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# Transporters and tubule crystals in the insect Malpighian tubule

**Carmen J Reynolds**[1], **Daniel R Turin**[1,2], **Michael F Romero**[1,3]

[1] Physiology and Biomedical Engineering, Mayo Clinic College of Medicine & Science, 200 First Street SW, Rochester, MN 55905, USA

[2] University of Minnesota-Rochester, 111 South Broadway, Suite 300, Rochester, MN 55904, USA

[3] Nephrology and Hypertension, Mayo Clinic College of Medicine and Science, 200 First Street SW, Rochester, MN 55905, USA

## Abstract

The insect renal (Malpighian) tubules are functionally homologous to the mammalian kidney. Accumulating evidence indicates that renal tubule crystals form in a manner similar to mammalian kidney stones. In *Drosophila melanogaster*, crystals can be induced by diet, toxic substances, or genetic mutations that reflect circumstances influencing or eliciting kidney stones in mammals. Incredibly, many mammalian proteins have distinct homologs in *Drosophila*, and the function of most homologs have been demonstrated to recapitulate their mammalian and human counterparts. Here, we discuss the present literature establishing *Drosophila* as a nephrolithiasis model. This insect model may be used to investigate and understand the etiology of kidney stone diseases, especially with regard to calcium oxalate, calcium phosphate and xanthine or urate crystallization.

## Introduction

The Malpighian tubules (MTs) are the renal organ for insects. These tubules provide osmoregulation, electrolyte balance, and waste-elimination in a manner analogous to the mammalian kidney. These functional similarities allow the insect to serve as a dynamic *in vivo* model to experimentally probe many translational scenarios. Experimental studies have focused on the *Drosophila melanogaster* model because (a) ~70% of their genes have human homologs; (b) the entire genome has been sequenced and annotated for >20 years [1•]; (c) FlyBase curates *Drosophila* databases, education, resources and tools; and (d) several international consortia (e.g. VDRC, Bloomington, Berkeley) maintain mutants and RNAi fly lines for every expressed gene in the genome. Additionally, insect experimental models can be maintained with minimal costs and produced in rapid generation timelines (e.g. two weeks for F1 adult). With these numerous benefits, the *Drosophila* model is a valuable tool for elucidating renal function and renal diseases which are typically investigated in mammals.

Corresponding author: Romero, Michael F (Romero.Michael@mayo.edu).

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Nothing declared.

Emerging evidence has revealed that insects produce renal crystals with remarkable similarity to mammalian kidney stones [2,3••,4••,5••]. Nephrolithiasis (i.e. kidney stones) affects approximately 10% of the United States population [6]. Additionally, nephrolithiasis is a care and longevity concern for companion animals [7,8•]. Presently, experiments on insect renal crystals have provided productive and translatable evidence for conditions already known to cause nephrolithiasis. Here, we review recent developments on renal crystals in *Drosophila* and reflect on potential avenues of research.

## Malpighian tubule crystals

Nephrolithiasis describes the presence of stones and calcifications that appear specifically in the mammalian kidney; however, the term does not apply to crystals in insect tubules. As insect and mammalian renal organs differ, one cannot refer to insect renal tubules as nephrons or kidneys, nor MTs crystals as kidney stones. Thus, crystallization in insect MTs are noted as tubulolithiasis, and the resulting crystals will be termed tubuloliths.

In the *Drosophila* model of nephrolithiasis, tubuloliths appear within the MT lumen. *Drosophila* have anterior and posterior MT pairs with each pair meeting and emptying into the gut through a common ureter [9,10]. All tubules consist of a single layer epithelium made up of two cell types [2,11]: principal and stellate cells. Principal cells are the most abundant cell type within MTs and transport ions though proton gradients created by $H^+$-ATPase on the apical and basolateral surfaces [12,13•]. The basolateral surface of principal cells also allows ion movement via $Na^+$- and $K^+$-ATPases and the $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger, NDAE1 [14•,15•]. Stellate cells are dispersed amongst the principal cells. The stellate cells express apical and basolateral transporters which allow for net salt movement from hemolymph to tubule lumen [13•,16••,17•]: aquaporins (Drip and Prip, respectively) and chloride transporters (secCl and Clc-a, respectively). Together the principal and stellate cells transport ions and water into the lumen which, under certain conditions, can promote solute precipitation and crystal, that is, tubulolith, formation.

Crystal accumulation within MTs is readily identified by various microscopic techniques. Calcium oxalate tubuloliths form as organized crystal lattice structures with birefringent properties making the crystals within dissected MTs easily visualized by polarized light microscopy [3••,4••,16••] (Figure 1a,b). In addition to light microscopy, crystal identification and quantification may be accomplished by scanning electron microscopy and energy dispersive X-ray spectroscopy [3••,4••,5••,16••]. Imaging of crystals in whole flies was shown using micro-computed tomography [4••]. The many analytical strategies and the simplicity for *ex vivo* assessments offer notable benefits of the *Drosophila* tubulolithiasis model.

Composition of tubuloliths is primarily determined by experimental conditions or genetic phenotypes. Analysis of crystal composition has been done by various methods including energy-dispersive X-ray spectroscopy [3••,18], X-ray diffraction [4••], high performance liquid chromatography-mass spectrometry, micro-X-ray fluorescence and inductively coupled plasma optical emission spectroscopy [5••]. Kidney stones are classified based on composition and, thus far, only three classes have been exploited for studies in the *Drosophila* model: calcium oxalate (CaOx), calcium phosphate, and xanthine crystals.

### Calcium oxalate crystals (Figure 1)

Oxalate is absorbed by the gut (e.g. from the diet) and generated by metabolism. Dietary intake and degradation of hydroxyproline or uptake and oxidation of a toxic precursor, for example, ethylene glycol, increases mammalian blood or insect hemolymph oxalate. Oxalate transported from hemolymph into the MT lumen is readily precipitated with luminal $Ca^{2+}$, particularly at alkaline pH, that is, due to exceeding CaOx solubility. The resulting CaOx crystals form rectangular prisms shapes that are easy to identify and quantify using birefringence (Figure 1b).

### Xanthine and urate crystals (Figure 2)

Purine degradation occurs via metabolism of hypoxanthine (to xanthine, then to urate) and ultimately yields allantoin. Each of these intermediates can precipitate with luminal metals (e.g. $Ca^{2+}$, $Zn^{2+}$, and $Mg^{2+}$). There is increased crystallization at low pH.

### Calcium phosphate crystals (Figure 3)

*Drosophila* manage excess inorganic phosphate from the diet by MT secretion. This secreted phosphate can readily precipitate with $Ca^{2+}$ to form hydroxyapatite[4] MT tubuloliths.

## Nutritional aspects of tubule crystals

Dietary composition and ultimately absorption influences whether or not $Ca^{2+}$ will precipitate in the MT lumen. Medical advice for the prevention of nephrolithiasis most often directs humans and companion animals toward diets low in oxalates or purines, high in citrate, and high in fluids to minimize precipitation [19,20]. Accumulating evidence finds that insects are just as likely to succumb to tubulolithiasis from dietary factors as are mammals.

The majority of kidney stones (70–85%) [21] are composed of calcium oxalate. If a fly ingests ~0.1 μg of $Ca^{2+}$ per day (0.1–0.5 mg/kg of body weight), this is comparable to 14 mg/kg intake recommended for adult humans (i.e. 1000 mg/day for 70 kg body weight) [22–24]. Approximately 30% of $Ca^{2+}$ in the fly is temporarily retained in lipid droplets at the blind ends of anterior MTs. This stored $Ca^{2+}$ does not normally result in crystallization, but once secreted into the lumen, it may precipitate with other substrates to initiate stone formation (Figure 1a) [22,25]. By consuming mostly fruit and vegetation, the fly's diet likely includes oxalate that is particularly insoluble with $Ca^{2+}$ [16••]. Naturally occurring oxalate is prominent in cocoa, tree nuts, and green leafy vegetables (e.g. spinach) with concentrations of 0.1% (dark chocolate) and up to 2% (fresh spinach leaves) [26,27]. While oxalate-induced tubulolithiasis in *Drosophila* is nutritionally possible, is more useful as model to study the etiology of kidney stones. Indeed, when wildtype *Drosophila* are fed a formula diet containing as little as 0.01% sodium oxalate (NaOx), the luminal $Ca^{2+}$ will precipitate with the oxalate to produce CaOx crystals in the MT lumen within three days (Figure 1a) [3••,4••,28•,29]. Feeding the amino acid precursors to oxalate, for example,

[4]Hydroxyapatite refers to a group of calcium and phosphate compounds: brushite ($CaHPO_4$*2H2O), monetite ($CaHPO_4$), octa-calcium phosphate $CaH(PO_4)_3$*2½ $H_2O$), and whitlockite $Ca_3(PO_4)_2$).

hydroxyproline, can elicit CaOx tubuloliths in flies similar to inducing nephroliths in rats [3••,30].

Other nutritional aspects of nephrolithiasis have also been studied in *Drosophila.* Calcium phosphate crystals accumulate in adult MTs when flies are fed a diet high in inorganic phosphate [31••]. Purine-induced tubulolithiasis results from interruptions in the purine degradation pathway, leading to accumulations of xanthine in secretions and the appearance of xanthine crystals [5••] (Figure 2). Xanthine crystal accumulation is exacerbated by high dietary zinc ($Zn^{2+}$) [5••]. CaOx precipitation and stone prevention strategies, such as citrate, have been tested in *Drosophila* [29,32•]. These studies revealed that pharmaceutical potassium citrate [29], but not citrate from commercial juices [32•], lessens CaOx crystals by 20%−50% compared to ethylene glycol-induced controls and by 50% compared to oxalate-induced controls [3••].

Fluid intake and secretion are hypothesized to exacerbate tubulolithiasis similar to urinary supersaturation in mammals. MTs secrete water to maintain osmotic balance in the hemolymph analogous to osmoregulation by the vertebrate nephron using similar water channels [33]. Osmoregulation in insects is managed by the aquaporins Prip (basolateral) and Drip (apical) in stellate cells, and by the aquaglyceroporins Eglp4 (basolateral) and Eglp2 (apical) in the principal cells [17•] (Figures 1–3). The principal cell aquaglyceroporins also transport small water-soluble molecules such as glycerol and urea (Figure 2). Knockdown of just one of the aquaporins or aquaglyceroporins can significantly decrease the fluid secretion rate through the MTs [17•,21]. Conversely, increasing fluid intake and water secretion would dilute primary urine and could prevent MT precipitation events.

## Detoxification and waste metabolism

Metabolized waste products are eliminated via the insect MT, similar to the mammalian nephron, and can initiate lithogenesis in the renal tubule. A notable, recent history example of a lithogenic toxin was due to the misuse of melamine. In 2007 and 2008, some international nutritional companies replaced protein with melamine in dog foods and infant formula. Melamine is nitrogen-rich and falsely increases protein assessments by the Kjeldahl protein assay. Despite inadequate protein content, these food products appeared to meet dietary nutrition standards. Subsequently, melamine was found to cause renal damage and nephrolithiasis to both dogs [34•] and infants [35] who had consumed the melamine-laced foods. Chen and colleagues later tested the toxic affect in *Drosophila* and found that the flies were also susceptible to melamine toxicity and CaOx crystal effects [18].

Historically, CaOx formation is known to be caused by ingestion of other non-nutritive components, for example, ethylene glycol, pharmaceuticals, and vitamins. Ethylene glycol is an additive for brake fluid and radiator fluid to lower the freezing point during cold weather. It is metabolized to oxalate for detoxification (liver) and elimination (kidney and intestine). Ethylene glycol fed to flies also induces CaOx tubuloliths [3••]. Pharmaceuticals such as N-acetyl-hydroxyproline and Baclofen[5] both inhibit the conversion of proline to

[5]β-(4-chlorophenyl)-γ-aminobutyric acid (β-(4-chlorophenyl)-GABA.

hydroxyproline (via hydroxyproline dehydrogenase 2) and, in *Drosophila* were shown to decrease tubuloliths [36]. Pyridoxine (vitamin B6) is needed to convert oxalate to glycine. It is used to treat primary hyperoxaluria type 1 (PH1) by promoting conversion of oxalate to glycine and thus prevent CaOx crystallization. In *Drosophila,* feeding pyridoxine lessoned CaOx crystal burden but only at high doses [36]. Together, these studies illustrate that several different causes of mammalian nephrolithiasis are closely mirrored by *Drosophila* tubulolithiasis.

## Genetic predisposition for tubule crystals

Genome-wide association studies (GWAS) in humans and other mammals have revealed protein mutations that cause nephrolithiasis [37]. Interestingly, many of the mutated proteins identified in mammalian nephrolithiasis share structural and functional homology with insect proteins (Table 1). Mutations of these homolog proteins can occur in the fly by natural genetic variation, by knocking-down the gene of interest, or by transgenic expression.

One of the first examples of a naturally occurring mutation is *rosy,* which affects purine degradation. Adenine and guanine degradation follow a common pathway in animals and some plants. Purines are oxidized to hypoxanthine and xanthine and then to uric acid for final secretion and elimination (Figure 2). Insects, including *Drosophila,* along with birds express a uricase that catabolizes urate to allantoin. *Aedes aegypti* have enzymes that further metabolize allantoin to urea and glyoxylic acid [38], but this pathway does not appear to exist in *Drosophila* as the catalytic histidines are mutated [39•]. In humans, mutations in the xanthine dehydrogenase/oxidase *(Xdh)* gene cause xanthinuria type 1 evidenced by elevations in urinary xanthine and xanthine stones. *Drosophila* stood out as a promising model for studying this pathway when the homologous Xdh enzyme, *rosy,* was mutated. These *rosy* flies have decreased urate secretion, increased hypoxanthine and xanthine secretion, and MT xanthine / urate tubuloliths [5••,40,41,42•]. *Drosophila* are now being used as a model to further understand xanthinuria and uric acid transport. Allopurinol is a Xdh inhibitor and is used to minimize urate crystals in synovial fluids in patients with gout. In wildtype *Drosophila,* allopurinol inhibits Xdh and causes tubulolithiasis similar to *rosy* [5••,42•]. The same study found that xanthine crystals contain a mixture of divalent metal ions (e.g. $Ca^{2+}$, $Zn^{2+}$, $Mg^{2+}$) and that $Zn^{2+}$ secretion promotes xanthine crystallization [5••]. Urate is downstream of xanthine metabolism and can form crystals as well. In humans and mice, mutations of the $Na^{+}/H^{+}$ exchange regulatory factor 1 (NHERF-1) increases uric acid excretion and the potential for urate crystals [43]. In *Drosophila,* mutations in the NHERF-1 homologue, Sip-1, not only increased uric acid excretion, but also increased urate crystallization by perturbing MT lumen pH [42•].

Another major benefit of using the *Drosophila* tubulolithiasis model is that investigators may use the simple genetics of the Gal4/UAS system [44••] for tissue-specific RNAi knockdown or transgenic expression of target lithogenic genes. This Gal4/UAS system, imported from yeast, allows crossing the promoter-driven, Gal4-expressing flies with flies that carry the upstream activating sequence (UAS) controlling the construct of interest (RNAi or transgenic protein) [44••]. Furthermore, tissue-specific regulatory elements can be placed before the Gal4 to target expression for testing a tissue-specific function of a protein.

By directing expression, the UAS/Gal4 system efficiently isolates the genetic manipulation without causing systemic impairments [45].

The Gal4/UAS system has been vital to understanding oxalate transport and CaOx crystal formation (Figure 1). One example is the Slc26a6 $Cl^-/Ox^{2-}$ exchanger being associated with human hyperoxaluria [46•] and mouse bladder stones [47•]. *Drosophila* have a homologous protein, dPrestin (Slc26a6), that is highly expressed in the principal cells of the MT [4••]. *In vivo* studies with knockdown of dPrestin confirmed that it too transports oxalate into the MT lumen. With the knockdown isolated to only the MT, dPrestin lessened the occurrence of CaOx crystals as expected [4••,28•]. In humans, PH1 is caused by mutations in alanine glyoxylate aminotransferase [48]. Again, *Drosophila* have a homologous transporter, dAGXT, that prevents CaOx tubulolith formation by diverting glyoxylate degradation to glycine rather than oxalate. As expected, dAGXT knock-down increased CaOx crystal proliferation [36]. Human PH also includes type 2 (Glyoxylate Reductase, GRHPR) and type 3, (4-hydroxy-2-oxoglutarate aldolase, HOGA1) [49]. PH2 has a potential homolog in *Drosophila* (Table 1). Dent Disease is another hereditary, kidney stone disease caused by mutations in either ClC-5, an endosomal $2Cl^-/H^+$ exchanger, or OCRL, an inositol phosphatase enzyme [50•,51]. Both Dent disease proteins have potential homologs in *Drosophila* (Table 1). Additional studies of these homologs are needed to understand the link between oxalate precursors and luminal crystallization.

Calcium phosphate crystals are the second most common crystal type in mammalian nephrolithiasis [21]. Emerging data in *Drosophila* reveals that a sodium-dependent phosphate transporter, $NaP_i$-T, plays a major role in secreting excess phosphate from the hemolymph into the MTs [31••] (Figure 3). $NaP_i$-T is thought to be on the basolateral membrane of the principal cells. Similar to control of mammalian phosphate transport, $NaP_i$-T activity may be regulated by the fibroblast growth factor, *branchless (bnl)* and the receptor dimer, *breathless (btl)* + *klotho* [31••]. Further investigation will be needed to identify the apical phosphate transporters and verify the hormonal regulation of phosphate transporters.

Bacterial infection and diversity in the kidney has been associated with kidney stones. Bacterial extracellular proteins may influence this lithogenic effect. Gram negative bacteria (e.g. *E. coli),* have been cultured from human-derived kidney stones and are suspected to influence stone formation or growth [52•]. The *Drosophila* protein, subdued, is a member of the TMEM16 protein family expressed in the MTs and was found to exhibit defense against the gram-negative bacteria *Serratia marcescens*. Further evidence showed that when *subdued* was knocked down, the fly was more susceptible to infection [53•]. The ability to alter bacterial defense can give a unique window into the relationship between bacterial infections and kidney stone formation.

## Summary

Malpighian tubulolithiasis has evolved into a realistic model for nephrolithiasis of the mammalian kidney. Numerous studies with *Drosophila* have confirmed nutritional, toxic, and genetic situations that promote crystals and correspond to mammalian kidney stone

etiologies. Known genetic mutations that cause hereditary kidney stone diseases often have homologs in *Drosophila.* These homologs can be exploited for mechanistic evaluation of stone formation. As more crystallization mutations are identified or suspected, we believe that the *Drosophila* tubulolithiasis model will be vital for fast and efficient evaluation of those candidates and possible therapeutic targets.

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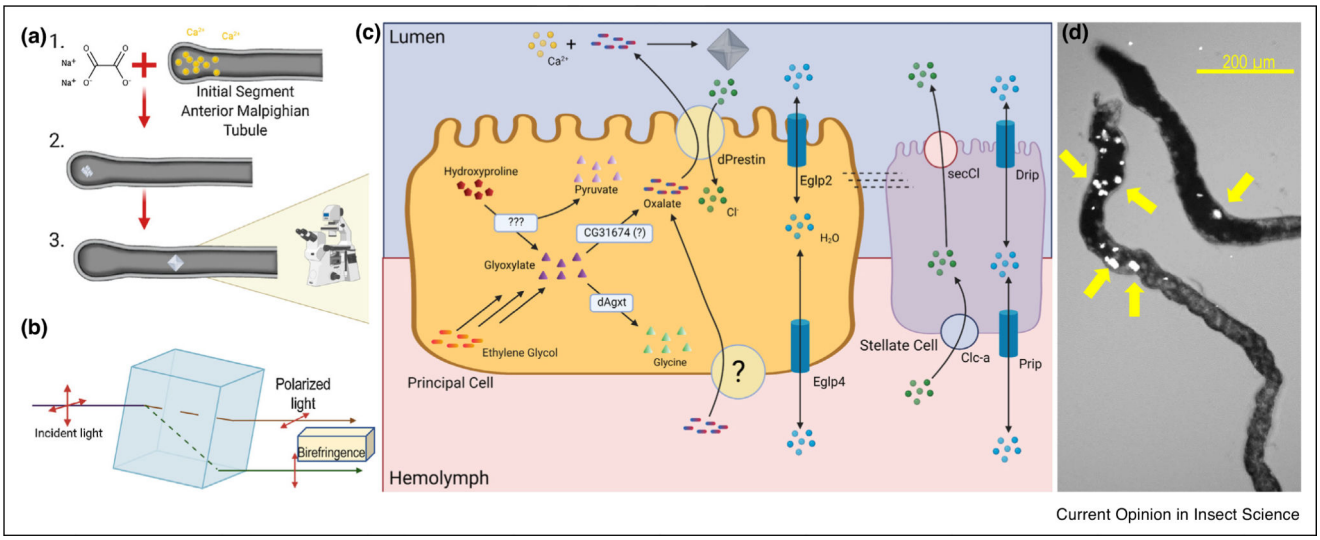


**Figure 1. Calcium oxalate (CaOx) crystallization in Drosophila renal tubules.**

Crystals in *Drosophila* Malpighian tubules are birefringent and easily visualized under polarized light microscopy: **(a)** Calcium ions that are secreted into the lumen may form precipitates that evolve into crystals. **(b)** Diagram depicting how polarized light passes through molecular crystal lattice. **(c)** Model of CaOx crystal formation. Oxalate can be transported into the principal cells from the hemolymph and/or produced from hydroxyproline or ethylene glycol precursors. Both hydroxyproline and ethylene glycol are degraded into glyoxylates in multistep pathways with hydroxyproline degradation also producing pyruvate. Glyoxylate is converted to oxalate by a proposed CG31676 protein or diverted to glycine by dAgxt. Oxalate is transported across the principal cell, apical membrane by dPrestin $Cl^-$ exchange. $Cl^-$ ions that drive oxalate export are supplied from stellate cell transporters, Clc-a (basolateral) and secCl (apical). Oxalate in the MT lumen precipitates with $Ca^{2+}$ (secreted from the blind ends of the tubule) to form CaOx crystals. Water transport by Prip, Drip, Eglp2, and Eglp4 may influence solute saturation and precipitation. **(d)** CaOx tubuloliths in anterior MT exhibiting birefringence due to polarized light. Yellow arrows indicate crystals, scale bar = 200 µm.

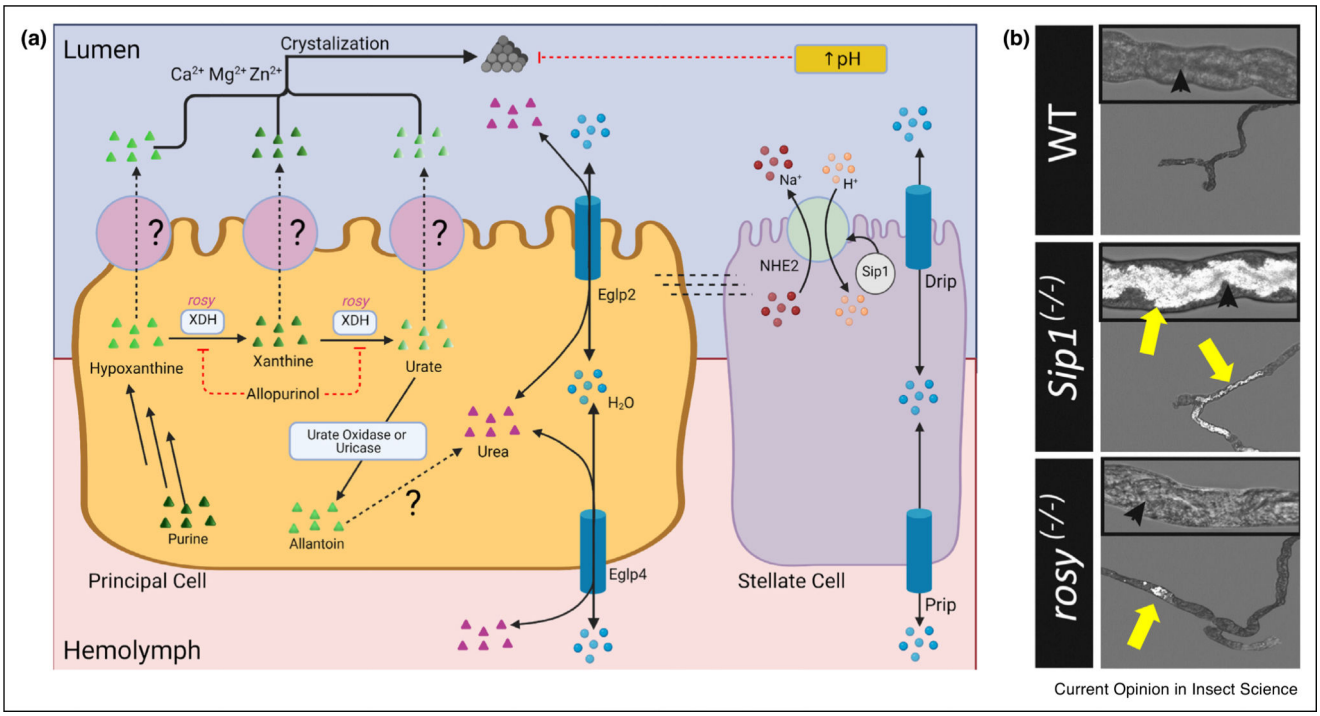


**Figure 2. Xanthine and Urate Crystallization in Drosophila renal tubules.**

**(a)** Model of xanthine and urate crystal formation. Purine degradation leads to the production of hypoxanthine. Xanthinuria dehydrogenase/oxidase (Xdh) converts hypoxanthine to xanthine, and then to urate. Urate is then oxidized by urate oxidase (i.e. uricase) to allantoin. Hypoxanthine, xanthine, urate, and allantoin are transported into the lumen by unknown transporter(s) on the apical membrane of principal cells. Allantoin is converted to urea in *A. aegypti* but this pathway has not yet been identified in other insects. Crystals can form from hypoxanthine, xanthine, or urate with the presence of $Zn^{2+}$, $Ca^{2+}$ or $Mg^{2+}$. Xdh is inhibited by Allopurinol. Crystal formation is inhibited by increased pH from NHE2 and/or Sip-1. Water transport by Prip, Drip, Eglp2, and Eglp4 may influence solute saturation and precipitation. **(b)** Urate tubuloliths in anterior MT of *Sip1*$^{(-/-)}$ mutants and xanthine tubuloliths in *rosy*$^{(-/-)}$ mutants exhibit birefringence due to polarized light (reproduced with permission from Ref. [42•]). Yellow arrows indicate the tubuloliths; black arrowheads mark MT lumen.

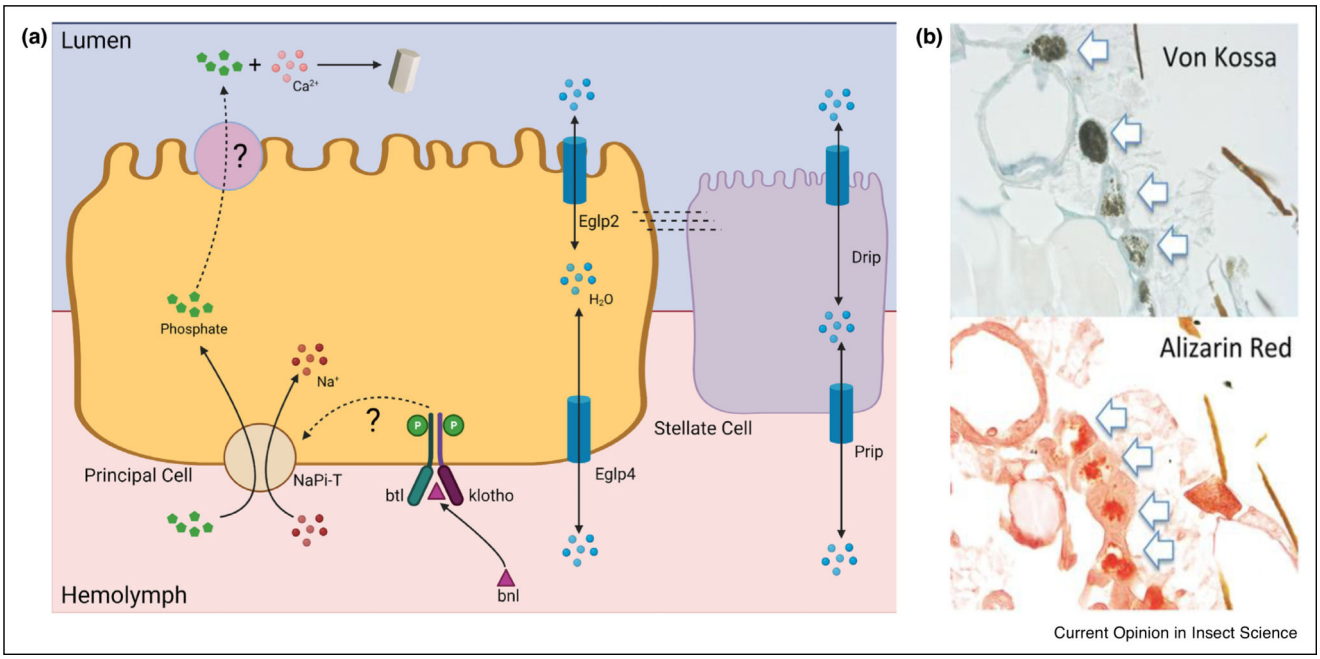


**Figure 3. Calcium phosphate crystallization in Drosophila renal tubules.**

**(a)** Model of calcium phosphate crystal formation. Phosphate is transported from the hemolymph to the tubule lumen by a basolateral $NaP_i$-T and an unknown apical phosphate transporter. Phosphate transport may be regulated by binding of fibroblast growth factor, *breathless* (btl), to a receptor complex, such as the *branchless* (bnl) + *klotho* dimer. Water transport by Prip, Drip, Eglp2, and Eglp4 may influence solute saturation and precipitation. **(b)** Paraffin sections of phosphate-fed *Drosophila* are stained with Von Kossa stain (phosphate, black precipitate; top) or Alizarin Red stain (calcium, red stain; bottom). Arrows indicate positive staining tubuloliths in anterior MT (reproduced with permission from Ref. [31••]).

**Table 1**

Conserved homologs of human kidney stone diseases in *Drosophila melanogaster*

| Diseases | Human Mutation | Fly Ortholog | References |
|---|---|---|---|
| PH1/PH2 | Slc26a6 | dPrestin | [4▪▪,28▪] |
| Xanthinuria | Xdh | Xdh (rosy) | [5▪▪,40,41,42▪] |
| Uric Acid Stones | NHERF-1 | Sip1 | [42▪] |
| Primary hyperoxaluria type 1 | AGXT | dAgxt | [4▪▪,36] |
| PH2 | GRHPR | CG31674[a], unconfirmed | X |
| PH3 | HOGA1 | Unknown | X |
| Dent Disease Type 1 | ClC-5 | Clc-c, unconfirmed | X |
| Dent Disease Type 2 | OCRL | OCRL, unconfirmed | x |

[a]CG31674 is most often predicted as a GRHPR-homolog but this is unconfirmed. Some algorithms do designate CG31674 as a HOGA1- homolog.