

Shaping up for action

The path to physiological maturation in the renal tubules of *Drosophila*

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Abbreviations: AM, alary muscle; BMP, bone morphogenetic protein; C-E, convergent extension; ChIP, chromatin immunoprecipitation; CPA, cation proton antiporter; CVM, caudal visceral mesoderm; DamID, DNA adenine methyltransferase identification; DH, diuretic hormone; ECM, extracellular matrix; EGF, epidermal growth factor; GAP, GTPase activating protein; GBE, germband extension; GFP, green fluorescent protein; HG, hindgut; HVN, hindgut visceral nerve; MG, midgut; MM, metanephric mesenchyme; MpT, Malpighian tubule; NHA, Na⁺/H⁺ antiporter; NHE, Na⁺/H⁺ exchanger; PC, principal cell; PCP, planar cell polarity; P-D, proximodistal; PDGF/VEGF, platelet-derived growth factor/vascular endothelial growth factor; RNAi, RNA interference; RNAseq, whole transcriptome shotgun RNA sequencing; SC, stellate cell; SLC, solute carrier; TMC, tip mother cell; UAS, upstream activating sequence; UB, ureteric bud

The Malpighian tubule is the main organ for excretion and osmoregulation in most insects. During a short period of embryonic development the tubules of *Drosophila* are shaped, undergo differentiation and become precisely positioned in the body cavity, so they become fully functional at the time of larval hatching a few hours later. In this review I explore three developmental events on the path to physiological maturation. First, I examine the molecular and cellular mechanisms that generate organ shape, focusing on the process of cell intercalation that drives tubule elongation, the roles of the cytoskeleton, the extracellular matrix and how intercalation is coordinated at the tissue level. Second, I look at the genetic networks that control the physiological differentiation of tubule cells and consider how distinctive physiological domains in the tubule are patterned. Finally, I explore how the organ is positioned within the body cavity and consider the relationship between organ position and function.

Introduction

The fastest fluid-secreting cell known belongs to an insect. Remarkably this cell, one of a few hundred that make up the renal or Malpighian tubule (MpT) of the blood-sucking bug *Rhodnius*, can secrete a volume of fluid equal to its own volume every 15 sec.¹ The hard-working MpT cells allow these bugs to eliminate urine at a rate equivalent to their original body weight every 20–30 min. This impressive feat is necessary because the prodigious blood meals the insects take—sometimes in quantities of up to 12 times their own body weight—leaves them with surplus

fluid and ions that must be excreted quickly to allow them to return to a more comfortable state. Bumblebee MpTs are equally industrious. The high water content of the nectar used to fuel flight coupled with the exertion of flying mean that bee tubules are required to work flat out to produce a continuous stream of urine to rid them of surplus fluid.² These extreme examples underscore the important homeostatic functions performed by the MpTs. In much the same way that our kidneys do for us, the insect's MpTs maintain water, ion and acid/base balance and rid the body of foreign and metabolic toxins to maintain a constant internal environment that is necessary for wellbeing, health and survival.

The development of the insect MpT has been studied extensively in *Drosophila*. This fly manages to assemble a set of fully functional MpTs from a group of undifferentiated cells in a little over 12 h. During this short period, the MpTs are specified, they grow and are shaped, tubule cells differentiate and the organ is positioned precisely within the body to achieve optimal function. In this article I consider MpT development focusing on three aspects that contribute directly to organ function: how tubule shape is sculpted; how cells within it specialize to produce different physiological cell types that underlie function, and; how the organ is positioned within the body cavity, with respect to other organs and tissues.

There are several reasons why research into insect MpTs is important. Insects as disease vectors and agents of pestilence are widespread and have considerable associated health and economic costs. Elucidation of the mechanisms of MpT epithelial transport and toxin clearance, and the ways in which they are regulated might lead to specific strategies for control. Besides this however, there is another more fundamental reason why studying MpTs is important: as a model for organogenesis. Many of our own tissues and organs, such as the kidney, vascular system, lungs and gut, to

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name only a few, are composed entirely or partly from epithelial or endothelial tubes some of which have transporting functions. Breakdown in tubular structure or function in these organs is often associated with human diseases such as polycystic kidney disease or cystic fibrosis. As MpTs are an extreme example of a transporting epithelium¹ they represent a particularly attractive model to study the process of epithelial transport and the mechanisms that underpin the development of transporting epithelia. Lessons learnt from this simple MpT model are likely to have wide relevance for other organs, including our own. I pinpoint key questions that still remain in the field and discuss the powerful tools that exist in the fly that will enable us to tackle fundamental questions about how organs develop.

The Insect Malpighian Tubule

MpTs are found in all insects apart from Thysanura and aphids, they are also found in other terrestrial arthropods—the spiders and myriapods (millipedes and centipedes). The first description of an insect Malpighian tubule was of the silkworm *Bombyx* (Fig. 1A) by Signor Marcello Malpighi in the seventeenth century (whose manuscripts are held in the library of the Royal Society in London).^{3,4} Their size, length and number can vary substantially between insect species, but their general form is similar (Fig. 1B). They are simple, single-cell layered epithelial tubes, which insert into the gut at the midgut-hindgut boundary. They float freely in the body cavity, bathed by the fluid they act upon—the insect blood or hemolymph. Primary urine is generated in the distal portion of the tubule by a secretory mechanism (see below), and subjected to modification as it is conveyed down the lumen toward the gut. Excreta are passed into the hindgut (where further modification such as retrieval of additional water can take place) before being expelled from the animal. In addition to these excretory roles, the tubules can mount an immune response and thus serve to protect the animal against pathogenic insult.^{5,6} Still more exotic activities have also been shown for tubules in some species, such as the production of light or of silk that are used to attract mates, make cocoons and capture prey.⁷⁻⁹

An Overview of MpT Development in *Drosophila*

Assembling the building blocks for organogenesis. A good place to start when considering how an organ develops is to ask where the cells used to build the organ originate. Potential sources of cells

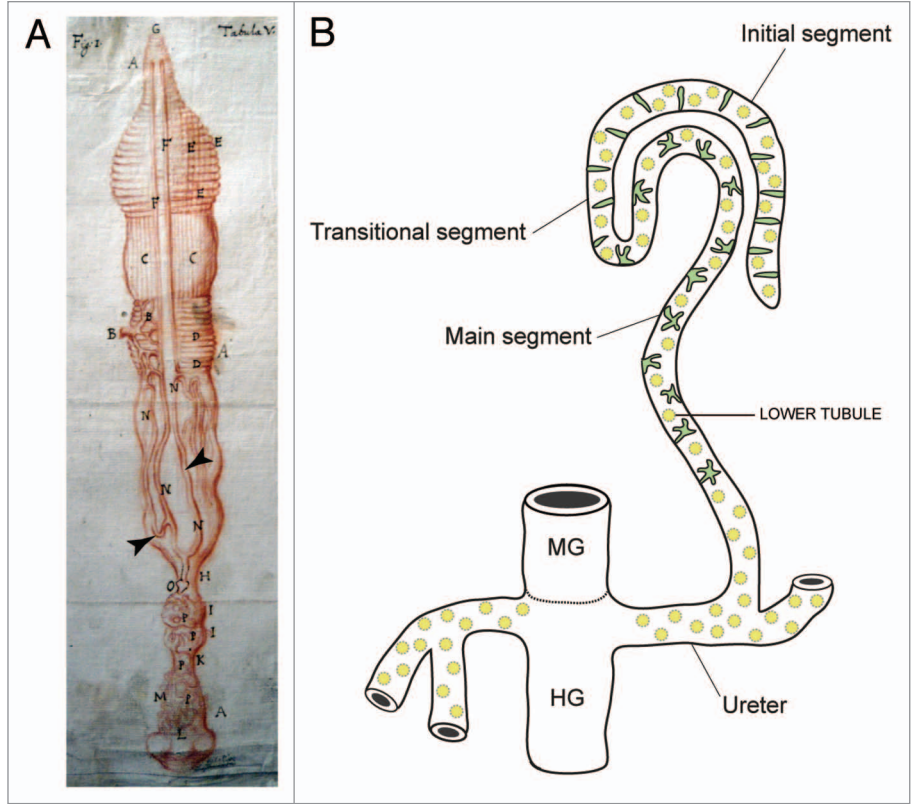


Figure 1. Insect Malpighian tubules (A), Malpighi’s original drawing of the MpTs (and gut) of the silkworm *Bombyx mori* (Malpighi, 1669). The MpTs are indicated with arrowheads. (B) Schematic representation of the MpTs of *Drosophila*. *Drosophila* has four MpTs, a longer anterior pair and a shorter posterior pair (a single anterior tubule is depicted). Each tubule is divided into four regions based on differences in cell structure and physiology along the proximodistal axis: initial, transitional, main segment and ureter. Two main physiologically distinctive cell types are known, principal cells (yellow) and stellate/bar cells (green). Midgut (MG) and hindgut (HG) are indicated. Image in A reproduced courtesy of the Royal Society of London.

for organ building include: (1) allocation of organ primordial cells, (2) cell proliferation within this population and (3) recruitment of cells from other sources. All three mechanisms are employed in the development of *Drosophila* MpTs. As these aspects of tubule development have been covered in detail elsewhere.^{10,11} I provide only an outline here. The tubules arise from an ectodermal primordium shared with the hindgut. At least three important signals are important for tubule cell specification and eversion: the Wnt (Wingless), and BMP (Decapentaplegic) proteins and an as yet unidentified signal from the midgut.¹² These signals converge and lead to the specification of four small clusters of about 20 cells, each marked by the expression of two transcription factors Cut and Krüppel both of which are important for early tubule development. The clusters evaginate as four little cylindrical buds and increase in size by cell proliferation (Fig. 2A and B). Tubule cell proliferation is controlled sequentially by the Wingless (Fig. 2A), then EGF signaling pathways (Fig. 2B), the later driven by EGF ligand secretion from the distal-most pair of cells—the tip cell and its sibling cell.¹³⁻¹⁶ By mid-embryogenesis cell proliferation is over, however the number of tubule cells is fortified by recruitment of mesodermal cells from the caudal visceral mesoderm (which also

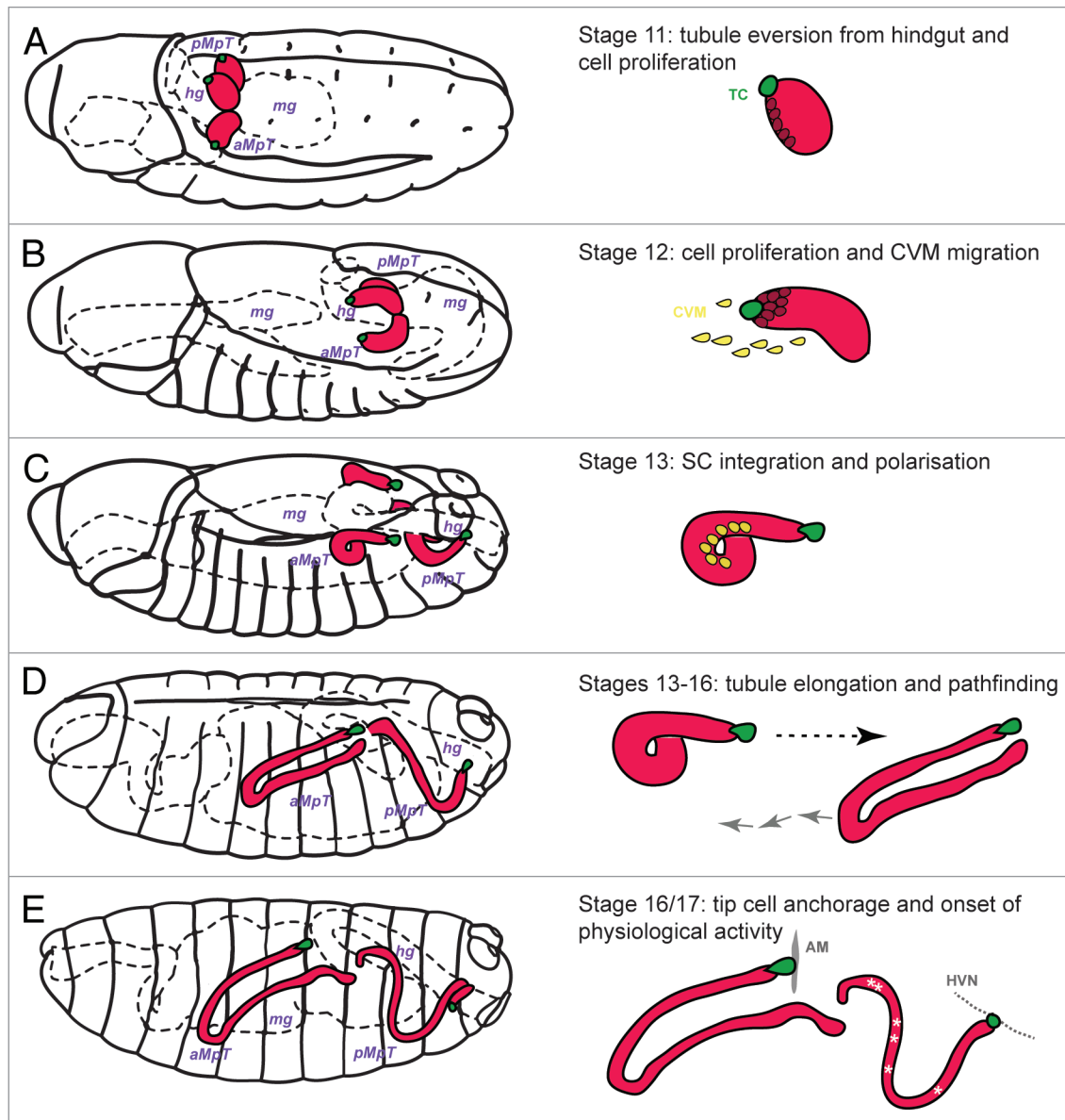


Figure 2. Embryonic development of *Drosophila* Malpighian tubules (A), Stage 11 (5h 15min–7h 15min) MpTs grow out as two then four small buds. Cell division occurs synchronously in all tubule cells and then in a subset of tubule cells regulated by Wingless. (B) Stage 12 (7h 15min–9h 15min) cell proliferation continues in a subset of tubule cell regulated by the EGF secretion from the tip cell (green cell) and sibling cell (not highlighted). The caudal visceral mesoderm (yellow cells) migrate toward the tubule. (C) Stage 13 (9h 15min–10h 15min) Cell division is complete. Stellate cells integrate into the tubule epithelium and develop apico-basal polarity. (D) Stages 13–16 (10h 15min–13h) Tubule elongation takes place by cell intercalation. MpTs migrate through the body cavity following highly stereotypical routes. (E) Stages 16/17 (13h–22h) the tip cell locates and anchors to its final position [alary muscle (AM) for anterior tubules and hindgut visceral nerve (HVN) for posterior tubules]. The onset of physiological activity becomes apparent as white crystals of uric acid appear in the lumen of the posterior MpTs.

form the founder cells of the midgut visceral muscle). These mesodermal cells migrate to the tubule, undergo a mesenchymal-to-epithelial transition and integrate into the epithelium, developing full apico-basal polarity (Fig. 2C).^{17,18} They begin to express the transcription factor called *teashirt* and ultimately differentiate into a physiologically distinctive subset of tubule cells known as stellate cells (SCs, see below). It has been shown that the basolateral and apical membranes and the adherens junctions of the ectodermally-derived principal cells (PCs), that make up the tubule epithelium, act as cues to establish and then stabilize SC polarity.¹⁸

Tubule morphogenesis, pathfinding and physiological maturation. The processes of tubule morphogenesis, path finding and physiological maturation that occur during stages 13–17 (Fig. 2D and E) are the main topics of this paper and are discussed in the following sections.

Post-embryonic development. The larva hatches after ~22 h of embryonic development; further growth of the tubule during larval stages occurs by an increase in cell size coupled with endoreplication of DNA. Differences in the appearance and structure of tubule cells allow four domains to be recognized: a distal

initial domain, a transitional domain, the main tubule segment and the lower tubule (Fig. 1B). These differences reflect functional differences, e.g., the main segment is secretory whereas the lower tubule is reabsorptive.¹⁹ Unlike most larval tissues the MpTs survive metamorphosis and are retained in the adult fly. During pupal development the tubule shrinks to half its former length with the initial segment expanding to form a bulb. The diameter of the tubule and its lumen also decreases.²⁰ Changes in cell shape also occur during pupation. For example, during the last 12 h of pupal development SCs transform from their cuboidal larval shape to adopt “bar-shaped” morphologies (in the initial and transitional segments) and “stellate-shaped” morphologies (in the main segment, Figs. 1B and 3B) characteristic of the adult tubule (Nan Hu, personal communication). After hatching, adult tubule domains resemble those of the larva, with the exception that the initial segment of the anterior tubule is much smaller than in the larva.²⁰

Malpighian Tubule Morphogenesis: Sculpting a Tubular Organ

Organs come in different shapes and sizes, tailored for particular physiological functions. In tubular organs like the MpTs or the vertebrate nephron it is important that appropriate tube dimensions are established during development and maintained throughout life for normal function. In circumstances where this fails, as it does in human polycystic kidney diseases, where nephron diameters are grossly enlarged,²¹ physiological function is severely compromised and this often leads to organ failure. However, the ways in which tubular epithelial are modeled in development are generally poorly understood. Here I describe the mechanisms that shape MpTs during development and discuss how this might be controlled at the tissue level.

Embryonic development, the period prior to the first larval stage, takes approximately 22 h at 25°C in *Drosophila*. From mid-embryogenesis, over the course of approximately 4 h the MpTs undergo a remarkable change in shape—increasing 4-fold in length along their proximo-distal (P-D) axis, while reducing circumferential cell number (the number of cells surrounding the lumen) from ~8–12 cells to just two (Figs. 2C–E, 4A and B). This transformation occurs in the absence of cell division and is underpinned by an organized pattern of cell intercalation. It represents a classic example of convergent-extension (C-E), where a tissue narrows (the cells converge) in one axis, and elongates (extends) in the orthogonal axis.^{22,23} What mechanisms underlie C-E in the tubules? We know that MpTs grown in isolated culture elongate normally (S. Bunt, personal communication)^{24,25} revealing that C-E is driven by internal forces and is independent of other tissues. As ordered cell intercalation lies at the heart of C-E, this is the process we would like to understand. In very simple terms we can break this down to ask: (1) what are the motile cell behaviors that drive intercalation, and (2) what is the nature of the global cues that control these behaviors?

Cell behaviors during intercalation. Genetic screens designed to isolate mutants where tubule elongation fails have

been particularly fruitful in revealing the molecular mechanisms that underpin MpT C-E movements. In mutants for the gene *crossveinless-c* (*cv-c*) tubule elongation fails completely.²⁶ In *cv-c* mutant tubules, cell intercalation does not initiate, so rather than undergoing P-D elongation and circumferential narrowing to produce four long thin tubules, the tissue coalesces into a single sack-like blister (A. Saxena, personal communication; Fig. 4E).²⁶ *Cv-c* encodes a RhoGAP (GTPase Activating Protein) that catalyzes the transition of Rho-family GTPases (Rho, Rac and cdc42) from their active to inactive state. In the tubules Rho appears to be the primary target for *cv-c*. Rho-family GTPase have established roles in regulating both actin and myosin, and consistent with this cortical actin is strongly disrupted in *cv-c* mutants during C-E movements.²⁶ Interestingly, elongation defects similar to those of *cv-c* are found in tubules mutant for *zipper*, the gene encoding the non-muscle myosin heavy chain, and *ribbon* and *raw*, two genes required for non-muscle myosin subcellular localization.^{27–29} These data indicate that an actin-myosin process underlies C-E movements. It has been shown that the *Cv-c* protein localizes to basolateral cortex and RhoGEF64 (a guanine exchange factor that has opposing, activating effects on RhoGTPases) to the apical cortex.³⁰ Together this suggests a model where subcellular differences in Rho activity, underpinned by the differential cellular distribution of its regulators, control polarized actomyosin-mediated processes in tubule cells to bring about orderly intercalation and tubule elongation.

The precise cellular mechanisms that bring about C-E in the tubules are not known. Two principal mechanisms that underpin C-E in other tissues have been described, and these may be informative to C-E in tubules: (1) local remodelling of cell junctions and (2) formation of lamelliform cellular protrusions used by cells to “walk” or “crawl.” During germband extension, which describes the developmental elongation of the *Drosophila* embryo, ordered cell intercalation proceeds through remodelling of the adherens junctions. Here, junctions are spatially reorganized within the plane of the epithelium following an ordered pattern of junction disassembly and reassembly. Junctional disassembly is dependent upon myosin II, and its planar polarized localization ensures directional cell intercalation to bring about axis elongation.^{31,32} Embryonic MpT cells are linked by adherens junctions and these are remodelled during elongation, however it is not known whether junctional remodelling via myosin II action is the driving force behind intercalation in this tissue. A requirement for actin, myosin and their regulators described above could be viewed as evidence for this mechanism.

An alternative mechanism, perhaps best characterized during mediolateral intercalation in frog mesodermal cells and the ascidian notochord,^{33–36} involves the formation of lamellipodial protrusions. These protrusions contact and exert traction on neighboring cells so that cells effectively crawl over one another. Because the protrusions are planar polarized—emanating from the lateral sides of the cell only—cell intercalation is directional. Similarly, during elongation in the *C. elegans* embryo dorsal hypodermal cells intercalate using medially-directed lamelliform protrusions.³⁷ These protrusions, which are positioned on the basal side of the cell, may use the extracellular matrix (ECM) in

addition to neighboring cells as a substrate upon which traction is gained. Active lamellipodial-like basal ruffles are found in tubule cells during elongation.³⁸ Furthermore, an ECM is deposited on the basal side of the tubules and is in place in time for the onset of tubule elongation,³⁸ suggesting a mechanism where tubule cells walk or crawl using the basement membrane as their substrate. In support of this hypothesis, work from our laboratory indicates that ECM components and their receptors, such as the integrins, are required for tubule elongation (Tarun Kumar, personal communication). Interestingly a similar mechanism may underlie the morphogenesis of the pronephros in zebrafish, where polarized basal lamellipodial protrusions from pronephric epithelial cells are used to walk along the basement membrane during the collective cell migrations that sculpt morphogenesis in the pronephric tubule.³⁹

Additional roles for the ECM in tubule morphogenesis. The ECM may be more than just a passive substrate to be walked or crawled upon. There is evidence the ECM plays an active and instructive role in the morphogenesis of some tissues. It has recently been discovered that the transformation of the *Drosophila* egg chamber from its initially near-spherical shape to an ellipsoid is dependent upon the surrounding ECM.⁴⁰ Here the ECM acts like a “molecular corset” restricting growth to produce the characteristic elongated shape of the mature chamber. A tightening corset of ECM around the tubule might also act to sculpt morphology during development. It is also possible that the ECM polymers and fibers have an intrinsic polarity that guide tubule cells as they intercalate, rather like the way railway tracks guide the movement of a train.

In addition to the basal ECM, there are also indications that apical or luminal ECM may contribute to tubule elongation. In tubules mutant for genes that support the formation of apical ECM such as *mummy/cystic* and *krotzkopf verkehrt*, which encode enzymes involved in the catalysis of chitin, the tubules are malformed, distended and fail to elongate.^{41,42} Clues to the function of these genes in tubulogenesis are revealed in studies of *Drosophila* tracheal development. *mummy/cystic* and *krotzkopf verkehrt* are both required for apical ECM formation and serve to regulate normal tracheal tube dimensions.⁴³⁻⁴⁶ It has been suggested that chitin is secreted into the tracheal lumen where it forms an expanding cylinder that coordinates the behavior of surrounding tracheal cells to ensure uniform tube expansion.⁴⁵ Furthermore, a general requirement for apical ECM in the morphogenesis of tracheal tubes and other epithelia is beginning to emerge.^{44,47-51} Could a luminal chitin or apical ECM cylinder regulate MpT morphogenesis? If it does the cylinder would need to begin wide and then contract (i.e., in the opposite direction to that hypothesized for the trachea), because the MpT lumen normally narrows during the elongation process. The presence of extracellular material in the lumen of elongating tubules supports this hypothesis.⁵² However, any role for luminal ECM in tubule C-E would have to be transient because it is known that mature MpTs are not cuticularized.

A more revealing picture of the cell biology underlying C-E in the tubules is within our grasp by using live-imaging techniques (Fig. 4C). This will allow us to catalog the precise

pattern of intercalation, and to quantify individual cell behaviors during the process (Fig. 4D). By following actinomyosin dynamics, cell membranes, junctions and ECM in real-time during elongation we will be able to visualize the dynamic cell behaviors that drive cell intercalation. These analyses can also be performed in mutant backgrounds, such as *cv-c*, to probe the molecular networks that underpin these cell behaviors during intercalation.

Orchestration of cell intercalation. MpT cell intercalation during C-E proceeds in a highly stereotypical fashion (Aditya Saxena, personal communication), suggesting there is a global signal that orchestrates the process. Recent evidence from our lab indicates that the EGF pathway is important in this respect (Denholm et al., in preparation). Briefly, an EGF signal is secreted from the distal tip of the tubule polarizing the tissue within the plane of the epithelium. When EGF signaling is perturbed the tissue does not polarize and C-E movements fail. It remains to be seen how this planar information is ‘read’ or acted on by cells to bring about orderly intercalation, however polarized remodelling of cell junctions like those that occur during GBE^{31,53} or the formation of directed cell protrusions like those that occur during C-E in the frog mesoderm and ascidian notocord^{34,36} are viable possibilities.

Planar cell polarity (PCP) is known to be indispensable for the morphogenesis and elongation of many different vertebrate tissues and tubular structures including the kidney tubule and the gut.^{54,55} Recently, Chung and colleagues (2009) have shown some of the core PCP proteins and the apically enriched PCP regulator, Serrano, are important in controlling tracheal tube size and elongation.⁵⁶ This study provides an example in flies where planar polarization regulated by PCP proteins is necessary for tubulogenesis. It is presently unclear if, as in the case of vertebrates, PCP is a general prerequisite for the elongation and morphogenesis of *Drosophila* tubular organs. Rigorous testing for planar polarization and its genetic regulation during morphogenesis of different fly epithelial tubes is required to address this issue. The MpTs can serve as one such organ system to test these ideas.

A genetic network for organ morphogenesis. Several other mutants have been described where MpT C-E fails. These include the transcription factors Buttonhead⁴¹ and Tracheless;⁴² the nuclear protein Lines;⁴¹ a cell adhesion molecule of the immunoglobulin superfamily called Faint Sausage;^{42,57} and Crooked neck (also known as Yok) a protein with a tetratricopeptide repeat protein interaction motif.⁴² As several of these are required for the morphogenesis of other tissues including salivary glands, tracheal system, heart, head, hindgut and dorsal epidermis it suggests that common genetic networks might operate to shape diverse tissues and organs. There is good evidence to suggest that a genetic network linking *trachealess*, EGF signaling and *cv-c* is common in shaping both the MpTs and trachea. *Trachealess* is required for normal MpT morphogenesis⁴² and for an early phase of tracheal development where the tracheal pits invaginate into the embryo by a two-part process involving apical constriction of invaginating cells and tissue remodelling. In the trachea, *tkh* links EGF-signaling and *cv-c* which are in turn both required for modifying the cytoskeleton to bring about pit invagination.⁵⁸ It is tempting

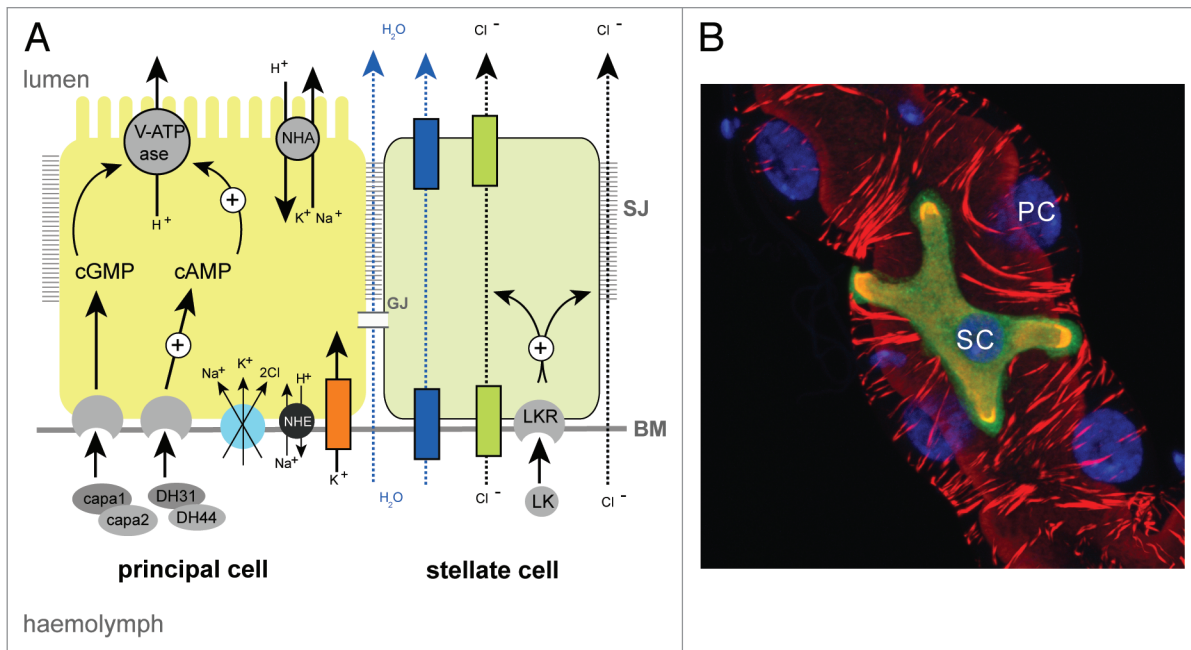


Figure 3. Principal and stellate cells (A), Drawing to show the major physiological activities performed by PCs (yellow) and SCs (green) in adult *Drosophila* tubules. Ion transport is driven by the activity of a H^+ -transporting vacuolar-ATPase (V-ATPase) located on the luminal membrane of principal cells. The V-ATPase, coupled with a cation/ H^+ antiporter (NHA), constitutes a cation pump that transports cations from the cytoplasm to the tubule lumen. Chloride ions (black dashed arrows) move into the lumen down an electrochemical gradient possibly through as yet unidentified chloride permeable channels (green) in SCs and via paracellular routes. Water (blue dashed arrows) follows by osmosis through water channels (dark blue) in SCs and via paracellular routes. Possible routes for basolateral cation entry include: K^+ channels (orange), cation coupled Cl^- cotransporters (light blue), and Na/H exchangers (NHE, black). Movement of ions between cells may occur through gap junctions (GJ). capa, capability peptides 1 and 2; DH, diuretic hormone; LK, leucokinin; LKR, leucokinin receptor; SJ, septate junction; BM, basement membrane. (B) Small section of an adult *Drosophila* MpT depicting a SC (green) and PCs (unlabelled). PCs and SC are indicated, the tubule is counterstained with phalloidin (red) to highlight the actin cytoskeleton and DAPI (blue) to highlight nuclei.

to speculate that this represents a genetic module that is utilized repeatedly during the morphogenesis of different organs. The challenge for the future will be to establish links between transcription factors, signaling pathways and the intracellular effectors of the cytoskeleton that bring about three-dimensional tissue shaping.

Physiological Maturation

The *Drosophila* MpT can be divided into several morphologically distinct domains the initial, transitional, main and lower segments (Fig. 1B) with distinct functional roles.^{19,20} Furthermore, physiologically distinctive cell types can be found within each domain (Fig. 1B). Based on the premise that differential gene expression equates to differential function, the work of Sozen and colleagues suggest that, in a tubule with average size of ~150 cells, there are at least 10 functionally different cell types.⁵⁹ How these cells differ, how their differentiation is controlled, and how they specifically align with respect to each other to ensure normal excretory function are important questions that will be explored below. To set the scene for this I first give a brief outline of insect tubule physiology.

Malpighian tubule physiology. Primary urine is generated by a secretory mechanism and this is coupled with active transport of nitrogenous wastes and specific toxins such as cardine

glycosides, in addition to the excretion of novel toxins by diffusion down paracellular routes.⁶⁰ MpT physiology has been studied in detail for many insect species and the transport processes that underlie urine production and its hormonal regulation are known in detail (Fig. 3A). Briefly, primary urine production is energised by a vacuolar-type H^+ ATPase (V-ATPase) located at the apical brush border of tubule principal cells (PCs).⁶¹⁻⁶⁴ This pump maintains a proton gradient across the apical membrane that drives the movement of alkali metal cations (Na^+ or K^+ , depending on the insect species) into the lumen through $Na^+/K^+/H^+$ exchange via members of the monovalent cation proton antiporter (CPA) transporter family. Rheault and colleagues characterized a member of the CPA2 family called NHA1 and show that it is expressed in MpTs of the *Anopheles* mosquito.⁶⁵ Further, the two *Drosophila* orthologs (NHA1 and NHA2) are co-expressed with the V-ATPase on the apical membrane of MpTs, and overexpression of NHA1 was shown to stimulate basal fluid secretion in tubules.⁶⁶ Together, this provides strong evidence that cation transport in insect MpT occurs by the action of a V-ATPase working in association with a NHA. Possible routes for cation movement across the basolateral membrane of PCs from the hemolymph include, potassium channels,⁶⁷⁻⁶⁹ cation coupled Cl^- cotransporters,⁶⁷ and Na/H exchangers (NHE).⁷⁰ The rates of cation transport are regulated by the diuretic

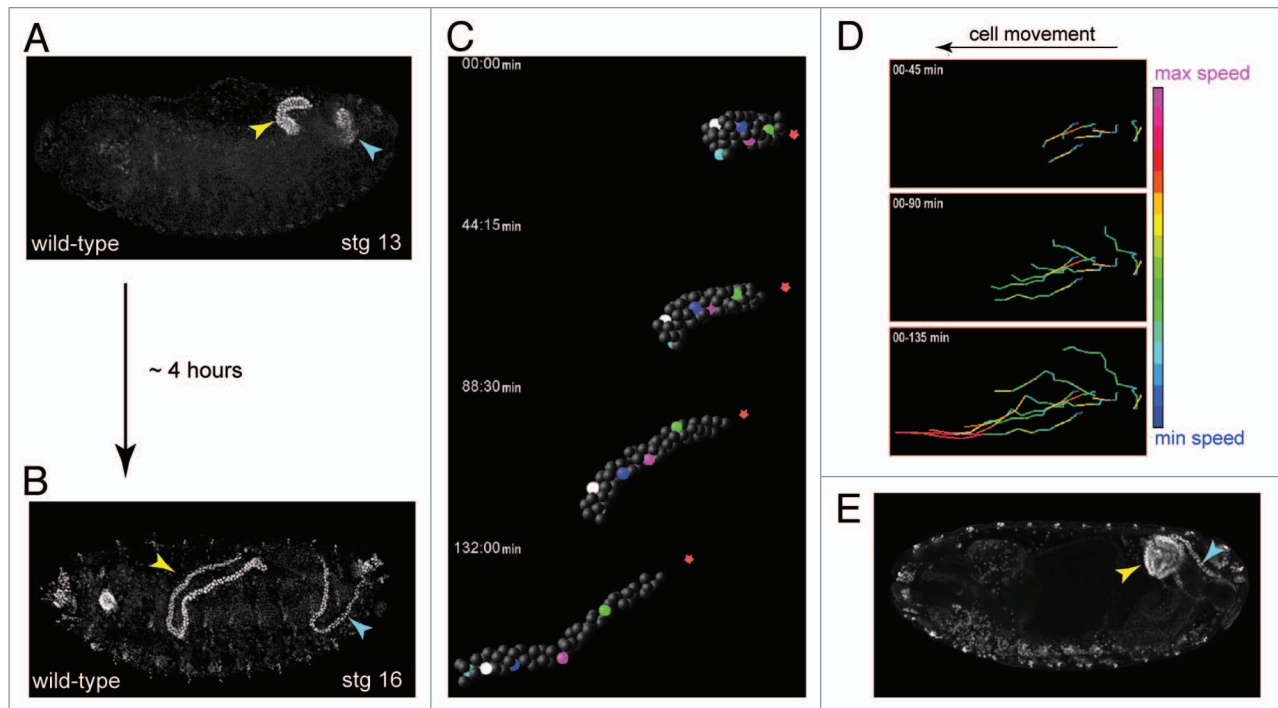


Figure 4. Shaping of the Malpighian tubule (**A and B**). During a period of about four hours MpTs undergo a dramatic change in shape that is driven by orderly cell intercalation, transforming from short, stubby tubules in mid-embryogenesis stage 13, (**A**) to long, elongated tubules in late embryogenesis stage 16 (**B**). (**C**) SIMI-Biocell assisted 3-D reconstruction of the distal portion of an anterior MpT. Five cells are marked in different colors and their positions tracked in XYZ over time. The distal-most tip cell is marked with red star. Such tracking can be used to quantify individual cell behaviors during tubule elongation (**D**). (**D**) Quantification of cell speed during intercalation. Cell speeds for the 5 cells marked in (**C**) are shown. The lines show cell tracks and color indicates cell speed. Net direction of cell movement is indicated by the arrow. (**E**) Orderly cell intercalation fails in *crossveinless-c* mutants. Anterior and posterior MpTs are indicated by yellow and cyan arrowheads in (**A**, **B** and **E**). Images in (**C** and **D**) courtesy of Aditya Saxena.

hormones DH31/44 and *capa1/2*, which act via cGMP and cAMP pathways to stimulate V-ATPase activity.⁷¹⁻⁷⁴

Active cation transport establishes a favorable electrochemical gradient for the movement of chloride ions across the epithelium. The route for chloride ions across the epithelium might be different depending on circumstance or species. In many, but possibly not all, insect species⁷⁵ chloride ion movement takes place across a distinct cell-type called the stellate cell (SC, also known as a type-II cell).⁷⁶ SCs are morphologically distinct and are interspersed between PCs along the main segments of the tubules (Fig. 1B and 3B), where they facilitate the transepithelial movement of chloride ions from the hemolymph into the lumen. The *Anopheles* and *Aedes aegypti* SLC4, a $\text{Cl}^-/\text{HCO}_3^-$ exchanger expressed in SCs, may be the route by which Cl^- enters the cell from the hemolymph.^{77,78} Evidence for apical Cl^- channels comes from patch-clamp experiments performed on the luminal surface of unstimulated *Aedes* MpTs.⁷⁹ This study found evidence for two types of Cl^- conductance channel: type I channels with intermediate conductance and slow kinetics and type II with low conductance and fast kinetics that operate in unstimulated MpTs. The molecular identity of these channels is unknown. However, two channels of the CIC family (CIC-a and CIC-c) have been identified in *Drosophila* as being enriched in MpTs,⁸⁰ and of these CIC-a is expressed highly in SCs in the embryo and adult,⁸¹ providing two promising candidates.

In addition to the transcellular passage of chloride ions, there is strong evidence for movement of chloride ions between cells in some insect species such as the mosquito *Aedes aegypti*, particularly under conditions of kinin-activated diuresis.⁸² This paracellular pathway is in effect a “transepithelial shortcut” bypassing cells of the epithelium to promote maximal transport under conditions of diuresis. The effects of leucokinin are extremely quick and reversible and it is thought that the post-transcriptional modification of one or more components of the septate junction—a ladder-like structure that adheres cells together and confers some permeability properties on the tubule⁸³—underpins the rate of transport through the paracellular pathway.⁸² Septate junctions consist of transmembrane proteins and cytoplasmic scaffolding proteins, linked into the actin/spectrin cytoskeleton. It has been hypothesized that modification to scaffold and cytoskeletal proteins results in the “unzipping” of the septate junction paracellular strands to bring about increased paracellular Cl^- conductance.⁸⁴ In support of this, actin, adducin and actin-depolymerising factors are mobilised after kinin stimulation.⁸⁴⁻⁸⁶

Similarly, movement of water occurs through both transcellular and paracellular routes (Fig. 3A).⁸⁷⁻⁹⁰ Transcellular movement occurs through water channels, with aquaporin water channels identified in MpTs of *Rhodnius*, the house cricket and *Drosophila*.^{87,91-93} Of note, the aquaporin channels *drip* and AQP1 are expressed specifically in the SCs of *Drosophila* and *Anopheles*

respectively, indicating a further important function for SCs in some species.^{87,94}

SCs have been found in tubules from a phylogenetically wide range of insects including Diptera, Lepidoptera, Coleoptera and Orthoptera, suggesting they are an ancestral feature of insect MpTs (reviewed in Dow, 2012).⁷⁵ However, they are not found in all insects. *Rhodnius* (Hemiptera) lack SCs, as do tsetse flies (a fairly close Dipteran relative of *Drosophila*).⁹⁵ In these species it has been suggested that cation and anion transport may occur through the same cell. The presence of SCs in the tubules of some insects but not others is curious and raises some interesting questions: What are the precise functions of SCs, and are they functionally equivalent in all insects? Are SCs a derived or ancestral character of insect tubules? Do those species without SCs have unrecognized cell physiological specializations, or does a single cell-type carry out all secretory functions in these insects? A wider sampling of species to determine the phylogenetic spread of SCs in insect tubules, in combination with functional analyses using RNAi will shed light on these questions.

Physiological differentiation of stellate cells. Work from our laboratory has begun to address the developmental mechanisms that underpin physiological maturation of SCs. We have recently found that the closely related *Teashirt/Tiptop* class of transcription factors are key regulators of SC differentiation in the tubules of the fly and other insects.⁸¹ In *Drosophila*, *teashirt* is expressed from mid-embryogenesis throughout the lifetime of the animal in SCs but not in PCs. *Teashirt* activity in SCs is required to regulate the expression of several genes associated with the mature physiological function of the SC including an aquaporin water channel, a chloride ion channel and a hormone receptor. Furthermore, *teashirt* is required for the regulation of cell shape. SC differentiation in the pupa is associated with a dramatic change in shape, where cuboidal larval SCs transform to adopt “bar-shaped” (in the initial and transitional segments) and “stellate-shaped” (in the main segment) morphologies characteristic of the adult tubule (Fig. 1B). The significance of these striking cell morphologies in the adult have not been explored but could be a mechanism to maximize contact with neighboring PCs. In the absence of *teashirt* SCs remain cuboidal, with a morphology similar to that found for larval SCs. These data indicate that *teashirt* controls multiple aspects of the SC phenotype and suggest that it occupies a pivotal position in the SC differentiation hierarchy. The role of *teashirt/tiptop* in SCs appears to be conserved in other insects because *tiptop* is expressed in the SCs of Coleopterans (the beetle, *Tribolium castaneum*) and the Othopterans (the cricket, *Gryllus bimaculatus*).^{81,96} These species are distantly related to *Drosophila* (a Dipteran), sharing a common ancestor approximately 280 (beetle) and 360 (cricket) million years ago, indicating that the activities of *teashirt/tiptop* in SCs are ancient and widespread. The genetic network operating downstream to *teashirt* is not known yet. However, mouse *Teashirt-3* regulates the expression of an aquaporin channel in the smooth muscle layer of the ureter in the mouse kidney, raising the intriguing possibility that *teashirt* targets may be conserved between widely divergent species.⁸¹

Physiological differentiation of principal cells. Less is known about the physiological maturation of PCs. The transcription factors *Cut* and *Krüppel* are expressed in PCs from early embryonic stages and are required for tubule morphogenesis and PC differentiation.^{97,98} In *Kr* mutants tubule eversion from the shared hindgut/Malpighian tubule primordium fails, and in *cut* mutants the tubules do not elongate; instead they form a multilayered cyst. Despite this, these mutant “tubule cells” still transport urates (a diagnostic feature for differentiated tubule cell function) revealing that PC differentiation can still occur.¹² However, in the absence of both *cut* and *Kr* urate transport is abolished, suggesting that both factors are required, perhaps redundantly, for PC physiological differentiation.¹² Although we know the repertoire of channels, pumps and receptors that define PC function (Fig. 3A), it is not yet known how these are linked with cell specification by transcription factors such as *Cut* and *Kr*.

The proximo-distal patterning of physiological activities. Domains with distinct physiological activities exist along the proximo-distal axis of the tubule. For example: the initial segment is geared toward calcium homeostasis and excretion;^{20,99,100} the transitional/main segments are secretory whereas the lower tubule is reabsorptive.¹⁹ Tubule cells with distinct physiological functions need to be aligned with respect to one another to ensure normal secretory function. For example, the interplay between PCs and SCs is key to the production of primary urine in some species, and their interspersed pattern is likely to be important for this. Therefore, tissue patterning and cell differentiation must take place to establish the correct arrangement of physiologically distinctive cell-types within the organ. Our understanding of these developmental mechanisms is limited. Distinct distal-proximal domains of gene expression arise during the specification of the tip cell lineage.^{15,101} During this process a cluster of cells expressing the proneural genes is established at the distal end of each tubule. Lateral inhibition, mediated by the neurogenic genes, selects a single cell—the tip mother cell (TMC)—in which proneural gene expression strengthens. In the remaining cells of the cluster proneural gene expression is dampened, however these low levels of expression are still sufficient to activate *seven up*, a mediator of the EGF pathway. In effect, this generates domains of differential gene expression along the distal-proximal axis: a distal tip cell with high levels of the proneural genes; a group of intermediate cells expressing *seven up*; and finally, a more proximal cell population that lack both proneural and *seven-up* gene expression.¹⁵ It is conceivable that these ‘tiers’ of gene expression lead to the differentiation of specialized cell-types along the distal-proximal axis of the tubule. A further role for the tip cell could be envisaged in which it secretes a morphogen to generate a morphogenetic field. In this scenario tubule cells would differentiate according to their distal-proximal position within this field.

In addition to these spatial mechanisms, there may be temporal control of differentiation. One could imagine a mechanism whereby cells adopt different physiological fates according to their “birth order” in much the same way that neurons differentiate according to neuroblast birth sequence.¹⁰² The pattern of cell proliferation within the tubule is tightly controlled and to a

large extent invariant;¹⁰³ the consequence of this is distal cells are born later in development than their counterparts in more proximal regions. This temporal birth order could be transformed into distinct spatial patterns of gene expression that go on to control physiological differentiation.

Structural differentiation. The subcellular structural changes that accompany maturation of physiological competence have been closely examined in the Malpighian tubule cells of the blood-sucking bug *Rhodnius*.¹⁰⁴ In these insects, tubule development occurs over a longer period making it easier to correlate changes in cell architecture with the development of physiological competence brought about by these changes and, by virtue of their size, it is possible to carry out *in vitro* physiology measurements in first instar MpTs. In *Rhodnius* there are two periods of functional maturation: a period beginning 3–4 d prior to hatching, where the transport of organic solutes such as urates and p-aminohippuric acid progressively increase in the tubule; and a second period, 1–8 d after hatching characterized by the onset of urine secretion. These phases in physiological maturation are accompanied by underlying changes in cell and tissue architecture. During the first phase, approximately 4 d before hatching septate junctions are assembled.¹⁰⁴ One possible function of septate junctions is to lower paracellular permeability by increasing the effective path length from the hemolymph to luminal sides of the tubule, and thereby to reduce bulk fluid flow between cells.⁸³ Further, the septate junction is an important structure that controls Cl⁻ conductance in *Aedes* MpTs.⁸² An increase in paracellular Cl⁻ conductance is expected to increase the transepithelial water flow. Lumen formation begins around this stage. At 4 d prior to hatching the tubules have no obvious lumen, however 2 d later a lumen is apparent, and continues to expand until approximately 8 d post-hatching. Lumen formation correlates with the onset of organic solute transport, and provides a space for its deposition.

Structural changes continue to occur until the eighth day after hatching. These changes include a striking amplification of cell membranes, apically to produce a carpet of microvilli, and basally to produce complex, interdigitating basal infoldings.¹⁰⁵ The amplification of the membranes provides increased surface area and is likely to be accompanied by their specialization, through the localization of specific receptor proteins, ion pumps and channels. At the same time microvilli and basal infoldings become highly populated with mitochondria, presumably to generate the energy needed to facilitate active transport. These changes correlate with the onset of urine secretion and together equip the animal for the prodigies of excretion called for by the immense size of their first blood meal. Structural and/or physiological maturation of MpTs may continue after eclosion in other insects. For example, Schepel and colleagues observe that responsiveness of *Aedes* MpTs to kinin stimulation sometimes develops only a few days after the adult hatches.¹⁰⁶ The authors suggest this delay may represent the continued development of the MpT after eclosion and/or be linked nutritional history of the mosquito in the larval stage.

The area of research at the interface between developmental biology and physiology is not yet fully explored for the majority

of organs. Our exquisitely detailed knowledge of insect renal tubule physiology, and the ease by which physiological activity can be measured on the one hand, coupled with our considerable understanding of tubule development (particularly for *Drosophila*) on the other, provide fertile ground for unpicking the mechanisms leading to physiological maturation. The resources to carry this out already exist and include: a complete transcriptome of Malpighian tubules⁸⁰ along with tools that allow cell specific gene knock-down, cell-specific transcriptional profiling and *in vivo* binding-site profiling.¹⁰⁷ Furthermore, the use of simple screens that reflect tubule physiological performance (e.g., uric acid deposition or osmoregulation in the intact animal) coupled with tubule-specific gene knockdown could be employed to uncover genetic mechanisms that underpin physiological maturation. Mechanisms uncovered in insect MpTs are likely to have wide significance for transporting epithelia more generally.

Organ Positioning Within the Body

Insects have an open circulatory system and effective excretion is dependent upon the tubules having wide coverage to remove waste. Accordingly, the mature tubules tend to adopt highly stereotypical positions within the body to maximize hemolymph sampling (Fig. 5A).^{38,108} In *Drosophila* one pair of tubules extend forward toward the head, and a second pair projects to the posterior. Further, one tubule of each pair lies to the right and one to the left within body cavity. This stereotypical arrangement is developmentally controlled: as tubules elongate through C-E movements they follow a highly reproducible course through the body cavity. The anterior tubules bend back on themselves so the apex of the bend “the elbow” or “kink region” extends forward, and this kink region leads the anterior tubules toward the head. Toward the end of embryogenesis the kink region changes course and dips ventrally.³⁸ The posterior tubules grow toward the tail of the embryo where, at the end of embryogenesis, their distal ends switch sides by crossing over the posterior region of the hindgut. Ultimately the distal-most cell in each tubule—the tip cell—makes specific contact with alary muscles of the heart for the anterior tubules, and a hindgut visceral nerve for the posterior tubules (Helen Weavers, personal communication).¹⁰¹ These movements can be seen in a movie (www.zoo.cam.ac.uk/zoostaff/skaer/links.htm).

The stereotypic navigation path taken by the tubules suggest that “guidepost signals” promote or inhibit extension into particular domains. These guidepost signals have been uncovered for anterior tubules to reveal a complex but fascinating picture with the involvement of multiple tissues and signaling cues.³⁸ The process is initiated by tubule cells through their secretion of PDGF/VEGF ligands to attract migrating blood cells known as hemocytes. Upon arrival the hemocytes begin to deposit the basement membrane component collagen IV that envelopes the growing tubules forming an extracellular collagen sheath. Here, as shown previously for other developing tissues in *Drosophila*,¹⁰⁹ collagen IV modulates BMP pathway activity, sensitizing a subset of tubule cells to local sources of the BMP ligand Decapentaplegic (Dpp), which act as attractant guidepost cues that propel the tubules

forward. At least three Dpp guidepost signals (from the dorsal epidermis, midgut visceral mesoderm and gastric cecal visceral mesoderm) are sequentially encountered to steer the tubules during their outgrowth. In the absence of Dpp signaling, in mutants for the Dpp receptor *punt* or pathway-induced transcriptional activator *schnurri*, the tubules locate abnormally in the body cavity.^{38,42} Further, an ectopic source of Dpp is sufficient to misroute tubule migration indicating that that Dpp is an instructive guidepost signal.³⁸ The ultimate position of the anterior tubules is likely to include additional signals. For example the ventral dip taken by the kink region during late development may be mediated by attractive cues from ventral tissues, or by repulsive cues emanating from dorsal regions.

Each tubule is capped at the distal end by a tip cell and its sibling cell. These cells also contribute important but distinct roles in tubule positioning. In tubules with two tip cells but no sibling cell, or two sibling cells and no tip cell the tubules do not course through the body cavity in the highly stereotypical fashion typical of wild-type embryos. Instead they appear to lose their way, with the distal ends randomly arranged within the body.¹¹⁰ During the migration phase the tip cell is replete with highly dynamic filopodia suggesting an active role in tubule pathfinding (Helen Weavers, personal communication; Fig. 5B). These studies demonstrate that positioning of tissues and organs within the body does not take place in a vacuum, but involve multiple signaling pathways and a complex interplay between several different cells and tissues, highlighting the importance of studying organogenesis within its normal developmental context.

Functional significance of organ position. The functional significance of tubule positioning is clear in some cases. A so-called “cryptonephridial” arrangement is common in most Lepidopteran larvae (butterflies and moths) and many Coleoptera (beetles) where the distal ends of the tubule associate intimately with the rectum, with which they are enclosed in a common membrane and sheath.^{111,112} Such an arrangement permits maximum water reabsorption from the hindgut and is often associated with insects living in particularly arid environments. In *Drosophila* the posterior pair of tubules also have intimate contact with the hindgut. The tubules loop over the lower region of the hindgut and the tip cell makes contact with the a9 abdominal ganglion nerve that runs up on either side of the hindgut.¹⁰¹ The significance of this is not known, but the authors speculate that tip cells make synaptic connections with a9 neurons on the surface of the hindgut. In support of this, a number of synaptic proteins such as Synaptotagmin; Synaptophysin and the Cysteine-string protein stain the tip cells of late stage embryos.¹⁰¹ Interestingly,

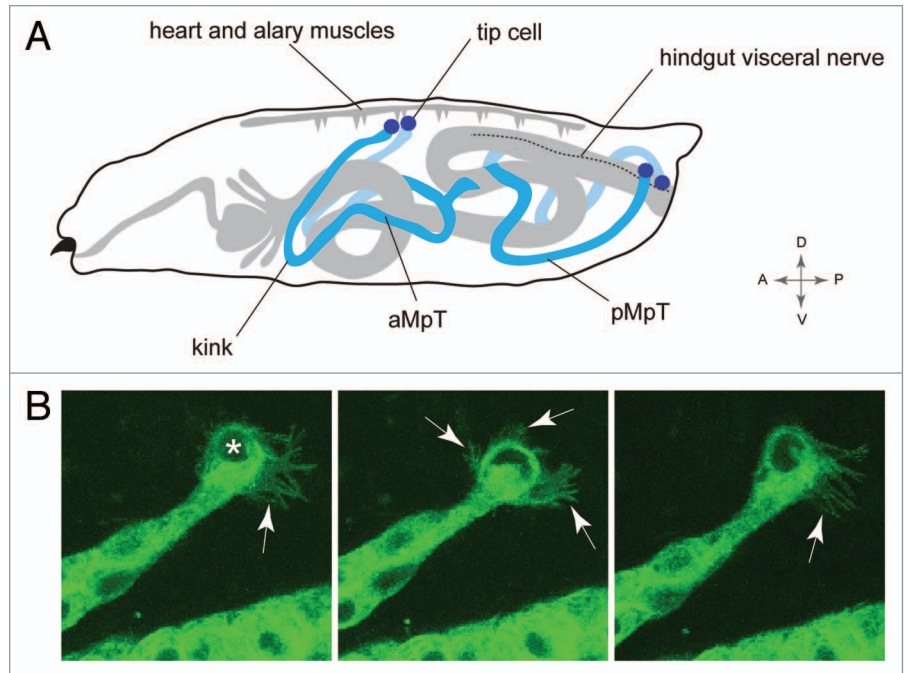


Figure 5. Positioning the tubule in the body cavity (A), Drawing of a *Drosophila* larva showing the final position of the MpTs relative to other internal structures. Anterior and posterior MpTs (aMpT and pMpT) are indicated, tip cells are shown in purple, gut and heart are shown in gray, the hindgut visceral nerve is depicted by a dashed line. Anterior, posterior, dorsal, ventral coordinates are given. (B) Still images from MpT movie showing the dynamic filopodial extensions (arrows) of the tip cell (indicated in first panel by asterisk). The time interval between images is 5 min. Images in (B) courtesy of Helen Weavers.

these neurons have been shown to control MpT physiology via release of the neuropeptide—pigment-dispersing factor (PDF).¹¹³ PDF is secreted from hindgut neurons and acts on the visceral muscle of the ureter to stimulate increased contractility. Another observation that may be significant shows that the hindgut of the crane fly (but apparently not of *Drosophila*) is innervated by leucokinin-positive efferent neurons.¹¹⁴ These observations suggest renal tubule physiology is under control of local circuits involving multiple tissues and cell-types. Further work will be necessary to characterize the details and directionality of these interactions.

Although anterior and posterior tubules in *Drosophila* have the same domain structure (Fig. 1B) and the rate of fluid secretion does not differ between them¹¹⁵ it is possible that the two pairs serve different functions by virtue of their position within the animal. A recent systematic investigation into the functional significance of tubule positioning has been performed using a transcriptomic approach suggests this may be the case.¹⁰⁰ In this study a comparison between gene expression in anterior vs. posterior tubules revealed that the expression of some genes were enriched in one pair relative to the other. These positional asymmetries appear to reflect physiological adaptations in the insect. For example the anterior tubules adjacent to the midgut are enriched for genes equipping them to deal with fluxes in overabundant ions (particularly calcium), xenobiotics and toxins that they encounter as a function of their proximity to the highly permeable midgut.¹⁰⁰ By contrast the posterior tubules, which lie

close to the hindgut, are enriched with genes allowing them to handle and be protected from elevated levels of ammonia from nitrogenous excretion. Some of the genes that pattern anterior vs. posterior tubule identity are known.¹² By manipulating these genes it would be interesting to generate animals containing only anterior or only posterior tubules and to determine how this affects the physiology of the animal.

The Insect Malpighian Tubule: An Experimental Toolkit and Future Perspectives

In this review I argue the value of the insect MpT as a model for organogenesis. The success of this model is rooted in its experimental tractability, particularly in vivo genetic analysis. Here I highlight some of the tools that have been so instrumental to its success:

Domain and cell-type specific expression. The Gal4-UAS system¹¹⁶ is an extremely powerful tool in the fly biologist's toolkit. The system exploits the yeast transcriptional activator, Gal4 and its cognate binding sites known as Upstream Activation Sequences (UAS). Gal4 "driver lines" are created by linking the Gal4 gene to tissue- or cell-specific promoters. Gal4 expression on its own has no phenotype. However, when this fly is crossed to another stock that has a gene of interest cloned downstream to UAS the progeny containing both elements will allow Gal4-mediated activation of the gene of interest only in those cells expressing Gal4. A large collection of driver lines with different expression patterns is available. Further temporal and spatial control of gene expression can be gained by using a temperature sensitive Gal80, an inhibitor of Gal4-mediated transcriptional activation.^{117,118} A large collection of Gal4 driver lines with tissue-, domain- or cell-specific expression patterns allows us to manipulate gene expression in the MpT with considerable resolution. For example there are lines that specifically drive in secretory regions or reabsorptive regions, there are other lines that drive in only PCs or SCs.⁵⁹

Gene knock-down using UAS-dsRNA. One of the most powerful tools to dissect gene function that has emerged in recent years is a genome-wide transgenic RNAi library for conditional gene inactivation.¹¹⁹ This is a UAS-GAL4 based system that allows gene knock-down for any gene in any tissue or cell. This can be performed in the intact animal or in organ culture (see secretory assay below) allowing the investigator to determine the physiological effects of gene function in specific cells/tissues at whole-animal and whole-organ levels.

Live imaging. The advent of live imaging has heralded a new era in developmental biology. *Drosophila* embryos are small and transparent, and it is possible to image the entirety of MpT embryonic development at high temporal and spatial resolution (Figs. 2C–E and 3B). Several lines that fluorescently label cell membranes, junctions, cytoskeleton, ECM can be used to illuminate the cell and molecular basis of organ development.

Organ transcriptome. The MpT transcriptome is known.^{80,120} This identifies those genes that are likely to be best studied in the tubule by virtue of their high enrichment. It also provides an important resource to target relevant genes when devising

reverse-genetic screening approaches. Interestingly, many human disease-causing genes are highly enriched in the tubule, suggesting that the MpT may be the best tissue to dissect the function of these genes.^{121,122}

Assay to directly measure organ function. The MpT secretion or "Ramsay" assay was pioneered by Alfred Ramsay in the 1950s.¹²³ In this simple set-up a tubule is placed in a little drop of saline, and covered with mineral oil. The proximal end of the tubule is teased out and anchored in place using a fine pin. Fluid secreted by the tubule emerges from a small nick in wall of the tubule or from the ureter into the oil. This can be measured over time to establish secretory rate. Hormones, drugs etc. can be added to the saline drop to monitor their effects on secretion. This assay has been highly informative in determining the mechanisms of fluid secretion and its hormonal control. This simple assay can now be combined with gene knock-down technology described above to provide a powerful tool to dissect the genes and genetic networks that underpin physiological function.

Simple genetic screens to assay physiology. Very simple screening methods exist to directly assess tubule function. For example, urate transport can be visualized in embryonic and larval tubules by simply looking down the microscope,²⁶ and osmoregulation can be assessed in intact adult animals by determining if they retain or lose too much water. These easily assessed phenotypes can be used to rapidly screen for: (1) genes encoding products directly involved in tubule physiology (channels, transporter and receptors); and (2) genes that underpin the developmental networks that establish physiological competence (transcription factors, signaling pathway molecules).

Uncovering the genetic networks of physiological differentiation. With the advent of next-generation sequencing technology, such as RNaseq, cell-specific transcriptional profiling is within grasp. This will allow us to determine exactly which genes are expressed in physiologically distinctive cell types, and how this is modified under conditions of genetic perturbation. In vivo tissue- and cell-specific binding-site profiling using DamID¹⁰⁷ or ChIP-chip allow transcription factors targets to be identified. Together these techniques will allow us to build a picture of the genetic networks that lead to physiological differentiation.

The combined application of these tools to the MpT model will continue to illuminate our understanding of organ development and function. For example, guided by MpT transcriptome datasets and using tubule- and tubule-cell-specific RNAi genetic knockdown, simple screens can be performed for genes effecting organismal physiology. Screens of this kind have the potential to reveal the genetic networks underpinning the development and physiology of this organ. Of particular interest will be those mechanisms that also have relevance for other organs in other species, especially those pertinent to mammalian organogenesis, human disease and regenerative medicine. It is worth considering the paths by which studies of MpTs can be translated to other systems. The techniques of in vitro culture of intact mammalian kidney cells and embryonic kidney rudiments are key techniques in the study of mammalian renal development. These techniques may provide one such route.

Grobstein,¹²⁴ and later Saxen,^{125,126} pioneered the kidney in vitro culture assay. In these assays explanted embryonic kidneys or kidney rudiments, when incubated on a filter above appropriate media, will grow for several days, during which time the early phases of kidney development such as ureteric bud (UB) branching and nephrogenesis take place in a manner closely approximating in vivo development. Experiments of this kind in which the UB was physically separated from the metanephric mesenchyme (MM) revealed the important reciprocal interactions that occur between the MM and UB during kidney development.¹²⁴⁻¹²⁶ More recently, this organ culture system has been coupled with live imaging and transgenic mouse strains expressing GFP in specific tissues. For example, using specific promoters to light up the UB, this approach was used to determine the pattern of UB branching, and to reveal the cellular and molecular mechanisms that underpin branching.¹²⁷⁻¹³⁰ Further innovations using viral vectors^{131,132} or small interfering RNAs (siRNA)^{133,134} for efficient gene knockdown allow functional analyses to be performed rapidly.

Taking advantage of a simple experimental system such as this, one can envisage testing candidate genes discovered in the “fly MpT model” to rapidly assess their relevance for mammalian kidney development. For example it would be interesting to determine whether conserved developmental programmes underlie tubular branching and tubular elongation in both MpTs and the mammalian kidney. The existence of deeply conserved cellular mechanisms for epithelial morphogenesis is suggested by a recent study showing that tubular elongation in the vertebrate kidney is driven by a myosin-dependent, multicellular rosette-based mechanism, similar to the mechanism driving GBE in flies.^{32,135} Using live imaging to follow: (1) developing MpTs in flies and (2) in vitro mammalian kidney explants, would reveal the extent of similarities between the cellular and molecular mechanisms underpinning tubule elongation across animal phyla.

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The work of Caubit and colleagues suggest the “fly MpT to kidney explant” will be a fruitful approach.¹³⁶ They set out to investigate a potential role for the Teashirt-family proteins in mammalian renal development, based on an earlier study highlighting a role for *tsb* in the fly MpT.¹⁷ They found that *Teashirt-3* (*Tshz3*) is required for smooth muscle differentiation in the mouse ureter, and by studying wild-type and *Tshz3* mutant explanted embryonic ureters were able to demonstrate a functional requirement for *Tshz3* in ureter peristalsis.¹³⁶

Concluding Remarks

The insect MpTs have provided valuable insight into the development of tubular epithelia. However, there are large gaps in our knowledge and understanding about the development of this organ, and indeed of the more complex organs of our own bodies. An important function of this review is to emphasize these shortcomings. This said, the genetic and experimental tractability afforded by the fly, coupled with the recognition that common molecular and cellular processes often underpin development in diverse organs, means that future studies of MpT development will continue to illuminate the important and fundamental processes by which organs are generated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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