Tube formation by complex cellular processes in *Ciona intestinalis* notochord

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In the course of embryogenesis multicellular structures and organs are assembled from constituent cells. One structural component common to many organs is the tube, which consists most simply of a luminal space surrounded by a single layer of epithelial cells. The notochord of ascidian *Ciona* forms a tube consisting of only 40 cells, and serves as a hydrostatic “skeleton” essential for swimming. While the early processes of convergent extension in ascidian notochord development have been extensively studied, the later phases of development, which include lumen formation, have not been well characterized. Here we used molecular markers and confocal imaging to describe tubulogenesis in the developing *Ciona* notochord. We found that during tubulogenesis each notochord cell established de novo apical domains, and underwent a mesenchymal–epithelial transition to become an unusual epithelial cell with two opposing apical domains. Concomitantly, extracellular luminal matrix was produced and deposited between notochord cells. Subsequently, each notochord cell simultaneously executed two types of crawling movements bidirectionally along the anterior/posterior axis on the inner surface of notochordal sheath. Lamellipodia-like protrusions resulted in cell lengthening along the anterior/posterior axis, while the retraction of trailing edges of the same cell led to the merging of the two apical domains. As a result, the notochord cells acquired endothelial-like shape and formed the wall of the central lumen. Inhibition of actin polymerization prevented cell movement and tube formation. *Ciona* notochord tube formation utilized an assortment of common and fundamental cellular processes including cell shape change, apical membrane biogenesis, cell/cell adhesion remodeling, dynamic cell crawling, and lumen matrix secretion.

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**Abstract**

In the course of embryogenesis multicellular structures and organs are assembled from constituent cells. One structural component common to many organs is the tube, which consists most simply of a luminal space surrounded by a single layer of epithelial cells. The notochord of ascidian *Ciona* forms a tube consisting of only 40 cells, and serves as a hydrostatic “skeleton” essential for swimming. While the early processes of convergent extension in ascidian notochord development have been extensively studied, the later phases of development, which include lumen formation, have not been well characterized. Here we used molecular markers and confocal imaging to describe tubulogenesis in the developing *Ciona* notochord. We found that during tubulogenesis each notochord cell established de novo apical domains, and underwent a mesenchymal–epithelial transition to become an unusual epithelial cell with two opposing apical domains. Concomitantly, extracellular luminal matrix was produced and deposited between notochord cells. Subsequently, each notochord cell simultaneously executed two types of crawling movements bidirectionally along the anterior/posterior axis on the inner surface of notochordal sheath. Lamellipodia-like protrusions resulted in cell lengthening along the anterior/posterior axis, while the retraction of trailing edges of the same cell led to the merging of the two apical domains. As a result, the notochord cells acquired endothelial-like shape and formed the wall of the central lumen. Inhibition of actin polymerization prevented cell movement and tube formation. *Ciona* notochord tube formation utilized an assortment of common and fundamental cellular processes including cell shape change, apical membrane biogenesis, cell/cell adhesion remodeling, dynamic cell crawling, and lumen matrix secretion.
fibrils (Cloney, 1964). The notochord is located in the central position of the larval tail, flanked on both sides by muscles. It functions presumably as a hydrostatic “skeleton” essential for the locomotion of the swimming larva (Keller, 2006; McHenry, 2005). The tension-resisting fibrils of the notochordal sheath allow the notochord to become rigid as the lumen inflates. To aid in swimming, this stiff yet flexible rod recoils the trunk and tail after each alternating stroke of the lateral muscles.

The development of Ciona notochord can be divided into two distinct phases. In the first phase, the notochord cells are induced at the blastula stage (Kumano and Nishida, 2007) and subsequently undergo a morphogenetic process during gastrula, neurula and early tailbud stages called convergent extension (C/E) that results in the formation of a columnar notochord of 40 cells in length and a single cell in diameter (Miyamoto and Crowther, 1985). This process has been studied in detail in another ascidian species Boltenia villosa (Munro and Odell, 2002). In the second phase, the morphogenesis of the notochord undergoes a series of dynamic changes which culminate in the formation of a central lumen by the swimming larval stage. While previous investigations have described cell shape changes during tubulogenesis (Cloney, 1964), conflicting results have been presented on whether lumen formation in ascidians is extracellular or intracellular (Burighel and Cloney, 1997; Miyamoto and Cloney, 2003). The GFP fragment was amplified by PCR (primers: memGFP5, 5′-GGATGATGATACACCCGG-3′ and memGFP3, 5′-TTTGCGGCCGCTTATTTGTATAGTTCATCCA-3′) and cloned into the pCR®8/GW/TOPO® vector (Invitrogen) to obtain an entry clone. This was recombined into the destination vector to create Minos-B3-eBra-bpFOG-B5′-Kozak-turboGFP-B1-hActin-B2 using the Gateway LR reaction (Invitrogen). The Dm-E-cadherin entry clone (a gift from Agnès Roure and Patrick Lemaire) was recombined into the destination vector to create Minos-B3-eBra-bpFOG-B5′-B1-Dm-E-cadherin-B2-mCherry.

Electroporation

Electroporation was performed as described previously with some modifications (Corbo et al., 1997). Eight-hundred μl of dechorionated fertilized eggs was mixed with 50–100 μg of plasmid DNA and electroporated in 4 mm cuvettes with a Gene Pulser Xcell System (Bio-Rad), using a square pulse protocol (50 V and 15 ms per pulse). After electroporation, embryos were allowed to develop at 16 °C to the desired stages for confocal observation.

Antibody production and immunohistochemistry

For preparation of an antibody to recognize the C. intestinalis homologue of SLC26-2 (C-iSLC26-2), a cDNA fragment encoding the carboxyl-terminal cytoplasmic region of C-iSLC26-2 (D658-N762) was amplified by PCR from the original cDNA clone cht022e16 using a pair of primers, 5′-AGAgatCTCGAGGACGCGCCT-3′ and 5′-TTaagCT- tCTACTGCTGTTAGCC-3′ (the small letters in the primer sequences indicate mismatched nucleotides introduced to generate restriction sites), and cloned into the pQE40 vector (Qiagen GmBH). The plasmid construct was introduced into the E. coli strain XL1-Blue (Stratagene). The carboxy-terminal region of C-iSLC26-2 was expressed as a fusion protein with dihydrofolate reductase and a histidine tag, then isolated and used to immunize mice. Ascidian embryos were fixed with 10% formalin in artificial seawater for 3 h at 4 °C. After fixation, the embryos were washed with PBS containing 0.1% Triton X-100 (PBST), and treated with 10% goat serum in PBST for 3 h. The embryos were then incubated overnight with the primary antiserum diluted 1000-fold with the blocking buffer and washed with PBST for 8 h at 4 °C. The specimens were then incubated with an Alexa 594-conjugated anti-mouse IgG goat antibody (Molecular Probes). The embryos were counter-stained with BODIPY phalloidin (488 nm excitation; Molecular Probes) for filamental actin. After rinsing several times with PBST, the specimens were mounted in Murray Clear for confocal observation (Horie et al., 2008).

Actin localization in notochord cells from embryos electroporated with the turboGFP-actin construct was visualized using anti-turboGFP antibody (Evrogen) and Alexa-488-conjugated secondary antibody (Invitrogen).

Actin inhibitor treatment

10 μM Latrunculin B was applied to embryos at 23 hpf for one hour then washed away. The embryos were cultured for two more hours before observation. Latrunculin B was substituted by DMSO in the control embryos.

Laser scanning confocal microscopy and 3D reconstruction

Confocal images were taken with a Leica TCS SP5 confocal laser scanning microscope (CLSM) equipped with 40× oil and 63× water immersion objectives (numerical aperture 1.25 and 1.40, respectively). Z-series were taken at intervals of 1–1.2 μm, resulting in stacks of 20–40 images. Image analysis and 3D reconstruction were performed with Leica TCS SP5 systems LAS AF software packages. Adobe Photoshop was used to pseudocolor the images.

Materials and methods

Ascidians and embryos

Adult C. intestinalis were collected from a fjord in Bergen, Norway. The animals were kept in the Sars Centre Ascidian Culture Facility, in running filtered seawater for seven days under constant light to accumulate gametes. Eggs were then dissected and mixed in seawater with sperm from other individuals. Five minutes after fertilization, the eggs were washed with seawater through a nylon filter to remove sperm and debris.

Plasmid construction

A membrane GFP notochord expression vector was constructed by modification of Ci-Bra′-GFP (Corbo et al., 1997; Deschet et al., 2003). The GFP fragment was amplified by PCR (primers: memGFP5, 5′-AAAAAGATCTGGACACCTTGTTGTATGAGGAAGAAAAAACAGTG-GAAAAAACGGATGAGAATCAGAAATAGTAAAGGAGAAGAATTTTTC-CACT-3′ and memGFP3, 5′-TTTGGCGGCCTATTTTTATAGTCATCCA-TGCCATGTTG-3′) with the first 20 amino acids of Ciona GAP-43 included in the 5′ primer to create a GAP-43-GFP fusion. This fragment was inserted back into Ci-Bra′-GFP at Bgl II and Not I sites to obtain Bra′-memGFP.

To create plasmids expressing actin and E-cadherin fluorescent fusion proteins in the notochord, we first modified pSP1.72BSPE-R3-ccdB/Cnr-R5′-RFA (Roure et al., 2007) to generate two destination constructs, Minos-B3-eBra-bpFOG-B5′-Kozak-turboGFP-R1-ccdB/Cnr-R2 and Minos-B3-eBra-bpFOG-B5′-R1-ccdB/Cnr-R2-mCherry.
Morphometric analysis

The diameter and length of consecutive segments (10–20) spanning the entire notochord were measured on confocal z-series images, where notochords were outlined by membrane GFP (Supplemental Fig. 1). At each developmental stage (14 hpf, 18 hpf, 36 hpf), five embryos or larvae were measured. The volumes of notochord and lumen were calculated by the following equations.

\[ V_{\text{notochord}} = \pi \left( \frac{d_0}{2} \right)^2 L_0 + \pi \left( \frac{d_1}{2} \right)^2 L_1 + \cdots + \pi \left( \frac{d_n}{2} \right)^2 L_n \]

\[ V_{\text{lumen}} = \pi \left( \frac{d'_0}{2} \right)^2 L'_0 + \pi \left( \frac{d'_1}{2} \right)^2 L'_1 + \cdots + \pi \left( \frac{d'_n}{2} \right)^2 L'_n \]

\[ V_{\text{notochord}}: \text{volume of entire notochord;} \quad V_{\text{lumen}}: \text{volume of entire lumen;} \quad d: \text{diameter of notochord;} \quad d': \text{diameter of lumen;} \quad L: \text{length of notochord segment;} \quad n: \text{the number of segments.} \]

Notochord cell volume was computed by

\[ V_{\text{cell}} = V_{\text{notochord}} - V_{\text{lumen}} \]

Notochord length (\( L_{\text{notochord}} \)) was the sum of all segments.

\[ L_{\text{notochord}} = \sum_{i=0}^{n-1} L_i \]

Individual notochord cell length (\( L_{\text{average single cell}} \)) was an average of 30 notochord cells from five to ten different embryos at each stage.

At the first two stages (14 hpf, 18 hpf), notochord cell average diameter (\( d_{\text{cell}} \)) was computed by

\[ d_{\text{cell}} = \frac{1}{n} \sum_{i=0}^{n} d_i \]

For the mature notochord tube, cell average thickness (\( d_{\text{cell thickness}} \)) was defined as

\[ d_{\text{cell thickness}} = \frac{d_{\text{notochord}} - d_{\text{lumen}}}{2} \]

At the last stage (36 hpf), diameter of notochord lumen (\( d_{\text{lumen}} \)) was calculated by

\[ d_{\text{lumen}} = \frac{1}{n} \sum_{i=1}^{n} d_i \]

Different membrane domains were measured (Supplemental Fig. 2) and surface areas were calculated by the following formula at four different stages (16 hpf, 18 hpf, 21 hpf, and 22 hpf). At each developmental stage, five cells in the middle section of notochords from five individuals were selected for measurement.

\[ S_{\text{apical}} = 4\pi \left( \frac{1}{2} d_{\text{lumen}} \right)^2 \]

\[ S_{\text{lateral}} = 2\pi \left( \frac{1}{2} d_{\text{cell}} \right)^2 - \frac{1}{2} \left( \frac{1}{2} d_{\text{lumen}} \right)^2 \]

\[ S_{\text{basal}} = \pi \times d_{\text{cell}} \times L \]

where \( S_{\text{apical}}, S_{\text{lateral}} \) and \( S_{\text{basal}} \) are total cell surface areas of apical, lateral and basal membrane domains respectively.

Results

Notochord cell behaviors after convergent extension

Fig. 1 summarized the developmental stages and timing of morphogenetic events described here, as they occurred in Ciona intestinalis embryos at 16 °C. For ease in later descriptions, we grouped these events into four stages. To be consistent with the previous staging of notochord morphogenesis (Munro and Odell, 2002), we designated the first stage after the completion of C/E (stage III) as stage IV.

![Fig. 1. Timeline of notochord morphogenesis. Shown are the major morphogenesis events occurring at 16 °C in Ciona intestinalis notochord following cell intercalation (from 14 h past fertilization, hpf). Other major concurrent events of Ciona embryogenesis are listed.](image-url)
Stage IV (14–18 hpf): cell elongation and narrowing

The morphogenesis at stages I–III, as described previously (Munro and Odell, 2002), resulted in the 40 notochord cells, which started as a single-layer sheet, converging and intercalating to form a column, one cell in diameter. At the beginning of stage IV, individual notochord cells had a “coin” shape (Figs. 2A and A′). During the next 4 h, cells elongated along the long axis and narrowed medially (Figs. 2B and B′). The nucleus was conspicuously located at the posterior end of each cell (see Fig. 2B), except in the posterior-most cell where it was anterior (Jiang et al., 2005). A constriction gradually appeared along the circumference of notochord cells midway from their anterior and posterior ends (arrow in Figs. 2B and B′). Throughout stage IV, cells
were radially symmetrical with respect to the cylindrical axis of the notochord (Figs. 2A’ and B’). No relative movement of notochord cells was observed at this stage, or the appearance of extracellular lumen.

Stage V (18–22 hpf): emergence of luminal domains and extracellular lumen formation

At the beginning of stage V, small extracellular lumens appeared between adjacent notochord cells (Fig. 2C). As the development proceeded, the extracellular luminal spaces increased, pushing the plasma membranes of adjacent notochord cells inward to give the cells a biconcave shape (Fig. 2D). In the cross section, the cell/cell contact interface changed from a disc to an annulus (compare Figs. 2C″ and D″ with B″). The posteriorly positioned nuclei were pushed anteriorly and sideways by the lumen, while still abutting the posterior membrane (Figs. 2C and D). Despite the growing lumens, the notochord cells remained relatively radially symmetrical with respect to the cylindrical axis (Figs. 2C′ and D′). Where extracellular lumens first appeared was stochastic, but usually among the 20 cells in the mid-section of the notochord. Among the most posterior notochord cells, the lumen formation lagged behind, or never occurred (data not shown).

Stage VI (22–24 hpf): loss of radial symmetry and complete circumferential contact with notochordal sheath

By the beginning of stage VI, as the extracellular lumens further increased in size, the notochord cells began to drastically change shape. At a certain point around the circumference of each biconcave cell, the anterior and posterior edges (designated as anterior trailing edge, ATE, and posterior trailing edge, PTE) retracted and moved closer to each other (Fig. 2E, red arrows indicate ATE and PTE of a single notochord cell). At the opposite point around the circumference the cells extended protrusions both anteriorly and posteriorly (designated as anterior leading edge, ALE, and posterior leading edge, PLE, white arrows in Fig. 2E). The direction of these movements was parallel to the cylindrical axis of the notochord. The radial symmetry of each notochord cell with respect to the cylindrical axis was broken (compare Fig. 2E′ with C′ and D′). Notochord cells continued to seamlessly line the entire inner surface of the sheath while this cell shape change took place. As the result of these movements, the luminal spaces were pushed and appeared tilted. Toward the end of stage VI, a small portion of each notochord cell remained in contact with the entire circumference of the notochordal sheath (Figs. 2E′″ and F′″). At the end of stage VI, the ATE and PTE of the same cell came together (designated as joint trailing edge, JTE, red arrow in Figs. 2F and F′). This resulted in the merging of the anterior and posterior luminal domains. A cross section at the point of the merging showed that this was the last remaining sectional plane along the longitudinal axis where the cell kept its complete circumferential contact with the notochordal sheath (compare Fig. 2F′″ with F′). The advance of two leading edges was never equal (compare ALE and PLE in Fig. 2E′), so that no mirror symmetry with respect to the cylindrical axis of the notochord (Figs. 2H′ and H″). A cross section of notochord cell at the plane(s) shown in (A–H) respectively. At the beginning of stage IV, notochord cells have a “coin” shape (A and A′). In the next four hours cells elongate along the A/P axis (B and B′) and narrow in cross section (compare A′ and B′). The nucleus in this elongated cell is localized at the posterior end (“N in B′), while a constriction can be observed midway (white arrow in B and B′). At stage V, lumens emerge (“L′ in C and C′) and enlarge (“L″ in D and D′). Cells remain radially symmetrical with respect to the cylindrical axis of the notochord (C′ and D′). The cell’s main body in cross section resembles a round disc (“C″ and D″), whereas the portion encompassing the lumen resembles a ring (“C′″ and D′″). During stage VI, a certain point on the anterior edge and its opposing point on the posterior edge (designated as anterior trailing edge, ATE, and posterior trailing edge, PTE, red arrows in E′) on each cell recedes along the A/P axis, while the opposite points around the circumference (designated as anterior leading edge, ALE, and posterior leading edge, PLE, white arrows in E″) advance along the A/P axis. The cell thus loses its radial symmetry (“E′ and E″). Some portion of the cell remains in basal contact with the notochordal sheath at the entire circumference (“E′″). At the end of stage VI, two trailing edges of the same cell meet and merge into one (designated as joint trailing edge, JTE, red arrow in F′). The only portion of the cell that retains basal contact around the entire circumference is at the level of the JTE (F′″), compared with F′″. This basal contact is lost at stage VII, as the JTE detaches from the basal lamina and splits into two trailing edges (designated as trailing edge 1 or TE1, and trailing edge 2, or TE2, red arrows in G′ and G″, also in H″) which continue to crawl across the basal lamina. The vacat basal area is taken over by either the anterior or the posterior neighbor cell. Concurrently, two lumens are allowed to connect (white arrow in G′). Notochord cells further flatten and eventually assume an endothelial-like morphology (H and H″). Cross sections, the notochord now has two cells surrounding a central lumen (“L′″ in H″–H″′), white arrows indicate cell–cell boundaries. Anterior is to the left. Scale bars: 10 μm.

Stage VII (24–36 hpf): cell flattening, lumen joining and tube formation

At the beginning of stage VII the JTE split into two trailing edges (designated as trailing edges 1 and 2, or TE1 and TE2, respectively) that continued to retract (red arrows in Figs. 2G–G″, now in a direction that was orthogonal to the long axis of the notochord, relinquishing the inner surface of the sheath to either the PLE of the anterior cell or ALE of the posterior cell. For the first time, the basal surface of a single cell did not contact the entire circumference of the inner surface of the notochordal sheath (compare Fig. 2G″ with F″). Thus, at any given point along the A/P axis, the walls of the forming lumen were composed of two cells. In 3-D reconstruction and cross section, we observed where the TE′s retracted the two adjacent lumens fused (white arrow in Fig. 2G″). Each notochord cell further flattened and assumed an endothelial-like morphology (Figs. 2H and H″), occupying a portion of circumference under the inner surface of notochordal sheath (Figs. 2H″–H″″). By the end of this stage the 40 notochord cells formed a tube with large central lumen.

Cell shape changes and elongation during tube formation

During tubulogenesis the notochord cells changed shape dramatically from “coin” shaped to short cylinders, then became biconcave shaped, and finally the cells lost their radial symmetry and became endothelial-like. To quantify individual cell transformations and characterize the overall changes in notochord size as the result of these morphological changes, we measured the notochord length (Fig. 3A), the individual cell length (Fig. 3B), and the notochord diameter (Fig. 3C) at three time points: (1) before the elongation of individual notochord cells (beginning of stage IV, 14 hpf); (2) before the emergence of extracellular lumen (end of stage IV, 18 hpf); and (3) at the completion of tubulogenesis (36 hpf). The diameter of the lumen at 36 hpf was also measured. The average diameter or thickness of individual notochord cells (Fig. 3D) at the three time points, the total volume of the cells at the three time points, and the total volume of luminal matrix in the tubular notochord at the last time point (Fig. 3E), were calculated.

As the result of cell elongation and narrowing during stage IV (14 hpf to 18 hpf), the overall length of notochord increased by a factor of 1.8 (from 256.4 μm to 466.6 μm, Fig. 3A). During the subsequent development (18 hpf to 36 hpf), the average notochord cell length increased by 4.3-fold (from 13.1 μm to 55.9 μm, Fig. 3B), but the total notochord length increased further only by 1.7-fold in the same period (from 466.6 μm to 795.2 μm, Fig. 3A, see Discussion). Thus the entire post-C/E morphogenesis brought about a further 3.1-fold increase in the total notochord length.

The average diameter of the notochord decreased by 1.3-fold prior to lumen formation (between 14 hpf and 18 hpf; Fig. 3C) as a result of individual cell narrowing (Fig. 3D). While this decrease of notochord diameter was reversed during the subsequent stages (stages V–VII, Fig. 3C), the notochord cells themselves continued to reduce their diameters as the result of epithelialization (Fig. 3D).

Fig. 2. Cell shape changes during notochord tube formation. (A–H) Confocal images of notochord cells expressing GFP. (A′–H′) 3-D reconstruction of the same cell(s) in (A to H). (A′–H′″″) Cross section of notochord cell at the plane(s) shown in (A–H) respectively. At the beginning of stage IV, notochord cells have a “coin” shape (A and A′). In the next four hours cells elongate along the A/P axis (B and B′) and narrow in cross section (compare A′ and B′). The nucleus in this elongated cell is localized at the posterior end (“N in B′), while a constriction can be observed midway (white arrow in B and B′). At stage V, lumens emerge (“L′ in C and C′) and enlarge (“L″ in D and D′). Cells remain radially symmetrical with respect to the cylindrical axis of the notochord (C′ and D′). The cell’s main body in cross section resembles a round disc (“C″ and D″), whereas the portion encompassing the lumen resembles a ring (“C′″ and D′″). During stage VI, a certain point on the anterior edge and its opposing point on the posterior edge (designated as anterior trailing edge, ATE, and posterior trailing edge, PTE, red arrow in E′) on each cell recedes along the A/P axis, while the opposite points around the circumference (designated as anterior leading edge, ALE, and posterior leading edge, PLE, white arrows in E″) advance along the A/P axis. The cell thus loses its radial symmetry (“E′ and E″). Some portion of the cell remains in basal contact with the notochordal sheath at the entire circumference (“E′″). At the end of stage VI, two trailing edges of the same cell meet and merge into one (designated as joint trailing edge, JTE, red arrow in F′). The only portion of the cell that retains basal contact around the entire circumference is at the level of the JTE (F′″), compared with F′″. This basal contact is lost at stage VII, as the JTE detaches from the basal lamina and splits into two trailing edges (designated as trailing edge 1 or TE1, and trailing edge 2, or TE2, red arrows in G′ and G″, also in H″) which continue to crawl across the basal lamina. The vacat basal area is taken over by either the anterior or the posterior neighbor cell. Concurrently, two lumens are allowed to connect (white arrow in G′). Notochord cells further flatten and eventually assume an endothelial-like morphology (H and H″). In cross sections, the notochord now has two cells surrounding a central lumen (“L′ in H″–H″′), white arrows indicate cell–cell boundaries. Anterior is to the left. Scale bars: 10 μm.
The volume of notochord did not increase significantly during stage IV, as notochord cells elongated but also narrowed. However, the total volume of the notochord increased significantly during the subsequent stages (by 2.9-fold, from 156334.6 μm³ to 455645.8 μm³, Fig. 3E), due primarily to the formation of extracellular lumen (from 0 to 213429.1 μm³). At 36 hpf cells made up approximately half of the notochord volume, the remainder being lumen.

Crawling behavior of individual notochord cells

During stage VI, when the biconcave notochord cells began to lose their cylindrical shape, they protruded long leading edges either primarily anteriorly (Figs. 4A–A'), or primarily posteriorly (Figs. 4B–B'), and eventually bi-directionally (Figs. 4C–C'). These long protrusions were always associated with a tilting of adjacent lumens (outlined in Figs. 4A–C). As a cell crawled along the inner surface of notochordal sheath, several subcellular regions were recognized. Large new extensions and lamellipodia were observed to make up the bulk of the ALE and PLE. These protrusions occupied the new inner surface of the sheath that was not occupied by the cell previously (the area previously occupied by the cell before protrusions were made is between two dashed lines in Fig. 4C'). Actin was concentrated at the leading edges (Fig. 4D and Supplementary movie), suggesting notochord cells actively crawled. These actin-based edges were decorated with fine “ruffles”, indicating a dynamic interaction taking place between neighboring cells as each of them moved longitudinally. To confirm that the crawling activity at this stage was essential for notochord tubulogenesis, we treated embryos with actin polymerization inhibitor Latrunculin B at 23 hpf, when lumens was enlarging but before notochord cells lost their cylindrical symmetry, for one hour and observed the notochord morphology at 26 hpf. We observed a complete blockage of cell

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**Fig. 3.** Biometric analysis of Ciona notochord during tubulogenesis. The calculations were done from measurements of five notochords at each of three time points. (A) Length of notochord. (B) Average length of individual notochord cells. (C) Average diameter of notochord. (D) Average diameter of notochord cells at 14 and 18 hpf, and average thickness of endothelial-like notochord cells at 36 hpf. (E) Total volume of entire notochord (black) and its two components, cells (purple) and lumen (white, at 36 hpf). For calculations see Materials and methods and Supplementary Fig. 1.
shape changes and tube formation in treated embryos (Fig. 4F, compared with Fig. 4D), while the lumens were unaffected.

**Emergence of luminal domains and luminal extracellular matrix formation**

To investigate the development of the lumen, we used an antibody that recognizes the *C. intestinalis* homologue of SLC26-2. Ci-SLC26-2 belongs to the large soluble carrier 26 family, whose members encode small ion transporters that occupy cell surfaces (Ohana et al., in press). The Ci-SLC26-2 antibody stained the apical surface of epithelial cells surrounding the lumen in the sensory vesicle of the tadpole (data not shown). At 18 hpf, before the appearance of lumens, staining with this antibody was observed in restricted regions at the center of the anterior and posterior membranes of notochord cells (arrowheads; Fig. 5A). As lumens between notochord cells began to form (20–22 hpf), SLC26-2 staining was observed only in membrane lining the lumen (Figs. 5B–C). Thus, at this stage three distinct membrane domains were recognized: (1) the luminal domain, which was SLC26-2 positive and adjacent to the extracellular lumen; (2) the lateral domain which was SLC26-2 negative and in contact with the neighboring notochord cell; and (3) the basal domain which contacted the basement membrane and notochordal sheath. The SLC26-2 positive domain enlarged concomitant with the increase of the lumen. At the end of stage VI (24 hpf), luminal domains of each cell began to fuse (Fig. 5D, arrow indicates the site of fusion). At stage VII the SLC26-2 expression was observed throughout the inner (luminal) membrane of the now endothelial-like cells (Fig. 5E).
Emergence and expansion of the luminal domain before and during lumen formation. Confocal images from the midline of notochord cells stained with antibody against Ciona homolog of SLC26-2 (red) for luminal domain, and BODIPY phalloidin for filamentous actin (F-actin, green) to highlight cell membrane. (A) SLC26-2 positive domains (arrowheads) first appear in the center of anterior and posterior cell surfaces. (B) The SLC26-2 positive domains enlarge and occupy the portions of notochord cell contacting extracellular lumens. (C) The luminal domains expand as the lateral domains abutting two notochord cells retract. (D) The anterior and posterior luminal domains of the same cell meet and fuse (arrow). (E) After the lumens have fused, the SLC26-2 positive domain occupies the luminal side of the endothelial-like notochord cell. Unknown structures that express SLC26-2 appear inside the luminal spaces (positive domain occupies the luminal side of the endothelial-like notochord cell. The same cell meet and fuse (arrow). (E) After the lumens have fused, the SLC26-2 abutting two notochord cells retract. (D) The anterior and posterior luminal domains of (B) The SLC26-2 positive domains enlarge and occupy the portions of notochord cell contacting extracellular lumens. (C) The luminal domains expand as the lateral domains abutting two notochord cells retract. (D) The anterior and posterior luminal domains of the same cell meet and fuse (arrow). (E) After the lumens have fused, the SLC26-2 positive domain occupies the luminal side of the endothelial-like notochord cell. Emergence and expansion of the luminal domain before and during lumen formation. (F) The emergence of luminal domain lags behind in the posterior-most notochord and predicts the temporal order of the lumen formation (arrowheads indicate lumen formation proceeds at different paces; arrows indicate that lumen has emerged. At 24 hpf, a new AJ (arrow in Figs. 7C and D) appeared to form between two previous separate cells a and c. The newly formed AJ domains ran perpendicular to the A/P axis of the notochord, while previous AJ domains (between cells a and b, and b and c in Fig. 7C) now formed an angle from the A/P axis.

At 36 hpf, once the notochord formed a tube with a single central lumen, notochord cells further flattened and became endothelial-like (Fig. B). At this stage E-cadherin was localized at junctions between the flattened cells (Figs. 8A and A′). A 3-D reconstruction of the notochord labeled with E-cadherin and mGFP is shown in Figs. 8B–B′, and the diagram of the cell edges is shown in Fig. 8C. At any cross section along the A/P axis two notochord cells made up the annular wall of the lumen. Each notochord cell resembled a rough hexagon and made contact with four neighbors (e.g., cell c with cells a, b, d and e in Fig. 8C).

**Cell adhesion junction remodeling during tubulogenesis**

As the lumens enlarged and luminal membrane domains expanded, the adhesion junctions between neighboring cells were remodeled. We analyzed this process by tracking the expression of the adherens junction (AJ) protein E-cadherin (Angst et al., 2001). E-cadherin was localized at the lateral membrane domains, where neighboring notochord cells contacted. When observed at 18 hpf, the lateral domains of notochord cells expressed a complete disk of E-cadherin (Figs. 7A–A′). As the lumens formed, beginning at approximately 20 hpf (Figs. 7B–B′), AJs began to disassemble from the center of the disc, transforming the lateral domain into an annulus (Fig. 7B″). The thickness of the E-cadherin expressing domain reduced as the lumen enlarged. At 24 hpf, a new AJ (arrow in Figs. 7C″ and D″) appeared to form between two previous separate cells a and c. The newly formed AJ domains ran perpendicular to the A/P axis of the notochord, while previous AJ domains (between cells a and b, and b and c in Fig. 7C″) now formed an angle from the A/P axis.

To quantify the dynamic changes of the different membrane domains during morphogenesis, we collected confocal images of notochord cells expressing a membrane-localized GFP. The surface area of the different domains were determined in the middle of stage IV (16 hpf), at the end of stage IV immediately before the appearance of lumens (18 hpf), and at stage V when the lumens enlarged (21 hpf and 22 hpf). In each time point only those cells at the center of the notochord, where cells were found to be more homogenous in shape and size, were used for these measurements. As the result of cell elongation and narrowing during stage IV, the total plasma membrane was greatly reduced (by 1.6-fold, from 2775.8 μm² to 1715.4 μm², Figs. 6A and B). This change was primarily due to the reduction of lateral surface (by 2.5-fold, 1818.1 μm² to 719.8 μm²), while the basal domain remained largely unchanged. Once lumens formed, the luminal domain of the membrane expanded by 2.2-fold in the course of 1 h (from 358.9 μm² to 805.9 μm², Figs. 6C and D), while the lateral domain reduced by 1.6-fold (from 491.2 μm² to 307.7 μm²), resulting in larger anterior and posterior surface areas of the notochord cells (by 1.3-fold). The lateral domains continued to diminish while the luminal domains expanded throughout stages V and VI.

**Fig. 5.** Emergence and expansion of the luminal domain before and during lumen formation.

**Fig. 6.** Biometric analysis of notochord cell membrane domains. The surface areas were calculated from measurements of 4 to 5 GFP-labeled cells located in the central region of notochord (see Materials and methods and Supplemental Fig. 2). (A) In the middle of stage IV (16 hpf) the lateral domain of notochord cells is larger than the basal domain. (B) After elongation (18 hpf), the total surface of notochord cells is reduced, and the basal domain is now larger than the lateral domain. (C) The lateral domain further reduces as the luminal domain emerges (21 hpf). (D) The luminal domain continues to enlarge while the lateral domain shrinks, as the lumens increase (22 hpf). The sum of both domains is larger than in the previous time point.
Discussion

Fig. 9 summarizes different processes that take place during Ciona notochord morphogenesis following C/E. The morphogenetic transformation begins with a simple process of cell elongation and narrowing at stage IV. How this cell shape change is initiated and achieved is not understood. In particular, the mechanism by which the lateral domain of each notochord cell is greatly reduced (compare Fig. 6B with A) is unknown. Starting from the end of stage IV, notochord cells exhibit dynamic cellular behaviors and dramatic morphological transformations ensue (Fig. 9A).

Mesenchymal–epithelial transition

The morphological processes of tube formation can be categorized broadly into two types. In the first type, tubes arise from a polarized epithelium by either wrapping or budding (Chung and Andrew, 2008; Lubarsky and Krasnow, 2003). In the second type, tubes are constructed from a cluster of cells or individual cells that are not epithelial, and the cells polarize as the tube forms. Ciona notochord tube formation clearly falls into the second category. Before the onset of tubulogenesis, individual notochord cells possess only two interfaces: notochord cell/cell interface and notochord cell/sheath interface. Shortly before the extracellular lumen forms, a third membrane domain arises at the center of juxtaposed notochord cells, and then expands to encompass the emerging lumen (Fig. 9B). As lumens form and accumulate, individual notochord cells possess three membrane domains: luminal domain (at notochord cell/lumen interface); lateral domain (at notochord cell/cell interface); and basal domain (at notochord cell/sheath interface). Ciona notochord cells can now be considered as modified epithelial cells, each with one circumferential basal domain, two lateral domains (one anterior; one posterior) and two apical domains (luminal domains; one anterior, and one posterior). Although the morphology of this epithelial cell is highly unusual, and unprecedented to our knowledge, we think our model is well justified for the following reasons. First, both apical (luminal) domains express the apical marker SLC26-2 and contact lumen. Second, the transformation from a modified epithelial cell with two apical domains at opposing ends to an endothelial-like cell lining the notochord tube is accomplished by the expansion and merging of the anterior and posterior apical domains. Topologically the two apical domains before the transformation and the single apical domain after are the same.

Apical/basal axis

Whereas conventional epithelial cells have a single linear A/B axis, we propose a model for the notochord cell in which the apical domains are initially present at the two ends of the cylindrical cell. These two apical domains are at right angles to the basal domain that comprises the circumference of the cell (see Fig. 9B). During stage VII the two apical domains merge as the trailing edges retreat and the segment of basal domain previously separating them is eliminated. At the completion of this process the A/B axis now appears more conventional with a single continuous apical domain facing the lumen and a single basal domain facing the basement membrane and sheath (Fig. 9B).

Lumen formation and biogenesis of apical membrane

The differentiation of apical membrane de novo at the center of lateral domains precedes the appearance of any visible lumen (see Figs. 5A and F). When the lumens begin to form, the apical domains grow and curve to the insides of the cells (Fig. 9B). We measured a 2.2-fold increase of the surface area of apical domain within one hour during stage V. This expansion cannot be due only to the conversion of lateral domain into apical domain because the lateral domain surface area only decreases by a factor of 1.6 (i.e., the sum of apical domain and

![Fig. 7. Adhesion remodeling as notochord cells undergo mesenchymal-epithelial transition.](image-url)
lateral domain has increased). Two processes may be responsible for the expansion of the apical domain. First, some lateral domain is likely converted by remodeling. Second, new membrane biogenesis may produce more apical domain to accommodate the expansion of extracellular lumen. Nascent membrane may be supplied and delivered to the apical site of notochord cells in small intracellular vesicles which also carry extracellular matrix. We speculate that the latter process, which couples both membrane biogenesis and extracellular matrix production simultaneously, and found in other tubulogenesis processes (Bryant and Mostov, 2008; Jayaram et al., 2008), is the primary cause of the apical domain expansion.

Reduction of lateral domains

The reduction of lateral domains occurs in two phases. First, during stage IV, the lateral domain surface area decreases as notochord cells change from a “coin” shape to a cylindrical shape, while the basal domain remains unchanged. The second phase occurs at stages V and VI, when the lateral domain is reduced by 57.2% within a course of 4 h. Trafficking of AJ complexes, especially cadherins, have been investigated extensively in other systems (Bryant and Stow, 2004). Cadherins can be endocytosed via several routes, including clathrin-dependent, and clathrin-independent pathways, and caveolae. Cadherin expression is also regulated by receptor tyrosine kinases (Cavallaro and Christofori, 2004). The precise molecular mechanism by which AJ complexes are removed from the notochord cell is unknown. It is intriguing to note that several relevant players are specifically expressed in Ciona notochord cells during tubulogenesis. These include the ascidian ortholog of caveolin, and several receptor tyrosine kinases (Ghost database, http://hoya.zool.kyoto-u.ac.jp/download.html). Further studies are required to examine their roles in this process.

Cell crawling

Our observation reveals that notochord cells exhibit dynamic movements during tubulogenesis. Each notochord cell initiates two types of crawling behavior: 1) ATE and PTE move toward each other; 2) ALE and PLE move away from each other (Figs. 9B and D). The direction of movement of two trailing edges is parallel and opposite to the direction of the movement of two leading edges. As a result, notochord cells simultaneously shorten at one point of the circumference and elongate at the opposite point. Their radial symmetry along the cylindrical axis is lost in the process. It is not known what process breaks this symmetry and initiates the movements at discreet points on the circumference of the cells. It is possible that these processes are determined stochastically, although there ultimately needs to be a degree of cell to cell coordination to make the fully enclosed lumen, suggesting that initial stochastic biases leading to protrusive activity may spread among the cells. It remains unknown if there is a correlation or a causal relationship between the points of advance and the points of retraction at the anterior (or the posterior) side of each cell. Our preliminary observations suggest the two points are usually 180° apart (data not shown). In addition, it is unknown if a correlation, or even a causal relationship, exists between the location of ALE and that of PLE (or between ATE and PTE) around the circumference. These parameters are important for understanding if and how the crawling behaviors of the 40 notochord cells are coordinated in their unique geometric context and if any correlation with the body axes, including dorsal/ventral and medial/lateral axes, might exist that suggests any influence from the notochord’s surroundings.

The crawling behavior can also be visualized in 2-D when we examine the movement of the basal surface of the cell on its substrate, the inner surface of the notochordal sheath (Fig. 9D). We suggest that
Fig. 9. Schematic views of Ciona notochord during tube formation. (A) Cartoon depicting the median section of three consecutive notochord cells (a, b, and c) at different stages of tubulogenesis. (B) Cartoon depicting the median section of cell a in (A). At the beginning of stage V, novel membrane domains (red) appear that encompasses the emerging extracellular lumens. From this point, each notochord cell possesses three membrane domains: apical/luminal domain (red), lateral domain (green), and basal domain (light blue). The cell can be viewed as a highly modified epithelial cell, with two separate apical domains and two separate lateral domains at the opposing ends, and curved A/B axes (brown arrows, only two are drawn). Concurrent with the enlargement of extracellular lumens, apical domains expand and lateral domains retract. At the end of stage V notochord cells in median section resemble steep biconcave lenses. The radial symmetry along the cylindrical axis of the notochord is still maintained. During stage VI, as the lateral domains of a notochord cell are reduced to a minimum, a certain point on the anterior rim (anterior trailing edge, ATE) and its opposing point on the posterior rim (posterior trailing edge, PTE) of an individual cell begin to retract (orange arrows). While at the opposite points in the circumferences, anterior and posterior edges (anterior leading edge, ALE, and posterior leading edge, PLE) of the same cell begin to protrude and extend (magenta arrows). The direction of these movements is parallel with the cylindrical axis of the notochord. The extent by which these edges retract and extend is not equal. As the result of these movements the radial symmetry of the cell with respect to the cylindrical axis is lost. At the end of stage VI, the ATE and PTE meet and merge (designated as joint trailing edge, JTE). At the same time, the basal domain previously located between ATE and PTE are eliminated, and the anterior and posterior apical domains join. At the beginning of stage VII, after the JTE has detached from the inner surface of the sheath and the vacated surface has been occupied by the leading edge of neighbor cell (b), lumens fuse. The existence of JTE is transient as it quickly splits into two trailing edges (designated as trailing edge 1 and 2, or TE1 and TE2, respectively). Both TE1 and TE2 retract across the inner surface of the sheath in opposite directions (orange arrows), effectively bringing themselves closer to each other. The endothelial-like notochord cells after tube formation occupy only a portion of the notochord circumference. (D) Schematic diagrams of the basal domains as viewed from the basal surface of the notochord. Each notochord cell simultaneously extends bi-directionally (to different extents) along the longitudinal axis at one point of the circumference (for example, at 180°), and retracts at the opposite point (at 0°). This movement transforms the shape of basal domain from a rectangle to an uneven hexagon. When both ATE and PTE merge and form a transient JTE, the shape of the basal domain is roughly a diamond at the end of stage VI. JTE quickly splits into TE1 and TE2 as it loses its contact with the notochordal sheath. The direction of retraction then turns from previously parallel, to the direction of extension of leading edges, to orthogonal. These movements eventually transform the shape of the basal domain back to a rough hexagon that is elongated along the longitudinal axis of the notochord. At stage VII, the cross section at any point along the tube is composed of two cells instead of one, as is the case at stages IV to VI. This configuration effectively reverses the result of intercalation that occurs prior to the stage IV, which arranges the notochord cells into a single file. (E) Schematic illustration of notochord cell/cell junctions viewed from the basal surface of the notochord flatten out onto a two dimensional space. New cell junction forms during stage VII between two notochord cells (a and c) which are previously separated (by cell b).
when the ATE and PTE merge, a transient single edge is formed (JTE in Fig. 9D, also see Figs. 2F and F'), which then splits into two new trailing edges (TE1 and TE2 in Fig. 9D; see also Figs. 2G and C'). These two trailing edges further regress on the inner surface of the sheath toward each other, and the direction is now orthogonal to both the direction of the previous ATE and PTE, and that of ALE and PLE which continue to advance along the A/P axis. The endothelial-like notochord cells during stage VII elongate bi-directionally along the A/P axis and narrow bi-directionally along the medial–lateral axis.

The advance of leading edges is captured in Fig. 4C'. We have demonstrated that cells at stages VI and VII extend long protrusions. The front of these cellular extensions consists of lamellipodium with ruffled edges. These membrane ruffles may represent dynamic interaction between the advancing edge of one cell and the receding edge of its neighbor. The notochord cells at stage VII resemble conventional motile cells such as fibroblasts and keratocytes crawling on flat substrata in vitro. The difference is that each notochord cell actively advances at two opposing fronts, while retracting at both flanks. Nevertheless, notochord cells may move across the inner surface of the notochordal sheath using the same conserved cytoskeleton machineries, and the similar basic mechanism of motile surface of the notochord cells of some ascidians change shapes and reorient themselves without making any lumen (Burighel and Cloney, 2001; Etienne-Manneville, 2004; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Rodriguez et al., 2003). These include: (1) actin-dependent extension of the leading edge; (2) formation and stabilization of new adhesive junctions between the cell and its substrate; (3) active contraction of the cortex and/or internal cytoplasm; and (4) regulation of actin filament and focal adhesion by microtubules. While the role of each of these mechanisms has to be verified in the notochord context, the intriguing question is how a notochord cell mounts a two-front advance (and retraction). One possibility is that the retraction of two trailing edges at the flanks of the cell results from the contraction of cell cortex and cytoplasm as a passive response to the advance of two leading edges.

It is unknown if the crawling behavior is directly related to the accumulation of extracellular matrix in the lumens. It is conceivable that the luminal matrix exerts force on the notochord cells and causes cell shape changes and cell movement. However, the result of our actin inhibitor study indicates that active crawling of individual notochord cells is responsible for the cell shape change and tube formation. Morpholino knockdown of Ciona ERM, a modulator of actin cytoskeleton which is highly expressed in Ciona notochord, results in a failure of notochord morphogenesis after C/E (Hotta et al., 2007). The essential role of intrinsic crawling behavior is further supported by the observation that notochord cells of some ascidians change shapes and reorient themselves without making any lumen (Burighel and Cloney, 1997; Jiang and Smith, 2007).

Remodeling of adhesion junction and reversal of intercalation

At the completion of C/E, notochord cells form two types of junction. The first is the junction between notochord cells; the second is the junction between notochord and basal lamina at the surface of the notochord. During subsequent development, as the leading and trailing edges of the notochord cells crawl along the inner surface of the sheath, two fundamental junctional changes take place. First, at the point where the ATE and PTE meet, each notochord cell loses its contact with the basal membrane. This is illustrated by the cell shown in cross section in Fig. 9C. Second, the inner surface of sheath vacated by each cell as the result of trailing (illustrated by cell b in Figs. 9D and E) is taken up by the PLE of the anterior cell a or the ALE of the posterior cell c, which for the first time forms cell/cell junction with the ALE of the posterior cell c or the PLE of the anterior cell a, respectively (Figs. 9D and E). Thus, throughout this process, the spatial relationships among three consecutive cells (a, b and c) are rearranged. The intercellular junction between two neighbor cells (a and b; b and c) is reduced, and a new intercellular junction between two cells (a and c) previously not in contact is established. The spatial reorganization of adhesion junctions during notochord tubulogenesis is the reverse of the cell intercalation process seen in Drosophila amnioserosa epithelial cells and tracheal system (Bertet et al., 2004; Jazwinski et al., 2003). We designate this morphogenetic process in Ciona notochord as reverse convergence because it effectively reverses the intercalation during stage III which results in notochord cells converging into a single file. As the result of reverse convergence at any given point along the A/P axis there are two notochord cells (Figs. 2H, 2I and 8B). This helps to explain how despite an approximately 4-fold increase of individual notochord cell length during stages V, VI, and VII (Fig. 3B), the total notochord length only increases by approximately 2-fold (Fig. 3A). This reverse convergence process involves relative cell movement along the A/P axis of the notochord, reflected by the anterior and posterior positioning of the lamellipodia in each notochord cell (Fig. 4C), in contrast to the medial/lateral cell movement of C/E. How the notochord cells initiate and accomplish these two cell intercalation processes along two orthogonal axes warrants future investigation. Whether the same polarity signals, which have been implicated in the early C/E movement in diverse systems including ascidians (Keller, 2002), or other positional cues are responsible for setting up this second intercalation, is unknown.

Ascidian notochord as a model system for tubulogenesis

The multicellular tubes that are found throughout the metazoans are constructed with different numbers of cells, have varying shapes, sizes, and branching patterns, and arise by apparently different developmental processes. Given this diversity, it remains to be determined if there are common underlying mechanisms of tubulogenesis, as has been suggested by several model systems (Bryant and Mostov, 2008; Chung and Andrew, 2008; Lubarsky and Krasnow, 2003).

Many tubes are constructed from cells that are initially not epithelial. Three mechanisms have been described. In the first mechanism, a thick cylindrical mass of cells creates a central cavity by eliminating cells in the center via apoptosis. This cavitation mechanism occurs, for example, during the formation of proamniotic cavity (Coucouvanis and Martin, 1995). In the second mechanism, cells organize into a thin cylindrical rod and then create a lumen with no cell loss. This cord hollowing process takes place in zebrafish and C. elegans gut formation (Bagnat et al., 2007; Leung et al., 1999) and tube formation of MDCK culture cells (Pollack et al., 1998). The third mechanism, cell hollowing, is seen in the formation of certain capillary vessels in the vertebrates (Folkman and Haudenschild, 1980; Kamei et al., 2006). Cell hollowing involves one cell producing a large intracellular vacuole that eventually opens to the exterior, although the details of this process remain controversial (Blum et al., 2008). Although the initial configuration of notochord cells (lining up in a single file) strongly resembles that of endothelial cells at the onset of capillary formation, we have not seen large intracellular vacuoles in notochord cells at any stage. There is no evidence that membrane fission and fusion, processes that are required for intracellular vacuoles to connect to the exterior, are taking place in the Ciona notochord. Despite the difference between the initial organization of cells in MDCK system, in which cells are assembled into thin rods 2 to 3 cells thick, and the Ciona notochord, there are some similarities in the subsequent morphogenetic events. First, in both cases apical domains arise de novo in the non-epithelial cells. Second, extracellular matrix is synthesized and deposited at the apical side to form the lumen of the tube. In neither cell type are intracellular matrix-containing vacuoles present. The difference is that, in the notochord, two apical domains are formed in each cell and extracellular lumens are deposited on both sides. The tubulogenic process in the notochord therefore requires additional steps: the joining of two apical domains,
and consequently the fusion of lumens on both sides. This is accomplished by the movement of two trailing edges toward each other which bring two apical domains to juxtapose.

In MDCK cells small exocytotic vesicles are thought to deliver both new membrane and matrix simultaneously (Bryant and Mostov, 2008; Vega-Salas et al., 1987). The same process may occur in Ciona notochord cells. Because the fate of these small exocytotic vesicles and that of the large intracellular vacuoles in endothelial cells of the capillaries are the same, being the apical membrane of the later endothelial (-like) cells, topologically they can be regarded as equal. Thus the cell hollowing process and the cord hollowing process, as well as Ciona notochord tubulogenesis, may be more similar than they appear. The difference may be simply that, in cell hollowing, small intracellular vesicles merge into a large vacuole before fusing to the plasma membrane, whereas in cord hollowing and in the notochord, small individual vesicles migrate and fuse to the plasma membrane directly. It is interesting to note that notochords of many chordates outside of Urochordate subphylum do not make tubes. Rather, the notochord cells possess large intracellular vacuoles. It is not known which of these notochord forms is ancestral, but as we have speculated previously (Jiang and Smith, 2007), the transition between these two forms may be as subtle as alternative trafficking of small intracellular vesicles to the plasma membrane versus fusion of these vesicles and retention within the cytoplasm.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.015.

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