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 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

**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.
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 zoning 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 (Terhazz et al. 2006). On its own, this would acidify the tubule lumen; however, a colocalized 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 α (ClC-a) on the basolateral side (Cabero 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 (Skær 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.
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).

**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) |

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 organisal homeostasis. Insect neuropeptides are usefully summarized in the online Database for Insect Neuropeptide Research (Yeo 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 prepro-peptide 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 corticortropin. 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-Leu-Gly-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 1P), 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 (Cabero 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 dipterinc to levels that are sufficient to kill bacteria. Overexpression of nitric oxide synthase in tubules also elevates Dipterinc levels (McGettigan et al. 2005). Dipterinc is not the only antimicrobial peptide with expression in the tubule; significant expression of attacin, Metchininikovin, 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 (Dabora 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 (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 (Brogiole 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 (Elzenga 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 (Dircks 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 (Gálková 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 (Swarmanydam 1737). While sexually dimorphic in species with highly specialized, sex-specific dietary needs such as mosquitoes (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 mosquitoes, ants, and the blowfly (Figure 1E, rectal papillar cells) (Gupta and Berridge 1966; Berridge and Gupta 1967; Hopkins 1967; Wiggesworth 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 highly 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 papillary 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 reabsors K⁺ to a greater extent than the ileum (Yerushalmi et al. 2018). Based on work in other insects such as mosquitoes 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 papillary 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. Usually 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 Krüppel (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, Tailess 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).
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 ~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.

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).
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 non-tip 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. The mature stellate cell is apicobasally polarized, with the posterior pair (Skaer and Martinez-Arias 1992).

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 dorsally, 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 apicolosal 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 opposed to midgut neuroendocrine cells that contain neuromodulators 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 Brachypterous/Brachury, 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; Skær 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 (Skær 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 (Soplo 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 pylorus expressing components of the JAK-Stat and Hedgehog pathways. Three transcriptional regulators: 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 (Tanioguchi 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 (Tanioguchi 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 epitelial 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 disrupted 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 papillary 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 polytene mitosis. This is currently the only known case where such divisions occur completely in flies, although subperineurial glia of the larval brain initiate polytoid divisions but fail cytokinesis (Unhavaithaya and Orr-Weaver 2012). Rectal papillary cell division requires elimination of polytene chromosome structure, which is a barrier to proper cell division. Polyten separation occurs in a process known as Separation Into Recent Sisters, or SIRS (Stormo and Fox 2016). To prepare for SIRS, papillary 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 anti-tumor chemotherapy agents (Levan and Hauschka 1953; Zybina and Zybina 1996; Sumner 1998). While it may seem laborious for papillary cells to build up polytene chromosomes only to then separate them later, endocycles
and polyplid mitosis are absolutely essential for rectal papillary development and tolerance of a high-salt diet in adult flies, underscoring the importance of papillary 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 sulfatase 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. 2003). 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).
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; Shao 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. 1996; Stover et al. 2005), RNA interference knockdowns showed that 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 chloride channel (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).

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).

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; Helmstädt 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, 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).

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Similarly, classical Bartter syndrome (type III) is associated with mutations in the CLCNKB gene, encoding the CLCK-b 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.

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; Miyaoaka 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; Takahashi 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 polyplody and compensatory cell proliferation (Losick et al. 2013; Tamori and Deng 2014). Similar to the pyloric injury response, both hypertrophy and polyplody 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 polyplody. 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/Cfzr 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. 2018).
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 micro-nuclei—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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