Mirror-symmetric microtubule assembly and cell interactions drive lumen formation in the zebrafish neural rod

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By analysing the cellular and subcellular events that occur in the centre of the developing zebrafish neural rod, we have uncovered a novel mechanism of cell polarisation during lumen formation. Cells from each side of the neural rod interdigitate across the tissue midline. This is necessary for localisation of apical junctional proteins to the region where cells intersect the tissue midline. Cells assemble a mirror-symmetric microtubule cytoskeleton around the tissue midline, which is necessary for the trafficking of proteins required for normal lumen formation, such as partitioning defective 3 and Rab11a to this point. This occurs in advance and is independent of the midline cell division that has been shown to have a powerful role in lumen organisation. To our knowledge, this is the first example of the initiation of apical polarisation part way along the length of a cell, rather than at a cell extremity. Although the midline division is not necessary for apical polarisation, it confers a morphogenetic advantage by efficiently eliminating cellular processes that would otherwise bridge the developing lumen.

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Introduction

Generation of epithelial tubes is a common requirement in many embryonic organs. These tubular structures can be different in complexity and function, but they all contain a single central lumen lined by apical membrane connected by cell–cell junctions. The position of the central lumen is mediated by the proper polarisation of the surrounding epithelial cells, which is crucial to lumen function. There

are a myriad of human disorders characterised by defects in epithelial cell polarity, such as polycystic kidney disease, cystic fibrosis and cancer (Wodarz and Nathke, 2007; Mellman and Nelson, 2008; Wilson, 2011). Understanding the cellular and molecular regulation of cell polarisation and lumen formation, and how this is coordinated with whole-tissue morphogenesis is therefore key, not only to further our understanding of normal development but also to determine what goes wrong in these diseases as well as to begin the possibility for engineering epithelial tubes outside the body with a view to tissue replacement and repair.

Although several of the molecular components required for lumen formation have been identified using cells lines in three-dimensional cultures (Desclozeaux *et al*, 2008; Jaffe *et al*, 2008; Rodriguez-Fraticelli *et al*, 2010), these systems lack the environmental and morphogenetic complexity of the *in vivo* situation. We study lumen formation in the context of whole-tissue morphogenesis using *in vivo* live imaging during neurulation in the transparent zebrafish embryo. During this process, neural progenitor (NP) cells first form a solid rod primordium in which cells from the left and right sides transiently interdigitate across the tissue midline (Hong *et al*, 2010). Cells then establish apical polarity at the tissue midline and subsequently the tissue cavitates to open a lumen at the tissue centre (Kunz, 2004; Lowery and Sive, 2004; Clarke, 2009). We and others previously identified a novel and dominant influence of oriented cell divisions in establishing the position and organisation of the developing lumen (Ciruna *et al*, 2006; Tawk *et al*, 2007; Quesada-Hernandez *et al*, 2010; Zigman *et al*, 2011). These C-divisions (for midline crossing divisions) occur close to the organ centre and generate mirror-symmetric daughters on either side of the nascent lumen. During the C-division, a GFP fusion for the polarity protein partitioning defective 3 (Pard3–GFP) is localised to the cleavage furrow between daughters. This results in the mirror-symmetric distribution of this protein to the region where daughters remain in contact at the midline (Tawk *et al*, 2007). This observation suggested that the division itself could be responsible for localising Pard3–GFP and related polarity proteins to the tissue midline. However, several papers have also shown that neural rods in which the midline division is inhibited can still polarise at the midline (Ciruna *et al*, 2006; Tawk *et al*, 2007; Quesada-Hernandez *et al*, 2010; Zigman *et al*, 2011). Thus, other factors must contribute to the establishment of midline polarity and the morphogenetic role of the C-division remains unclear.

Here, we uncover a division-independent mechanism that organises cell polarisation at the tissue midline. Apical polarity is established at the point where cells intersect the midline and depends on a mirror-symmetric microtubule cytoskeleton and cell–cell interactions across the midline. We also show that although the C-division is dispensable

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for midline polarisation, it confers a morphogenetic advantage to the cell remodelling required for lumen formation over non-dividing cells.

Results

Apical polarisation of cells at the tissue midline begins prior to the C-division

We analysed the C-division and the initiation of Pard3-GFP localisation at higher spatial and temporal resolution than previously (Tawk *et al*, 2007). Most cells interdigitate across the midline prior to the C-division and we find that small puncta of Pard3-GFP first appear broadly localised to the region where cells overlap at the midline (Figure 1A) in advance of the C-division. This suggests that cells recognise the tissue midline prior to division.

The broad localisation of Pard3-GFP puncta around the midline is maintained through metaphase and early telophase

as cells undergo mitosis. However, cells do not all lie precisely at the midline during cytokinesis (Figure 1B) and this results in some variability in Pard3-GFP distribution during cleavage. Cells dividing exactly at the tissue centre localise Pard3-GFP across the middle of the dividing cell and it accumulates in the cleavage furrow from early stages of telophase (Figure 1C). However, cells whose metaphase plate is lateral to the midline have an asymmetric location of Pard3-GFP towards their medial side that does not accumulate evenly across the cleavage furrow (Figure 1D). Despite this, even in cells in which Pard3-GFP is initially asymmetrically localised, Pard3-GFP always accumulates on either side of the cleavage plane at later stages of division, as previously reported (Figure 1A, Supplementary Movie S1) (Tawk *et al*, 2007). These results show that Pard3-GFP localisation is initiated prior to the C-division and its sub-cellular distribution through cytokinesis is related to cell position relative to the midline.

Figure 1 Apical polarisation of cells at the tissue midline begins prior to the C-division. Dotted lines: midlines. Dashed lines: basal edges. (A) Time-lapse sequence showing a neural rod cell prior to, during and following C-division. Prior to division, the cell extends across the tissue centre and Pard3-GFP puncta broadly localise around the region where the cell intersects this point. Pard3-GFP puncta are biased to the medial side of the cell at metaphase but subsequently are found at the cleavage plane between daughters (17/17 cells from six embryos) and later more precisely to the nascent apical surface (arrow). The first and last images are duplicated with the bright field shown in grey. The Pard3-GFP channel is shown separately and enlarged to the right. See also Supplementary Movie S1. (B) Dot plot showing distribution of midline crossing divisions from three embryos relative to their tissue midline (zero on y axis). Over time, the location of divisions near the midline becomes more precise. (C) Pard3-GFP puncta localise to the cleavage furrow in cells dividing very close to the midline (11/12 cells from six embryos). (D) Pard3-GFP puncta are biased to the medial side of cells that divide lateral to the midline (18/20 cells from six embryos). Pard3-GFP then progressively localises to the cleavage plane between the two daughter cells.

Apical polarisation of cells at the tissue midline is independent of cell division

To better understand the division-independent mechanisms of cell polarisation, we blocked C-divisions between 9 and 22 h post fertilisation (h.p.f.) (Supplementary Figure S1). We found that most cells in the division-blocked neural rod extended across the midline and 43% cells spanned the whole width of the neural rod from the left to right hand side (Figure 2A). Blocking cell division thus exaggerated the extent of cell interdigitation across the tissue midline and allowed us to observe the generation of polarity within cells more easily than in wild-type embryos. Remarkably, Pard3-GFP puncta were first localised around the point where the cells intersected the tissue midline, irrespective of whether cells spanned the whole width of the rod, or only part of it (Figure 2B, C, D, Supplementary Movie S2). Immunostaining revealed that the apical tight junction marker zonula occludens 1 (ZO1) was also localised to the region where cells intersected the tissue midline (Figure 2E). Division-blocked cells that spanned the midline initially showed no morphological specialisations at the midline. However, we later observed a dynamic and complex rearrangement of microtubules between the main cell body and the contralateral process (Figure 2F) as they built the apical end-foot that will

form part of the lumen surface. In addition, in many cells the contralateral process narrows and retracts back towards the neural midline (Figure 2F).

These results confirm that localisation of Pard3-GFP protein at the organ midline is independent of cell division and occurs despite cell extension across the midline. Furthermore, cell morphology can be remodelled at the midline to form an apical surface in a division-independent manner.

A mirror-symmetric microtubule cytoskeleton is organised around the tissue midline

Apical protein localisation and cytoskeletal rearrangement around the point where division-blocked cells intersect the midline rather than at the cell's extremity suggests that the machinery of cell polarity may be organised around this point. Since centrosomes are characteristically found at the apical pole of neuroepithelial cells (Taverna and Huttner, 2010) and it has previously been suggested that centrosomes gradually locate to the rod midline during the transition from keel to rod (Hong *et al*, 2010), we have used live imaging to determine whether this centrosomal location is dependent on cell division. We analysed wild-type cells before division, while they still interdigitate across the centre of the tissue. We find that, prior to division, centrosomes gradually locate to

Figure 2 Apical polarisation of cells at the tissue midline is independent of cell division. Dotted lines: midlines. (A) Division-blocked cells in *emi1*MO embryos frequently stretch completely across the width of the neural rod (36/84 from 5 *emi1*MO embryos, compared to 18/112 cells before C-division in six control embryos). Arrows indicate left and right sides of rod. (B–D) Time-lapse sequences of Pard3-GFP puncta localising close to the region where division-blocked cells intersect the tissue midline. Pard3-GFP localises to the tissue midline irrespective of the length of the cells' contralateral process (44/44 cells from seven embryos: B=16 cells, C=10 cells and D=18 cells). See also Supplementary Movie S2. (E) Single Z-section demonstrates that puncta of ZO1 protein also appear at the region where division-blocked cells intersect the midline. The mGFP channel is shown separately with the outlines of individual example cells highlighted. (F) Time-lapse sequence of a division-blocked cell labelled with DCX-GFP. 23/23 cells from five embryos that extended beyond the tissue midline underwent microtubule reorganisation (arrow) close to the point at which the cell intersects the midline. Seven of these cells consequently retracted their contralateral microtubule bundles. See also Supplementary Figure S1.

whichever part of the cell that lies over the tissue midline, despite the main body of the cell often remaining laterally located (Figure 3A). This centrosomal location corresponds broadly with the location of Pard3-GFP puncta seen in cell processes that interdigitate across the midline prior to C-division (Figure 1). Before the cells enter mitosis the nucleus also moves towards the midline, the duplicated centrosomes separate and the spindle for division is assembled close to the midline (Figure 3A). This suggests that the localisation of centrosomes to the midline is independent of cell division and may be instrumental in specifying the correct location for the C-division. To confirm that centrosome location is independent of cell division, we analysed this in division-blocked cells and also found that centrosomes were located very close to where these cells intersected the midline, rather than at the cells' extremity (Figure 3B).

The location of centrosomes part way along the cell suggested that microtubule polarity might be organised around this point. To test this, we analysed expression of End-binding protein 3-GFP (EB3-GFP), a marker of the plus-ends of growing microtubules (Stepanova *et al*, 2003) in division-blocked cells. EB3-GFP microtubule tips were found to grow mirror-symmetrically away from the microtubule organising centre (MTOC), close to where each cell intersected the tissue midline (Figure 3Ci and ii and Supplementary Movie S3). Microtubule polarity is thus reversed around the point at which division-blocked cells intersect the midline. We also observed the same mirror-reversal of microtubule polarity in wild-type cells that protrude across the neural rod midline before C-division (Figure 3Di and ii, Supplementary Movie S4). The spindle for division is then assembled close to the midline and, following C-division, the MTOCs are gradually repositioned towards the midline, where the daughters remain attached to each other (Tawk *et al*, 2007) (Figure 3Diii).

These results demonstrate that mirror-symmetric microtubule polarity within individual cells is organised around the neural rod midline prior to and independent of C-division, and suggest that microtubule-dependent processes could underlie the delivery of proteins required for lumen formation to the apical midline.

Pard3 fusion proteins are mislocalised following nocodazole treatment

In order to test whether apical polarisation at the neural rod midline is dependent on microtubule-mediated transport, we depolymerised microtubules in division-blocked embryos using a 1.5 h nocodazole treatment from the 6-somite stage. In untreated embryos, Pard3 fusion protein begins to accumulate at the neural midline in some cells in the early neural keel and is localised to the middle of the rod in all cells by 17–19-somite stages (Figure 2B–D). While Pard3 fusion protein was present at the neural rod midline in many cells before and shortly after nocodazole treatment, by the 10-somite stage 88% cells in treated embryos had localised some (Figure 4Bi) or all (Figure 4Bii) Pard3 fusion proteins ectopically to their basal side (Figure 4C). At this stage, microtubules had been extensively depolymerised (Figure 4A). However, cells were able to recover after nocodazole wash-out: Pard3-GFP puncta gradually moved from the basal to apical sides of cells, along microtubule-like structures (Figure 4Dii arrows), and the Pard3-GFP domain was re-

established at the apical side of cells (Figure 4D). A previous study (Hong *et al*, 2010) has suggested that Pard3 polarisation is independent of microtubules, but we suggest that their shorter nocodazole treatment may be insufficient to reveal the importance of microtubules. Our results demonstrate that apical localisation of Pard3 is dependent on microtubule-mediated transport, apparently with a plus to minus end directionality. This suggests that the intracellular reversal of microtubule polarity around the midline is a key step in establishing the correct localisation of apical proteins.

Pard3 and Rab11a are necessary for lumen formation

In order to test the importance of microtubule-mediated protein transport in lumen formation, we first expressed a mutant form of Pard3, Pard3-Δ6-EGFP, which lacks amino acids 688–1127, including the aPKC-binding domain. This results in binding to microtubules and a lack of specific apical localisation (von Trotha *et al*, 2006; Tawk *et al*, 2007). Embryos expressing Pard3-Δ6-EGFP had severely disrupted ventricle morphology in comparison to wild-types (Figure 5A and B), demonstrating that Pard3 function is necessary for normal lumen formation.

Next, we considered the small GTPase Rab Protein 11a (Rab11a). Previous work has suggested that Rab11a is required for apically directed traffic and lumen formation in MDCK cell cysts and *Drosophila* embryos (Lock and Stow, 2005; Desclozeaux *et al*, 2008; Roeth *et al*, 2009; Schluter *et al*, 2009; Bryant *et al*, 2010). Furthermore, apical traffic of Rab11a-positive endosomes *in vitro* is dependent on microtubules (Schluter *et al*, 2009; Xu *et al*, 2011) and it has been suggested that Rab11a endosomes are necessary to target Pard3 to the apical domain (Bryant *et al*, 2010). To determine whether Rab11a has a role in lumen formation in zebrafish, we expressed dominant-negative Rab11a (Rab11a S25N, abbreviated to Rab11aDN) specifically in all cells in rhombomeres 3 and 5 from approximately the 6-somite stage. This resulted in a complete loss of lumen formation in rhombomeres 3 and 5 (Figure 5C–H). Since Rab11 is known to be required for abscission (Skop *et al*, 2001; Fielding *et al*, 2005; Wilson *et al*, 2005; Yu *et al*, 2007; Pohl and Jentsch, 2008), the loss of lumen opening could result from a lack of abscission between sister cells at the midline. However, when we blocked cell division in these embryos, lumen opening remained absent in Rab11aDN rhombomeres but was present in adjacent rhombomeres (Figure 5I). Thus, lumen opening is independent of cell division and loss of Rab11a function must prevent lumen opening by inhibiting other processes.

To determine the role of Rab11a in the delivery of proteins to the apical domain and in junctional organisation, we analysed the expression of Pard3-GFP, ZO-1, aPKC and Crb2a in Rab11aDN rhombomeres and of Pard3-RFP in RAB11ADN-EGFP cells. Surprisingly, Pard3-RFP is localised normally to the apical end feet of Rab11aDN-EGFP cells (Figure 5J) and the localisation of all these proteins to the neural midline was initially normal in rhombomeres 3 and 5 at late rod stages. However, their distribution within the plane of the midline was slightly less homogenous than in controls and the level of Crb2a staining was reduced compared to controls (Figure 5F and G and Supplementary Figure S2). At later stages, when the lumen is just about to open, the relative protein expression levels of aPKC are further reduced and the distribution of proteins along the midline plane

Figure 3 A mirror-symmetric microtubule cytoskeleton is organised around the tissue midline. Dotted lines: midlines. Dashed lines: basal edges. **(A)** Time-lapse sequence of centrosomal and nuclear movement within two cells from a wild-type embryo. The centrosomes (small arrows) from both cells gradually move towards the tissue midline. The initially lateral nuclei then relocate medially to the centrosomes, the centrosomes duplicate (arrowheads) and division occurs close to the midline (e.g., see nucleus from the lower cell, marked with an asterisk). The duplicated sister cells then extend towards the basal sides of the neural rod, locating their cleavage planes more precisely at the tissue midline and the centrosomes locate just laterally to each side of the midline (arrowheads). Of 24 cells from five embryos that extended across or near the tissue midline, 19 cells located their centrosomes close to the tissue midline and divided at this location. The division location of the five cells that divided more laterally was still coincident with the location of their centrosomes. **(B)** A division-blocked cell labelled with CENTRIN-GFP. Six out of six cells that extended beyond the tissue midline from three embryos localised their centrosomes close to the point at which they intersected the midline. Two spots of CENTRIN-GFP represent the duplicated centrosomes resulting from the *emi1*MO-blocking M-phase entry. **(C, D)** EB3-GFP-labelled cells showing plus-end-directed growing microtubule comets. **(Ci)** A single z-plane of two division-blocked cells at a single time point. **(Cii)** A projection of 20 sequential time points from a single z-plane. Microtubule comets grew from MTOCs, located close to midline. Arrows mark the path taken by selected microtubule comets. Seven out of seven cells that extended across the middle of the tissue from one embryo had a mirror reversal of microtubule polarity close to the tissue centre (see also Supplementary Movie S3). **(Di)** A z-projection of one wild-type cell at a single time point prior to division. **(Dii)** A projection of 29 sequential time points from a stack of six z-planes. A similar location of MTOCs close to the midline and a reversal of microtubule polarity around the tissue midline were seen in control cells prior to division. Arrows mark the path taken by selected microtubule comets. Fourteen out of fourteen cells from six embryos that extended across the middle of the tissue had a mirror reversal of microtubule polarity close to the tissue centre (see also Supplementary Movie S4). **(Diii)** A time-lapse sequence of the same cell as it carries out the C-division. After division, the MTOCs are gradually repositioned towards the midline. Fourteen out of fourteen pairs of cells from seven embryos repositioned their MTOCs close to the midline.

Figure 4 Pard3 fusion proteins are mislocalised basally with nocodazole treatment. Dotted lines: midlines. Dashed lines: basal edges. (A) Dorsal view of DCX-GFP-labelled NP cells within the hindbrain of a division-blocked embryo treated with nocodazole from the 6-somite stage. After 10 min treatment, the microtubule cytoskeleton is still intact, with long DCX fibres present along the whole length of the cells. After 95 min, the microtubules are depolymerised, resulting in disorganised and fragmented DCX fibres. (Bi) Low-magnification dorsal view of right-hand side of neural rod. Some Pard3-RFP is present apically before treatment as well as 15 min after treatment with nocodazole. However, Pard3-RFP appears at the basal end of cells (dashed line to right) after 105 min of nocodazole treatment. (Bii) Pard3-GFP is initially located at the apical pole (arrow) of this individual cell before treatment as well as 15 min after treatment with nocodazole. However, Pard3-GFP appears at the basal pole (arrowhead) within 75 min of nocodazole treatment. (C) The percentage of cells expressing Pard3-FP only apically or not at all (blue) or at least partially basally (red) before and after nocodazole treatment. After nocodazole addition, 88% of cells contained some basal Pard3-FP, as opposed to 12% of cells in control embryos ($P < 0.0001$, Fisher's exact test). $n = 35$ cells from three treated embryos and 28 cells from three control embryos. (Di) Recovery of apical Pard3-GFP (arrow) from basal (arrowhead) following nocodazole washout. In this cell, Pard3-GFP puncta were seen to decorate and travel along filamentous structures (arrows). We monitored 16 out of 26 cells from four embryos that repositioned Pard3-GFP from a basal to apical position following nocodazole washout. The remaining cells either had an unclear morphology ($n = 3$), delaminated from the epithelium ($n = 4$) or died ($n = 3$).

becomes irregular and characterised by clumps of immunoreactivity separated by distinct stain-free zones (Supplementary Figure S2), reminiscent of the mislocalised crumbs localisation seen in the zebrafish hindbrain previously (Clark *et al*, 2011). By 31 h.p.f., apical proteins were increasingly disorganised and no longer confined to the midline in Rab11aDN rhombomeres (Figure 5L). Together, this data suggests that Rab11a is required for the maintenance of the coherent planar organisation of apical protein complexes at the tissue midline, and that this disruption to junctional organisation inhibits lumen opening.

RAB11A traffic progressively localises to the tissue midline and is mislocalised following nocodazole treatment

Having established the importance of Rab11a as a key player in lumen assembly, we investigated whether Rab11a traffic is also directed to the point where cells intersect the tissue midline. In wild-type cells, RAB11A-EGFP endosomes were initially broadly localised around the region where cells intersected the midline. As cells entered mitosis, their nuclei moved to the RAB11A-EGFP domain and, following division, the RAB11A-EGFP endosomes became redistributed around

the apical pole of each sister cell (Figure 6A, Supplementary Movie S5). We next analysed RAB11A-EGFP localisation in division-blocked cells. At early neural rod stages, RAB11A-EGFP vesicles were similarly broadly localised around the region where cells intersected the midline. The distribution of RAB11A-EGFP vesicles became increasingly restricted over

time until, at late neural rod stages, a precise focus of RAB11A-EGFP vesicles was formed near the midline of the neural rod (Figure 6B, Supplementary Movie S6). This restriction of RAB11A-EGFP vesicles coincided temporally and spatially with the rearrangement of microtubules where they intersected the midline (Figure 6B arrow and see Figure 2F).

Figure 6 RAB11A traffics to the point where cells intersect the tissue midline and is mislocalised following nocodazole treatment. Brightfield images are shown in grey. Dotted lines: midlines. Dashed lines: basal edges. **(A)** Time-lapse sequence of a control cell expressing RAB11A-EGFP and dividing across the tissue midline. RAB11A-EGFP puncta broadly accumulate within a 15 μ m region close the tissue midline before C-division ($n=15$ cells from four embryos, s.e. = 1.127 μ m). The nucleus then moves to this point and the cell divides across the midline. RAB11A-EGFP puncta then redistribute around the apical ends of the sister cells. See also Supplementary Movie S5. **(B)** Time-lapse sequence of a division-blocked cell expressing RAB11A-EGFP. RAB11A-EGFP puncta are initially broadly distributed within an 18 μ m region near the midline ($n=13$ cells from two embryos at 12 somites, s.e. = 2.18 μ m). Puncta then progressively accumulate more precisely to a 6- μ m region near where the cell intersects the midline ($n=9$ cells from two embryos at 17 somites, s.e. = 1.18 μ m). This accumulation coincided spatially and temporally with the appearance of the cell reorganisation near the nascent apical surface (arrow). See also Supplementary Movie S6. **(C)** Division-blocked embryos were treated with nocodazole from early neural keel stages. The EGFP channel is shown separately. Before nocodazole treatment, RAB11A-EGFP was broadly distributed around the tissue centre. RAB11A-EGFP was basally mislocalised following nocodazole treatment (arrowheads).

Figure 5 Pard3 and Rab11a are necessary for lumen formation. Dotted lines: midlines. Dashed lines: basal edges. **(A, B)** Three-dimensional reconstructions of dextran-filled brain ventricles from 28 h.p.f. wild-type **(A)** and Pard3- $\Delta 6$ -EGFP **(B)** embryos. Ventricule morphology is severely disrupted in Pard3- $\Delta 6$ -EGFP embryos. **(C–H)** A *Krox20-RFP-KalTA4* control embryo **(C–E)** and a *Krox20-RFP-KalTA4xUAS:mCherry-Rab11a-S25N* embryo **(F–H)** labelled with Pard3-GFP in horizontal orientation. A z-projection of each embryo is shown at the 17-somite stage **(C, F)**, with the GFP channel shown separately **(D, G)**. Pard3-GFP is able to localise to the apical midline in both embryos but intensity levels appear slightly lower in DNRab11a rhombomeres 3 and 5 **(F, G)**. A montage of images for each embryo is shown 8 h and 30 min later (26 h.p.f.), illustrating a single z-plane at the dorsal-most surface of the opening lumen **(E, H)**. While the lumen opens normally in control embryos **(E)**, opening does not occur in Rab11aDN rhombomeres 3 and 5 in dominant-negative embryos **(H)** ($n=15/15$ control embryos and 16/16 Rab11aDN embryos). **(I)** A projected stack of a division-blocked *Krox20-RFP-KalTA4xUAS:mCherry-Rab11a-S25N* 28-somite-stage embryo labelled with GFP-ZO1, H2B-RFP and CAAX-CHERRY in horizontal orientation. Disorganised lumen opening has started to occur in control rhombomeres 2, 4 and 6 but not in Rab11aDN rhombomeres 3 and 5. **(J)** Z-projection of mosaicically labelled RAB11ADN-EGFP cells in a 22-somite stage neural rod. Pard3-RFP is localised normally to the apical end feet of the cells (e.g., arrow). **(K–L)** A projected stack of a *Krox20-RFP-KalTA4* 31 h.p.f. control embryo **(K)** and a *Krox20-RFP-KalTA4xUAS:mCherry-Rab11a-S25N* 31 h.p.f. embryo **(L)** labelled with ZO1 and sytox in horizontal orientation. White lines indicate the approximate position of the basal surfaces. A smooth lumen fully opens in control embryos **(K)**, while in Rab11aDN embryos lumens lined by apical junctions were present in rhombomeres 2, 4 and 6 but no lumens were formed in rhombomeres 3 and 5 and junctional proteins were mislocalised (e.g., arrows) **(L)** ($n=7/7$ control embryos and 6/6 Rab11aDN embryos). See also Supplementary Figure S2.

In line with previous literature (Schluter *et al*, 2009), we found RAB11A trafficking to be microtubule dependent, since treatment with nocodazole resulted in the ectopic localisation of RAB11A-EGFP to the basal end of cells, supporting recent *in vitro* results (Xu *et al*, 2011) (Figure 6C). Together, these results demonstrate that RAB11A-positive endosomes carry cargo necessary for lumen formation and are targeted to the point at which the cells intersect the tissue midline by a microtubule-dependent mechanism. This process is independent of cell division.

Cells integrate anti-basal signals with cell-cell interactions to determine localisation of apical complexes

The assembly of apical complexes around the point where cells intersect the midline could be determined by a cell autonomous mechanism that somehow measures where in the cell these complexes should be positioned. Alternatively, interactions between cells that meet at the midline could determine the position of apical complex assembly. To test the latter hypothesis, we prevented interactions between cells from the left and right sides by physically dividing the neural plate along the midline using tungsten knives. This physical intervention slows the convergence of cells to the midline and often prevents them meeting their contralateral counterparts. In these circumstances, wild-type cells undergo ectopic C-divisions and generate ectopic lumens (Tawak *et al*, 2007). However, when division was blocked, cells assembled apical complexes at the most superficial surface of the neural tissue rather than at some point along the cell length (Figure 7A). This indicates that, in the absence of interactions with contralateral cells, neural cells have an underlying propensity to assemble apical complexes at their most anti-basal extremity.

If interactions with contralateral cells do organise the distribution of apical complexes, then markers of cell-cell junctions should coincide spatially with zones of left-right interdigitation in the neural keel and rod. To test this, we analysed nascent cadherin-based cell-cell interactions using Gt(Ctnna-citrine)ct3a transgenic embryos (Zigman *et al*, 2011), in which we blocked division. During neural keel stages, ctnna-citrine puncta were present along the cells' lateral membrane rather than confined to the cells' anti-basal extremity (Figure 7Bi). Ctnna-citrine puncta were accurately localised within the zone of cell interdigitation across the tissue midline (Figure 7Biv). This supports the view that a zone of left-right interactions defines the initial localisation of cell-cell junctions and can counteract the default anti-basal localisation of apical complexes.

We hypothesised that extracellular matrix (ECM) interactions with the basal ends of cells might be responsible for mediating the underlying anti-basal polarisation of NP cells since ECM has recently been implicated in specifying intercellular junctional position (Tseng *et al*, 2012) as well as centrosome position and lumen formation (Rodríguez-Fraticelli *et al*, 2012). To test this, we knocked down Laminin C1 using a morpholino (Parsons *et al*, 2002) to disrupt Laminin 1 incorporation into the basement membrane. In support of *in vitro* data (O'Brien *et al*, 2001; Yu *et al*, 2005; Myllymaki *et al*, 2011), this resulted in the ectopic basal accumulation of normally apically localised GFP-ZO1 in the hindbrain (Figure 7C). This suggests that ECM components are at least partially responsible for the underlying anti-basal localisation of apical complexes.

Lumen surface is disrupted without division

Our observations of apical polarisation within the middle of division-blocked cells demonstrate that cells can build apical

Figure 7 Cells integrate anti-basal signals with cell-cell interactions to determine localisation of apical complexes. **(Ai)** Cartoon depicting physical separation of the two halves of the neural plate to delay convergence. Blue line is the plane of orientation for **(Aii)**. **(Aii)** A single horizontal z-plane of the hindbrain of a division-blocked embryo at the 18-somite stage in which convergence has been delayed. Cells were labelled with mGFP and H2B-RFP and subsequently stained for ZO1 immunoreactivity. Where the left and right halves do not meet, ZO1 lines the superficial surface (arrowed) of the developing neuroepithelium. The superficial surface is seen *en face* on left-hand side (arrowhead). **(Aiii)** Reconstruction in the transverse plane of left-right separated tissue (approximately at level of dotted line in **Ai**), showing ZO1 at the superficial tip of neural cell. Arrow = midline. Quantification showed 33/33 cells from five embryos localised ZO1 strongly at their most anti-basal tip, situated at the superficial surface. **(B)** Horizontally orientated hindbrain of a division-blocked embryo at the 8-somite stage, labelled with Ctnna-citrine. **(Bii)** Cells on one side of the neural keel were mosaically labelled and the signal intensity increased to clearly show the cell outline. **(Biii)** Cell morphologies (red) were mirrored (yellow) to create a predicted zone of interdigitation, overlying a z-projection of Ctnna-citrine. **(Biv)** The zone of interdigitation is indicated by dashed lines and closely reflects the zone over which cells localise Ctnna puncta. Between 10 and 25 interdigitating cells were used to define the zone of interdigitation in each embryo ($n = 8$ embryos). **(C)** Horizontal 10- μ m z-projection of 15-somite stage embryo hindbrains injected with control or Laminin C1 morpholino and labelled with ZO1 and DAPI. Laminin C1 morphants had large areas of basally mislocalised ZO1, (arrowheads, $n = 15$), while control embryos never had basally located ZO1 ($n = 7$).

specialisations close to the centre of the neural rod independently of the C-division, therefore suggesting that the role of C-division is not to confer appropriate apico-basal polarity to neuroepithelial cells. Although not necessary to organise cell polarity in the neural rod, the C-division may none the less confer a morphological advantage to lumen formation. Therefore, we analysed the structure of lumens built with and without the C-division. While the nascent lumen in 18 h.p.f. control embryos was outlined by two continuous ZO1 domains at the hindbrain midline, in division-blocked embryos the domains of ZO1 immunoreactivity were interrupted by cell nuclei that remained straddling the midline (Figure 8A and B). This cell bridging phenotype was especially prevalent at rhombomere boundaries in the hindbrain. However, analysis at spinal cord levels also revealed discontinuous ZO1 domains, thus demonstrating that this phenotype is not unique to rhombomere boundaries. By 22 h.p.f., lumen opening was disorganised and the forming lumen surface was ragged in division-blocked embryos compared to wild types (Figure 8C). The lumen was still not fully inflated by 24 h.p.f. and ventricle opening was particularly restricted at rhombomere boundaries (Figure 8C, arrows), coinciding with the

highest prevalence of cell bridges seen at 18 h.p.f. Analysis of individual division-blocked cells demonstrated that in 58% of cells the contralateral process became very narrow and then retracted back to the midline by 16.5 h.p.f. (Figure 8D), while the remaining 42% of cells bridging the midline failed to retract their contralateral process by the end of our time-lapse analyses at ~19 h.p.f., and remained straddling the midline. By contrast, at this time in wild-type embryos, C-division has finished and we have not observed any remaining cell processes that span the midline. Thus, although cells can resolve interdigitation across the midline in the absence of the C-division, this process is less efficient. These results demonstrate that the C-division is not required for lumen formation but helps to remove nuclei and cell processes that would otherwise remain bridging the tissue midline and disrupt lumen opening.

Discussion

A novel mechanism of lumen formation at the tissue midline

In tissues that generate a lumen from a solid primordium, two important processes must occur. First, cells must assemble

Figure 8 Lumen surface is disrupted without division. (A) Maximum z-projections of control and division-blocked embryos showing the hindbrain at 18 h.p.f. and the spinal cord at 24 h.p.f. ZO1 immunoreactivity is shown separately in white and the otic vesicles are marked with asterisks. Control embryos had uninterrupted ZO1 along the midline. However, in division-blocked embryos the midline was interrupted by cell nuclei, resulting in gaps in ZO1 staining (arrows). This was found particularly prevalently but not exclusively at rhombomere boundaries. (B) Quantification of the number of gaps in ZO1 immunoreactivity at all dorsal-ventral levels in a 150 µm region of each embryo adjacent to the otic vesicle. A two-tailed unpaired *t*-test was used. There were significantly more gaps in *emil1*-MO embryos (3.1) than control-MO embryos (0.86). $P < 0.0001$. $n = 7$ for both groups. Data are represented as a mean $\pm$ s.e.m. (C) Maximum projections of dorsally oriented 22 and 24 h.p.f. control and division-blocked embryos showed that lumen opening was disrupted and the lumen surface was ragged in division-blocked embryos. Lumen opening was particularly restricted at rhombomere boundaries (e.g., arrows). (D) Time-lapse sequence of a division-blocked cell showing the retraction of the contralateral process back to the shoulder region where the cell intersects the midline. This occurred in 58% (21/36) cells from five embryos. Bright field is shown in grey. The tissue midline is indicated by a dotted line.

apical membrane and cell–cell junctions at the centre of the organ primordium. Second, cells that intersect the organ centre must be removed or remodelled to allow the lumen to open. We and others previously identified a dominant influence of oriented cell divisions in establishing the position and organisation of the developing lumen (Ciruna *et al*, 2006; Tawk *et al*, 2007; Quesada-Hernandez *et al*, 2010; Zigman *et al*, 2011). These C-divisions (for midline crossing divisions) occur close to the organ centre and generate mirror-symmetric daughters on either side of the nascent lumen. Now in order to reveal any division-independent mechanisms that underlie polarisation and lumen formation at the centre of the neural rod, we have inhibited the C-division. Our analyses reveal several novel cellular and subcellular events that underlie these processes *in vivo*. We show that although the C-division is normally co-ordinated spatially and temporally with apical polarisation, it is not required to assemble apical complexes at the neural midline (Figure 9Ai). Independently of division, cells in the neural rod are, surprisingly, able to assemble the apical and junctional machinery necessary for lumen formation at the point where they intersect the tissue midline. The ability of junctions to relocate along the length of epithelial cells has been demonstrated recently (Wang *et al*, 2012) but to our knowledge this is the first example of the assembly of epithelial junctions part way along the length of a cell, rather than at a cell extremity.

C-divisions and the onset of apical polarisation may be regulated by developmental time, since both occur ‘on time’ but lateral to the midline when the neural plate cells are delayed from reaching the midline. In this case, cells polarise and divide before they have interdigitated with their contralateral counterparts. This results in duplicate ectopic ‘midlines’ because the ectopic dominant C-divisions generate aligned mirror-symmetric pairs of cells to the left and right of the actual midline (Figure 9Aii and Tawk *et al*, 2007). However, in our current study, we have blocked the C-division at the same time as surgically delaying convergence of the neural plate. Cells therefore begin to polarise before they have interdigitated with their contralateral counterparts but are unable to divide and this results in the assembly of apical proteins at their anti-basal extremity (Figure 9Aiii). This underlying anti-basal drive is at least partially mediated by the ECM at the basal ends of NP

cells (Figure 9B). In addition, we show that the propensity to assemble apical complexes at the anti-basal extremity of cells is normally modified by left–right interactions between cells that interdigitate across the midline: when cell interdigitation is allowed to occur, apical complexes no longer assemble at the anti-basal extremity of cells; instead these complexes assemble within the zone of interdigitation around the tissue midline (Figure 9Ci).

We therefore propose that the novel process of cell polarisation at the point where cells intersect the tissue midline occurs via the following mechanism: we suggest that nascent adhesions form between interdigitating cells from each side of the neural rod and determine the coarse location of apical polarisation to this region within the cells (Figure 9Ci,ii). We suggest this coarse distribution of apical protein might then be responsible for localisation of centrosomes to the midline (see also Hong *et al*, 2010), which is likely to be upstream of the mirror-symmetric microtubule organisation that we demonstrate occurs around this point (Figure 9Cii). This then reinforces and refines the delivery of microtubule-dependent apical proteins, such as Pard3 and Rab11a (the function of which we show is necessary for lumen formation), to the midline and an organised planar apical epithelium is formed. This circulatory loop is not dissimilar to that proposed for the localisation of Par protein domains during cytokinesis of the one-cell stage *Caenorhabditis elegans* embryo (Schenk *et al*, 2010).

Once the apical epithelium has successfully formed, it is necessary to resolve cell interdigitation across the midline in order to allow efficient lumen opening. In normal embryos, this occurs via oriented cytokinesis across the midline (Tawk *et al*, 2007; Quesada-Hernandez *et al*, 2010; Zigman *et al*, 2011), when localisation of the cleavage plane to the tissue midline ensures that cells no longer bridge across the tissue centre (Figure 9Di). In the absence of cell division, we find that cell processes that bridge the tissue midline are retracted back to the point in the cells where they are assembling apical and junctional machinery. However, this process is a less efficient mechanism to clear cellular bridges and nuclei from the midline than the process of division (Figure 9Dii). Therefore, although we demonstrate that the specialised midline division is not necessary for apical domain formation, it does mediate the efficient reorganisation of cells at the midline, and is therefore necessary for organised lumen

Figure 9 Graphical model of results. **(A)** Summary of results for the role of interdigitation in polarisation. When division-blocked cells interdigitate normally (**Ai**), they localise apical proteins at the tissue midline at neural keel/rod stages. When convergence is delayed to prevent cells from interdigitating at the midline, cells polarise and divide ectopically on either side of the midline to generate ectopic duplicated apical planes on either side of the midline (**Aii**). However when convergence is delayed to prevent cells from interdigitating at the midline and cell divisions are also blocked, then cells assemble apical proteins at their most anti-basal extremity, coincident with the superficial surface (**Aiii**). This suggests that the underlying apical polarisation of cells is anti-basal and that interdigitation is required to specifically localise this anti-basal polarisation around the point where cells intersect the tissue midline. **(B)** Summary of results for the role of the ECM in polarisation. When a normal ECM is present (**Bi**), apical proteins localise precisely to the tissue midline at neural rod stages. However, when ECM structure is disrupted (**Bii**), apical proteins are mislocalised basally. This suggests that the underlying anti-basal polarisation of NP cells is at least partly mediated by the ECM. **(C)** Model for a polarisation feedback loop. When cells interdigitate at the tissue midline at keel stages, we suggest that nascent adhesions are formed between contralateral cells (**Ci**). These could then recruit apical polarity protein puncta to the broad region of the midline (**Cii**), which in turn could recruit centrosomes. Centrosomes could then organise a mirror-symmetric microtubule cytoskeleton, which would reinforce and refine the localisation of apical proteins to the midline (**Ciii**), allowing the formation of mature junctions. **(D)** Summary of results for the role of division and the microtubule cytoskeleton in polarisation. **(Di)** In wild-type embryos, cells undergo the C-division near the midline, efficiently redistributing sister cells on either side of the developing rod and localising apical proteins to the nascent lumen surface at their point of connection at the midline. This therefore allows normal lumen opening. **(Dii)** When C-division is blocked, cells localise apical proteins to the tissue midline and some cells retract ectopic cell processes to the midline. However, this process is not efficient and many cells remain straddling the tissue midline. This therefore interrupts normal lumen opening. **(Diii)** If microtubules are depolymerised in division-blocked cells using nocodazole, apical proteins localise ectopically at the basal surface. If microtubules are allowed to repolymerise by washing out nocodazole, apical proteins relocate to the apical surface and lumen opening occurs.

opening. This work therefore provides further evidence for the importance of regulating cytokinesis during morphogenesis (Grosshans and Wieschaus, 2000; Baena-Lopez *et al*, 2005; da Silva and Vincent, 2007; Woolner and Papalopulu, 2012).

Although the orientation of junction-mediated polarity differs in different cell types, cadherin- and catenin-based junctional formation has been shown to be important in mediating the intracellular organisation necessary for cell

polarisation in various cell types (Capaldo and Macara, 2007; Nejsum and Nelson, 2007; Desai *et al*, 2009; Dupin *et al*, 2009; Yang *et al*, 2009; Chilov *et al*, 2011; Zigman *et al*, 2011), therefore supporting our hypothesis that nascent adhesions between interdigitating cells might initiate apical organisation within the interdigitation zone. The coordination of adhesion with polarity during early junctional maturation has also recently been demonstrated in keratinocytes (Gladden *et al*, 2010), and nascent cell-cell

adhesion clusters between zebrafish NP cells have been shown to be important in defining the division angle of C-divisions (Zigman *et al*, 2011).

We suggest that the correct localisation of the centrosome at the midline of the tissue is likely to be a key event in reinforcing correct apico-basal polarity. It is known to play a role in initial microtubule organisation of epithelial cells (Bellett *et al*, 2009) and a recent study in *C. elegans* intestinal epithelia suggests that the centrosome is also necessary to establish later nucleation of microtubules at the apical surface (Feldman and Priess, 2012). We show that centrosome location at the midline precedes nuclear migration to and mitotic spindle formation at the midline. Centrosome localisation could therefore play the dual role of directing apical proteins to the correct location and coordinating this with the location of the midline mitoses that play such a powerful role in lumen organisation (Tawk *et al*, 2007; Quesada-Hernandez *et al*, 2010; Zigman *et al*, 2011). Previous work has shown that apical proteins, such as Pard3, are necessary for determining centrosome positioning and spindle position in several different cell types (Kemphues *et al*, 1988; Grill *et al*, 2001; Cai *et al*, 2003; Schmoranzer *et al*, 2009; Hong *et al*, 2010; Feldman and Priess, 2012), and aPKC has also recently been implicated in centrosomal positioning and subsequent lumen formation in MDCK cells (Rodriguez-Fraticelli *et al*, 2012). This therefore supports our hypothesis that initial broad apical protein localisation may drive centrosomal movement to the tissue midline, which then organises the microtubule cytoskeleton around this point and reinforces the trafficking of Pard3 and Rab11a.

Rab11a is necessary for lumen opening but not for initial midline formation

We demonstrate that functional Rab11a is required for neural lumen opening and the maintenance of a coherent planar-organised apical epithelium *in vivo*. Like Pard3, RAB11A-EGFP endosomes are targeted to the point at which the cells intersect the tissue midline via a microtubule-mediated and division-independent mechanism. Therefore, RAB11A trafficking provides another example of a process that is organised around the cells' intersection with the midline in order to assemble a lumen. There are several paralogues of Rab11 in zebrafish. We chose to abrogate Rab11a function since this paralogue shows ubiquitous expression (Clark *et al*, 2011) and Rab11a-S25N has previously been shown to cause polarity defects in other systems (Lock and Stow, 2005; Desclozeaux *et al*, 2008; Schluter *et al*, 2009; Bryant *et al*, 2010). However, the very high protein identity between zebrafish paralogues of Rab11 (Clark *et al*, 2011), especially in the region that is mutated in Rab11a-S25N, suggests that the S25N construct may interfere with the function of all Rab11 paralogues.

Surprisingly, despite the lower levels of Crb2a staining in Rab11aDN rhombomeres at the late rod stage, the initial location of apical proteins is at the midline in all rhombomeres. It is only at later developmental stages that protein staining at the midline significantly decreases (especially aPKC staining), the apical epithelium becomes disorganised and the lumen fails to open within Rab11aDN rhombomeres 3 and 5. The early downregulation of Crb2a staining is in line with a study in *Drosophila*, which shows that a loss of Crb

from the cortex of the embryonic ectoderm as a consequence of Rab11DN expression precedes adherens junction destabilisation (Roeth *et al*, 2009) and supports data suggesting that Rab11a is directly necessary for Crumbs localisation to apical surfaces (Roeth *et al*, 2009; Schluter *et al*, 2009; Fletcher *et al*, 2012). There are many interactions between the Crumbs and Par polarity complexes. For example, loss of Crumbs or of the retromer complex that traffics it in *Drosophila* embryos prevents the apical localisation of aPKC (Pocha *et al*, 2011), and a feedback loop between Crumbs and aPKC maintenance at the plasma membrane has also been suggested (Fletcher *et al*, 2012). Our data therefore suggests that Rab11a is not necessary for the initial polarised delivery of apical junctional components but is necessary for the maintenance of epithelial organisation, at least partly through localising Crumbs proteins to the apical domain.

The lack of lumen opening in Rab11aDN rhombomeres could be downstream of Rab11a's known role in abscission (Skop *et al*, 2001; Fielding *et al*, 2005; Wilson *et al*, 2005; Yu *et al*, 2007; Pohl and Jentsch, 2008) since a lack of abscission between sister cells bridging the neural midline could explain the lack of lumen opening seen on expression of a dominant-negative Rab11a. However, our results suggest that the lack of lumen opening is not inherently related to cytokinesis. It is not clear whether the loss of apical complex proteins that we found in Rab11aDN rhombomeres may mediate the lumen opening phenotype and/or the mislocalisation of junctional proteins later in development. It is possible that there is a separate requirement for Rab11a in resolving junctional disassembly, which is likely necessary for the separation of contralateral cells during lumen opening. Determining the molecular and cellular basis of Rab11a's role in neural tube formation is an important future goal.

Conclusions

Our work advances our understanding of lumen formation *in vivo* by identifying a novel process of cell polarisation, the location of which is determined by cell interdigitation across the tissue midline during convergence. Midline polarisation of proteins necessary for lumen formation, such as Pard3-GFP and RAB11A-EGFP, occurs prior to and independent of the midline division and is dependent on a mirror-symmetric microtubule cytoskeleton. Although division is dispensable for cell polarisation, it confers a morphogenetic advantage to cell remodelling and lumen formation over non-dividing cells.

Materials and methods

Blocking cell divisions

To block cell divisions during neurulation, embryos were injected with 0.5–1 nl of 0.5 mM *emi1* morpholino (*emi1*MO) at a one- to four-cell stage. Blocking *emi1* was previously shown to arrest cells in the G2 phase of the cell cycle (Zhang *et al*, 2008; Rhodes *et al*, 2009). This efficiently blocked cell division between 9 and 22 h.p.f. (Supplementary Figure S1). Control embryos were injected with standard control morpholino at the same concentration and stage.

Nocodazole treatment

To break down the microtubule cytoskeleton during neurulation, embryos were treated with 5 µg/ml (17 µM) nocodazole from seven somites. This concentration range is above stoichiometric levels (µM range) and causes depolymerisation of microtubules (Jordan and Wilson, 1998; Gallo and Letourneau, 1999). One effect of

nocodazole treatment is to arrest dividing cells in prometaphase, resulting in the persistent rounding up of cells. To isolate the effects of nocodazole on polarisation from these complicating effects on division, we also blocked division using *emi1*MO. This allowed most cells to maintain an elongated morphology, and the apico-basal location of Pard3 fusion proteins could therefore be assessed within cells that had a disorganised microtubule cytoskeleton. Embryos remained in nocodazole solution for 1 h and 30 min during imaging until the equivalent of the 10-somite stage.

Dextran injection

To enable visualisation of ventricular morphology, a small volume of 4% rhodamine dextran was injected into the hindbrain ventricles of 28 h.p.f. embryos.

Abrogating Rab11a function

We crossed a UAS-inducible dominant-negative Rab11a line of zebrafish Tg(UAS:mCherry-Rab11a S25N)^{mw35} (Clark *et al*, 2011) with a line of zebrafish in which the optimised Gal4-activator, KalTA4, is driven by Krox20 specifically in rhombomeres 3 and 5 from approximately the 6-somite stage tg(Krox20-RFP-KalTA4) (Distel *et al*, 2009). This resulted in expression of Rab11a-S25N specifically in rhombomeres 3 and 5. The S25N version of Rab11a does not bind GTP and so is maintained in the GDP form and inhibits recycling.

Preventing left-right interdigitation

Prior to convergence, at 10 h.p.f., *emi1*MO-injected embryos were mounted in agarose and the neural plate was bisected at the midline using a tungsten needle. Embryos were allowed to heal in E2 embryo medium with penicillin/streptomycin (1%, Invitrogen) for 3 h, removed from agarose and incubated until the 16–18-somite stage.

Data analysis

Division position analysis. To measure the position of C-divisions, time-lapse movies of nuclei labelled with H2B-RFP were taken at the level of the hindbrain between the otic vesicles. The midline position was defined as the midpoint between the left and right edges of the neural tube as observed in bright-field. Division position was measured from the midline to the metaphase plate of each division from both horizontal and transverse sections.

Pard3-FP subcellular location during nocodazole treatment. Embryos were selected from different experiments; some of which were mosaically labelled with Pard3-GFP and some with Pard3-RFP

to ensure that conditions were not bias towards one experiment. Contingency tables were drawn to compare the number of cells containing some basal Pard3-FP with the number containing exclusively apical Pard3-FP or no Pard3-FP in nocodazole-treated and control embryos. This was done for the 6-somite stage (before nocodazole treatment) and at the equivalent of the 10-somite stage (1 h and 30 min after nocodazole treatment). These tables were used to carry out Fisher's exact test.

Nascent lumen surface analysis. Tissue bridges across the neural rod midline were visualised by ZO1 immunostaining and a sytox nuclei counter stain. The number of midline bridges was counted along the entire dorsal-ventral axes in a horizontal 150-µm region surrounding the otic vesicle for seven embryos from each group (*emi* MO vs control MO). A two-tailed unpaired *t*-test was used to compare numbers of bridges between each treatment group.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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