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The *Drosophila* Malpighian Tubule as a Model for Mammalian Tubule Function

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Abstract

Purpose of review—Studies of the genetic model organism, *Drosophila melanogaster*, have unraveled molecular pathways relevant to human physiology and disease. The Malpighian tubule, the *Drosophila* renal epithelium, is described here, including tools available to study transport; conserved transporters, channels, and the signaling pathways regulating them; and fly models of kidney stone disease.

Recent findings—Tools to measure Malpighian tubule transport continue to advance, including use of a transgenic sensor to quantify intracellular pH and proton fluxes. A recent study generated an RNA sequencing-based atlas of tissue-specific gene expression, with resulting insights into Malpighian tubule gene expression of transporters and channels. Advances have been made in understanding the molecular physiology of the WNK (With No Lysine)-SPAK/OSR1 (Ste20-related proline/alanine rich kinase/oxidative stress response) kinase cascade that regulates epithelial ion transport in flies and mammals. New studies in *Drosophila* kidney stone models have characterized zinc transporters and used Malpighian tubules to study the efficacy of a plant metabolite in decreasing stone burden.

Summary—Study of the *Drosophila* Malpighian tubule affords opportunities to better characterize the molecular physiology of epithelial transport mechanisms relevant to mammalian renal physiology.

Keywords

epithelial ion and water transport; signaling; nephrolithiasis; *Drosophila* genetics; organismal physiology

Introduction

Thomas Hunt Morgan's pioneering studies in the early 20th century began 110 years of *Drosophila melanogaster* research, and established the common fruit fly as a powerful

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Conflicts of interest

None.

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genetic model organism (1). Six Nobel prizes have recognized *Drosophila* studies on fundamental genetic mechanisms, early embryonic development, odorant receptors and the olfactory system, innate immunity, and circadian rhythms, illuminating molecular mechanisms conserved in mammalian physiology. Ongoing work has exploited the fly for study of the nervous system, metabolism, cardiovascular function, cancer, and inflammation (2,3). This review will highlight studies on the *Drosophila* renal epithelium, the Malpighian tubules, with a focus on epithelial transport.

The *Drosophila* toolbox

The *Drosophila* genome, which contains approximately 14,000 genes, was the first animal genome sequenced using a whole-genome shotgun approach (1). Germline mutations exist for many *Drosophila* genes, and gene disruption and gene-silencing techniques can target additional genes (2). Genes can be expressed or silenced in a temporally and spatially restricted fashion, using the GAL4/UAS system (4,5) (Figure 1).

UAS-RNAi lines targeting nearly every *Drosophila* gene are available from publicly accessible collections (10,11), or can be generated in individual laboratories using established protocols (12,13), as can transgenic UAS lines to allow expression of wild-type or mutant genes (3,13,14*). Transgenic animals can be made in 2–3 months.

FlyBase (http://flybase.org) is a rich *Drosophila* resource, including information on genes and the genome, orthologs, references, available tools, and expression data. “Omics” efforts have provided a detailed map of gene expression across developmental timepoints and in different tissues. In particular, FlyAtlas provides information on gene expression in larval and adult Malpighian tubules, as well as many other tissues. The first iteration was based on an Affymetrix microarray platform (15), while FlyAtlas 2 used RNA sequencing technology (16**). Both FlyAtlas (http://flyatlas.gla.ac.uk/flyatlas/index.html) and FlyAtlas2 (http://flyatlas.gla.ac.uk/FlyAtlas2/index.html) have publicly accessible search engines that allow queries for expression patterns of genes of interest. Analysis of these datasets has identified tubule-enriched genes (17,18).

Assays for study of Malpighian tubule function

While *Drosophila* has podocyte-like cells, called nephrocytes, that also have some of the endocytic functions of mammalian proximal tubule cells (19–21), these are anatomically separate from the four Malpighian tubules, which lie in the abdominal cavity in direct contact with the hemolymph (plasma).

Malpighian tubules are genetically and functionally segmented. GAL4 expression patterns, driven by endogenous genomic enhancers, define a distal initial and transitional segment, a main segment, a proximal lower segment, and an upper and lower ureter (6,17). Urine generation by the blind-ended tubules occurs in the main segment (22), with subsequent modification in the lower segment and hindgut (23–25*) (Figure 2).

Transepithelial ion and water fluxes can be measured in isolated Malpighian tubules, as first pioneered by Ramsay in stick insects (30), and later adapted for *Drosophila* tubules (22).

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Drosophila tubules, which are nearly 3 mm long (25*), are easily dissected under a stereomicroscope, and 20–30 tubules can be studied in a single experiment using the Ramsay assay (31,32). When paired with the use of ion-specific electrodes (31), the Ramsay assay allows measurement of transepithelial fluxes of inorganic and organic ions, including sodium, potassium, and calcium (29); ammonium (33); salicylate (34); and tetraethylammonium (35). When paired with confocal microscopy, the Ramsay assay can measure transport of fluorescent organic anions and cations (36). *In vitro* tubule perfusion has been performed in *Drosophila* tubules (37), and allows control over luminal perfusate. Ion secretion and reabsorption have also been measured using self-referencing ion-selective microelectrodes positioned in the unstirred layer (27,35,38).

Transgenic sensors, expressed in the tubule using the GAL4/UAS system, have been used to measure intracellular (9) and mitochondrial calcium (39) in tubule epithelial cells, as well as intracellular chloride (14*,40) and pH (41**). cAMP (adenosine 3’5’-cyclic monophosphate), cGMP (guanosine 3’5’-cyclic monophosphate) and calcium signaling have been manipulated in a cell-specific fashion using optogenetic techniques and by expressing exogenous receptors coupled to these signaling pathways and exposing tubules to their ligands (42,43).

Malpighian tubule ion and water transport

Although the configuration of transporters and channels in the Malpighian tubule differs from the mammalian tubule, in many cases the transporters, and the signaling pathways regulating them, are conserved.

Fluid secretion by the main segment

In the fluid-secreting main segment, transepithelial cation flux occurs through principal cells, while chloride flux occurs through the neighboring stellate cells (40,44,45). Fluid secretion is energized by the apical vacuolar proton ATPase (V-ATPase) (22,46–49), a multi-subunit transporter homologous to the mammalian V-ATPase in the collecting duct intercalated cell that is mutated in patients with distal renal tubular acidosis (50,51). The V-ATPase generates a lumen-positive transepithelial potential of ~40 mV (37,44), which is thought to drive proton/cation exchange across the apical membrane (Figure 3).

In the mammalian kidney, SLC12 cation-chloride cotransporters, including the sodium-potassium-2-chloride (NKCC) and sodium-chloride (NCC) cotransporters, play important roles, and are the target of commonly used diuretics (62). Both NKCC2 and NCC are mutated in human salt-losing tubulopathies, as are the inwardly rectifying potassium channels, ROMK (renal outer medullary potassium channel) and Kir4.1 (63). Functional roles in the principal cell have been demonstrated for the *Drosophila* NKCC (28), encoded by *Ncc69* (64,65), and two inwardly rectifying potassium channels, Irk1 and Irk2, expressed in the tubule (37,66–68). While a third putative inwardly rectifying potassium channel, *Irk3*, is expressed at very high levels in the tubule (17), there was no functional consequence of knocking it down on fluid secretion or potassium flux (37).

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The basolateral sodium/potassium-ATPase (Na^+/K^+-ATPase) (56,69) is required for transepithelial potassium flux, by recycling sodium entering the principal cell through the NKCC (28). Na^+/K^+-ATPase activity is also required for hormonally-stimulated fluid secretion (70), and provides the driving force for sodium-dependent transport of organic anions like salicylate and para-aminohippuric acid (71,72).

CLC family chloride channels are important for chloride transport in the mammalian kidney, and are mutated in human patients with salt-losing tubulopathy (73). A CLC chloride channel in *Drosophila*, encoded by *Clc-a*, is required in stellate cells for transcellular chloride secretion in hormonally stimulated tubules (40). Chloride transport mechanisms in unstimulated tubules are not defined. A paracellular pathway for chloride transport has been demonstrated in *Aedes aegypti* mosquitos (74,75); whether a similar pathway exists in *Drosophila* tubules is unknown.

There are 8 aquaporin (AQP) family genes in *Drosophila* (76). Transcripts for *Drip* have been localized to the stellate cell of the adult Malpighian tubule, while transcripts for *CG17664* and *CG4019* have been localized to the principal cell (77), suggesting that transcellular water transport could occur in both cell types. Knockdown of the *CG4019 Aedes aegypti* homolog, *AaAqp5*, which is expressed on the apical and basolateral membranes of the larval mosquito principal cell, results in decreased tubule fluid secretion (78).

Calcium, magnesium and phosphate transport

Calcium transport occurs predominantly in the initial/transitional segment, which is larger in anterior tubules compared to posterior tubules (6,38,79–81). Intracellular calcium- and magnesium-rich concretions are found in the distal tubule of *Drosophila hydei* larvae (82), and active magnesium transport has been demonstrated in Malpighian tubules of larval *Aedes campestris* mosquitos (83). Mammalian TRPM (transient receptor potential cation channel, subfamily M) channels are important for epithelial magnesium transport (84–87). A *Drosophila* TRPM channel is predominantly expressed in the initial/transitional segment, and has been implicated in magnesium transport (88), although tubule magnesium fluxes have not been measured in *Drosophila.*

At least one proven phosphate transporter, MFS13 (major facilitator superfamily 13), is enriched in the Malpighian tubule. Malpighian tubule phosphate fluxes have not been measured, but ablation of tubule epithelial cells in the main segment results in higher hemolymph phosphate concentrations in animals fed a high-phosphate diet (89,90). However, while 42 of 46 human solute carrier (SLC) families are found in insects, the SLC34 family, which includes the mammalian proximal tubule NaPi (sodium phosphate) cotransporters Npt2a and Npt2c, is not found in insects (91).

Other transporters expressed in the Malpighian tubule

Many other transporters, exchangers and channels are also expressed in the Malpighian tubule (17); a few examples will be reviewed here. The SLC5 family of sodium/glucose cotransporters, which includes the mammalian proximal tubule transporters SGLT1 and SGLT2, is comprised of 15 genes in *Drosophila* (92), 7 of which are enriched in expression

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in the adult tubule in FlyAtlas 2. Addition of glucose to the bathing medium increases fluid secretion by the tubule (26), but glucose transport has not otherwise been examined.

An important function of the proximal tubule is the secretion of small molecules, including medications and endogenous solutes like uremic toxins (93,94). Similarly, the Malpighian tubule secretes organic anions and cations, including tetraethylammonium, para-aminohippuric acid, ouabain, and human therapeutics like methotrexate, daunorubicin, and salicylate (34–36,70–72,95). Transporter families involved in this process in the mammalian proximal tubule include the ABCC (ATP-binding cassette C, also known as the MRP, or multidrug resistance, transporters), SLC22 (which includes the OAT1 and OAT3 organic anion transporters), and SLCO (also known as OATP, or organic anion transporter P) families (94). These transporter classes are well-represented in the *Drosophila* genome, and many family members are enriched in expression in the Malpighian tubule in FlyAtlas 2, including nine of fourteen ABCC, eleven of twenty-five SLC22 (76,91,96), and six of eight SLCO/Oatp transporters (70). A functional role for Oatp58Db has been demonstrated in ouabain transport (70), and the roles of dMRP, MET (Methoprene-tolerant, an SLC46 family member), and Oatp58Dc have been explored in organic anion and cation transport (95,97,98).

Signaling pathways regulating ion transport

Signaling pathways regulating epithelial transport are also conserved between flies and mammals. For example, nitric oxide signaling regulates sodium transport in multiple nephron segments in the mammalian kidney (99–102), and also regulates transport in the Malpighian tubule (103–105).

Another conserved regulatory pathway is the WNK (With No Lysine)-SPAK/OSR1 (Ste20-related proline/alanine rich kinase/oxidative stress response) kinase cascade, which regulates ion transport in the thick ascending limb and distal convoluted tubule of the mammalian nephron (106). There are four WNK paralogs in mammals, and WNK1 and WNK4 are mutated in a human syndrome of hypertension and hyperkalemia (107). WNKs phosphorylate two related kinases, SPAK and OSR1, to activate them (108,109). Activated SPAK and OSR1 then phosphorylate SLC12 transporters, including NCC, NKCC1 and NKCC2, to activate them (110–115). *Drosophila* has a single *WNK* homolog, which phosphorylates the fly SPAK/OSR1 homolog, encoded by *Fray* (116–118). Fray phosphorylates fly NKCC (Ncc69), and *WNK* and *Fray* regulate Malpighian tubule transepithelial ion transport via NKCC in the principal cell (13). Thus, WNK-SPAK/OSR1 regulation of renal epithelial ion transport is conserved from flies to mammals.

Chloride binds to the active site of WNKs and inhibits their autophosphorylation and activation *in vitro* (119,120). Acute decreases in intracellular chloride in Malpighian tubule epithelial cells result in WNK activation over 30 to 60 minutes, with stimulation of transepithelial ion flux (13,14*). These findings are relevant to chloride regulation of transport in the mammalian distal convoluted tubule to maintain potassium homeostasis (121,122). A role for the scaffold protein mouse protein 25 (Mo25)/calcium-binding protein 39 to achieve maximum pathway stimulation and ion transport was also demonstrated (14*).

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Mo25 is expressed in the mammalian distal convoluted tubule and thick ascending limb (123), but its functional role in those nephron segments has so far been unexplored.

The Malpighian tubule also affords opportunities to study mammalian genes in the context of a transporting renal epithelium. For example, knockdown of *Drosophila Mo25* was rescued by expression of mouse *Mo25α* (14*). Knockdown of *Drosophila* WNK can be rescued by expression of mammalian WNKs, but the three kidney-expressed mammalian WNKs (1, 3 and 4) behave differently (Rodan AR, unpublished data). Because mammalian WNKs compensate for one another (124–126), and also interact (127–129), the ability to express individual or specific combinations of WNKs may allow better definition of the roles of individual WNK paralogs or their combinations.

Malpighian tubule kidney stone models

Kidney stones are increasing in incidence and prevalence, with an associated increase in cost, and are associated with substantial morbidity (130). The *Drosophila* Malpighian tubule has been developed as a model of stone formation using dietary and genetic approaches (Table 1) (131). These include high oxalate diet, which can contribute to stone formation in humans (132); melamine, which resulted in infant stone disease due to tainted milk powder in China (133,134); and knockdown of *Xanthine dehydrogenase* (*Xdh*), which results in stone formation when mutated in humans (xanthinuria type I) (135). Stones can be visualized with light microscopy with polarizing light, microscopic computed tomography or scanning electron microscopy (132–134).

The SLC26 anion exchanger family includes nine *Drosophila* genes (136), six of which show enrichment in the Malpighian tubule in FlyAtlas and FlyAtlas 2, including *dPrestin*. dPrestin mediates chloride exchange with oxalate, sulfate, thiosulfate and formate (132,136,137). Principal cell knockdown of *dPrestin* decreased crystal formation in the high-oxalate diet model (132) and the inhibitory effect of sulfate and thiosulfate feeding on stone formation (137). Thus, dPrestin is an important mediator of Malpighian tubule oxalate transport.

A recent study used *Drosophila* to examine effects of the plant metabolite 3,4,5-tri-O-galloylquinic acid methyl ester (TGAME) on stone formation, and found decreased calcium oxalate formation *ex vivo* when tubules were incubated in TGAME-containing baths (138**). Thus, this rapidly performed assay may have utility for prioritizing compounds of interest for further study.

Analysis of the concretions in *Xdh* knockdown flies revealed significant amounts of zinc, which was also found in human samples of Randall's plaques (precursors for calcium-based kidney stones) and human xanthine kidney stones. Increasing dietary zinc increased Malpighian tubule stone formation, while dietary supplementation with a zinc chelator decreased stones (135). There are two major families of zinc transporters. Transcripts of five of seven SLC30 family genes, and five of ten SLC39 family genes, are enriched in the Malpighian tubule in FlyAtlas 2. Knockdown of three of the tubule-enriched SLC30 family members reduced tubule stone formation (135). *CG10006* (*Zip10*) is an SLC39 zinc

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transporter highly enriched in the Malpighian tubule. Immunohistochemistry demonstrated apical membrane staining of the *Drosophila* transporter in the principal cell of the Malpighian tubule, and of the human transporter in the apical membrane of the proximal tubule and cortical collecting duct (139**). *Drosophila* is thus a useful model to study the role of zinc in stone formation.

Conclusion

The strength of *Drosophila melanogaster* as a model organism relevant to human physiology has derived from conservation of molecular pathways. Complex interactions between mammalian nephron segments (140), or between the kidney and other organs, may be better modeled in organisms with kidney structures and hormonal signaling more similar to humans (141). On the other hand, as discussed here, transporters and channels important in the mammalian kidney are also present in the *Drosophila* Malpighian tubule, and some have been functionally characterized. Similarly, signaling pathways relevant to ion transport regulation in the mammalian kidney also regulate transport in the Malpighian tubule. *Drosophila* thus affords opportunities for ongoing characterization of the molecular physiology of epithelial transport. Unbiased genome-wide forward genetic screening may identify novel pathways interacting with known transporters and signaling pathways, and opportunities also exist for drug screening (3) and characterization (138**).

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Key points

- *Drosophila melanogaster* has a sophisticated genetic and physiological toolkit to characterize transport processes in the fly renal epithelium, the Malpighian tubule.
- Many classes of transporters, exchangers and channels important in mammalian kidney function have *Drosophila* homologs that are expressed in the Malpighian tubule.
- Signaling pathways regulating transport are also conserved in *Drosophila*, such as the WNK-SPAK/OSR1 pathway, and recent studies have characterized the molecular physiology of these pathways.
- The Malpighian tubule has also been developed as a model for kidney stone formation, including testing of pharmacological agents for stone treatment, and characterizing the role of zinc in stone formation.

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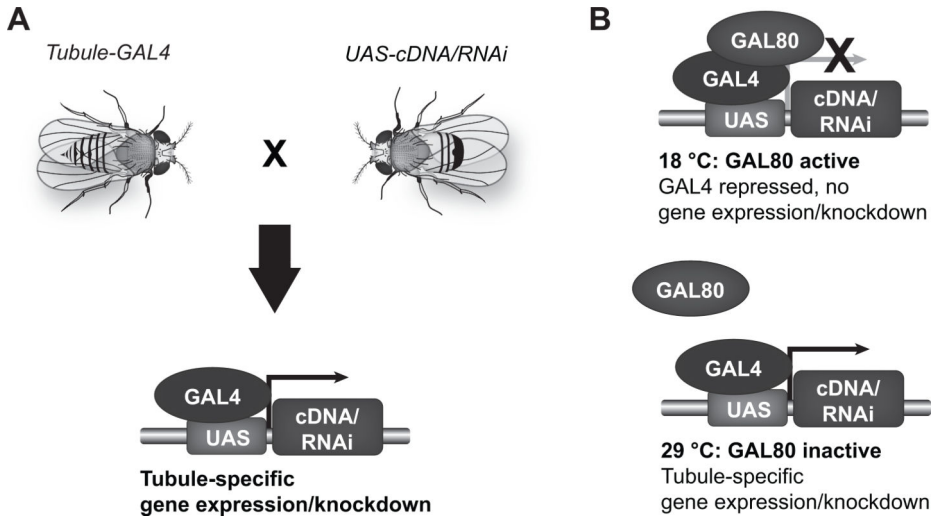

Figure 1. Temporally and spatially restricted gene expression using the GAL4/UAS system.

A) The yeast transcription factor, GAL4, is transgenically expressed in a tissue or cell type of interest under the control of endogenous genomic enhancers, or engineered cell type-specific promoters. For example, GAL4 lines targeting specific subsets of Malpighian tubule epithelial cells have been generated (6–9). The GAL4-expressing fly is mated to a second fly containing the transgenically expressed GAL4 DNA-binding domain, UAS (Upstream Activating Sequence). In progeny containing both the GAL4 and UAS transgenes, GAL4 binding to UAS results in the transcription of DNA cloned downstream of the UAS. This allows cell-specific expression of a wild-type or mutant *Drosophila* or mammalian gene. Alternatively, GAL4/UAS-driven transcription of an RNA that is processed into an interfering RNA (RNAi) allows cell-specific gene knockdown. B) Introduction of a temperature-sensitive GAL80 transgene allows temporal control: at 18 °C, GAL80 is active, and represses GAL4; at 28 °C, GAL80 is inactive, allowing GAL4 expression. For example, to achieve adult-specific gene knockdown or expression, flies are reared at 18 °C throughout development (GAL4 off), and then switched to 28 °C in adulthood (GAL4 on).

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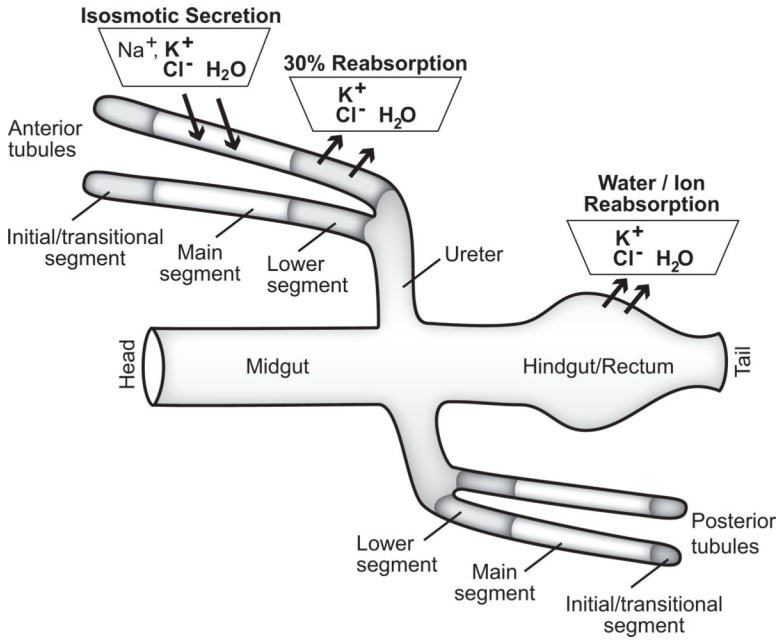

Figure 2. Schematic of *Drosophila* iono- and osmoregulatory epithelia.

The two pairs of Malpighian tubules (anterior and posterior), together with the hindgut, regulate ionic and osmotic homeostasis in the fly. Urine is generated by the transepithelial movement of ions and water across the main segment of the anterior and posterior tubules, resulting in isosmotic secretion of potassium chloride-rich fluid; secreted fluid also contains sodium, and secretion can occur in potassium-free medium (22,25*–28). Urine then flows through the downstream lower segment, where ~30% of the potassium and water secreted by the main segment are reabsorbed (29). The urine then passes through the ureter and enters the hindgut, where further ion and water fluxes occur to match the composition of excreta to the animal's physiological need (23–25*).

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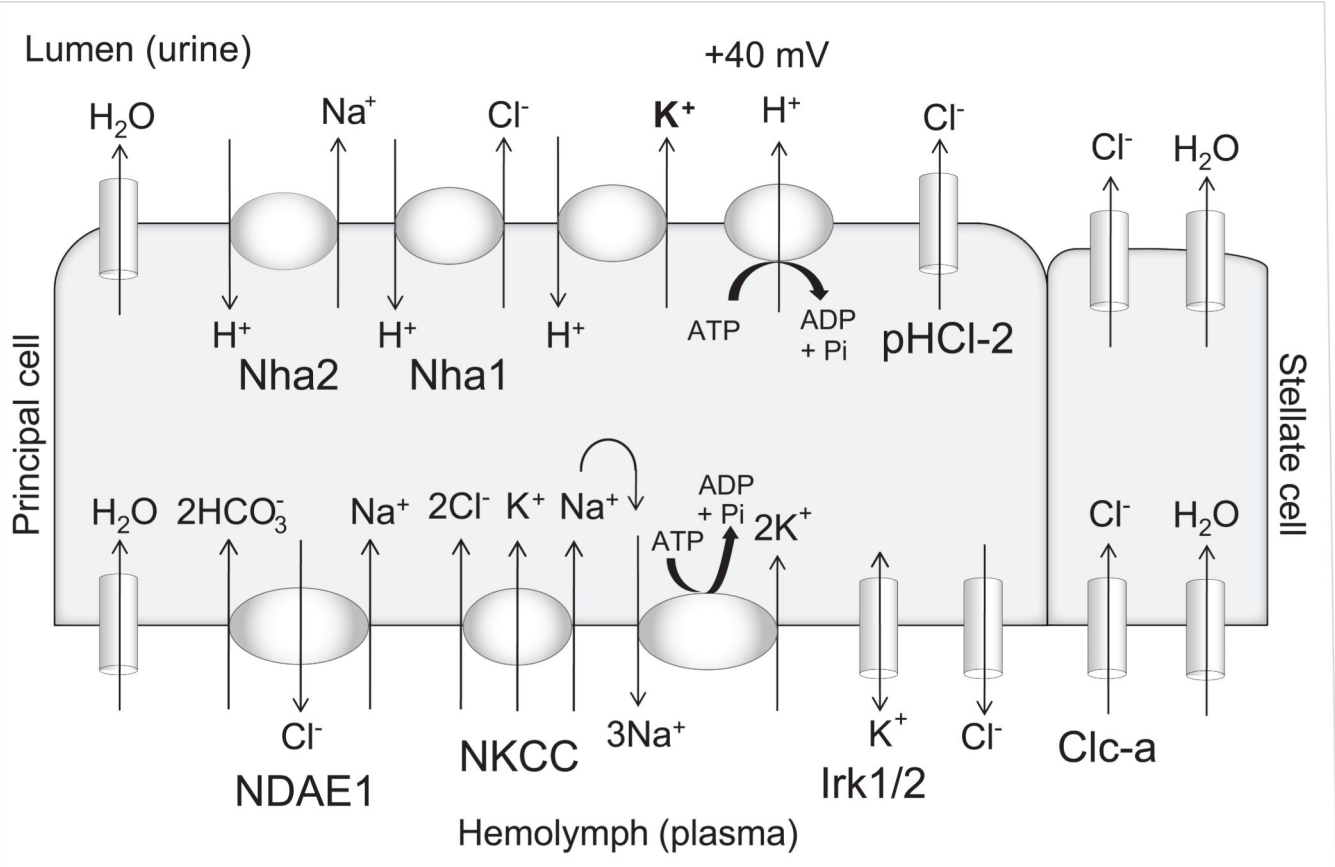

Figure 3. Cell model of the fluid-secreting Malpighian tubule main segment.

Transporters and channels described in the text are shown in the cation-conducting principal cell and anion-conducting stellate cell. In addition, Nha1 and Nha2 are apically-expressed chloride/proton and sodium/proton exchangers, respectively (52,53); the potassium/proton exchanger is unknown. The apically-expressed pentameric ligand-gated chloride channel, pHCl-2, has a functional role in cAMP-stimulated fluid secretion (54). NKCC and the sodium/potassium-ATPase (Na^+/K^+-ATPase) are required for normal transepithelial potassium flux (28). The SLC4 family sodium-driven anion exchanger NDAE1 is localized to the basolateral membrane of the principal cell (55,56). Basolateral potassium and chloride conductances have been demonstrated (57); the identity of the chloride channel is unknown, while inwardly-rectifying potassium channels, *Irk1* and *Irk2*, have a demonstrated functional role in transepithelial ion flux and fluid secretion (37). One study has demonstrated a small cell-to-bath driving force for potassium across the basolateral membrane (57), while others have suggested that basolateral potassium channels allow uptake of potassium from the hemolymph into the principal cells (32), as occurs in the *Formica* (ant) tubule when extracellular potassium is high (58,59). The direction of potassium flow through the basolateral potassium channels may therefore depend on extracellular potassium concentration, which in normal conditions is ~26 mM (60,61). Principal cell knockdown of *Irk1* and *Irk2* has additive effects with ouabain on decreasing fluid secretion and

transepithelial potassium flux, suggesting that these channels are not solely recycling potassium entering through the Na^+/K^+-ATPase (37).

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Table 1.

Malpighian tubule kidney stone models.

Model	Stone type	Chemical Analysis	References
High oxalate feeding	Calcium oxalate	X-ray diffraction	132
Bathing tubules in Na oxalate (*ex vivo*)	Calcium oxalate	Not done	137
Ethylene glycol feeding	Calcium oxalate	Energy-dispersive X-ray spectroscopy	133
Melamine feeding	Chemically complex (carbon, oxygen, phosphate, chloride, calcium)	Energy-dispersive X-ray spectroscopy	133, 134
Knockdown of *xanthine dehydrogenase* or allopurinol feeding	Xanthine, hypoxanthine, and hydroxyapatite	Fourier transform infrared spectroscopy, high performance liquid chromatography-mass spectrometry, micro X-ray absorption near edge spectroscopy	135