The *Caenorhabditis elegans* Excretory System: A Model for Tubulogenesis, Cell Fate Specification, and Plasticity

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ABSTRACT The excretory system of the nematode *Caenorhabditis elegans* is a superb model of tubular organogenesis involving a minimum of cells. The system consists of just three unicellular tubes (canal, duct, and pore), a secretory gland, and two associated neurons. Just as in more complex organs, cells of the excretory system must first adopt specific identities and then coordinate diverse processes to form tubes of appropriate topology, shape, connectivity, and physiological function. The unicellular topology of excretory tubes, their varied and sometimes complex shapes, and the dynamic reprogramming of cell identity and remodeling of tube connectivity that occur during larval development are particularly fascinating features of this organ. The physiological roles of the excretory system in osmoregulation and other aspects of the animal's life cycle are only beginning to be explored. The cellular mechanisms and molecular pathways used to build and shape excretory tubes appear similar to those used in both unicellular and multicellular tubes in more complex organs, such as the vertebrate vascular system and kidney, making this simple organ system a useful model for understanding disease processes.

KEYWORDS *Caenorhabditis elegans*; tubulogenesis; excretory–secretory system; WormBook

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10.1534/genetics.116.189357
Manuscript received December 1, 2015; accepted for publication March 7, 2016.
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Introduction to the Excretory System and Unicellular Tubes

Organs are made up of networks of tubes that transport fluids or gases and mediate exchange of nutrients and waste products with surrounding tissues. Each tube type in the network has a distinctive size and shape that facilitates its particular function. Many biological tubes are constructed from polarized epithelial cells, with the apical surface forming the lumen, while the basal surface forms the outside of the tube facing other tissues or body cavity (Figure 1). Wider tubes are formed from multiple cells whose lateral surfaces bind to each other via adherens and tight junctions to maintain the patency of the lumen (Figure 1A). The smallest tubes are unicellular, made up of single cells (Figure 1, B–D). Tubes are often dynamic and can convert from one type to another during development (Ribeiro *et al.* 2004; Denker *et al.* 2013; Lenard *et al.* 2013). Cells within tubes can also delaminate, change identity, and give rise to other cell types (Kalluri and Weinberg 2009; Zuryn *et al.* 2012). Biological tubes make up a vast proportion of cells in all eukaryotic organisms, and tube abnormalities underlie many birth defects and diseases in humans, yet the mechanisms that determine tubule identity, size, shape, and connectivity are incompletely understood (Lubarsky and Krasnow 2003; Iruela-Arispe and Beitel 2013; Sigurbjornsdottir *et al.* 2014).

The *Caenorhabditis elegans* excretory system is a very simple tubular organ that consists of just three unicellular tubes (canal, duct, and pore) connected in tandem to form a continuous lumen (Nelson *et al.* 1983) (Figure 2). Dynamic changes in pore identity and tube connectivity occur during development (Figure 3, Figure 4). Two fused gland cells (Figure 3E) form a syncytium attached to the canal–duct junction (Nelson *et al.* 1983), and two canal-associated neurons (Figure 2A, Figure 3F) have axons that run along the basal surface of the canal cell and have been proposed to regulate canal function (Hedgecock *et al.* 1987; Forrester and Garriga 1997). The system appears to function in both osmoregulation and secretion, and it shares many molecular features with osmoregulatory or renal systems in other organisms, and with other epithelial and endothelial tube networks. The simplicity of the excretory system, and its amenability to genetic analysis and live imaging, make it a very attractive model for studying the cell biology of tubulogenesis, tube dynamics, and other aspects of organ development and physiology.

Unicellular tubes

Excretory system tubes are unicellular, meaning that the apical or luminal domain is located inside of the cell (Figure 1, B–D). Unicellular tubes are also found in tissues as diverse as vertebrate capillaries (Bär *et al.* 1984; Iruela-Arispe and Davis 2009; Lenard *et al.* 2015; Yu *et al.* 2015) and glia (Simons and Trotter 2007; Jessen *et al.* 2015), *Drosophila* tracheal tubes (Samakovlis *et al.* 1996; Schottenfeld *et al.* 2010; Maruyama and Andrew 2012), plant pollen tubes (Geitmann 2010), and the contractile vacuoles of single-cell protozoa (Plattner 2015). Understanding how unicellular tubes are formed and maintained has significant health relevance, given the prevalence of such tubes in the microvasculature and nervous system and their involvement in cardiovascular disease, stroke, and neuropathies (Feihl *et al.* 2008; Govani and Shovlin 2009; Vallat *et al.* 2013; Draheim *et al.* 2014; Haffner *et al.* 2016). Several different mechanisms for forming unicellular tubes have been proposed, although none are very well understood at the molecular level.

Wrapping: An individual cell can form a tubule of defined luminal diameter by wrapping its plasma membrane around an extracellular core of matrix or other material and forming an autocellular junction (AJ) or "seam" to itself (Figure 1B). Seamed unicellular tubes are generally open at both ends. Examples of seamed unicellular tubes include the *C. elegans* excretory pore cell (Nelson *et al.* 1983); *Drosophila* tracheal stalk cells, which wrap around a core of chitin (Ribeiro *et al.* 2004; Tonning *et al.* 2005; Luschnig *et al.* 2006); many vertebrate capillaries (Yu *et al.* 2015), especially in the brain (Bär *et al.* 1984); and many invertebrate glial socket cells that wrap around neurons (Freeman 2015; Shaham 2015). A related topology is observed in other wrapping glia, such as mammalian Schwann cells, which do not form actual junction seams, but instead adhere to themselves via secreted myelin (Simons and Trotter 2007; Jessen *et al.* 2015).

Wrapping and autofusion: A seamed tube formed by wrapping can subsequently autofuse to eliminate the autocellular junction and become a "seamless" tube that has no junctions running along its length (Figure 1C). Autofusion occurs in the *C. elegans* excretory duct (Stone *et al.* 2009) and pharyngeal valve cells (Rasmussen *et al.* 2008), some capillaries in the zebrafish vascular system (Lenard *et al.* 2015), and mammalian Madin-Darby canine kidney (MDCK) cells grown on micropillar arrays in culture (Sumida and Yamada 2013, 2015).

Hollowing: As an alternative to cell wrapping, a single cell can create a seamless tube through "cell hollowing," in which endocytic (plasma membrane-derived) and/or exocytic (Golgi-derived) vesicles coalesce to form an internal apical surface that connects to the plasma membrane (Figure 1D). This mechanism was first described in mammalian endothelial cells grown in culture (Folkman and Haudenschild 1980; Davis and Camarillo 1996; Sacharidou *et al.* 2012). The initial nucleating event for intracellular lumen formation is not clear, but *in vivo*, lumen formation often initiates near a junction with another epithelial tube cell and then appears to grow inward from that point, suggesting polarized vesicle trafficking toward the newly established apical domain (Figure 1D), similar to exocytosis-driven cord hollowing in multicellular tubes (Datta *et al.* 2011; Eaton and Martin-Belmonte 2014) (Figure 1A). Seamless tubes can be closed at

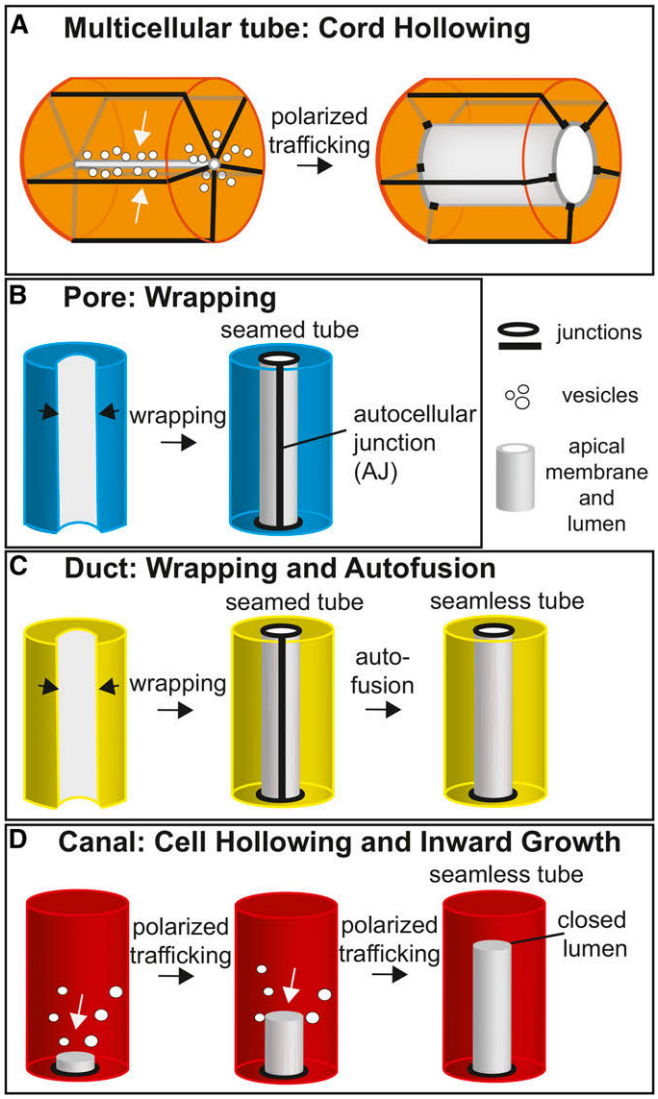

Figure 1 Tube topologies and models for unicellular tube formation. (A and B) In multicellular tubes (A) and seamed unicellular tubes (B), adherens junctions (in black) separate and delineate the apical and basal domains. (C and D) In seamless unicellular tubes, junctions appear only at the end(s) of the tube, at the site of connection to another cell. Some multicellular tubes form by a cord-hollowing mechanism (A) involving polarized vesicle trafficking toward the lumen (arrows) (Datta *et al.* 2011). The excretory pore cell (in blue) (B) forms a seamed tube by wrapping and forming an autocellular junction (AJ). The excretory duct cell (in yellow) (C) initially forms a seamed tube and then converts to a seamless tube via autofusion to remove the AJ (Stone *et al.* 2009). The canal cell (in red) (D) forms a seamless tube, likely through a cell-hollowing mechanism involving polarized vesicle trafficking toward the lumen. The origin of relevant vesicles is not known. The intercellular junction with the duct may provide a polarizing cue that directs vesicle targeting to this region. For all tubes, both basal and apical sides may secrete extracellular matrices. For all diagrams, lumen is shown in lighter color.

one end or open at both ends, depending on where nucleation of apical surface occurs. Examples of seamless tubes thought to form by a hollowing mechanism include the *C. elegans* excretory canal cell (Buechner 2002; Berry *et al.* 2003), *Drosophila* tracheal terminal and fusion cells

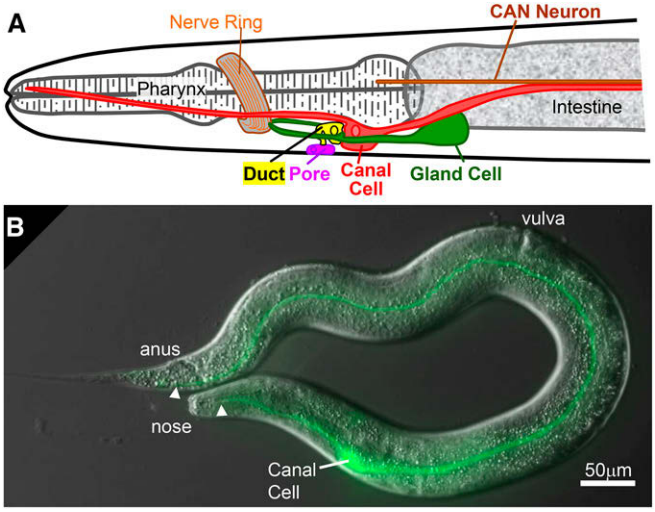

Figure 2 The *C. elegans* excretory system. (A) Diagram of cells of the *C. elegans* adult excretory system. (B) DIC with fluorescence photograph of *qpIs11*[$P_{vha\text{-}1}$::*gfp*] adult expressing GFP in the canal cytoplasm. Arrowheads mark anterior and posterior ends of the canals running the length of the organism.

(Samakovlis *et al.* 1996; Gervais and Casanova 2010; Gervais *et al.* 2012), and many capillaries (Wolff and Bar 1972; Bär *et al.* 1984; Yu *et al.* 2015), including those that fuse via anastomosis (Herwig *et al.* 2011; Denker *et al.* 2013; Lenard *et al.* 2013; Caviglia and Luschnig 2014).

Overview of excretory system development

The *C. elegans* excretory system is an excellent model for the study of unicellular tubes, since it contains tubes that form by each of the above mechanisms: (1) a seamed pore tube formed by wrapping (Figure 1B and Figure 3, C and D); (2) a seamless duct tube formed by wrapping and autofusion (Figure 1C and Figure 3B); and (3) a seamless and branched canal tube likely formed by hollowing (Figure 1D and Figure 3A). The excretory system also has been used to study other important steps of organ development, including long-range cell migration, cell fate determination, epithelial delamination, and cell fate reprogramming.

Figure 4 shows a timeline of excretory system development. Cells of the excretory system are born about one-third of the way through embryogenesis and derive from the AB lineage (Sulston *et al.* 1983) (Figure 3 and Figure 4, A–C). The presumptive duct, G1 pore, and glands are initially born in lateral locations far from the canal cell and then migrate to their final positions during ventral enclosure (Figure 4A). Junction and lumen formation begin only after the canal, duct, pore, and gland cells come in physical contact, around the lima bean stage of embryogenesis (Abdus-Saboor *et al.* 2011) (Figure 4, D and E and Figure 5A). The tubes, especially the duct and canal, subsequently elongate and change shapes, concomitant with embryo elongation (Suzuki *et al.* 2001; Stone *et al.* 2009) (Figure 4F). During this time period, the CAN neurons also migrate to their positions near the

Cell Type	Lineage origin	Major TF regulators or markers^
A excretory canal cell 	ABplpappaap	Lineage Identity: LAG-1/CSL CEH-36/Otx + UNC-30/Pitx Epithelial Identity: LIN-26/C2H2 Fate Execution: PROS-1/Prox1^ CEH-6/POU NHR-31/HNF4
B duct 	ABplpaaaapa	Lineage Identity: CEH-36/Otx + UNC-30/Pitx MLS-2/Hmx Epithelial Identity: LIN-26/C2H2 NHR-23/ROR NHR-25/Ftz-F1 Duct identity: LIN-1/Ets* + EOR-1/PLZF* Fate Execution: LIN-48/Ovo^
C G1 pore 	ABprpaaaapa	Lineage Identity: CEH-36/Otx + UNC-30/Pitx MLS-2/Hmx Epithelial Identity: LIN-26/C2H2 NHR-23/ROR NHR-25/Ftz-F1 G1 pore identity: LIN-1/Ets
D G2 pore or G2p pore 	ABplapaapa, ABplapaapap	Epithelial Identity: LIN-26/C2H2 NHR-23/ROR NHR-25/Ftz-F1 G2 identity: LAG-1/CSL
E exc gland L and gland R 	ABplpapapaa, ABprpapapaa	Unknown: LIM-6/LIM^
F CANL and CANR neurons 	ABalapaaapa, ABalappappa	CAN Identity: CEH-10/Paired Fate Execution: UNC-39/Six Unknown: CEH-23/homeodomain^

Figure 3 *C. elegans* excretory system parts list. Schematic representations of the cell types that contribute to the excretory system, their lineal origins (Sulston *et al.* 1983), and major transcription factor (TF) regulators or markers. Colors represent specific cell types throughout the figures. Anterior is to the left. Junctions (as visualized with AJM-1, DLG-1, or HMR-1/cadherin reporters) are represented with heavy black rings or lines. (A) Canal cell (ventral view). Two ring-shaped junctions connect the canal lumen to the duct and gland. (B) Duct (lateral view). Ring-shaped junctions connect the duct to the canal cell (right), and pore (left). (C and D) G1, G2, or G2p pore (lateral views). An AJ (arrow) seals the tube, and ring-shaped junctions connect the pore to the duct (top) and hypodermis (bottom). (E) Binucleate excretory gland (ventral view). A small ring-shaped secretory–excretory junction (SEJ, arrowhead) connects the gland to the canal cell. (F) CAN neurons (ventral view). *, putative phosphorylation. ^, markers suitable for visualizing the mature cells.

posterior canal arms (Sulston *et al.* 1983; Forrester and Garriga 1997), and the system begins functioning in excretion, as judged by edema formation in mutant embryos (Stone *et al.* 2009). During the L1 larval stage, the G1 pore delaminates and is replaced as pore by the epidermal cell G2 (Sulston *et al.* 1983; Parry and Sundaram 2014) (Figure 4G). In the L2 larval stage, G2 divides and its daughter G2p becomes the permanent pore.

Epithelial organization in excretory tube cells

Excretory tubes exhibit many typical epithelial characteristics (Figure 3). The apical (luminal) surface of each tube cell is marked by CRB-1/Crumbs (Bossinger *et al.* 2001) and the apical PAR proteins PAR-3, PAR-6, and PKC-3 (Mancuso *et al.* 2012; Armenti *et al.* 2014). The tubes are joined by ring-shaped apical junctions containing both the Cadherin–Catenin complex and the DLG-1–AJM-1 complex (Stone *et al.* 2009; Abdus-Saboor *et al.* 2011; Parry and Sundaram 2014; Pasti and Labouesse 2014). Apical junctions and submembranous domains are enriched for F-actin; this is particularly true of the pore cell, which has a strong F-actin signal along its autocellular junction (Parry and Sundaram 2014), and the canal cell, which has a prominent terminal web containing ACT-5/actin and cortical actin-binding proteins (Buechner *et al.* 1999; Gobel *et al.* 2004; MacQueen *et al.* 2005; Praitis *et al.* 2005) (Figure 5B). During tube development, the lumen is filled with a fibrous apical extracellular matrix (aECM) visible by means of transmission electron microscopy (TEM) (Buechner *et al.* 1999; Mancuso *et al.* 2012) (Figure 5A). In the duct and pore, this luminal matrix disappears prior to hatching and is replaced by a cuticular lining (Nelson *et al.* 1983).

Excretory system functions and phenotypes

One important role of the *C. elegans* excretory system is in osmoregulation. Long tubes of the canal cell extend throughout the length of the animal and are thought to collect excess water to be transported to the duct cell and then excreted through the pore cell in the head (Figure 2). Osmoregulatory function is inferred from morphological and molecular features of the canal cell (Figure 2, Figure 5, Table 1) and from laser ablations of the canal cell, duct, pore, or CAN neurons, which cause characteristic Clear (Clr) and rod-like larval lethal (Lvl or Let) phenotypes in which the pseudocoelom swells with accumulating fluid (Nelson and Riddle 1984; Forrester and Garriga 1997). Many mutations that perturb

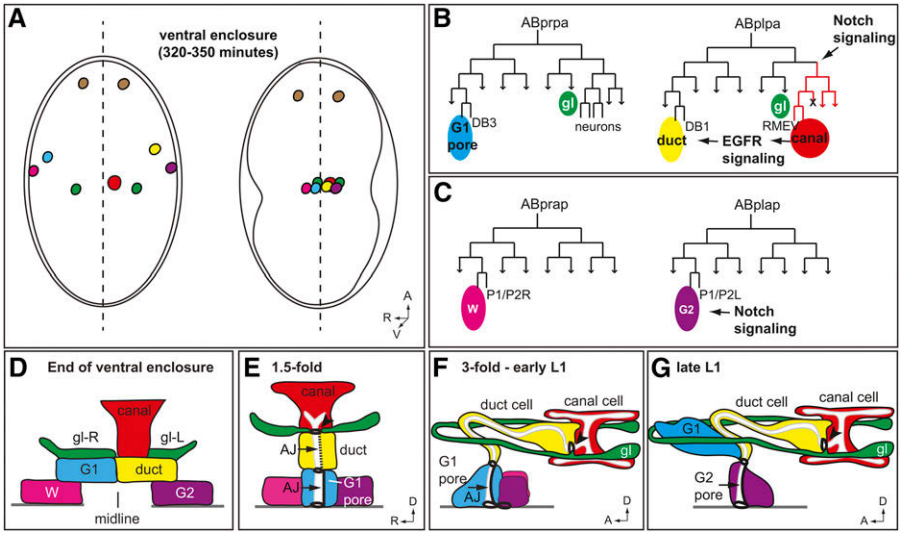

Figure 4 Overview of excretory system development. (A) Excretory system cells are born in disparate locations of the developing embryo and migrate to meet at the ventral midline during ventral enclosure (Sulston *et al.* 1983). Colors represent cell types as in Figure 3. Dashed line, ventral midline. (B) Sequential Notch and EGFR signaling break symmetry in the ABplpa *vs.* ABprpa lineages that give rise to the canal, duct, G1 pore, and gland cells (Sulston *et al.* 1983; Moskowitz and Rothman 1996; Abdus-Saboor *et al.* 2011). (C) LIN-12/Notch signaling breaks symmetry in the ABplap *vs.* ABprap lineages to promote the G2 pore *vs.* W neuroblast fate (Greenwald *et al.* 1983; Sulston *et al.* 1983). (D) Near the completion of ventral enclosure, the tube progenitor cells are in contact but have not yet formed junctions or lumen (Abdus-Saboor *et al.* 2011). Note asymmetric position of the canal cell with respect to the ventral midline. (E) By the 1.5-fold stage, the tubes have junctions consisting of AJM-1, DLG-1, and HMR-1/cadherin (heavy black lines) and a continuous lumen (white) that extends from the canal cell, through the duct and pore, and is open to the extraembryonic environment (Stone *et al.* 2009). Arrowhead, canal–duct intercellular junction. The gland cells also connect to the canal and duct at this region. Arrows, duct and pore AJs. The duct AJ autofuses at this stage (dashed lines) (Stone *et al.* 2009). (F) By late embryogenesis, the tubes have undergone morphogenesis to adopt their characteristic shapes. (G) By late in the first larval stage (L1), the G1 pore has delaminated and lost all its prior junctions and lumen, and the G2 cell has replaced it as the pore (Sulston *et al.* 1983; Parry and Sundaram 2014). Note that in F and G, the canal cell arms are drawn much shorter than actual length, and rotated relative to their actual lateral positions, to show cell shape; in particular the canal arms reach the midbody by late embryogenesis and the rectum by late L1 stage.

these cells cause a similar lethal phenotype (Yochem *et al.* 1997; Liegeois *et al.* 2007; Stone *et al.* 2009; Mancuso *et al.* 2012), while mutants with milder excretory system abnormalities often appear pale and slightly bloated and/or show increased sensitivity to osmotic challenge (Buechner *et al.* 1999; Wang and Chamberlin 2002; Hahn-Windgassen and Van Gilst 2009).

The Excretory Canal Cell

Canal cell structure and ultrastructure

The excretory canal cell is the largest and one of the most distinctively shaped mononucleate cells in the worm (Figure 2, Figure 3A). The cell body and nucleus are located just ventral to the posterior bulb of the pharynx. The nucleus is very large, which suggests that, as for intestinal nuclei, the chromosomes are endoreduplicated (Hedgecock and White 1985). The cell sends out four hollow tubules or "canals," two posterior and two anterior, forming an "H-shape" (Nelson *et al.* 1983). The canals are closed at their tips and extend the entire length of the adult animal. The canal cell is a seamless tube—it does not have adherens junctions along most of its length, but only at its connection to the excretory duct cell and gland.

The lumen of the canals is easily observed via DIC (Figure 5C) or TEM (Figure 5B). The lumen diameter is narrowest near the canal tips and widens steadily toward the excretory canal cell body. The lumen is also wider in the posterior canals than in the anterior canals. In cross-section, the basolateral surface of the canals extends between the lateral hypodermis and the basement membrane, and the canal and hypodermal membranes merge where gap junctions are present (Figure 5B). The cytoplasmic terminal web is a thick filament-rich coating adjacent to the luminal membrane (Figure 5B).

Surrounding the lumen and filling most of the cytoplasm are a series of smaller tubes or vesicles called canaliculi (Nelson *et al.* 1983) (Figures 5, B and D). EM tomography has shown that the canaliculi can be separate vesicles or attached to each other and to the lumen (Khan *et al.* 2013) (Figure 5B). Canalicular vesicles are 50–100 nm in diameter and differ from other vesicles such as endosomes in having an electron-dense coat, which at high magnification exhibits "lollipop" structures characteristic of ATPases (Figure 5D). Canaliculi appear to store water, and regulated docking of the canaliculi with the apical membrane has been proposed as a major driver of both axial and circumferential canal lumen growth (Khan *et al.* 2013; Kolotuev *et al.* 2013; Armenti *et al.* 2014) (see below).

Surrounding and interspersed with the canaliculi are multiple microtubules (MTs) running along the length of the canals (Figure 5B). The canals are rich in TBG-1/γ-tubulin, which marks MT organizing centers (MTOCs) (Zheng *et al.* 1991), and Venus::TBG-1 is visible as myriad puncta throughout the canals (Shaye and Greenwald 2015) (Figure 6D). Outside of the canalicular region, other larger diameter vesicles, as well as mitochondria, endoplasmic reticulum (ER), and Golgi, are prominently visible by TEM (Hahn-Windgassen and Van Gilst 2009; Khan *et al.* 2013; Lant *et al.* 2015) toward the basal surface of the canals (Figure 5B).

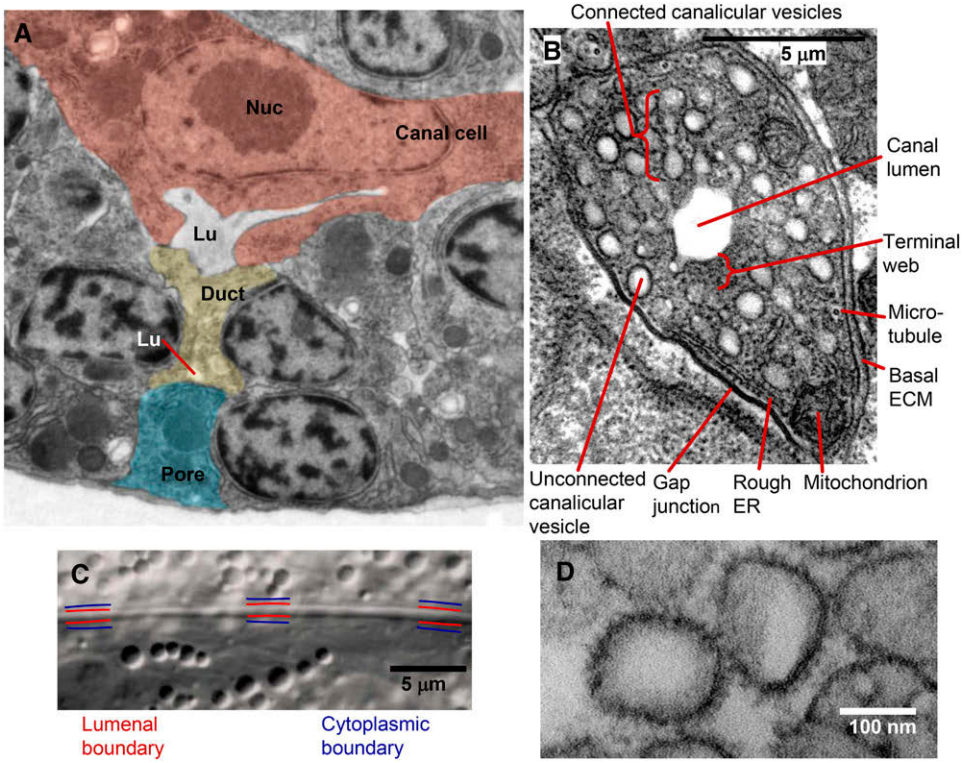

Figure 5 Excretory canal cell structure and ultrastructure. (A) TEM of 420 min (comma stage) embryo canal, duct, and pore (tinted as in previous pictures) shows canal lumen developing at the point where canal contacts the duct (TEM courtesy of Shai Shaham, The Rockefeller University). Nuc, cell nuclei; Lu, lumen. (B) TEM of transverse section of an adult anterior canal, shows multitude of canalicular vesicles, some connected to the round central lumen and/or to each other, while others are disconnected. MTs surround the canaliculi, as do ER and mitochondria. The left side of this section is almost entirely gap junction, while the right side is separated from muscle by basal extracellular matrix. The terminal web is stained lightly in this section and surrounds the central lumen (M. Buechner, D. Hall, E. Hedgecock, unpublished results). Note that the membrane of the canalicular vesicles stains more darkly than does the plasma membrane or luminal membrane. (C) DIC micrograph of a section of the posterior canal in N2 adult (M. Buechner and E. Hedgecock, unpublished results). Boundary between lumen and cytoplasm (red guidelines) is highly refractive and shows up well in DIC. Boundary between cytoplasm and hypodermis (blue guidelines) is more difficult to see. (D) High-resolution TEM of canalicular vesicles in adult *exc-7(rh252)* mutant, in which the cytoplasmic contents appear more dilute. Each canalicular vesicle is surrounded by proteins protruding into the lumen, that appear reminiscent of the lollipop structure of ATPase (M. Buechner, D. Hall, E. Hedgecock, unpublished results).

Lineal origin and Notch dependency of the canal cell

Notch signaling promotes left/right asymmetry in *C. elegans* (Priess 2005), as in other organisms (Kato 2011), and is required to generate the asymmetrically positioned canal cell (Lambie and Kimble 1991; Moskowitz and Rothman 1996) (Figure 4, A and B). The ABpl and ABpr lineages generate mostly bilaterally symmetric descendants (Sulston *et al.* 1983), with some exceptions that depend on signaling by the Delta-like ligand LAG-2 through the Notch receptors LIN-12 and GLP-1 and the downstream CSL [CBF1/Su(H)/LAG-1] family transcription factor LAG-1 (Lambie and Kimble 1991; Moskowitz and Rothman 1996; Priess 2005). LAG-2 is expressed by interior MSap descendants on the left side of the embryo and activates Notch signaling in various ABpl (but not ABpr) descendants. Notch signaling promotes the identity of ABplpapp (Moskowitz and Rothman 1996), which gives rise to the canal cell and a mix of other epithelial and neuronal descendants. In the absence of Notch signaling, ABplpapp adopts an ABprpapp-like identity and generates mostly neuronal descendants; no canal cell is generated. The only known downstream target of this signaling is the Hes-related transcription factor REF-1 in ABplpapp descendants, though *ref-1* mutants do not have defects in this cell fate decision (Neves and Priess 2005).

Within the ABplpapp lineage, multiple asymmetric cell divisions generate the canal cell (Sulston *et al.* 1983) (Figure 4B). The canal cell's grandmother, ABplpappa, generates a larger anterior daughter that survives and a smaller posterior daughter that undergoes apoptosis. In *ceh-36*; *unc-30* double mutants, this asymmetry can be reversed (Walton *et al.* 2015). In *pgl-1 ced-3* double mutants, the posterior daughter can survive to generate an extra canal cell (Denning *et al.* 2012). The next asymmetric division, of the canal cell's mother ABplpappaa, generates a small anterior daughter that becomes the RMEV neuron and a larger posterior daughter that becomes the excretory canal cell. Since a noncanonical Wnt pathway involving WRM-1/β-catenin promotes many anterior/posterior asymmetries in the *C. elegans* lineage (Sawa and Korswagen 2013; Zacharias *et al.* 2015), this pathway is a good candidate for controlling such asymmetries relevant to canal cell identity.

Transcription factors important for canal cell differentiation

No single transcription factor has been found to specify canal cell identity, but the following are important for canal differentiation (Figure 3A):

LIN-26 is a nematode-specific C2H2 zinc finger protein important for epithelial differentiation and maintenance, whose loss leads to epidermal degeneration as well as variable defects in canal morphology (Labouesse *et al.* 1994, 1996). The final cell division that generates the

Table 1 Membrane channel or transporter proteins expressed in the excretory canal cell

Canal-expressed genes	Human homologs (if present)	Associated vertebrate diseases or traits	*C. elegans* gene reference(s)	Disease references
INX-3, 5, 12, 13	None (invertebrate innexin gap junction proteins)		Altun *et al.* 2009	
AQP-2, 3, 8	Aquaporin 3, 9, 10	GIL blood group	Huang *et al.* 2007; Khan *et al.* 2013	Rumsey and Mallory 2013
VHA-1, 5, 8, 12, 13, 19	Vacuolar ATPase (ATP-dependent proton pump)	Renal distal tubular acidosis, osteopetrosis	Oka *et al.* 1997; Liegeois *et al.* 2006, 2007; Hahn-Windgassen and Van Gilst 2009; Knight *et al.* 2012	Breton and Brown 2013
APTS-2, Y70G10A.3	SLCO4A1, C1 solute carrier organic anion transporters	Reduction of renal-failure-induced hypertension	Sherman *et al.* 2005	Suzuki *et al.* 2011
SULP-4, 5, 8	SLC26A4, A6 sulfate permease anion exchanger, (pendrin)	Pendred syndrome	Sherman *et al.* 2005	Everett *et al.* 1997; Soleimani 2015
NHX-9	NHE3 cation proton antiporter and ezrin linker	Congenital Na^+ diarrhea	Nehrke and Melvin 2002	Donowitz *et al.* 2013
MRP-2, PGP-3, 4, 12	ATP-binding cassette (ABC) transporters ABCB1, ABCB4	Chemotherapeutics resistance, cholestasis	Zhao *et al.* 2005; Armstrong and Chamberlin 2010	Srivastava 2014
NAC-2	SLC13A3 Na^+-coupled dicarboxylate transporter	Association with Canavan disease, hypertension	Armstrong and Chamberlin 2010	Bergeron *et al.* 2013
TWK-36	KCNK4 (TRAAK,TWiK) mechanosensitive potassium channel		Salkoff *et al.* 2001	Brohawn 2015
CLH-3, 4	CLCN1 voltage-sensitive chloride channel 1	Bartter syndrome (hyponatremia), myotonia congenital disease	Schriever *et al.* 1999; Hisamoto *et al.* 2008	Andrini *et al.* 2015; Imbrici *et al.* 2015
EXC-4	CLIC4 chloride intracellular channel 4	X-linked intellectual disability	Berry *et al.* 2003; Berry and Hobert 2006	Takano *et al.* 2012; Jiang *et al.* 2014
GTL-2	TRPM3,6,7 transient receptor potential magnesium channels	Hypomagnesemia	Teramoto *et al.* 2010; Wang *et al.* 2014a	Li *et al.* 2006; de Baaij *et al.* 2015

canal partitions epithelial and neuronal fates (Figure 4B), and activation of LIN-26 in the canal is likely to be an important early step in epithelial determination.

PROS-1 (formerly called CEH-26 or RDY-3) is a Prospero-related homeodomain transcription factor (Kolotuev *et al.* 2013). In *pros-1* mutants, there is a nearly complete failure of canal cell and lumen outgrowth, although many aspects of canal cell identity appear intact. Downstream (direct or indirect) targets of PROS-1 include the aquaporin gene *aqp-8*, the guanine nucleotide exchange factor gene *exc-5*, the germinal center kinase gene *gck-3*, and the intermediate filament gene *ifb-1*; the last appears to be a direct target (Kolotuev *et al.* 2013). The mammalian homolog Prox1 promotes development of the lymphatic system (Wigle and Oliver 1999), suggesting possible similarities between the canal tube and lymphatic vessels (Kolotuev *et al.* 2013).

CEH-6 is a POU-family homeodomain transcription factor related to mammalian Brn1, which is required for development and function of the distal tubule of the mouse kidney (Burglin and Ruvkun 2001; Nakai *et al.* 2003). *ceh-6* mutants have canal cell and outgrowth defects similar to those of *pros-1* mutants, but less severe. Downstream (direct or indirect) targets of CEH-6 include multiple transporter- or channel-encoding genes, including *aqp-8*, *clh-4*, *mrp-2*, *nac-2*, *pgp-3*, *pgp-4*, *pgp-12*, *sulp-4*, *sulp-5*, *sulp-8*, *twk-36*, *vha-12*, *vha-13*, and Y70G10A.3; *aqp-8* and *nac-2* appear to be direct targets (Mah *et al.* 2007; Armstrong and Chamberlin 2010).

NHR-31 is a nuclear hormone receptor related to mammalian HNF4α. NHR-31 is required for expression of *vha* genes encoding subunits of vacuolar ATPase and appears to restrain and coordinate both apical and basal membrane growth (Hahn-Windgassen and Van Gilst 2009).

Neural guidance cues and basement membrane steer canal outgrowth

To adopt its distinctive H-shape, the canal cell undergoes an extensive program of elongation and branching that is regulated by neural guidance cues such as netrins, Wnts, and FGFs and by other factors in the extracellular matrix such as collagens and laminins (Hedgecock *et al.* 1987; Buechner 2002; Oosterveen *et al.* 2007; Polanska *et al.* 2011). Once born, the canal cell begins to grow out left and right over the ventral muscle quadrants from the cell body to the lateral

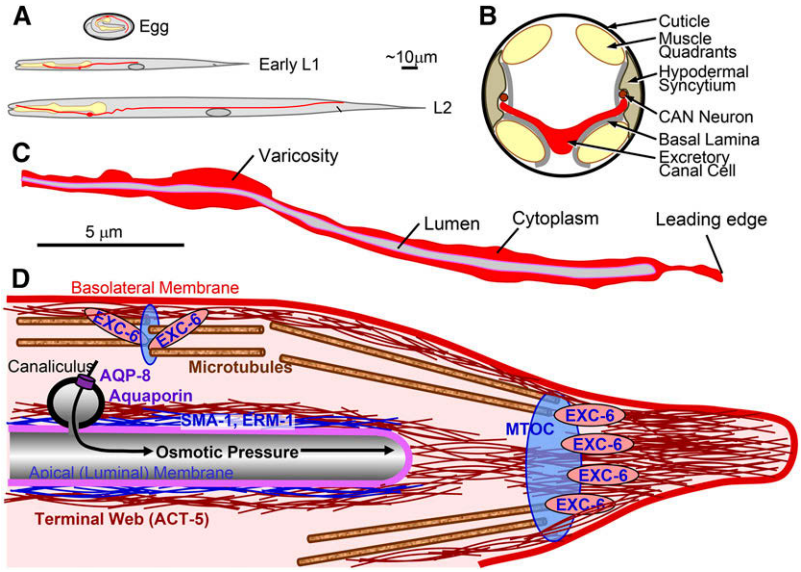

Figure 6 Canal cell outgrowth. (A) Canal length extends greatly during the L1 stage. Diagrams to scale of animals with left canals shown in red. At hatch, the posterior canals extend only as far as the gonad (light gray). During the next 8 hr the canals extend to the anus (black diagonal line) and have reached full length by the beginning of L2 stage, while the animal has also lengthened considerably. (B) Transverse section of the animal near the canal cell body. The cell body (red) stretches across left and right muscle quadrants beneath the pharynx and extends canals to the hypodermis. Some basal extracellular matrices (basal lamina) are shown, incompletely, in gray. Canal arms must cross the hypodermal basal lamina to extend posteriorward alongside the hypodermis and the CAN neuron (brown). (C) Growth of the tip of a canal during wild-type L1 stage, adapted from fluorescence micrograph (Kolotuev *et al.* 2013). The basolateral surface of the canals precedes the end of the lumen (apical surface) by several micrometers. In addition, large varicosities of the cytoplasm appear at regular intervals along the canal during growth, while the lumen diameter is less variable. (D) Model for canal tip outgrowth, adapted from Shaye and Greenwald (2015) and Khan *et al.* (2013) (not to scale). EXC-6/INF2 formin is spread throughout the canal and concentrated at varicosities and especially at the canal tip, where it helps to form a large actin patch to push the membrane forward. EXC-6 is also associated with MTOCs that nucleate MTs growing predominantly toward the cell body. Actin filaments at the tip may extend to connect the basolateral surface to the terminal web of the apical surface, providing guidance for growth of that surface as well. Growth of the apical surface may occur through ERM-1, RAL-1, and exocyst (not shown; see Figure 7)-mediated fusion of canaliculi to the luminal surface, which allows water to pass through aquaporin AQP-8 into the canal lumen; increased osmotic pressure drives luminal expansion.

surface. Both left and right extensions branch, and each extends the wide posterior-directed canals as well as the smaller anterior-directed canals. These branches are visible in GFP-marked cells by the time of the threefold stage (Suzuki *et al.* 2001) (Figure 6A). At hatch, the posterior canals reach approximately the V3/V4 interval in the midbody. The canal arms reach their target positions at the V6/T boundary by the end of the L1 stage (Fujita *et al.* 2003). While extension along the hypoderm then ceases, the canals continue to lengthen to maintain their positions as the animal grows to adulthood.

The canals are positioned between the hypodermis and a basement membrane (Figure 6B). It is unknown whether this basement membrane is synthesized by the canal cell or if the canals insinuate themselves between the hypodermis and its own basement membrane. Supporting the second possibility, the canal synthesizes several matrix metalloproteases (Spencer *et al.* 2011) that could be used for cutting through the basement membrane. In addition to the guidance cues mentioned above, multiple other genes and pathways that affect the extent of canal outgrowth appear to function in the hypodermis or in other nearby tissues over which the canal grows (Polanska *et al.* 2011; Marcus-Gueret *et al.* 2012).

Relatively little is known about molecular pathways that direct basal canal branching, but a few mutants have been observed that exhibit extra canal arms at low penetrance. These include mutants for *unc-6*/Netrin or *unc-5*/Netrin receptor (Hedgecock *et al.* 1987); *nck-1*(*mig-1*)/Nck kinase (Mohamed and Chin-Sang 2011); and *axl-1*, an axin-like molecule (Oosterveen *et al.* 2007).

Cytoskeletal organization in the growing canals

Extracellular guidance cues likely regulate cytoskeletal organization to direct canal outgrowth, similar to what occurs during neurite outgrowth (Dent *et al.* 2011). The growing tip of the canals is wider than the canal behind it, in appearance similar to a growth cone of a neuron (Suzuki *et al.* 2001). Canal basal membrane outgrowth slightly precedes apical membrane outgrowth, such that the growing tips of each canal arm initially lack lumen (Kolotuev *et al.* 2013) (Figure 6C). The growing tips are marked by a cluster of MTOCs and by a patch of F-actin that accumulates basolaterally rather than apically (Figure 6D), which may be important for basal outgrowth (Shaye and Greenwald 2015).

Mutations in several cytoskeletal regulators result in shortened canals without disrupting the formation or structure of the lumen.

Integrins PAT-3, PAT-2, and INA-1 link laminins and other extracellular matrix factors to the cytoskeleton (Gettner *et al.* 1995; Baum and Garriga 1997). While null mutations in *pat-3* are embryonic lethal, hypomorphs and canal mosaics show length defects (Hedgecock *et al.* 1987).

UNC-53, homologous to vertebrate NAV2, is an actin- and MT-binding protein that affects many anterior–posterior cell and axon migrations, as well as canal outgrowth (Stringham *et al.* 2002). UNC-53 acts cell autonomously and in a common pathway with UNC-71 (ADAM), MIG-10 (Lamellipodin), ABI-1 (Abelson Interactor-1), WVE-1 (WAVE), ARX-2 (Arp2), and the Rho-specific guanine nucleotide exchange activity of UNC-73 (TRIO) (Schmidt *et al.* 2009; Marcus-Gueret *et al.* 2012; McShea

et al. 2013; Ghosh and Sternberg 2014; Wang *et al.* 2014b). The contactin RIG-6 also functions cell autonomously in the canal, potentially as part of the UNC-53 pathway (Katidou *et al.* 2013). Finally, PLR-1, a putative E3 ubiquitin ligase, interacts with UNC-53 to regulate outgrowth (Bhat *et al.* 2015).

Kinesins are motor proteins that transport vesicles along MTs (Caviston and Holzbaur 2006). UNC-116/kinesin-1 heavy chain and KLC-2/kinesin light chain are expressed in the canal, and mutation of either gene results in short canals (Patel *et al.* 1993; Shaye and Greenwald 2015).

The canal cell likely forms a seamless tube through a hollowing mechanism

Canal lumen formation initiates around the lima bean stage of embryogenesis, near the newly formed junction with the excretory duct cell (Stone *et al.* 2009; Abdus-Saboor *et al.* 2011; Mancuso *et al.* 2012) (Figure 4E, Figure 5A). Apical PAR proteins (Achilleos *et al.* 2010; Mancuso *et al.* 2012; Armenti *et al.* 2014) and the ezrin/radixin/moesin ortholog ERM-1 (Khan *et al.* 2013) are among the earliest markers of the nascent canal lumen, which suggests that recruitment of these proteins may be involved in lumen nucleation. One model consistent with existing data is that PAR proteins recruit the exocyst complex (Armenti *et al.* 2014) to promote vesicle docking at the canal–duct interface, while ERM-1 organizes actin to coat these vesicles and promote their fusion with the apical membrane (Khan *et al.* 2013), which then grows inward (Figure 1D). However, most studies have focused on postembryonic stages of canal outgrowth, and it remains unknown whether the aforementioned gene products are actually required for lumen nucleation.

Models for canal elongation and maintenance

Possible models for canal elongation and maintenance are shown in Figure 6D and Figure 7. Elongation requires ERM-1 and the exocyst, which recruit canaliculi and other vesicles to the apical membrane by interacting with vesicle-residing proteins such as AQP-8 or RAL-1 (Khan *et al.* 2013; Armenti *et al.* 2014). The vacuolar ATPase (V-ATPase) and the aquaporin AQP-8 drive water into canaliculi (Khan *et al.* 2013; Kolotuev *et al.* 2013). Canaliculi may fuse into the canal apical membrane (Kolotuev *et al.* 2013; Armenti *et al.* 2014), or they may dock only transiently to dump water into the lumen and thereby increase hydrostatic pressure to push the lumen outward (Khan *et al.* 2013). Other Golgi-derived or endocytic vesicles follow MT and/or actin tracks to reach their proper destinations and deliver apical or basal membrane and other cargo. In particular, EXC-1/5/9 and the CCM-3–STRIPAK complex regulate CDC-42 to promote recycling endosome trafficking for apical membrane maintenance. Actin filaments of the terminal web, linked to the apical membrane by ERM-1 (Gobel *et al.* 2004; Khan *et al.* 2013) and SMA-1/β-spectrin (Buechner *et al.* 1999; Praitis *et al.* 2005), influence trafficking and also cooperate with intermediate filaments (Woo *et al.* 2004; Kolotuev *et al.* 2013) to provide structural support and restrain lumen diameter, guiding growth in the axial direction. At the growing canal tips, apical and basal membranes are linked by cytoskeletal filaments and the formin EXC-6 to coordinate apical *vs.* basal outgrowth (Shaye and Greenwald 2015).

During periods of rapid canal growth, such as during the L1 larval stage or during recovery from hyperosmotic shock, basally protruding cytoplasmic bulges, variously called "pearls," "varicosities," or "periluminal cuffs," form at regular intervals along the length of the canal arms (Figure 6C). Pearls are rich in organelles such as ER and Golgi (Hahn-Windgassen and Van Gilst 2009), and most docking of canaliculi to the apical membrane occurs in these pearl regions (Kolotuev *et al.* 2013). Osmotic stress stimulates pearl formation, canalicular docking, and aquaporin-mediated water influx into the lumen, thereby providing a mechanism to speed lumen growth in response to physiological demands (Khan *et al.* 2013; Kolotuev *et al.* 2013). This proposed role of aquaporin-induced water influx is similar to that of blood flow in stimulating lumen growth in vascular capillaries (Herwig *et al.* 2011).

These models are based on studies of the following groups of genes, which are important for canal lumen growth, shape, or maintenance. Only mutants with the most severe canal defects are lethal, whereas many mutants are viable but have cystic lumens (Exc phenotype).

Gene products required for most or all lumen growth

There are only a few known gene products in this category, and their analysis so far has been limited to larval stages.

PROS-1: The transcription factor PROS-1 upregulates many genes important for canal lumen growth, including the intermediate filament gene *ifb-1* (Kolotuev *et al.* 2013) (see above).

ERM-1: ERM proteins are membrane-actin linkers with broad roles in cortical membrane organization (Neisch and Fehon 2011) and intracellular tubulogenesis (JayaNandanan *et al.* 2014; Jiang *et al.* 2014). ERM-1 localizes near the apical membrane and is essential for terminal web organization and for both apical and basal canal outgrowth (Khan *et al.* 2013) (Figure 6D, Figure 7). ERM-1 is necessary and sufficient to recruit canaliculi containing the Aquaporin AQP-8 to the apical membrane (Figure 6D), but also has AQP-8-independent functions, which may involve recruiting other classes of vesicles (Khan *et al.* 2013).

RAL-1 and the exocyst: The small GTPase Ral helps direct assembly of the exocyst, a multiprotein complex that tethers exocytic vesicles to the plasma membrane for subsequent SNARE-mediated membrane fusion (Liu and Guo 2012). RAL-1 on canaliculi was proposed to interact with the exocyst on the apical membrane to promote canalicular fusion and lumen growth (Armenti *et al.* 2014)

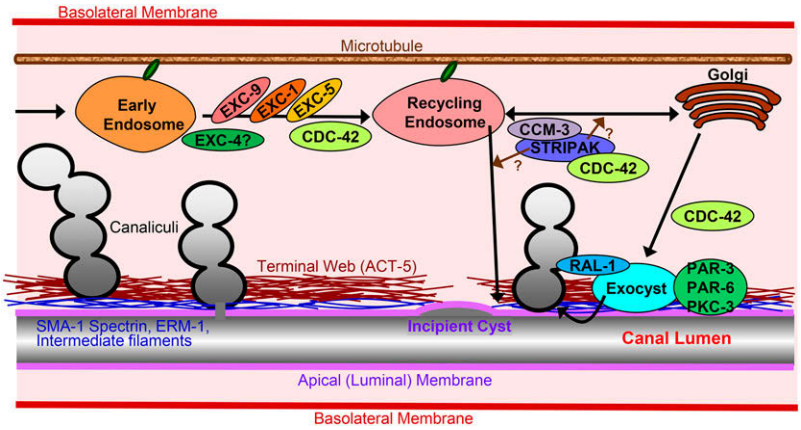

Figure 7 Model for vesicle trafficking in the canal cell. Speculative model of factors allowing extension and maintenance of the luminal surface. Intermediate filaments, ACT-5/actin, ERM-1, and SMA-1/β_H-spectrin surround the luminal surface (Gobel *et al.* 2004; Praitis *et al.* 2005; Khan *et al.* 2013; Kolotuev *et al.* 2013), where they maintain the smooth diameter of the lumen. ERM-1 interacts with AQP-8 on canaliculi (Khan *et al.* 2013), and RAL-1 GTPase on canaliculi and the PAR complex on the apical surface attract the exocyst to fuse canaliculi to the apical surface (Armenti *et al.* 2014), which could allow the activity of vacuolar ATPase to drive water into the lumen via AQP-8 (Figure 6D, Figure 8) (Khan *et al.* 2013). Various marked endosomes can be viewed moving anteriorward and posteriorward throughout the length of the canals (H. Al-Hashimi and M. Buechner, unpublished results). The canal cytoplasm is rich in MTs (Shaye and Greenwald 2015), which presumably form tracks for this movement. By analogy to its role in vertebrate kidney proximal tubules (Chou *et al.* 2016), EXC-4/CLIC may mediate vesicle movement in growing canals. In addition, two sets of protein cascades promote trafficking through recycling endosomes: EXC-9/CRIP (Tong and Buechner 2008), EXC-1/IRG (K. Grussendorf, D. Hall, M. Buechner, unpublished results), and EXC-5/FGD (Mattingly and Buechner 2011) activate CDC-42 (Olson *et al.* 1996; Gao *et al.* 2001) to stimulate growth of actin filaments and/or transport of endosomes along those filaments. Similarly, CCM-3 and the STRIPAK complex also activate CDC-42 to promote trafficking, possibly toward or from the Golgi (Lant *et al.* 2015). Presumably, some feature of a weakened or growing luminal cytoskeleton activates EXC-9 and/or CCM-3 to strengthen the cytoskeleton at these points. The recycling endosomes are hypothesized to allow maintenance and/or growth of the lumen, presumably by supplying lipid and/or membrane proteins either directly to the luminal membrane, or indirectly via the canalicular vesicle membrane.

(Figure 7). The canal lumen appears short and misshapen in *ral-1* or *sec-5* zygotic mutants, and nearly absent in *ral-1* maternal-zygotic mutants. Conversely, ectopic lumen pockets form under conditions of RAL-1 overexpression. RAL-1 may also play a role in production or stabilization of canaliculi, since *ral-1* mutants have severely reduced numbers of canaliculi, and some canaliculi appear aberrantly docked at the basal plasma membrane.

Cytoskeletal factors and the terminal web

The cytoskeleton helps direct lumen growth to appropriate locations and provides structural support to maintain lumen shape and integrity.

ACT-5 (*actin*) is expressed almost exclusively in the terminal webs of the canal cell and intestine (MacQueen *et al.* 2005) (Figure 6D, Figure 7). In *erm-1*(RNAi) conditions that slow lumen formation, ACT-5 coats large-diameter vesicles as they coalesce (Khan *et al.* 2013). No phenotype has yet been attributed to *act-5* loss, probably due to redundancy with other actin paralogs, but *act-5* genetically interacts with other genes such as *erm-1* and *sma-1* (Gobel *et al.* 2004).

SMA-1 is homologous to the F-actin cross-linker β_H-spectrin (Bennett and Healy 2008). SMA-1 is located at the apical membrane of the canal cell (Praitis *et al.* 2005) and is needed to maintain the terminal web at the apical membrane (Buechner *et al.* 1999) (Figure 6D, Figure 7). Strong *sma-1* mutants often exhibit a canal lumen that appears like a very large sac, a single cyst surrounded by myriad canalicular vesicles. *sma-1* genetically interacts with both *erm-1* and *act-5* for this phenotype (Gobel *et al.* 2004). *sma-1* mRNA is bound at its 3′ UTR by EXC-7, a member of the ELAV/Hu family of RNA-binding proteins, which regulate RNA stability and translation (Fujita *et al.* 2003; Simone and Keene 2013). Null *exc-7* mutants have the same canal phenotype as does mild knockdown of *sma-1* (Buechner *et al.* 1999; Fujita *et al.* 2003). These data suggest a model in which EXC-7 binding stabilizes *sma-1* mRNA to be carried to the canal distal tips to be translated, analogous to the role of *Drosophila* ELAV and mammalian Hu proteins in stabilizing neural mRNAs (Bronicki and Jasmin 2013; Simone and Keene 2013).

EXC-6 is homologous to human INF2, a formin with both actin polymerization and MT-binding activities (Shaye and Greenwald 2015). Human mutations in INF2 cause inherited forms of the kidney disease focal segmental glomerulosclerosis (Brown *et al.* 2010) and can also cause Schwann cell actinopathy (Mathis *et al.* 2014). EXC-6 associates with MTs and affects both actin localization and MT dynamics at the growing tips of the canal arms (Shaye and Greenwald 2015) (Figure 6D). *exc-6* mutants have short canals with multiple lumens (Buechner *et al.* 1999), consistent with EXC-6 promoting basal membrane growth and restricting apical membrane growth to the leading edge.

TBB-2 is a β-tubulin expressed in the canals (Spencer *et al.* 2011). *tbb-2* mutants exhibit a similar canal phenotype to that of *exc-6*, while *tbb-2* gain-of-function mutations suppress the effects of *exc-6* mutation (Shaye and Greenwald 2015).

Intermediate filament proteins in the cytoplasm provide mechanical strength to tissues (Carberry *et al.* 2009). IFB-1 is located at the periapical region of the canal and is required for canal outgrowth and integrity (Woo *et al.*

2004; Kolotuev *et al.* 2013). IFB-1 has two protein isoforms, IFB-1A and IFB-1B; both are expressed in the canal cell, but IFB-1B appears more critical for canal cell function (Woo *et al.* 2004). IFB-1 forms heterodimers with multiple other intermediate filament proteins, including IFA-1/2/3/4 (Karabinos *et al.* 2003). At least two of these, IFA-1 and IFA-4, are also expressed in the excretory canal cell (Karabinos *et al.* 2003).

Regulators of recycling endosome trafficking

Recycling endosomes are important for later stages of canal outgrowth and for maintenance of the terminal web. The relevant cargo in these endosomes, or from where it originates, is unknown, but a reasonable model is that apical membrane proteins and lipids are being continuously endocytosed and recycled, as has been shown in many epithelia (Eaton and Martin-Belmonte 2014). Recycling endosomes may return material directly back to the apical membrane or may traffic material to the Golgi for subsequent sorting and exocytosis (Figure 7).

CDC-42 is a Rho-family GTPase that plays widespread roles in apical cell polarity and cytoskeletal organization, in part by regulating vesicular transport to and from the Golgi network (Harris and Tepass 2010; Park *et al.* 2015). Both in *C. elegans* and mammalian cells, CDC-42 is colocalized with the recycling endosome marker RME-1 and promotes retrograde transport of certain cargoes from recycling endosomes to Golgi (Balklava *et al.* 2007; Bai and Grant 2015). In the canal cell, CDC-42 is critical for lumen extension and maintenance and appears to act via effects on endocytic recycling (Mattingly and Buechner 2011; Lant *et al.* 2015). CDC-42 activity is regulated by at least two sets of gene products, EXC-1/5/9 and CCM-3/STRIPAK.

cdc-42 dominant-negative mutants or *cdc-42*(RNAi) animals have shortened canals with lumen dilations or cysts that are depleted for Golgi and recycling endosome markers (Mattingly and Buechner 2011; Lant *et al.* 2015), consistent with a role for CDC-42 in endocytic recycling (Figure 7). Expression of constitutively active CDC-42 results in a convoluted lumen phenotype, in which a long lumen of near-normal diameter forms within a canal cell of greatly reduced basal surface area (Mattingly and Buechner 2011), which suggests that CDC-42 also influences the balance between apical-directed and basolateral-directed trafficking.

EXC-1, EXC-5, and EXC-9: EXC-1 is homologous to the mammalian immunity-related GTPase (IRG) family, a set of dynamin-related GTPases that are found at intracellular membrane compartments, some of which target parasite-containing vacuoles for autophagy and destruction (K. Grussendorf and M. Buechner, unpublished results) (Howard 2008; Petkova *et al.* 2012). EXC-5 is homologous to the mammalian FGD family of guanine nucleotide exchange factors (GEFs), regulators of CDC-42 activity that are mutated in faciogenital dysplasia (FGD) and Charcot-Marie-Tooth (CMT) disease type 4H (Olson *et al.* 1996; Gao *et al.* 2001; Suzuki *et al.* 2001; Delague *et al.* 2007). FGD5 also is required for capillary formation in culture (Kurogane *et al.* 2012). EXC-9 is homologous to mammalian cysteine-rich intestinal protein (CRIP), a cytoplasmic LIM-domain protein of unclear biochemical function (Lanningham-Foster *et al.* 2002; Tong and Buechner 2008); a vertebrate homolog is expressed in the pronephros (Hempel and Kuhl 2014) and a *C. elegans* homolog, VALV-1, appears to aid in the function of several epithelial valves (Tong and Buechner 2008).

exc-1, *exc-5*, or *exc-9* loss-of-function mutants have similar phenotypes in which canal length is reduced and the canal lumen becomes progressively dilated or cystic starting in late embryogenesis (Buechner *et al.* 1999). In *exc-5* mutants, cyst regions show reduced apical accumulation of F-actin, and the leading edge of the canal arms also lacks its usual basolateral F-actin patch, consistent with actin organization being a key downstream readout of EXC-5 function (Shaye and Greenwald 2015). The cystic regions are enriched for the early endosome marker EEA-1, but depleted of the recycling endosome marker RME-1, while Golgi markers remain unchanged (Mattingly and Buechner 2011). Overexpression of *exc-1*, *exc-5*, or *exc-9* all result in a convoluted-lumen phenotype (Suzuki *et al.* 2001; Tong and Buechner 2008; Mattingly and Buechner 2011; K. Grussendorf and M. Buechner, unpublished results) similar to that of constitutive CDC-42 activation. Epistasis experiments based on the loss-of-function and overexpression phenotypes indicate that EXC-9 acts upstream of EXC-1, and both act upstream of EXC-5. Yeast-2-hybrid results additionally indicate that EXC-9 binds to wild-type EXC-1. These results suggest that EXC-1/5/9 act upon CDC-42 in a common pathway for apical membrane recycling (Mattingly and Buechner 2011; K. Grussendorf, C. Trezza, A. Salem, B. Mattingly, H. Al-Hashimi, D. Kampmeyer, L. Khan, V. Göbel, D. Hall, B. Ackley, M. Buechner, unpublished results), but do not affect trafficking to Golgi (Figure 7).

CCM-3 is the worm homolog of CCM3/PDCD10, a scaffold protein that is part of several protein complexes, including the striatin-interacting phosphatase and kinase complex (STRIPAK) (Hwang and Pallas 2014; Lant *et al.* 2015). A key role of CCM-3 is to recruit GCKIII family kinases to promote Golgi assembly and polarization (Fidalgo *et al.* 2010; Kean *et al.* 2011). Mammalian mutations in CCM-3 cause an autosomal-dominant form of cerebral cavernous malformation (CCM), a neurovascular disease associated with swelling and rupture of brain capillaries (Draheim *et al.* 2014). Mutants for *ccm-3*, *gck-1*, *cash-1*/striatin, *farl-11*/STRIP, or *mrck-1*/MRCK (myotonic dystrophy-related CDC-42-binding kinase) have shortened canals and a cystic lumen phenotype similar to that of *exc-1*, *exc-5*, or *exc-9* mutants, although the

phenotype originates later in development (Lant *et al.* 2015). The cyst regions in *ccm-3* mutants do not accumulate early endosome markers, but are depleted for Golgi, ER, and recycling endosome markers and are enriched in canaliculi and other abnormal vesicles. *ccm-3* mutation also results in reduced activation of CDC-42. CCM-3 is therefore concluded to act with GCK-1 and other STRIPAK homologs to activate CDC-42 (Lant *et al.* 2015). Based on the differences in early endosome and Golgi marker accumulation, CCM-3 and STRIPAK may act on CDC-42 at a later step of recycling than do EXC-1/5/9 (Figure 7).

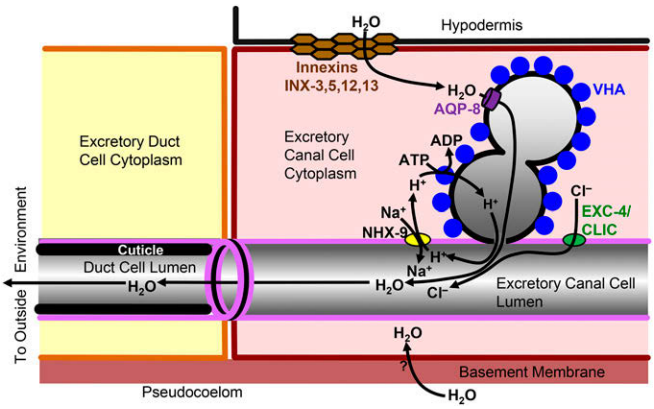

Figure 8 Speculative model of osmoregulatory function of the excretory canals. Excess organismal liquid may flow from the hypodermis into the canals via the many gap junctions between the two tissues and/or may cross the basement membrane from the pseudocoelom to the canals. Canalicular vesicles contain vacuolar ATPase and aquaporin AQP-8 (Khan *et al.* 2013; Kolotuev *et al.* 2013). Depending on environmental osmolarity, canaliculi may store water or transport water into the lumen. ATPase pumps protons into the lumen, which may attract a counter-ion into the lumen via EXC-4/CLIC or another associated channel (Berry *et al.* 2003; Berry and Hobert 2006; Ulmasov *et al.* 2009). The sodium-proton exchanger (NHX-9) is hypothesized to prevent acidification of the lumen, increasing osmolarity of NaCl sufficient to draw water into the lumen of the channel. Water then flows through the excretory duct and pore to the outside environment. In this model, the duct cuticle and membrane are impermeable to water. Pumps and channels in the duct might retrieve the NaCl to prevent its loss; or pumps and channels in the hypodermis could replenish Na^+ and Cl^- from the environment.

Other potential trafficking regulators

The following genes also may influence trafficking, but the specific step(s) affected is unknown.

EXC-4 is homologous to mammalian chloride intracellular channel (CLIC) proteins (Berry *et al.* 2003). Mutants have severely truncated canals with cystic lumens (Buechner *et al.* 1999). Vertebrate CLIC proteins have been proposed to act in many capacities, including as: chloride channels (allowing counter-ion transport, during vesicle acidification in hollowing endothelial cells) (Ulmasov *et al.* 2009); enzymes (homologous to GST) (Littler *et al.* 2010); cytoskeletal adaptors (associating with ERM proteins) (Jiang *et al.* 2014); or a combination of these, since the proteins can convert between cytosolic and integral membrane forms (Littler *et al.* 2010; Jiang *et al.* 2014). Mouse CLIC4 is needed for endolysosome formation during Cdc42-dependent lumen formation in kidney proximal tubule cells, prior to becoming progressively enriched at the luminal surface (Chou *et al.* 2016), which may represent a conserved role for EXC-4/CLIC4 in promoting vesicle trafficking during luminogenesis (Figure 7). *C. elegans* EXC-4 is constitutively membrane-associated, acts as an ion channel at pH below 6 (Littler *et al.* 2010), is required during early lumen elongation (Berry *et al.* 2003), and is found at the luminal membrane of adult canals (Berry *et al.* 2003; Berry and Hobert 2006). The site of its action in excretory cell luminogenesis has not been determined; an attractive hypothesis is that EXC-4 might acidify vesicles for transport to promote trafficking-mediated lumen elongation (Figure 7). An alternative, and not mutually exclusive, model is that EXC-4 at the luminal surface provides counter-ions to V-ATPase-driven protons that promote osmotic-mediated lumen elongation (Kolotuev *et al.* 2013), and this function is also required for lumen maintenance (Figure 8).

DAF-6 and CHE-14 are homologs of Patched and Dispatched, respectively, transmembrane proteins involved in Hedgehog signaling, endocytosis, and polarized secretion (Tukachinsky *et al.* 2012; D'Angelo *et al.* 2015). DAF-6 and CHE-14 are localized to apical membranes and function redundantly to promote canal lumen extension (Perens and Shaham 2005). Although *C. elegans* does not have a standard Hedgehog (Hh) signaling pathway, it does contain various "Warthog" and "Groundhog" proteins related to Hedgehog (Burglin 1996), several of which are expressed in the canal cell (Hao *et al.* 2006). Based on molecular identities, DAF-6 and CHE-14 might regulate endocytosis or secretion of specific apical factors, although it is not known if the Hh paralogs are relevant cargoes.

RDY-2 is a nematode-specific tetraspan protein located in apical membranes of the canal, duct, and pore cells (Liegeois *et al.* 2007; H. Gill and M. Sundaram, unpublished results). In addition to canal defects, *rdy-2* mutants share several additional phenotypes with *vha-5* mutants, including defects in amphid channel morphology and hypodermal alae formation, which indicates a possible role in apical trafficking.

Canalicular factors

Canaliculi docking with the apical membrane presumably increase lumen growth (Figure 6D), but the origin of canaliculi is unknown and further studies are needed to determine if they contribute apical membrane or only water. Besides RAL-1 (above), the following gene products are localized to canaliculi:

AQP-8 is an aquaporin, a multipass transmembrane protein that forms pores or channels permeable to water but

impermeable to ions and other charged molecules (King *et al.* 2004). AQP-8 is located on canalicular vesicles and may draw water into those vesicles, which is later released into the lumen upon ERM-1-dependent docking with the apical membrane (Khan *et al.* 2013) (Figure 6D, Figure 7, Figure 8). Significant expansions in lumen diameter occur when ERM-1 or AQP-8 are overexpressed. *aqp-8* null mutants have only a mildly shortened canal lumen, however, which suggests that aquaporin-mediated water flux may not be the key driver for lumen growth under stable physiological conditions.

Vacuolar ATPase (V-ATPase) is a proton pump important for acidification of exocytic and endocytic vesicles (Lee *et al.* 2010). It is composed of two multisubunit sectors: V0 embedded in the membrane and the ATP-utilizing V1 proton pump (Lee *et al.* 2010). The V0 sector also has a V1-independent function in apical secretion (Liegeois *et al.* 2006). *C. elegans* V-ATPase subunits (encoded by *vha* genes) are highly expressed in the canal cell and excretory duct (Liegeois *et al.* 2006, 2007; Hahn-Windgassen and Van Gilst 2009; Knight *et al.* 2012). The V0-*a* subunit VHA-5 was identified by means of immunoEM in canalicular vesicles (Kolotuev *et al.* 2013). In *vha-5* mutants, pearl formation and canaliculi density are increased, and multiple separate lumens form (Liegeois *et al.* 2006, 2007; Hahn-Windgassen and Van Gilst 2009). One interpretation of this phenotype is that VHA-5 promotes efficient docking or fusion of canalicular vesicles with the mature apical membrane to create a central lumen. Another interpretation is that the V-ATPase plays a broader role in inhibiting both apical and basal membrane production and canal cell growth.

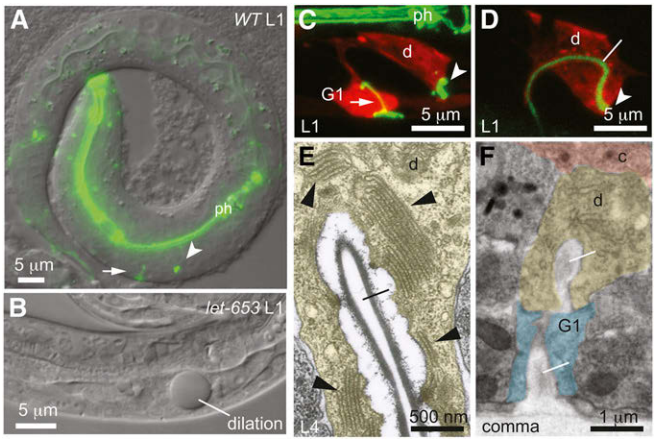

Figure 9 Excretory duct and pore structure and ultrastructure. (A) Early L1 larva expressing apical junction marker AJM-1::GFP (Koppen *et al.* 2001). The duct and pore are located just anterior and ventral to the posterior bulb of the pharynx (ph). Arrow, pore cell junctions; arrowhead, junction between duct and canal. (B) *let-653(cs178)* early L1 larva. The spherical dilation near the duct–canal junction is characteristic of mutants with a block in duct or pore lumen continuity (Stone *et al.* 2009; Mancuso *et al.* 2012). (C) Duct and G1 pore marked with *dct-5promoter*::mCherry (cell bodies) and AJM-1::GFP (apical junctions). (D) Duct marked with *lin-48promoter*::mRFP (cell body) and LET-653::GFP (lumen, line) (H. Gill, J. Cohen and M. Sundaram, unpublished results). Photos in C and D are courtesy of Fabien Soulavie (University of Pennsylvania). (E) TEM of duct lumen in an L4 hermaphrodite. Arrowheads point to membrane stacks (lamellae) that surround the apical membrane. Line indicates lumen, surrounded by darkly staining cuticle, which has detached from the apical membrane in this specimen. TEM image is courtesy of John Sulston (Medical Research Council). (F) TEM of duct and G1 pore in a 420-min (comma stage) embryo. Lines indicate fibrous apical ECM in the lumen. TEM image is courtesy of Shai Shaham (The Rockefeller University).

Ion transport and fluid flow

Studies of AQP-8 (above) showed that water influx can drive canal lumen expansion (Khan *et al.* 2013). Flow from the canal lumen into the lumens of the adjacent duct and pore tubes is also important for lumen shape, as mutations in genes such as *let-4* and *let-653*, which are not expressed in the canal cell, but affect duct and pore tube patency, cause severe dilations and cysts in the canal (Buechner *et al.* 1999; Mancuso *et al.* 2012) (H. Gill, J. Cohen and M. Sundaram, unpublished results) (Figure 9B). Finally, ion channels or channel modulators also affect lumen shape, possibly by affecting fluid flow (Figure 8).

WNK-1, GCK-3, and CLH-3: Mammalian with-no-lysine (WNK) serine/threonine kinases are critical regulators of ion transport in the kidney and other tissues and are mutated in familial hypertension syndromes (McCormick and Ellison 2011; Alessi *et al.* 2014). One important set of Wnk substrates are GCK-VI kinases, which in turn regulate various ion channels (McCormick and Ellison 2011; Alessi *et al.* 2014). WNK-1 is the sole Wnk-family kinase in *C. elegans*, and it positively regulates the GCK-3 kinase, which negatively regulates CLH-3 chloride channel activity (Denton *et al.* 2005; Hisamoto *et al.* 2008). This pathway appears to function in multiple tissues to affect cell volume (Denton *et al.* 2005; Choe and Strange 2007). *wnk-1* mutants have shortened canals, while *gck-3* mutants exhibit short and wide canal lumens despite normal basal outgrowth, consistent with a role for ion channel activity in modulating canal lumen growth (Hisamoto *et al.* 2008). However, *clh-3* mutants have normal canal morphology, and cell autonomy of *wnk-1* or *gck-3* canal phenotypes has not been rigorously established.

Future questions

Many questions remain about how the canal cell is formed and shaped. For example, how is the intracellular apical domain initially established and connected to that of the duct tube? What is the origin of canaliculi or other vesicles that contribute to the apical membrane? On what specific vesicle compartments do the EXC and CCM-3 gene products act? Studies of earlier embryonic stages and live imaging of the relevant vesicular compartments will be important for answering these questions. Most of the key players involved in canal outgrowth have homologs in vertebrates, many of which affect tubule

shape and integrity in the vascular system, kidney, or glial Schwann cells, so a better understanding of how these genes function in the canal cell is likely to lead to broadly relevant insights.

The Excretory Duct and Pore Cells

Duct and pore tube structure and ultrastructure

The excretory duct and pore are smaller unicellular tubes that connect the canal cell lumen to the exterior of the animal to allow fluid excretion and secretion (Figure 3, B–D, Figure 4, Figure 9). The duct tube channel is essential for viability: in mutants lacking a duct or having a discontinuous duct lumen, the upstream canal lumen becomes grossly dilated (Figure 9B), and larvae die with a turgid, rod-like morphology (Stone *et al.* 2009; Mancuso *et al.* 2012). On the other hand, mutants can survive quite well without a pore cell, because the duct can connect directly to the external hypodermis to provide an open channel for excretion (Abdus-Saboor *et al.* 2011).

The mature duct is an elongated, seamless tube with an asymmetric shape and a narrow cellular process that connects it to the pore (Figure 3B, Figure 9, C and D) (Nelson *et al.* 1983; Stone *et al.* 2009). The duct lumen takes a looping path through the cell (Figure 9D). The duct apical membrane is attached to surrounding parallel stacks of lamellar membranes (Figure 9E) that greatly increase its potential surface area and have been speculated to facilitate selective reuptake of ions (Nelson *et al.* 1983). The vacuolar ATPase subunit VHA-5 localizes to these lamellar stacks (Kolotuev *et al.* 2010).

The pore has an autocellular junction, a relatively simple, conical shape, and few or no lamellae (Nelson *et al.* 1983; Stone *et al.* 2009) (Figure 3, C and D, Figure 9C). The pore tube further has the amazing property that its structure and function is preserved through several changes in pore cell identity (Figure 4G; see *The G1-to-G2 Pore Swap* below).

EGF-Ras-ERK signaling specifies the duct vs. G1 pore fate

The duct and G1 pore are another example of left/right asymmetry within the ABp lineage (Figure 4, A and B) (Sulston *et al.* 1983), but in this case the asymmetry is due to EGF-Ras-ERK signaling (Abdus-Saboor *et al.* 2011). Asymmetry of EGF-Ras-ERK signaling depends on the earlier, Notch-dependent asymmetry in canal cell position, because the canal cell is a major source of LIN-3/EGF (Figure 10A).

The logic behind the duct *vs.* G1 pore fate decision is similar to that of tip *vs.* stalk cell fate decisions in more complex, branched tubular organs such as the vertebrate vascular system, lung, or kidney, where cells compete for a tip cell fate in response to growth factor signaling (Siekmann *et al.* 2013). The duct and G1 pore are left/right lineal homologs (Figure 4B) that are initially equivalent (Sulston *et al.* 1983). These cells are born in lateral positions in the embryo and migrate toward each other and the excretory canal cell during ventral enclosure (Figure 4A). As the duct and G1 pore progenitors migrate ventrally, both cells express LET-23/EGFR and appear to compete for access to the LIN-3-secreting canal cell (Abdus-Saboor *et al.* 2011) (Figure 10A). Whichever cell "wins" this competition becomes the duct and adopts a canal-proximal position, sinking dorsally into the interior of the embryo; the remaining cell becomes the G1 pore and adopts a more ventral position on the outside surface of the embryo. In wild-type embryos, the competition is biased such that the left precursor always wins and becomes the duct, probably because it starts out closer to the canal cell and thus gets earlier and/or higher levels of LET-23/EGFR stimulation. Signaling of LET-23/EGFR through LET-60/Ras and MPK-1/ERK is both necessary and sufficient for the duct fate (Figure 10B). In the absence of signaling, both cells adopt a G1 pore fate, whereas in the presence of constitutive signaling, both cells adopt a duct fate (Yochem *et al.* 1997; Abdus-Saboor *et al.* 2011) (Figure 10, C and D).

In wild-type animals, there is always one duct and one G1 pore, but if the presumptive duct is removed via laser ablation, the presumptive G1 pore instead adopts a duct fate (Sulston *et al.* 1983). Furthermore, in mosaic animals, loss of LET-60/Ras from the ABpl lineage, but not ABpr lineage, causes these cells to switch fates: the presumptive ABpl-derived duct adopts the G1 pore fate, and the presumptive G1 pore adopts the duct fate (Yochem *et al.* 1997) (Figure 10E). Together, these data show that the duct prevents G1 from also adopting a duct fate through lateral inhibition.

Lateral inhibition presumably prevents G1 from sensing or responding to LIN-3/EGF signaling (Figure 10A), but the molecular basis of the inhibition is not known. Unlike many other lateral inhibition mechanisms in *C. elegans* (Sundaram 2005), or in vertebrate tip *vs.* stalk cell decisions (Siekmann *et al.* 2013), Notch signaling does not appear to be involved; instead, morphological data suggest that the duct physically prevents G1 from contacting the canal cell and in this way reduces its access to the LIN-3/EGF ligand (Abdus-Saboor *et al.* 2011).

Ras signaling is required at or before the 1.5-fold stage to specific duct identity, but has additional roles beyond this time. Experiments with a temperature-sensitive (ts) allele of *sos-1* (encoding the Ras guanine nucleotide exchange factor) (Chang *et al.* 2000) showed that upshifting to restrictive temperature after the 1.5-fold stage does not perturb duct identity, but causes other abnormalities in duct lumen morphology and integrity (Abdus-Saboor *et al.* 2011). One later requirement for EGF-Ras signaling is in proper execution of the G1-to-G2 pore swap (Parry and Sundaram 2014) (see below).

Transcription factors important for duct or G1 pore differentiation

The following transcription factors are important for duct and/or G1 differentiation (see also Figure 3, B and C, Figure 10B):

CEH-36/Otx and UNC-30/Pitx are related bicoid-type homeodomain factors expressed in the ABp(l/r)p lineages that

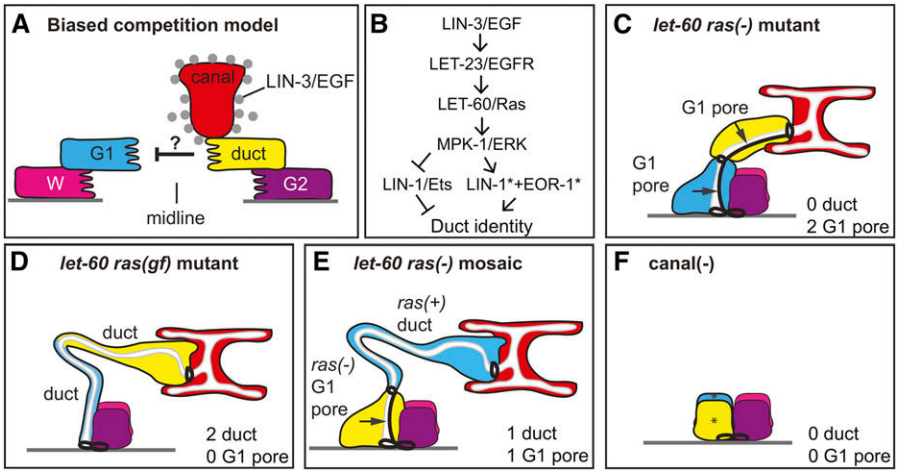

Figure 10 EGF-Ras-ERK signaling promotes duct *vs.* G1 pore fate. (A) The biased competition model postulates that asymmetry in canal position results in the left member of the duct/G1 equivalence group receiving more canal-derived LIN-3/EGF signal and therefore becoming the duct cell (Abdus-Saboor *et al.* 2011). An unknown lateral inhibitory mechanism (Sulston *et al.* 1983) prevents the cell on the right from receiving or responding to the EGF signal. (B) The EGF-Ras-ERK pathway acts via LIN-1/Ets and EOR-1 to promote duct fate (Howard and Sundaram 2002; Abdus-Saboor *et al.* 2011). Asterisks indicate potential ERK-dependent phosphorylation (Jacobs *et al.* 1998; Howell *et al.* 2010). (C and D) Reduced EGF-Ras-ERK signaling causes a duct-to-G1 pore fate transformation. Constitutive signaling causes a G1 pore-to-duct fate transformation; the two ducts fuse to make a binucleate duct (Yochem *et al.* 1997; Abdus-Saboor *et al.* 2011). (E) In mosaics lacking *let-60*/Ras activity in the ABpl lineage, the duct and G1 pore progenitors switch fates (Yochem *et al.* 1997). (F) Physical or genetic ablation of the canal cell prevents duct and G1 pore tubulogenesis (Abdus-Saboor *et al.* 2011).

give rise to the canal, duct, and G1 pore cells (Walton *et al.* 2015). CEH-36 and UNC-30 function redundantly to upregulate *mls-2* to promote proper migration of the duct and pore cells to the ventral midline.

MLS-2 is an Hmx-family homeodomain transcription factor expressed in the ABp(l/r)paaa lineages that give rise to the duct and G1 pore (Abdus-Saboor *et al.* 2012). After the final set of divisions in this lineage, MLS-2 protein disappears from the DB1 and DB3 neurons, but remains present in the duct and G1 pore cells during their migration and early differentiation. In *mls-2* mutants, the duct and pore cells show variable defects in migration to the ventral midline and in final tube shape (Abdus-Saboor *et al.* 2012; Walton *et al.* 2015). MLS-2 cooperates with Ras signaling to express duct-specific genes such as lin-*48*/Ovo (Abdus-Saboor *et al.* 2012). MLS-2 is also important for glial differentiation in *C. elegans* (Yoshimura *et al.* 2008), suggesting molecular similarities between duct/pore and glial tube formation.

LIN-26, the general epithelial C2H2 factor, is expressed in both the excretory duct cell and G1 pore, and its loss leads to variable defects in duct and pore morphology (Labouesse *et al.* 1994, 1996). As for the canal cell (see above), the final division that generates the duct and G1 pore partitions epithelial from neuronal cell fates (Sulston *et al.* 1983) (Figure 4B). Expression of *lin-26* in the duct and G1 is likely to be one important early step in epithelial determination.

NHR-23 and NHR-25 are nuclear hormone receptors expressed in most external (cuticle-lined) epithelia, including the duct and pore (Gissendanner and Sluder 2000; Kostrouchova *et al.* 2001; Murray *et al.* 2012; M. V. Sundaram, unpublished data). In the epidermis, NHR-23 and NHR-25 are required for cyclical expression of collagens and other secreted ECM factors, and for proper molting (Gissendanner and Sluder 2000; Hayes *et al.* 2006; Kouns *et al.* 2011). These transcription factors are likely to play similar roles in the duct and pore.

LIN-1 and EOR-1 are two MPK-1/ERK targets that, in the presence of Ras signaling, function redundantly to promote excretory duct *vs.* G1 pore fate specification (Figure 10B). LIN-1 is an Ets transcription factor related to mammalian Elk1 (Beitel *et al.* 1995; Jacobs *et al.* 1998). EOR-1 is a BTB-Zinc Finger protein related to mammalian PLZF (Howard and Sundaram 2002; Howell *et al.* 2010). *eor-1 lin-1* double mutants resemble *let-60 ras* mutants and have two G1 pore-like cells and no duct (Abdus-Saboor *et al.* 2011). In the absence of Ras signaling, LIN-1 also has an opposing function to inhibit the duct fate (Figure 10B). *lin-1* single mutants resemble *let-60*(*gf*) mutants and have a binucleate duct cell and no G1 pore (Abdus-Saboor *et al.* 2011).

LIN-48 is a C2H2 zinc finger transcription factor related to *Drosophila* and mammalian Ovo (Johnson *et al.* 2001), which play roles in epithelial differentiation and shaping (Menoret *et al.* 2013; Roca *et al.* 2013; Aue *et al.* 2015). It is expressed continuously in the duct from mid–late embryogenesis through adulthood and is therefore a convenient duct fate marker. Ras signaling and MLS-2 cooperate to stimulate *lin-48* expression in the duct (Abdus-Saboor *et al.* 2012). *lin-48* mutants have subtle changes in pore position, possibly due to changes in duct shape (Wang and Chamberlin 2002). Interestingly, *C. briggsae* lacks *lin-48* duct expression, and its pore cell is located posterior to the position of the *C. elegans* pore; forced expression of *lin-48* in *C. briggsae* moves its pore cell anteriorly (Wang and Chamberlin 2002, 2004).

CES-2 and ATF-2 are bZIP factors related to the mammalian proline- and acid-rich (PAR) family of bZIP transcription factors (Metzstein *et al.* 1996). CES-2 and ATF-2 function

are required for correct timing of *lin-48* expression, and mutants in either *ces-2* or *atf-2* exhibit subtle changes in pore position, similar to the phenotype of *lin-48* mutants (Wang and Chamberlin 2002; Wang *et al.* 2006).

The duct and G1 pore form tubes through a wrapping mechanism

Following ventral migration and contact with each other and with the canal cell, and concomitant with their cell fate decisions, the duct and G1 pore cells form tubes with autocellular junctions (Stone *et al.* 2009; Abdus-Saboor *et al.* 2011) (Figure 1, B and C, Figure 4E). Mechanisms of duct and G1 pore wrapping are not understood, but wrapping is closely coupled to *de novo* junction formation.

The excretory canal cell is required for duct cell and G1 pore cell wrapping and stacking (Abdus-Saboor *et al.* 2011). Removal of the canal cell by means of laser ablation or Notch mutation prevents tube formation; the duct and G1 pore still epithelialize but do not form tubes, and instead take adjacent positions and adopt an epidermal-like morphology (Figure 10F). Partial reduction of *let-60*/Ras activity (in hypomorphic mutants) can also lead to adjacent epithelial cells, only one of which forms a tube (Abdus-Saboor *et al.* 2011), which suggests that Ras signaling also influences the wrapping process.

AFF-1-dependent duct autofusion converts an autocellular tube to a seamless tube

Although the duct initially has an autocellular junction, this junction disappears at or soon after the 1.5-fold stage (Figure 4E). Autocellular junction removal requires the plasma membrane fusogen AFF-1 (Stone *et al.* 2009). AFF-1 is both necessary and sufficient for several cell–cell fusions that generate syncytia (Sapir *et al.* 2007). In the case of the duct, AFF-1 mediates autofusion to convert a seamed autocellular tube into a seamless tube (Figure 1C). The junctions connecting the duct to the adjacent pore and canal cells are not affected by the autofusion.

EGF-Ras-ERK signaling promotes *aff-1* expression to promote duct autofusion (F. Soulavie and M. V. Sundaram, unpublished results). Normally, only the duct expresses *aff-1* and autofuses, whereas the G1 pore retains its autocellular junction (Figure 4). In the absence of signaling, neither cell expresses *aff-1*, and both retain their autocellular junctions (Figure 10C). In mutants with constitutive Ras signaling, both cells express *aff-1*, and the two cells fuse to make a binucleate duct (Abdus-Saboor *et al.* 2011) (Figure 10D).

Luminal matrix maintains lumen shape and tube integrity during morphogenesis

Subsequent to tubulogenesis and autofusion, the duct elongates and adopts its characteristic asymmetric looping shape (Figure 4F). The pore also elongates, but to a much lesser degree. Molecular pathways involved in duct cell and lumen elongation have not yet been described. *erm-1* does not appear to be expressed or have phenotypes in the duct (Gobel *et al.* 2004; Mancuso *et al.* 2012), so the mechanisms of lumen growth and maintenance may differ significantly between the excretory duct and canal cells.

Duct morphogenesis occurs in the context of a fibrous luminal matrix that is visible via transmission electron microscopy (Mancuso *et al.* 2012) (Figure 9F). At the completion of morphogenesis, this luminal matrix disappears, and the duct and G1 pore secrete a luminal cuticle (Figure 9E) that is contiguous with the epidermal cuticle that covers the outside of the animal. This cuticle is shed and resynthesized at each larval molt (Nelson *et al.* 1983). The luminal matrix and rigid cuticle in the duct and pore may serve analogous functions to the terminal web in the canal cell, to maintain apical domain shape and integrity. Several mutations that disrupt luminal matrix organization lead to duct lumen breaks and dilations (Figure 9B) and to physical separation of the duct and G1 pore cells around the time of hatch (Mancuso *et al.* 2012; H. Gill, J. Cohen and M. Sundaram, unpublished results). Proteins involved in duct luminal integrity and shape include:

LET-653 is a Zona Pellucida (ZP)-domain and mucin-related glycoprotein (Jones and Baillie 1995). ZP proteins form fibrils and contribute to many apical matrices (Plaza *et al.* 2010), including luminal matrices of the *Drosophila* tracheal system (Jazwinska *et al.* 2003), mammalian vascular system (Govani and Shovlin 2009), and kidney (Rampoldi *et al.* 2011). Mucins form gel-like protective barriers within many tubular epithelia (Johansson *et al.* 2013; Lillehoj *et al.* 2013). LET-653 is one key component of the early luminal matrix in the duct and pore (H. Gill, J. Cohen and M. Sundaram, unpublished results).

LET-4 and EGG-6 are extracellular leucine-rich repeat only (eLRRon) proteins (Mancuso *et al.* 2012), part of a family of secreted and transmembrane glycoproteins with diverse roles, including collagen fibril assembly, synaptic adhesion, and signaling regulation (Brose 2009; Merline *et al.* 2009). LET-4 and EGG-6 are located in or near the duct and pore apical membrane and are predicted to extend their LRR domains into the lumen. *let-4* and *egg-6* mutants have defects similar to those of *let-653* mutants along with widespread defects in apical ECM organization and cuticle barrier function, which suggests that these transmembrane proteins may interact with luminal matrix factors and help organize matrix deposition or cross-linking.

LPR-1 is a lipocalin (Stone *et al.* 2009); these are cup-shaped secreted proteins that bind lipophilic cargoes and have a broad range of functions related to cargo transport, sequestration, and signaling (Flower 1996; Sun and Kawaguchi 2011; Paragas *et al.* 2012). *lpr-1* mutants strongly resemble *let-653*, *let-4*, and *egg-6* mutants, but *lpr-1* is capable of acting cell nonautonomously when provided from outside of the excretory system (Stone *et al.* 2009), and therefore may act indirectly to affect membrane or matrix integrity.

Future questions

The duct and pore provide opportunities to study important aspects of tube development such as molecular control of tube wrapping and autofusion, roles of receptor tyrosine kinase signaling in tube shaping, and the role of luminal matrix in tube shaping and integrity. Furthermore, because the duct is a permanent epithelial cell, while the G1 pore undergoes reprogramming, these cells also provide a model for understanding the factors that determine fate plasticity.

The G1-to-G2 Pore Swap

Pore identity changes several times during development (Figure 3, C and D). In the middle of the L1 stage, the G1 pore cell delaminates from the excretory system (Figure 4G), reenters the cell cycle, and divides to generate two neuronal daughters, the RMHL and RMHR motor neurons (Sulston and Horvitz 1977; Sulston *et al.* 1983; Parry and Sundaram 2014). As G1 departs, the G2 epidermal cell undergoes wrapping tubulogenesis around the central lumen cuticle to form a replacement pore (Figure 4G). During the L2 stage, G2 divides and its posterior daughter (G2.p) becomes the permanent excretory pore (Sulston and Horvitz 1977; Sulston *et al.* 1983). These dynamic changes in pore identity require significant remodeling of cell–cell and cell–matrix attachments within the excretory organ.

Lineal origins and Notch-dependent fate specification of G2 and W

G2 and W provide another example of Notch-dependent left/right asymmetry in the *C. elegans* embryo (Figure 4C), again to make an epithelial *vs.* neuroblast fate decision. G2 and W are derived from left/right lineal homologs that are initially equivalent and compete for the G2 fate (Sulston *et al.* 1983). LIN-12/Notch promotes the G2 (left) over the W (right) fate; in *lin-12*(*lf*) mutants, both cells adopt a W neuroblast fate, whereas in *lin-12*(*gf*) mutants, both cells adopt a G2 pore cell fate (Greenwald *et al.* 1983).

Dynamic behavior of G2 and W parallels that of G1

G2 and W start out as embryonic epidermal cells, but change their identities to pore tube or neuroblast, respectively, in the L1 stage (Sulston *et al.* 1983). During ventral enclosure, G2 and W cells migrate to the ventral midline in unison with other ventral epidermal cells and with the duct and G1 cells (Figures 4, A and D). As the duct and G1 cells form tubes, G1 inserts itself between the encroaching G2 and W cells and the anterior epidermis to contact the outside environment (Figure 4E); eventually, the ventral apical junction ring of the G1 pore contacts those of G2 and W at a tricellular junction (Abdus-Saboor *et al.* 2011) (Figure 11A). At embryonic stages after 1.5-fold, G2 and W are easily recognizable, because they are the only ventral epidermal cells in the anterior central region of the embryo that do not fuse to join the hyp7 syncytium (Podbilewicz and White 1994). These cells retain long, rectangular apical junction domains throughout much of embryogenesis (Figure 11B), but their apical junctions shrink to small rings by the time of hatching (Figure 11C).

At mid-L1, W (also known as P0.a) delaminates to become a neuroblast and undergoes several rounds of division to generate four neuronal descendants, including the VB2 (W.aap), VA1 (W.pa), and VD1 (W.pp) ventral cord motor neurons, plus one of the AVF interneurons (W.aaa) (Sulston and Horvitz 1977). Shortly after W delaminates and divides, G1 also delaminates, and G2 becomes the replacement excretory pore tube (Sulston *et al.* 1983; Parry and Sundaram 2014) (Figure 4G, Figure11D).

Cellular events of the G1-to-G2 pore swap

G1 delamination and G2 wrapping tubulogenesis occur in the context of a cuticle-lined pore channel, and the cells appear to migrate over the luminal cuticle as they change positions (Parry and Sundaram 2014); therefore, remodeling of apical cell–matrix connections is likely to be important for delamination. The presence of the cuticle may also help the channel to remain open throughout the remodeling process.

Changes in the actin cytoskeleton precede G1 delamination (Parry and Sundaram 2014). In the early L1 stage, F-actin localizes apically along the G1 autocellular junction, but just prior to G1 delamination, this F-actin disperses. Since F-actin strengthens junctions (Huveneers and de Rooij 2013), actin relocalization may loosen the adherens junction to allow subsequent dynamic changes. After actin relocalization, G1 appears to stretch dorsally and "unzip," converting its autocellular junction to an intercellular junction with G2 (Parry and Sundaram 2014) (Figure 11D). G2 concomitantly forms an autojunction and "zips up" beneath G1, such that a region of two-celled tube is formed at their intersection. Subsequently, G1 junctions gradually shrink and disappear, and G1 then divides. Relatively little is known yet about the molecular control of these various G1 and G2 cell behaviors, but Ras signaling in the duct is required for G1 junction loss (Figure 11D; see below). Gene expression changes that must accompany the epithelial-to-neuronal transition remain to be characterized.

G1 and G2 behave independently

Although G1 delamination and G2 intercalation are closely coordinated during wild-type development, G1 and G2 can execute these behaviors independently. For example, in *lin-12*(*lf*) mutants, there is no G2 cell (Greenwald *et al.* 1983), but G1 still delaminates, and the duct then connects directly to the hypodermis to maintain a functional system (Abdus-Saboor *et al.* 2011) (Figure 11F). In *let-60*(*gf*) mutants, there is no G1 cell to delaminate, but G2 still wraps around the base of the duct to form a pore tube (Abdus-Saboor *et al.* 2011) (Figure 11G). In *let-60*(*lf*) mutants, where two G1 cells but no duct are present, both G1 cells appear to initiate delamination behaviors, which suggests that the duct is not needed to instruct these behaviors (Abdus-Saboor *et al.* 2011; Parry

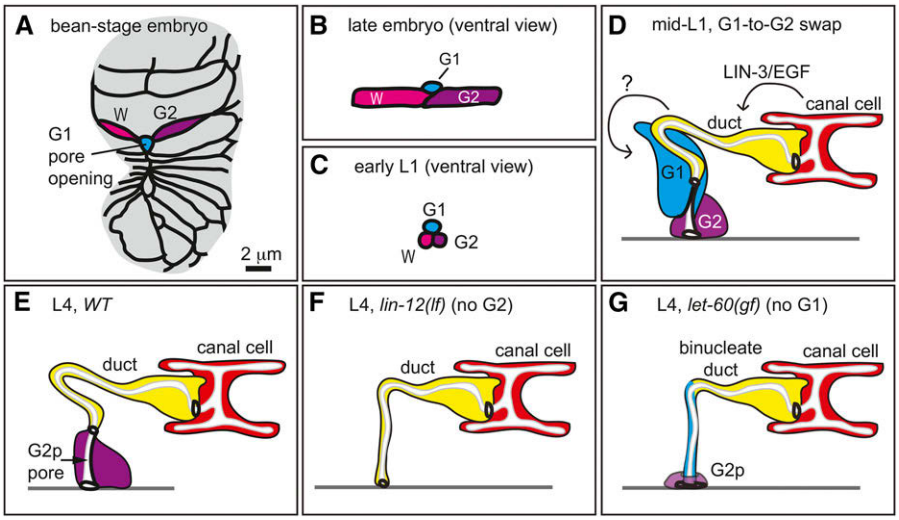

Figure 11 G2 and W epidermal cells and the G1-to-G2 pore swap. (A–C) Schematic ventral views of apical junctions in the pore region, with G2 and W flanking the G1 pore opening, based on Abdus-Saboor *et al.* (2011). (A) Lima bean-stage embryo, when the pore tube first forms. (B) Threefold embryo. G2 and W have rectangularly shaped apical junctions. (C) Early L1. G2 and W junctions are ring shaped. (D) At mid-L1, during the G1-to-G2 swap, G1 appears to stretch dorsally and "unzip," while G2 concomitantly forms an autojunction and "zips up" to form a replacement pore (Parry and Sundaram 2014). An unknown Ras-dependent signal (?) from the duct may promote G1 junction loss (Parry and Sundaram 2014). (E–G) L4 excretory system. (E) In wild type, G2p is the pore. (F) In the absence of G2, as in *lin-12(lf)* mutants, there is no longer a pore after G1 delaminates, and the duct connects directly to the hypodermis (Abdus-Saboor *et al.* 2011). (G) In the absence of G1, as in *let-60(gf)* mutants, the duct connects directly to the hypodermis (see Figure 10D). G2p usually wraps around the base of the duct tube and occasionally inserts to form a true pore (Abdus-Saboor *et al.* 2011).

and Sundaram 2014). G1 and G2 may be executing intrinsically programmed behaviors, or they may be responding to cues from the canal cell or from somewhere outside of the excretory system.

A cell-nonautonomous role for LET-60/Ras signaling in G1 pore delamination

Ras signaling in the duct cell is required for successful execution of the final steps of G1 delamination (Parry and Sundaram 2014) (Figure 11D). SOS-1 is the guanine nucleotide exchange factor that activates LET-60/Ras during most Ras signaling events (Chang *et al.* 2000). In temperature-sensitive *sos-1* mutants that are upshifted to restrictive temperature as early L1 larvae, the pore swap initiates normally, but the G1 cell junctions do not shrink and disappear, and G1 remains stuck within the excretory system (Parry and Sundaram 2014). This delamination defect can be rescued by expressing constitutive LET-60(gf) in the duct, but not in the G1 pore, showing that signaling acts cell nonautonomously. Potential models are that Ras signaling in the duct triggers production of a secreted factor that then acts on G1 or its matrix (Figure 11D), or that Ras signaling in the duct acts more directly to modulate duct–G1 adhesion. These results suggest that Ras signaling might also have nonautonomous effects on epithelial-to-mesenchymal transitions during tumor metastasis.

Why swap?

Why does the worm bother with these complex and potentially dangerous cellular gymnastics? The functions of the G1 and G2 pores do not differ in any known way. The function of the RMH neurons is still unknown—they presumably act in the motor circuit based on their pattern of synaptic connections (White *et al.* 1986), but *let-60*(*gf*) mutants, which lack G1 (Abdus-Saboor *et al.* 2011) (and thus presumably lack the RMH neurons), are viable and have not been reported to have motility defects. Presuming that the RMH neurons do have some more subtle yet important function, why generate them in this way? The rationale is not clear, but many neuroblasts in *C. elegans* and other organisms are generated from epithelial progenitors (Sulston *et al.* 1983; Colman 1999; Technau *et al.* 2006; Jarriault *et al.* 2008; Lassiter *et al.* 2014), as are many other cell types in mammals (Kalluri and Weinberg 2009), suggesting that epithelial cells have a general propensity for such reprogramming. Along with other similar examples in the worm (Zuryn *et al.* 2012), the excretory pore provides a simple and tractable model for studying epithelial reprogramming and delamination.

The Excretory Gland

Excretory gland structure and ultrastructure

The excretory gland is an A-shaped binucleate cellular syncytium that connects to the canal cell immediately adjacent to the canal–duct junction; this connection point has been termed the secretory–excretory junction (SEJ) (Nelson *et al.* 1983) (Figure 3E, Figure 12, A and B). As seen under TEM, the gland cytoplasm contains many electron-dense putative secretory granules in close proximity to Golgi complexes and the SEJ; these granules disappear in dauer larvae. The gland presumably secretes material into the duct channel for passage to the outside environment and/or secretes material into the canal lumen for transport to other parts of the body, but the nature of any such secretions is unknown. The anterior gland processes appear to make synapses with the nerve ring, suggesting possible neuronal control of secretion (Nelson *et al.* 1983).

Lineal origin and development of the excretory gland

The gland is derived from two left/right lineal homologs that migrate to the ventral midline during ventral

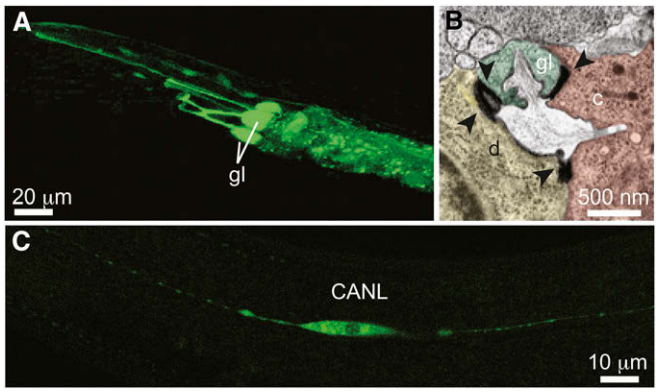

Figure 12 The excretory gland and CAN neurons. (A) The A-shaped excretory gland (gl) visualized with B0403.4promoter::GFP (Hunt-Newbury *et al.* 2007). Image is courtesy of Don Moerman (University of British Columbia). (B) The gland empties into the canal sinus at the secretory junction. Note luminal matrix (gray). Black arrowheads, junctions. TEM image of late threefold embryo is courtesy of Richard Fetter and Cornelia Bargmann (The Rockefeller University). (C) CAN neuron (lateral view) visualized with *ceh-23*promoter::GFP (Forrester and Garriga 1997).

enclosure and then fuse at two separate points (Nelson *et al.* 1983; Sulston *et al.* 1983) (Figure 3E, Figure 4). The first fusion event occurs between processes linking the cells near their junction to the canal and duct. The second fusion event occurs between the tips of two narrow processes that the gland cells extend toward the nerve ring (these form the tip of the A-shape). It is not yet known if gland fusion requires the fusogens AFF-1 or EFF-1.

The LIM homeodomain transcription factor LIM-6 is expressed in the excretory gland cell and serves as a useful marker for its morphology, but the impact of *lim-6* mutation on the gland is unknown (Hobert *et al.* 1999) (Figure 3E).

Possible functions of the excretory gland

One hypothesized function for the gland is the production of hormones. The gland stains with the neurosecretory marker paraldehyde-fuschin (PAF) (Nelson *et al.* 1983) and with the sterol-binding agents dehydroergosterol (DHE) and filipin (Merris *et al.* 2003), consistent with steroid hormone production. In the absence of hermaphrodites, male *plep-1* mutants attempt to copulate with the excretory pore of other males (Noble *et al.* 2015), consistent with some attractive secretion emanating from the pore, and possibly originating in the gland. Singh and Sulston (1978) and Nelson and Riddle (1984) investigated the effects of gland ablation in larvae and found no disruption in osmoregulation, molting, dauer formation, or dauer exit, but other aspects of the animal's physiology or behavior were not examined. Several other functions have been proposed for the gland in other nematodes (see below), including secretion of the epicuticle (Page *et al.* 1992a), but this function remains untested in *C. elegans*.

The Canal-Associated Neurons

CAN structure

The canal-associated neurons CANL and CANR are named for their close physical association with the excretory canal cell arms (White *et al.* 1986) (Figure 2, Figure 3F). The two CANs are born in the head region (Figure 4A) and then migrate posteriorly during late embryogenesis to their final positions in the midbody, immediately adjacent to the two posterior canal arms (Hedgecock *et al.* 1987). CAN neurons extend one axon into the head, and a second posteriorward into the tail, between the excretory canal and basement membrane (White *et al.* 1986) (Figure 6B, Figure 12C).

At birth, the CAN cell bodies resemble those of other neurons. After migration, the cell bodies swell to a much larger size (Figure 12C) and may form gap junctions to the excretory canals (White *et al.* 1986). This observation, plus the fact that the CANs do not make obvious synapses with other neurons, suggests that the function of these cells may be neurosecretory (White *et al.* 1986).

Transcription factors important for CAN differentiation

Transcription factors expressed in the CANs (Figure 3F) include:

CEH-10 is a paired-type homeodomain factor (Svendsen and McGhee 1995) required for CAN differentiation (Forrester *et al.* 1998; Manser and Wood 1990). In *ceh-10* null mutants, the CANs fail to express CEH-23::GFP and do not migrate to their correct positions; it is not clear if the mutant CANs adopt an alternative identity or simply fail to differentiate.

CEH-23 is a divergent homeodomain factor (Wang *et al.* 1993). CEH-23::GFP is often used as a CAN marker (Figure 12C), but *ceh-23* deletion mutants are viable and do not have apparent defects in CAN development or function (Altun-Gultekin *et al.* 2001).

UNC-39 is a Six5-related homeodomain factor required for CAN migration and axon pathfinding (Manser and Wood 1990; Yanowitz *et al.* 2004).

Regulators of CAN migration and axon outgrowth

Many studies of the CAN neurons have focused on their long-range migration during embryogenesis. Mispositioning of both CAN cells, or more specifically, failure of the posterior CAN axons to reach their normal posterior regions, leads to a characteristic withered tail (Wit) phenotype in which the posterior half of the animal is much thinner than the anterior half; this phenotype has facilitated genetic screens for migration mutants (Manser and Wood 1990; Forrester *et al.* 1998). Many CAN migration mutants also have defects in axonal extension and/or pathfinding, but the two defects are separable (Lundquist *et al.* 2001; Yanowitz *et al.* 2004).

Among the genes important for CAN migration are many that are also important for excretory canal outgrowth, such as *epi-1/*laminin alpha, *ina-1/*integrin alpha, *unc-34/*Enabled, *mig-10/*Lamellipodin, *unc-73/*Trio, and *vab-8/*Kinesin-like (Manser and Wood 1990; Forrester *et al.* 1998). Multiple Rac genes are also important and likely act downstream of UNC-73 (Lundquist *et al.* 2001). Proper CAN migration also requires the ROR family receptor tyrosine kinase CAM-1 and multiple Wnt-related ligands (Forrester *et al.* 1999).

Possible functions of the CAN neurons

Laser ablation or genetic removal of a single CAN cell has no obvious effect on the animal, but removal of both CAN cells causes a rod-like lethal phenotype (Forrester and Garriga 1997; Forrester *et al.* 1998). Based on the similarity of this lethal phenotype to that observed after ablation of the excretory canal, duct, or pore cells, the CANs were proposed to regulate excretory system function, but this has not been demonstrated.

Functions of the Nematode Excretory System

Adenophorean vs. Secernentean systems

The functions of the nematode "excretory–secretory" (ES) system have been a source of debate for well over a century, since not all nematode species contain the canal cell, but it appears that all species contain an excretory gland cell (Bastian 1866; Schneider 1866; Chitwood and Chitwood 1974). A simpler "Adenophorean" system is found particularly in marine species such as *Enoplus* and *Monhystera* (van de Velde and Coomans 1987; Turpeenniemi and Hyvarinen 1996), which live in an environment where environmental osmolarity is generally stable. In these animals, the ES system consists of a single large ventral gland (or "renette" cell) leading directly to a cuticle-lined duct and/or pore located ventrally anywhere from behind the terminal bulb forward to just behind the buccal cavity, depending on the species (Chitwood and Chitwood 1974; van de Velde and Coomans 1987) (Figure 13).

The *C. elegans* excretory system is an example of the more complicated "Secernentean" ES system found in most terrestrial species that experience large changes in osmolarity (Chitwood and Chitwood 1974). Secernentean species all contain a branched canal cell, but variations between species include the number and position of canals (Chitwood and Chitwood 1974; de Grisse 1977; Bird and Bird 1991), the number of excretory cell nuclei (Wharton and Sommerville 1984), fusion of the duct and pore cells (Narang 1972), and even the presence of muscles associated with the system in the parasite *Brugia malayi* (Moreno *et al.* 2010).

Secretory function of the ES system

The ubiquitous presence of gland cells suggests that secretion is a critical function of the ES system in all nematodes. Excretion of mucilaginous material has been observed from the excretory pore of many species (Maggenti 1962; Premachandran *et al.* 1988; Bird and Bird 1991). Proposed functions of this material include:

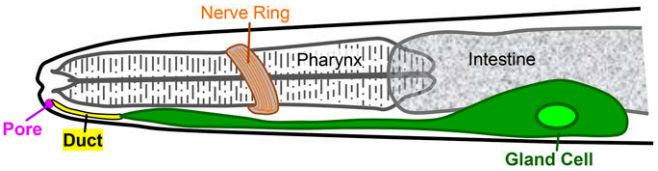

Figure 13 Adenophorean ES system. Diagram of ES system from *Monhystera disjuncta*, based on drawings by Bird and Bird (1991) and van de Velde and Coomans (1987).

To help digest prey (Lee 1970; van de Velde and Coomans 1987).

To effect changes in the animal's life cycle (Bird and Bird 1991), such as molting (Singh and Sulston 1978) or entrance or exit from the dauer or dispersive phase of nematode growth (Nelson and Riddle 1984).

To coat the cuticle with physically or osmotically protective material (Blaxter *et al.* 1992; Page *et al.* 1992a,b).

To help parasitic nematodes infect a host organism and evade its immune response (Blaxter *et al.* 1992; Moreno *et al.* 2010). Consistent with such a role, ES system-associated muscles in *Brugia malayi* appear to be the relevant target of the nematocide ivermectin (Moreno *et al.* 2010).

Efforts in the parasitic nematode research community have characterized secreted ES products aiding in host infection (Hewitson *et al.* 2009), but whether these originate from the ES gland or from other bodily sources has been addressed in only a few cases (Page *et al.* 1992a; Moreno *et al.* 2010). A better understanding of excretory gland development and function in *C. elegans* could have important implications for the role of the gland in other nematodes and suggest new ways to prevent and treat parasitic nematode infections.

Osmoregulatory function of the excretory system

Nematodes maintain a hyperosmotic coelomic fluid to inflate the body cavity and keep the hypodermis rigid for effective muscle attachment and movement (Harris and Crofton 1957; Nelson and Riddle 1984; Wright 2004). A body cavity osmolarity higher than that of the environment attracts water that leaks through the tissues exposed to the environment, especially cuticle (Perry 1977; Wharton *et al.* 1988; Bird and Zuckerman 1989) and intestinal cells (Stephenson 1942; Wright and Newall 1976), so the animal must eliminate excess water to maintain homeostasis.

The excretory canals are ideally located to collect this excess water, as they stretch along the entire body adjacent to the pseudocoelom, separated solely by a basement membrane. Water may enter the canal cell by diffusion or via gap junctions with the hypodermis (Nelson *et al.* 1983; Altun

et al. 2009), and then be transported via canaliculi to the lumen for excretion (Figure 8). Since water follows osmotic gradients, the canaliculi and lumen must concentrate ions to attract water. The canal cell expresses multiple types of membrane channels and transporter proteins for regulated movement of water and ions (Spencer *et al.* 2011) (Table 1). Ion-selective electrical measurements at the excretory pore show effluxes of Mg^{++}, K^{+}, H^{+}, Na^{+}, and Ca^{++} (Teramoto *et al.* 2010; Adlimoghaddam *et al.* 2014; Wang *et al.* 2014a), as would be expected from concentration and release of these ions by the excretory system.

A speculative model for transport of water through the canaliculi and lumen is presented in Figure 8, in analogy to the well-studied vacuolar ATPase/acid-driven excretion of water by the tubular water vacuole of the freshwater protozoan *Paramecium* (Plattner 2015). The V-ATPase proton pump concentrates cations, while anion channels provide anions to neutralize the acidic charge while increasing osmolarity. AQP-8/aquaporin channels allow excess water to cross membranes easily from the cytoplasm to the areas of high osmolarity. Upon docking with the apical membrane, canaliculi release the water and ions into the lumen.

The morphological structure of the canals shares features with the vertebrate lymphatic system, in that both contain narrow tubes reaching throughout the organism to collect fluid, and both require the PROS-1 transcription factor (Kolotuev *et al.* 2013). In addition, the osmoregulatory function of the canals shares features with the vertebrate renal system. Although the primary pump driving ion homeostasis in the kidney nephrons is the sodium/potassium exchanger (Ross *et al.* 1974; Geering 1997), V-ATPase plays critical roles in intercalated cells especially of the tubular epithelia of the collecting duct (Brown *et al.* 2012). The protons pumped into the lumen are used to drive water to be excreted from the plasma into the collecting duct lumen via aquaporins through the neighboring principal cells. Regulation of water permeability is modified via regulated trafficking of vesicles carrying aquaporin to the plasma membrane (Kwon *et al.* 2009; Nedvetsky *et al.* 2009). Loss of either collecting duct aquaporin or V-ATPase function causes renal tubular acidosis and osteopetrosis (Borthwick and Karet 2002). Other *C. elegans* excretory system proteins with direct vertebrate renal homologs include proteins discussed above: NHR-31 (homolog to human HNF-4**α**) (Hahn-Windgassen and Van Gilst 2009; Menezes *et al.* 2012); CEH-6 (mouse BRN1) (Burglin and Ruvkun 2001); EXC-6 (formin INF2) (Shaye and Greenwald 2015), and channels listed in Table 1.

In addition to the excretory system, the hypodermis and other tissues in *C. elegans* play key roles in fluid homeostasis, a topic that has been reviewed separately (Choe and Strange 2007). The physiology of the excretory system, and how it functions coordinately with other tissues, are relatively unexplored areas that warrant further study.

The *C. elegans* Excretory System as a Model for Understanding Human Diseases

The morphology of the *C. elegans* excretory system is very different from that of the kidney or other vertebrate multicellular tubular organs. Excretory tubes do not have cilia, and their shaping does not involve cell division or cell death, so the system cannot be used to model those important processes. Nevertheless, as highlighted above, many aspects of signaling, cytoskeletal and matrix organization, and vesicle trafficking are conserved between different tube types, and between *C. elegans* and vertebrates. Furthermore, unicellular excretory tubes are topologically similar to endothelial capillaries and to wrapping glia such as Schwann cells, and are particularly good models for studying the mechanisms that form, shape, and stabilize these types of narrow tubes.

Examples of specific disorders that might be better understood through study of the excretory system include:

Ion channel-associated diseases, as listed in Table 1.

Achlorhydria: Loss of hydrochloric acid secretion of the stomach parietal cells is caused by knockdown of the ERM-1 homolog ezrin (Tamura *et al.* 2005; Yoshida *et al.* 2016). In this condition, tubulovesicular structures of these cells fail to fuse with the central canaliculus to secrete stomach acid, reminiscent of the effects of *erm-1* mutation on canalicular vesicle fusion.

Cerebral cavernous malformation (CCM): is a stroke-predisposing syndrome associated with abnormal brain capillary bed structure (Draheim *et al.* 2014). Studies in both *C. elegans* and *Drosophila* suggested that unicellular tubes (such as 75% of brain capillaries) (Bär *et al.* 1984) are particularly sensitive to CCM3 loss (Song *et al.* 2013; Lant *et al.* 2015). CCM-3 orthologs act with STRIPAK to affect Golgi organization and/or vesicle trafficking in multiple cell types (Fidalgo *et al.* 2010; Kean *et al.* 2011; Song *et al.* 2013; Lant *et al.* 2015). The precise roles of CCM in mediating CDC42 activity and apical membrane recycling, however, are still unclear.

Hereditary hemorrhagic telangiecstasia (HHT): is another stroke disorder with more widespread defects in capillaries throughout the body (Govani and Shovlin 2009). Mutations in the luminal ZP protein endoglin are a frequent cause of HHT (McAllister *et al.* 1994). A key role of endoglin is to bind and influence signaling by TGFβ family receptors (Guerrero-Esteo *et al.* 2002). The mechanism by which the ZP protein LET-653 and other luminal factors affect excretory duct tube integrity is not yet known, but studies in both *C. elegans* and *Drosophila* suggest that ZP proteins within luminal matrices are especially critical for maintaining patency of small unicellular tubes (Jazwinska *et al.* 2003; H. Gill, J. Cohen and M. Sundaram, unpublished results).

Focal segmental glomerulosclerosis (FSG): is a kidney disease associated with loss and scarring of glomeruli, where capillary beds interface with kidney podocytes

(D'Agati *et al.* 2011). Mutations in the EXC-6 homolog INF2 are one cause of FSG (Brown *et al.* 2010). Studies of EXC-6 confirmed that the disease-causing mutations are dominant gain of function and showed that both actin and MT-binding activities are important for function (Shaye and Greenwald 2015).

Charcot-Marie-Tooth (CMT) disease: These peripheral neuropathies often involve aberrant structure of glial Schwann cells that ensheath peripheral neurons (Vallat *et al.* 2013). Mutations in the EXC-5 ortholog FGD4 cause a particular CMT syndrome in which Schwann cell growth is initially normal, but fails during the period of rapid nerve growth during puberty (Delague *et al.* 2007). The requirement for EXC-5 in recycling endosome trafficking and apical domain maintenance (Mattingly and Buechner 2011) suggests that FGD4 may also play a similar role. Mutations in the EXC-6 ortholog INF2 also cause CMT in addition to glomerulosclerosis (Mathis *et al.* 2014).

Though few in number, the cells of the *C. elegans* excretory system use a wealth of conserved mechanisms and molecules to create a complex and vital organ. Continued studies to determine how the nematode integrates all of the elements discussed above to specify, form, maintain, and use these cells will inform our knowledge of basic cell biological processes as well as our understanding of many disease mechanisms.

ACKNOWLEDGMENTS

We thank David Hall and the Center for *C. elegans* Anatomy (Yeshiva University, Albert Einstein College of Medicine), Edward Hedgecock (Johns Hopkins University), Don Moerman (University of British Columbia), Erik Lundquist (University of Kansas), John Sulston (Medical Research Council), Shai Shaham (The Rockefeller University), and Richard Fetter and Cornelia Bargmann (The Rockefeller University) for providing archival images; anonymous reviewers; and John Murray, Amin Ghabrial, and members of our labs for comments on the manuscript. M.S. was supported by National Institutes of Health grant GM58540 and National Science Foundation (NSF) grant 1257879. M.B. is supported by the NSF.

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Communicating editor: M. Labouesse