Physiology, Development, and Disease Modeling in the *Drosophila* Excretory System

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ABSTRACT The insect excretory system contains two organ systems acting in concert: the Malpighian tubules and the hindgut perform essential roles in excretion and ionic and osmotic homeostasis. For over 350 years, these two organs have fascinated biologists as a model of organ structure and function. As part of a recent surge in interest, research on the Malpighian tubules and hindgut of *Drosophila* have uncovered important paradigms of organ physiology and development. Further, many human disease processes can be modeled in these organs. Here, focusing on discoveries in the past 10 years, we provide an overview of the anatomy and physiology of the *Drosophila* excretory system. We describe the major developmental events that build these organs during embryogenesis, remodel them during metamorphosis, and repair them following injury. Finally, we highlight the use of the Malpighian tubules and hindgut as accessible models of human disease biology. The Malpighian tubule is a particularly excellent model to study rapid fluid transport, neuroendocrine control of renal function, and modeling of numerous human renal conditions such as kidney stones, while the hindgut provides an outstanding model for processes such as the role of cell chirality in development, nonstem cell–based injury repair, cancer-promoting processes, and communication between the intestine and nervous system.

KEYWORDS colon; *Drosophila*; excretion; hindgut; kidney; large intestine; Malpighian tubule; FlyBook

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Physiology

The Drosophila excretory system: overview

The goal of excretion is to maintain physiological homeostasis through the elimination of potentially harmful substances (Nation 2015). As in humans, a kidney-like organ (Malpighian tubules) and a large intestine-like organ (hindgut) are principally involved in insect excretion by the alimentary canal (Figure 1, A and B), although we note that other specialized cell types outside the gut (*e.g.*, the nephrocytes; Helmstädter and Simons 2017) perform specific roles related to sequestration from the hemolymph. Here, we focus on the renal system and hindgut excretory.

The structure and function of the excretory system can be conveniently modeled by the Berridge analysis of gut function (Berridge 1970). As the cuticle is highly impermeable, exchanges of everything except oxygen, carbon dioxide, and water vapor must take place along the length of the alimentary canal. Of the three regions, the foregut is lined with highly impermeable cuticle, and the hindgut with cuticle of restricted permeability. The midgut is considered to provide the absorptive cycle, in which digestion and uptake of nutrients takes place, whereas the excretory cycle features the generation of primary urine by the Malpighian tubules, followed by selective reabsorption by the hindgut (Berridge 1970). Within *Drosophila*, the alimentary canal is arranged in a stereotypically looped structure, and the tubules and hindgut have carefully specified locations in the body cavity of both larvae and adults (Figure 1A).

The four Malpighian tubules first secrete a primary urine from the open circulatory system or hemolymph, which is added to the midgut contents as they pass posteriorly into the hindgut. The hindgut processes this material and forms waste material, or excreta, while also selectively reabsorbing other hindgut contents back to the hemolymph (Figure 1B). Both the Malpighian tubules and hindgut contain specialized anatomical regions and cell types with unique structural features (Figure 1, C–E) that aid in distinct aspects of excretion.

Malpighian tubule physiology

Overview of tubule structure and function: Insect renal tubules were first described and named by Marcello Malpighi in the 17th century (Malpighi 1669). *Drosophila* has two pairs of tubules, with each pair feeding into a short common ureter

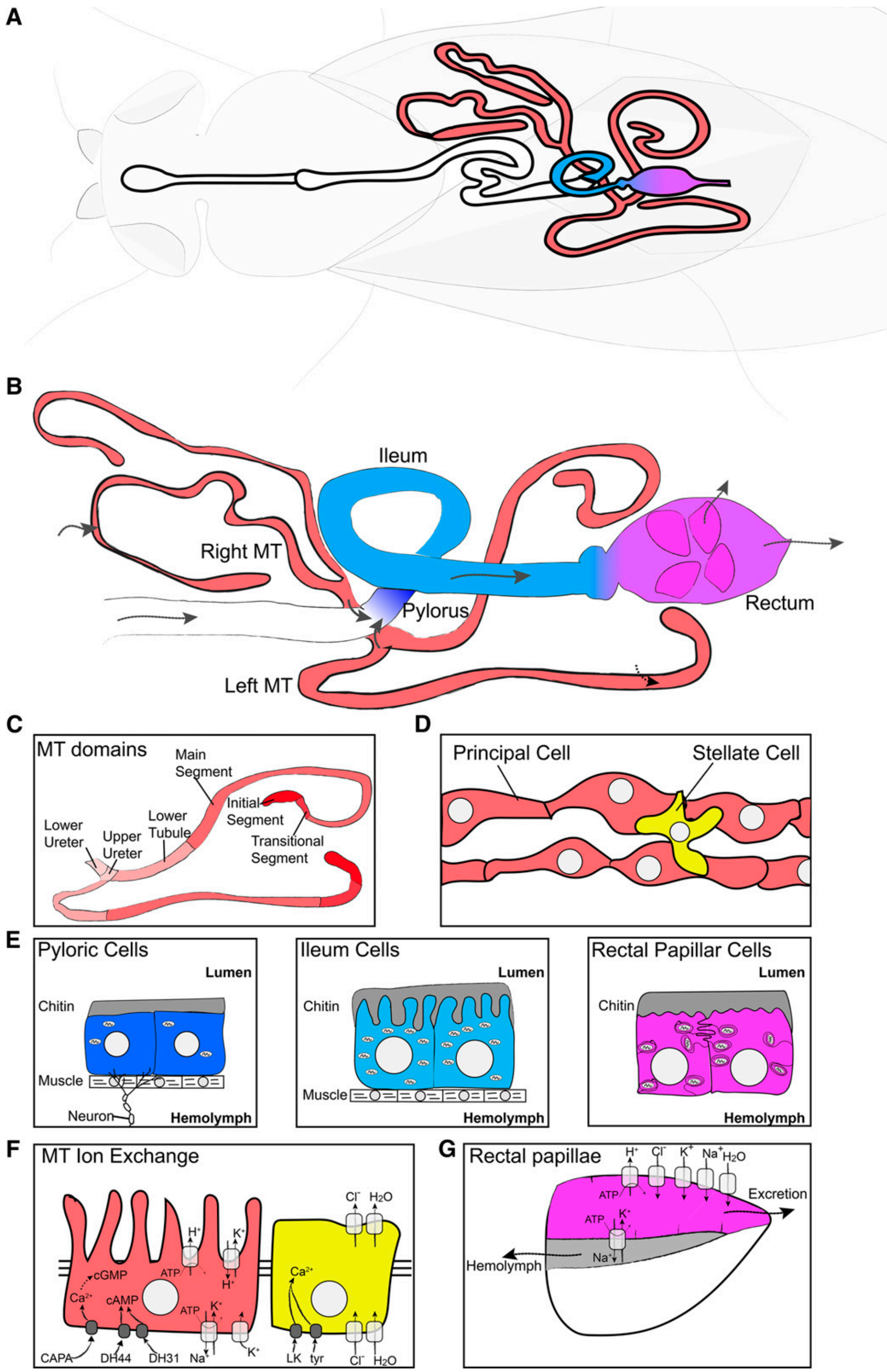

Figure 1 Physiology of the Malpighian tubules and hindgut. (A) Location of the Malpighian tubules and hindgut in adult *Drosophila*. Tubules are in red and hindgut is blue/purple. (B) Diagram of flow of contents into and out of the *Drosophila* Malpighian tubules and hindgut. Coloring as in A. (C) Domains of the Malpighian tubules. (D) Major cell types of the Malpighian tubules. Nuclei are indicated. (E) Major cell types of the hindgut. Mitochondria and nuclei are indicated. (F) Overview of Malpighian tubule ion exchange in principal and stellate cells. Key ions, transport regulators, and second messengers discussed in the text are highlighted. (G) Overview of rectal papillar reabsorption and excretion, with select exchange of ions and water indicated. A is adapted from Chintapalli *et al.* (2012). C, D, and F are adapted from Dow (2009). MT, Malpighian Tubule.

that connects to the junction of the midgut and hindgut, just ahead of the pylorus. The tubules are nonidentical: the pair on the right is longer and always ramifies anteriorly, associating with the anterior midgut, whereas the pair on the left is shorter, ramifies posteriorly, and associates loosely with the hindgut. The tubule plan is established by the time the insect hatches from the embryo and persists into adulthood. This persistence through metamorphosis is unusual for a *Drosophila* tissue (see *Hindgut development* section for comparison). Although the tubule physiologically shuts down during pupation (as evidenced by loss of apical microvilli), it does not undergo extensive remodeling from larva to adult, and cell number does not change. As the cells get larger, they increase their ploidy, rather than divide.

Despite their tiny size (1.5–3mm long, 35 μm wide, and each containing ~200 cells; Wessing and Eichelberg 1978; Sözen *et al.* 1997; Yerushalmi *et al.* 2018; Martínez-Corrales *et al.* 2019) the tubules transport fluid at a record-breaking rate (Dow *et al.* 1994), so generating a primary urine that is acted on by the lower tubule and hindgut. This rapid flux facilitates the rapid removal of wastes and toxic solutes, at the cost of ion, water, and solute loss that must be balanced by selective hindgut reabsorption.

Structural insights from enhancer trapping: Despite their small size, the tubules are remarkably sophisticated, and show structural zonation that is borne out by functional specialization (Table 1). Classical morphology had suggested that the posterior tubule was uniform, whereas the longer anterior tubules had a concretion-filled initial segment, joined to the rest of the tubule by a narrow transitional segment (Wessing and Eichelberg 1978). However, enhancer trapping has the potential to reveal the organism's (rather than the experimenter's) view of the tissue organization. In fact, both anterior and posterior tubules have six domains and six cell types (Sözen *et al.* 1997). There are miniature initial and transitional regions in the posterior tubule, reflecting their more obvious orthologs in the anterior pair. Additionally, the main part of the tubule can be subdivided into a main segment and a lower tubule, and the ureter can be further subdivided into two regions (Figure 1, B and C). Although multiple cell types can be delineated, the two predominant cells are the large, metabolically active principal cells and the smaller stellate cells (Figure 1C); together, these are responsible for most of the secretory function of the tubule. Remarkably, the number of cells of each type in each region is almost invariant (Sözen *et al.* 1997). These genetically defined domains are not mere curiosities. The tubule is also an unusually straightforward system in which to study function, and in every case where functions have been mapped to the tubule, they align with one of the enhancer-determined domains.

There is thus unusual confidence in the authority of the enhancer-trap derived map in this tissue. Of course, such complexity in a small space could prove daunting for physiological analysis; however, the enhancer traps were part of a large-scale GAL4 screen (Yang *et al.* 1995; Sözen *et al.* 1997), and so it is also possible to manipulate gene expression in any of the domains reported. Useful tubule Gal4 drivers are listed in Table 2.

It is worth noting that there is not a "clean" GAL4 line that marks all cell types in the tubule with no expression in other tissues.

An epithelium specialized for rapid transport: Water is not directly transported into the tubule, but follows an osmotic gradient; therefore, to secrete fluid, it is necessary to move solutes first. In insects, the Malpighian tubules are "driven" by very high levels of proton pumping vacuolar ATPase (V-ATPase). In *Drosophila*, the V-ATPase is located in the apical microvilli of the principal cells (Terhzaz *et al.* 2006). On its own, this would acidify the tubule lumen; however, a colocated K^+/H^+ exchanger allows the proton gradient to drive net excretion of K^+ from the principal cells (Figure 1F) (Day *et al.* 2008). To allow net excretion of K^+ from hemolymph to tubule lumen, K^+ must also be allowed to enter the basolateral membrane of the tubule (Figure 1F). Several mechanisms have been shown to be important for this flux; inward-rectifier K+ channels (Evans *et al.* 2005; Y. Wu *et al.* 2015), the Na^+/K^+ ATPase (Figure 1F) (Torrie *et al.* 2004), and the $Na^+/K^+/2Cl^-$ cotransport (Y. Wu *et al.* 2014).

The net transepithelial flux of potassium across the principal cell constitutes a major charge imbalance, and so chloride flows to balance the charge (Figure 1F). This is mediated by chloride channels in the stellate cell: Chloride channel a (Clc-a) on the basolateral side (Cabrero *et al.* 2014), and SecCl apically (Feingold *et al.* 2019). The transepithelial flux of K^+ and Cl^- corresponds to a net movement of salt, and osmotically obliged water follows (Figure 1F).

This method of fluid secretion by active cation transport is in marked contrast to the mammalian kidney, where the primary urine is effectively an ultrafiltrate through leaky capillaries, the glomerular basement membrane, and tightly controlled spaces between finger-like processes of specialized podocytes in Bowman's capsule. A corollary of this difference is that the default in the kidney is for all smaller solutes to be excreted, and so desired solutes must subsequently be rescued. By contrast, the *Drosophila* Malpighian tubule is a "tight" epithelium in which paracellular spaces are guarded by highly convoluted smooth septate junctions (Skaer and Maddrell 1987; Tepass and Hartenstein 1994); therefore, undesirable solutes must be actively transported to the tubule lumen. This is accomplished by highly expressed organic solute transporters; indeed, nearly every class of ABC and other transporter shows enriched expression in the tubule (Wang *et al.* 2004). As many of these transporters can carry a broad spectrum of solutes, the system can be effective at excreting both expected solutes and xenobiotics that *Drosophila* might not have encountered in nature. For example, the Na^+/K^+ ATPase inhibitor ouabain is actively excreted by tubules, masking its pharmacological effect (Torrie *et al.* 2004). Organic anion transport peptides have also been shown to transport a range of fluorescent dyes (Chahine *et al.* 2012). The classic *Drosophila* gene *white* encodes an ABC transporter that in the tubules, in addition to transporting visual pigment precursors, also transports cyclic GMP (cGMP) (Evans *et al.* 2008). The overall effect of the multiple transporters in the tubule is thus to form a system that achieves the effect of the mammalian kidney, but under much tighter control. This may provide specific advantages, for example in limiting water loss. Although these differences should be borne in mind, as discussed later in the *Modeling renal disease in the Malpighian Tubules* section, there is nonetheless potential in modeling human disease in the tubule.

Table 1 Validation of genetic domains by mapping of functional properties in the Malpighian tubule

Function	Tubule region	Reference
Fluid secretion	Main segment	O'Donnell and Maddrell (1995)
Fluid reabsorption	Lower tubule	O'Donnell and Maddrell (1995)
Rapid calcium excretion	Initial segment of anterior tubules	K. A. Dube *et al.* (2000), Terhzaz *et al.* (2005)
Alkaline phosphatase	Lower tubule	Sözen *et al.* (1997)
Ion transport by V-ATPase	Main segment principal cells	Sözen *et al.* (1997)
Chloride shunt conductance through channels	Stellate cells	Cabrero *et al.* (2014), Feingold *et al.* (2019)
α-HRP binding (surrogate for neuronal isoform of Na^+, K^+ ATPase)	Tiny cells	Sözen *et al.* (1997)
Receptors for kinin neuropeptide	Stellate cells	Radford *et al.* (2002)
Calcium-mediated signaling by Capa neuropeptide	Principal cells	Rosay *et al.* (1997)

Most of the discussion above has been of the main segment of the tubule (Sözen *et al.* 1997), as this is the region that generates the primary urine. Less is known about the other tubule regions (see Table 1); however, painstaking mapping of fluid production by different regions of the tubule showed that the lower tubule is reabsorptive (O'Donnell and Maddrell 1995). This domain corresponds with the expression pattern of c507, a GAL4 driver under control of the alkaline phosphatase gene *Alp4*, and histochemistry confirms that alkaline phosphatase is expressed in lower tubule (Yang *et al.* 2000), although the functional significance is not clear.

The initial segment contains large cells as well as narrow, bar-shaped cells that are marked by stellate cell drivers, and so are presumably related (Sözen *et al.* 1997). This region contains abundant white calcium-rich concretions, or spherites, that form intracellularly and move to the lumen (Wessing and Zierold 1999). Indeed, the tubule is capable of excreting calcium at a high rate, and this function is concentrated in the initial segments (K. Dube *et al.* 2000). The vesicles are bound by a membrane with contains Spock, a secretory pathway Ca^{++} ATPase that is necessary for concretion formation (Southall *et al.* 2006). This sequestration may be a form of storage excretion, allowing the insect to store calcium until a time of future need (for example reproduction).

Neuroendocrine control: Terrestrial insects are under significant risk of desiccation, and so it is not surprising that urine production is under neurohormonal control. Several secretagogues, mainly neuropeptides, have been identified and their intracellular signaling and targets identified; recent progress has provided suggestions for the conditions under which they are released to optimize organismal homeostasis. Insect neuropeptides are usefully summarized in the online Database for Insect Neuropeptide Research (Yeoh *et al.* 2017).

Capa peptides are related to the CAP2b neuropeptide originally discovered in the tobacco hornworm *Manduca sexta* (Tublitz *et al.* 1992). In *Drosophila*, Capa1 and Capa2 (together with unrelated Capa3) are encoded by the prepropeptide gene *capability* (Kean *et al.* 2002). Their receptor, encoded by *CapaR* (Iversen *et al.* 2002), is expressed in principal cells, and only at a very low level in some other tissues (data retrieved from flyatlas.org) (Chintapalli *et al.* 2007; Robinson *et al.* 2013). Capa1 or Capa2 trigger a complex cascade in principal cells that ultimately stimulates fluid production (Figure 1F). CapaR elevates intracellular calcium in only principal cells, from 80 to 300 nM, as measured with the luminescent probe apoaequorin (Figure 1F) (Rosay *et al.* 1997). Tubule principal cells contain nitric oxide synthase, and the calcium signal stimulates nitric oxide production, which activates a soluble guanylate cyclase to produce cGMP and thus activate the apical V-ATPase (Davies *et al.* 1995, 1997; MacPherson *et al.* 2004). In parallel, sustained elevation of intracellular calcium activates the apical mitochondria, so providing ATP directly to the V-ATPase (Terhzaz *et al.* 2006). A physiological role for Capa1 is becoming clearer, as it is associated with survival under cold or desiccation stress (Terhzaz *et al.* 2012, 2015, 2018; Davies *et al.* 2013; MacMillan *et al.* 2015). *Aedes* Capa has also been argued to inhibit the response to the diuretic neuropeptide kinin in *Drosophila* (see below) (MacMillan *et al.* 2018).

Two large peptide hormones act very similarly through cyclic AMP (cAMP). DH44 is a 44-aa diuretic peptide, distantly related to vertebrate corticotropin. This acts to stimulate fluid secretion by elevating cAMP in principal cells (Figure 1F) (Cabrero *et al.* 2002; Johnson *et al.* 2005; Hector *et al.* 2009; Cardoso *et al.* 2014). DH31 is a 31-aa diuretic peptide, distantly related to vertebrate calcitonin (Coast *et al.* 2001). Again, this acts through cAMP in principal cells to stimulate the apical V-ATPase (Figure 1F) (Coast *et al.* 2001). Most DH44-expressing neurons carry receptors for DH31, suggesting cross-talk between these signals (Johnson *et al.* 2005).

Two ligands are known for the stellate cells, kinin and tyramine (Tyr). Kinin is a short diuretic peptide found in most insects, and even in snails (Elekes *et al.* 1994). In *Drosophila*, its sequence is Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide, and is encoded by the gene *pp* (Terhzaz *et al.* 1999). The leucokinin receptor Lkr (Radford *et al.* 2002) is found in several tissues, but at particularly high levels in just the tubule stellate cells (Figure 1F), a pattern observed in other Diptera (Radford *et al.* 2004; Lu *et al.* 2011). It acts through intracellular calcium (Radford *et al.* 2002) to rapidly activate the chloride shunt conductance (Figure 1F) (O'Donnell *et al.* 1996), and so restore electroneutrality in the tubule lumen. Although the mechanism of calcium activation is not yet known, the targets are the basolateral chloride channel Clc-a (Cabrero *et al.* 2014) and the

Table 2 Some useful GAL4 drivers for the Malpighian tubule

Line	Region	Associated with	Reference
c42	Principal cells of main and lower tubule (also bar-shaped cells)	?	Rosay *et al.* (1997)
uro-GAL4	Main segment principal cells of only third instar and adult	Synthetic construct with Urate oxidase control region	Terhzaz *et al.* (2010)
capaR-GAL4	Main segment principal cells	Synthetic construct with Capa receptor control region	Terhzaz *et al.* (2012)
c710	Stellate cells	Teashirt	Sözen *et al.* (1997)
c724	Stellate cells	Teashirt	Sözen *et al.* (1997)
Clc-a-GAL4	Stellate cells	Synthetic construct with Clc-a control region	Cabrero *et al.* (2014)
C649	Bar-shaped cells	?	Sözen *et al.* (1997)
c507	Lower tubule cells	Alk4	Sözen *et al.* (1997)

apical SecCl channel (Feingold *et al.* 2019). Tyr is a biogenic amine that has been shown to act to stimulate chloride flux through stellate cells (Figure 1F) (Blumenthal 2003). This signal, although carried through a different receptor, appears functionally indistinguishable from that of kinin (Cabrero *et al.* 2013). However, Tyr can be produced by tyrosine decarboxylase in neighboring principal cells, suggesting a possibility for cross-talk between the two cell types (Blumenthal 2009).

As a functional analog of the renal system, and with the role of maintaining ionic and osmotic homeostasis, it is not surprising that the tubule expresses many genes identified as receptors (Wang *et al.* 2004). However, in addition to the familiar G protein–coupled receptors, the tubule also expresses several receptor guanylate cyclases, which act directly to raise cGMP. One of these, Gyc76C, was deorphaned by showing that it was a receptor for the novel neuropeptide NPLP1-VQQ, encoded on the *Nplp1* gene (Overend *et al.* 2012). The neuropeptide signaling pathway was shown to modulate innate immunity in the tubule (discussed below) in response to salt stress (Overend *et al.* 2012).

As well as these extensively researched molecules, there is evidence that the tubule receives a multiplicity of signals from the rest of the insect. In a meta-analysis of the tubule transcriptome, enriched expression was detected for several G protein–coupled receptors with ligands not previously described in tubule function (Chintapalli *et al.* 2012). For example, both neuropeptide F and short neuropeptide F were shown to have modest but significant effects on tubule signaling. Although the role of these signals is not known, both neuropeptides have been implicated in multiple roles, such as feeding and stress (Nässel and Wegener 2011), so it is quite reasonable that the tubule should receive information about such significant events. Surprisingly, high levels of sex-peptide receptor were found in male tubules (Chintapalli *et al.* 2012); although sex peptide is transferred to the female during copulation, it emerges that the sex-peptide receptor is actually a better receptor for myoinhibitory peptide/allatostatin B (Kim *et al.* 2010). It is thus reasonable that the tubule is receiving signals from the latter peptide, associated for example with satiety or ecdysis (Lange *et al.* 2012).

Although ligand-mediated signaling in stellate cells so far has operated only through calcium, it appears that the tubule uses each of the second messengers cAMP, cGMP, and calcium in both cell types. By ectopically expressing receptors for ligands that do not normally affect tubules (serotonin and natriuretic peptide A), it was possible to elevate and monitor cAMP, cGMP, and calcium in principal and stellate cells separately, and further to show that in each case, fluid secretion was significantly elevated (Kerr *et al.* 2004). These results are consistent with what is already known in principal cells; cAMP is invoked by DH31 and DH44, whereas Capa acts through calcium and cGMP (Figure 1F). However, in stellate cells, only calcium has been implicated in Kinin and Tyr signaling so far, suggesting that signaling pathways that employ cyclic nucleotides in these cells have yet to be discovered.

The epithelial cells of the ureter show the classic structural adaptations required for transport, with apical microvilli and basal membrane infoldings both in close association with mitochondria (Wessing and Eichelberg 1978). However, it is also surrounded by longitudinal and circular muscle, and is visibly contractile; it can thus be considered to act as an analog of the bladder. Pigment-dispersing factor (PDF), a neuropeptide that modulates the circadian clock (Yoshii *et al.* 2009), alters the rate of contraction of the ureter, although PDF neurons do not directly innervate the ureter, suggesting a gut/tubule communication (Talsma *et al.* 2012). In showing both central and visceral roles, PDF shares many commonalities with mammalian vasoactive intestinal peptide (Talsma *et al.* 2012).

Other roles for the tubule: The tubules ramify throughout the body cavity, and their excretory nature exposes them to blood-borne molecules that might provide early warning of problems. Given that there are not enough insect tissues to map 1:1 with mammalian organs, it is not surprising that the tubule might play roles additional to ion transport and solute excretion. Two of these are innate immunity and xenobiotic defense; that is, the tubule shows some properties associated with the immune system (Buchon *et al.* 2014) and liver.

Innate immunity: The observation that the tubule employed nitric oxide signaling (something also involved in immune response; Nappi *et al.* 2000) suggested a possible role for tubules in detecting and signaling, or even directly defending against, bacterial pathogens. In fact, the tubule contains a complete innate immune response pathway (McGettigan *et al.* 2005). Bacterial invasion is detected by PGRP-LC (Kaneko *et al.* 2006), which signals through the Imd pathway to elevate levels of the antimicrobial peptide diptericin to levels that are sufficient to kill bacteria. Overexpression of nitric oxide synthase in tubules also elevates *Diptericin* levels (McGettigan *et al.* 2005). Diptericin is not the only antimicrobial peptide with gene expression in the tubule; significant expression of *attacin, Metchninikowin, and Drosomycin* is also found (Chintapalli *et al.* 2012).

Detoxification: The insect excretory system must be capable of handling, not just predictably toxic molecules, but also those that it might not have experienced previously, such as insecticides. High expression rates of ABC transporters, such as the multidrug resistance transporter, in tubule has been documented (Wang *et al.* 2004), as has the tubule's functional role in excretion of unfamiliar molecules (Chahine *et al.* 2012). FlyAtlas reports that the tubule also expresses high levels of detoxifying enzymes of the cytochrome P450 and glutathione S-transferase families (Yang *et al.* 2007). One such abundantly expressed gene, *Cyp6g1*, has been implicated in resistance to the insecticide DDT (Daborn *et al.* 2002). When *Cyp6g1* levels were downregulated in just tubule principal cells, the whole fly showed increased sensitivity to DDT; when similarly overexpressed, the fly shows increased resistance. In the adult fly, then, the tissue with the highest expression of *Cyp6g1*—the tubules—plays a key and limiting role in xenobiotic defense.

Circadian regulation: Like humans, insect activity varies over the course of a day. The human kidney shows diurnal variation in urine production (strictly "diuresis" refers to daytime urination) and it is reasonable that insect renal function might show similar variation. This could be slaved to the central nervous system, in that the brain could exert neuroendocrine control over the tubule; however, the tubule actually contains all elements of the circadian clock (Giebultowicz and Hege 1997), which can operate autonomously *in vitro* in isolation from the fly (Giebultowicz *et al.* 2000). In fact, in adult flies, one clock-associated gene (*cryptochrome*) shows the highest expression in tubule (Chintapalli *et al.* 2007). It is thus likely that the tubule maintains its own time, to optimize its function in anticipation of the insect's needs over a day.

Hindgut physiology

The pylorus: an intestinal gatekeeper and immune signaling hub: As first described by classic entomologists (*e.g.*, Snodgrass 1935), the hindgut of many insects (including *Drosophila)* consists of three major regions, termed the pylorus, ileum, and rectum (Figure 1B). Each region contains a single layer of distinctly different epithelial cell types that contact the intestinal lumen, which are surrounded by circular muscle fibers (Figure 1E) (Hartenstein 2005). Much like the human ileocecal valve connecting the small and large intestines, the pylorus functions as a contractile sphincter (Snodgrass 1935; Vanderveken and O'Donnell 2014) that connects the midgut and hindgut. Contraction of the pylorus is controlled by the hindgut-expressed neuropeptide proctolin (Johnson *et al.* 2003; Miguel-Aliaga and Thor 2004; Vanderveken and O'Donnell 2014). Important neuronal/gut interactions likely occur in this intestinal region, as compared to other parts of the *Drosophila* intestinal tract, both muscle and epithelial cells of the pylorus are heavily innervated by sensory and efferent neurons from both the peripheral and central nervous system (Figure 1E, pyloric cells). This innervation may enable the pylorus to function as an intestinal checkpoint for further passage of gut contents (Brogiolo *et al.* 2001; Miguel-Aliaga and Thor 2004; Miguel-Aliaga *et al.* 2008; Cognigni *et al.* 2011). These contents include the primary urine from the Malpighian tubules, which empties into the intestinal lumen just anterior to the midgut/pyloric junction (Figure 1B). Perhaps as a consequence of changing intestinal contents, the gut increases in acidity at this junction (Cognigni *et al.* 2011). The transition from the posterior midgut epithelium to the hindgut pyloric epithelium is noticed ultrastructurally by the absence of apical microvilli projecting into the lumen. Instead, cells of the hindgut pyloric epithelium contact the lumen through an electron-dense chitinous layer (Murakami and Shiotsuki 2001; Sawyer *et al.* 2017). Pyloric epithelial cells are diploid and much smaller than the polyploid epithelial cells of other posterior segments of the hindgut and contain few striking intracellular ultrastructural features (Figure 1E, pyloric cells) (Murakami and Shiotsuki 2001; Fox and Spradling 2009; Fox *et al.* 2010; Sawyer *et al.* 2017). However, as the pylorus progresses from the anterior, midgut-facing side to the posterior, ileum-facing side, distinct domains of gene expression are observed (Murakami *et al.* 1994; Takashima *et al.* 2008, 2013; Fox and Spradling 2009; Sawyer *et al.* 2017; Tian *et al.* 2018, 2019) The function of each gene expression domain remains to be fully determined; however, as discussed in the ***Hindgut injury and repair: whole-scale organ regeneration and repair by polyploidy*** section, the anterior-most pyloric cells engage in interorgan communication with the midgut and may be especially important in maintaining the midgut/hindgut boundary following pyloric injury.

In addition to functioning as an intestinal valve, the pylorus is also an important zone of interaction between the *Drosophila* host environment and its microbiota, both symbiotic and pathogenic. A recent *FlyBook* chapter (Miguel-Aliaga *et al.* 2018) reviewed recent progress on *Drosophila* intestinal microbiota. In-depth examination of hindgut-specific microbe interactions remains to be performed. However, it is worth noting that the cuticle of the pyloric region of several insects and related diplopods contains cuticular microspines, which are thought to serve as sites of enriched microbial communities within the intestinal tract (Elzinga 1998; Nardi *et al.* 2006; X. Wang *et al.* 2018). The pylorus is also an immune signaling hub in the insect gut. Production of the pigment melanin is a major component of the insect innate immune response (Wu *et al.* 2016). p38 MAPK signaling may act as a first line of *Drosophila* hindgut defense to pathogenic bacteria, whereas melanization, mediated in part by JNK signaling, may act as a second line of defense in the absence of p38 signaling (Chen *et al.* 2010; Seisenbacher *et al.* 2011). Evidence for the importance of melanin in hindgut immunity comes from both *Drosophila* and other insects. Following feeding of silkworms with pathogenic bacteria, prophenoloxidase, a component of the melanization process, is activated specifically in the feces when passing through the hindgut pylorus (Shao *et al.* 2012). Honeybees infected with a pathogenic bacterium exhibit melanin scar formation in the pylorus (Engel *et al.* 2015). Further, feeding *Drosophila*, silkworms, or cotton bollworms with toxic plant phenolic compounds activates a melanization response in the hindgut. This pyloric melanization response is thought to be a last chance for the infected host to clear bacteria or toxic substances before excretion (Shao *et al.* 2012; K. Wu *et al.* 2015). The *Drosophila* hindgut, and the pylorus in particular, is also prone to melanization following genetic alterations in immune responses, cell signaling, or cell cycling (Reed and Orr-Weaver 1997; Takashima *et al.* 2008; Chen *et al.* 2010; Seisenbacher *et al.* 2011; Pan and Jin 2014). The accumulation of microbes and acute immune sensitivity of the pylorus argue that this hindgut region may be an ideal location for future exploration of gut immunity mechanisms.

The ileum and rectum: critical sites of reabsorption: Reabsorption is critical in animals with a high surface-to-volume ratio, such as *Drosophila*. The hindgut is the last chance for water and nutrient recycling to the hemolymph following primary urine formation in the Malpighian tubules (Nation 2015). In the hindgut, reabsorption occurs in the ileum and rectum. Following the pylorus, the majority of the anterior-posterior length of the *Drosophila* hindgut is made up of the ileum. The epithelium of the ileum is a single layer of large polyploid enterocytes, which are 64C in the larva and 8C in the adult (Fox and Spradling 2009). Underneath an apical cuticle, these enterocytes contain long, microvillar-like, apical plasma membrane infoldings that are closely associated with mitochondria (Murakami and Shiotsuki 2001) (Figure 1E, ileum cells). These infoldings are important for increasing surface area available for reabsorption, and are found in other insects such as ants (Villaro *et al.* 1999). In the ileum and rectum, selective reabsorption or secretion occurs to maintain ion and water homeostasis. Major resorbed ions include Na^+, Cl^-, and K+ (Figure 1G).

Reabsorption in the ileum is a highly regulated process. The larval *Drosophila* ileum exhibits phenotypic plasticity in response to dietary salt stress, as dietary increases in NaCl concentration cause the epithelium of the ileum to switch from absorbing Na^+ to secreting it (Naikkhwah and O'Donnell 2012). Studies in the desert locust established that ion reabsorption in the ileum is under antidiuretic hormonal control, principally by the Cl^- transporting neuropeptide ion transport peptide (ITP; Audsley *et al.* 1992; Meredith *et al.* 1996). *Drosophila* contains a single ITP gene, and *ITP*-expressing neurons from the abdominal ganglia innervate the hindgut (Dircksen *et al.* 2008). *Drosophila* adults lacking *ITP* function exhibit a diarrhea-like phenotype, with a dysregulated pace of transit of food through the digestive tract. ITP also regulates thirst, appetite, and water storage, providing a functional analog of the human vasopressin and renin-angiotensin systems (Gáliková *et al.* 2018). In addition to hormone control, transporters are obviously key to hindgut reabsorption function. The solute carrier 6A family transporter *inebriated (ine)* is expressed in the basolateral membrane of cells in the adult *Drosophila* ileum, where it colocalizes with a subunit of the Na^+/K^+ ATPase. Ine is critical for systemic water homeostasis under conditions of high dietary Na^+ or K+ (Luan *et al.* 2015). In addition to ion transport, water transport is also a critical component to reabsorption. Both humans and flies contain aquaporin water channels (Kaufmann *et al.* 2005). Several aquaporin family genes are expressed highly in the hindgut, especially the classical water channels *Drip* and *Prip* (Chintapalli *et al.* 2013).

The rectum is the final site of reabsorption, and the site of some of the most elaborate cell membrane networks documented anywhere in nature. To aid in efficient recycling of contents to the hemolymph, *Drosophila* and other dipterans contain elaborate epithelial infoldings known as rectal papillae, also referred to as rectal pads or rectal glands. These prominent intestinal structures were first described in honeybees in 1737 (Swammerdam 1737). While sexually dimorphic in species with highly specialized, sex-specific dietary needs such as mosquitos (Hopkins 1967), both male and female adult *Drosophila* contain four cone-shaped papillae, which project into the intestinal lumen from defined points in the bulbous rectum (Bodenstein 1950). A conserved rectal papillar ultrastructure has been well defined in *Drosophila* and other insects, including mosquitos, ants, and the blowfly (Figure 1E, rectal papillar cells) (Gupta and Berridge 1966; Berridge and Gupta 1967; Hopkins 1967; Wigglesworth 1972; Wessing and Eichelberg 1973; Garayoa *et al.* 1999; Chapman 2012; Nation 2015). While the apical surface of each papillar enterocyte contacts the intestinal lumen, the basal side organizes around a central canal, which directly contacts the hemolymph (Figure 1E, rectal papillar cells, Figure 1G). The central canal is rich in tracheal structures with branches that directly insert into papillar enterocytes, implying a high demand for oxygen. Similar to enterocytes of the adult ileum, *Drosophila* rectal papillar enterocytes are polyploid, at 8C or 16C (Fox *et al.* 2010). Papillar enterocytes are also similar to those of the ileum in that they contain an apical cuticle, which covers elaborate internal microvillar-like projections. But unlike enterocytes of the ileum, insect papillar enterocytes display heavily folded regions of lateral membrane stacks with tightly associated mitochondria. These stacks are thought to greatly increase basolateral membrane surface area available for ion transporter localization and function, with the neighboring mitochondria providing energy for active ion transport. Ions destined for reabsorption into the hemolymph would then be absorbed from the intestinal lumen, and then transported through the papillar membrane stacks into an intermembrane space that ultimately leads to the central canal and hemolymph (Gupta and Berridge 1966; Berridge and Gupta 1967; Hopkins 1967; Wessing and Eichelberg 1973; Garayoa *et al.* 1999; Nation 2015) (Figure 1, B and E, rectal papillar cells, Figure 1G). From this torturous membrane architecture, which vastly increases membrane surface area, it is clear that insect rectal papillae are structures shaped by evolution to be highly efficient resorptive structures.

The importance of *Drosophila* rectal papillae in regulation of organismal ion balance can be underscored by the fact that adult flies with malformed papillae (but no other anatomical defects) die upon feeding a high NaCl diet, while control flies are completely tolerant (Schoenfelder *et al.* 2014). The *Drosophila* rectum also reabsorbs K^+ to a greater extent than the ileum (Yerushalmi *et al.* 2018). Based on work in other insects such as mosquitos and midges, the Na^+/K^+ ATPase (known as P-ATPase) and V-ATPase are required for K^+ transport in the rectum (Figure 1G). In these species, P-ATPase localizes to papillar enterocyte basolateral membranes, while the V-ATPase is found in both cytoplasmic and apical membrane regions (Patrick *et al.* 2006; Jonusaite *et al.* 2013). Along with the pylorus, the rectum is one of the most highly innervated regions of the *Drosophila* intestinal tract. Both the papillae and the rectal musculature are innervated (Cognigni *et al.* 2011). A subset of these neurons are insulin-producing, suggesting cross-talk between metabolic signaling and hindgut function (Miguel-Aliaga *et al.* 2008). Innervation also plays a role in the final step of excretion following reabsorption, defecation, which in larvae occurs in a stereotypical behavior and is regulated by the TRP channel NOMPC in a single mechanosensitive sensory neuron in the anal slit (Zhang *et al.* 2014). Going forward, the extensive interactions between the nervous system and the muscles and epithelia of the hindgut argue that the hindgut is an essential model in *Drosophila* for enteric nervous system study. Given the genetic strengths, relatively simple anatomy, and accessible assays for function such as live observation of food passage and hindgut contractions (Cognigni *et al.* 2011; Vanderveken and O'Donnell 2014; Zhang *et al.* 2014), excretion in the *Drosophila* hindgut may provide an accessible model for human enteric nerve conditions such as Hirschsprung's disease.

Unlike in the adult, the tubular larval *Drosophila* rectum does not contain obvious structures that are adapted for absorption (Murakami and Shiotsuki 2001). However, just posterior to this region are two papillae-like anal pad structures containing cells with structural features of absorptive cells (Jarial 1987). Anal pad morphology is noticeably altered under conditions of altered salinity (Jarial 1987; Keyser *et al.* 2007). Mutant larvae of the *Drosophila* homolog of the human nuclear receptor nuclear factor of activated T cells are sensitive to a high-salt diet and have enlarged anal pads in hypotonic solution (Keyser *et al.* 2007). As discussed below, the larval rectum plays a critical role in adult hindgut development and is a source of chromosomally unstable cell divisions similar to those seen in human cancers.

Development

Malpighian tubule development

Overview of development: The formation of the tubules is intertwined with that of the hindgut (Figure 2), which is described in the following section. Formed as pouches at the tip of the proctodeal invagination during gastrulation, the tubules are mainly ectodermal in origin, but with extra added mesenchyme late in embryonic development. Unusually for a *Drosophila* tissue, and in contrast to the rest of the hindgut, the tubule of the newly hatched insect is maintained for life, without extensive remodeling through pupation. There are further reviews available on tubule development (Jung *et al.* 2005; Beyenbach *et al.* 2010; Denholm 2013).

Specification: Specification (Figure 2A) marks out groups of cells that will in the future take on a particular role, in advance of visible differentiation of a tissue. In gastrulation, the ectodermal foregut and hindgut invaginate and join with the endodermal future midgut to form a single tube. The future tubule cells are ectodermally derived at the junction of midgut and hindgut (Hartenstein 1993). Although the future tubule is ectodermal, the midgut is necessary for the specification; in mutants for *huckebein* and *serpent*, where the midgut fails to develop (Bronner and Jackle 1991; Abel *et al.* 1993), tubules fail to be specified (Ainsworth *et al.* 2000). The nature of the signal from the midgut is not yet known. The gap gene and transcription factor *Kruppel (Kr)* is broadly expressed in the hindgut, and is also necessary for tubule specification, as tubules fail to form in *Kr* mutants (Gloor 1950). Hatton-Ellis *et al.* (2007) took the formation of uric acid crystals as diagnostic of differentiated tubule function, and showed that Kr and its target, the homeodomain protein Cut, interact to specify tubule identity. *Kr* initially shows broad expression, which is refined within the hindgut by the action of Forkhead, Tailless and Wingless (Wg), to a group of cells that subsequently express *cut* (Gaul and Weigel 1990). Although tubules fail to form in *Kr* mutants, there is evidence of differentiated clusters of cells in the anterior hindgut and the formation of uric acid crystals (Hatton-Ellis *et al.* 2007); the *Kr* defect is thus of eversion, not specification. By contrast, in *Kr*/*cut* double mutants, no crystals of uric acid form in the hindgut, whereas ectopic expression of *cut* in the *Kr*-expressing foregut is sufficient to generate uric acid crystals there (Hatton-Ellis *et al.* 2007). Kr/Cut cooperation thus suffices to specify a future tubule identity (Liu and Jack 1992).

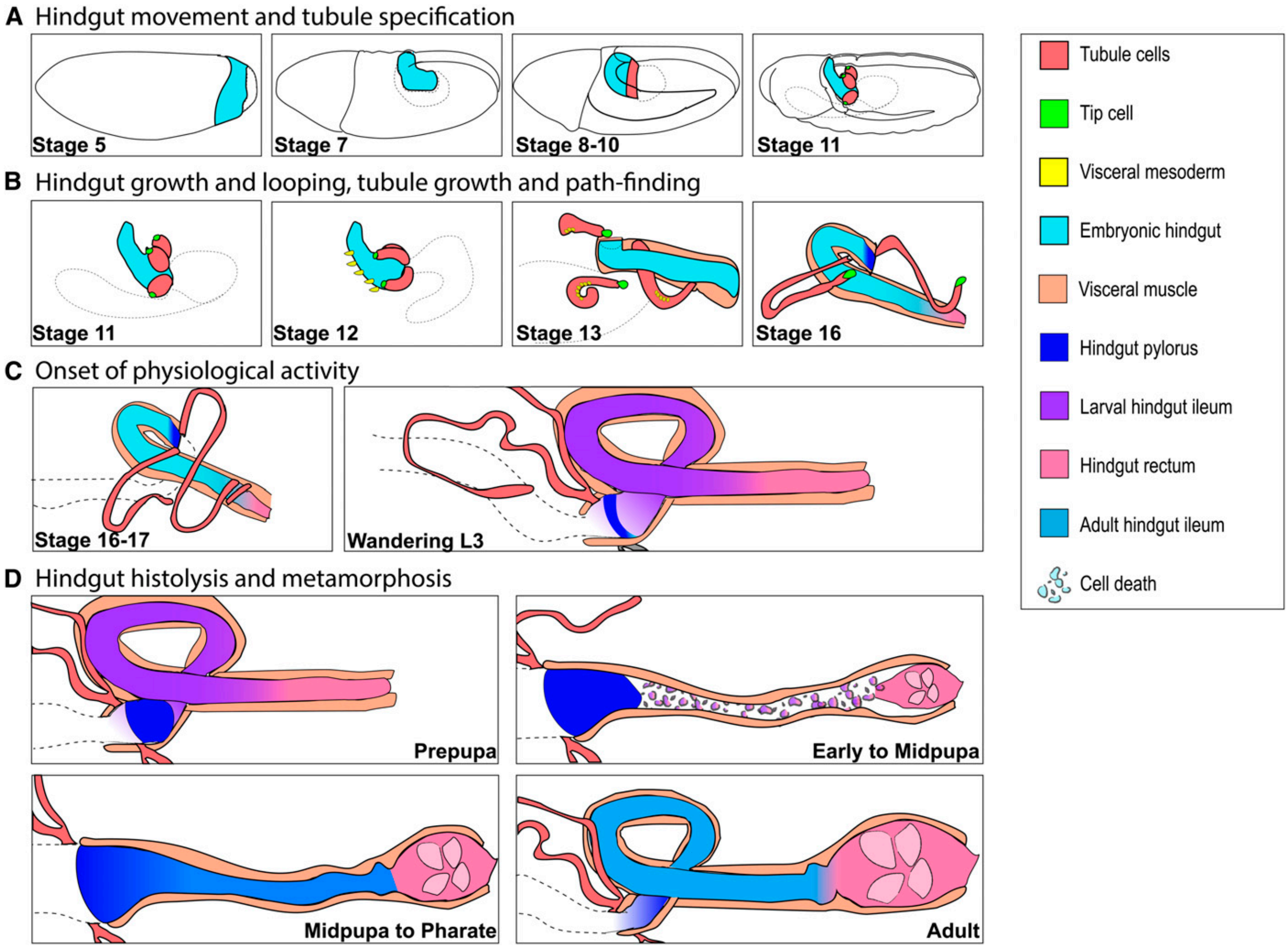

Figure 2 Overview of Malpighian tubule and hindgut development. Major cell types (indicated in the key) and developmental events are diagrammed in the embryo (A–C), wandering third instar larva (C), pupa (D), and adult (D). Individual substages are indicated in each panel. For the embryo panels, an entire embryo is shown for reference, while only tissues of interest are shown for the remaining stages. Anterior is to the left in all panels. Tubule diagrams are adapted from Beyenbach *et al.* (2010).

Eversion: As *Kr*-expressing cells resolve into four clusters, they start to rearrange into buds. The ventral pair of buds, marked by *brinker*, project posteriorly toward the caudal mesoderm and become the posterior tubules, while the lateral pair, marked by *Dorsocross*, ramify anteriorly and become the anterior tubules. The characteristic lateral asymmetry of the tubules is thus specified early as a dorsoventral pattern under control of *Decapentaplegic (Dpp)*; subsequent rotation of the gut means that the anterior pair is always found on the right, and the posterior pair on the left. This asymmetry persists throughout the life of the animal, both morphologically (the anterior tubules have an extended initial segment; Wessing and Eichelberg 1978) and functionally (anterior and posterior tubules show overlapping but distinct patterns of gene expression; Chintapalli *et al.* 2012).

Division: After cellularization, the tubule/hindgut anlage undergoes a synchronous division. A second division is confined to the tubules, and a third to just a subset of tubule cells, requiring Wg (Skaer and Martinez-Arias 1992). These divisions produce only about half the cell count required for a tubule. Further division requires the emergence of the tip cell (Figure 2B, stages 11 and 12), which then directs mitosis through the action of EGF-like Spitz (Sudarsan *et al.* 2002).

The allocation of the tip cell is a classic story of multiple signals and lateral inhibition. Initially, a cluster of $\sim$6 cells in each tubule start to express proneural genes such as *achaete* (Hoch *et al.* 1994).The pattern is refined to a single cell (the tip mother cell) in each cluster by lateral inhibition through the action of Delta on its receptor Notch. This cell then divides to form the tip cell and its sibling, which start to express the EGF family regulators *rhomboid* and *Star*, allowing them to secrete Spitz (Kerber *et al.* 1998). Meanwhile, the remaining cells express the EGF receptor, and are so able to respond by dividing. One might predict that the tip cell is essential for the later divisions, and this is the case; if the tip cell fails to form through interference with the neurogenic gene cascade (Hoch *et al.* 1994), or by ablation (Skaer 1989) of the tip cell progenitor, then the tubule develops with about half the normal number of cells.

The tip cell and its sibling are not equivalent: although they divide from a common progenitor cell, one receives more Numb protein than the other (Wan *et al.* 2000). As in neuronal development, Numb inhibits the action of Notch (Spana and Doe 1996), and so this cell becomes the tip cell. As predicted, in *numb* mutants, two nontip daughter cells differentiate, and in *numb*-overexpressing animals, two tip cells are generated (Wan *et al.* 2000). Interestingly, the final tubule cell number in both cases is wild type, suggesting that both the tip cell and its sibling are capable of secreting Spitz to trigger mitosis in the neighboring cells (Wan *et al.* 2000). However, despite equivalence in function in controlling mitosis, the tip cell and its sibling must both be present for the tubules to find their correct final positions in the body, and tend to remain clumped together (Ainsworth *et al.* 2000; Weavers and Skaer 2013, 2014). A similar loss-of-direction phenotype has been seen in mutants for *myoblast city*, which is homologous to *Caenorhabditis elegans CED-5*, which encodes a regulator of the small GTPase Rac, which directs the migration of the gonad within the body. There is thus a hierarchy of permissions to undergo mitosis, which helps to provide robustness in cell number and organization (Sudarsan *et al.* 2002). After division is complete, there are 144 ± 10 cells in the anterior pair of tubules, and 103 ± 8 in the posterior pair (Skaer and Martinez-Arias 1992).

Arrival of the stellate cells: By stage 13, division is complete. Meanwhile, a group of migratory caudal visceral mesoderm cells have set out on a journey, and arrive at the tubules, intercalate between the ectodermal cells, and undergo a mesenchymal-to-epithelial transition and characteristically express the nephrin ortholog *hibris* and the transcription factor *teashirt*, so establishing the stellate cell population (Figure 2B, stage 13) (Denholm *et al.* 2003; Campbell *et al.* 2010). The mature stellate cell is apicobasally polarized, and it takes its apicobasal cues from its neighboring principal cells (Campbell *et al.* 2010).

Elongation: By the end of division, the tubules are short and stubby. Between stages 13–16, they then undergo a phase of elongation by cell rearrangement through a convergent-extension process requiring multiple genes (Figure 2B, stages 13 and 16) (Jack and Myette 1999). This process of tubular elongation is seen in other systems, such as the salivary gland and trachea. In mutants for the Rho-GAP *crossveinless*, elongation fails completely (Denholm *et al.* 2005). Ribbon and Raw regulate cytoskeletal changes; myosin II (the heavy chain encoded by *zipper*) accumulates at the basolateral side of the tubule cells and causes that surface to produce pulsatile shortening, so causing cells to slide over one another, and producing a long, thin tubule (Saxena *et al.* 2014). Mutations in any of *ribbon*, *raw*, or *zipper* produce an elongation phenotype similar to *crossveinless*. The distal-to-proximal gradient of EGF signaling from the tip cell conveys the necessary planar polarity information without the involvement of traditional planar cell polarity genes (Saxena *et al.* 2014).

The mechanism of rearrangement is not completely clear; it must involve dissolution and reformation of cell junctions. Additionally an extracellular matrix has been deposited basolaterally by hemocytes in response to vascular endothelial growth factor/platelet-derived growth factor–related ligands from the tubule cells by the time of elongation, and there is evidence for lamellipodial ruffles in the cells as they move, suggesting a crawling mechanism (Bunt *et al.* 2010).

Organ positioning: The elongation process produces a tubule of the familiar shape, but it must also be positioned correctly in the body. The left tubules always ramify posteriorly and the right ones anteriorly, but this apparent left-right asymmetry is caused by a rotation of the gut: the tubules originate dorsoventrally, and when the gut rotates, the dorsal pair become the right-hand pair. As the anterior pair move forward, they develop a bend, or kink, approximately at the site of the future transitional segment, and this kink region draws the tubules toward the head (Bunt *et al.* 2010). This stereotyped movement depends on being able to read guidepost signals of TGF-β/Dpp, in turn from the dorsal epidermis, the midgut visceral mesoderm, and the gastric caeca; mutations in *dpp* or its receptor cause abnormal positioning (Bunt *et al.* 2010). Similarly, ectopic expression of *dpp* causes the tubules to misroute (Bunt *et al.* 2010). Meanwhile, the posterior tubule moves backward, and tubule positioning is complete when the tip cells of the anterior tubules have made contact with the alary muscles of the heart, and those of the posterior tubule with a hindgut visceral nerve (Denholm 2013; Weavers and Skaer 2013).

Development of functional competence and subsequent function: By the time the insect hatches, the tubules contain their first crystals of uric acid (Figure 3B). This is a metabolic byproduct of purine catabolism (Dow 2012), and so implies apicobasal polarity, with basal transporters for purines, correct assembly of the 13-subunit V-ATPase (Allan *et al.* 2005) on newly formed microvilli, and an apical transporter for urate. In mutants for any subunit of the plasma membrane isoform of the V-ATPase, the larvae fail to thrive, and lack of functional ATPase fails to acidify the lumen and so precipitate uric acid (Davies *et al.* 1996; Allan *et al.* 2005). This competence continues throughout larval life; however, in the pupae, the apical microvilli disappear and transport function is lost, only reappearing as the adult prepares to emerge (Halberg *et al.* 2016). The maintenance of the microvilli depends on the famous neuronal developmental gene and cell adhesion molecule, *fasciclin 2 (fas2)*: in *fas2* knockdowns, the microvilli are shorter, and in *fas2* overexpressors, they are longer. Transport function is proportional to microvillar length (Halberg *et al.* 2016). Critically and unusually, however, the cell numbers laid down in the embryo appear not to change throughout life (Sözen *et al.* 1997); although the tubules change shape somewhat, and physically grow throughout the life of the animal, this is by an increase in cell size, reflected by a steady increase in ploidy, and not by cell division. This is despite the presence of cells in the lower tubule identified as stem cells (Singh *et al.* 2007).

Stem cells occupy the lower tubule/ureter domains during metamorphosis. Although they are not thought to move further into the tubule, respecting the main segment/lower tubule boundary (Sözen *et al.* 1997), it is likely that they participate in the formation of the adult ureter. The nephritic stem cells derive from a population of adult midgut progenitor cells (AMPs) in the posterior midgut that move into the ureter during metamorphosis (Takashima *et al.* 2013), Overexpression of a dominant negative form of Rac1 in the AMPs causes the absence of nephritic stem cells in the ureter (Takashima *et al.* 2013). The future nephritic stem cells appear to be selected by a combination of a steep Wnt/Wg morphogen gradient, and a pulse of ecdysone hormone (Xu *et al.* 2018). The transcription factor GATAe is necessary for maintenance, differentiation and migration of intestinal stem cells (ISCs; Takashima *et al.* 2013); however, it shows enriched expression in tubules (Wang *et al.* 2004), and plays further roles. Knockdown of expression of the transcription factor *GATAe* in tubule principal cells caused a tumorous overproliferation phenotype, while knockdown in stellate cells affected physiological function (Martínez-Corrales *et al.* 2019). GATAe in stem cells is also necessary for correct migration to the ureter (Martínez-Corrales *et al.* 2019). Stem cell maintenance further requires the action of the transcription factor Shavenbaby, post-translationally modified by Polished rice, to activate Yorkie, an effector of the Hippo pathway, to prevent apoptosis (Bohère *et al.* 2018).

The anterior and posterior tubules are substantially similar in their physiology, but nonetheless show significant differences in their transcriptomes, perhaps reflecting the roles imposed by their differing location in the body (Chintapalli *et al.* 2012). For example, calcium handling is very much a function of the anterior tubules, perhaps reflecting the need to mop up excess calcium as it is taken up by the midgut (Chintapalli *et al.* 2012). The anterior tubules are also closely apposed to midgut neuroendocrine cells that contain neuropeptides to which the tubules are known to respond (Veenstra 2009).

The tubules also differ significantly between males and females, reflecting the different physiological demands placed upon them. For example, male and female tubules show distinct patterns of expression of antimicrobial peptide genes (Chintapalli *et al.* 2012).

Finally, although the tubule development has been told in terms of the two main cell types, it is important to note that enhancer trap mapping of domains in the tubule identifies six domains and at least six cell types (Sözen *et al.* 1997), suggesting that the development of this system is richer than we have identified to date. For example, the main length of the tubule can be divided into a secretory main segment and a reabsorptive lower tubule; stellate cells are only found in the former and tiny cells [the stem cells of Singh *et al.* (2007)] are only in the latter (Sözen *et al.* 1997), so this domain boundary must already be in place when the stellate cells arrive and intercalate.

Hindgut development

Nobel physicist Arthur Leonard Schawlow once remarked, "anything worth doing is worth doing twice." Hindgut development is exactly this way, as it is built during embryogenesis, then mostly destroyed during metamorphosis and remade from specialized imaginal progenitors. Both the larval and adult hindgut contain similar overall cellular organization and are organized into a pylorus, ileum, and rectum. We note here that much of the literature on the embryonic *Drosophila* hindgut instead refers to the pylorus as the small intestine and the ileum as the large intestine. Given that this terminology is not used in any other insect outside of *Drosophila*, is not commonly used in the adult hindgut literature, and the similarity of stem cell–based renewal in the *Drosophila* midgut and the human small intestine, we suggest that going forward only the terms pylorus, ileum, and rectum are used in the *Drosophila* hindgut field. We will use these more standard terms here for uniformity of discussion.

Embryogenesis: building the larval hindgut: Rudimentary gut structures appeared at the advent of multicellularity (Stainier 2005). A highly conserved feature of gut structures is the division into three major regions (foregut, midgut, and hindgut). In insects, the foregut and hindgut are ectodermally derived, while the midgut is endodermally derived. The *Drosophila* embryonic hindgut forms from a group of several hundred ectodermal cells in the posterior embryo, called the proctodeal primordium. These cells are specified by a well-defined cascade of gene expression changes downstream of the maternally supplied receptor tyrosine kinase Torso, which include transcriptional and cell signaling regulators (*e.g.*, the homeodomain transcription factor Caudal/Cdx, the transcription factor Forkhead/HNF-3, the T-box transcription factor Brachyenteron/Brachyury, and the signaling ligand Wg/Wnt) that play evolutionarily conserved roles in gut development from *C. elegans* to sea urchin to mouse (Weigel *et al.* 1989; St Johnston and Nüsslein-Volhard 1992; Kispert *et al.* 1994; Hoch and Pankratz 1996; Wu and Lengyel 1998; Iwaki and Lengyel 2002). The proctodeal primordium is internalized by involution after posterior midgut invagination during gastrulation (Figure 2A) (Harbecke and Janning 1989; Skaer 1993; Campos-Ortega and Hartenstein 1997). Involuted hindgut primordia do not undergo an epithelial to mesenchymal transition, but rather establish an apical/basal polarity while organizing into an epithelial hindgut tube (Skaer 1993). Initial lumen and hindgut tube expansion is regulated by the secreted glycoprotein Tenectin, which functions to stretch the tube wall (Syed *et al.* 2012). After embryonic germband extension, the hindgut epithelium begins to gradually associate with cells of the visceral mesoderm, which will later differentiate into the circular muscle fibers that surround the hindgut (Figure 2B, stages 12 and 13) (Bate 1993). Signaling from the visceral mesoderm to the epithelial cells of the ileum, carried out by the Slit/Roundabout (Robo) pathway, is critical for proper length of microvillar-like structures in the differentiating ileum epithelium (Soplop *et al.* 2012). Underscoring the opinion of noted developmental biologist Lewis Wolpert that gastrulation "is truly the most important time in your life" (Wolpert and Vicente 2015), following this event cells of the hindgut primordia have already found their position within the embryo and have initiated regional differentiation.

Once the primordia is internalized, the hindgut begins to resemble its mature larval form. Localized JAK/STAT signaling at the anterior hindgut is required for proper mediolateral cell elongation, which extends the newly formed tubular hindgut (Johansen *et al.* 2003a). Patterned gene expression differences in the anterior/posterior axis begin to form the pylorus, ileum, and rectum. Expression of cell signaling regulators is distinct between these hindgut regions in the embryo and have been reviewed previously (Skaer 1993; Lengyel and Iwaki 2002). Briefly, at the boundary of the midgut and hindgut, a ring of the anterior-most cells of the pylorus expresses the Wnt homolog *wg* (hereafter the Wg^{+} ring). This expression is maintained into the larva and adult (Takashima and Murakami 2001; Takashima *et al.* 2008; Fox and Spradling 2009; Sawyer *et al.* 2017; Tian *et al.* 2019). The rest of the pylorus expresses components of the JAK-Stat and Hedgehog (Hh) pathways, an expression pattern that again is seen in the adult hindgut (Takashima and Murakami 2001; Takashima *et al.* 2008). The ileum is enriched in expression of the homeodomain transcription factor *engrailed* and components of the Dpp and Notch pathways, while the rectum expresses components of the Hh and Notch pathways. Three transcriptional regulators: the zinc finger proteins Drumstick and Bowl and the nuclear protein Lines, control localization of such signaling regulators, and mutants in these three regulators disrupt regional hindgut patterning, especially in the pylorus and ileum (Iwaki *et al.* 2001; Green *et al.* 2002; Johansen *et al.* 2003b; Hatini *et al.* 2005; Uddin *et al.* 2011). The human *bowl* homolog ZKSCAN3 is a driver of colorectal cancer, suggesting possible conserved links in molecular regulation of the human/fly colon/hindgut that affect disease progression (Yang *et al.* 2008a,b). The larval hindgut ileum is the only portion of the *Drosophila* gut appreciated to exhibit dorsal/ventral patterning. The dorsal ($Engrailed^{+}$) and ventral (Notch ligand $Delta^{+}$) domains are separated by two rows of boundary cells, which exhibit distinct cell polarity regulation relative to neighboring enterocytes of the ileum (Kumichel and Knust 2014). Specification of the dorsal and ventral ileum and boundary cells is controlled by Notch signaling (Fuss and Hoch 2002; Iwaki and Lengyel 2002; Takashima *et al.* 2002), as well as two independent dorsal and ventral gene regulatory systems (Hamaguchi *et al.* 2012). The ileum also further differentiates from the pylorus and rectum by becoming the only embryonic hindgut region to initiate ploidy- and cell size–increasing endocycles. These cycles, which are programmed by Dpp signaling and transcriptional regulation from the zinc finger proteins Knirps and Knirps-like, expand the size of this gut region (Smith and Orr-Weaver 1991; Fuss *et al.* 2001).

Recent progress on the embryonic hindgut highlights its utility as a model of the newly appreciated role of cell chirality in development. As the hindgut elongates, it also undergoes a stereotypic dextral looping relative to the established embryonic anterior/posterior axis (Figure 2B, stage 16, Figure 2C, stages 16 and 17) (Hayashi *et al.* 2005). This looping reflects the acquisition of left/right (L/R) asymmetry. The *Drosophila* hindgut was the first system in which it was shown that chirality at the level of cells drives L/R asymmetry (Taniguchi *et al.* 2011). Just before rotation of the hindgut tube, hindgut epithelial cells exhibit L/R asymmetry in their apical surface, with leftward-tilting boundaries more frequent than rightward-tilting boundaries. Because the mirror three-dimensional image of these cells cannot be superimposed, this satisfies the definition of cell chirality (Inaki *et al.* 2018b). This rightward-tilting morphology is reflected in polarized localization of centrosomes, the adherens junction component DE-Cadherin, and the Rho GTPase guanine exchange factor Pebble (Taniguchi *et al.* 2011; Nakamura *et al.* 2013). Computer simulations, corroborated by live imaging, suggest this tilted morphology facilitates chiral sliding during hindgut looping (Inaki *et al.* 2018a). Critical to proper curvature of the hindgut is JAK/Stat signaling, which asymmetrically activates the cell adhesion molecule FasIII, which provides the appropriate level of tubular stiffness needed to achieve the proper hindgut tube curvature (Wells *et al.* 2013). Directionality of cell tilting, and therefore gut looping, is regulated by the class I myosin MyoID. *MyoID* mutants exhibit hindgut looping, but in the opposite direction. Given the colocalization of MyoID with the actin cytoskeleton in the hindgut, and the similarity of *MyoID* mutant phenotypes with dominant negative mutants in the actin-regulating Rho family GTPases Rho, Rac, and Cdc42, it is likely that the actin cytoskeleton plays a critical role in L/R hindgut asymmetry (Hozumi *et al.* 2006; Spéder *et al.* 2006). Additional cell chirality factors continue to be identified, including the transcriptional regulator Extra MacroChaetae and its binding partner Daughterless (Ishibashi *et al.* 2019). It will be interesting to determine whether unique segments of the hindgut drive looping. Another key question in this field regards what molecules establish the earliest cellular symmetry breaking events. One early cue appears to be the Hox gene *Abdominal-B (Abd-B)*. Abd-B binds to regulatory sequences of MyoID and controls its hindgut expression, and Abd-B mutants exhibit no symmetry breaking (Coutelis *et al.* 2013). Going forward, further study of hindgut looping hold promise to unravel the fascinating mechanisms of cell chirality.

Cellular chirality is also appreciated to play a key role in vertebrate development, and studies in both flies and vertebrates are likely to inform future work. Chick embryonic cardiac cells exhibit intrinsic cell chirality prior to looping, which ensures a dominant clockwise rotation. Like the *Drosophila* hindgut, these cells exhibit polarized Cadherin and Myosin molecules prior to cardiac looping (Ray *et al.* 2018). Further, it is known that L/R asymmetry in vertebrates is dictated by the floor plate, an analogous embryonic landmark to the *Drosophila* midline cells. Fly embryos mutant for the midline regulator *single minded* exhibit hindgut looping defects (Maeda *et al.* 2007). Future studies on this relatively newly appreciated yet clearly fundamental property will unveil new principles governing organ morphogenesis.

Metamorphosis: developmental hindgut regeneration: Holometabolic insect development frequently involves the programmed histolysis of larval intestinal organs and their reconstruction. These events take place during metamorphosis (Robertson 1936). The *Drosophila* hindgut epithelium undergoes such whole-scale organ remodeling, but in a manner completely different from the neighboring midgut epithelium. The midgut is remodeled by dispersed islands of AMPs (Jiang and Edgar 2009; Mathur *et al.* 2010), whereas adult hindgut progenitors reside at the far ends of the organ, both anterior and posterior. Cells of the larval pylorus and larval rectum are the only epithelial cells to survive metamorphosis (Figure 2D). These two regions are the source of progenitors of the adult hindgut epithelium, while the larval ileum and anal pads do not persist into adulthood. The overlying hindgut musculature persists during this epithelial remodeling.

The larval pylorus expands significantly in cell number between hatching and metamorphosis (Takashima *et al.* 2008; Fox and Spradling 2009; Yang and Deng 2018). The initial phase of these divisions are under the control of Notch signaling (Yang and Deng 2018). The larval pylorus is the source of both the adult pylorus (which expands further in cell number during metamorphosis), as well as the adult ileum. While the pylorus remains diploid, the adult ileum cells eventually endocycle to reach a ploidy of 8C (Fox and Spradling 2009). Wg and Hh signaling are required during metamorphosis for proper adult hindgut cell number and morphology (Takashima *et al.* 2008), as is mitochondrial fusion, mediated by conserved fusion regulators Opa1 and MARF (Deng *et al.* 2018). MyoID again controls establishment of L/R asymmetry and looping of the adult hindgut, with the atypical cadherin Dachsous playing an important role in oriented hindgut cell polarity in this process during metamorphosis (González-Morales *et al.* 2015). Also during metamorphosis, the pylorus remains in contact with the remodeling midgut. Long-range Wg signaling at the midgut/hindgut border, which acts in part through Dpp signal activation, is important for epithelial cell fate establishment, proliferation control, and proper muscle architecture (Sawyer *et al.* 2017; Tian *et al.* 2019). Disruption of long-range Wg signaling during adult hindgut development disrupts a signature fold in the intestine at the midgut/hindgut border (Tian *et al.* 2019). Gene expression at the midgut/hindgut border is also highly dynamic during metamorphosis, with some cells at the border exhibiting gene expression markers that are normally specific to only one of the two organs. Currently, it is unclear whether this dual marker expression reflects the *trans*-differentiation of some hindgut cells into midgut cells, or whether cells originally expressing only hindgut markers transiently adopt a hybrid midgut/hindgut gene expression pattern (Takashima *et al.* 2013; Sawyer *et al.* 2017). As the new adult pylorus and ileum emerge from anterior proliferation in the pylorus, macrophages appear to engulf the dying larval ileum (Aghajanian *et al.* 2016). During this whole-scale remodeling of the hindgut epithelium, the overlying visceral musculature remains intact, leaving a sleeve-like scaffold within which the newly forming adult hindgut epithelium develops. Ablation of the visceral muscle disrupts the removal of the larval hindgut and construction of the adult hindgut, underscoring important muscle-epithelium cross-talk during this whole-scale organ remodeling event (Aghajanian *et al.* 2016).

In parallel to pylorus and ileum development, during metamorphosis the rectum is also undergoing significant remodeling. Previously, it was suggested that the adult rectal papillae are derived from the genital disc, which lies just posterior to the larval rectum (Robertson 1936; Skaer 1993). However, it was subsequently shown larval rectal cells undergo Notch-dependent remodeling into adult papillae during metamorphosis (Fox *et al.* 2010). Further, lineage tracing with a genital disc promoter showed that these cells do not contribute to the adult papillae, but instead form the outer rectal sac, which envelopes the forming papillae (Fox *et al.* 2010). Rectal papillar precursors (larval rectal cells) undergo a highly distinctive cell-cycle program. During second larval instar, these cells undergo a variant of endocycle known as a premitotic endocycle (Schoenfelder *et al.* 2014). This endocycle variant differs from that of many endocycling tissues as it involves retention of centrosomes and initiation of late-S phase sequences (Mahowald *et al.* 1979; Fox *et al.* 2010; Nordman and Orr-Weaver 2012; Schoenfelder *et al.* 2014). During metamorphosis, the now octoploid rectal cells undergo two rounds of polyploid mitosis. This is currently the only known case where such divisions occur completely in flies, although subperineurial glia of the larval brain initiate polyploid divisions but fail cytokinesis (Unhavaithaya and Orr-Weaver 2012). Rectal papillar cell division requires elimination of polytene chromosome structure, which is a barrier to proper cell division. Polytene separation occurs in a process known as Separation Into Recent Sisters, or SIRS (Stormo and Fox 2016). To prepare for SIRS, papillar cells transiently eliminate cohesins between sister chromatids during each round of the premitotic endocycle (Stormo and Fox 2019). SIRS-like processes are also described in the placenta of some mammals, as well as in specific tumors or cells treated with antimitotic chemotherapeutic agents (Levan and Hauschka 1953; Zybina and Zybina 1996; Sumner 1998). While it may seem laborious for papillar cells to build up polytene chromosomes only to then separate them later, endocycles and polyploid mitosis are absolutely essential for rectal papillar development and tolerance of a high-salt diet in adult flies, underscoring the importance of papillar cell cycles to adult hindgut physiology (Schoenfelder *et al.* 2014).

The adult hindgut: no constitutive or injury-induced intestinal stem cells: When the midgut was shown to contain adult stem cells (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006), it seemed possible that the neighboring hindgut also contained such proliferating cells. Proof of adult stem cell activity requires lineage marking techniques which demonstrate the output of a single cell during adulthood. Leakiness of clonal marking, which is a common technical pitfall of lineage marking approaches (Fox *et al.* 2008), led to the initial claim that the adult pylorus of the hindgut contains constitutive adult stem cells that repopulate the entire pylorus and ileum during adulthood (Takashima *et al.* 2008). However, using nonleaky labeling systems, multiple groups showed that there is no evidence of constitutive stem cell activity in the adult hindgut (Fox and Spradling 2009; Fernández-Hernández *et al.* 2013). Apoptotic injury to the hindgut did induce mitotic activity in a region near the midgut/hindgut border (Fox and Spradling 2009), but a definitive lineage experiment remained to be performed to determine if the adult hindgut contained reserve injury-induced stem cell activity. When this experiment was performed, along with a high-resolution analysis of the cell population at the midgut/hindgut border, it was shown that hindgut injury does induce cell division, but not in the hindgut. Instead, neighboring midgut organ boundary intestinal stem cells (OB-ISCs) are induced to divide following hindgut injury (Sawyer *et al.* 2017). It is now clear that the adult *Drosophila* hindgut contains no stem cells and no proliferative cells in either the uninjured or injured state. Therefore, the term "hindgut proliferation zone/HPZ" can and should only be used in reference to hindgut development, and the term "hindgut intestinal stem cells/ISCs" should not be used. However, as discussed next, the hindgut is a valuable model for a stem cell alternative repair process that is now appreciated to occur frequently throughout nature, including in mammals.

Modeling Disease Processes

Modeling renal disease in the Malpighian tubules

Although there are significant differences in the origin and function of Malpighian tubules and the mammalian nephron, it is still possible to model a range of renal diseases in *Drosophila*. This is because the two systems are functionally analogous; they both generate and process a primary urine, facilitating the maintenance of ionic and osmotic homeostasis, while allowing the excretion of waste compounds. Additionally, there tends to be close sequence homology between many *Drosophila* renal-enriched genes and their human orthologs, because there are simply not many ways to build a transport ATPase, exchanger, or channel.

Diseases of metabolism: One simple way to investigate plausible models of renal disease is to sort human renal disease genes for enriched expression in Malpighian tubules (Wang *et al.* 2004; Chintapalli *et al.* 2007). Conspicuous in such lists is the gene for xanthine oxidase/dehydrogenase (XO), a single-copy gene in both humans and flies, which when mutated in humans causes the inborn error of metabolism, xanthinuria type I (Dent and Philpot 1954; Ichida *et al.* 1997). Xanthine oxidation is a necessary step in the catabolic pathway for purines toward urate, allantoin and urea, and nulls for XO cause the build-up of such high levels of hypoxanthine and xanthine that it crystallizes in the kidney, forming stones. The fly homolog is *rosy*, the second *Drosophila* mutant to be described (after *white*). Remarkably, the same phenotype is observed in Malpighian tubules; they become bloated as the lower tubules are blocked with orange concretions, and the null is considered semilethal (Glassman and Mitchell 1959; Mitchell and Glassman 1959). Recent metabolomic analysis of *rosy* mutants shows significant changes up to five metabolites away from the metabolic lesion, with large increases in levels of hypoxanthine and xanthine, and undetectable levels of the downstream metabolite, uric acid (Figure 3A) (Hobani *et al.* 2009). This finding offers the possibility of more detailed study, for example by pharmacology. Although XO causes a loss of uric acid, metabolic excess of urate causes ectopic crystals to form in the joints, a painful condition known as gout. Although most cases are idiopathic, there can also be genetic causes (Kelley *et al.* 1967; Curto *et al.* 1998). Gout is treated with a simpler diet (to lower purines) and with allopurinol, which phenocopies the XO mutation by blocking the XO enzyme. Allopurinol indeed has the corresponding action in *Drosophila*; addition to the diet increases xanthine and hypoxanthine, and decreases urate and allantoin (Al Bratty *et al.* 2011). A number of quantitative trait loci associated with gout have been identified in humans(Cheng *et al.* 2004; Li *et al.* 2007; Cummings *et al.* 2010; Matsuo *et al.* 2011; Lee *et al.* 2019), and it will also be interesting to see whether *Drosophila* orthologs of these genes also play a role in maintaining fly urate levels.

XO is one of a family of molybdoenzymes (including aldehyde oxidase and sulfite oxidase) that depend on a molybdenum-containing prosthetic group (Kamdar *et al.* 1994). It could be predicted that upstream genes in this synthetic pathway would also produce xanthinuria-like symptoms, but would have a more severe phenotype because other molybdoenzymes would also be affected. This is exactly what is found: in humans, mutation of the upstream gene molybdenum cofactor sulfurase produces xanthine stones, but as a part of a more widespread disease, xanthinuria type II (Ichida *et al.* 2001; Zannolli *et al.* 2003). This disease is also a problem in cattle herds (Watanabe *et al.* 2000). The corresponding *Drosophila* gene *maroon-like* also causes renal defects and rosy-like eyes (Mitchell and Glassman 1959), and metabolomics confirms a similar metabolic disruption (Kamleh *et al.* 2009).

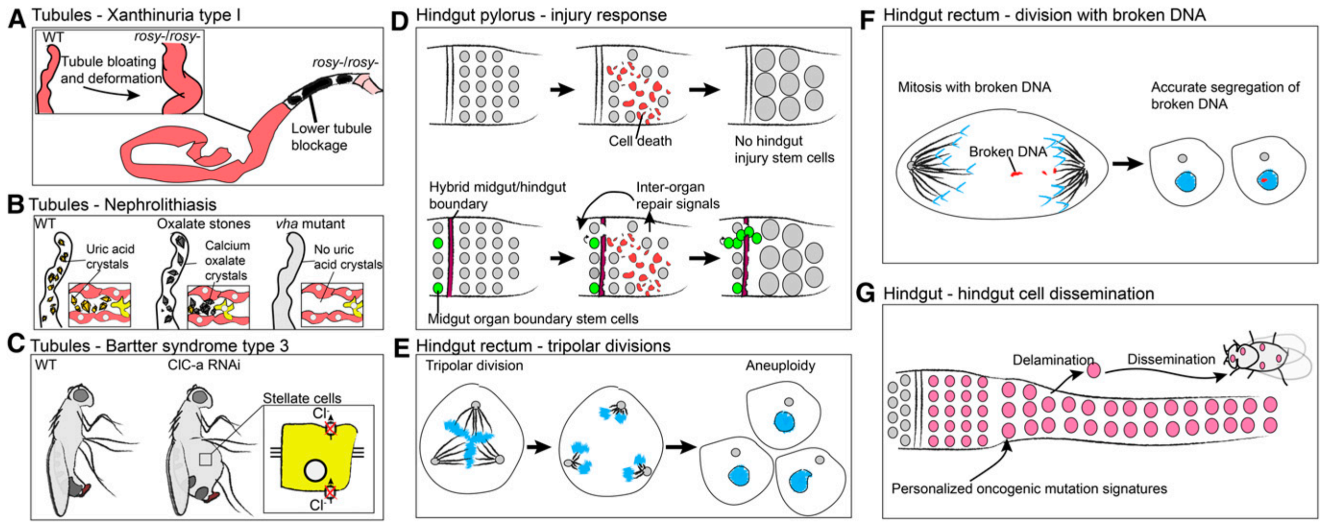

Figure 3 Examples of human disease process modeling in the Malpighian tubules and hindgut. (A) *rosy* mutants enable modeling of the disease Xanthinuria type I in the Malpighian tubules. (B) Feeding oxalate rich media or examining mutants in vacuolar ATPase genes (*vha* mutant) enable modeling of excessive or absent renal crystal structures in the Malpighian tubules. (C) RNA interference (RNAi) of the *Clc-a* gene cripples chloride transport in the Malpighian tubule stellate cells, enabling modeling of Bartter syndrome type III. (D) Adult hindgut epithelial injury enables modeling of tissue injury repair by compensatory hypertrophy. Additionally, the midgut/hindgut boundary facilitates modeling of the role of interorgan signaling responses. (E) The division of pupal hindgut rectal cells enables study of tripolar divisions and resulting aneuploidy. (F) Division of pupal hindgut rectal cells also enables study of mitosis with persistent DNA damage. (G) Expression of personalized oncogenic mutation signatures can mimic cancer cell dissemination in the hindgut. WT, wild type.

Xanthinuria is one example of a set of human diseases called inborn errors of metabolism (IEMs). These diseases typically show Mendelian recessive inheritance, and are overwhelmingly seen in populations where consanguineous marriage is practiced (Milne 1970; Saadallah and Rashed 2007; Tadmouri *et al.* 2009; Al-Gazali and Ali 2010). It is not unusual for such diseases to have renal sequelae, as a defective enzyme will lead to hyperaccumulation of its substrate, and the kidney may struggle to excrete it. Nephrolithiasis is thus a common finding in IEMs (Milne 1970; Cochat *et al.* 2010). Given that 70% of genes are conserved between fly and human, there is the possibility that several of these diseases could be modeled using the fly tubule (Dow and Romero 2010).

Nephrolithiasis: Nephrolithiasis (stones in the kidney) and urolithiasis (stones in the urinary tract) are serious and painful conditions, responsible for 250,000 emergency room admissions annually in the United States alone. Although some of these cases can be attributed to rare IEMs, most are idiopathic, and the most common form of stone is of calcium oxalate (Figure 3B) (Ramello *et al.* 2000; Worcester and Coe 2008; Gisselman *et al.* 2009; Shoag *et al.* 2015). Treatment of oxalate stones is relatively crude and of limited effectiveness, because of the lack of good animal models. However, oxalate stones can be modeled easily and reproducibly in flies, simply by supplementing the diet with oxalate; birefringent crystals of oxalate can be seen to form within a day (Dow and Romero 2010; Miller *et al.* 2013). Although this model initially met with some resistance (Knauf and Preisig 2011), its appropriateness has been borne out by further studies. Contamination of food with either ethylene glycol (Lyon *et al.* 1966; Hebert *et al.* 1983; Hanif *et al.* 1995; Besenhofer *et al.* 2011) or melamine (Brown *et al.* 2007; Guan *et al.* 2009; Hocking 2009) can produce catastrophic renal sequelae in humans, pets, or livestock. Both of these compounds also trigger the formation of stones in *Drosophila* tubule (Chen *et al.* 2011, 2012). One of the limited treatments available for humans is consumption of citrate, as metal citrate salts tend to be highly soluble. Administration of citrate (Chen *et al.* 2011; Ho *et al.* 2013), thiosulfate, or sulfate (Landry *et al.* 2016) in *Drosophila* similarly reduces stone burden and extends lifespan.

These similarities in stone causation and treatment offer the possibility that *Drosophila* tubules could be used for chemical screens to identify inhibitors of stone formation—something that would be almost impossible in humans or mammalian models. Such screens are underway and have already identified natural compounds that appear effective in treating lithiasis (S. Y. Wu *et al.* 2014; Ali *et al.* 2018; Yang *et al.* 2018). Similarly, genetic screens have the potential to identify new genes that could cause nephrolithiasis in humans. In one such case, the product of the *Drosophila* gene *prestin* was shown to transport oxalate in tubules, and knockdown of *prestin* expression in the tubules reduced stone formation (Hirata *et al.* 2010, 2012). In another study, *Drosophila* mutants of the gene *NHERF/Sip1* were found to carry a massive stone burden of uric acid crystals (Ghimire *et al.* 2019). The loss of naturally occurring uric acid crystals was also used as a screen to identify those V-ATPase subunits that formed the plasma membrane proton pump in *Drosophila* tubule principal cells (Allan *et al.* 2005).

Zinc is found in many kidney stones in humans, implying a role in nucleation or early growth (Negri 2018). Zinc has also long been known to be present in the concretions found in *Drosophila* tubules (Zierold and Wessing 1990; Schofield *et al.* 1997). Now that the similarity with human stones is apparent (Chi *et al.* 2015; Dow 2017), the genetic tools available in *Drosophila* can be applied to identify further genes that might be associated with risk for stone formation (Yin *et al.* 2017; Tejeda-Guzmán *et al.* 2018). As a result of these advances across multiple classes of stones, the utility of the *Drosophila* approach in the study of lithiasis is becoming widely accepted (Miller *et al.* 2013; Sayer 2017).

Diseases of ion transport: The tubule is energized by an apical plasma membrane V-ATPase, a massive 300 kDa assembly of at least 13 subunits (Allan *et al.* 2005). In humans, plasma membrane V-ATPase isoforms are found in the intercalated cells of the collecting duct (Breton and Brown 2013). Mutations in different V-ATPase subunits can thus cause distal renal tubular acidosis or sensorineural deafness, or both, depending on the subunit (Karet *et al.* 1999; Stover *et al.* 2002). In *Drosophila* tubules, failure to clear acid from the hemolymph in *vha55* mutants corresponds to failure to acidify the tubule lumen sufficiently to precipitate uric acid crystals from transported urate (Figure 3B) (Davies *et al.* 1996). This telltale sign is found with mutants for genes for all other subunits in the tubule, but not for those expressed elsewhere (Allan *et al.* 2005).

Bartter syndrome is the umbrella term for a group of diseases that have impaired salt resorption in the thick ascending loop of Henle. The $Na^+/K^+/2Cl^-$ cotransporter (SCL12A1) is mutated in antenatal Bartter syndrome type I (Simon *et al.* 1996). The *Drosophila* ortholog is found in Malpighian tubules, and knockdown compromises tubule secretion (Rodan *et al.* 2012). Rescue with wild-type $Na^+/K^+/2Cl^-$ restores function (Rodan *et al.* 2012).

Antenatal Bartter syndrome type II is caused by homozygous or compound heterozygous mutations in the ROMK/KCNJ1 inwardly rectifying potassium channel (Finer *et al.* 2003). There are three highly similar inward rectifier genes in *Drosophila*, *ir*, *irk2*, and *irk3*, of which *ir* is the most closely similar to ROMK; all three are highly expressed in *Drosophila* tubules (Wang *et al.* 2004; Evans *et al.* 2005). Tubules are highly sensitive to sulfonylureas and barium (Evans *et al.* 2005), and RNA interference knockdowns showed that Ir and Irk2 carried a significant fraction of transported K^+; together with the basolateral Na^+/K^+ ATPase, they accounted for 75% of flux (Y. Wu *et al.* 2015).

Similarly, classical Bartter syndrome (type III) is associated with mutations in the CLCNKB gene, encoding the CLCK-b kidney epithelial chloride channel (Simon *et al.* 1997). Of the three CLC chloride channel genes in *Drosophila*, the most similar to CLCNKB is *Clc-a*, which is also the most highly expressed in tubules (Cabrero *et al.* 2014). RNA interference knockdown of this gene in just the stellate cells of the tubule (using the GAL4/UAS system), completely abolished hormone-stimulated fluid secretion, confirming its essential role in *Drosophila* renal function (Figure 3C) (Cabrero *et al.* 2014). Overall, it can be seen that key players in ion transport in the mammalian kidney are frequently highly conserved in *Drosophila*, show enriched expression in Malpighian tubules, and can be seen to play key roles in tubule function.

Continuing challenges in modeling human disease: The major challenge for clinical nephrology is chronic kidney disease, which progresses until, in end-stage kidney disease, only dialysis or transplant can help (Tonelli *et al.* 2006; Murtagh *et al.* 2007). There are multiple causes for progressive kidney failure; glomerular defects may be usefully modeled with the *Drosophila* nephrocyte, as discussed elsewhere (Weavers *et al.* 2009; Helmstädter and Simons 2017).

One of the most common genetic causes of renal failure is polycystic kidney disease (PKD), in which a progressive accumulation of fluid filled cysts compromises kidney function (Harris and Torres 2009). Symptoms are highly variable, from neonatal death to minimal kidney dysfunction in adult. In autosomal dominant PKD, the most common form of PKD, mutations are commonly found in two genes: polycystin 1, a large transmembrane protein associated with primary cilia; and polycystin 2, a TRP-family channel. The orthodoxy is thus that defects in the primary cilium of renal cells confers problems with apicobasal polarity (Yoder 2007; Dell 2015), although the subtlety and complexity of the connection between cilia and PKD is only now becoming clear (Dell 2015). Unfortunately, while *Drosophila* has a gene similar to Pkd2, it lacks primary cilia in most cells, so a direct renal model is not available. However, in humans PC1 and PC2 are thought to form a mechanosensory channel, which acts through intracellular calcium to modulate Wnt, JAK/STAT, and TOR pathways, which do exist in *Drosophila*. It may thus be possible to model some aspects of the disease in flies. Indeed, mutants in *Bicaudal C*, the homolog of a human gene BICC1 implicated in cystogenesis, develop cyst-like swellings in Malpighian tubules, accompanied by activation of the TOR pathway (Gamberi *et al.* 2017). Inhibition of the TOR pathway by rapamycin ameliorated the cyst-like symptoms (Gamberi *et al.* 2017).

As an alternative approach, outside the tubule, it is possible to study ciliary dysfunction in cell types where they are present, such as sperm. The sperm flagella is effectively a primary cilium, and contains Pkd2 protein at its distal tip; mutants of this gene have reduced fertility (Watnick *et al.* 2003). Thus, *Drosophila* may be able to offer insight into the most common kidney disease.

Modeling injury repair and cancer initiation in the hindgut

Hindgut injury and repair: whole-scale organ regeneration and repair by polyploidy: Tissues of the digestive and excretory systems are bombarded with external stresses such as ingested pathogens, alterations in microbiota, and accumulation of free oxygen radicals. These insults render such tissues prone to injury and cell loss. As a result, digestive and excretory tissues frequently activate tissue injury repair responses to maintain and restore organ function. However, there is a diversity in tissue injury responses. For example, in the intestine of several mammals, stem cell divisions are active during repair. In contrast, in the liver, some modes of injury trigger few cell divisions, but instead activate ploidy and cell size increasing hypertrophy events (Poccia 1986; Miyaoka *et al.* 2012; Gentric *et al.* 2015). It remains to be determined how distinct cell types and organs activate different injury responses. In this regard, the hindgut has emerged as a model of how an organ is programmed to undergo distinct and evolutionarily conserved injury repair responses.

As discussed in the physiology section, in the hindgut of several insects, scarring is caused by persistent pathogen infection or compromised immune responses, as well as by alterations in cell cycling or signaling. This scarring (as indicated by accumulation of the pigment melanin) occurs specifically in the epithelium of the adult pylorus (Heimpel and Angus 1960; Reed and Orr-Weaver 1997; Takashima *et al.* 2008; Berliner 2009; Pan and Jin 2014). Over the past decade, the acute sensitivity of the hindgut pyloric epithelium to injury has been studied further, providing an accessible model for studying evolutionarily conserved injury responses. These include tissue injury responses involving cellular ploidy and size increases, as well as responses occurring at organ boundaries.

As discussed in the adult hindgut section, the *Drosophila* hindgut does not contain injury responsive stem cells. In response to injury by apoptotic gene expression (*head involution defective*, *reaper*) or toxin induction (ricin, dithiothreitol), cells of the adult pylorus leave a quiescent state and enter S phase (Fox and Spradling 2009; Sawyer *et al.* 2017; Cohen *et al.* 2018). Rather than activating a stem cell response following injury, the pyloric region of the hindgut instead provides an excellent model of a tissue injury repair response that, in recent years, has been reported in numerous tissues in both flies and mammals. This response does not involve repair by cell division and creation of new cells, but instead involves cell and genome enlargement of cells that remain following injury (Figure 3D) (Fox and Spradling 2009; Losick *et al.* 2013; Sawyer *et al.* 2017; Cohen *et al.* 2018). These processes are known as wound induced polyploidization and compensatory cell proliferation (Losick *et al.* 2013; Tamori and Deng 2014). Similar to the pyloric injury response, both hypertrophy and polyploidization have been observed in other *Drosophila* tissues (Tamori and Deng 2013; Losick *et al.* 2016), as well as in the mammalian kidney, liver, bladder, and cornea (Ikebe *et al.* 1988; Duncan 2013; Losick *et al.* 2016; Lazzeri *et al.* 2018; J. Wang *et al.* 2018). The hypertrophic hindgut injury response is not an aberrant response to tissue injury. Rather, it is highly tunable to the level of injury induced in the adult hindgut (Cohen *et al.* 2018). With increasing severity of injury, cells undergo proportional rounds of endocycles and polyploidization. The ability to regulate entry into the endocycle and return to quiescence following recovery implicates a tightly regulated compensatory response, similar to that observed in regenerating tissues (Guo *et al.* 2013; Ayyaz *et al.* 2015).

In contrast to the adult pylorus, larval pyloric cells do not undergo endocycles but instead undergo compensatory proliferation/mitosis in response to injury. Ablation of up to 75% of larval pyloric cells drives additional rounds of mitotic cell cycles during larval/pupal development. The pylorus, which acts as the imaginal ring of the adult hindgut, thus possesses similar regenerative activity as imaginal discs (Hadorn *et al.* 1949; Ursprung 1959; Hadorn and Buck 1962; Schubiger 1971; Haynie and Bryant 1977; Smith-Bolton *et al.* 2009; Bergantiños *et al.* 2010). As pyloric cells are maintained throughout metamorphosis (Fox and Spradling 2009; Aghajanian *et al.* 2016; Sawyer *et al.* 2017; Cohen *et al.* 2018), the ability of this tissue to first regenerate and then later switch to repair through endocycles establishes the pylorus as a model for studying how a single-cell population alters its injury responses across development. During pupation, the pylorus begins to express a negative regulator of mitotic cyclins, *fizzy-related*. Fizzy-related is an activator of the anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase. APC/C^{fzr} has been previously implicated in developmentally programmed mitotic to endocycle switches (Nakayama *et al.* 1997; Deng *et al.* 2001). Following its expression, *fizzy-related* is required in the pylorus for the developmentally programmed switch from injury-mediated cell division to injury-mediated endocycles. The identification of *fizzy-related* as a regulator of the switch between injury repair programs in the pylorus enabled study of the purpose of such a switch. In injured *fizzy-related* adult animals, the pylorus is capable of complete regeneration of the tissue through cell division instead of endocycles. However, this more regenerative mode of tissue injury repair in *fizzy-related* animals causes problems under conditions of chronic injury (driven by constitutive growth signaling through activation of the Ras pathway). Under these conditions, *fizzy-related*, but not wild-type animals, become susceptible to epithelial malformations and barrier leakage (Cohen *et al.* 2018). This finding suggests that regeneration may not always be the ideal outcome in injured tissues. Future studies focusing on the effects of restoring regenerative capacity to the hindgut pylorus responses in both chronic and acute injury conditions can provide insights into the potential benefits of nonregenerative responses.

While injury to the pylorus does not drive cell division anywhere in the adult hindgut, its close proximity to the highly regenerative midgut presented a model to study how tissue injury responses are regulated in the complex environment of organ boundaries. Injury to the adult hindgut pyloric epithelium triggers a mitotic response in the adjacent midgut OB-ISCs (Sawyer *et al.* 2017) These ISCs reside 0–30 μm from the adult hindgut Wg$^+$ ring, and are distinguished by both expression of the Wg effector *frizzled3* and a lower rate of proliferation than immediately anterior ISCs (Tian *et al.*

2016, 2019; Sawyer *et al.* 2017). Injury to the adult pylorus (including the Wg$^+$ ring) induces hindgut expression of Upd3 cytokines of the JAK-Stat pathway. These cytokines nonautonomously promote increased proliferation of OB-ISCs (Figure 3D) (Sawyer *et al.* 2017). These findings underscore how study of injury to one organ can affect a physically adjacent organ.

The distinct function of OB-ISCs awaits further investigation. Lineage analysis suggests these cells may possess the ability to repopulate the hindgut Wg$^+$ ring (Sawyer *et al.* 2017), which contains cells of both midgut ISC daughter cell and hindgut pyloric cell gene expression, and were thus termed a "hybrid zone" (HZ; Figure 3D). Interestingly, similar HZ populations were recently described as sites at risk for cancer progression in the mammalian gut (at the stomach-esophagus border; Jiang *et al.* 2017) and *Drosophila* salivary gland imaginal ring (similar anatomy and function as the hindgut Wg$^+$ ring (Yang *et al.* 2019), and were also identified as playing important roles in rib injury repair in the mouse skeletal system (Kuwahara *et al.* 2019). Under uninjured conditions, the HZ may repress OB-ISC division, as severe HZ injury causes extreme OB-ISC hyperplasia and causes OB-ISCs to cross the midgut/hindgut boundary. Similar cross-tissue injury responses appear to occur in other animals following both injury (Joseph *et al.* 2018) and disease (Badreddine and Wang 2010; Hvid-Jensen *et al.* 2011). Going forward, the midgut/hindgut boundary provides an accessible model to study how tissues respond to injury across organ boundaries, and how disruption of such responses may influence disease progression.

Different hindgut segments respond differentially to injury. The larval ileum is resistant to cell death induced by myriad stressors, including salt, SDS, oxidative stress, UV exposure, heavy metals, and cold exposure (Seisenbacher *et al.* 2011; MacMillan *et al.* 2017). While inhibition of JNK-mediated apoptosis has been implicated in the resistance to chronic salt stress and SDS feeding, the mechanisms conferring resistance to additional stressors of the larval ileum remains unknown. It is also unclear if these resistance mechanisms are maintained in the adult ileum following histolysis and remaking of the ileum by the injury-prone pyloric cells. Overall, with new genetic tools to induce injury in the hindgut and its different compartments, the *Drosophila* hindgut has become a useful model to study nonstem-cell injury responses, injury across tissue boundaries, as well as stress and apoptotic resistance.

Cancer: the hindgut as a model for its initiation and a tool for drug discovery: Crucial events in tumor initiation are elevated rates of genetic change (genomic instability) and cell dissemination. The *Drosophila* hindgut provides an accessible model to follow these processes as they arise *in vivo*. Further, the genetic accessibility of flies and their amenability to large-scale *in vivo* drug screening enable discovery of critical molecular mechanisms that drive these tumor progression properties, as well as potential drug interventions.

Genomic instability has many sources. One source that is highly relevant to cancer is whole-genome duplication, or polyploidy. Polyploidy is a driver of elevated genomic instability in numerous contexts, where it promotes unfaithful cell divisions through several mechanisms (Davoli and de Lange 2011; Fox and Duronio 2013; Storchova 2014; Tanaka *et al.* 2018). Further, polyploidy is thought to be the underlying cause of roughly one-third of altered karyotypes in human cancers (Carter *et al.* 2012; Zack *et al.* 2013; Bielski *et al.* 2018). As discussed in the hindgut development section, hindgut rectal papillar cells naturally acquire polyploidy and then undergo mitotic division. As in other cases of polyploid divisions, papillar divisions are highly error-prone, even during wild-type fly development (Fox *et al.* 2010). Similar chromosome segregation errors are seen in dividing polyploid subperineurial glia nuclei (Unhavaithaya and Orr-Weaver 2012). In papillar cells, one source of mitotic errors is centrosome amplification. Such amplification causes multipolar spindle formation, which frequently causes tripolar divisions, a known source of chromosomal imbalances (aneuploidy). Although aneuploidy is detrimental in many cases (Santaguida and Amon 2015), experimentally increasing papillar tripolar division has no detectable effect on papillar development or ion balance physiology, and papillar cells from these animals do not form noticeable tumors (Figure 3E) (Schoenfelder *et al.* 2014). Future work can determine whether papillar cells have evolved a mechanism to counteract the numerous disease-promoting properties of aneuploid cells. A second source of papillar mitotic errors is chromosome breakage (Fox *et al.* 2010; Bretscher and Fox 2016). Papillar cells lack canonical apoptotic and cell-cycle arrest responses to DNA breaks, and as a result these breaks persist into mitosis. However, during mitosis, broken chromosome fragments lacking microtubule attachment sites still manage to segregate, in a process that depends on the conserved Fanconi anemia family DNA repair proteins. Fanconi anemia protein–deficient animals fail to segregate chromosome fragments, which end up in micronuclei—highly detrimental cytosolic DNA structures attributed to cancer progression and cell death (Figure 3F) (Bretscher and Fox 2016). These studies demonstrate that papillae are a model to understand the genesis of polyploid genomic instability, as well as a model for how nature evolved to sidestep the negative aspects of this cancer promoting cellular property.

Cell dissemination from a primary tumor site is a critical step in cancer progression. In the intestine of flies expressing oncogenic active mutations in the Ras GTPase, cells from an anterior location of the hindgut, but not the midgut, are prone to dissemination into distant sites in the body (Figure 3G). This dissemination is enhanced by the presence of pathogenic bacteria and requires innate immune signaling from the Imd pathway (Bangi *et al.* 2012). As genetic factors driving cancer properties such as dissemination are often multigenic, this model was extended to include additional cancer driver mutations. Using the dissemination phenotype as a readout, it was shown that specific multigenic mutation combinations found in human cancers cause common cellular cancer phenotypes, including hyperproliferation, epithelial multilayering, and apoptotic evasion. Tumors in the hindguts of these animals also exhibit drug resistance, which enabled screening in the *Drosophila* hindgut to identify mechanisms of resistance to currently used therapeutics and to devise new combination therapies (Bangi *et al.* 2016). Most recently, this model was used to derive personal colon cancer therapies. After sequencing a patient's tumor, nine cancer driver mutations were targeted in the hindgut using either overexpression or small interfering RNA transgenes. Following this, a robotics-based, high-throughput screening of 121 drugs was performed for compounds that suppressed tumor-like phenotypes originating from the hindgut. Excitingly, the top-performing drug combination from this *Drosophila* screen led to a significant antitumor response in the original patient (Bangi *et al.* 2019). These studies underscore the utility of the *Drosophila* hindgut in directly contributing to advances in human disease therapy.

Summary and Future Outlook

Here, we have summarized advances in our understanding of the *Drosophila* excretory system, which has especially highlighted an accelerated pace of discovery in the last decade. Insights and experimental tools from study of the now well-understood development of the tubules and hindgut can continue to be applied to the less well-understood area of physiology. Numerous aspects of excretion, which are widely conserved in metazoans, can be accessibly modeled in *Drosophila*. Further, improved understanding of both developmental and physiological processes can promote further use of these tissues as models of human disease conditions.

Both the tubule and hindgut contact the posterior midgut, which has seen an explosion of recent interest in the *Drosophila* field. As all three of these organs work (and likely signal) together to regulate intestinal physiology, we argue that the function of each distinct organ should always be considered when studying the other. Further, interesting variants of midgut biology are found in the excretory system as well. For example, just as the adult midgut epithelium is attractive for its ability to model the role of stem cells in tissue homeostasis and repair, numerous tissues from insects to humans engage in the hindgut version of nonstem cell tissue repair through ploidy increases. Additionally, just as the midgut provides a model for stem cell–associated disease, modeling of renal conditions such as kidney stones and the complex cancer landscape of colorectal genomes is possible in the *Drosophila* excretory system. In addition to highlighting the work of those already using the tubules and hindgut as model organs, we hope that this review can serve as an introductory guide for those interested in joining this field of study. There is no shortage of interesting work to be done.

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We note that a recent preprint manuscript (Wang and Spradling 2019) reports new characterization of renal stem cells that replenish principal cells in only the Malpighian tubule.

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