

Multicellular Rosette Formation During Cell Ingression in the Avian Primitive Streak

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Cell movements are a fundamental feature during the development of multi-cellular organisms. In amniote gastrulation, cells ingress through the primitive streak, which identifies the anterior-posterior axis of the embryo. We investigated the cytoskeletal architecture during these morphogenetic processes and characterized microtubule organisation in whole chick embryos. This revealed the distribution of cells with polarized and radial microtubule (MT) arrays across different regions of the embryo. Cells in the epiblast usually displayed radial MT-arrays, while the majority of cells in the primitive streak had polarized MT-arrays. Within the primitive streak, many cells organized into groups and were arranged in rosette-like structures with a distinct centre characterized by an accumulation of actin. Extended confocal microscopy and three-dimensional image reconstruction identified tips of polarized cells that were protruding from the plane of rosettes, usually from the centre. We propose that organization into higher order structures facilitates cell ingression during gastrulation. *Developmental Dynamics* 237:91–96, 2008.

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INTRODUCTION

Gastrulation in avian embryos involves highly coordinated morphogenetic movements beginning with the establishment of the primitive streak in the midline. The streak has been shown to form through the large-scale movement of epiblast cells in two counter-rotating streams, which merge at the site of streak formation (Cui et al., 2005; Graeper, 1929; Vakaet, 1970). As the streak forms, it elongates in both the anterior and posterior directions and prospective mesoderm cells begin to ingress (Lawson and Schoenwolf, 2001; Mikawa et al., 2004). Some of the molecular signals

driving these processes in amniotes have been characterized and work in mouse and chick embryos implicates both FGF and Wnt pathways (Bertocchini et al., 2004; Chuai et al., 2006; Ciruna and Rossant, 2001; Kelly et al., 2004; Stern, 2004; Sun et al., 1999; Yang et al., 2002).

These signals ultimately control cell behaviour and have to integrate a variety of cellular responses including cell shape changes, cell polarity and directed migration. Central to this are dynamic changes in cyto-architecture, in particular the actin and microtubule cytoskeleton, which are essential to cell motility. The actin cytoskeleton

provides the driving force for migration whereas microtubules are important for establishing direction. In migrating cells, actin filaments polymerize in the protruding front of the cell whereas they contract in the cell body resulting in retraction of the cell's rear. The dynamic organization of the actin cytoskeleton is regulated by small GTPases of the Rho family, in particular Rac1, RhoA, and Cdc42 (Wittmann and Waterman-Storer, 2001). In recent years, the interplay between the actin and microtubule cytoskeletons has become better characterized and it seems that microtubules influence actin filament organization by modulating the

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Rho GTPases (Watanabe et al., 2005). In migrating cells, microtubules form polarized bundles and the centrosome is often repositioned towards the leading edge. Microtubule plus-end capture and anchorage at the cell cortex within the advancing lamellipodium seems to be critical for both of these processes. Microtubule plus-end tracking proteins, also known as +TIPs, such as EB1, CLASPs, and APC, and cortical receptors such as IQGAP1 and Dlg1, appear to be important for establishing cortical contact in migrating cells (Etienne-Manneville et al., 2005; Mimori-Kiyosue et al., 2007; Wittmann and Waterman-Storer, 2005). In addition, a lack of APC seems to cause a decrease in cell migration (Kroboth et al., 2007) and correlates with overall changes in microtubule stability (Akiyama and Kawasaki, 2006; Gundersen, 2002; Lansbergen et al., 2006; Watanabe et al., 2004, 2005). In contrast, non-migrating cells have a radial microtubule array, which is anchored at a centrally located centrosome.

Here we investigated the cytoskeletal organization in gastrulating avian embryos focussing on microtubules and actin. We established a fixation and staining protocol in whole embryos and have examined microtubule arrays in primitive streak stage chick embryos from early to late Hamburger-Hamilton stage 3 (HH3) (Hamburger and Hamilton, 1951). We found distinct patterns of cellular organization in different regions of the embryos. Cells in the epiblast contained radial microtubule arrays while the majority of cells in the primitive streak were polarized with microtubule bundles oriented in the direction of migration. In addition, we show that in the primitive streak many cells were arranged in rosette-like structures containing multiple cells with an accumulation of actin in the rosette centre. Using confocal imaging and 3D-reconstruction, we detected tips of cells that were protruding from the centre of rosettes, consistent with cells in the process of ingression.

RESULTS

Microtubule Organisation in Gastrulating Chick Embryos

Using confocal microscopy, we characterised microtubule organisation in

cells along the length and width of the primitive streak and the surrounding epiblast in chicken embryos ranging from HH stage 3 to late stage 3 (Fig. 1A). At the tip of the streak, the microtubule network was disorganised and the cells were densely packed. The microtubule network in the majority of the cells at the tip lacked a central focus and displayed non-radial arrays, suggesting that most of the microtubules were not anchored at the centrosome (Fig. 1B,C). A number of different microtubule organisations were observed posterior to the tip and in the central region of the primitive streak (Fig. 1D–G). A few cells showed distinct radial arrays with microtubules presumed to be anchored at a centrally located centrosome and their plus-ends projecting towards the periphery of the cell (Fig. 1E). The majority of the cells in this region had polarised arrays with microtubules organized in bundles that stretched along the entire length of the cell with some of the bundles tapering towards one pole (Fig. 1D,F,G). This organization is typical for migratory cells. Cells containing radial arrays were interspersed with the polarized ones (Fig. 1D). Most interestingly, many of the cells with a polarised microtubule array organized into groups of six or more cells, which were arranged in a rosette-like structure, resulting in a regular pattern of cellular organization within the central streak. (Figs. 1D, 2). There were approximately 20 rosettes along the length of the streak, most of them residing in the central region with a few in the anterior and occasionally one or two in the very posterior regions. All embryos examined between HH3 and HH4 ($n = 15$) contained rosettes, and in all cases the rosettes were distributed across the entire width of the streak with no obvious orientation towards a “midline.” In addition, some were on the outer edge of the streak in the boundary region between streak and epiblast. Not all cells with a polarized MT array were organised into rosettes as they were also seen between rosettes, among cells containing radial arrays. It was not always possible to decide which cells belonged to a distinct rosette. Often, cells with a polarized MT array could potentially be part of either one or other of two neighbouring

rosettes, suggesting that these multicellular structures form and resolve in a dynamic fashion.

The pattern of cellular organisation of the central primitive streak changed in an anterior to posterior direction. More anteriorly, most cells were organised in groups with many rosette structures present, while in the posterior part of this region cells became slightly less organised with fewer groups of cells organised into rosettes. This trend continued into the posterior region of the embryo (Fig. 1H). Rosette structures were rarely observed in the posterior streak and the majority of cells in this region exhibited radial arrays.

Cells in the epiblast exhibited a uniform organisation throughout, giving rise to a very regular appearance of this region. The majority of cells had a radial microtubule array, with some interspersed cells that were dividing (Fig. 1I,J). In close proximity to the streak, there was a transition from epiblast organisation to streak organisation, in particular, epiblast cells were often polarised and sometimes participated in the formation of rosettes. Overall, there was no exact boundary between the epiblast and streak and the change in patterns of microtubule arrays was gradual. However, further away from the streak fewer cells with a polarised microtubule array were seen.

Confocal Analyses of Rosette Structures

Cells organized in rosettes were prominent in the central region of the primitive streak, and rosettes contained mainly cells with polarised arrays but also some cells with radial microtubule arrays (Figs. 1D, 2A). Three-dimensional reconstructions of the microtubule networks within the rosette structures (based on confocal Z stacks) showed that the tips of some cells were protruding from the plane of the rosette towards the hypoblast (Fig. 2B–F). 3D reconstructions in Velocity highlight a group of cells protruding towards the epiblast side (Fig. 2C–F). In a view from the side, a distinct funnel-like shape, containing the tips of three cells, can be distinguished. In addition, adjacent to this is another funnel-like structure containing two

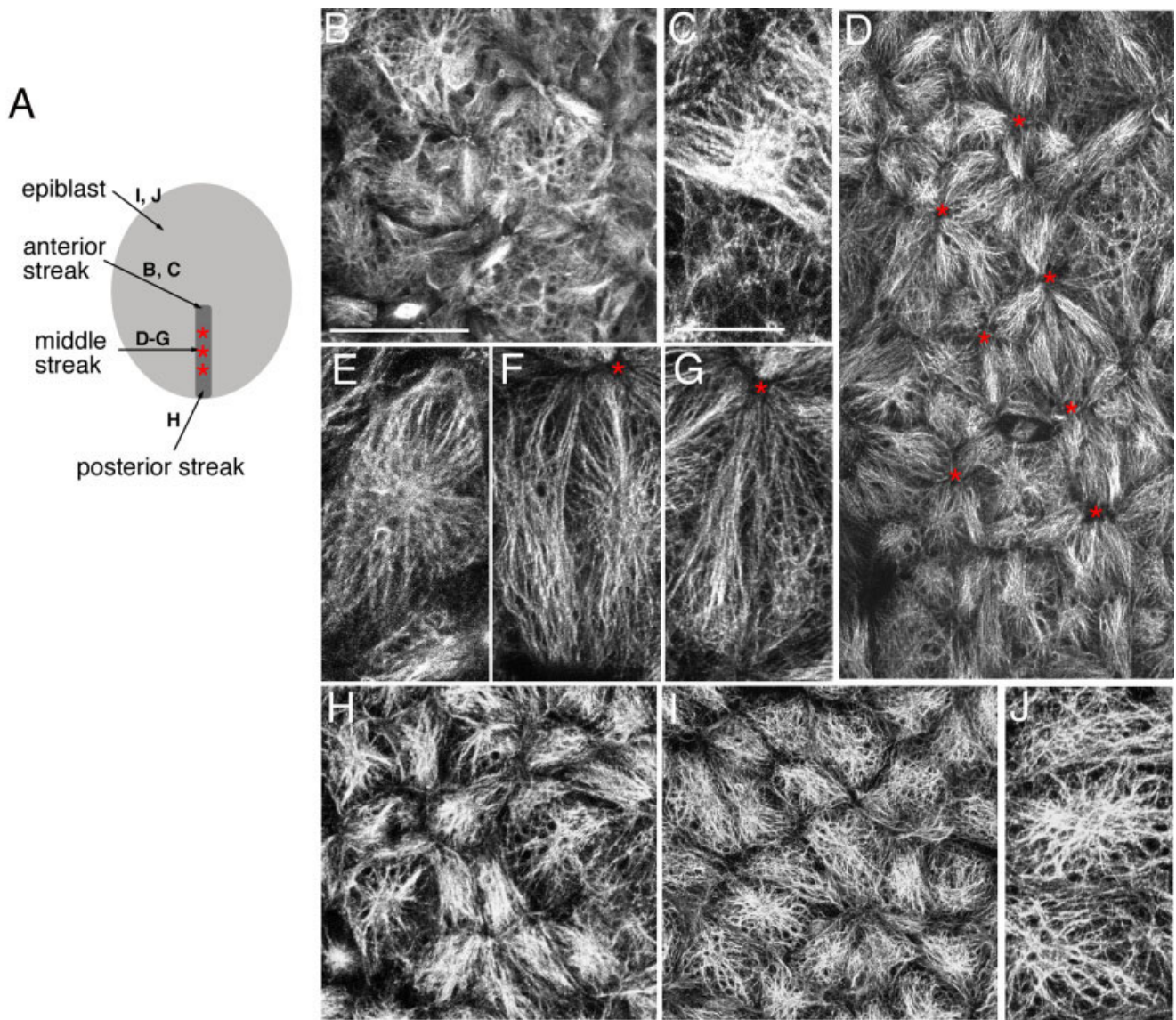


Fig. 1. Overview of microtubule organisation in an HH3 embryo stained with α -tubulin in whole mount. **A:** Schematic illustration of the regions shown in B–J. **B:** Cells at the tip of the streak were densely packed and contained disorganized arrays. **C:** Higher magnification of cells at the tip of the streak in another sample. **D:** The central region of the primitive streak contained mainly cells with polarized arrays, which seem to organize into “rosettes,” and some interspersed cells with radial arrays. **E:** Higher magnification images of radial arrays. **F,G:** Higher magnification images of polarized arrays. **H:** The posterior streak contained mainly cells with radial arrays. **I:** The epiblast contained mainly cells with radial arrays. **J:** Higher magnification of cells in I. Red asterisks (*) identify rosette centres. Scale bar in B = 50 μm (for B,D,H,I); scale bar in F = 20 μm (for C,E–G,J).

cells (Fig. 2E,F) (see Supplemental Movie 1, which can be viewed at www.interscience.wiley.com/jpages/1058-8388/suppmat). To visualize the actin filament organization within cells in the primitive streak, chick embryos were stained with rhodamin-conjugated phalloidin. This confirmed the organization of six or more polarized cells into rosettes (Fig. 3A). Furthermore, it showed a distinct accumulation of actin at the leading edge of the cells, which converged at the centre of the rosettes (Fig. 3A,B). 3D reconstruction

and rotation reveals that actin containing tips protruded towards the hypoblast (Fig. 3C).

DISCUSSION

Microtubule Organisation in the Chick Primitive Streak Reveals a Higher Order Organisation of Cells During Ingression

Our characterization of microtubule arrays in gastrulating chick embryos

reveals for the first time different patterns of cyto-architecture across different regions of whole embryos. The organization of the microtubule cytoskeleton is highly dynamic and intimately related to cellular behaviour and function. Most undifferentiated cells have a radial microtubule array that is anchored at a centrally located centrosome. However, many differentiated cells including polarised epithelial cells, neurons, and skeletal muscle cells have a non-radial array. For example, cell-to-cell contact and polari-

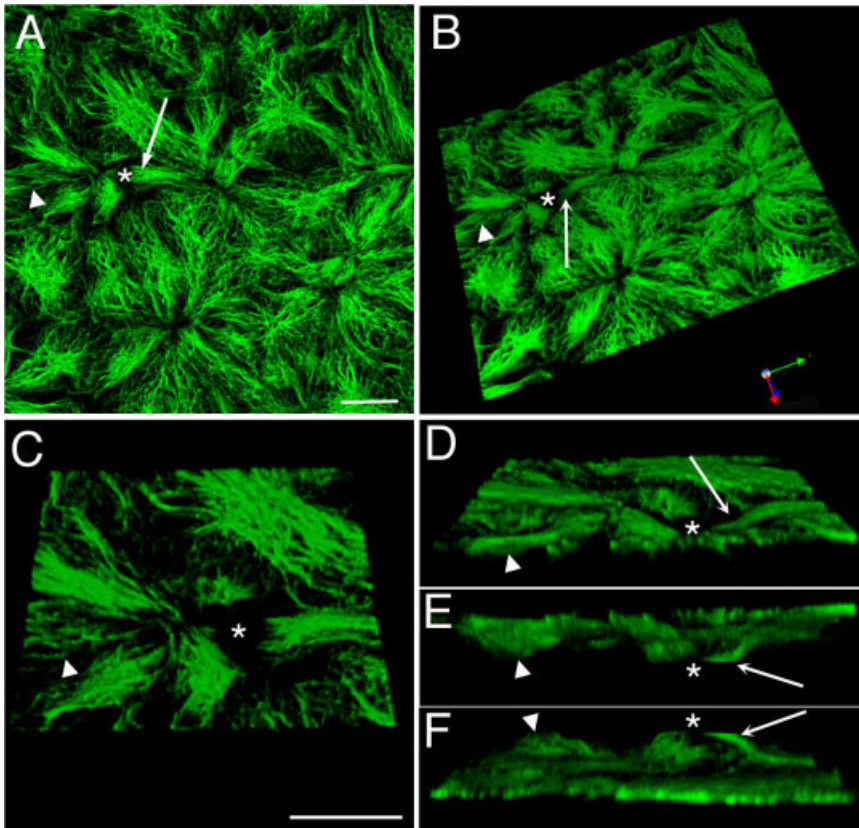


Fig. 2. Three-dimensional reconstruction of rosettes in the primitive streak. **A:** Projection of a Z stack showing the extended focus of cells stained for microtubules. Groups of cells forming rosettes are apparent. **B–F:** Rotation of the three-dimensional reconstruction in A, (B) dorsal view, (C,D) dorso-lateral views, (E,F) side views. Arrows in A–F indicate the tip of a cell protruding from the centre of the rosette. Asterisks and small triangles in E,F indicate sites of cell ingressions. Scale bars in A,B = 20 μ m.

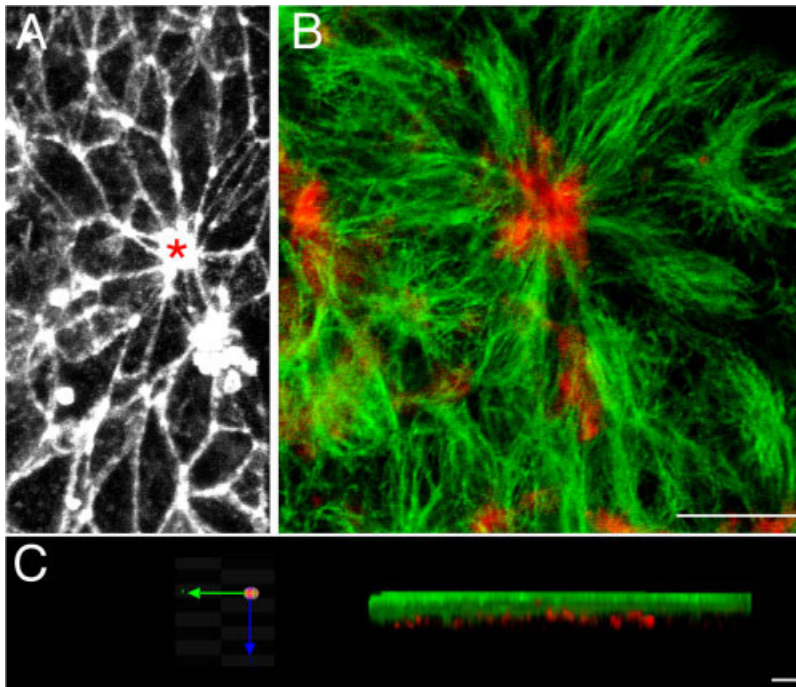


Fig. 3.

sation of epithelial cells like those of the intestine, kidney, and inner ear lead to the formation of an apico-basal microtubule array no longer anchored at the centrosome, which provides mechanical rigidity to the epithelium (Mogensen 1999; Mogensen et al., 2000). Cells that migrate typically form bundles of microtubules, which give the cell polarity and provide essential direction.

Using a specifically adapted fixation and staining protocol for microtubules in whole embryos, we investigated the cytoskeletal architecture and organization of cells in the epiblast and the primitive streak during chick gastrulation. High-resolution images of the microtubule network revealed the distribution of cells with radial or polarized microtubules in both the primitive streak and surrounding epiblast. The detailed mapping of microtubule organisation revealed different patterns of cellular arrangements across different regions of early chick embryos. In the epiblast, cells had a radial microtubule-array, while in the streak the majority of cells had a polarized microtubule-array consistent with cells undergoing directional migration. In addition, cells in the streak organized into rosette-like structures, suggesting that this may facilitate the co-ordinated movement and ingression of groups of cells through the streak. The highest proportion of polarized cells was found in the central portion of the streak and we propose that these cells are migratory. This is in agreement with 3-D analysis and Z-stacks of confocal images show tips of cells protruding from the plane of the rosette, often in its centre. In addition, the accumulation of actin in the centre of rosettes leads us to speculate that motile cells group together in the streak with their leading edges

Fig. 3. Actin staining confirms the formation of rosette structures in the primitive streak. **A:** Phalloidin staining (white) visualizes actin along cell edges and accumulating in the centre of a rosette, indicated by red asterisk (*), in the primitive streak. **B:** Higher magnification image of a different sample with microtubule staining in green and actin staining in red. **C:** Side view of a HH3 primitive streak anterior region, with microtubule staining in green and actin staining in red. Actin-rich parts are protruding towards the hypoblast. Scale bars = 20 μ m.

converging. We did not notice a particular polarization of cells toward the streak midline and cells appear to ingress across the whole width of the streak.

Rosettes were notably enriched in the middle primitive streak (Fig. 1D). The tip region consisted of densely packed cells (Fig. 1B) while cells in the posterior of the streak were more loosely arranged and mainly contained radial arrays (Fig. 1H). The functional significance of multi-cellular rosettes in the primitive streak is not clear at present; however, it is interesting to note that similar structures have been reported to form during germ band extension in *Drosophila* (Blankenship et al., 2006). In this work, computational analysis showed that a majority of germband cells transiently formed rosettes containing between 6 and 11 cells. The rosettes formed and resolved in a dynamic and directional fashion and mutant analysis suggested that this process is crucial for proper axis extension (Blankenship et al., 2006). Different cellular movements are used during ingression of cells following an epithelial to mesenchymal transition at the avian primitive streak, and during germ band extension, which is driven by convergent extension. Interestingly, multi-cellular rosettes seem to play a role in both cases (Blankenship et al., 2006; this report). Therefore, we speculate that global tissue reorganization during morphogenesis may generally involve the formation of higher-order cellular structures to coordinate cell behaviours.

EXPERIMENTAL PROCEDURES

PFA/PHEMO Fixation of Embryos in EC Culture

Embryos were cultured in EC culture (Chapman et al., 2001) until HH3-4 and were kept on the filter paper throughout the fixation and staining protocol to facilitate transfer between solutions. Embryos were washed in PBS before fixing with PHEMO fix solution (68 mM PIPES, 25 mM HEPES, 15 mM EGTA, 3 mM MgCl₂, 3.7% PFA, 0.05% Glutaraldehyde, 0.5% triton X) for 20 to 40 min. This fixative maintained microtubule (MT) struc-

ture and the integrity of the embryos. Embryos were rinsed twice in PHEMO buffer solution (68 mM PIPES, 25 mM HEPES, 15 mM EGTA, 3 mM MgCl₂, 10% [v/v] DMSO, pH 6, with 10M KOH), twice with PBS and then incubated with 50 mM NH₄Cl/PBS for 10 min, washed with PBS, and then blocked with 10% goat serum (Sigma) in PBS for 30 min at RT.

We found that embryo quality was critical for subsequent staining with anti- α -tubulin antibody (M α T, Molecular Probes), 1:1,000 in 1% goat serum/PBS. Primary antibody was applied for 1 hr up to 2.5 days. After washing in 1% goat serum twice for 30 min, embryos were incubated with secondary antibody for 30 min to overnight. We used anti-mouse Alexa fluor 488 (green), or 464 (red), both 1:1,000 (Molecular Probes). For detection of actin, embryos were stained with phalloidin (1:150, National Diagnostics) in 1% goat serum/PBS in a humidified chamber in the dark for 30 min. For microscopy, embryos were removed from filter paper and mounted in hydromount (National Diagnostics).

Scanning Confocal Microscopy, Image Capture, and Processing

All confocal microscopy was carried out on a Zeiss LSM 510 META confocal microscope with Zeiss LSM software. Fluorescent images were recorded using lasers, Argon/Iron and Helium/Neon giving lines at 464 and 488 nm. Optimal scan speed, binning, and pinhole size were determined for each sample. Optical sections were analysed using the Volocity software package (Improvision). This allowed 3-dimensional reconstructions to be made from sequential 2-dimensional data. Processed 3D-images are shown alongside original data, shown as extended focus (flattened Z-stack images).

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REFERENCES

- Akiyama T, Kawasaki Y. 2006. Wnt signaling and the actin cytoskeleton. *Oncogene* 25:7538–7544.
- Bertocchini F, Skromne I, Wolpert L, Stern CD. 2004. Determination of embryonic polarity in a regulative system: evidence for endogenous inhibitors acting sequentially during primitive streak formation in the chick embryo. *Development* 131: 3381–3390.
- Blankenship JT, Backovic ST, Sanny JS, Weitz O, Zallen JA. 2006. Multicellular rosette formation links planar cell polarity to tissue morphogenesis. *Dev Cell* 11: 459–470.
- Chapman SC, Collignon J, Schoenwolf GC, Lumsden A. 2001. Improved method for chick whole-embryo culture using a filter paper carrier. *Dev Dyn* 220:284–289.
- Chuai M, Zeng W, Yang X, Boychenko V, Glazier JA, Weijer CJ. 2006. Cell movement during chick primitive streak formation. *Dev Biol* 296:137–149.
- Ciruna B, Rossant J. 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev Cell* 1:37–49.
- Cui C, Yang X, Chuai M, Glazier JA, Weijer CJ. 2005. Analysis of tissue flow patterns during primitive streak formation in the chick embryo. *Dev Biol* 284:37–47.
- Etienne-Manneville S, Manneville JB, Nicholls S, Ferenczi MA, Hall A. 2005. Cdc42 and Par6-PKC zeta regulate the spatially localized association of Dlg1 and APC to control cell polarization. *J Cell Biol* 170:895–901.
- Graeper L. 1929. Die Primitiventwicklung des Hühnchens nach stereokinematographischen Untersuchungen kontrolliert durch vitale Farbmarkierung und verglichen mit der Entwicklung anderer Wirbeltiere. [Chick primitive streak stage development following vital dye labelling and videomicroscopy studies and comparison to the development of other vertebrates.] *Wilhelm Roux Arch. Entwicklungsmech* 116:382–429.
- Gundersen GG. 2002. Microtubule capture: IQGAP and CLIP-170 expand the repertoire. *Curr Biol* 12:R645–647.
- Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. *J Morphol* 88:49–92.
- Kelly OG, Pinson KI, Skarnes WC. 2004. The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* 131:2803–2815.
- Kroboth K, Newton IP, Kita K, Dikovskaya D, Zumbunn J, Waterman-Storer CM, Nathke IS. 2007. Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall

- changes in microtubule stability. *Mol Biol Cell* 18:910–918.
- Lansbergen G, Grigoriev I, Mimori-Kiyosue Y, Ohtsuka T, Higa S, Kitajima I, Demmers J, Galjart N, Houtsmuller AB, Grosveld F, Akhmanova A. 2006. CLASPs attach microtubule plus ends to the cell cortex through a complex with LL5beta. *Dev Cell* 11:21–32.
- Lawson A, Schoenwolf GC. 2001. New insights into critical events of avian gastrulation. *Anat Rec* 262:238–252.
- Mikawa T, Poh AM, Kelly KA, Ishii Y, Reese DE. 2004. Induction and patterning of the primitive streak, an organizing center of gastrulation in the amniote. *Dev Dyn* 229:422–432.
- Mimori-Kiyosue Y, Matsui C, Sasaki H, Tsukita S. 2007. Adenomatous polyposis coli (APC) protein regulates epithelial cell migration and morphogenesis via PD2 domain-based interactions with plasma membranes. *Genes Cell* 12:219–233.
- Mogensen MM. 1999. Microtubule release and capture in epithelial cells. *Biol Cell* 91:331–341.
- Mogenson MM, Malik A, Piel M, Bouckson-Castaing V, Bornens M. 2000. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. *J Cell Sci* 113:3013–3023.
- Stern CD. 2004. *Gastrulation: from cells to embryos*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sun X, Meyers EN, Lewandoski M, Martin GR. 1999. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* 13:1834–1846.
- Vakaet L. 1970. Cinematographic investigations of gastrulation in the chick blastoderm. *Arch Biol (Liege)* 81:387–426.
- Watanabe T, Wang S, Noritake J, Sato K, Fukata M, Takefuji M, Nakagawa M, Izumi N, Akiyama T, Kaibuchi K. 2004. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. *Dev Cell* 7:871–883.
- Watanabe T, Noritake J, Kaibuchi K. 2005. Regulation of microtubules in cell migration. *Trends Cell Biol* 15:76–83.
- Wittmann T, Waterman-Storer CM. 2001. Cell motility: can Rho GTPases and microtubules point the way? *J Cell Sci* 114:3795–3803.
- Yang X, Dormann D, Munsterberg AE, Weijer CJ. 2002. Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. *Dev Cell* 3:425–437.