Dsh, and Mud loss-of-function randomizes spindle orientation. During zebrafish gastrulation, the Wnt11-Frizzled-Dishevelled PCP pathway orients spindles along the animal-vegetal axis, and reducing NuMA levels disrupts spindle orientation. Overall, we describe a Frizzled-Dishevelled-NuMA pathway that orients division from Drosophila to vertebrates.

INTRODUCTION

Oriented cell division is important for the specification of cell fate and for tissue morphogenesis (Keller, 2006; Lecuit and Le Goff, 2007; Siller and Doe, 2009). The orientation of cell division relies on intrinsic or extrinsic cortical polarity cues, which specify the orientation of the mitotic spindle. In both vertebrates and invertebrates, the Frizzled (Fz) planar cell polarity (PCP) pathway acts to orient the cell division relative to extrinsic cues within a tissue. By doing so, the Fz PCP pathway has fundamental roles in body plan specification, cell fate determination, and tissue elongation (reviewed in Ségalen and Bellaïche, 2009).

Here we study the mechanism of Fz PCP regulated mitotic spindle orientation in Drosophila and zebrafish. During the development of the Drosophila adult peripheral nervous system, the sensory organ precursor (pl) divides with an anterior-posterior planar polarity to produce a posterior cell, pIIa, and an anterior cell, pIIb, which will further divide to generate, respectively, the external and internal cells of the adult mechanosensory organs (Gho and Schweisguth, 1998; Gho et al., 1999; Fichelson and Gho, 2003). The transmembrane receptor Fz and its cortical effector Dishevelled (Dsh) are localized to the apical posterior cortex of the pl cell. They specify the posterior localization of the Par complex (Bazooka [Baz]; Par-6; atypical protein kinase C [aPKC]) and the anterior localization of the Discs large (Dlg)/Partner of Inscuteable (Pins) complex, which are both necessary to promote anterior localization of the cell fate determinants Numb and Neuralized, as well as the adaptor Partner of Numb (Pon) (Bellaïche et al., 2001a, 2001b; Le Borgne and Schweisguth, 2003; Wirtz-Peitz et al., 2008). Spindle orientation along the anterior-posterior polarity axis does not require the Par complex, Pins, or Dlg; in contrast, Fz and Dsh are both required for spindle orientation along this polarity axis and thereby promote the correct specification of the pl daughter cells (Bellaïche et al., 2001a; Bellaïche et al., 2001b; David et al., 2005).

In vertebrates, the function of PCP signaling as a regulator of mitotic spindle has been established for symmetric cell division during zebrafish gastrulation. In the epiblast, which gives rise to the neural ectoderm and the epidermis, the Wnt11-Frizzled-Dishevelled PCP pathway orients spindles along the animal-vegetal axis, and reducing NuMA levels disrupts spindle orientation. Overall, we describe a Frizzled-Dishevelled-NuMA pathway that orients cell division from Drosophila to vertebrates.
understanding of how PCP links to the mitotic spindle is an important step for understanding cell fate specification and embryo morphogenesis in invertebrates and vertebrates.

Although little is known about how the PCP pathway regulates spindle orientation, there has been recent progress in understanding spindle orientation mechanisms in other contexts (reviewed in Siller and Doe, 2009). In particular, the mechanisms linking intrinsic Par complex polarity cues to the mitotic spindle depend on the conserved GoLoco domain proteins Pins/LGN/AGS3/GPR1/2 (Drosophila Pins, Partner of Insuteable; vertebrates LGN and AGS3; Caenorhabditis elegans GPR-1/2; for review see Gönczy [2008] and Siller and Doe [2009]). Once localized at the cell cortex, Pins/LGN/AGS3/GPR1/2 directly bind the coiled-coil domain protein mushroom body defective (Mud)/nuclear mitotic apparatus (NuMA)/Lin-5 that recruits the dynein complex to regulate mitotic spindle orientation during Drosophila and C. elegans asymmetric cell division and spindle oscillation in human cultured cells (Grill et al., 2001, 2003; Srinivasan et al., 2003; Du and Macara, 2004; Schmidt et al., 2005; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Nguyen-Ngoc et al., 2007; Park and Rose, 2008; Siller and Doe, 2008; Cavernard and Doe, 2009). In addition, Drosophila Pins can interact with the mitotic spindle via the Dlg-Khc73 pathway (Siegrist and Doe, 2005; Johnston et al., 2009). Here we show that Drosophila Dsh interacts with Mud protein, linking the conserved Fz-Dsh planar cell polarity pathway to the Mud/NuMA-Dynein spindle orientation pathway. Furthermore, we show that the combined Fz-Dsh-NuMA/Mud pathway regulates spindle orientation during both Drosophila asymmetric cell division and zebrafish tissue morphogenesis. Hence our work identifies a conserved mechanism controlling mitotic spindle orientation by extrinsic polarity cues.

RESULTS AND DISCUSSION

The Dsh DEP Domain Is Sufficient for Spindle Orientation in Drosophila S2 Cells

To directly examine the role of Dsh in mitotic spindle orientation, we used a recently developed “induced cell polarity” system (Johnston et al., 2009). This system allows the generation of Dsh cortical asymmetric localization in the otherwise unpolarized Drosophila S2 cell line. It therefore allows us to test whether Dsh asymmetric distribution is sufficient to orient the spindle and if so, to identify putative Dsh effectors needed to regulate mitotic spindle orientation. Briefly, Dsh was fused in frame to the cytoplasmic terminus of the homophilic cell adhesion molecule Echinoid (Ed::Dsh), expressed in the normally nonadherent S2 cell line, and gently shaken to induce Ed-dependent cell clustering. This results in the localization of Dsh to a defined cortical domain at the site of cell-cell contact. The ability of the Dsh cortical domain to orient the mitotic spindle was assayed by measuring the mitotic spindle angle relative to the center of the Dsh cortical domain (Figure 1A) (Johnston et al., 2009), and represented as a cumulative plot of spindle angle from 0° (perfectly oriented) to 90° (not oriented). In this system, random spindle orientation would give an average spindle angle of ~45° and show a linear diagonal cumulative plot spanning 0°–90°, whereas a cortical domain with spindle orientation ability should give a lower average spindle angle, and a leftward deflection of the cumulative plot due to overrepresentation of small spindle angle data points.

We confirmed our previous findings that control S2 cells expressing Ed::GFP have randomized spindles (Figures 1B and 1M and Table 1) (average angle = 53° ± 23°; linear cumulative plot) (Johnston et al., 2009). In contrast, fusion of the full length Dsh protein to Ed resulted in an Ed::Dsh cortical domain that had significant spindle orientation activity (Figures 1C and 1M and Table 1) (average angle = 28° ± 18°; left-shifted cumulative plot). We next used this system to identify the Dsh domain responsible for spindle orientation. Dsh harbors at least three conserved domains, a DIX domain, a PDZ domain and a DEP domain (Figure 2A). The DIX and PDZ domains are sufficient to mediate canonical Wnt signaling, and the DEP domain is essential for Dsh PCP signaling activity (Axelrod et al., 1998). We found that the PDZ domain had no spindle orientation activity (Figures 1D and 1M and Table 1) (average spindle angle = 49° ± 24°); in contrast, the C-terminal region containing the DEP domain provided excellent spindle orientation (Figures 1E and 1M and Table 1) (average angle = 17° ± 15°). Expression of the N-terminal DIX domain did not result in reliable cortical targeting, thus preventing direct assessment of this domain in isolation. The finding that the isolated DEP domain is better at spindle orientation than the full length Dsh protein suggests that there may be intramolecular or intermolecular interactions in the Dsh protein that partially limit the ability of the DEP domain to orient the spindle. We next examined the ability of Fz, the canonical receptor-mediated activator of Dsh, to induce spindle orientation. Interestingly, the Fz1 C-terminal cytoplasmic domain fused to Ed did not orient the spindle in the S2 assay (Figures 1F and 1N and Table 1) (average angle = 46° ± 29°), despite the presence of endogenous Dsh protein in S2 cells (data not shown). However, we found that a similar fragment of Fz4 elicited spindle orientation indistinguishable from full-length Dsh (Figures 1G and 1Q and Table 1) (average angle = 24° ± 12°). Fz4 was also capable of recruiting Dsh to the cortical Ed crescent (Figures 1K–1L). Fz1 may require cell type-specific post-translational modifications for Dsh-mediated spindle orientation not available in S2 cells. We conclude that the Dsh DEP domain acts downstream of Fz signaling and is sufficient for spindle orientation in Drosophila S2 cells.

Dsh Acts via the Mud-Dynein Pathway to Promote Spindle Orientation

We next sought to delineate the downstream signaling pathways responsible for Dsh-mediated spindle orientation. We had previously shown that two pathways are needed for spindle orientation in Drosophila S2 cells and neuroblasts: a Pins-Dlg-Khc-73 pathway, and a Pins-Mud-Dynein pathway (Johnston et al., 2009). We examined the requirement for each of these pathways in Dsh-mediated spindle orientation using Dlg and Mud inactivation by RNAi. Whereas treatment with RNAi against Dlg did not reduce Dsh spindle orientation activity, knockdown of Mud completely abolished Dsh-mediated spindle alignment (average angle, 46° ± 27°) (Figures 1H, 1L, and 1O and Table 1). RNAi against Lis-1, a functional component of the dynein complex during asymmetric cell division (Nguyen-Ngoc et al., 2007; Siller and Doe, 2008), also abrogated proper spindle alignment to the Ed::Dsh crescent (Figures 1J and 1O and Table 1) (average angle = 50° ± 27°).
Figure 1. Dissection of the Dsh-Mud-Dynein Pathway in S2 Cells

(A) Schematic representation of the division angle measured in S2 cells. The angle of division (α) is defined by the vector perpendicular to the middle of Ed::GFP crescent and the spindle axis.

(B–J) Dsh domains were fused in frame to Echinoid tagged with GFP (green) (C–E) and (H–J) and transfected into S2 cells. C-terminal domain of Fz1 from amino acids (aa) 553–581 (F) and C-terminal domain of Fz4 from aa 560–705 (G) were fused in frame to Echinoid (Ed) (green) and transfected into S2 cells. Cells were stained for α-tubulin (red). Ed::GFP control (B); Ed::Dsh corresponds to Ed::GFP fused to Dsh full length (FL) (C); Ed::Dsh(PDZ) corresponds to Ed::GFP fused to the Dsh(PDZ) (D); Ed::Dsh(DEP) corresponds to Ed::GFP fused to the Dsh(DEP) (E); Ed::Fz1 corresponds to Ed::GFP fused to Fz1 C-terminal domain (F); Ed::Fz4 corresponds to Ed::GFP fused to Fz4 C-terminal domain (G); Ed::Dsh + dlg RNAi corresponds to Ed::GFP fused to Dsh FL and RNAi againstDlg (H); Ed::Dsh + mud RNAi corresponds to Ed::GFP fused to Dsh FL and RNAi against mud (I); Ed::Dsh + lis1 RNAi corresponds to Ed::GFP fused to Dsh FL and RNAi against Lis1 (J). The scale bar in (B) represents 2 μm.

(K–L) Localization of Dsh::Myc (red in K, K', L, and L') in S2 cells expressing Ed::GFP (green in K' and K'') or Ed::Fz4C (green in L and L'). The scale bar in (K) represents 2 μm.

(M) Cumulative graph of angles measured in the S2 cell “induced polarity” assays in Ed::GFP (black line), Ed::Dsh (blue line); Ed::Dsh(DEP) (green line); and Ed::Dsh(PDZ) (red line).
Dsh and the Mud C Terminus Can Associate in a Protein Complex

To explore the mechanisms by which Dsh orient the mitotic spindle via Mud, we tested whether these proteins could associate with each other. We determined that a C-terminal region of Mud from amino acid 1825 to 2475 specifically immunoprecipitates Dsh::Myc (Figures 2B and 2C) from lysate of HEK293 cells, usually used to analyze Drosophila PCP protein interactions (Jenny et al., 2005). This region (thereafter referred to as MudC) includes the Pins binding domain and the MT-binding domain of Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Furthermore, and in agreement with the results of the spindle orientation assay, the C-terminal Dsh domain containing the Dsh DEP domain is immunoprecipitated with MudC whereas the Dsh Dix-PDZ domains only weakly interact with MudC (Figure 2D). We conclude that the Dsh DEP dictates association with Mud, consistent with a role of the Dsh DEP domain in engaging the Mud-Dynein pathway for spindle orientation.

Dsh Recruits Mud to the Posterior Cortex During pl Asymmetric Cell Division

To explore the in vivo function of the Fz-Dsh-Mud spindle orientation pathway, we first analyzed the respective localization of Mud and Dsh during the asymmetric cell division of the Drosophila sensory organ precursor cell (pl; identified by the expression of Senseless [Ss]) (Nolo et al., 2000).

In the epithelial cells of the papal dorsal thoracic imaginal disc, Fz and Dsh are planar polarized and they accumulate at the apical posterior cell cortex at the level of the adherens junctions (AJs) stained by Armadillo ([Arm], Drosophila β-catenin). Fz and Dsh also strongly accumulate at the apical posterior cortex of pl cells in late interphase and prophase (Figures 3A, 3A’, 3A”, 3C’, and 3E’; data not shown) (Bellaïche et al., 2004; David et al., 2005). There, they colocalized with Mud protein (80% of the cells; n = 24) (Figures 3C, 3C”, 3E, and 3E”). We next tested whether Dsh or Mud regulate the localization of each other in interphase/prophase pl cells. We found that the apical posterior localization of Dsh was not affected in mud mutant pl cells (Figures 3B–3B”), whereas the apical localization of Mud was lost in dsh pl mutant pl cells (n = 16) (Figures 3D, 3D’, 3D”, 3F, 3F’, and 3F”). The dsh allele abrogates only the Dsh PCP function (Axelrod et al., 1998) and contains a missense mutation in the DEP domain, consistent with our biochemical data showing MudC-Dsh DEP domain interaction (see above). We conclude that Dsh recruits Mud to the apical posterior cortex in interphase/prophase pl cells. Mud could also be found to accumulate with Pins at the anterior lateral or basal cortex in early prophase in wild-type and dsh mutant pl cells (not shown; Figures 3F’–3F”).

In prometaphase and metaphase pl cells, Dsh accumulates at the apical posterior cortex whereas Pins accumulates at the anterior lateral cortex (Figures 3G–3G”). In addition, the mitotic spindle is aligned along the anterior posterior axis whereas Pins accumulates at the anterior lateral cortex (Figures 3G–3G”). At the posterior cortex, Mud was partially colocalized with Dsh at the apical posterior pl cell cortex at the level of the AJs, and it is also present laterally along the pl cell cortex (n = 13) (Figures 3I, 3I”, 3I, 3K, and 3K”). At the anterior cortex, Mud is located laterally with Pins (n = 14) (Figures 3I, 3I”, 3I, 3K, 3K, 3K’, and 3K”). Furthermore the posterior and anterior cortical Mud enrichments were found in close vicinity of the posterior and anterior centrosomes of the dividing pl cell, respectively (arrowheads in Figures 3I, 3I”, 3I, 3K, 3K, 3K’, and 3K”). In pins mutant pl cells, the Mud anterior localization was reduced or lost (n = 30) (Figures 3L–3L”) in agreement with the previously described interaction between Mud and Pins in neuroblasts (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). In contrast, Mud apical posterior accumulation was maintained in pins null mutant pl cells in prophase (n = 8) (Figures 3H–3H”). In dsh mutant prometaphase pl cells, Mud apical and lateral enrichment opposite to the Pins accumulation was reduced or lost (n = 16) (Figures 3J–3J”), similar to the phenotype at interphase/prophase. Altogether, we conclude that in the dividing pl cells, Mud is recruited to the anterior lateral cortex by Pins, and, consistent with the observed interaction between the Dsh DEP domain and the Mud C-terminal domain, Mud is recruited to the posterior cortex by Dsh.

Mud Is Required for Anterior-Posterior Spindle Orientation in pl Cells

The orientation of the mitotic spindle in the pl cell is strictly controlled along both the anterior-posterior axis and the apical-basal axis (Figure 4A). Fz and Dsh orient the mitotic spindle along the anterior-posterior axis but their apical localization tends to tilt the spindle relative to the apical-basal axis (Gho
and Schweisguth, 1998; Bellaiche et al., 2001a; David et al., 2005). In contrast, Pins and heterotrimeric G protein (HGP) pathway, which act at the anterior lateral cortex, are only needed to counterbalance the apical-basal tilt induced by the Fz PCP pathway; thereby they maintain the mitotic spindle in the plane of the epithelium (David et al., 2005). Thus, in cells lacking the Fz PCP pathway, the spindle is misaligned relative to the anterior-posterior axis and parallel to the plane of the epithelium, whereas in pins mutant pl cells, the spindle is correctly aligned along the anterior-posterior axis but is strongly tilted relative to the apical-basal axis (Figure 4A).

To characterize the role of Mud in the pl cell spindle orientation, we first tested whether mud mutants have normal pl cell polarity. Wild-type mitotic pl cells have Numb, Pon, and Pins localized to the anterior cortex and Baz localized to the posterior cortex (Figures 4B, 4B', 4D, 4D', 4F, 4F', 4H, and 4H'). In mud mutant mitotic pl cells, all four proteins showed normal polarized localization (Figures 4C, 4C', 4E, 4E', 4G, 4G', 4I, and 4I'; see Figures S1A–S1C available online). Thus, mud mutant pl cells have normal anterior-posterior cortical polarity, permitting us to analyze the function of Mud in spindle orientation.

We assayed pl mitotic spindle orientation in living Drosophila pupa by expressing the microtubule-associated Tau::GFP fusion protein in pl cells using the neuralized-GAL4 driver (Bellaiche et al., 2001a; David et al., 2005). We measured the angle of the spindle relative to both the anterior-posterior axis ($\alpha_{AP}$) and the angle of the spindle relative to the plan of the epithelium ($\alpha_{AB}$) (Figure 4A) (David et al., 2005) in wild-type, dsh$^+$, mud or pins mutant pl cells. In mud mutant pl cells, the mitotic spindle was parallel to the plane of the epithelium (mean $\alpha_{AB/mud}$ = 3.2° ± 19°, $p_{wt/mud}$ = 0.003; Figure 4J); a similar phenotype was observed for dsh and fz mutant pl cells ($p_{dsh/mud}$ = 0.56, $p_{fz/mud}$ = 0.34; Figure 4J and not shown). This phenotype is distinct from the one observed in pins or HGP pathway mutant pl cells ($p_{pins/mud}$ = 0.0001 and $p_{Gul/mud}$ = 0.0001) (Figure 4J; data not shown).

Furthermore, the orientation of the mitotic spindle was randomized relative to the anterior-posterior axis in mud mutant pl cells ($p_{wt/mud}$ = 0.0009) (Figure 4K) similar to dsh or fz mutant pl cells ($p_{mud/dsh}$ = 0.31, $p_{mud/fz}$ = 0.47) (Figure 4K; data not shown). This suggests that Mud does not function downstream of Pins, as previously reported (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), but also acts in the same pathway as Fz and Dsh. The observation that Dsh recruits Mud to the apical posterior cortex, and that fz, dsh, and mud mutants share a common spindle orientation phenotype, leads us to propose that Mud acts downstream of the Fz-Dsh PCP pathway to regulate anterior-posterior mitotic spindle orientation in pl cells.

To analyze the cell fate consequences of mud mutant spindle orientation defects in pl cells, we analyzed the segregation of Partner of Numb::GFP (Pon::GFP) in pl cells expressing Histone2B::mRFP by time-lapse microscopy. In wild-type cells, Pon::GFP accumulates at the anterior cortex of the pl cell in metaphase and segregates only into the anterior daughter cell in telophase (n = 17) (Figure 5A). In 27% of mud mutant pl cells in telophase (n = 15), Pon::GFP failed to exclusively segregate in one of the two daughter cells (Figure 5B). Accordingly, the loss of Mud function was associated with pllb to plla cell fate mis-specification as marked by the presence of sensory organs.
only composed of external sensory cells at the expense of the internal sensory cells (Figures 5C–5F). Thus, Mud is required for the proper specification of p1 daughter cells as an effector of Fz signaling during asymmetric cell division.

Mud has been shown to function downstream of Pins in Drosophila neuroblasts. To analyze whether Mud could also function downstream of Pins in absence of PCP activity, we compared the orientation of the mitotic spindle relative to the Pins crescent in dsh1, mud mutant pl cells (Figures S1D–S1I). Whereas the mitotic spindle was aligned with the Pins crescent in most of the dsh1 mutant pl cells in metaphase, the mitotic spindle failed to align with the Pins crescent in dsh1, mud double mutant pl cells in metaphase. We conclude that Mud functions downstream of Fz signaling to regulate mitotic spindle orientation and that, in absence of Fz activity, Mud is needed downstream of Pins to orient the mitotic spindle in the pl cell.

NuMA Is Required for Dsh-Dependent Mitotic Spindle Orientation during Zebrafish Gastrulation

The vertebrate NuMA protein, ortholog of Drosophila Mud protein, has never been tested for a role in spindle orientation during tissue development. NuMA is a good candidate for regulating spindle orientation in vertebrate embryos because it is known to regulate mitotic spindle oscillation in cultured cells (Du and Macara, 2004), it is asymptomatically localized during skin and neuron progenitor asymmetric cell division (Lechler and Fuchs, 2005; Lake and Sokol, 2009), and its invertebrate orthologs are known to regulate spindle orientation (Srinivasan et al., 2003; Du and Macara, 2004; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Park and Rose, 2008; Cabernard and Doe, 2009). Having established the role of Mud in the Fz-Dsh PCP spindle orientation pathway in Drosophila, here we analyze the function of NuMA in the zebrafish epiblast where the Wnt11 function downstream of Pins in absence of PCP activity, we compared the orientation of the mitotic spindle relative to the Pins crescent in dsh1, mud mutant pl cells (Figures S1D–S1I). Whereas the mitotic spindle was aligned with the Pins crescent in most of the dsh1 mutant pl cells in metaphase, the mitotic spindle failed to align with the Pins crescent in dsh1, mud double mutant pl cells in metaphase. We conclude that Mud functions downstream of Fz signaling to regulate mitotic spindle orientation and that, in absence of Fz activity, Mud is needed downstream of Pins to orient the mitotic spindle in the pl cell.

To determine whether zebrafish Dishevelled (Dvl) could orient the mitotic spindle using a pathway analogous to Drosophila Dsh, we first tested if the zebrafish Dvl3 DEP domain has spindle orientation activity in the S2 induced polarity assay. Strikingly, Ed::Dvl3(DEP) domain orients the mitotic spindle and this spindle orientation activity requires Mud function (Figures 6A–6C). This indicates that zebrafish Dishevelled acts via its DEP domain to activate the Mud-Dynein spindle orientation pathway in S2 cells. We next sought to determine whether Dvl3 could associate with zebrafish NuMA and whether they might colocalize in zebrafish epiblast cells. We identified the zebrafish NuMA gene, which is encoded by a 7.1 kb mRNA. Zebrafish NuMA, like Dvl3, is ubiquitously expressed in the epiblast during gastrulation (Thisse and Thisse, 2004) (Figures S2A–S2F). We determined that a C-terminal fragment of zebrafish NuMA from amino acid 1508 to 2382 (NuMAC) specifically immunoprecipitates Dvl3::Myc (Figure 6D) from lysate of HEK293 cells. In interphase and dividing epiblast cells Dvl3::GFP was mostly localized in the cytoplasm. In interphase, HA::NuMAC was localized in the nucleus and did not colocalize with Dvl3::GFP (Figures 6E–6E”; Figures 6F–6F”). During cell division, HA::NuMAC was mostly cytoplasmic (Figures 6E–6E”). We could nevertheless observe localization of HA::NuMAC at the cell cortex in ~50% of the cells (Figures 6E–6E”). In division Dvl3::GFP was mostly cytoplasmic (Wallingford et al., 2000) (Figure 6E), preventing assessment of its colocalization with HA::NuMAC (Figure 6E”). Strikingly, coinjection of Fz7 induced the recruitment of Dvl3::GFP to the cell cortex as well as a translocation of HA::NuMAC from the cytoplasm to the cell cortex, where it was colocalized with Dvl3::GFP (Figures 6F–6F”). In this context, Dvl3::GFP and HA::NuMAC were also found to colocalize in cytoplasmic structures such as the mitotic spindle (Figure 6F”) We also analyzed the colocalization between HA::NuMAC and Dvl2::GFP, finding that HA::NuMAC colocalized with Dvl2::GFP at the cell cortex and on the mitotic spindle, even without Fz7 injection (Figures 6G–6G”).

To test whether NuMA might function downstream of the PCP pathway in vivo, we then compared the effects of Dvl Morpholino (MO) and NuMA MO injection on the orientation of the mitotic spindle along the animal-vegetal axis during zebrafish gastrulation. To measure the orientation of cell divisions in the epiblast, the cell membranes were labeled with a GFP fused to a Ras-phylation domain (hereafter membrane::GFP) and cell division orientation was determined by measuring the angle between the long axis of late anaphase cells and the animal-vegetal axis (Figure 6H). We confirm previous observations (Gong et al., 2004) that cell divisions are preferentially oriented along the animal-vegetal axis in wild-type embryos (Figure 6K). In contrast, injection of a mRNA coding for a dominant-negative form of Xenopus Dishevelled (Xdd1) led to the misorientation of the mitotic spindle relative to animal-vegetal axis in embryos (P(Xdd1) = 0.003) (Figure 6K). The function of Dvl in the regulation of mitotic spindle orientation along the animal-vegetal axis was further analyzed by the coinjection of MO against the three identified zebrafish Dishevelled, Dvl2, Dvl3, and Dvl2-like. Triple Dvl MO randomized the orientation along the animal-vegetal axis (P(0.001) (Figures 6L, 6K, and 6M). In vertebrates, NuMA has essential functions in nuclear organization, mitotic spindle formation and mitotic exit (Radulescu and Cleveland, 2010). We therefore induced a partial loss of function of NuMA by injection of 0.6 pmol of NuMA MO allowing the study of the role of NuMA during gastrulation. At this concentration, NuMA MO does not lead to any defect in mitotic spindle morphology (Figures S2I–S2J”) or to any gross morphological defects during embryonic development. In particular embryo elongation was normal suggesting that NuMA loss of function does not induce obvious convergence-extension defects (not shown). On injection of 0.6 pmol of NuMA MO, the mitotic spindle was significantly less oriented along the animal-vegetal axis, whereas the mitotic spindle orientation was normal in embryos injected with a control 5-mismatch MO (9 embryos for a total of 339 divisions analyzed, P(MO/ NuMA MO) = 0.0001) (Figures 6J and 6L). The specificity of the defects in spindle orientation induced by NuMA MO was further established by showing that a second independent MO, targeting a distinct sequence of NuMA, produces the same phenotype (Figure 6L). Thus, triple Dvl MO and the NuMA MO both disrupt mitotic spindle orientation along the animal-vegetal axis, although the NuMA MO phenotype is slightly less severe. This less severe phenotype is likely due to the partial loss of NuMA function induced by MO, however, we cannot rule out that other regulators of mitotic spindle orientation function downstream of Fz PCP in zebrafish (Figure 6M).
Figure 3. The Localization of Mud Is Dependent on Pins and Dsh

(A–B') Localization of Dsh (red in A, A', B, and B') and Armadillo (blue in A', A', B', and B') at the apical cortex of wild-type (A–A') and mud mutant (B–B') interphase cells; pI cells identified by the accumulation of Senseless (Ss) (Nolo et al., 2000). In epithelial cells of the dorsal thorax, Dsh is planar polarized and accumulates weakly at the posterior apical cortex of all epithelial cells and strongly at the apical posterior cortex of the pI cells. Therefore, Dsh appears strongly enriched at the posterior cortex of the pI cell and weakly enriched at the posterior cortex of the epithelial cells located anterior to the pI cells.

(C–D') Apical localization of Mud (green in C, C', D, and D'), Dsh (red in C' and C'), Armadillo (Arm, blue in D' and D'), Senseless (Ss, blue in C', D', and D') and Pins (red in D' and D') in wild-type (C–C') and dsh^1 mutant (D–D') in pl cells in interphase. Note that, Mud does not accumulate with Dsh at the anterior apical cortex of the epithelial cells located anterior to the pl cells suggesting that the colocalization between Dsh and Mud is specific to the pI cells, in agreement with the notion that the Fz-Dsh pathway regulates mitotic spindle orientation in the pl cells and not in the epithelial cells (Gho and Schweisguth, 1998).

(E–F') Localization of Mud (green in E, E', and F–F'), Dsh (red in E' and E'), Armadillo (Arm, blue in E', F, and F'), Senseless (Ss, blue in E', F, and F') and Pins (red in F' and F') in wild-type (E–E') and dsh^1 mutant (F–F') in pl cells in prophase. Apical confocal sections are shown in (E–F'). Basal confocal section is shown in (F'). The bracket in (F') indicates the accumulation of Pins and Mud at the basal-lateral cortex of the dsh^1 mutant in prophase.

(G–G') Localization of Dsh (red in G and G'), Armadillo (Arm, blue in G' and G'), Senseless (Ss, blue in G' and G') and Pins (green in G') in wild-type pl cell in metaphase. Apical confocal sections are shown in (G) and (G') and a basal confocal section is shown in (G').

(H–H') Apical localization of Mud (green in H and H'), Dsh (red in H' and H'), Armadillo (Arm, blue in H'), Senseless (Ss, blue in H') in pins pl cell in prophase. The red arrowhead indicates the apical centrosome in vicinity of the apical accumulation of both Mud and Dsh.
Altogether, we conclude that NuMA is required downstream of the Fz-PCP pathway to orient symmetric cell division in zebrafish.

**Conclusion**

Oriented cell division has a fundamental role in cell-fate specification, tissue morphogenesis, and homeostasis. The orientation of cell division has been extensively characterized downstream of the Pins/LGN/AGS3/Lin-5 protein family that regulates spindle orientation in response to cell intrinsic cues during asymmetric cell division in *Drosophila* and *C. elegans*. In contrast the mechanisms regulating mitotic spindle relative to extrinsic polarity cues associated with cell-cell contact or epithelial planar cell polarity are poorly understood. Here we have deciphered the mechanisms of mitotic spindle orientation in response to the PCP signaling. Using the recently developed “induced polarity” S2 cell system, we show that the Dsh DEP domain is sufficient for spindle orientation, and that it requires Mud and Dynein complex function. We show that the Dsh DEP domain can immunoprecipitate the Mud C-terminal domain, and that the Dsh DEP domain is required to recruit Mud to the apical posterior cortex of pI cells. We propose that *Drosophila* pI cells use a Fz-Dsh-Mud-Dynein pathway for anterior-posterior spindle orientation; this pathway operates in parallel to the previously identified Pins-Mud-Dynein pathway regulating apical-basal spindle orientation. Finally, we document the functional relevance and conservation of this pathway in vertebrates. Our work generalizes the role of NuMA as regulator of mitotic spindle orientation in response to intrinsic and extrinsic polarity cues by establishing a molecular and functional characterization of a Fz-Dsh-NuMA pathway orienting cell division both in *Drosophila* and zebrafish.

**EXPERIMENTAL PROCEDURES**

**S2 Cell Experiments**

S2 cells were grown and cultured at room temperature in Schneider's Insect Media (Sigma) supplemented with 10% fetal bovine serum. The “induced cell polarity” assay was carried out as previously described (Johnston et al., 2009). Briefly, ~1 x 10⁶ cells were transiently transfected with Ed fusion constructs (~400 ng) using Effectene (QIAGEN) reagent according to manufacturer protocol. After 24–48 hr transfection, cells were induced with CuSO₄ (500 μM) for 24 hr to allow for Ed fusion protein expression. Cells were harvested and resuspended in fresh media and allowed to shake (175 RPM) for 2–3 hr to induce Ed-mediated cell-cell clusters. Clustered cells were plated on glass coverslips and allowed to incubate for 3 hr to allow for optimal mitotic index. Cells were fixed (4% PFA in PBS for 15 min) and stained using standard techniques (Johnston et al., 2009) before imaging.

**Immunoprecipitation**

HEK293T cells were grown in DMEM supplemented with 10% fetal calf serum. Immunoprecipitation were carried out as previously described in Langevin et al. (2005b). Briefly, HEK293T were transfected with Drosophila Dsh or Mud fusion constructs or with zebrafish Dvl3 or NuMA fusion constructs. Cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 5 mM β-glycerophosphate, 1% Triton X-100 supplemented with Complete (Roche) protease inhibitor cocktail. Mud::GFP fusion protein were immunoprecipitated with mouse Anti-GFP (Roche) and magnetic GA-Sepharose agarose beads (Ademtech). After three washes in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, immunoprecipitates were analyzed by western blot using rabbit anti-Myc antibody (Santa Cruz).
Figure 4. Mud Controls Mitotic Spindle Orientation Downstream of Fz PCP

(A) Schematic of the localization and function of the Fz-Dsh and the Pins for the regulation of the mitotic spindle orientation in the pI cells according to David et al. (2005). pI cells are in white, surrounding epithelial cells are in gray, mitotic spindle is in green. In the pl cell, Fz and Dsh (dark blue) are accumulated at the apical posterior cortex, Pins (orange) is located at the anterior lateral cortex. At the apical posterior pole of the cell, Fz and Dsh control the anterior-posterior orientation of the mitotic spindle. As Fz and Dsh are localized at the apical cortex of the pl cell, they pull the posterior centrosome of the spindle inducing a tilt of the spindle along the apical-basal axis. At the anterior lateral pole of the pl cell, Pins and the HGP pathway, composed of Ric-8, Gαi, and Gγ1, act in opposition to Fz and Dsh to maintain the spindle in the plane of the epithelium. In fz mutant cells, the spindle is randomly oriented along the anterior-posterior axis and more planar than in wild-type cells. In pins mutant cells, there is no defect along the anterior-posterior axis but the spindle is more tilted than in wild-type cells. Because the activity of Pins and the HGP pathway are only needed to counterbalance the Fz PCP pathway, the fz, pins double mutant and the fz mutant pl cells have a similar phenotype. Schematic of the $\alpha_{AP}$ and $\alpha_{AB}$ angles measured as described in David et al. (2005).

(B–G) Localization of the polarity markers: Pins (green in B–C'), Bazooka (Baz) (green in D–E'), and Numb (Nb) (green in F–G') in wild-type (B and B') n = 26 for Pins localization; (D and D') n = 32 for Baz localization; (F and F') n = 32 for Numb localization; mud mutant (C and C') n = 30 for Pins localization; (E and E') n = 46 for Baz localization; (G and G') n = 46 for Numb localization; pl cells in metaphase. pl cells are labeled by Senseless (blue, B–G).

(H–I) Localization of Pon::GFP (green) in wild-type (H, n = 17) and mud mutant (I, n = 15) pl cells in metaphase. pl cells are labeled by the expression of Histone2B::mRFP (His::mRFP, red in H and I). Pon::GFP and His::mRFP were expressed under the control of the neuralized-Gal4 driver.
morpholino induces a developmental arrest. Only embryos showing a normal development were analyzed. 1.2 pmol of each Dvl2, Dvl3 (Angers et al., 2006), and Dvl2-Like MO (GGTATATGATTTTAGTCTCCGCCAT) were coinjected. The morpholinos were synthesized by Gene Tools (Philomath, OR). Zebrafish Fz7 mRNA, zebrafish Dvl2::GFP mRNA and zebrafish Dvl3::GFP mRNA were injected at 50 ng/μl. HA::NuMAC mRNA was injected at 40 ng/μl. Imaging was performed on Leica DMR 6000 or Nikon Ti spinning disk microscopes equipped with a HQ2 Ropper Camera.

Statistics
Student tests were used to compare the division angle distribution in zebrafish embryos. Chi-square tests were used to compare the cortical localization of Mud.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.devcel.2010.10.004.

ACKNOWLEDGMENTS
We thank W. Chia, F. Matsuzaki, A. Merdes, H. Nash, R. Scott, M. Semeriva, D.L. Shi, T. Uemura, C. Vesque, A. Wodarz, the Developmental Studies

Figure 5. Mud Loss of Function Is Associated with Pon::GFP Misegregation and Cell Fate Specification Defects
(A and B) Time-lapse of Pon::GFP (green) and Histone2B::mRFP (His::mRFP, red) localizations in a wild-type pI cell (A) and a mud mutant pI cell (B). Pon::GFP fails to segregate exclusively in one daughter cell in 26.6% of the mud mutant cells (n = 15). Pon::GFP and His::mRFP were expressed under the control of the neuralized-Gal4 driver. Time in min:s.

(C) The pI cell lineage. The pI cell divides asymmetrically to produce a pilla cell and a pilib cell. The pilla generates the external socket and shaft cells. The pilib gives rise to the neuron and the sheath as well as a cell that undergoes apoptosis. Numb, Pon, and Neutralized are inherited by the pilib cell, the apoptotic cell, the neuron and the shaft cell.

(D) Schematic representation of the adult sensory organ. The wild-type sensory organ is composed of four cells: the external socket and shaft cells; the internal neuron and sheath cell. Each sensory cell expresses the Cut nuclear marker (blue). The socket cell expresses the Su(H) marker (green) and the neuron expresses the HRP marker (red).

(E and F) Sensory organs in wild-type (E–E0) and mud mutant (F–F0) pupae at 24 hr APF stained for Cut (blue in E, E', F, and F'), Su(H) (green in E, E', F, and F') and HRP (red in E and F). All panels are z maximal projections. The wild-type organ (E) is composed of four different cells. The external subepithelial socket and shaft cells are identified based on their large subepithelial polyploid nucleus stained by Cut (dashed outline in E). The socket cell is identified by the expression of the Su(H) marker (dashed outline in E and E0). The sheath cell and the neuron are identified by their basal position and their smaller nucleus (solid outline in E0). The Cut positive sheath cell is located next to the neuron identified by its strong HRP staining (red). A mud mutant organ (F) is composed of four large Cut positive nuclei (dashed outlines in F), none of which are strongly HRP positive (F); two socket cells identified by Su(H) staining (green, dashed outlines in F and F') and two subepithelial Cut positive cells identified as a shaft cell based on their large subepithelial polyploid nuclei. The percentage of cell fate transformation is 1.6% in mud mutant pupae (n = 180 organs).

Scale bars represent 2 μm; anterior is left.

morpholino induces a developmental arrest. Only embryos showing a normal development were analyzed. 1.2 pmol of each Dvl2, Dvl3 (Angers et al., 2006), and Dvl2-Like MO (GGTATATGATTTTAGTCTCCGCCAT) were coinjected. The morpholinos were synthesized by Gene Tools (Philomath, OR). Zebrafish Fz7 mRNA, zebrafish Dvl2::GFP mRNA and zebrafish Dvl3::GFP mRNA were injected at 50 ng/μl. HA::NuMAC mRNA was injected at 40 ng/μl. Imaging was performed on Leica DMR 6000 or Nikon Ti spinning disk microscopes equipped with a HQ2 Ropper Camera.

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Figure 6. NuMA Controls Mitotic Spindle Orientation Downstream of the Fz PCP Pathway during Zebrafish Gastrulation

(A and B) Dvl3(DEP), C-terminal region of Dvl3 containing the DEP domain was fused in frame to Echinoid tagged with GFP (green) and transfected into S2 cells. Cells were stained for α-tubulin (red). Ed::Dvl3(DEP) (A); Ed::Dvl3(DEP) + mud RNAi corresponds to Ed::GFP fused to Dvl3(DEP) and RNAi against mud (B). Scale bar in (A) represents 2 μm.

(C) Cumulative plot of angles measured in the S2 cell “induced polarity” assays in Ed::GFP (black line), Ed::Dvl3(DEP) (red line), and Ed::Dsh(DEP) and mud RNAi (yellow line).

(D) Anti-Myc antibody western blot of GFP::NuMAC immunoprecipitates from extracts of HEK293T cells expressing full-length Dvl3::Myc. Arrowhead indicates Dvl3::Myc.

(E–G) Localization of Dvl and NuMAC in epiblast cells during gastrulation. Localization of Dvl3::GFP (green in E and E’) and HA::NuMAC (red in E’ and E”) in interphasic and dividing epiblast cells without Fz7 coinjection. Localization of Dvl3::GFP (green in F and F’) and HA::NuMAC (red in F’ and F”) in interphasic and dividing epiblast cells with Fz7 coinjection. Cortical enrichment of HA::NuMAC was observed in 7 of 14 epiblast cells in division in absence of Fz7, whereas most of the dividing epiblast cells (12 of 14) have a strong cortical localization and cytoplasmic depletion of HA::NuMAC when Dvl3::GFP was strongly enriched at the cortex on Fz7 coinjection. Scale bar in (E) represents 10 μm.

(H–J) Confocal time-lapse images of embryos labeled with membrane-GFP and injected with the control morpholino (H) or the triple Dvl MO (I) or the NuMA ATG morpholino (J). Observations are done on the dorsal side, from shield stage to 80% epiboly, and limited to the neuro-ectoderm. Scale bars in (H) represent 10 μm; animal pole is to the top. Time is in minutes.

(K–M) Cumulative plot of αm in embryos injected with the Dvl control morpholino (n = 311 divisions in four embryos), or the Xdd1 construct (n = 66, in one embryo, already shown by Gong et al. [2004]) or the three Dvl morpholinos (n = 183, in four embryos) (K); Cumulative plot of αm in embryos injected with the NuMA control morpholino (n = 304 divisions in five embryos), the NuMA “ATG” morpholino (n = 339 divisions in nine embryos), the NuMA “ATG” morpholino (n = 118 divisions in three embryos) (L); Cumulative plot of αm in embryos injected with the NuMA control morpholino (n = 311 divisions in four embryos), the Dvl control morpholino (n = 183 divisions in four embryos), the NuMA “ATG” morpholino (n = 339 divisions in nine embryos) and the NuMA “ATG” morpholino (n = 118 divisions in three embryos) pooled together or triple Dvl morpholino (n = 183, in four embryos) (M). *p < 0.001 relative to control experiments.
Hybridoma Bank, and the Bloomington and Exelexis Stock Centers for strains and antibodies. We also thank the members of the Lhomm and of the Developmental Biology Curie Imaging (PICT-IBISA@BDD, UMR3215/U934) facilities for their help and advice with confocal microscopy. We thank P.-L. Bardet, J.-R. Hyuhn, A.-M. Lennon, A. Leibfried, J. Matthieu and A. Molla-Herman for critical comments on the manuscript. Y.B. thanks A. Molla-Herman for help with the figures. This work was supported by grants to Y.B. from the Association pour la Recherche sur le Cancer (ARC 4830), the ANR (BLAN07-3-207540), the CNRS, INSERM, ERC Starting Grant (CeoPoDo 208718), and the Curie Institute. M.S. was supported by a predoctoral fellowship from ARC and from French Research Ministry. This work is also supported by the FRM grant 26329 and the INCA grant 4731. C.A.J. is supported by a Damon Runyon postdoctoral fellowship; K.E.P. by the National Institutes of Health (NIH GM068032), and C.Q.D. by the Howard Hughes Medical Institute.

Received: April 19, 2010
Revised: August 27, 2010
Accepted: October 2, 2010
Published: November 15, 2010

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