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Updates on ion and water transport by the Malpighian tubule

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The Malpighian (renal) tubule is capable of transporting fluid at remarkable rates. This review will focus on recent insights into the mechanisms by which these high rates are achieved and controlled, with particular reference to the tubules of *Drosophila melanogaster*, in which the combination of physiology and genetics has led to particularly rapid progress. Like many vertebrate epithelia, the *Drosophila* tubule has specialized cell types, with active cation transport confined to a large, metabolically active principal cell; whereas the smaller intercalated stellate cell controls chloride and water shunts to achieve net fluid secretion. Recently, the genes underlying many of these processes have been identified, functionally validated and localized within the tubule. The imminent arrival of new types of post-genomic data (notably single cell sequencing) will herald an exciting era of new discovery.

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A model for tubule function

Insect survival depends on maintaining a constant internal environment in the face of external variation. Much of this homeostatic heavy lifting is performed by the Malpighian (renal) tubules, in conjunction with the hindgut. Tubule function has been extensively reviewed over the last decade, both as a transport phenomenon, but also with reference to possible modelling of human renal diseases [1–7]. Such comprehensive coverage allows this short article to focus on recent insights into our understanding of insect renal tubules in general, and those of *Drosophila melanogaster* in particular. However, space will not allow coverage of some other interesting growth areas, such as the role of tubules in maintaining ionic homeostasis during insect adaptation to cold [8–10], or the promise of modelling human diseases such as nephrolithiasis [11–18].

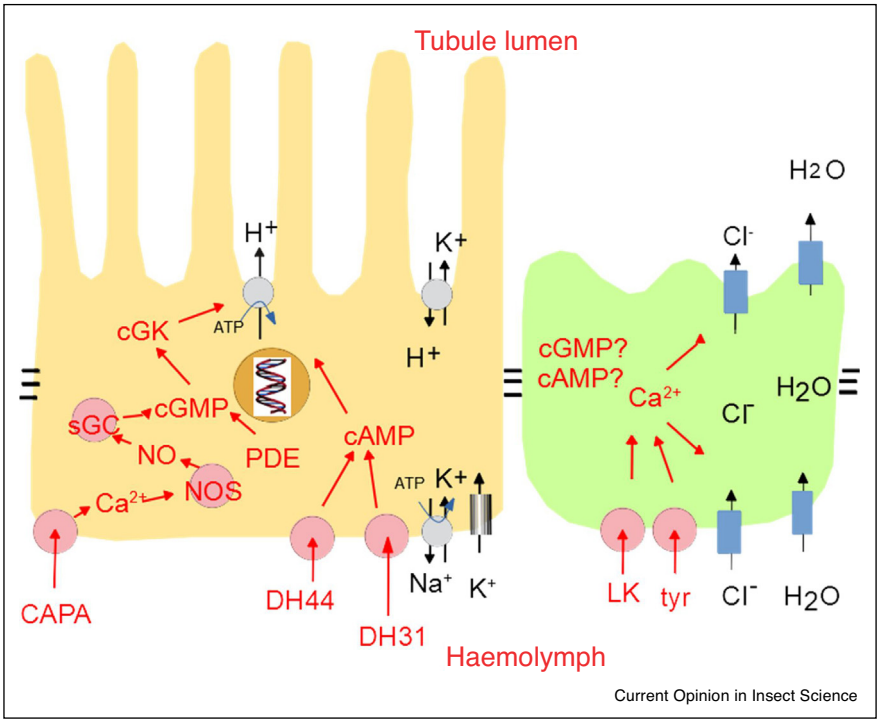
The basic model, discussed below, segregates active cation transport into a large, metabolically active principal cell, energized by the large H^+ motive V-ATPase complex, while chloride flows through channels in smaller stellate cells to balance the charge. This accomplishes a net flux of salt, and so osmotically obliged water follows to accomplish a bulk flow of approximately isotonic saline (Figure 1).

This article will address each of these stages in turn.

Cation transport

The role of H^+ V-ATPases in tubule function is well-documented, and mutation of any of the 13 genes encoding the plasma membrane isoform of the V-ATPase confers lethality and a characteristic tubule phenotype [19]. The V-ATPase is thought to drive an apical K^+/H^+ exchanger of the NHA family [20], accomplishing an apical secretion of K^+. Basolateral entry of K^+ could be through several routes: for example through the Na^+, K^+ ATPase [21] or the NKCC cotransport [22]. However, the tubule expresses very high levels of all three inward rectifier K^+ channels, that allow flux of K^+ into, but not out of, the cell; these have been localized to principal cells, and tubule fluid secretion has accordingly been shown to be potently inhibited by antidiabetic sulphonylurea drugs [23]. *Irk1* and *Irk2* play semi-redundant roles in secretion, as knockdown of both is required to significantly impact secretion rates, whereas *Irk3* knockdowns were without effect [24]. Similarly, the *Aedes aegypti* orthologues *Kir1* and *Kir2B* were expressed on the basolateral membranes of principal cells, and the intracellular *Kir3* did not allow K^+ currents when expressed in *Xenopus* oocytes [25]. This is intriguing, because *Irk3* is expressed strongly and uniquely in Malpighian tubules of *Drosophila*. Similar tubule enrichment was observed in the Hemipteran bedbug, *Cimex lenticularis*, where knockdown of the *Irk3* orthologue did not impact survival [26]. In the lepidopteran pest *Chilo suppresalis* (the Asiatic rice borer), knockdown of inward rectifiers with Flonicamid, or of the tubule-enriched CsKir2B by dsRNA, reversibly inhibited growth [27].

Figure 1



Overview of the two-cell model for insect tubule fluid secretion and its control.

Although there are other routes for basolateral K^+ entry, the effect of combined knockdown of *Irk1* and *Irk2* suggests that these are major players in basolateral K^+ entry. A slightly different model was suggested for *Aedes*; there, Irk1 and Irk2 localise to the basolateral membranes of principal and stellate cells, respectively [25]; stellate cells could provide K^+ to neighbouring principal cells via gap junctions. By contrast Irk3 is intracellular, which would also explain the lack of impact of *Irk3* knockdowns in *Drosophila* secretion assays. Nonetheless, the phylogenetically conserved tubule-specific expression of Irk3 and its orthologues suggest that it plays an important role in tubule function. Given that the tubule contains multiple specialized vesicles, for example to store visual pigment [28], a functional role for Irk3 may be found with more subtle phenotypes.

Chloride flux

Chloride flux is under direct control of kinin and tyramine signalling, both of which act through intracellular calcium signals in stellate cells [29] to collapse the normally luminal positive potential in seconds by activating the chloride shunt conductance [30–32]. The route for stimulated chloride flux has been controversial, but has been resolved in two recent papers. Chloride entry is via the Clc-a chloride channel, which was localized immunocytochemically to the basolateral membrane of only stellate cells. Knockdown of the *Clc-a* gene in stellate cells did not affect basal secretion rates, but abolished kinin-stimulated secretion. Knockdown also led to a characteristic bloated phenotype in adult flies, as urination was compromised [33]. Using a transgenic intracellular chloride sensor, ClopHensor [34], it was possible to demonstrate a transient in intracellular choride within the stellate cells when they were stimulated [33]. Similar work has identified the pentameric cys-loop chloride channel SecCl as the apical partner to Clc-a in stellate cells [35]. There is thus a complete transepithelial pathway for hormone-sensitive chloride movement across stellate cells. Nonetheless, all tubule cell types are likely to have finite chloride permeability. Another pentameric chloride channel, pHCl-2, has been localized to the apical surface of principal cells, where it is thought to be gated by extracellular (luminal) pH, rather than by chemical ligands. Knockdown only slightly impacts cAMP-induced fluid secretion [36], suggesting that this channel may play a somewhat subtle, regulatory role.

Water flux

Now that there is a sound understanding of cation transport and balancing chloride flux, it remains to be established how osmotically obliged water follows the net salt flux. Tubules are known to express genes for several members of the Major Intrinsic Protein (MIP) family,

that includes the classical aquaporins as well as the aquaglyceroporins, that also facilitate fluxes of several small organic solutes. In principle, then, water could flow transcellularly through the principal cells or the stellate cells, or intercellularly through the septate junctions (see next section), or through any combination of these. To address this, a recent paper identified the route of water flux by incubating tubules with an impermeant high molecular weight fluorescent dextran, that could be drawn into intercellular clefts, or into basal infoldings of either cell type. As tubules do not allow such large molecules to pass, they would accumulate at the site of water flux. In this way, it was shown that water moves preferentially through the stellate cells [37]. Tubules express very high levels of four members of the MIP family, two classical aquaporins (*Drip* and *Prip*), and two aquaglyceroporins (*Eglp2* and *Eglp4*). Functional studies in *Xenopus* oocytes showed that Drip and Prip were classical water channels, whereas Eglp2 and Eglp4 additionally allowed flux of small molecules such as urea and glycerol [37]. Immunocytochemistry showed that Prip was basolateral, and Drip apical, in just the stellate cells; whereas Eglp4 was basal, and Eglp2 apical, in just the principal cells [37]. Cell-specific knockdowns confirmed the roles of aquaporins in tubule water flux. Although the possibility that some water flows through the principal cells cannot be excluded, there is a clear, functioning transcellular route for water through the stellate cells.

Although these precise genetic interventions are not easy beyond *Drosophila*, there is also evidence for a functional role for MIPs in other species. The aquaporin family of the Dengue vector mosquito *A. aegypti* has been characterised [38], and an aquaglyceroporin has been localised to principal cells [39]. An aquaporin has also been demonstrated in midgut and tubules of the malaria vector *Anopheles gambiae* [40]. It seems likely, then, that a transcellular route for water flux may be general, especially considering the formidable barrier of the septate junctions.

Junctions, cytoarchitecture and the paracellular route

Like all epithelia, intercellular spaces in Malpighian tubules are guarded by tight junctions to prevent leakage of solutes, and so dissipation of gradients established by vectorial transport. In insects they are termed septate junctions, based on their electron microscopic appearance. Tubules, in common with midgut, have smooth (rather than pleated) septate junctions. Recent progress (reviewed elsewhere in this volume [64] has identified several key players in tubule junctions. Knockdown of known septate junction proteins Mesh [41] or Tetraspanin 2A [42] in tubule principal cells results in measurably leaky epithelia with compromised fluid secretion and bloated phenotype. Similar effects were seen with knockdown of *Snakeskin*, encoding another junctional protein [43]. Additionally, progressive disruption in epithelial organisation was observed with aging [43]. The overall impression therefore, is that the septate junction is indeed a functional analogue of the vertebrate tight junction, and that its barrier function is necessary to allow successful secretion.

Successful junctions are required for correct epithelial organization, and one conspicuous property of the principal cells is an abundant field of apical microvilli, loaded with V-ATPase and each containing a long mitochondrion. Given that the tubule secretes faster on a per-cell volume basis than any other epithelium in biology, the microvilli are likely to be under significant shear stress, and may require structural reinforcement. A well-known protein in morphogenesis, Fasciclin II, plays this role [44]. Knockdown of Fas2 shortens the microvilli, whereas overexpression lengthens them, compared to wild-type. Fluid secretion is proportional to microvillar length, providing direct evidence for what had previously been assumed; that microvilli extend surface area and allow higher transport rates in an epithelium [44].

Regulation

The hormonal control of tubule secretion by neuropeptides Capa, kinin, DH31, DH44 and Nplp1-4 have been well-reviewed elsewhere [1,2]. However, in mammalian kidney there are also internal homeostatic mechanisms. For example, in vertebrate nephron, autophosphorylation of the 'with no lysine' (WNK) kinase is reduced by chloride. WNK phosphorylates two further kinases, SPAK and OSR1, which then phosphorylate the sodium chloride cotransporter NCC and the sodium–potassium-2 chloride cotransporters NKCC1 and 2 [45]. There are *Drosophila* orthologues for most of these components, so could a similar system apply? In *Drosophila* tubule, WNK is activated by hypotonicity, which would act to stimulate fluid secretion and so correct the imbalance. Knockdown of *Drosophila* WNK or SPAK/OSR1/fray reduces K^+ flux through the tubule, and it was shown that Fray phosphorylates NKCC *in vitro* [46]. The scaffold protein Mo25 also impacts on WNK signalling in tubule [47]. Although the tubule is directly controlled by the nervous system, mechanisms of direct intracellular homeostasis are now being uncovered.

Comparative

The genetic tools and genomic resources unique to *Drosophila* have hugely accelerated research into tubule function, but it is critical to establish whether the models being developed in fly have more general applicability. There is evidence, for example, that functional properties associated with stellate cells in *Drosophila* are associated with similar subpopulations of cells in other organisms. The kinin receptor, highly specific to stellate cells in fly, shows a similar distribution in *Anopheles* and *Aedes* [48]. Similarly, *tiptop*, a paralogue of the *Teashirt* transcription

factor that drives the stellate cell fate in flies [49,50], is found in specialized cell subtypes in in the beetle *Tribolium castaneum* and the cricket *Gryllus bimaculatus* [49].

Given that the diuretic neuropeptides Capa, DH31 and DH34 are all thought to act to stimulate fluid secretion by activating principal cell V-ATPase, and kinin by activating chloride flux in stellate cells, a direct test of the two-cell model would be to map receptors in tubules of other insects. Both immunocytochemistry and in situ hybridization would require species-specific informatics and generation of reagents; however, direct labelling of neuropeptides with a fluor allowed visualization of cell-specific binding to tubules across representatives of 90% of insect biodiversity [51]. In this way, it was shown that kinin indeed labelled a subpopulation of small cells in

Table 1

Provisional single cell transcriptomics of main segment principal and stellate cells of adult *Drosophila*

Principal cells			Stellate cells		
Gene	Log FC	Function	Gene	Log FC	Function
CG8492	4.4	Lysozyme	*tutl*	7.7	Cell–cell adhesion mediator
Hs3st-A	4.1	Heparan sulfate 3-*O* sulfotransferase-A	*CG6282*	7.6	Oxidoreductase
Chinmo	3.9	Nucleic acid binding	*twz*	6.4	Potassium channel
CG42235	3.7	Symporter	*NetB*	6.0	Neuron guidance
lncRNA:CR43768	3.7	—	*Nha2*	5.9	Na^+/H^+ exchanger
Cpr92A	3.6	Structural constituent of cuticle	*dac*	5.7	Transcription factor
List	3.5	Neurotransmitter:sodium symporter	*Octalpha2R*	5.5	Biogenic amine receptor
CG14397	3.4	—	*CG30116*	5.1	—
CG4928	3.4	Potassium channel regulator	*Doc2*	5.1	Transcription factor
CG8785	3.3	Amino acid transmembrane transporter	*Alk*	5.0	Transmembrane receptor protein tyrosine kinase
CG4467	3.3	Metalloaminopeptidase	*lh*	4.8	Potassium channel
nompC	3.2	Calcium channel	*tsh*	4.8	Transcription factor
Snmp2	3.2	Scavenger receptor	*ETHR*	4.8	Neuropeptide receptor
Dh31-R	3.2	Diuretic hormone receptor	*Ldh*	4.7	Lactate dehydrogenase
ouib	3.2	Transcription factor	*CG4607*	4.6	Carbohydrate:proton symporter
Pu	3.2	GTP cyclohydrolase I	*tok*	4.5	Zinc metallopeptidase
CG7342	3.2	Transmembrane transporter	*Doc1*	4.5	Ectodermal transciption factor
Slc45-1	3.2	Sucrose transporter	*Pde1c*	4.5	Phosphodiesterase
CG8028	3.1	Monocarboxylic acid transporter	*SK*	4.4	Potassium channel
wtrw	3.1	Calcium channel	*asRNA:CR43454*	4.4	—
CG7882	3.1	Transmembrane transporter	*CG2145*	4.4	Endoribonuclease
ppk13	3.1	Sodium channel	*Lim3*	4.3	Transcription factor
CG2187	3.0	Symporter	*Ten-a*	4.3	Cell adhesion
CG16727	3.0	Transmembrane transporter	*CG13323*	4.2	Transcription regulator
Ptx1	3.0	Transcription factor	*ed*	4.1	Cell adhesion
Ugt302K1	3.0	Hexosyl transferase	*svp*	4.0	Transcriptional regulator
CG31663	3.0	Transporter	*Or94b*	3.9	Odorant receptor
Cyp6t3	2.9	Oxidoreductase	*CG44325*	3.9	Septate junction
CG4286	2.9	—	*CG9674*	3.9	Glutamate synthase
CG34426	2.9	Chitin binding	*Lkr*	3.9	Kinin neuropeptide receptor
Selected enriched >2x				Selected enriched >2x	
Irk1	2.8	Potassium channel	*Nep2*	3.8	Peptidase
NaPi-T	2.2	Phosphate transporter	*hth*	3.3	Transcription factor
Eglp4	2.1	Aquaglyceroporin	*Ace*	3.0	Acetylcholine esterase
cad	2.1	Transcription factor	*norpA*	2.5	Phospholipase C
Fas3	2.1	Cell adhesion	*Pde11*	2.1	Phosphodiesterase
pHCl-2	2.0	Chloride channel	*rdgA*	2.0	Diacylglycerol kinase
subdued	2.0	Chloride channel	*sdt*	2.0	Junctional polarity

Genes are ranked by their log fold change above an average of all cells in the lower tubule dataset [55]. Below the top 30, further genes with significant enrichments above 2.0x are selected for interest.

tubules of the endopterygote Diptera, Hymenoptera and Lepidoptera; but labelled tubules of the exopterygote Orders Hemiptera and Orthoptera more generally. It thus seems that the two-cell model may be general to the 'higher' insect Orders. Conspicuously though, the Coleoptera showed no binding of kinin, nor the presence of kinin or its receptor in any species studied at the time [51]; it has since become clear that only very few beetles from the suborder Adephaga have kinin signalling, and it may have been lost secondarily through the rest of the Order [52–54].

Single cell sequencing

Drosophila has provided clear evidence for the segregation of active cation transport from the anion and water shunts (see updated model in graphical abstract), but the transcriptomic signature of this separation is masked by the necessity for whole tissue transcriptomics. However, the relatively new technique of single-cell sequencing can radically change this landscape. In a recent paper, the lower tubules of *Drosophila* were painstakingly dissected in order to characterize their stem cell population [55]. The physical separation of lower tubules from main segments is necessarily approximate, and the authors provided evidence that they might have adventitiously included some main segment principal and stellate cells [55]. The original dataset was heavily filtered to enrich for the small stem cells; so realizing that principal and stellate cells differed hugely in both ploidy and mitochondrial content, we reanalysed their kindly provided raw datasets with fewer filtering restrictions. This produced more cells of each type; and we identified the main segment principal cells as expressing high $CAPA_R$ but low *Aph4* (a marker of lower tubule in *Drosophila* and other insects [56]), and stellate cells as containing high *lkr* [29], *Drip* [37] and *Clc-a* [33]. The most enriched genes in each cell type (relative to the average lower tubule transcriptome) are shown in Table 1.

While it should be emphasized that this is an imperfect analysis (a nuclear single cell transriptome for the whole tubule is imminent as part of the FlyCellAtlas project), we now have a first view of what makes principal and stellate cells unique. Confidence in the dataset is increased by the observation of enriched genes previously characterized in tubule, for example *DH31R* [57], *Irk1* [24], *NaPi-T* [58], *Eglp4* [37] and *pHCl-2* [35] in principal cells and *Nha2*, *tsh*, *lkr* and *Nep2* in stellate cells. However, there are also surprises; for example, an enrichment of genes associated with the termination of cell signals (*Pde1c* [59], *Pde11* [59], *Nep2* [60], *Ace* [61], *norpA* [62] and *rdgA* [63]) in stellate cells.

Perhaps the most exciting outcome, however, is that many of the genes with expression most highly enriched in principal and stellate cells have yet to be named (Table 1), suggesting that, even in this extraordinarily well-studied epithelium, there is much still to be learned. As more detailed post-genomic datasets come onstream, there is likely to be an exciting step change in our understanding of this remarkable tissue, and thus of insect success.

Conflict of interest statement

Nothing declared.

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