Polarized basolateral cell motility underlies invagination and convergent extension of the ascidian notochord

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SUMMARY

We use 3D time-lapse analysis of living embryos and laser scanning confocal reconstructions of fixed, staged, whole-mounted embryos to describe three-dimensional patterns of cell motility, cell shape change, cell rearrangement and tissue deformation that accompany formation of the ascidian notochord. We show that notochord formation involves two simultaneous processes occurring within an initially monolayer epithelial plate: The first is invagination of the notochord plate about the axial midline to form a solid cylindrical rod. The second is mediolaterally directed intercalation of cells within the plane of the epithelial plate, and then later about the circumference of the cylindrical rod, that accompanies its extension along the anterior/posterior (AP) axis. We provide evidence that these shape changes and rearrangements are driven by active extension of interior basolateral notochord cell edges directly across the faces of their adjacent notochord neighbors in a manner analogous to leading edge extension of lamellapodia by motile cells in culture. We show further that local edge extension is polarized with respect to both the AP axis of the embryo and the apicobasal axis of the notochord plate. Our observations suggest a novel view of how active basolateral motility could drive both invagination and convergent extension of a monolayer epithelium. They further reveal deep similarities between modes of notochord morphogenesis exhibited by ascidians and other chordate embryos, suggesting that cellular mechanisms of ascidian notochord formation may operate across the chordate phylum.

Key words: Notochord, Cell motility, Morphogenesis, Ascidian

INTRODUCTION

The active rearrangement of cells along one or more axes within a tissue to cause its extension along another, orthogonal axis is one of the fundamental morphogenetic engines of metazoan development. Examples of this convergence and extension engine appear throughout metazoan phylogeny in both mesenchymal and epithelial tissues including, primordial insect limbs (Fristom and Fristom, 1976), axial mesoderm and neural tissues in chordates (Keller et al., 1989; Keller et al., 1992; Keller et al., 1985; Miyamoto and Crowther, 1985; Schoenwolf and Alvarez, 1989; Thorogood and Wood, 1987; Trinkaus et al., 1992; Warga and Kimmel, 1990) and the sea urchin archenteron (Ettensohn, 1985; Hardin, 1989).

In the above examples, direct observations have established convergent extension as an autonomous process in which the tissue itself produces locally the forces responsible for deforming it. Observations of cell motility during rearrangements in situ reveal many similarities to how isolated cells move in vitro (Cooper and Kimmel, 1998; Elul et al., 1997; Hardin, 1989; Miyamoto and Crowther, 1985; Shih and Keller, 1992a; Trinkaus et al., 1992). These observations, and the phylogenetic conservation of core molecular machinery responsible for cell motility and adhesion, suggest that universal cellular mechanisms may underlie convergent extension in divergent embryonic contexts.

However, convergent extension movements within embryos are necessarily collaborative efforts in which every cell simultaneously senses, exerts forces upon, and experiences forces from, all neighboring cells. To understand these collaborative rearrangements, we must understand how cell-cell interactions and tissue geometry constrain and organize the forces generated by individual cells to produce specific global patterns of cell rearrangement and tissue deformation. A fundamental step towards achieving this goal is to characterize, in specific case studies, the morphogenetic properties of cells in relation to the embryonic context in which they operate, and the global patterns of morphogenetic movement they collectively produce. Unfortunately, the size, cell number or opacity of many embryos makes doing so impossible.

Here, we exploit unique features of ascidian embryos to describe the three-dimensional patterns of cell motility, shape change and rearrangement that accompany notochord formation in the ascidians Boltenia villosa and Corella inflata. The ascidian notochord consists of exactly forty cells that transform in only 6 hours without cell divisions from a monolayer epithelial sheet into a rod of cells stacked end to end to form the structural core of the larval tadpole tail (Cloney,
MATERIALS AND METHODS

Animal collection and embryo culture

We collected Boltenia villosa and Corella inflata adults from Puget Sound. We isolated Boltenia gametes according to the method of Coombs et al. (Coombs et al., 1992). We removed chorions from mature eggs enzymatically by brief incubation in 1% sodium thioglycolate and 0.1% protease at pH 10, followed by 3 rinses in filtered sea water (FSW). We then fertilized the eggs in FSW at pH 10 by adding 0.5-1 ml concentrated sperm. We maintained developing embryos in FSW at 9-13°C in Petri dishes coated with Sylgard or a 10% of maximum intensity and the brightest pixels were just under the resulting stacks into NIH Image for further analysis (counting) of saturation. Fig. 9 shows sample optical sections. We then imported DIC focus sweeps into NIH Image, and deduced time/sweep numbers/times, and to either re-record them on Hi8 tape as time-lapse movies, or to save them as a digital image stack that could be imported to other programs for further analysis. Fig. 6 shows typical optical sections.

Reconstructions of cell trajectories and fates

To compile cell trajectories at different focal heights (Fig. 7), we imported DIC focus sweeps into NIH Image, and deduced approximate cell outlines near dorsal and ventral surfaces by ‘focussing’ up and down through those surfaces. We then used time-lapse sequences at each focal height to establish a correspondence between identified cells at successive time points.

To extract morphometric parameters from DIC focus sweeps we used NIH Image to trace a polygonal outline for cellular cross sections at dorsal, middle and ventral levels. From this polygonal data, we used NIH Image to trace a polygonal outline for cellular cross sections.

3D time-lapse microscopy

We observed living embryos using a modified Kiehart chamber (Kiehart, 1982). All glass surfaces were coated with a thin layer of agarized sylgard or 0.1% gelatin/0.1% formaldehyde (Sardet et al., 1989) to prevent embryos from sticking. A standard temperature control circuit (Horowitz and Hill, 1989) supplied current to a peltier cooling chip (Melcor Thermoelectrics, Trenton NJ) mounted on the chamber so as to maintain a fixed temperature at a thermocouple lead placed next to the embryo.

To collect 3D time-lapse data, we used a computer controlled image acquisition system designed and built around a Zeiss WL Standard upright microscope in our laboratory by Garrett Odell and Victoria Foe. We imaged embryos with standard Nomarski optics using 40× (NA=0.9), or 63× (NA=1.2) plan neofluor multi-immersion lenses adjusted for water immersion. We collected images using a Hamamatsu C2400 CCD camera and recorded frames processed through a digital frame grabber board (MaxVision, Databuce Inc, Peabody Ma) directly onto Hi8 video tape at 30 frames/second using a Sony EVO-9650 animation recorder. An IBM PC computer controlled both the frame grabber board and a digital stepper motor affixed to the fine focus knob of the microscope. Custom-written software synchronously controlled the stepper motor and the frame grabber board so as: (i) To produce a fixed user-specified increment in focus (approx. 0.3 μm) per video frame, phased to occur between frames. (ii) To pass each digitized frame through an internal buffer to be stamped with a binary grayscale pattern, written in the overscan region of the image, encoding the focal height, sweep number, and absolute time associated with that frame.

This system allows us to complete an entire focus sweep in approx. 3-10 seconds, collecting 30 optical sections each second. The specific time depended on the incremental step size and the depth of the focus sweep. We then wrote software controlling the animation recorder from a NeXT computer through an RS232 interface to address, retrieve, and digitize frames from any desired series of focal heights/sweep numbers/times, and to either re-record them on Hi8 tape as time-lapse movies, or to save them as a digital image stack that could be imported to other programs for further analysis. Fig. 6 shows typical optical sections.

Histochemistry and confocal microscopy

We fixed embryos for 20-30 minutes at room temperature in 4% EM grade formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in a buffer containing 50 mM EGTA, 100 mM Pipes, and 400 mM sucrose, adjusted to pH 6.9. We then rinsed embryos 3 times with phosphate-buffered saline (PBS) and incubated them in Bodipy-phalloidin (1 unit/200 μl; Molecular Probes, Eugene OR) in PBS + 0.2% Triton X-100 (PBST) either overnight at 4°C or at room temperature for 1-2 hours. We then rinsed embryos 3× in PBS, attached them to poly-l-lysine coated coverslips, inverted them over tape spacers onto 3×5 inch glass microslides, dehydrated them through an isopropanol series, and cleared them using Murray clear.

Image collection

We collected laser scanning confocal microscopy (LSCM) images on a Biorad model 600 LSCM attached to an upright Nikon microscope using a Nikon 60× Plan Apochromat oil immersion lens (NA 1.4). In general, we used a Kalman average of 6-10 3/4-second scans. Additional collection parameters appear in figure legends. We used Adobe Photoshop to colorize the images shown in Fig. 2, Fig. 3, Fig. 4.

To visualize F-actin rich protrusions, we collected LSCM z-series at 0.2 μm or 0.3 μm intervals (section thickness 0.3-0.4 μm), with gain and offset adjusted so the dimmest pixels were at approximately 10% of maximum intensity and the brightest pixels were just under saturation. Fig. 9 shows sample optical sections. We then imported the resulting stacks into NIH Image for further analysis (counting) of protrusions and to produce the stereo pairs shown in Fig. 10.
Scanning electron microscopy

We fixed embryos for SEM in 2% gluteraldehyde in FSW for 2-4 hours at room temperature, rinsed them several times in FSW, and then post-fixed them in 1% osmium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA) in FSW for 1-2 hours at room temperature. We then rinsed them briefly in distilled water, dehydrated them in ethanol, and critical point dried them at room temperature in hexamethyl disilizane (Sigma), mounted them onto SEM stubs coated with a single layer of double stick tape, and fractured them using a small piece of razor blade affixed to a wooden applicator. In some cases, we fractured embryos following the primary fixation step and then processed them as described above. We imaged specimens on a JEOL JSM-35 SEM microscope and captured selected images on Polaroid 55 film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film. To prepare final figures, we scanned either the Polaroid film.

RESULTS

Confocal analysis of fixed and staged Boltenia villosa embryos

Fig. 1 summarizes the developmental period and the schedule of developmental events considered here, as they occur in Boltenia embryos at 10°C. For descriptive clarity, we group these events into three stages.

Stage I (early neurulation: 15-16 hours): oriented cell divisions and contraction of the posterior notochord boundary establish the initial shape of the notochord plate

The final round of notochord cell divisions begins about 30 minutes after neurulation starts (15 hours after fertilization; AF). At this time, the presumptive notochord comprises an epithelial sheet of exactly 20 cells arranged as two semi-circular arcs around the anterior lip of the blastopore (Fig. 2). The middle eight cells in each arc [the primary lineage: descendants of the A4.1 blastomeres (Conklin, 1905; Nishida, 1987)] divide first, then the lateralmost cells [the secondary lineage: descendants of the B4.1 blastomeres (Conklin, 1905; Nishida, 1987)] divide approximately 30 minutes later. All cells divide perpendicular to the original arcs, transforming 2 rows into 4 (Fig. 3, left column). During this same time, the apices of primary notochord cells at the blastopore lip gradually constrict and disappear, and the lateral ends of anterior notochord arcs bend posteriorly and towards the midline to enclose these cells within the plate’s interior, joining the strands of secondary cells at the posterior midline (Fig. 3A). Also during the final division, the lateral edges of the notochord plate fold ventrally to lie beneath the main plate, possibly driven by the lateral spreading of notochord cell arcs as they divide (Fig. 3J). Often cells lying at or near the lateral fold position withdraw their apices into the notochord interior but retain contact with the basal notochord surface; otherwise all notochord cells remain within a contiguous monolayer.

Stage II (middle-late neurula: 16-18.75 hours). The notochord invaginates to form a cylindrical rod while individual cells elongate and intercalate within the monolayer and perpendicular to the AP axis

During stage II, the notochord begins to extend along the AP axis (Fig. 3, top 3 rows). Below the apical surface, cells elongate perpendicular to the AP axis, shorten along it, and repack in a convergent extension fashion (Fig. 3A-C). Similar...
shape changes occur across the entire width of the notochord plate and around the lateral folds (compare Fig. 3B and 3H). Cell apices also repack, but they remain roughly isodiametric and their areas decrease as the notochord plate invaginates (Fig. 3D,E).

At the same time, the notochord invaginates about the axial midline to make a cylindrical rod (Fig. 3J-L). At the anterior, where neural folds are less pronounced, curvature increases steadily and uniformly across the width of the notochord plate and individual cells within the plate become increasingly wedge shaped in cross-sectional profile (Fig. 3J-L). At the posterior, the outer (basal) boundary of the notochord plate, initially sharply folded at its lateral edges, adopts a progressively more uniform curvature (not shown). Also the lateral folds move towards and fuse at the ventral midline, first at the posterior and then at progressively more anterior positions (Fig. 4).
Cell rearrangements during ascidian notochord formation

Stage III (early tailbud, 18.75 – 21 hours). All cells intercalate about the circumference of the notochord cylinder as it elongates

When stage III starts, the notochord is a cylindrical rod except its most anterior end which remains flattened (Fig. 3, right column). Individual notochord cells have adopted ‘pizza slice’ shapes with their original basal ends forming the outer notochord boundary and their original apices lying near the notochord center along the original apical notochord surface, which persists well into stage III as a single densely phalloidin-stained line along the center of the notochord axis (not shown). Over the next 3 hours, the number of cells seen in cross section at a given axial position decreases steadily as each cell’s basal end extends around the cross-sectional circumference of the notochord until each cell is coin-shaped and all cells stack single file along the rod (Fig. 5). Subsequently individual cells vacuolate and swell, driving further extension of the notochord and tail (Cloney, 1964; Miyamoto and Crowther, 1985).

Mediolateral cell intercalation within a monolayer epithelium drives notochord extension

To analyze cell intercalations during notochord formation, we used 3D time-lapse microscopy (see Materials and Methods) to identify and follow every notochord cell within a single Corella embryo from early stage I to just before the end of stage III (Fig. 6). Fig. 7 shows outlines of identified cells near the dorsal and ventral surfaces at early stage I, late stage II, and late stage III. Cells intercalated perpendicular to the AP axis within the dorsal plate, between dorsal plate and lateral folds and, when lateral folds fused at the ventral midline, across that midline. In all cases, intercalations occurred between immediately adjacent neighbors within the plane of the original notochord monolayer. Even at later stages, when the apical surface has been internalized, notochord cells rarely if ever extended across this apical centerline until they come to occupy a full half of the notochord circumference, at which point they extended rapidly across the rest.

Existing models for cell rearrangement within epithelia make different predictions about where the first contact between intercalating cells should be established (Fristrom, 1982; Jacobson et al., 1986). Fristrom’s biased apical contraction model (Fristrom, 1988; Fristrom, 1982) implies that contacts should be established first at the apical surface, while Jacobson et al.’s Cortical Tractor Hypothesis (Jacobson et al., 1986) implies that contacts should initiate near the basal surface and then propagate apically. We therefore examined where contact is first established between intercalating cells in the notochord plate. In all intercalation events we examined (n=19), the first contact between intercalating cells occurred well within the interior of the notochord plate, and then propagated towards the basal and apical surfaces.

Notochord cell extension and intercalation is polarized relative to both the AP and apicobasal axes of the notochord plate

To further characterize mediolateral intercalation, we measured changes in cross-sectional cell shape and position at three heights along the apical-basal axis: just below the apical surface (apical); just above the basal surface (basal); and...
equidistant between the apical and basal surface (middle) (Fig. 8; see Materials and Methods). Cross-sectional length/width ratios increased steadily within basal (and middle) cross sections from 1.43 (and 1.36) near the end of stage I to 2.3 (and 2.09) near the end of stage II. Cell lengths increased and cell widths decreased, while cross-sectional areas decreased slightly. In contrast, apical cross-sectional length/width ratios remained roughly constant while cross-sectional lengths, widths and areas decreased steadily (Fig. 8).

To quantify relative movements of cells during intercalation, we computed the average distance between centers of mass of neighboring cells during late stage I and early stage II \((n=15\) cell pairs), dividing by cell lengths to normalize for changes in center distance produced by coordinate shape change rather than relative cell movement (e.g. convergence of apical cell centers produced by uniform shrinkage of all cell apices; see Materials and Methods for details). The resulting normalized center distance decreased steadily throughout late stage I and II (Fig. 8), showing that the emergence of polarized cell shape changes are accompanied from the outset by relative movements of cells across one another’s surfaces. Normalized
distance changes were very similar at apical, middle and basal levels, implying a greater absolute relative movement of cells at middle and basal levels.

The organization of F-actin in ascidian notochord cells reveals active basolateral crawling

Our DIC time-lapse movies revealed a general jostling of cells similar to that described by Myamoto and Crowther (Myamoto and Crowther, 1985). But we were unable to resolve localized motile behaviors that might account for the cell movements and shape changes we observed. Because filamentous actin (F-actin) has been implicated in the generation of motile force in nearly all cell types and is enriched in cellular structures associated with active protrusion and/or contraction, we decided to characterize its sub-cellular organization in ascidian notochord cells during active rearrangement.

Fig. 9A shows a medial LSCM section at early stage II when notochord cells are actively rearranging. At the basolateral cortex, F-actin appears as a patchy ring of intense stain, which grazing sections reveal to be a dense meshwork of interconnected fibres (Fig. 9C). 3D reconstructions of entire notochord plates reveal a similarly patchy cortical distribution across the entire basolateral surfaces of all notochord cells, but little or no difference in average density of F-actin along the apicobasal axis. Adjacent endoderm cells, which do not rearrange at these stages, exhibit a similar density and organization of internal actin, but the cortical actin is both more uniform and less dense, with an intensity of signal similar to that seen along the least dense sections of notochord cell cortices.

The brightest accumulations of F-actin within the notochord plate occur at interior junctions made by three or more notochord cells, where dense bands of F-actin run the apical-to-basal length of each notochord cell. Similar accumulations occur in the mid-gastrula stage notochord and in anterior endoderm cells but they are far brighter in notochord cells when they are rearranging. Transverse grazing sections which pass through three cell junctions (Fig. 9B,C), or 3D reconstructions of notochord plates (Fig. 10), show these accumulations lie within lamelliform extensions of individual interior notochord cell edges. These extensions become larger and more numerous as cells begin to converge and extend, and they also become more obviously biased to medial and lateral edges (compare Fig. 10A,B; Table 1). Between late stage I and mid stage II, the fraction of basolateral edges bearing detectable protrusions increased from 0.27±0.04 (n=5
embryos) to 0.46±0.11 (n=5 embryos). At stage I, there was a weak but non-significant (\(P=0.08\), Student’s \(t\)-test) bias towards protrusions being oriented mediolaterally. By early-mid stage II, however, a clear bias had emerged (\(P<0.02\)). By early stage III when cells have adopted their typical pizza slice morphology, nearly every medial or lateral edge bears a broad flattened lamellar protrusion (Fig. 10B).

**SEM analysis of interior and surface protrusions**

To corroborate our confocal observations, we viewed transverse fractures of fixed embryos using scanning electron microscopy (SEM) (Fig. 11A,B). In fractures that pass cleanly between neighboring cells, we could often see flat lamelliform protrusions extending across the faces of adjacent cells. These protrusions, devoid of yolk granules, have roughly the same dimensions as the F-actin-rich protrusions seen in confocal preparations.

At the apical surface, cells make very short protrusions across the surfaces of adjacent cells that tend to interlock with one another (Fig. 11D). Basally, (at least at early stage III when we were able to obtain clean fractures) protrusions extend more continuously along boundaries between adjacent cells so one cell tends to continuously overlap its neighbor (Fig. 11C). In a given embryo, most overlapping protrusions were oriented in the same direction along the AP axis giving the basal surface a shingled appearance. However, the direction of overlap differs from one embryo to the next.

**DISCUSSION**

**Ascidian notochord forms by simultaneous invagination and convergent extension within a monolayer epithelium**

Our results show that ascidian notochord formation involves two simultaneous processes within a monolayer epithelium (Fig. 12A): invagination of the notochord plate to form a solid cylindrical rod; and convergent extension driven by active mediolateral intercalation of cells, first within the notochord plate and then later about the circumference of the solid rod. The final result is a single file of cells stacked end-to-end along the AP axis.

This mode of notochord formation is strikingly similar to that seen in many other chordate embryos including amphioxus (Conklin, 1928), urodele amphibians (Brun and Garson, 1984; Lofberg, 1974), and mouse (Sulik et al., 1994). In all of these cases, the notochord forms by invagination of the superficial

**Table 1. Distribution of interior protrusions within the notochord plate during early stages of notochord formation**

<table>
<thead>
<tr>
<th>Stage</th>
<th>F(_{\text{total}})</th>
<th>F(_{\text{perp}})</th>
<th>F(_{\text{par}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>0.27±0.04</td>
<td>0.32±0.06</td>
<td>0.22±0.09</td>
</tr>
<tr>
<td>Stage II</td>
<td>0.46±0.11</td>
<td>0.59±0.08</td>
<td>0.32±0.15</td>
</tr>
</tbody>
</table>

Perpendicular edges are interior edges whose orientation lies within 45° of perpendicular to the AP axis. Parallel edges are interior edges that lie within 45° of parallel to the AP axis. F\(_{\text{total}}\): fraction of total interior edges bearing F-actin-rich protrusions. F\(_{\text{perp}}\): fraction of perpendicular edges bearing F-actin-rich protrusions. F\(_{\text{par}}\): fraction of parallel edges bearing F-actin-rich protrusions. Data taken from 5 embryos at each stage.
Cell rearrangements during ascidian notochord formation

Fig. 12. (A) Invagination and convergent extension lead to formation of a cylindrical intermediate. Arrows at left indicate convergent extension movements of cells within the notochord plate and its invagination, which occur simultaneously during late stage I and stage II. Dorsal is down and ventral is up. (B) Schematic view of an early-mid stage II notochord plate showing how individual cells extend their interior edges across the faces of adjacent notochord cell neighbors. (C). Textbook view of how an isolated cell crawls on a flat external substratum. (1) Localized actin-dependent protrusive forces (blue arrows) cause the leading edge to extend relative to adhesive contacts with the underlying substratum; (2) New adhesive contacts (green ovals) form at the leading edge with the underlying substratum, and subsequently stabilize through various mechanisms, including lateral clustering of adhesion proteins, and association with the underlying cortical cytoskeleton; (3) actin/myosin-dependent contractile forces within the cortical or interior cytoplasm (red arrows) set up a tug of war between different sites of attachment to the substratum. Directional movement occurs when this tug of war is biased to favor consolidation of leading edge attachments and release of adhesions at the rear (Chen, 1981; Jay et al., 1995; Palecek et al., 1996). (D) How the same machinery might operate within a monolayer epithelium. Each polygonal cell represents a cross section through an epithelial cell somewhere below the apical surface, each vertex represents an interior (basolateral) edge, analogous to the leading edge in (C), which attempts to extend (blue arrows) between adjacent neighbors. Homophilic associations between cadherin proteins replace the integrin-based adhesion used by most mesenchymal cells, but the underlying mechanics are entirely analogous. For simplicity, we consider contractile forces only within the cortex. (E) Mediolaterally biased protrusion (blue arrows) drives cells away from their preferred circular cross-sectional shapes. The cortical contractile forces that act to restore these shapes within each cell (red arrows) are joined by adhesive contacts to make contractile chains that span the width of the notochord plate and cause it to become longer and narrower.
epithelium that forms the roof of the embryonic archenteron. This suggests that invagination coupled to convergent extension is an evolutionarily conserved mode of notochord morphogenesis and that insights we gain from studying notochord morphogenesis in the relatively simple context of the ascidian embryo may be applicable to other chordates.

**Mechanisms of cell rearrangement within epithelia**

Attempts to understand the mechanistic basis for active cell rearrangements within epithelia have focused on two basic questions (Fristrom, 1988; Gumbiner, 1996; Kolega, 1986; Speigel and Speigel, 1986): Where and how are the active forces responsible for cell movements and shape changes generated? And how do these forces bring together the boundaries of non-adjacent cells to cause the neighbor exchanges necessary for cell rearrangements while maintaining the close adhesive associations characteristic of epithelial sheets?

Direct observations of motile activity in epithelia have focussed on the exposed basal surfaces. These observations have revealed a range of local protrusive structures and behaviors, but it has been difficult to envision how they could produce observed patterns of cell shape change and rearrangement, leading some to suggest that the real action may occur elsewhere (Fristrom, 1988; Fristrom, 1982; Jacobson et al., 1986; Keller and Hardin, 1987). Jacobson and colleagues proposed a cortical tractor model in which a time-averaged cortical flow carries adhesive contacts from basal and basolateral regions towards the apical surface (Jacobson et al., 1986). They suggest cellular protrusions, which they and others have observed, extend across the basal or basolateral surfaces of the epithelium to establish novel contacts between non-adjacent cells which the cortical flow would then propagate to the apical surface. In this model, apical junctions turnover continuously and are replaced by junctional proteins that are inserted into basal and lateral membranes and carried apicwards by the cortical flow, providing an elegant mechanism by which junctions between one pair of cells can replace those between another gradually and without loss of mechanical integrity or relative impermeability of the epithelium. This model is consistent with numerous observations of cortical flow in cultured cells (Bray and White, 1988) and polarized insertion/turnover of adhesion proteins and other cortical elements (Lawson and Maxfield, 1995; Palecek et al., 1996; Schmidt et al., 1995; Schmidt et al., 1993), but remains to be established for epithelial cells in situ.

An alternative hypothesis proposed by Fristrom (Fristrom, 1988; Fristrom, 1982) suggests that contractions of circumapical filament bundles, biased to specific apposing cell faces, could pull non-adjacent cell edges together to make novel contacts through special 4-cell junctional intermediates. In this view, cells rearrange without any shear between adjacent cell boundaries because they extend or shorten their common boundaries in a coordinated fashion. Fristrom’s hypothesis is based on her own observations of cell rearrangements and junctional morphology underlying imaginal disc evagination in *Drosophila* (Fristrom, 1982), and is consistent with the demonstrated contractility of circumapical filament bundles (Owaribe et al., 1981).

Neither of these models is consistent with our observations. In the ascidian notochord, contacts between non-adjacent cells typically form interior to the notochord and then propagate towards both the apical and basal poles, rather than flowing only basal-to-apical as the cortical tractor hypothesis asserts, or initiating apically as Fristrom’s hypothesis implies. The short flattened basal protrusions that we observe at the basal surfaces of notochord cells do not extend far enough to establish novel contacts between non-adjacent cells as the cortical tractor hypothesis suggests. On the other hand, we find no evidence for an especially dense circumapical microfilament ring as others have described elsewhere, or for the biased accumulation of F-actin to particular interior cell faces.

**A working hypothesis for active cell rearrangements within a monolayer epithelium**

Our observations suggest an alternative hypothesis: that notochord cells move and change shape by crawling directly across the interior surfaces of their adjacent notochord neighbors using the same conserved cytoskeletal machinery, and the same basic mechanisms of motile force generation, that many other cells (e.g. fibroblasts and keratocytes) use to crawl across flat substrata in vitro (Fig. 12B-D).

Fig. 12C illustrates the current textbook view of how motile cells advance across an external planar substratum through a combination of, (1) actin-dependent extension of the leading edge; (2) formation and stabilization of new adhesive contacts; and (3) active contraction of the cortex and/or internal cytoplasm (Alberts et al., 1994; Bray, 2000; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Sheetz, 1994). Fig. 12D shows how the same processes might operate to drive convergent extension in the very different cellular, mechanical, and geometric context of a close-packed polarized monolayer epithelium. In this view, actin-dependent protrusive forces cause interior basolateral edges to extend across and between the interior faces of adjacent neighbors. To do so, the extending edge must displace existing adhesive connections between, and establish new adhesive contacts with, each of those neighbors. At the same time, cortical contractile forces operating away from the leading edge attempt to contract the cell boundary around an incompressible fluid volume.

Absent protrusive activity, contractile and hydrostatic forces will force cells towards isodiametric shapes. When protrusive forces deform a cell away from its preferred shape, contractile forces attempt to restore that shape. As local protrusive extension becomes biased to medial and lateral interior edges (Table 1), the contractile restoring force acting within each cell will also become biased perpendicular to the AP axis. Because contractile force will be roughly the same for neighboring cell boundaries, boundary shortening can occur without the need to make or break adhesive bonds. The only place where adhesive connections need break and reform is at or near three-cell junctions, where cells actively extend between neighbors.

The local result will be a steady movement of neighboring cells past one another through a combination of active local extension at three-cell junctions (which drives cells away from their preferred shapes), and coordinate contraction of neighboring cell boundaries elsewhere (which pulls cells back towards their preferred shapes). The global result will be chains of contractile cells, perpendicular to the AP axis, spanning the notochord plate’s width, which contract to converge the notochord plate’s width and thereby necessarily extend its length (Fig. 12E). As the ventral folds fuse, these contractile side-to-side chains become contractile rings encircling the
cylindrical rod, which squeeze it and cause it to extend anteriorly and posteriorly.

So long as mediolaterally biased protrusive extension persists, and unless resisted by some greater external force, mediolateral contraction and axial extension of the notochord will continue inevitably until every locally extending interior edge disappears (i.e. until every cell itself spans the entire width of the notochord plate or cross-sectional area of the notochord cylinder).

We have made and analyzed a mathematical model for cell rearrangements within epithelial sheets that incorporates detailed representations of the local protrusive, contractile and adhesive mechanics hypothesized above (Munro and Odell, unpublished). The results confirm our intuitive predictions outlined above and provide additional insights into the mechanics of cell rearrangement within epithelia. For example, they imply that the basal to apical flow of cortical and adhesive structures postulated by the cortical tractor model (Jacobson et al., 1986) would automatically result if protrusive activity were stronger basally than apically. Similarly, the biased coordinate contraction postulated by Fristrom (Fristrom, 1982) emerges as a secondary consequence of biased protrusive extension within the epithelial plane. Thus, rather than contradicting previous hypotheses, ours parsimoniously reconciles them within a single framework.

**Active basolateral forces drive non-autonomous rearrangement of notochord cell apices**

Our observations suggest that active forces generated below the apical surface drive a secondary ‘passive’ rearrangement of notochord cell apices. Apical domains elongate in the direction of tissue extension, a characteristic of non-autonomous forms of epithelial cell rearrangement (Honda et al., 1982; Keller and Hardin, 1987; Keller, 1978). Basolateral domains move relative to one another first and faster than apical domains even though the fractional rates (the absolute rate normalized by the cross-sectional cell length) are roughly equal, and the F-actin rich protrusions, which accompany and presumably drive basolateral extension, rarely if ever extend to the apical surface.

Apical rearrangement could be purely passive: i.e. active movement of basolateral domains towards (mediolaterally) or away from (along the AP axis) one another might simply pull the corresponding apices towards or away from one another. However if the apical cortex were contractile, then contractile forces could help drive rearrangements by forcing stretched apices back towards more isodiametric shapes as proposed above (Honda et al., 1984; Weliky and Oster, 1990).

**Polarized basolateral extension may contribute to invagination**

We have shown that active basolateral extension begins with, and continues through, the period of notochord plate invagination. If extension forces are stronger in the basolateral domain than apically as our data suggests, then they should contribute to invagination, for they will counteract the contractile forces which shorten cell boundaries more strongly basally than apically. If cortical contractile forces are everywhere equal, this would lead to a greater net constriction in apical cross sections which would help force an invagination.

**Comparison to cellular mechanisms of convergent extension in Xenopus laevis**

In *Xenopus laevis*, as in chick (Bancroft and Bellairs, 1976) and many of the teleost fishes (e.g. Cooper and Kimmel, 1998; Wood and Thorogood, 1994), the notochord forms from, and convergent extension occurs within, deep mesenchymal mesoderm that condenses secondarily during neurulation to form a cylindrical rod. Nevertheless, the cellular mechanisms underlying convergent extension of an epithelial sheet in ascidians are strikingly similar to those that occur in a mesenchymal context during gastrulation in *Xenopus laevis* [reviewed by Keller et al. (Keller et al., 1992)]. During gastrulation, somitic and chordamesodermal cells extend local mediolaterally directed protrusions across the surfaces of their neighbors, and the biased cellular traction forces that arise perpendicularly to the AP axis give rise to contractile arcs of cells spanning the involuting marginal zone. These arcs form within pre-involutio transparent tissue and subsequently move over the dorsal lip of the blastopore as part of the involution front. Shih and Keller suggest their contraction may drive involution as well as convergence and extension of dorsal mesoderm (Keller et al., 1992; Shih and Keller, 1992b). Later, when the *Xenopus* notochord becomes a cylindrical rod, these arcs become constriction rings as we have described here (Keller et al., 1989).

Together, the results we report here and those from Keller’s lab point to a very general cellular mechanism of convergent extension, one conserved within the chordate phylum if not more broadly, and one that transcends differences between mesenchymal and epithelial germ layers. Our ‘chains of contractile cells ‘above are Shih and Keller’s ‘arcs’. We suggest that this reflects an even deeper underlying conservation of the contractile, protrusive and adhesive machinery that cells use to move and change shape within embryos.

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