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The polarity protein Pard3 is required for centrosome positioning during neurulation

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ABSTRACT

Microtubules are essential regulators of cell polarity, architecture and motility. The organization of the microtubule network is context-specific. In non-polarized cells, microtubules are anchored to the centrosome and form radial arrays. In most epithelial cells, microtubules are noncentrosomal, align along the apico-basal axis and the centrosome templates a cilium. It follows that cells undergoing mesenchyme-to-epithelium transitions must reorganize their microtubule network extensively, yet little is understood about how this process is orchestrated. In particular, the pathways regulating the apical positioning of the centrosome are unknown, a central question given the role of cilia in fluid propulsion, sensation and signaling. In zebrafish, neural progenitors undergo progressive epithelialization during neurulation, and thus provide a convenient *in vivo* cellular context in which to address this question. We demonstrate here that the microtubule cytoskeleton gradually transitions from a radial to linear organization during neurulation and that microtubules function in conjunction with the polarity protein Pard3 to mediate centrosome positioning. Pard3 depletion results in hydrocephalus, a defect often associated with abnormal cerebrospinal fluid flow that has been linked to cilia defects. These findings thus bring to focus cellular events occurring during neurulation and reveal novel molecular mechanisms implicated in centrosome positioning.

Introduction

Epithelial and mesenchymal cells exhibit distinct forms of cell polarity. Epithelial cells are polarized along the apico-basal axis, which is manifested by the localized distribution of junctional proteins, the apical position of the centrosome, the organization of the microtubule (MT) and actin cytoskeleton, the transport of cellular components and solutes across the epithelium and the presence of basal lamina at the basal surface. Mesenchymal cells are often migratory and have a front-to-back polarity (Hay, 2005; Lee et al. 2006; Thiery and Sleeman, 2006). The ability of cells to establish polarization is essential, not only for their function but also for proper morphogenesis of the tissues and organs of which they are a part. Polarization is typically studied in epithelial cells or mesenchymal cells but fewer studies have focused on how cells that are transitioning between the two states rearrange their polarity. In particular, the changes that occur during mesenchymal-to-epithelial transitions (MET) are poorly understood.

MTs are dynamic polar filaments with fast-growing plus ends and slow-growing minus ends that are key regulators of cell polarity. In migratory cells, the majority of MTs are anchored by their minus ends at the centrosome or microtubule-organizing center (MTOC), resulting in a radial MT array with plus ends facing towards the cell cortex. Radial MT tracks are thought to deliver activators of actin polymerization to the leading edge of the cell, thereby promoting polarized migration (Siegrist and Doe, 2007). By contrast, in epithelial cells, MTs are mostly noncentrosomal and align along the apico-basal axis. Polarity of MTs in these cells is manifested by the orientation of the minus ends towards the apical surface and the plus ends facing the basal domain. This polarized organization facilitates directional vesicular transport to the apical and basolateral domains of the cell (Musch, 2004). Key to the acquisition of epithelial MT organization is the release of MTs from the centrosome (Keating et al., 1997). The latter is positioned at the apical surface in most epithelial cells and functions as a basal body, templating the growth of a ciliary axoneme (Satir and Christensen, 2007).

The mechanisms that mediate centrosome/basal body positioning in epithelial cells are poorly understood (Dawe et al., 2007) and yet essential, as cilia carry out functions in sensation, signaling and fluid flow across the surface of the epithelial sheet (Satir and Christensen, 2007). The role of the cytoskeleton in centrosome migration has been investigated in multi-ciliated cells using drugs that disrupt the cytoskeleton (Dawe et al., 2007). Disruption of MTs in these cells did not directly prevent centrosome migration, whereas disruption of the actin–myosin network did, highlighting a central role for the actin cytoskeleton in this process. The requirement for either the MT or actin network in cells with only one (primary) cilium on their surface is unknown (Dawe et al., 2007). Given the increasing evidence that proteins of the Par6–Par3–aPKC complex organize the MT network (Manneville and Etienne-Manneville, 2006), it is likely that these

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molecules are also implicated in centrosome positioning, however direct evidence for this is lacking.

Neurulation in the zebrafish provides a convenient *in vivo* setting in which to investigate how cell polarity is established, as neural progenitor cells are known to undergo progressive epithelialization. At the onset of neurulation, the zebrafish neural plate is composed of two cell layers. Deep cells are columnar and maintain contact with the basement membrane throughout neurulation (Hong and Brewster, 2006; Papan and Campos-Ortega, 1994). Superficial cells lie directly below the enveloping layer (Hong and Brewster, 2006). During neural convergence, an early stage in neurulation resulting in the formation of the neural rod, deep and superficial cells converge towards the dorsal midline while simultaneously intercalating amongst one another to create a single cell layered neuroepithelium. Both convergence and intercalation are active processes, mediated by the formation of polarized membrane protrusions (Hong and Brewster, 2006). Following neural convergence, cells establish a clearly defined apico-basal axis, marked by the presence of apical junctional complexes and primary cilia that extend into the luminal space (Geldmacher-Voss et al., 2003; Hong and Brewster, 2006; Kramer-Zucker et al., 2005). The establishment of apico-basal polarity in the zebrafish neural tube is thought to be intimately linked to a unique mode of cell division, known as a C-Division, during which one daughter cell remains on the ipsilateral side of the neural keel while the other crosses the midline (Kimmel et al., 1994; Papan and Campos-Ortega, 1994). Following these divisions, daughter cells acquire mirror-image polarity, which is essential for defining the midline of the neural tube (Clarke, 2009; Tawk et al., 2007). The importance of these divisions is highlighted by the severe morphological defects that arise when they occur in ectopic positions (Ciruna et al., 2006; Tawk et al., 2007). Pard3, also known as ASIP/Par3/Bazooka (Geldmacher-Voss et al., 2003), is an essential regulator of C-Divisions, as the orientation of the mitotic spindle is defective in Pard3-depleted embryos (Geldmacher-Voss et al., 2003) and midline crossing is not observed (Clarke, 2009; Tawk et al., 2007). While C-Divisions are clearly important for regulating cell polarity, other mechanisms that do not involve cell division are likely to be in place. Indeed, not all neural cells undergo C-Divisions (Concha and Adams, 1998) and blocking cell division does not prevent proper neural tube morphogenesis or epithelialization (Ciruna et al., 2006; Tawk et al., 2007).

In order to gain a better understanding of the mechanisms regulating cell polarity during neurulation, we investigate here the role of MTs and Pard3. We observe that MTs transition from a radial to a linear organization during neural convergence, consistent with a progressive epithelialization process, akin to MET. We further demonstrate overlapping functions for MTs and Pard3 in positioning the centrosome at the apical cortex, where it templates a primary cilium. Pard3 depletion results in hydrocephalus, a defect often associated with abnormal cerebrospinal fluid flow and cilia defects. Together, these findings bring to focus cellular events implicated in cell polarization during neurulation and reveal novel molecular mechanisms required for centrosome positioning. Since cell behaviors that drive neurulation in zebrafish are similar to those occurring during neural tube morphogenesis in *Xenopus* (Davidson and Keller, 1999) and secondary neurulation in amniotes (Harrington et al., 2009) the mechanisms revealed in this study are likely to be conserved.

Materials and methods

Zebrafish strains

Studies were performed using wild-type (AB) strains and *linguini* (*lin*) mutants. The stages of neurulation (neural plate/tb-1 som, neural keel/4–5 som, neural rod/12–13 som and neural tube/24 hpf and older) are as previously defined for the hindbrain region (Hong and Brewster, 2006).

Immunohistochemistry and imaging

Immunohistochemistry and imaging were carried out as previously described (Hong and Brewster, 2006) with the following modifications. Embryos were fixed overnight at 4 °C in BT fixative (4% paraformaldehyde, 0.15 mM $CaCl_2$, 4% sucrose in 0.1 M PO_4 buffer) or at room temperature with either 4% paraformaldehyde or Prefer fixative (Anatech). Embryos that were treated using the BT fixative were washed with 0.1 M PO_4 and H_2O for 5 min each and treated with acetone at −20 °C for 7 min, followed by sequential washes with H_2O and 0.1 M PO_4. Fixed embryos were sectioned through the hindbrain region and processed for immunolabeling. Sections were imaged either with a Zeiss 510 META or a Leica SP5 confocal microscope.

The following antibodies were used: mouse-anti-ZO-1 (Zymed laboratories) at 1:200; mouse-anti-acetylated tubulin (Sigma) at 1:200; mouse-anti-β-tubulin (Sigma) at 1:200; rabbit-anti-β-tubulin (Abcam) at 1:200; mouse-anti-γ-tubulin (Sigma) at 1:200; rabbit-anti-Glu-tubulin (Millipore) at 1:500; mouse-anti-GM130 (Sigma) at 1:150; rabbit-anti-GFP (Invitrogen) at 1:1000. Secondary antibodies conjugated to Alexa 488 or Cy3 (Molecular Probes) were used at a 1:200 dilution. DAPI (Molecular probes) was used according to manufacturer's instructions.

pard3 MO-injected embryos displaying the hydrocephalus phenotype and uninjected controls were imaged live, using Nomarski optics, under a Nikon SMZ-1500 dissecting microscope and a Nikon Ri1 Color Camera.

Transmission electron microscopy

Transmission electron imaging was performed as described by Brosamle and Halpern (2002).

DNA, RNA and morpholino injections

Plasmids encoding mGFP (courtesy of R. Harland) and *pard3-GFP* (von Trotha et al., 2006) were prepared using a Qiagen Maxi Prep Kit. *pard3-GFP* was linearized and transcribed using mMESSAGE mMACHINE (Ambion). *mGFP* DNA was injected at the one cell stage and *pard3-GFP* RNA was injected mosaically at the 8–16 cell stage.

The *pard3* MO1 (5′ TCAAAGGCTCCCGTGCTCTGGTGTC 3′) and ASIP MO (5′-ACACCGTCACTTTCATAGTTCCAAC-3′) are targeted against the 5′UTR of the *pard3* mRNA (Geldmacher-Voss et al., 2003; Wei et al., 2004). *pard3* MO1 was injected at 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml concentrations. ASIP MO was injected at 20 mg/ml. *mGFP* DNA was injected at a 40 ng/μl concentration and *pard3-eGFP* RNA at 70 ng/μl for expression studies. Approximately 2 nl was injected into each embryo. For rescue experiments, a total of 130 pg or 260 pg of *pard3-GFP* RNA were injected.

Nocodazole treatment

Nocodazole (Sigma, cat # M1404) was prepared according to the manufacturer's instructions and diluted to 20 μg/ml in embryo medium. Because survival was poor following exposure to nocodazole, the treatments of (dechorionated) embryos were limited to 15–30 min at 28 °C and the embryos fixed immediately after drug-exposure. Analysis of centrosome position: embryos that were injected with *mGFP* DNA at the one cell stage were exposed to nocodazole for 30 min beginning at the 9 som stage. Analysis of Pard3-GFP localization: embryos were injected mosaically with *pard3-GFP* mRNA at the 8–16 cell stage and exposed to nocodazole at 6 som.