The cellular and molecular mechanisms of the notochord tubulogenesis in the marine invertebrate *Ciona intestinalis*

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By

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ABSTRACT

The biological tube is the fundamental structural design of most internal organs and body systems. Tubulogenesis involves a series of coordinated cellular processes, failure of any of which can cause severe dysfunctions in the organism. Therefore, the study of tube formation can increase our knowledge of the cellular and developmental mechanisms of morphogenesis, and furthers our understanding of a number of human diseases.

Among various models for tubulogenesis in animal development, *Ciona intestinalis* notochord development features a unique cellular morphogenesis that constructs a simple tube. The mechanism underlying the notochord tubulogenesis is largely unexplored, and is what this thesis wishes to address.

First, we explore the transcriptome of the *Ciona* notochord by microarray analysis, and expand the current inventory of notochord-specific genes. Second, employing 3D live confocal imaging, we demonstrate that components of the cytoskeletons, especially actin filaments, are involved in several cellular morphogenetic events during notochord tube formation. Third, we characterized a notochord-specific anion transporter, *Ci*-Slc26aα, and reveal that its transport activity is essential for notochord lumen formation. Detailed analysis indicates that lumen formation is independent from other processes leading to notochord tubulogenesis.

Overall, this work contributes to the understanding of molecular and cellular mechanisms of *Ciona* notochord tubulogenesis, and the roles of a Slc26 family transporter in the context of lumen formation.

1. GENERAL INTRODUCTION

1.1. Tubulogenesis

1.1.1. Biological tubes

Biological tubes can be found in most multi-cellular organisms and present a vast diversity of sizes, shapes, and connecting patterns. Tubular systems can traverse the whole body, sprout into organs, or form the basic structural unit of internal organs. They confer important aspects of function by transporting and exchanging essential biological materials like air, water and nutrients, or by providing a specific internal compartment for physiological processes (West et al., 1999; Lubarsky and Krasnow, 2003; Baer et al., 2009). Tubulogenesis, the formation of a tube, involves sequential steps of coordinated cellular morphological changes. Improper tube development leads to malfunction or loss of the tubular structures, and causes pathological changes of internal organs or systems, such as polycystic kidney disease (Boletta and Germino, 2003). The study of tubulogenesis is therefore of interest from both biological and medical perspectives.

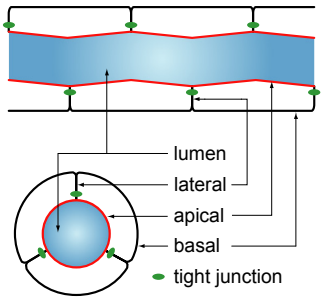

Fig. 1. The structure of a simple biological tube. It is composed of a single layer of epithelial cells (white areas) that encloses a central lumen (blue area). Upper, longitudinal view; lower, transverse view. black line, lateral and basal membrane of the epithelial cell; red line, apical membrane. If not specified, the colour settings in this figure also apply to other figures in this thesis.

In its simplest configuration, a biological tube is composed of a single layer of epithelium (white areas, Fig. 1) that encloses a central lumen (blue area, Fig. 1). The membrane surface of the epithelial cells that is in direct contact with the lumen is usually specified as the apical (luminal) domain (red line, Fig. 1). The apical domain provides an interface where the lumen exchanges essential materials with the cytoplasm, and through the latter further exchanges

these with the surrounding environment (Lubarsky and Krasnow, 2003; Bryant and Mostov, 2008). In the rest of this chapter, I will briefly introduce recent researches and current understanding of tubulogenesis, as inferred from a number of tubular systems in metazoans.

1.1.2. Models of tubulogenesis

The process by which the tubular epithelium and the lumen are built defines the tubulogenesis models, which have been systematically categorised in two reviews (Lubarsky and Krasnow, 2003; Baer et al., 2009).

The tubular epithelium can be reorganized from a pre-existing epithelium or generated *de novo* from mesenchymal cells. A pre-existing epithelium builds a tube either by *wrapping* itself around a central space to enclose the lumen, for example in vertebrate neural tube formation (Colas and Schoenwolf, 2001), or by *budding* from a pre-established tube through a branching process, for example in *Drosophila* trachea and salivary gland system (Kerman et al., 2006).

De novo tube formation involves a mesenchymal-epithelial transition (MET) to create an epithelium with nascent apical/basal (A/B) polarity and the production of a lumen at the apical membrane. During *cavitation* in mammalian salivary gland formation (Tucker, 2007), a central cavity is created after the cells in the central region are eliminated in a thick cylindrical mass of cells. In *cord hollowing*, as in zebrafish gut development and MDCK cell tube formation (Pollack et al., 1998; Belting and Affolter, 2007), an intercellular space is created by separating the attached plasma membranes apart, without eliminating any cell. The *cord hollowing* also features *de novo* formation of apical membrane and lumen, which will be further discussed in this section.

In addition to the above modes of tubulogenesis, some of the simplest tubes are formed through *cell hollowing* and *cell wrapping, which* are similar to *cord hollowing* and *wrapping* respectively, but distinct in that the morphogenetic processes occur in single cells (Lubarsky and Krasnow, 2003; Baer et al., 2009).

Recent studies have recognized some common cellular processes, such as cell polarization (Bryant and Mostov, 2008), apical domain and lumen formation (Iruela-Arispe and Davis, 2009), and lumen connection, which are involved in all *de novo* tubulogenetic processes.

1.1.3. Apical-basal polarization

The first step of tube formation begins with sensing and defining the external (related to the surrounding environment) and the internal (luminal) orientations of a cluster of cells ready for tubulogenesis. This process is called apical-basal polarization (Pollack et al., 1998; Lubarsky and Krasnow, 2003). Tube formation continues with specifying and sustaining the basal (outward) and apical (luminal) membrane surfaces of the epithelial cells. In order to establish a same orientation of the cell-polarity axis, the polarization must be concerted among all the cells. It has therefore been suggested that, the asymmetrical distribution of polarity complexes, cytoskeletal and junctional components, as well as the overall intracellular trafficking, must also follow the same pattern, presumably coordinated by molecular signalling (Bryant and Mostov, 2008).

The polarity signal originates from outer environment cues, such as the extracellular matrix (ECM), which provides positional information and differentiation cues for the tissues, and ultimately influences cell polarity. Cells detect the ECM characters through an interaction mediated by receptors (*e.g.*, β1-integrin), or through communication with neighbouring cells with adhesion molecules such as cadherins (Kass et al., 2007). β1-integrin and cadherins are required for controlling the biogenesis of phosphatidylinositol (PtdIns), which is fundamental in generating and maintaining cell basolateral identity, and for the maintenance of polarity through small GTPase mediated activation of PtdIns signalling cascades. During cell polarization, members of the Rho family of GTPases, RhoA, RAC1 and CDC42, interact with the PAR complex and act as a signalling hub in controlling various aspects of asymmetric cellular organization and polarity. The GTPases therefore play integral roles in essential polarization events, like cell cytoskeletal arrangement, membrane trafficking and ECM interactions (Jaffe and Hall, 2005). The PAR signalling results in the *de novo* acquisition of epithelial characteristics such as A/B polarity, epithelial-type junction remodelling and accumulation of apical materials, and eventually transforms the

mesenchymal cells into epithelial cells, a process called mesenchymal-epithelial transition (MET) (Bryant and Mostov, 2008).

1.1.4. Cytoskeletons in tubulogenesis

The activity of PAR complex triggers a series of well-defined signal transduction pathways mediated primarily by Rho GTPases, leading to specified cellular events through the formation and reorganization of the cytoskeleton (Jaffe and Hall, 2005). Cytoskeletons are mainly composed of three kinds of filaments: actin filaments, microtubules, and intermediate filaments. These filaments interact with hundreds of accessory proteins, which help organise the cytoskeletons into complex and dynamic networks (Alberts et al., 2008). The actin filament and microtubule networks provide mechanical support to the fragile plasma membrane and protect cells from environmental stresses. They are also responsible for cell shape changes and cell mobility in nearly every morphogenetic process (Fig. 2) (Alberts et al., 2008; Pollard and Cooper, 2009).

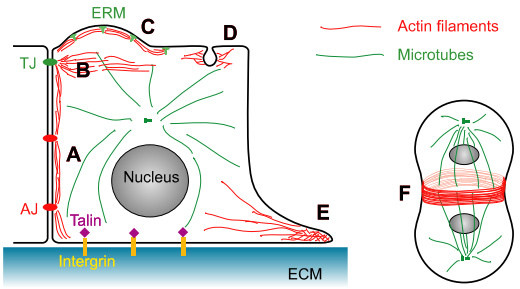

Fig. 2. Several populations of actin filaments in an endothelial cell (left) and in a dividing cell (right). (A) Cortical actin filaments provide mechanical support to maintain the cell shape. (B) Circumferential actin filaments contribute to the formation and maintenance of tight junction (TJ). (C) Apical actin filaments and ERM help shaping the apical membrane. (D) Actin patches are involved in endocytosis. (E) Actin filaments form the 2-dimensional network structure lamellipodium at the leading edge of the cell protrusion. (F) Actin filaments form a contractile ring at the centre of a cell during cytokinesis. AJ, adherens junction; ECM, extracellular matrix.

Actin filaments are highly concentrated in the cortical region beneath plasma membrane. Polymerization of the actin filaments and their interaction with the motor protein myosin cause cortical contraction and result in cell shape changes (Fig. 2B,D) (Pollard and Cooper, 2009), such as in cytokinesis (Fig. 2F) (Alberts et al., 2008). In motile cells, actin projection on the leading edge of the cell forms a two-dimensional mesh structure called lamellipodium

(Fig. 2E), which propels the cell across a substrate (Ananthakrishnan, 2007). In tubulogenesis, actin filaments in collaboration with myosin are involved in processes like cell elongation, separation of apical membranes for lumen formation, cell edge crawling and migration (Lubarsky and Krasnow, 2003; Pollard and Cooper, 2009).

The ERM (ezrin/radixin/moesin) protein mediates interaction between the cortical actin and transmembrane proteins, and is essential for membrane domain stabilization and remodelling (Fig. 2C) (Alberts et al., 2008), thereby determining cell shaping, cell migration, endo- and exocytosis, and other membrane based processes (Bretscher, 1999; Fehon et al., 2010). Talin protein mediates both intracellular and extracellular signals, by interacting with the transmembrane protein integrin at the basal domain and cell-cell junctions, and regulates the formation and maintenance of focal adhesions that link to actin cytoskeleton (Nayal et al., 2004; Critchley and Gingras, 2008).

Microtubules are usually organized in a radial array from the centrosome to the entire cytoplasm (Fig. 2) (Alberts et al., 2008). They provide structural support within the cell, position the internal organelles, and transport secretory vesicles and domain-specific transmembrane proteins (Goode et al., 2000; Alberts et al., 2008). During tubulogenesis, microtubules are involved in targeted luminal membrane recruitment and lumen formation (Hogan and Kolodziej, 2002; Cohen et al., 2004).

The precise role of the cytoskeletons in tubulogenesis and how they interact with other cellular components is not fully understood.

1.1.5. Apical membrane biogenesis

After apical/basal (A/B) polarity is established, neighbouring cells coordinate to deliver apical membrane components to a common site in the centre of the cell cluster, and generate luminal membranes *de novo* (Fig. 3A) (Datta et al., 2011). The apical domain emerges at the cell surface distal to the ECM. In a unicellular tube system like in *endothelial cell hollowing*, the luminal membrane emerges in the centre of the cell, where the apical identity has been established. The biogenesis of apical membrane and the localization of apical proteins at the

membrane further strengthens and stabilizes the apical-basal polarity (Lubarsky and Krasnow, 2003; Bryant and Mostov, 2008).

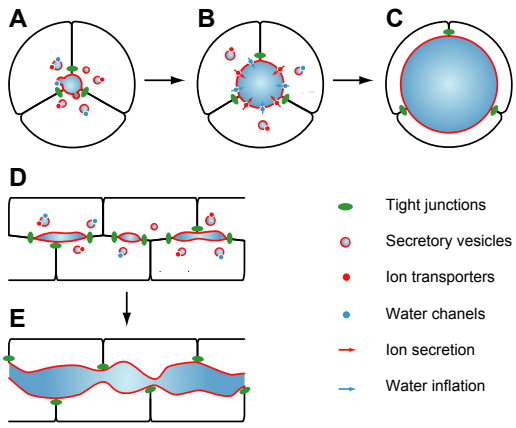

Fig. 3. Apical membrane and lumen formation in MDCK cell *chord hollowing*. (A) Secretory vesicle trafficking contributes to the apical membrane biogenesis and initial lumen formation. (B) Apical-targeted ion transporters and water channels drive ion and water accumulation in the lumen, which further expand the lumen to its mature size (C). (D) Small lumen pockets expand and coalesce to larger lumens, and finally connect to create a single tube (E).

In *chord hollowing* models like in MDCK cells and in zebrafish intestine, the apical domain is small at the beginning, and gradually grows to its mature size and shape (Fig. 3A-C) (Lubarsky and Krasnow, 2003; Baer et al., 2009). This requires an expansion of the apical domain and a reciprocal reduction of the lateral domain. The apical domain is normally demarcated by a ring of tight junctions (TJs), which expands concurrently as the apical region enlarges (Fig. 3A-C). The TJs separate the apical region from the surrounding lateral domain, and isolate the lumen from the surrounding ECM by preventing permeation of the paracellular liquid (Lubarsky and Krasnow, 2003; Bryant and Mostov, 2008). Exocytosis and endocytosis have been suggested to contribute to apical membrane expansion and lateral shrinking respectively (Fig. 3A). There is a possibility of direct transition of lateral membrane into apical membrane at the boundary of the two domains, but this has not yet been reported (Bryant and Mostov, 2008; Denker and Jiang, 2012).

1.1.6. Lumen formation and connection

Except for cases in which a pre-existing extracellular matrix is enclosed by an epithelial sheet, *de novo* creation of lumen is the critical process during tube morphogenesis. In *cell hollowing* and *cord hollowing*, intracellular vacuoles and extracellular lumen pockets emerge,

respectively, as the result of the continuous apical secretion of small secretory vesicles to the polarized membrane (Kamei et al., 2006). The newly formed lumen separates the apical membranes and strengthens apical identity of the membrane region. Together with the ECM it also maintains the apical/basal polarity (Kass et al., 2007; Bryant and Mostov, 2008). During mammalian lung formation, the lumen inflation has been suggested to stimulate and drive the apical membrane expansion and tube growth (Lubarsky and Krasnow, 2003).

In *cord hollowing*, lumen formation by vesicle secretion requires the trafficking of luminal materials to the apical/luminal membrane (Fig. 3A), mediated by microtubules and actin filaments (Rodriguez-Boulan et al., 2005; Mellman and Nelson, 2008). Lumens are initially small, but grow by fusing into larger pockets (Fig. 3D,E). Although secretory trafficking contributes to the initial formation of the apical membrane and lumen (Fig. 3A), additional mechanisms involving transmembrane transport are also needed for further lumen expansion. Osmotic gradient-based hydrostatic pressure, created by the activity of pumps and channels at the apical membrane, is thought to account for the luminal expansion by driving water efflux (Fig. 3B) (Datta et al., 2011; Denker and Jiang, 2012).

Individual lumen pockets need to connect to each other to create a tube. In endothelial tube formation, the expanding vacuoles extend and finally span the length of each cell before eventually merging with vacuoles of the neighbouring cells to form a connected lumen (Kamei et al., 2006). In MDCK cells or other systems undergoing multicellular *cord hollowing*, the lumen pockets expand and push their way into the intercellular space, and eventually coalescence into a single lumen spanning the whole cord (Fig. 3D,E) (Lubarsky and Krasnow, 2003). However, in the zebrafish dorsolateral anastomotic vessel (DLAV) and in the *Ciona* notochord, the tube develops from a single rod of cells and the lumens are completely separated by the cells (Fig. 9B,C). To connect these separated lumens, dramatic cell shape changes or remodelling are required (discussed later) (Herwig et al., 2011; Denker and Jiang, 2012).

Most tubulogenesis models share the common morphogenetic events including cell polarization, apical specification, lumen formation and connection, which are presumably associated to the function of the cytoskeleton. How these processes are integrated and

practiced by the cytoskeleton is not fully understood.

1.2. The *Ciona* notochord as a model for tubulogenesis

1.2.1. The ascidian Ciona intestinalis

Ciona intestinalis is one species of ascidians, which are marine invertebrate chordates and commonly known as sea squirts. In metazoan phylogeny, ascidians constitute one branch of the tunicates, the sister group of the vertebrates (Delsuc et al., 2006). The adult *Ciona* animals are sessile filter feeders usually living in shallow waters and bear few obvious morphological similarities to vertebrates (Fig. 4A). In contrast, the tadpole-shaped larvae are free swimmers that resemble vertebral tadpoles (Fig. 4B), in particular because of the presence of an axial structure called the notochord, a major shared feature of chordates. The fast embryonic development (completed within 24 hours) and the transparent body structures allows convenient *in vivo* observation of the embryonic development of *Ciona* with microscope (Satoh, 2003). Transgenic embryos can be obtained by two well established approaches: electroporation and microinjection (Zeller et al., 2006; Christiaen et al., 2009a, 2009b). The transgenes are usually mosaically expressed (Corbo et al., 1997; Zeller et al., 2006). Antisense morpholinos have been successfully used to knockdown the gene of interest (the treated animal is called a morphant) (Ekker, 2000; Satou et al., 2001a).

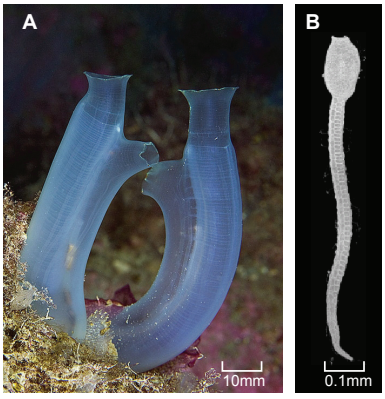

Fig. 4. The adult animals (A) and a larva (B) of *Ciona intestinalis*. Image credit: A, Jim Greenfield; B, Hotta et al. (Hotta et al., 2007a)

The ascidian embryos possess the basic developmental and morphologic features of the

vertebrate embryos but has the simplicity of the invertebrate embryos in terms of cell biology and genomics (Corbo et al., 2001). The *C. intestinalis* tadpole has a simple chordate body plan with approximately 2600 cells. It consists of 6 major tissues: the epidermis, the endoderm, the trunk mesenchyme, muscles, a dorsal neural tube, and an axial notochord in the tail, of which the last two are the defining characters of the chordate. With 36 muscle cells, 40 notochord cells, less than 150 neurons, and invariant cell lineages, the simplicity of *Ciona* larva is comparable to that of the nematode *Caenorhabditis elegans* (Corbo et al., 2001; Imai and Meinertzhagen, 2007). In spite of the significant divergence among the adults of different ascidians, their embryos share almost identical developmental patterns, therefore knowledge obtained from one species can be applied in other species (Lemaire, 2009). In addition, recent studies have demonstrated extensive molecular homologies between ascidians and vertebrates (Corbo et al., 2001). Therefore, the study of ascidian development offers a simple model to understand vertebrate development, and provides insights into the evolutionary origins of the chordates from an ancestral deuterostome, and into the emergence of the vertebrates within chordates (Satoh, 2003).

Ascidians have served as model organisms in developmental and evolutionary biology for over a century. *Halocynthia roretzi* has been used to identify the cell lineages and to construct a fate map in the early embryonic development of ascidians (Nishida, 1987; Satoh, 2003). *Boltenia villosa*, *Corella inflata* and others have been used for the study of cellular morphogenesis (Munro and Odell, 2002; Munro et al., 2006), while *C. intestinalis* has been used to study the molecular mechanisms of the nervous system, notochord and heart development (Meinertzhagen and Okamura, 2001; Satoh, 2003; Jiang and Smith, 2007; Christiaen et al., 2008). The genomes of two ascidians (*Ciona intestinalis* and *Ciona savignyi*) have been sequenced (Dehal et al., 2002; Small et al., 2007). Together with genome-wide surveys for early developmental genes and regulatory elements (Hotta et al., 1999, 2003; Hino et al., 2003; Hamada et al., 2007), the signalling pathways and regulatory networks underlying the embryo development of ascidians have been unveiled in great detail (Imai et al., 2004; Shi et al., 2005).

1.2.2. Morphology of ascidian notochord

The notochord is an axial structure of mesoderm origin. It emerges during the early development and persists through the larval stage, but disappears at adult stages in most chordate groups. The biological implications of the notochord are denoted from structural and inductive aspects (Satoh, 2003). The first and most conserved function is that, during embryonic development, the morphogenesis of the notochord provides a driving force for the elongation of the whole embryo, and in the tadpole stage it serves as a stiff backbone supporting the body during locomotion (Stemple, 2005). Second, especially in vertebrates, it acts as a source of inductive signals for the patterning of the neural tube and other surrounding tissues (Jiang and Smith, 2007; Satoh, 2003).

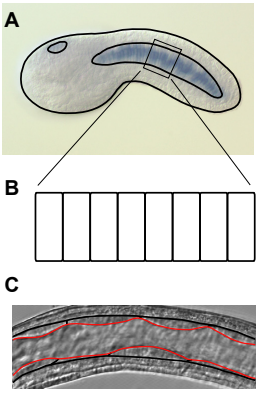

Fig. 5. Morphology of the *Ciona* notochord. (A) A *Ciona* embryo at early tailbud stage, side view. The dark region (shown by *in situ* hybridization of *Ci-Slc26aα*) in the tail presents where the notochord is located. (B) A diagram of the cells in a section of the notochord at this stage. (C) A section of notochord tube in a *Ciona* larva, longitudinal view. Red line marks apical membrane and black line marks basal membrane of the notochord cells.

The notochord is a central and rod-shaped structure in the tail of ascidian larvae (Nishida, 1987; Satoh, 2003; Jiang and Smith, 2007). In early- and mid-tailbud stage of the *Ciona* embryo (Fig. 5A), the columnar notochord is composed of 40 cells stacked end to end along the midline (a section shown in Fig. 5B), surrounded by a collagen-rich sheath (Stemple, 2005). At the larval stage, the notochord elongates and becomes a simple tube consisting of a thin layer of endothelial-like cells enclosing a central lumen (Fig. 5C, Fig. 6A) (Jiang and Smith, 2007; Dong et al., 2009). The change is mainly due to the cell and tissue remodelling events occurring between these two stages. Interestingly, the ascidian notochord exhibits diverse morphologies in different species (Fig. 6). Many ascidians including *Botryllus schlosseri* and *Aplidium californicum* posses the same tubular notochord structure as in *Ciona* (Fig. 6A) (Jiang and Smith, 2007). In other species such as *H. roretzi* (Gaku Kumano from Osaka University; personal communication) and *Clavelina huntsmani* (Burighel and Cloney,

1997; Jiang and Smith, 2007), individual lenticular lumens form among notochord cells but they never connect (Fig. 6B). In yet other species including *Styela plicata*, no lumen forms in the notochord at all, and the notochord cells change shape and remodel into an intercalated saw tooth pattern (Fig. 6C) (Jiang and Smith, 2007). In *Okamia thileni*, neither lumen formation nor cell remodelling occurs in the notochord development, thus the notochord never progresses beyond the single row of cells (Fig. 6D) (Burighel and Cloney, 1997).

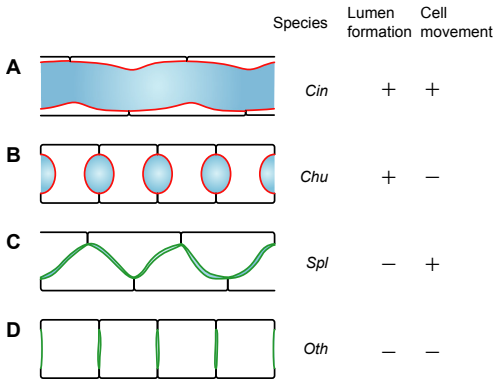

Fig. 6. Diverse notochord morphology among ascidian larvae. (A) A tubular notochord. (B) A notochord with discrete lumen pockets that never connect. (C) A notochord lacking visible lumen, while the cells become intercalated. (D) A notochord that doesn't form a lumen or undergo cell remodelling. Green line, undefined domain of the cell membrane. *Cin*, *Ciona intestinalis*; *Chu*, *Clavelina huntsmani*; *Spl*, *Styela plicata*; *Oth*, *Okamia thileni*.

1.2.3. Ascidian notochord development

In ascidians, the specification of the notochord lineage completes before gastrulation. The early notochord morphogenesis involves cell divisions and convergent extension. At the 112-cell stage, the 10 notochord precursor cells form a semicircular arc in the region anterior to the dorsal blastopore (Munro et al., 2006), and then undergo two rounds of cell division to form a layer of 40 cells. At the gastrula stage, these cells fold into the interior of embryo and rearrange into a cluster. During the neurula stage, convergent extension rearranges and transforms this cluster into a column of 40 stacked cells (Munro and Odell, 2002; Jiang and Smith, 2007).

The subsequent morphogenesis of ascidian notochord has recently been described in *C. intestinalis*, and is characterized by axial elongation and tube formation (Jiang and Smith, 2007; Dong et al., 2009). After convergent extension, from 14h (post fertilization) to 18h, the notochord cells elongate along the body axis and narrow medially, resulting in a lengthening

of the notochord and the tail (Fig. 7, 14, 17 & 17.5h). Next, small extracellular lumen pockets appear between adjacent notochord cells, where they proceed to increase in size and attain a double-convex shape (Fig. 7, 19 & 22h). Subsequently, the notochord cells start a bi-directional crawling movement (22-26h, Fig. 7, 24h), by which the cells are stretched and transformed into an endothelial-like morphology (Fig. 7, 28h). Also as the result of the cell movement, the lumen pockets tilt (Fig. 7, 24h) and eventually connect to each other (Fig. 7, 26h) to form a continuous lumen surrounded by a wall of the flattened notochord cells (Fig. 7, 28h) (Dong et al., 2009). Following the continuous lumen expansion, the notochord expands only along the longitudinal axis, possibly due to the constriction exerted by the stiff notochord sheath (Stemple, 2005).

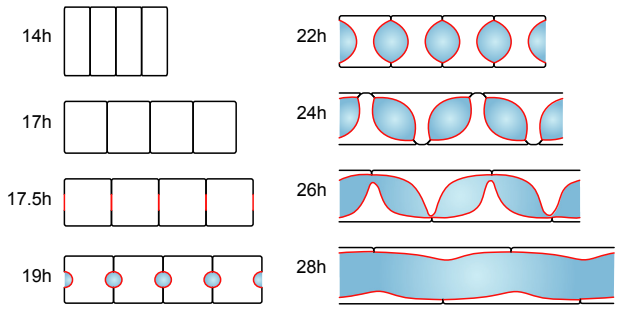

Fig. 7. Diagram of the late stages of *Ciona* notochord development at 16°C. "h", hours post fertilization.

1.2.4. Cellular events during Ciona *notochord tubulogenesis*

Several prominent cellular events take place during the *Ciona* notochord tubulogenesis (Dong et al., 2009). First, after 16h, a circumferential constriction gradually develops in the equatorial region of the basal surface of the notochord cells. This constriction is associated with the presence of an actomyosin ring at the same location (Fig. 8A), and the actomyosin activity is essential to the formation of the constriction and the elongation of the notochord cells (Dong et al., 2011). However, how this actomyosin ring is established has not been reported.

Second, unlike in conventional MET where a single apical membrane is created after the cells have become epithelialized (Fig. 9A), in *Ciona* notochord development, two apical domains emerge in the central region of the lateral domains of both the anterior and the posterior ends

of the notochord cells (Fig. 7 17.5h, Fig. 8B). The development of each apical domain follows common apical-basal (A-B) polarization processes, involving dynamic re-localization of the Par3/6/aPKC complex and the Lgl/Scribble/Dlg complexes, in concert with the remodelling of tight and adherens junctions (Denker et al., 2012).

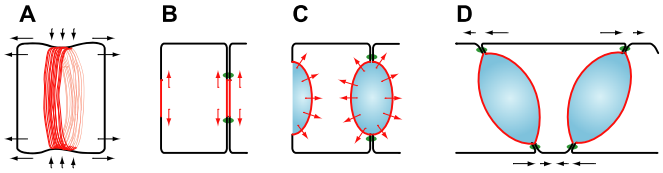

Fig. 8. Cellular events during *Ciona* notochord tubulogenesis. (A) The equatorial constriction of notochord cell is associated with an actomyosin ring (thin red lines). (B) The apical domains form and expand at the onset of epithelialisation. (C) Lumen and apical membrane expansion. (D) Bidirectional movement at the cell edges causes tilting of the lumen. Short black arrow, direction of retraction; long black arrow, direction of protrusive movement; red arrow, direction of apical membrane and lumen expansion.

Third, small extracellular lumen pockets emerge between adjacent cells at the apical domains, and continue to increase in size following the apical membrane expansion (18-22h, Fig. 7, 19-22h, Fig. 8C). During this process the lumen pockets present largely a spherical shape, implying that a high hydrostatic pressure is present in the lumen and pushes the apical membrane toward the cytoplasm proper of the notochord cell. The lumen expansion process lasts for approximately four hours, during which each lumen grows to approximately the same volume as the cells (Dong et al., 2009). However, the mechanism of the lumen expansion has not been previously studied.

Fourth, after lumen expansion, at each end of the notochord cell, the basal-lateral edge protrudes on one side while retracting on the other. The neighbouring notochord cells move reciprocally in a reversed manner (Fig. 7, 24h, Fig. 8D). This coordinated movement causes the shape of the notochord cells to change from cylindrical to trapezial, and the lumen pockets to tilt (Fig. 8D). The inclined pockets then approach each other, until neighbouring lumens meet and connect. Finally, all lumens in a notochord merge into a single one, while the notochord cells are flattened and form a layer of endothelia-like cells surrounding the

lumen (Fig. 7, 28h) (Dong et al., 2009). However, how these cell movements are initiated and regulated, and how different regions of the cell edges are coordinated are largely unknown.

1.2.5. Comparison of Ciona *notochord tube formation to other tubular systems*

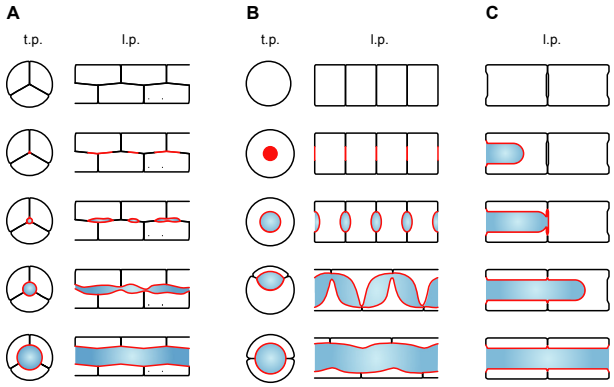

Fig. 9. Diagrams of different tubulogenesis strategies. (A) *Cord hollowing* in MDCK cells. (B) *Cord hollowing* in a *Ciona* notochord. (C) *Cord hollowing* in some of DLAV cells. t.p., transverse plane; l.p. longitudinal plane.

The overall process of *Ciona* notochord tubulogenesis follows a similar cellular design as that of *cord hollowing* in MDCK cells (Fig. 9A) (Lubarsky and Krasnow, 2003; Dong et al., 2009). Both start with a solid rod of cells, then initiate steps of tubulogenesis involving apical membrane specification, and *de novo* formation of extracellular lumen in the interior of the cell mass. However, an important difference is that the *Ciona* notochord is composed of a single line of cells and that the luminal pockets are initially separated from each other by the individual notochord cells (Fig. 9B). This configuration is similar to that in zebrafish DLAV formation (Herwig et al., 2011). Some DLAV cells perform cell migration in a similar fashion as the *Ciona* notochord cells, through which the unicellular rod of cells remodels into a multicellular tube, and the lumen pockets connect through cell migration to form a common intercellular space surrounded by the neighbouring cells. It is worth noting that cells in certain segment of DLAV adapt a different strategy to form tube. There the apical domain on one end of the endothelial cells invaginate and fuse with the apical domain at the opposite end to create a through tube (Fig. 9C) (Herwig et al., 2011; Denker and Jiang, 2012).

1.3. The *Ciona* genome and genes involved in notochord formation

1.3.1. The Ciona *genome*

In 2002, an international consortium using a whole-genome shotgun approach determined the sequence of the gene-rich regions of the *Ciona intestinalis* genome, and identified 16000 gene models (Dehal et al., 2002). Later, the annotated genomes of another ascidian, *Ciona savignyi*, and one appendicularian, *Oikopleura dioica*, have also been published (Small et al., 2007; Denoeud et al., 2010). The most recent update (Aniseed V4.0, 2012) of the ANISEED database released the genomic sequences for four ascidians, *Halocynthia roretzi*, *Halocynthia aurantium*, *Phallusia mammillata* and *Phallusia fumigate* (Tassy et al., 2010).

At an estimated 160 million base pairs (Mbp), the *Ciona* genome is only one-twentieth the size of the human genome, and less than half the size of the smallest vertebrate genome, *Tetraodon nigroviridis* (340Mbp) (Jaillon et al., 2004), but larger than those of *Drosophila melanogaster* (123Mbp), *C. elegans* (100Mbp) and *O. dioica* (70Mbp) (The C. elegans Sequencing Consortium, 1998; Adams et al., 2000; Seo et al., 2001). The compact size of the *C. intestinalis* genome has mainly been attributed to the absence of the genome duplication that occurred in vertebrate lineages (Dehal and Boore, 2005; Delsuc et al., 2006), and to a possible simplification of tunicate genome from a more complex ancestor (Dehal et al., 2002; Denoeud et al., 2010; Chavali et al., 2011).

The *Ciona* genome is densely packed, with an average of one gene per 10kbp (kilobase pairs). Analyses suggest that almost 62% of the *Ciona* genes have a detectable protostome homolog, presumably constituting an ancient core of genes common to bilaterian animals. Sixteen percent of the *Ciona* genes have homologs only in the vertebrates, presumably comprising a set of genes originated from the chordate innovation. Twenty-one percent of the genes have no homolog either in protostomes or in vertebrates, suggesting that they are either fast evolving or ascidian-specific (Dehal et al., 2002; Cañestro et al., 2003). The result of whole-genome comparisons among three chordate groups (tunicates, lancelets and vertebrates) does not support the traditional view of a close relationship between lancelet and vertebrates, and favours the idea that tunicates are actually the closest sister group of vertebrates, while lancelets are placed at a more basal position in the chordate phylogeny (Delsuc et al., 2006;

Putnam et al., 2008).

1.3.2. Ciona *genes involved in embryogenesis*

The draft genome of *Ciona* contains 2500 genes for which the functions of the vertebrate homologs are unknown (Dehal et al., 2002; Satou et al., 2002). Studies of these genes in *Ciona* might inform the functions of their vertebrate homologs. Two studies have employed antisense morpholino knockdown to screen a total of 504 genes and obtained embryonic morphological phenotypes in 111 of them (Yamada et al., 2003; Hamada et al., 2007). The phenotypic defects were found in all six major embryonic tissues, suggesting the existence of undiscovered pathways or mechanisms in nearly all aspects of embryogenesis, many of which probably have universal implications for embryonic development in all chordates (Hamada et al., 2007).

Microarray analysis has been widely used to identify tissue- or organ-specific genes and to study their regulation. In *Ciona,* whole genome cDNA microarrays and oligo-probe microarrays have been used to study the global gene expression profiles in eggs, individual blastomeres in early divisions, and whole embryos at different development stages (Azumi et al., 2004, 2007; Yamada et al., 2005). Since the *Ciona* embryo is small, individual tissues are difficult to isolate for microarray analysis. A recent work therefore employed fluorescence-activated cell sorting (FACS) to isolate *Ciona* heart precursor cells, which were then used for microarray analysis to identify heart-specific genes. The data were further used to construct the gene regulatory network that controls heart formation and serves as an interface between gene regulation and cellular processes leading to migration (Christiaen et al., 2008).

1.3.3. The ascidian notochord specification

The molecular mechanisms underlying cell differentiation during early ascidian embryogenesis have been extensively studied. The notochord is derived from mesoderm, the specification of which from mesoendoderm is mainly regulated by the FGF signalling pathway, through its effecter Twist-like 1 (Imai et al., 2003; Kumano and Nishida, 2007;

Lemaire, 2009). During mesoderm differentiation, the nuclear localization of maternal β-catenin activates various targets, including *FoxA*, *FoxD*, and *FGF*. Of these, the first two are required for the production of Zic in the primary notochord lineage (A-line). Zic together with FoxA confers notochord-specific responsiveness to FGF signalling and activates *brachyury* (Kumano and Nishida, 2007). FGF might also up-regulates *Nodal*, which is required for the secondary notochord (B-line) fate specification. Where the Nodal signals either directly restrict the fates of the notochord and mesenchyme common precursors, or through Delta2/Notch signalling activate *brachyury* by binding to the Su(H) binding site in the *brachyury* upstream region (Satoh, 2003; Hudson and Yasuo, 2006). Brachyury is a T-box transcription factor controlling notochord differentiation in both vertebrates and ascidians. During *C. intestinalis* notochord specification, *brachyury* begins to be expressed in the notochord precursor cells at the 64 cell stage (Corbo et al., 1997).

1.3.4. Brachyury-downstream notochord genes

In ascidians, the spatial and temporal expression pattern of *Brachyury* coincides with the restriction of the notochord lineages (Corbo et al., 1997). It has been shown that in *Ciona* embryos, ectopic expression of *Ci-Brachyury* (*Ci-Bra*) is able to convert endodermal cells to notochord cells (Yasuo and Satoh, 1998), while knockdown of *Ci-Bra* blocks notochord formation (Takahashi et al., 1999). This suggests the critical roles of *brachyury* in ascidian notochord specification and in metazoan evolution in general (Satoh et al., 2012). A subtractive cDNA library from *Ciona* embryos with ectopically expressed *Ci-Bra* identified 501 genes as potential brachyury targets, among which, 38 are specifically expressed in the notochord (Takahashi et al., 1999). Half of these notochord genes have known homologs belonging to several functional categories, including components related to the extracellular matrix, cytoskeletal architecture, cell signalling, and metabolic enzymes. One of them, clone 309h, show sequence similarity to sulfate transporters (Takahashi et al., 1999; Hotta et al., 2000). So far, this is the only anion transporter specifically expressed in the *Ciona* notochord. Further investigations on this gene will be presented later in this thesis.

Morpholino knockdown analysis has revealed that these genes function in different aspects of *Ciona* notochord morphogenesis (Hotta et al., 2007b). For example, the notochord cells in

Ci-PTP or *Ci-ACl* morphants fail to converge and extend, while knockdown of *Ci-prickle* causes a failure of intercalation (Hotta et al., 2007b), consistent with the phenotype of the *Ci-prickle* natural mutant (Jiang et al., 2005). Suppression of *Ci-netrin*, *Ci-trop*, *Ci-noto3* or *Ci-ASAK* results in a partial defect of notochord intercalation (Hotta et al., 2007b). When *Ci-ERM* and *Ci-pellino* are suppressed, notochord cells fail to elongate even though they complete convergent extension normally (Hotta et al., 2007b). However, these genes have only been analysed at the early stage of notochord morphogenesis, and no functional study of their roles in notochord tubulogenesis has been reported.

A further study on the EST clones of the *Ci-Bra* subtractive library has validated 450 non-redundant genes that are positively regulated by *Ci*-Bra (Hotta et al., 2008). Among these, 73 show single tissue- or organ-specific expressions (17 in the notochord), while 194 exhibit multiple tissue-specific expression patterns (42 in the notochord). Expression outside of the notochord tissue for a major portion of the *Ci-Bra* downstream genes suggests that these may be the indirect targets of *Ci*-Bra, and that the notochord can potentially function as a source of signalling to the surrounding tissues (Hotta et al., 2008). In addition, this work identified 11 wnt/PCP components as *Ci-Bra* downstream genes, suggesting a linkage between brachyury and wnt/PCP signalling (Hotta et al., 2008).

Notochord-specific expression of many *Ci*-Bra regulated genes start within a short period of the embryonic development, implying that these genes are either coordinately controlled or directly regulated by brachyury. However, the 43 genes analysed show no evidence of gene clustering in the chromosomes, excluding the possibility that they are under common cis-regulation (Takahashi et al., 2010). Reporter assays detected minimal enhancers with notochord-specific activity in the 3kb upstream regions of 8 of 20 *Ci-Bra* downstream genes. These minimal enhancers are more complex than common *Ci*-Bra binding motifs, suggesting that most of the *Ci-Bra* downstream genes examined may be the indirect targets of *Ciona* brachyury (Takahashi et al., 2010).

1.3.5. Notochord genes identified in other surveys

Other efforts have been made to identify genes expressed in the *Ciona* notochord. In a screen

of 182 genes known to be expressed in vertebrate notochords, twenty were found expressed in the *Ciona* notochord. These include transcription factors (*Ci-XBPa* and *Ci-miER1*) and extracellular matrix proteins (*laminin*). *In situ* hybridization in embryos ectopically expressing *Ci-Bra* suggests that a subset of these genes are not regulated by *Ci*-Bra, supporting the existence of (an) alternative regulatory mechanism(s) in *Ciona* notochord development (Kugler et al., 2008).

1.3.6. Notochord genes in other tunicates

Some of the *Ciona* notochord genes are absent in notochords of other tunicates. A recent study on the *Oikopleura* genome found apparent counterparts for only half of the *Ciona* notochord genes, and less than half of these conserved genes showed clear notochord expression (Kugler et al., 2011). This was anticipated by the previous finding that 3 of the 6 posterior *Hox* genes in *O. dioica* were lost in *Ciona* (Seo et al., 2004). This result suggested that *Ciona* and *Oikopleura*, in spite of their morphological similarity in the notochord, have developed divergent sets of notochord genes after their split from a common tunicate ancestor (Kugler et al., 2011). It is also consistent with the finding that, in ascidians, the invariant cell lineages and conserved embryonic structures are underpinned by divergent gene regulatory networks and cellular morphogenetic mechanisms (Lemaire, 2009).

Among the 16000 annotated *Ciona* genes, the above-mentioned works have only identified a total of 80 genes specifically expressed in notochord, and most of them are *brachyury* downstream genes. The list of *Ciona* genes involved in the notochord development is far from complete.

1.4. Anion transporters in tubular systems

1.4.1. Channels and transporters involved in tubulogenesis

Channels and transporters are extensively involved in the physiological activities of mature tubular systems (Matthay et al., 2002; Dorwart et al., 2008), but so far only a few such proteins have been studied in the context of tubular development. The Na-K-ATPase and the

claudin family of tight-junction proteins have emerged as important regulators of luminal hydrostatic pressure and paracellular permeability, respectively. They function in concert to mediate lumenogenesis and the coalescence of multiple small lumens into bigger lumens (Datta et al., 2011). Control of the chloride transport appears to be essential for fluid transport into lumens in several systems (Montesano et al., 2009; Datta et al., 2011). Activity of chloride transporters regulates the pH of the vacuole liquid in endothelial cell tubulogenesis (Ulmasov et al., 2009), or provides an osmotic gradient that draws water into the lumen across the pulmonary epithelium in lung development (Hooper and Harding, 1995). Except these examples, other ion transporters have not been implicated in tubulogenesis during development.

1.4.2. The Slc26 anion transporter family

The Slc26 (solute carrier 26) gene family encode anion exchangers that transport a wide variety of monovalent and divalent anions, and play essential roles in animal physiology. Ten Slc26 (SLC26 in human) members have been identified in human and in other vertebrates. Several members have been found in invertebrates, bacteria and fungi (Mount and Romero, 2004). Fourteen Slc26 homologs exist in *Arabidopsis*, making up the plant sulfate transporter family (Hawkesford, 2003; Dorwart et al., 2008). The sequence identity among members of Slc26 transporters of the same species is generally low (21-43%) (Dorwart et al., 2008). The conservation level between fly/worm and mammalian Slc26 homologs are only 25%-40%, therefore it is difficult to discern individual orthologs between invertebrates and vertebrates (Mount and Romero, 2004).

Slc26 family members are large trans-membrane proteins. N-terminal two thirds of the protein is hydrophobic and spans the plasma membrane 10-14 times. Analysis of one bacterial, one teleost, and two mammalian Slc26 transporters suggests that they exist and function as dimers on the membrane (Mount and Romero, 2004). The C-terminal region is cytosolic and contains a “sulfate transporter and anti-sigma” (STAS) domain that is related to the SpoIIAA anti-sigma factor antagonists (Campbell et al., 2002). Structure of the cytosolic portion of rat Slc26a5 (prestin) has been solved (Pasqualetto et al., 2010). Recently a low-resolution structure of a bacterial solute carrier, YeSlc26A2, has also been obtained

(Compton et al., 2011). A high-resolution structure of a full-length eukaryotic Slc26 protein is not yet available.

1.4.3. Functional diversity of the Slc26 transporters

Members of the Slc26 family were also called sulfate transporters, since its first member Slc26a1, or rat sat-1, was found to transport sulfate (SO_4^{2-}) (Bissig et al., 1994; Everett and Green, 1999). The substrates of this family of transporters are now known to be various combination of an expanding number of monovalent and divalent anions (Table 1), including SO_4^{2-}, chloride (Cl^-), iodide (I^-), formate, oxalate, hydroxyl ion (OH^-) and bicarbonate (HCO_3^-) etc (Mount and Romero, 2004). Extensive diversities exist in the transport mode and in the stoichiometry of different Slc26 transporters, or even of the same transporter transporting different substrates (Dorwart et al., 2008). For example, mouse Slc26A6 can exchange Cl^- with HCO_3^-, oxalate or formate in coupled transport mode, or carry on uncoupled transport of NO_3^- and SCN^- as an anion channel (Ohana et al., 2011). It is impossible to assume function of a Slc26 transporter directly from protein sequence similarity; the properties of individual members have to be explored experimentally (Dorwart et al., 2008).

1.4.4. Slc26 transporters in epithelial ion transport

Most human SLC26 members localize on the luminal (apical) or basolateral membranes of secretory epithelia cells in internal organs, and are involved in important physiological processes of the epithelium (Dorwart et al., 2008). For example, SLC26A3 (or down−regulated in adenoma, DRA) mediates the absorption of chloride in the intestinal epithelium through its Cl^-/HCO_3^- exchange activity, and maintains the physiological pH at the luminal surface (Hoglund et al., 1996; Melvin et al., 1999). SLC26A4 functions as an I^- transporter in the thyroid (Scott et al., 1999), while in epithelial cells of the inner ear and in the distal convoluted tubule of kidney, it serves as a Cl^-/HCO_3^- exchanger and controls vascular volume and pH in the kidney tubule structures (Wangemann et al., 2007; Dorwart et al., 2008). SLC26A6 functions in various systems associated with fluid secretion. Its $Cl^-/2HCO_3^-$ exchange activity is essential to the secretion of HCO_3^--rich fluids in pancreatic duct, and probably also in salivary glands and in the airway (Steward et al., 2005; Bryant and

Mostov, 2008). Animal experiments showed that Slc26A6 is related to intestinal oxalate secretion, and is important for the balancing of the plasma and renal oxalate load (Jiang et al., 2006)

Table 1. Six members of the human SLC26 family

Members	Aliases	Substrates	Distribution	Associated diseases
SLC26A1	Sat-1	SO_4^{2-}/Cl^-, OH^-, Ox	liver, kidney, etc.	Renal oxalate homeostasis
SLC26A2	DTDST	SO_4^{2-}, Cl^-	intestine, crtilage, etc.	Diastrophic dysplasia
SLC26A3	DRA, CLD	SO_4^{2-}, Cl^-/HCO_3^-	duodenum, colon	Congenital chloride diarrhea
SLC26A4	Pendrin	Cl^-/I^-, HCO_3^-, Fo	thyroid, kidney, cochlear	Pendred syndrome
SLC26A5	Prestin	* Cl^-/ SO_4^{2-}, Ox	outer hair cells in inner ear	Non-syndromic deafness
SLC26A6	PAT-1, CFEX	SO_4^{2-}, Cl^-, Ox, OH^-, HCO_3^-	widespread	Impaired pancreatic fluid secretion

*, only applicable to non-mammalian ortholog. Ox, oxlate; Fo, formate.

1.4.5. Diseases associated with SLC26 mutations

Due to their diverse functions in trans-epithelial ion transport, and their wide presence, dysfunctions of SLC26 family members can cause a series of severe human diseases (Table 1) (Everett and Green, 1999). The clinical importance of the *SLC26* gene family has been appreciated through the identification of a number of pathogenic mutations in at least four family members, *SLC26A2*, *A3*, *A4* and *A5*, which give rise to different clinical phenotypes (Dawson and Markovich, 2005).

SLC26A2 is linked to diastrophic dysplasia (DTD) and is also known as DTD sulfate transporter (DTDST). DTD features a normal-headed but short-limbed dwarfism together with skeletal defects (Hästbacka et al., 1994). More than 30 disease-associated mutations have been identified in the *SLC26A2* gene, causing different levels of severity including early

neonatal death (Karniski, 2001; Dawson and Markovich, 2005).

At least 30 mutations in *SLC26A3* have been associated with congenital chloride diarrhea (CLD), a heritable recessive disorder causing premature birth and secretory diarrhoea, most prevalent in Finnish population but also occurring world wide (Hoglund et al., 1996; Dawson and Markovich, 2005)

Over 80 *SLC26A4* mutations have been identified in patients with Pendred syndrome (PS) or non-syndromic hearing impairment. Most PS individuals are prelingually deaf and with enlarged vestibular aqueducts, and have developed thyromegaly (Campbell et al., 2001; Dawson and Markovich, 2005).

Mutations in *SLC26A5* cause non-syndromic hearing loss. In mammals, Slc26a5 serves as a motor protein in the outer hair cells of the cochlea (Zheng et al., 2000; Dallos et al., 2006). However, in non-mammalian vertebrates, Slc26a5 works simply as a sulfate transporter without the motor function (Schaechinger and Oliver, 2007).

1.4.6. Experimental approaches to anion transporter study

Functional studies of Slc26 proteins have mainly focused on determining their effective substrates and the mode and stoichiometry of the transport by electrophysiological methods. The Slc26 ransporter of interest is expressed in cultured cells such as CHO, and is then targeted to the cell membranes. Whole cell patch-clamp recordings are carried out to record the electrogenic transport current of candidate ions crossing the membrane. The current plot over the voltage ramp is analysed to reveal the transport properties of the expressed transporter (Schaechinger and Oliver, 2007).

The molecular function and structural characteristics of Slc26 transporters is important for our understanding of the transport biology and pathogenesis of the related diseases. Artificial chimera and mutated transporters have been generated to investigate the transport roles of individual domains or residues, in combination with electrophysiological methods (Ohana et al., 2011; Schaechinger et al., 2011). For example, a glutamate residue (E357) in mouse

Slc26a6 has been found to be essential in controlling transport modes. Neutralizing the charge of this residue (E357A) eliminates coupled transport without affecting the uncoupled current, while reversing it (E357K) results in the inhibition of both modes of transport (Ohana et al., 2011). However, until now, no systematic *in vivo* functional analysis of the physiological properties of the Slc26 family proteins has been carried out.

1.4.7. Known Slc26 family transporters in C. intestinalis

Three sulfate transporters have been annotated in the *C. intestinalis* genome: *Slc26a2*, *Slc26a6* and *Slc26a11* (Dehal et al., 2002; Satou et al., 2005). However, none of these have been experimentally verified, and their classification is mostly based on sequence similarities. The fourth member, clone 309h (renamed *Ci-Slc26aα* in this thesis), was isolated in the *brachyury* downstream gene survey (Takahashi et al., 1999). Its presence in the notochord has been verified using immunostaining, showing a specific localization at the apical membrane during notochord lumen formation (Dong et al., 2009). We have also identified another Slc26 family member, *Ci-Slc26aβ*, which is not expressed in the notochord and is excluded from this study. This thesis will present a functional study of *Ci-Slc26aα* in lumen formation during notochord tubulogenesis, and related its physiological properties to the SLC26 functional defect in human diseases.

2. AIMS OF THE STUDY

This thesis aims to understand the cell biology and developmental mechanisms of the notochord tube morphogenesis in *Ciona* embryos. I have focused my efforts on the following three aspects: gene expression in notochord development, visualisation of cellular morphologenetic events during notochord tubulogenesis, and the roles of individual notochord genes and the molecular mechanism of their functions.

The first aim is to provide a more complete transcriptome of the *Ciona* notochord undergoing tubulogenesis. Toward this goal, I employed whole genome microarray analysis on notochord cells obtained from fluorescence-activated cell sorting, and discussed the potential functions of notochord genes in terms of tubulogenesis.

The second goal is to provide a detailed analysis of cellular behaviours during different stages of tubulogenesis, by tracing the dynamic distribution and movement of cytoskeleton components, especially the actin filaments, in *Ciona* notochord development, using confocal live imaging and transient gene transfers.

The third and main aim of my thesis is to decipher the role of an anion transporter during tube formation. I performed functional analysis on *Ci*-Slc26aα to understand how this protein contributes to *Ciona* notochord tubulogenesis and what mechanism is underlying its function. Phenotypic analyses of embryos with *Ci-Slc26aα* morpholino knockdown, in combination with rescue experiments using wild-type and a series of mutant *Ci-Slc26aα*, were carried out.

3. LIST OF PUBLICATIONS

Paper I

José-Edwards, Diana S, Pierre Kerner, Jamie E Kugler, **Wei Deng**, Di Jiang, and Anna Di Gregorio. 2011. "The Identification of Transcription Factors Expressed in the Notochord of *Ciona* Intestinalis Adds New Potential Players to the Brachyury Gene Regulatory Network." *Developmental Dynamics* 240 (7): 1793–1805.

Paper II

Dong, Bo, **Wei Deng**, and Di Jiang. 2011. "Distinct Cytoskeleton Populations and Extensive Crosstalk Control *Ciona* Notochord Tubulogenesis." *Development* 138 (8): 1631–1641.

Paper III

Ivonne M. Sehring, Bo Dong, Punit Bhattachan, Elsa Denker, **Wei Deng**, and Di Jiang. 2012. "A cytokinesis-like actomyosin contractile mechanism drives cell elongation" (submitted)

Paper IV

Deng, Wei, Florian Nies, Anja Feuer, Dominik Oliver, Di Jiang. 2012. "A Slc26 anion transporter is essential for lumen expansion during tubulogenesis" (submitted)

Note:

I have been involved in several collaborations that lead to the above publications. I made partial contributions to the first three papers and was the major contributor to the last one. The following two sections (Results and Discussion) mainly contain a presentation of the work that I have contributed to the published or unpublished results, and each contribution is referred by a quotation of the respective publication (Paper I-IV). When work contributed by others is included, it is referred by the name of the contributor(s) and a quotation of the respective publication. When general conclusions and ideas presented in these publications are discussed in this thesis as the basis for further research, the respective papers are cited as references.

4. SUMMARY OF RESULTS

4.1. Microarray analysis of genes expressed in the *Ciona* notochord

By fluorescence-activated cell sorting (FACS), we successfully isolated notochord cells from trypsin-treated *Ciona* embryos. The notochord cells were distinguished from other cells by the fluorescence of a notochord marker eBra-bpFOG::memGFP (Dong et al., 2009), which begins to express in the notochord at the neurula (Neu) stage. After the mid-tailbud (MTB) stage the embryos become difficult to dissociate due to the presence of cellulose at the surface, we therefore only sampled Neu and MTB embryos. Total RNAs from either sorted notochord cells or whole embryos (as a control) were extracted for microarray analysis (see supplementary document for details on the microarray analysis).

The microarray design includes 26000 Kyotograil2005 *Ciona* transcriptome gene models (Christiaen et al., 2008). Of these we found 4735 showing positive hybridization signals (intensity greater than 2 in log10, see also supplementary document for microarray analysis) in MTB samples. This number increases to 5500 when results from both the MTB and Neu samples are combined. The most strongly expressed genes are mainly housekeeping genes (ribosomal proteins, cytoskeleton proteins), and genes encoding ECM of notochord and components of the notochord sheath (collagens, laminins, Supplementary table 1) (Stemple, 2005). Among the 5500 genes, 353 showed more than two-fold up-regulation in the MTB notochord. The most highly up-regulated genes included *1,4-galactosyltransferase*, *potassium voltage-gated channel*, *neurotrypsin precursor*, *goti-1*and *fucosyltransferase* (Supplementary table 2), and previously characterized notochord genes *leprecan-like 1, Ci-bra, ERM, Ci-noto2,4,8, and netrin* (Hotta et al., 2000). Among the 353 up-regulated genes, 103 do not have any sequence similarity to known genes, or ontology evidence. These genes add to the list of candidates for the study of novel mechanisms for notochord development (Supplementary table 2). Some of the genes identified from this work have been used in several following projects, and made essential contribution to these works.

The notochord expression of seven transcription factors identified from our microarray analysis have been validated by whole-mount *in situ* hybridization, and have been further

characterized (contributed by our collaborators from Anna Di Gregorio's lab, Paper I). Among these, *Spalt-like-a*, *Lmx-like*, and *STAT5/6-b* have previously been found in the notochord of other animals, thus their expression in the *Ciona* notochord emphasizes the conservation of notochord expression among chordates. Four others, *Fos-a*, *NFAT5*, *AFF* and *Klf15*, have not been reported in the notochord of any organism before, and are associated with notochord development for the first time. At lease five of these genes are regulated by brachyury, thus expanding the brachyury regulatory network, whereas the expression of *Klf15* appears to be independent from brachyury, suggesting the involvement of an additional signalling pathway in notochord development.

Among the top up-regulated genes there are several components of the glycoprotein biosysthesis pathway, including *beta 1,4-galactosyltransferase* and *sulfotransferase*. This result and previous finding of other enzymes in this pathway in the notochord inspired us to propose a glycoprotein hydration model in luminal space expansion (Paper IV). Similarly, high-level expression of homologs of potassium channels and Na^{+}/Ca^{2+} exchangers suggests an alternative mechanism of lumen inflation that utilizes an ion-based osmotic pressure gradient (Paper IV).

Most of the major cytoskeleton components can be found in the list of highly abundant genes in the notochord. Some of them are also highly enriched in the notochord. These include *talin* and *ERM* (annotated as *moesin*), which were further studied in Paper II.

4.2. Morphological processes during notochord tubulogenesis

Using actin markers, we found several populations of actin filaments in the notochord cells during development. Their localization and dynamic movement suggest specific roles of actin in notochord morphogenesis.

4.2.1 Cortical flow of actin filaments at the basal constriction

We have shown the presence of an actomyosin ring in the equatorial region of the basal membrane, and suggested that this actomyosin ring causes the formation of a constrictive

furrow (Dong et al., 2009, 2011). We were interested to explore the mechanism responsible for the formation of this actin ring. Using 3-D time-lapse imaging of embryos expressing mCherry-UtrCh, we found that a population of actin filaments emerge from both sides of the basal equatorial region and flow in the cortex towards the equator (Fig 2A in Paper III, illustrated in Fig. 10A). At the equator, the actin filaments gradually disappear. Experiments using lifeact-mEGFP and mCherry-MRLC (myosin regulatory light chain) confirmed the cortical flow of the actin and myosin filaments, respectively (Fig 2B in Paper III, contributed by Ivonne Sehring).

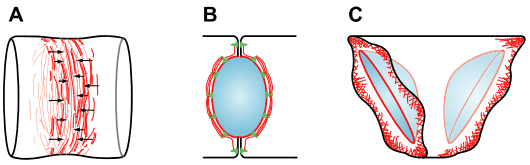

Fig. 10. Dynamic actin filaments in notochord development. (A) Cortical flow of actin filaments towards equator of the cell (indicated by the arrows) contribute to the formation of an actomyosin contractile ring. (B) Apical actin filaments and ERM (green) are essential for lumen formation. (C) Dynamic movement of lamellipodia contributes to notochord cell remodelling.

4.2.2 Actin and ERM are required for lumen formation

We utilized the fusion construct mCherry-Talin A I/LWEQ (mCherry-Talin A), which contains an cortical actin-binding module from talin (Singiser and McCann, 2006), as an *in vivo* actin marker. In the study presented in paper II, we found that before lumen formation (17.5h, hours post fertilization), mCherry-Talin A forms a circumferential band in the equatorial region at the basal side of the notochord cells, and is also localized at the entire lateral domain except the centre, where the future apical domain will emerge (Fig 6A,A' in paper II). After lumen has expanded (20h), the circumferential signal decreases at the basal region, whereas a new signal accumulates at the apical domain, indicating the presence of an apical cortical actin population during lumen formation (Fig 6E,E' in paper II, illustrated in Fig. 10B). To confirm this we used two other actin markers, lifeact-mEGFP and mCherry-UtrCh (Burkel et al., 2007; Riedl et al., 2008). Like mCherry-Talin A, both showed the presence of an apical cortical actin population (Fig 6F-G' in paper II). These results

suggest that actin polymerization is involved in the formation and maintenance of the apical membrane and the lumen.

Ciona ERM is highly expressed in notochord cells (Hotta et al., 2007b), and localizes at the lateral and apical membranes before and during notochord lumen formation, respectively (Fig 8A in Paper II, contributed by Bo Dong). We used a morpholino to disrupt ERM expression and found that the notochord in the morphant did not produce any lumens at 22h, when the control notochord developed large lumens (Fig 8C,D in Paper II). This result supports similar data showing that the expression of a dominant negative ERM mutant significantly blocks lumen formation (Fig 8B in Paper II, contributed by Bo Dong).

4.2.3 Dynamic movement of lamellipodia at the notochord cell edges

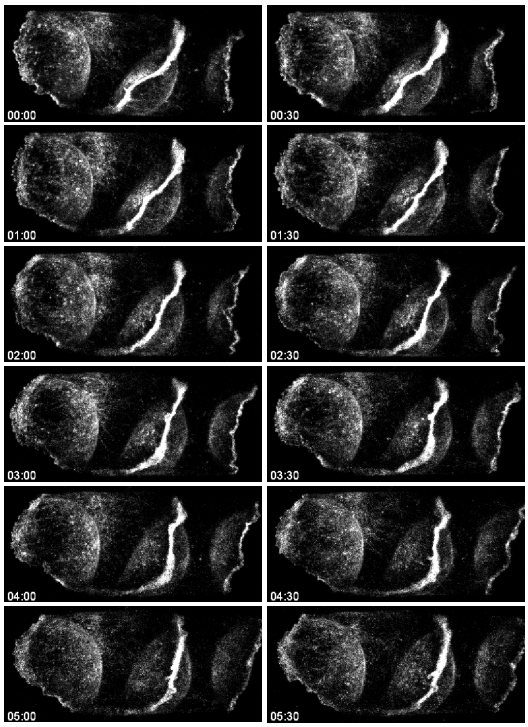

Fig. 11. Dynamic movement of lamellipodia in a *Ciona* notochord. Images are confocal projections of the actin filaments marked by mCherry-UtrCh. Strong signals at the basal-lateral boundary of the cell represents lamellipodia. The image series show their dynamic movement.

In the *Ciona* notochord, lumen tiling and connection are the consequence of cell movements. We used mCherry-UtrCh to visualize the actin populations associated with cell motility,

which are located at the crawling edge of notochord cells. After 22hpf, actin filaments become densely enriched at the cell edges, forming the protrusive structure called lamellipodium. We subsequently recorded a dynamic wave-like movement of the lamellipodia at the crawling edge of the membrane, going back and forth along the longitudinal axis of the notochord (Fig. 11, illustrated in Fig. 10C). We did not see any significant bias of the actin signal at the protruding edge compared to the retracting edge, indicating that both protrusion and retraction are actin-based processes. Nevertheless, the accumulated result is a steady movement of the membrane in one direction, which finally connects the edges of two notochord cells originally separated by an intervening cell.

4.3. Functional study of *Ci-Slc26aα* in notochord tube formation

(Paper IV)

4.3.1. A Slc26 family anion transporter in the Ciona *notochord*

We have identified *Ci*-Slc26aα as a member of the Slc26 family proteins based on following observations. First, Pfam analysis of *Ci*-Slc26aα detects a unique structure consisting of three conserved domains that are common to all SLC26 family members (Fig. 13A; Fig1A in Paper IV) (Dorwart et al., 2008). Second, a phylogenetic tree composed of 134 sulfate transporters show that *Ci*-Slc26aα resides in the Slc26 clade (Fig. 15; Fig1B in Paper IV). Third, the patch clamp recordings show that *Ci-Slc26aα* conducts transport of sulfate and oxalate in exchange for Cl^- or HCO_3^- in CHO cells (contributed by collaborators from Dominik Oliver's lab, Fig1C-D in Paper IV).

Ci-Slc26aα introduced by transient transgenesis is targeted to the apical domains of the notochord cells at all luminal stages (Fig2E-H in Paper IV), confirming previous immunostaining results (Dong et al., 2009). This apical localization suggests a role of this transporter in lumen formation.

4.3.2. Slc26aα knockdown causes lumen formation defect in the Ciona *notochord*

We used two independent morpholino oligos to knockdown the *Ci-Slc26aα* gene in *Ciona*

embryos, both causing significant and identical defects in the notochord and in sensory vesicles (Fig3 in Paper IV). In the morphant notochord, extracellular lumen formation was markedly inhibited, and the lumen only expanded to a narrow space even after prolonged observation time (Fig. 12B,B'; Fig3A'-D',E in Paper IV). In comparison, a control morpholino did not cause any phenotype (Fig. 12A,A'; Fig3K in Paper IV). Expression of *Ci*-Slc26aα in embryos treated with the splicing morpholino, which only affects the endogenous gene, rescued normal lumen formation (Fig. 12C; Fig3J,J' in Paper IV). These results suggest that the activity of *Ci*-Slc26aα is essential and specific to lumen formation.

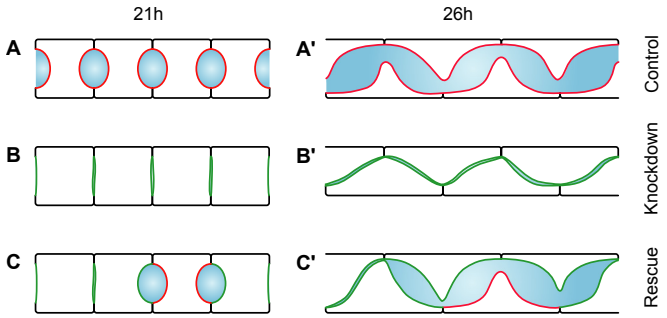

Fig. 12. *Ci*-Slc26aα is essential for lumen formation. Lumen formation (A,A') in the *Ciona* notochord is inhibited by morpholino knockdown of *Ci-Slc26aα* (B,B'). This phenotype can be partly rescued in notochords mosaically expressing a *Ci-Slc26aα* transgene (C,C'). Red line, apical domain expressing *Ci*-Slc26aα; green line, apical domain lacking Ci-Slc26aα expression.

To further characterize the *Ci*-Slc26aα knockdown phenotype, we examined other aspects of notochord tubulogenesis in the morphant embryos. First, we checked whether the lumen formation defect is the result of abnormal cell polarization, by following the membrane localization of two other apical markers, *Ci*-caveolin (Scheiffele et al., 1998), and a mutant of *Ci*-Slc26aα (E419K, see below), which targets to the apical membrane in normal notochord cells but is otherwise non-functional. We found that both markers were recruited to a large area at the centre of the anterior and posterior membranes (Fig3I-M' in Paper IV), indicating that the centre of the original lateral domain had already been specified as apical prior to the emergence of a visible lumen (Fig3M' in Paper IV). Second, we noticed that in morphant embryos with mosaic expression of *Ci*-Slc26aα, cells that did not express *Ci*-Slc26aα also developed normal sized and shaped apical domains on the side facing a *Ci*-Slc26aα-positive cell (Fig. 12C; Fig3J in Paper IV), suggesting that apical membrane expansion is not dependent on the expression of *Ci*-Slc26aα in the same cell. Third, the lumen expansion

defect did not affect the crawling movement of the notochord cells; the significantly reduced intercellular lumen pockets eventually tilt and connect to each other, forming a single narrow tube (Fig. 12B'; Fig3E in Paper IV).

4.3.3. The transport activity of Slc26aα is essential for lumen formation

We took advantage of the fact that the lumen formation defect after morpholino knockdown can be rescued by the introduction of wild type *Ci-Slc26aα* and developed an *in vivo* assay to test whether the transport activity of *Ci*-Slc26aα is required for the lumen formation. We first generated a series of deletion constructs, in which one or two protein domains were eliminated, and found that all these proteins failed to target to the plasma membrane (Fig. 13). We next created seven *Ci*-Slc26aα point mutants, each carrying a single amino acid substitution. The mutated residues are located in the highly conserved region of the Slc26 proteins, and correspond to mutations in human SLC26 family members that have been associated with SLC26 diseases (Table 2) (Dawson and Markovich, 2005). We expected that some of these mutations would cause transport defects, and wanted to test if they could rescue the lumen formation defect caused by morpholino.

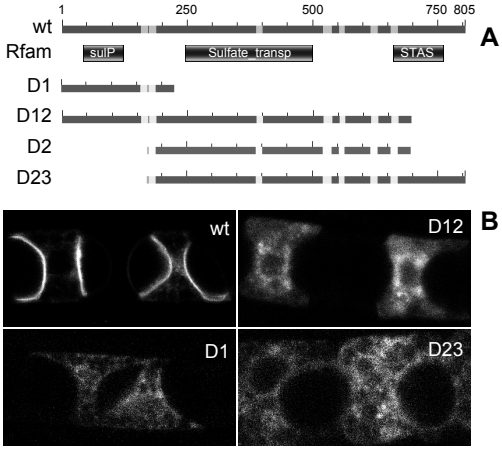

Fig. 13. All domains of *Ci*-Slc26aα are essential for membrane targeting. (A) Schematic diagram of domains in *Ci*-Slc26aα and the domain deletion constructs (D1, D12, D2 and D23). (B) Truncated *Ci*-Slc26aα do not target to the plasma membrane. D2 did not produce any signal (not shown). wt: wild type.

Florian Nies and co-workers from Dominik Oliver's lab tested six of the mutants for their transport properties in CHO cells (Table 2). Two of them, E419A and E419K, with mutations at the glutamic acid residue homologous to E357 in mouse Slc26a6 (Ohana et al., 2011), were

not targeted to the CHO cell membrane, precluding the possibility of examining their transport function. The remaining four were all targeted to the membrane. Among these, Y591C functioned similarly to the wild type, R444C maintained a partial transport activity, whereas the transport activity of L485P was mostly eliminated, and the activity of G245V was completely abolished (Fig 4F,G in Paper IV, Dominik Oliver and co-workers).

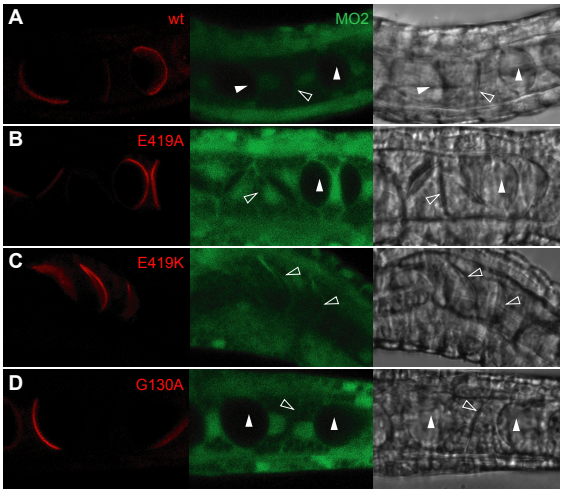

Fig. 14. *In vivo* activity of *Ci*-Slc26aα mutants. Mosaic expression of wild type (A) and *Ci*-Slc26aα point mutants (B-D) in the notochords of *Ci*-Slc26aα morphants. White hollow arrowhead indicates a substantial reduction of lumen; white solid arrowhead indicates a lumen recovery.

We examined the ability of all seven mutants to rescue lumen formation in the *Ciona* notochord cells depleted of *Ci*-Slc26aα (Table 2). All mutants localized on the apical domain at the onset of lumen formation and did not cause any abnormality in normal embryos (data not shown). In embryos injected with morpholino, Y591C, G130A and E419A mutants restored the lumen to normal size, while R444C partially rescued the lumen formation, and G245V, L485P and E419K failed to restore the lumens (Fig. 14; Fig 4A-E in Paper IV). The *in vivo* activity of the *Ci*-Slc26aα mutants in rescuing lumen formation defect correlates well with their *in vitro* transport activity, indicating that the specific transport activity of *Ci*-Slc26aα is essential to lumen formation (Table 2; Fig 4 in Paper IV).

Table 2. Design and characterization of the *Ci-Slc26aα* mutants

Referred members	Referred mutations	Associated diseases	Ci-Slc26aα Mutants	Target noto. M.	Lumen rescue	Target CHO M.	Anion transport
SLC26A4	G139A	None	G130A	+	++	N.T.	N.T.
SLC26A4	G209V	PD HSHL	G245V	+	−	+	−
Slc26a6	E357A	N.A.	E419A	+	+	−	N.T.
Slc26a6	E357K	N.A.	E419K	+	−	−	N.T.
SLC26A4	R409H/P	PD	R444C	+	+	+	+
SLC26A2	L483P	ACG1B	L485P	+	−	+	−
SLC26A4	Y556C/H	PD	Y591C	+	++	+	++

noto. notochord cells; M. membrane; N.T. not tested; N.A. not available. PD, Pendred syndrome; HSHL, non-syndromic hearing loss; ACG1B, achondrogenesis type1B.

5. DISCUSSION

The results of this work contribute to our understanding of several aspects of *Ciona* notochord tubulogenesis. First, the identification of a number of genes likely involved in notochord development forms the basis for further studies on the gene regulation and molecular pathways underlying tubulogenesis. Second, our observations on several dynamic populations of actin filaments associated with distinctive morphological changes provide novel insights on the contribution of the cytoskeleton to morphogenesis. Finally, the functional study of *Ci*-Slc26a*α* establishes the first clear example that ion transport is essential for lumen formation. Our detailed analysis mechanistically decouples lumen expansion from other aspects of tubulogenesis.

5.1. Microarray analysis of notochord genes

Previous works have identified around 80 genes specifically expressed in the *Ciona* notochord during development (Takahashi et al., 1999; Hotta et al., 2000, 2007b, 2008; Kugler et al., 2008). Most of these were obtained from whole mount *in situ* hybridization screens of notochord- or brachyury-related *Ciona* genes, and they account for a small portion of the genes assayed in the screens (Satou et al., 2001b, 2005). Microarray analyses have previously been applied to the transcriptomes of *Ciona* whole embryos, individual blastomeres (Yamada et al., 2005; Azumi et al., 2007), and heart precursor cells (Christiaen et al., 2008). Our work is the first attempt to apply microarray analysis to *Ciona* notochord gene expression.

Due to experimental limitations, we only assayed notochord samples at the Neu and MTB stages. Since significant morphological events take place at these stages of notochord development (Jiang and Smith, 2007), extensive change in the notochord gene expression is expected. We estimated, based on a rough threshold, that as many as 5500 genes are expressed in the notochord, many of which being housekeeping genes or genes generally involved in embryonic development. Nonetheless, 353 up-regulated genes are potentially either notochord specific or highly enriched in the notochord, and are particularly interesting since many of them have not previously been associated with notochord development.

Previously identified notochord genes only sum up to around 80 (Hotta et al., 2000, 2008; Kugler et al., 2008). The discovery of 103 unclassified genes potentially expressed in notochord development suggests unexpected mechanisms of notochord morphogenesis and new genes in development.

We note several caveats in our experiments. Microarrays are generally used to assess relative changes rather than absolute levels of gene expression (Ness, 2006). The accuracy of determining absolute expression from the raw data can be influenced by variations arising from hybridization efficiency, probe design and so on, while the influence of these variations can be largely reduced in an analysis comparing the expressions of two samples. Our experimental procedure requires the cells remain outside their physiological environment for more than 2 hours before RNA is extracted. It is possible that this, as well as the trypsin treatment, the sorting procedure, and the exposure of cells to the lysate solution, may influence the gene expression. Thus, we can neither assume that genes obtained in this work are indeed expressed in the notochord, nor can we consider that our list contains a complete set of highly expressed notochord genes. Nonetheless, this analysis provides a list of candidates for the study of notochord tubulogenesis, which can be individually validated by independent methods, such as *in situ* hybridization (Paper I). Therefore, these data not only made an essential contribution to this work, they may also be instructive for further studies on notochord tubulogenesis.

5.2. The cytoskeleton is involved in several aspects of tubulogenesis

Actin is a central player in cell movement and cell shape changes (Pollard and Cooper, 2009). Our results show that actin filaments are dynamically involved in several aspects of notochord tubulogenesis. In *Ciona* notochord cells, the basal actin filament population spatially coincides with the circumferential constriction, and temporally coincides with cell elongation before lumen expansion, suggesting that the contraction of the actin ring is the underlying mechanism of the constriction and cell elongation. It is plausible that the basal circumferential constrictive force is transferred to the anterior-posterior axis to drive the axial elongation of the notochord cells (Dong et al., 2009). In cytokinesis, the actomyosin ring can form either through cortical flow of pre-existing actin filaments towards the equator, or

through *de novo* formation of ring elements at the equator (Cao and Wang, 1990). Our observation indicates that cortical flow of actin filaments contributes significantly to the formation of the equatorial ring structure in the notochord cells. Whether this is the only mechanism operating in this process requires further study.

Immediately before lumen formation (~17.5h), the actin signal disappears from the central region of the lateral membrane, suggesting a loss of the adherens junctions usually found at the lateral domain and a specification of the apical domain in this region (Dong et al., 2009; Denker et al., 2012). When the lumen pocket becomes visible, actin filaments begin to accumulate at the apical cortex. We suggest that actin filaments play a role in delivering nascent membrane and apical components to the apical domain, which contributes to the apical expansion (Dong et al., 2011). After the individual lumens have expanded to a considerable size (22h), actin filaments gradually disappear from the apical cortex, while the apical membranes continue to expand until the lumens connect to each other. Therefore, other mechanisms may also be involved in targeting the apical components to the membrane. Another possible function of the cortical actin filaments is that they are contractile, and contribute to an increase in surface tension that counterbalances the expansive force of the lumen (Clark and Paluch, 2011). As a consequence, the luminal pressure is directed to the apical-lateral boundary, where it causes disassociation of the adherens junctions (Pollard and Cooper, 2009).

Our investigations on cytoskeleton components suggest that cortical actin and ERM are important for lumen formation, as both drug treatment of actin filaments and disruption of the ERM expression disable lumen formation in the notochord (Dong et al., 2011). The inhibition of lumen formation could explain the tail-elongation failure in ERM morphants, as observed previously (Hotta et al., 2007b) and in this work. Actin filaments are involved in various aspects of morphogenetic events and are distributed in different regions of a cell and in different tissues of the embryo (Alberts et al., 2008). Consequently, it is difficult to attribute the inhibition of lumen formation to the disruption of any specific population of actin filaments. On the other hand, *Ciona* ERM is specifically expressed in the notochord and is localized at the apical/lateral membrane domain, so it seems reasonable to implicate the apical actin population in the lumen formation.

In the *Ciona* notochord, lumen tilting and connection requires cell movement and its associated changes in cell shape and tissue remodelling. We hypothesized that like other crawling cells (Ananthakrishnan, 2007), notochord cell motility also involves actin-based lamellipodia at the leading edge (Dong et al., 2011). This is confirmed by our observation that a dense population of actin filaments accumulates at the crawling edges of the notochord cells, forming cellular protrusive structures identical to lamellipodia (Alberts et al., 2008). The lamellipodia dynamically move back and forth, and this movement is also observed at the retracting edge of notochord cells, suggesting that the protrusion and retraction are not simple and steady movements, but constitute many waves of small advances and retreats. The observation that adjacent edges of neighbouring cells both exhibit protrusive activity, but eventually one will move forward, whereas the other will retreat, suggests a possible biochemical and biophysical interaction between neighbouring cells that determines the outcome of local intercellular movement (Parker et al., 2002).

5.3. Characterization of Ci-Slc26aα

Our results clearly show that the previously reported sulfate transporter homolog (*Ci*-Slc26aα) is a member of Slc26 family anion transporters (Hotta et al., 1999; Dong et al., 2009). However, we found no evidence to consider *Ci*-Slc26aα as a *Ciona* ortholog of any known vertebrate Slc26 member. In our phylogenetic analysis we found that *Ci*-Slc26aα is more closely related to the vertebrate Slc26a1-9 clades than to the Slc26 branches of *Drosophila*, bacteria or plants (Fig. 15). We therefore designated it as *Ci*-Slc26aα with a suffix "α" to reflect the lack of basis to assign this protein to any vertebrate Slc26 subgroup.

Ci-Slc26aα exchanges the divalent anions sulfate or oxalate with Cl^- or bicarbonate in a coupled transport manner. A similar transport mode and spectra of substrates was reported for the mammalian Slc26a6 and the non-mammalian vertebrate Slc26a5 (Schaechinger and Oliver, 2007; Dorwart et al., 2008). However, *Ci*-Slc26aα does not exhibit the uncoupled transport activity (paper IV) common to Slc26A5 and Slc26A6 (Ohana et al., 2011; Schänzler and Fahlke, 2012). This underscores both the conservation and diversification of transporter function that is seen in the evolution of the Slc26 family and their plant homologs (Takahashi

et al., 2012).

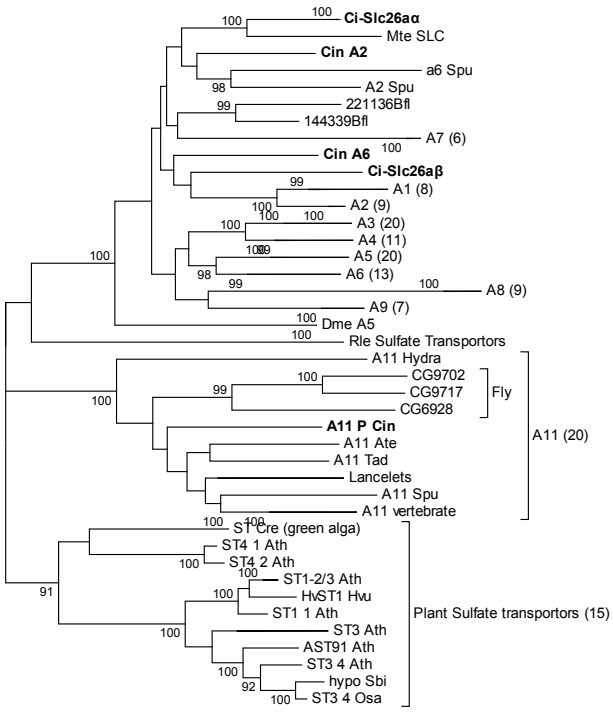

Fig. 15. A maximum-likehood phylogeny tree of 134 Slc26 family transporters. All vertebrate orthologs with clear orthologous relationship are compressed. The number in parentheses indicates the number of orthologs included in a compressed clade. Numbers at each node are bootstrap values of 100 replicates (shown only when greater than 90). Bold, *Ciona* Slc26 proteins. Ath, *Arabidopsis thaliana*; Bfl, *Branchiostoma floridae*; Cin, *Ciona intestinalis*; Dme, *D. melanogaster*; Mte, *Molgula tectiformis*; Rle, *Rhizobium leguminosarum*; Spu, *Strongylocentrotus purpuratus*.

5.4. Function of Slc26aα in lumen expansion

Our results demonstrate that the anion transporter *Ci-Slc26aα* is essential for notochord lumen formation, and extend the functional importance of Slc26 family transporter to a developmental context. This developmental function should not come as a surprise, since Slc26 family proteins are extensively involved in regulating lumen homeostasis in many adult internal organs (Dorwart et al., 2008). Slc26 transporters have not been reported in the notochords of other chordates. It is conceivable that, in other ascidians with tubular notochord morphology, lumen formation is also associated with a Slc26 transporter.

Our *in vivo* analysis and *in vitro* electrophysiological experiment together suggest that the ion transport activity is required for the *Ci*-Slc26aα function in lumen formation. However, in terms of the *in vivo* function, it is still unknown whether the electrogenic activity of

Ci-Slc26aα is essential, and which transported substrates are relevant. Additional rescue experiments using other Slc26 proteins or chimeric transporters with better characterized and more restricted transport properties may be helpful to address these questions (Schaechinger et al., 2011).

The immediate consequence of anion transport in the context of lumen expansion is also poorly understood. Hydrostatic pressure has been thought to account for part of lumen formation in many tissues (Matthay et al., 2002; Datta et al., 2011). It is therefore conceivable that, in the *Ciona* notochord, concerted activities of *Ci*-Slc26aα and other transporters (suggested by microarray data) generate an osmotic gradient across the apical membrane, which drives a fluid efflux and accomplishes the extensive lumen expansion.

An alternative model is that the sulfate transport facilitates the production of high molecular weight proteoglycans that expand in the luminal space upon hydration. This hypothesis envisions that *Ci*-Slc26aα shuttles sulfate into the cytoplasm to facilitate the sulfation of proteoglycans. This is supported by the finding that many components of the glycosaminoglycan synthesis pathway are present in the *Ciona* notochord (Paper IV; microarray and *in situ* hybridization) (Kamiyama and Nishihara, 2004).

5.5. An in vivo *assay system for the study of Slc26 transport properties and SLC26 pathogenesis*

We intended to find mutants that could only transport a subset of substrates or a single anion, and to use these to identify which anion is physiologically relevant to the lumen formation in the *Ciona* notochord. In mouse Slc26a6, mutation E357A disrupts coupled transport while E357K eliminates both transport modes (Ohana et al., 2011). For *Ci*-Slc26aα, the homologous mutant E419A is able to rescue the lumen formation in defected embryos, whereas E419K is not, suggesting that this residue is a conserved structural element for the function of Slc26 family. Since *Ci*-Slc26aα does not exhibit the same uncoupled transport activity as the mouse Slc26a6 does, it would be important to determine which coupled transport activity, which is essential to the lumen formation, is lost in E419K, but is retained in E419A. Unfortunately, although E419A/K can localise at the apical membrane in *Ciona*

notochord cells *in vivo*, they are not targeted to the plasma membrane of CHO cells, precluding the possibility of determining their transport properties. Our attempt therefore failed to reveal the relevance of transport property to the *in vivo* functionality of E419 mutants. This difference in the ability of E419 mutants to target the plasma membrane in the two systems is paradoxical. It is possible that the biogenesis, the stabilization, or membrane targeting of the E419 mutants are differentially affected, or the defects are differentially compensated in *Ciona* notochord and mammalian cells.

Our rescue experiments demonstrate that the *Ciona* notochord can serve as a useful model system to assay the function of Slc26 proteins and mutants. Since no high resolution structure is available for any Slc26 family member (Compton et al., 2011), mutational analysis provides valuable insights into the structural and biochemical basis of the transport function of this group of transporters. In *Ci*-Slc26aα, mutations at different residues cause various extents of transport activity loss or do not, in accordance with their physiological ability to rescue lumen formation (Table 2). Although we have so far only analysed *Ciona* mutants, human SLC26 mutants can also be directly used for phenotypic analysis in our *in vivo* system. Such combined knowledge can potentially help us to understand the molecular pathogenesis of SLC26 diseases and offer improved prognosis in clinical settings (Dawson and Markovich, 2005).

5.6. Lumen formation can be uncoupled from other processes of tubulogenesis

Although lumen expansion and apical membrane biogenesis occur simultaneously in the normal notochord, our functional analysis of *Ci-Slc26aα* shows that they are mechanistically independent processes. During lung formation, lumen inflation is required to stimulate apical expansion and tube growth (Lubarsky and Krasnow, 2003), however, the relationship between lumen formation and apical expansion in this and other developmental contexts has not been fully addressed. In *Ciona* embryos injected with the *Ci-Slc26aα* morpholino, the expansion of the notochord apical domain takes place normally, in spite of the absence of lumen expansion. A small amount of lumen can be produced due to the vesicle secretion and incomplete knockdown of *Ci-Slc26aα*, but this appears to be undetectable in our experiment. This therefore highlights the importance of transmembrane transport mechanism for lumen

formation. Cells lacking *Ci-Slc26aα* expression develop normal luminal membrane at the side facing a fully inflated lumen, but fail to develop lumen next to cells also lacking *Ci-Slc26aα* expression, suggesting that apical expansion is triggered by the inflation of the lumen, rather than the presence of *Ci-Slc26aα* on the membrane.

In wild type *Ciona* embryos, tilting of the lumen pockets starts at the time when their expansion is maximal and the lateral domain is significantly reduced (Dong et al., 2009). This correlation seems to indicate that a critical size of the lumen pockets is sensed by the cells and acts as a trigger to initiate cell movement and lumen tilting. However, our analysis shows that cell movement proceeds independently from the lumen expansion, suggesting that the former is triggered by a signal other than a critical lumen size.

Taken together, we suggest that lumen formation can be mechanistically uncoupled from the other three processes of notochord tubulogenesis: apical specification, apical domain expansion, and cell movement.

5.7. Evolutionary aspects of ascidian notochord morphology

Ascidian notochords exhibit a variety of morphologies in cellular organisation (Jiang and Smith, 2007). Our analysis of embryos with a disrupted *Ci-Slc26aα* expression uncouples cell movement from lumen formation, two processes that are essential in determining notochord morphology in ascidians. *Ciona* notochord development undergoes both procedures sequentially and constructs a tubular structure (Fig. 6A). In *Clavelina huntsmani,* individual lumen pockets form in the notochord but never connect to each other (Jiang and Smith, 2007), suggesting the presence of lumen formation and the absence of cell movement (Fig. 6B). The notochord in *Botrylloides violaceus* does not produce any lumen, but remodels from a stack of columnar cells to a pack of intercalated cells (Jiang and Smith, 2007), suggesting the presence of cell movement but the absence of lumen formation (Fig. 6C). A similar pattern is seen in the *Ciona* notochord after *Ci-Slc26aα* knockdown (Fig. 12B'; Fig2 in Paper IV). We therefore speculate that these two development processes were differentially recruited in ascidian evolution to create diverse notochord morphologies, allowing different ascidian larvae to adapt to their environment.

6. CONCLUSION

This study presents two major findings. First, the *Ciona intestinalis* notochord tubulogenesis employs the cytoskeletal mechanisms to complete a series of specific cellular processes, which lead to the unique morphogenesis of the notochord tube. Second, we have shown that lumen formation is associated with the anion transport activity of *Ci*-Slc26aα and that it can be uncoupled from other morphogenetic steps of the notochord tubulogenesis. Both findings contribute to our understanding of the *Ciona* notochord tubulogenesis in specific, and how cellular behaviours determine animal morphology in general.

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