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Kidney Stones and the Zip10 Transporter: An Analysis of Calcium Oxalate Stone Formation Using Frog and Fruit Fly Models

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Abstract

Kidney stones are a health ailment (~1% population occurrence), yet the mechanism and initiation of stone formation remains incompletely explained. Calcium oxalate (CaOx) stones seem to initially form on sites called Randall's Plaques that are high in zinc content. A suggested correlation between stone disease and the Zip10 transporter in a GWAS (genome wide association study) on Miniature Schnauzers allowed me to investigate the role of zinc and Zip10 on CaOx stone formation. Our results from radioactive ^{63}Zn uptakes in injected frog (*Xenopus laevis)* oocytes indicate that (human, canine and *Drosophila*) Zip10 transport Zn^{2+}. The Zip10 clones had highest function at pH 7.5, with lower uptakes at both acidic pH and alkaline pH. The effects of Zn^{2+} and Zip10 on CaOx stone formation in a fruit fly (*Drosophila*) model are also presented in this paper. *Drosophila* Zip10 knockdown (KD) flies were created and compared to wild type (WT) flies in tubule experiments using *in vitro* Malphigian Tubule (MT) experiments. MTs taken from WT and KD flies and immersed in sodium oxalate (NaOx) solutions develop CaOx crystals (the fly form of stones) within 45 minutes. When Zn^{2+} was added to immersion solutions, crystal volume in both WT and KD flies increased. Cd^{2+} (cadmium, another group IIB transition metal) addition to immersion solutions also increased crystal volume. KD flies formed larger crystals than WT flies among all solutions in tubule immersion experiments. WT flies formed more crystals than KD flies among all solutions besides the Cd^{2+} + Zn^{2+} + oxalate solution during tubule immersion experiments. In feeding experiments, KD flies formed smaller crystals with Zn^{2+} present than without Zn^{2+}. The difference in results between feeding and tubule immersion experiments is likely attributed to gut absorption of solutes. Our results indicate that WT flies formed larger crystals when zinc was present in an oxalate rich diet, and KD flies formed larger crystals when an oxalate rich diet lacked Zn^{2+}. Both a knockdown of Zip10 and the addition of Zn^{2+} to immersion solutions and diet altered crystal formation. The present study suggests that Zip10 and Zn^{2+} play a role in CaOx crystal formation. Our results support previous studies on the correlation between kidney stones and Zn^{2+} intake. Further studies should determine the exact mechanism of Zn^{2+}/Cd^{2+} transport by Zip10, as well as the relationship between the Zn^{2+}/Cd^{2+} transporter and CaOx stones.

Introduction

Kidney stones are a painful and expensive health ailment and 12% of United States residents can expect to have a kidney stone in their lifetime (Sierakowski et al., 1978). About 1.3 million people in the United States labor force (ages 18-64) receive kidney stone treatment per year, which accounts for 4.5 billion dollars spent on assessment, hospitalization and treatment annually (Saigal et al., 2005). Kidney stones are associated with other long-term health complications such as hypertension, myocardial infarction, diabetes, and stroke (Domingos and Serra, 2011). Up to 80% of all kidney stones are composed of calcium oxalate (CaOx) (Coe et al., 2005).

Like humans, dogs can also develop stones, the most susceptible being Miniature Schnauzers, for which the incidence is ~40% (Furrow, unpublished data). Furrow (unpublished data) conducted a Genome Wide Association Study (GWAS) on both control and CaOx stone-forming Miniature Schnauzers and found a critical region on canine chromosome 37 with 18 possible protein-coding genes, all of which were found on human chromosome two. Among these genes was Slc39a10 (Zip10). The ZIP (Slc39) family of transmembrane transporter proteins are involved in the uptake of zinc, iron and manganese by various cell types (Nam and Knutson, 2012); however, not all of the Slc39 proteins have been demonstrated to transport Zn^{2+}. Since kidney stone disease has been associated with dietary zinc intake in humans, the mechanism of Zn^{2+}transport by Zip10 has become important to understand (Tang et al., 2012).

CaOx stones are associated with complexes called Randall's Plaques (found in the interstitium of the kidney), which are high in zinc (Zn^{2+}) content (Evan et al., 2003; Blaschko et al., 2013). Thus, the possibility that Zip10 transports Zn^{2+} makes it an intriguing and important protein to study for understanding stone formation. Homology in structure and sequence between the Zip10 intracellular domain and that of infectious prion proteins has been found through *in silico* analysis of protein domains (Schmitt-Ulms et al., 2009). This suggests, that if in fact Zip10 is involved with CaOx stone formation, initiation sites may be formed by a mechanism incorporating self-aggregation of the transporter protein. Although the known role and function of Zip10 remains incomplete, these characteristics make it attractive to study in the context of CaOx stone formation. For this thesis, I hypothesized that Zip10 is a contributing factor in CaOx kidney stone formation.

In the present study, to analyze the effect of Zip10 on kidney stone formation, a RNAi knockdown line of fruit flies (*Drosophila*) was created using the GAL4/UAS system, with a specific knockdown restricted to the principle cells of the Malpighian tubule (MT) (analogous to the human kidney). The *Drosophila* renal system, composed of two Malpighian tubules (**Figure 1**), is a legitimate model to study human renal disease because ion and fluid homeostasis is a highly conserved process within animals (Dow and Romero, 2010). In these experiments, *Drosophila melanogaster* was used as model to view, manipulate, and analyze CaOx crystal formation (Hirata et al., 2012; Blaschko et al., 2013). CaOx crystals can be induced to form either by dietary intake of oxalate or through immersion of micro-dissected

Malpighian tubules (MT) in oxalate-containing solutions. CaOx crystal formation in micro-dissected Malpighian tubules can be viewed in real time and quantified.

The effects of zinc, or other transition metals, on CaOx crystal formation in wild type (WT) *Drosophila* have not previously been reported, nor has the effect of a Zip10 knockdown on crystal formation in oxalate or oxalate plus metal solutions. In the present study both WT and *Drosophila* Zip10 (dZip10) (CG10006) knockdown mutant flies were used for *ex vivo* tubule and *in vivo* feeding experiments in both oxalate and experimental solutions including cadmium, zinc, and manganese. On average, CaOx crystals form in *ex vivo* experiments after 45 minutes and in *in vivo* experiments after 48 hours (**Figure 2**). Unlike the formation of stones in mammals, which occurs over several years, *Drosophila* develop crystals quickly (tens of minutes rather than months) and thus provide an expedited model to view and analyze the process of CaOx stone formation.

Since the exact mechanism of Zip10 transport remains unknown, I attempted to elucidate the Zip10 transport mechanism using oocytes from the African-clawed frog (*Xenopus laevis)*. Moreover, I used *Drosophila* as a model of CaOx stone formation to determine if Zn^{2+} and other metals affected CaOx crystal formation in wild type and dZip10 knockdown mutant flies. I hypothesized that a knockdown of Zip10 as well as the addition of Zn^{2+} to the diet and MT immersion solutions would alter CaOx crystal formation in the *Drosophila* model of kidney stones.

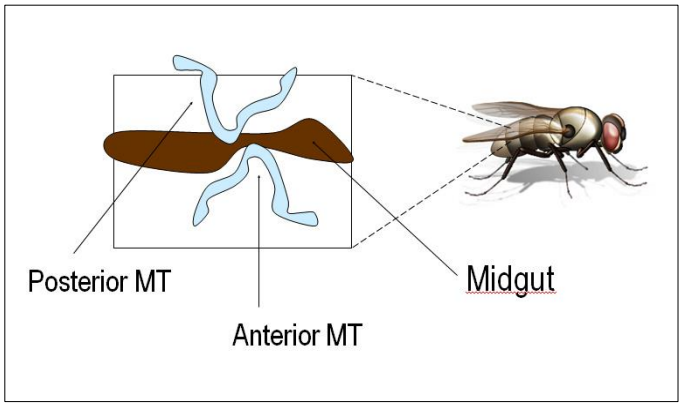

Figure 1: Anterior and posterior Malpighian tubules (MT) are removed via micro-dissection.

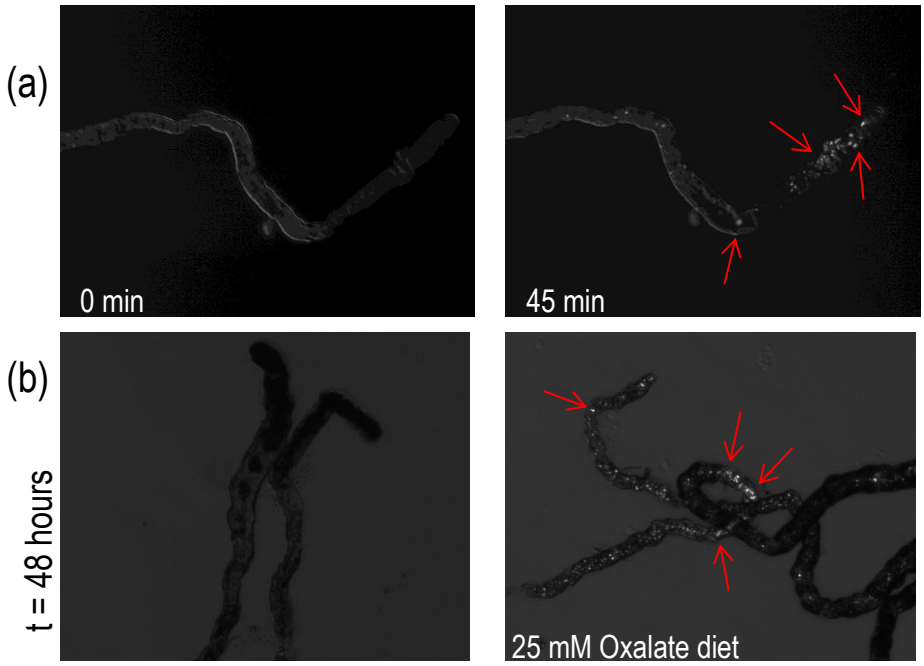

Figure 2: Preliminary results: (a) CaOx crystals appear in WT Malpighian tubules (MT) 45 minutes post- dissection when incubated in an oxalate solution. (b) MT from flies 48 hours after being placed on oxalate specific diets. CaOx crystals can be visualized and quantified with AxioVision software on a Zeiss microscope. Red arrows indicate examples of CaOx crystals.

Materials and Methods

Drosophila Zip10 Cloning

The *Drosophila* homologue of Zip10, dZip10, was amplified using primers (TH366-365 and TH612-612) and cloned into the *Xenopus* oocyte expression vector pGEMHE. The new construct was then used to transform *E.coli*, and colonies with the ampicillin resistance gene were selected. A single *E.coli* colony was expanded in Laria Broth (LB) plus 50 μg/mL ampicillin for 16-18 h at 37°C. The liquid culture was pelleted, and plasmid DNA was isolated via QIA Mini and Maxi prep procedures. Copy-RNA (c-RNA) was synthesized after the plasmid was linearized (using *Not I*) and purified using a Gel/PCR DNA fragment extraction kit. dZip10 cRNA concentration was measured with a Nanodrop by Optical Absorbance at 260 nm. The cRNA solution was then diluted to 0.5μg/μL for oocyte injection.

$^{63}Zn^{2+}$ uptake experiments: Xenopus laevis oocytes injected with cRNA from human, canine and fruit fly

Human Zip10 (hZip10) and canine Zip10 (dogZip10) cRNA were obtained from the Romero Lab (Mayo Clinic). One hundred *Xenopus* oocytes were injected with water (25 nL/oocyte), 100 with 12.5 ng human Zip10 (hZip10) (25 nL 0.5 μg/μL cRNA per oocyte) and 100 with 12.5 ng *Drosophila* Zip10 (dZip10) (25 nL 0.5 μg/μL cRNA per oocyte). Uptake experiments were performed three days post injection. Oocytes were pre-incubated in ND96 (96 mM NaCl, 2mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) and then incubated for 30 minutes in ND96 containing 10 μM ZnCl and radioactive ^{63}Zn (PET isotope), with varying pHs of 6, 7.5 and 9 (Hirata et al., 2012). Uptake conditions included ND96+10μM $ZnCl_2$+

$^{63}Zn^{2+}$ and varying pH solutions of pH 6.0 (MES buffer), pH7.5 (HEPES buffer), pH 9.0 (Tris buffer). Oocytes were then washed four times with ND96 containing 1 mM $ZnCl_2$ and $^{63}Zn^{2+}$ uptake was directly measured using a Perkin Elmer Wizard 2480 Gamma Counter. To determine if bicarbonate might be transported with Zn^{2+}, similar experiments were repeated using *h*Zip10- injected oocytes with ND96 in which 96 mM NaCl was replaced with 96 mM $NaHCO_3$ (pH 8.3). To test if high pH rather than HCO_3^- affects Zip10 transport, the ND96 uptake solution was titrated to pH 8.3.

Zip10 Knockdown in Drosophila

Uro signifies urokinase which is a gene only expressed in the principle cells of the MT. Uro-Zip10 knockdown flies were created using the Gal4/UAS system. Uro/Gal4 virgin females were crossed with UAS-RNAi CG10006.101031/KK males obtained from the Vienna RNAi center (http://stockcenter.vdr.at/control.main.). In order to select for virgin females, larvae were isolated and flies were separated by gender just hours after emerging from pupae. Male F1 offspring were collected and used for CaOx crystallization experiments. Male flies were chosen for data collection in order to expedite the process, as there are no eggs present during dissection that interfere with removal of tubules. Dissections of both male and female flies showed that they respond similarly to solutions.

Ex Vivo Drosophila tubule dissections from tubules placed in experimental solutions

Malpighian tubules (MT) were micro-dissected from adult wild type (WT) and dZip10 KD (KD) male flies and directly placed in insect PBS (121.5 mM NaCl, 20 mM KCl, 20 mM glucose, 8.6 mM HEPES, 10.2 mM $NaHCO_3$, 4.5 mM NaH_2PO_4)

adjusted to pH 7.4 for initial (0 minute) pictures. Tubules were then immersed in various experimental solutions:

a) 7.5 mM Na-oxalate

b) $ZnCl_2$

c) 7.5 mM Na-oxalate plus 10 μm $ZnCl_2$

d) 7.5 mM Na-oxalate plus 10 μm $CdCl_2$

e) 7.5 mM Na-oxalate plus 10 μm $ZnCl_2$ plus10 μm $CdCl_2$

f) 7.5 mM Na-oxalate plus 10 μm $MnCl_2$ and

g) 7.5 mM Na-oxalate plus 10 μm $ZnCl_2$ plus 10 μm $MnCl_2$.

Pictures were taken in 30 or 45 minute increments using a Zeiss Observer microscope and Axiovision software. CaOx crystal counts and sizes were quantified using Axiovision software. Lengths (l) and widths (w) of the crystals were measured. Since height could not be measured, crystal volumes were calculated in three different ways; l*l*w; w*w*l, and l*w*(l*w/2). Averages of the three volumes were used for analysis.

In Vivo Drosophila tubule dissection from flies placed on experimental diets

Experimental diets included 1.9 grams of dry food plus 7.3 mL of 25 mM NaOx, 25 mM NaOx plus 0.05 mM $ZnCl_2$ or 0.05 mM $ZnCl_2$. Tubules were dissected from WT and KD adult male F1 flies 24, 48, and 72 hours after placement on experimental diets (3-4 flies per diet). Control WT and KD flies were placed on generic diets including 1.9 grams of dry food mixed with 7.3 mL water. Tubules were placed in insect PBS and pictures were taken immediately to analyze size and number of crystals.

Results

$^{63}Zn^{2+}$ uptake experiments: Xenopus laevis oocytes injected with cRNA from human, canine and fruit fly

Both *Drosophila* and human Zip10 transporters were shown to transport Zn^{2+}. *Drosophila* Zip10 parallels the function of human Zip10. The function of Zip10 was altered by a change in pH (**Figure 3**). Oocytes accumulated more $^{63}Zn^{2+}$ when Zip10 (human and *Drosophila*) was incubated in solution at a pH of 7.5 than at a pH of 8.5.

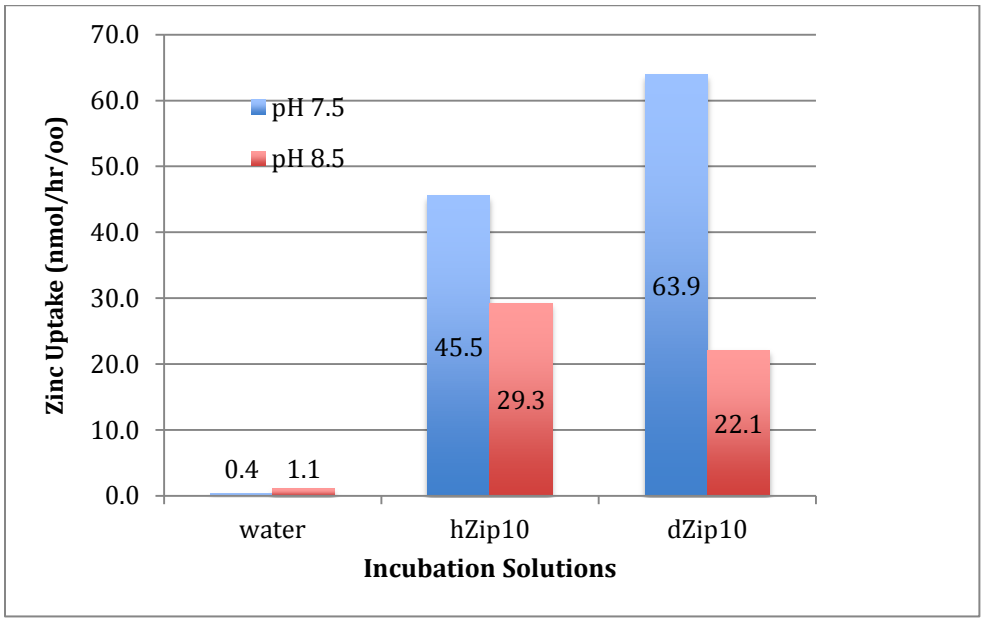

Figure 3: *Xenopus* oocyte ^{63}Zn uptake data in *Xenopus* oocytes containing human Zip10 (hZip10) and *Drosophila* Zip10 (dZip10). Oocytes incubated in solutions of pH 7.5 (blue) accumulated more $^{63}Zn^{2+}$ than solutions of pH 8.5 (red). Water (control), injected oocytes did not accumulate Zn^{2+}. Standard deviations for water at pH 7.5 and 8.5 were 0.1 and 0.2, respectively. Standard deviations for hZip10 at pH 7.5 and 8.5 were 5.6 and 4.1, respectively. Standard deviations for dZip10 at pH 7.5 and 8.5 were 8.1 and 4.1, respectively.

Although not shown in Figure 2, $^{63}Zn^{2+}$ transport by human Zip10 decreased when bicarbonate was present. Transport of zinc in solution containing bicarbonate,

at pH 8.0, and solutions lacking bicarbonate (pH 7.5) were 39 (+/-7) and 69 (+/-7) nmol/hr/oocyte, respectively.

Ex Vivo Drosophila tubule dissections from tubules placed in experimental solutions

Crystal formation, in terms of type and size, appeared to differ visually among experimental solutions as well as between fly types (**Figure 4**). Crystals appear to be larger and more prominent in dZip10 KD mutants.

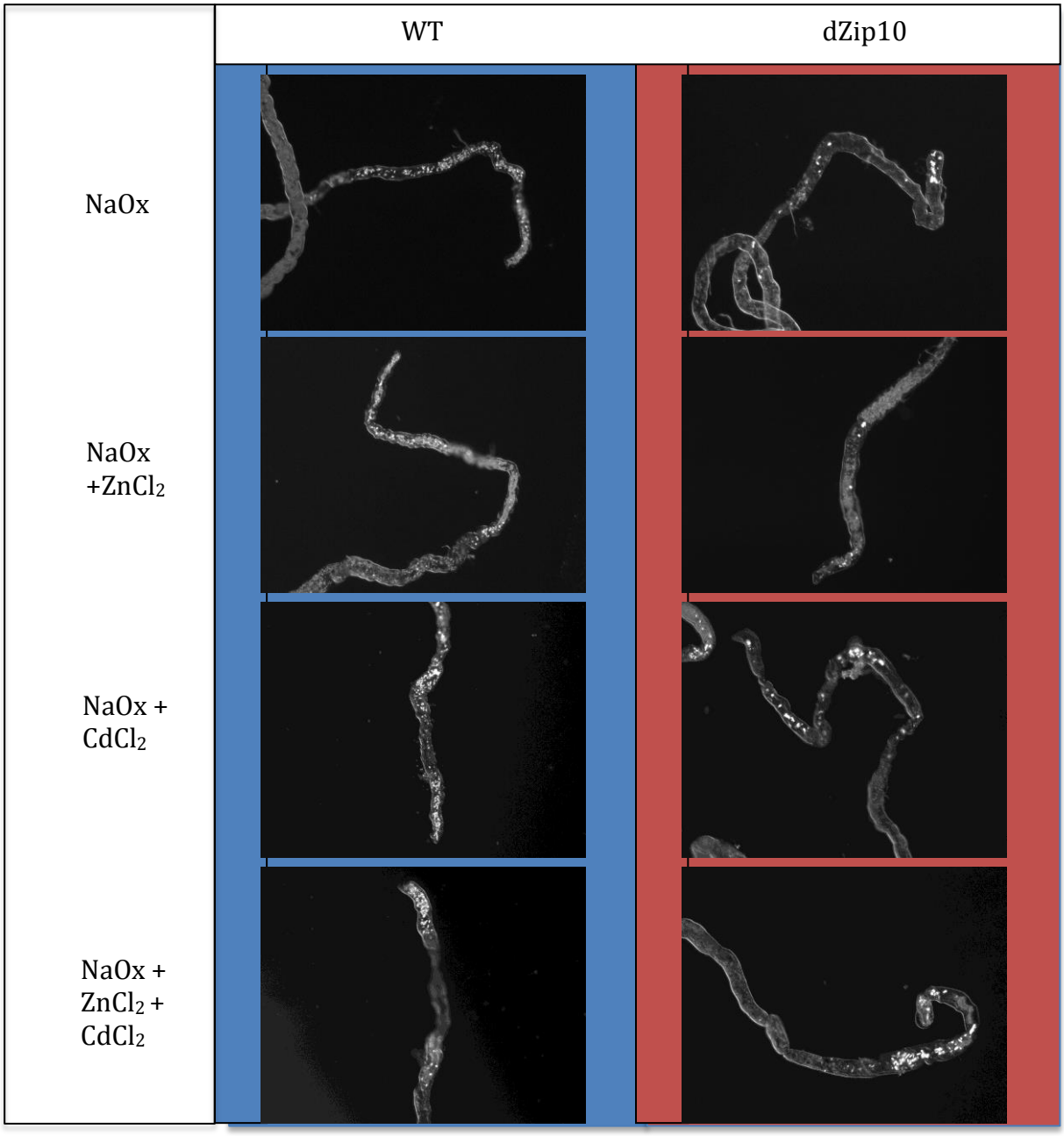

Figure 4: Tubules on the left are from male wild type flies (blue), and on the right are from male knockdown flies (red), lacking the Zip10 gene. The concentrations of the solutions are as follows: (a) 7.5 mM NaOx, (b) 7.5 mM NaOx + .05 mM $ZnCl_2$, , (c) 7.5 mM NaOx + .05 mM $CdCl_2$, (d) 7.5 mM NaOx + .05 mM $ZnCl_2$+ .05 mM $CdCl_2$.

The amount and volume of crystals also varied among solutions and between fly types (**Figure 5**). The dZip10 KD mutant flies formed very large crystals in solutions containing $CdCl_2$. Tubules in solutions containing 0.05 mM $ZnCl_2$ formed a

greater distribution of larger crystal volumes in WT flies. The distribution of crystal volumes was larger in dZip10 KD mutant flies than in wild type flies. Crystals formed in Na-oxalate solutions are more consistent and are overall smaller than crystals formed in other solutions.

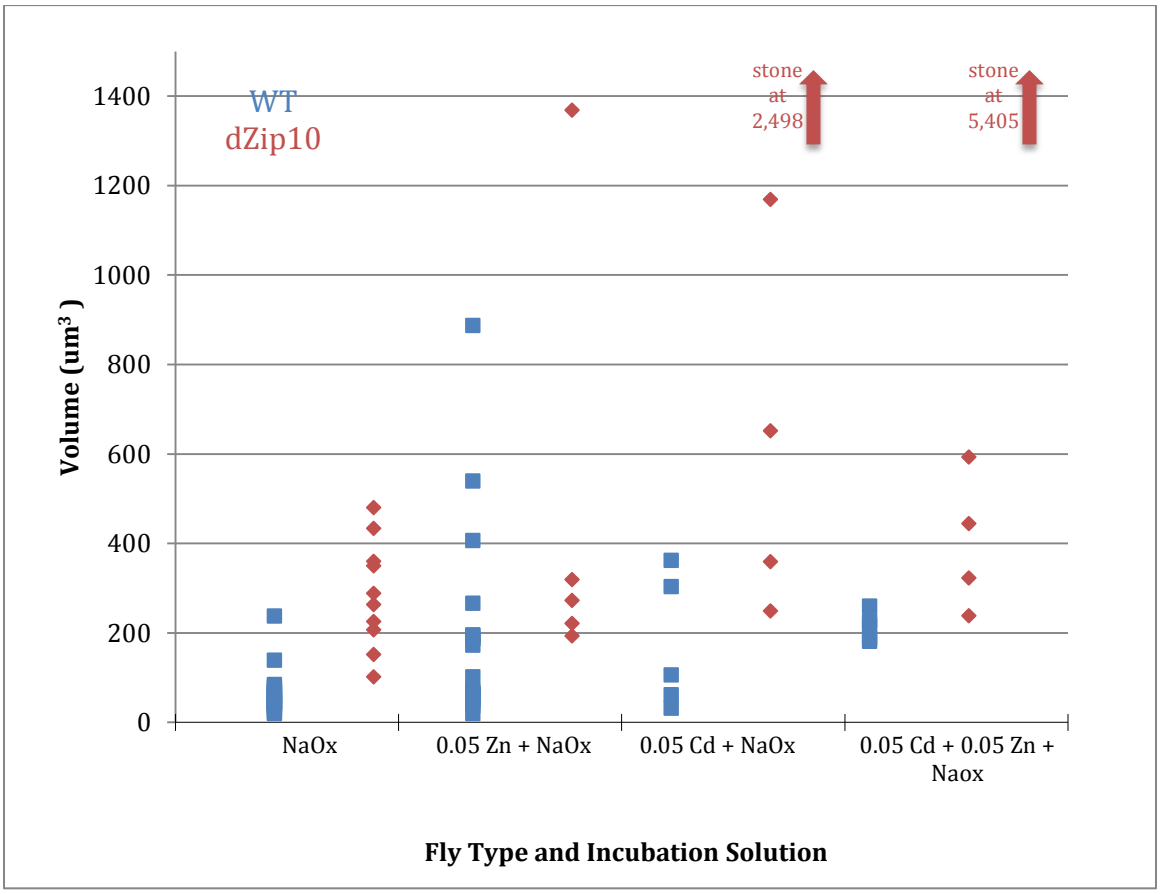

Figure 5: Individual distribution of crystal volumes between wild type (red boxes) and Zip10 knock down flies (lacking the Zip10 gene) (blue diamonds) among various solutions. Two crystals from dZip10 knockdown flies in solutions including cadmium do not appear on graph, as indicated with arrows. Larger crystals are formed in dZip10 knockdown flies. The addition of metals to solutions appears to increase crystal volume.

Average crystal volumes between solutions including oxalate only and Zn^{2+} plus oxalate are compared in **Figure 6**. Crystal volumes are larger in KD flies than in

WT flies among all solutions. The addition of 0.05 mM $ZnCl_2$ to immersion solutions also increased crystal volume.

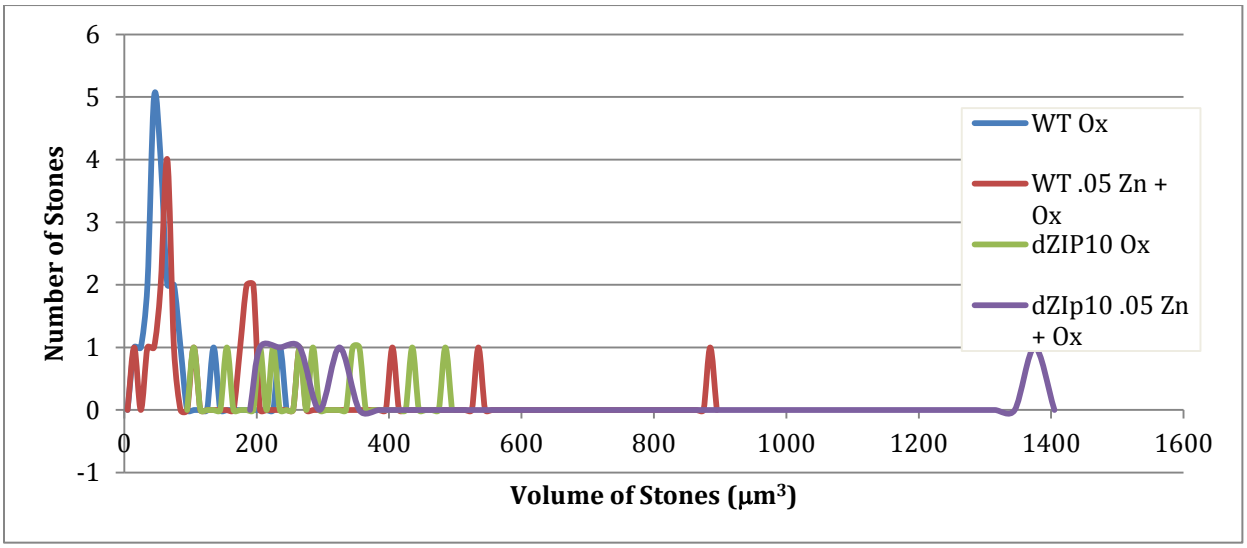

Figure 6: The distribution of crystal volumes among various solutions. Wild type flies (red and blue) form crystals at smaller volumes, while knockdown flies (purple and green) (flies lacking Zip10 gene) form crystals at larger volumes, and appear to be more variable in size. The addition of Zn^{2+} to solutions (red and purple) appeared to increase crystal volume in both wild type and knockdown flies.

KD mutant flies form larger crystals than WT flies among all solutions. The addition of $CdCl_2$ to immersion solutions greatly increases crystal volume (**Figure 7**).

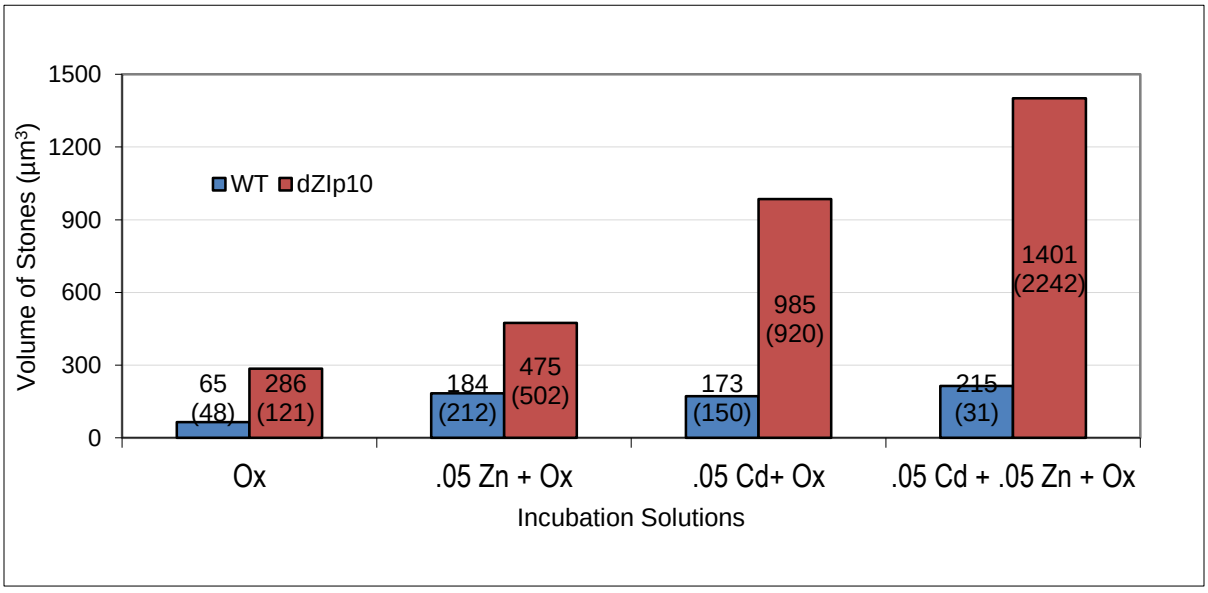

Figure 7: Average crystal volumes among solutions. Knockdown fly tubules (lacking Zip10 gene) formed larger crystal than wild type fly tubules in all solutions. Tubules incubated in solutions containing 0.05 mM Zn^{2+} and 0.05 mM Cd^{2+} formed larger crystals than tubules in control solutions (oxalate only). The addition of Cd^{2+} to incubation solutions greatly increased crystal volume in both wild type and knockdown flies. Sample size was five for all solutions containing metals and therefore standard deviations are large and indicated in parenthesis below the volumes. The presence of very large crystals in tubules contributes to high standard deviations.

The number of crystals present per 600 μm tubule differed among solutions and between types of fly as well (**Figure 7, Figure 8**). These data suggest that the average amount of crystals is greater in wild type flies than in dZip10 KD mutant flies. These data also suggest that a greater crystal count occurs in solutions containing $CdCl_2$. WT flies had a greater amount of crystals present per 600 μm tubule than KD flies.

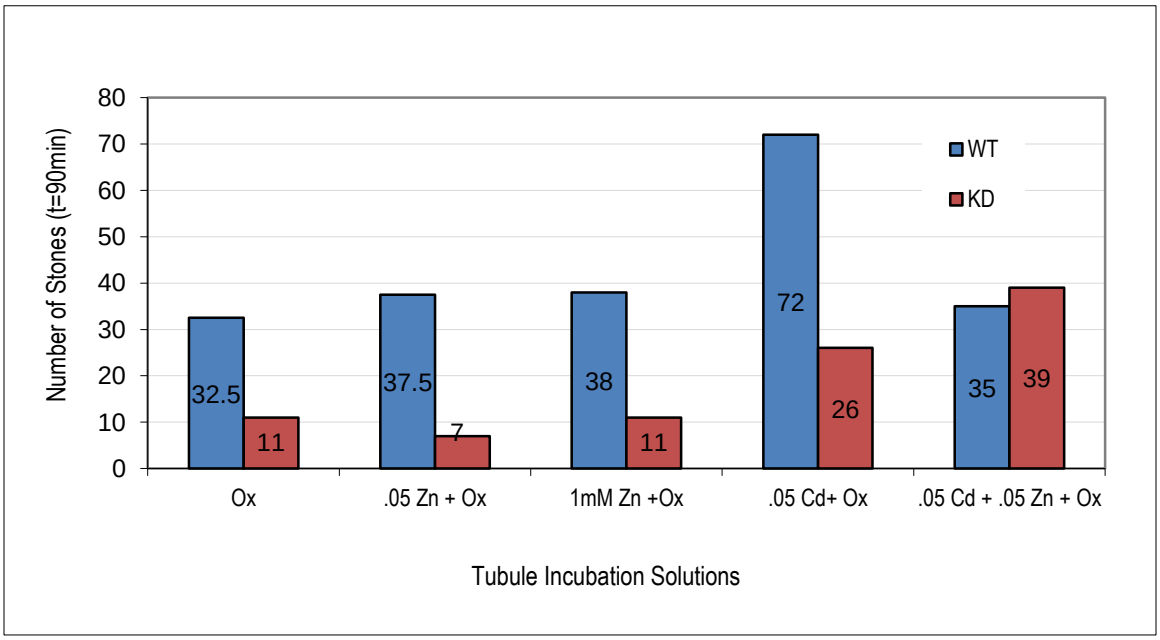

Figure 8: Average number of crystals in wild type and knockdown (lacking Zip10 gene) flies in various solutions. Tubules from wild type flies accumulated more crystals among most solutions (besides 0.05 Cd^{2+} + 0.05 Zn^{2+} + Ox) than knockdown flies. The addition of Cd^{2+} to tubule incubation solutions increased crystal accumulation in both fly types. Statistical analysis is shown below.

Statistical Analysis of Ex-Vivo Drosophila

Wild type flies formed more crystals than knockdown flies among all solutions (p-value = .037). Knockdown flies formed larger crystals than wild type flies among all solutions (p-value= .047). In wild type flies, crystal volumes were statistically smaller when incubated in solutions containing (a) oxalate than when incubated (b) Zn^{2+} plus oxalate (p-value = 0.019).

In Vivo Drosophila tubule dissection from flies placed on experimental diets

Crystal size appears to visually differ between experimental diets as well as between fly types (**Figure 9**). *d*Zip10 KD flies appear to respond differently to experimental diets than WT flies.

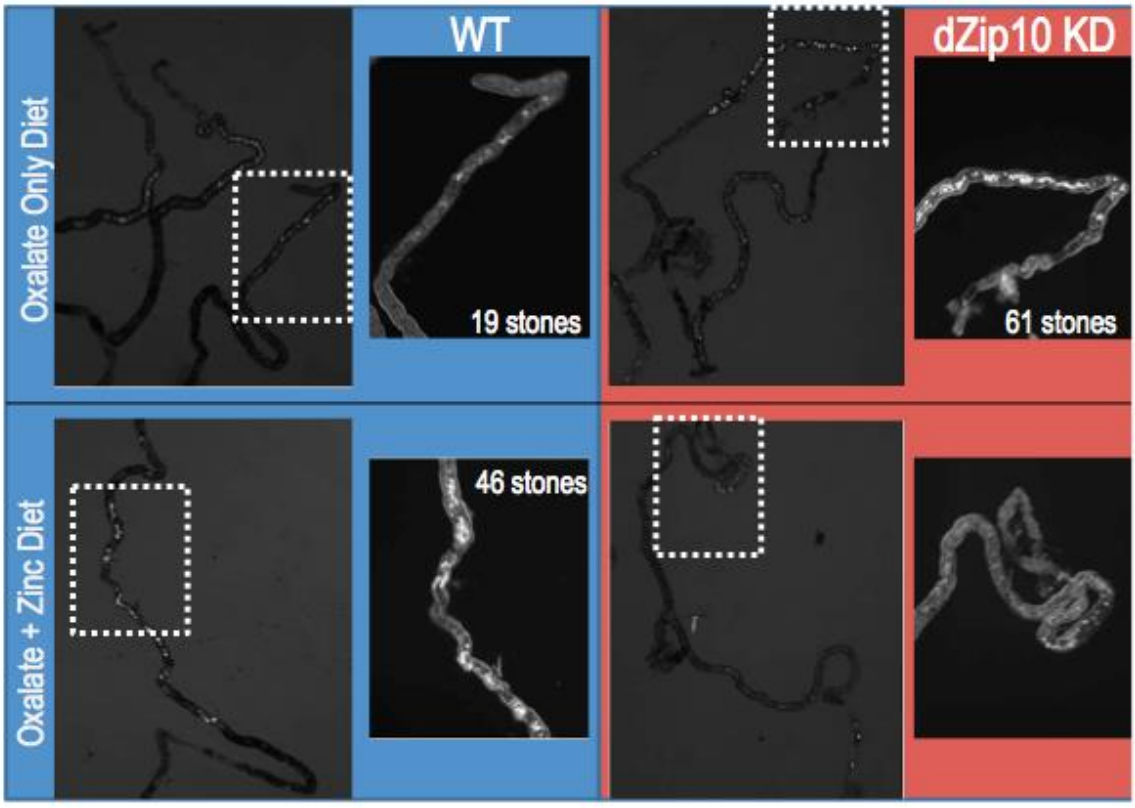

Figure 9: Pictures from tubules dissected from flies on oxalate (top) and zinc + oxalate (bottom) diets. Wild type fly tubules are in blue and knockdown (lacking the Zip10 gene) fly tubules are in red. More crystals appear to accumulate when zinc is

present in wild type fly diets, while fewer crystals appear to accumulate when zinc is present in knockdown fly diets.

WT flies form larger crystals than KD when placed on diets containing Zn^{2+}, while KD flies form larger crystals than WT when placed on diets without Zn^{2+} (**Figure 10**).

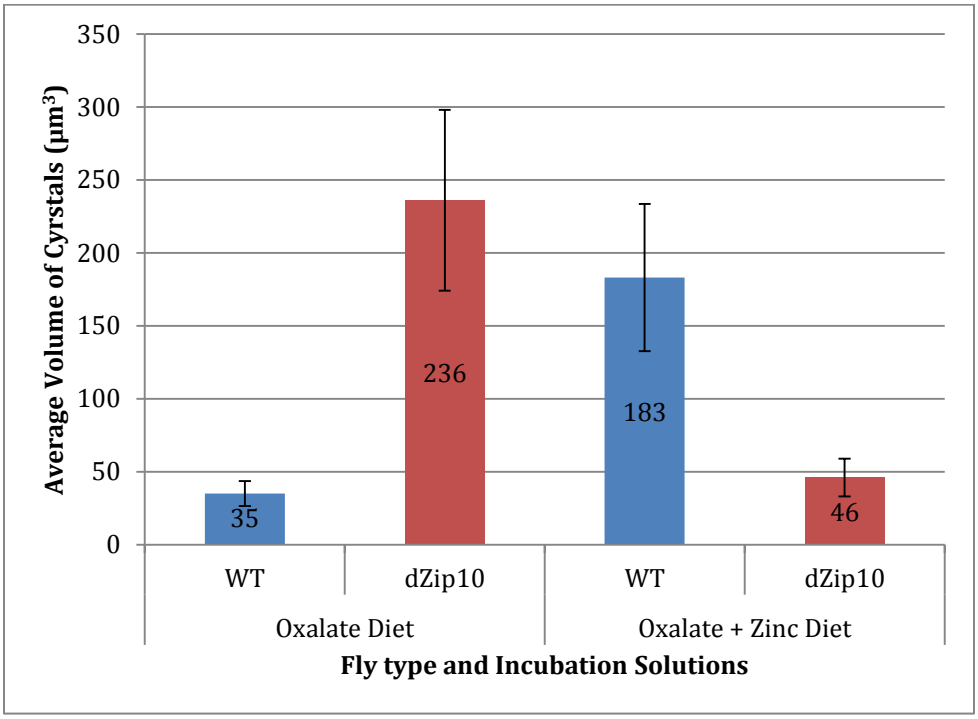

Figure 10: *In vivo* experimental data of wild type (WT) and knockdown (lacking the Zip10 gene) (KD) flies on oxalate and zinc plus oxalate diets. WT flies formed larger crystals on diets including Zn^{2+}, while KD flies formed larger crystals on diets lacking Zn^{2+}.

In Vivo and Ex Vivo Comparison of Results

In vivo feeding experimental results were not always the same as *ex vivo* MT results. Consistent results, however, were present in the comparison between the response of WT flies to solely oxalate and oxalate plus Zn^{2+} (**Figure 11**). Wild type flies formed larger crystals with Zn^{2+} present in solutions or food.

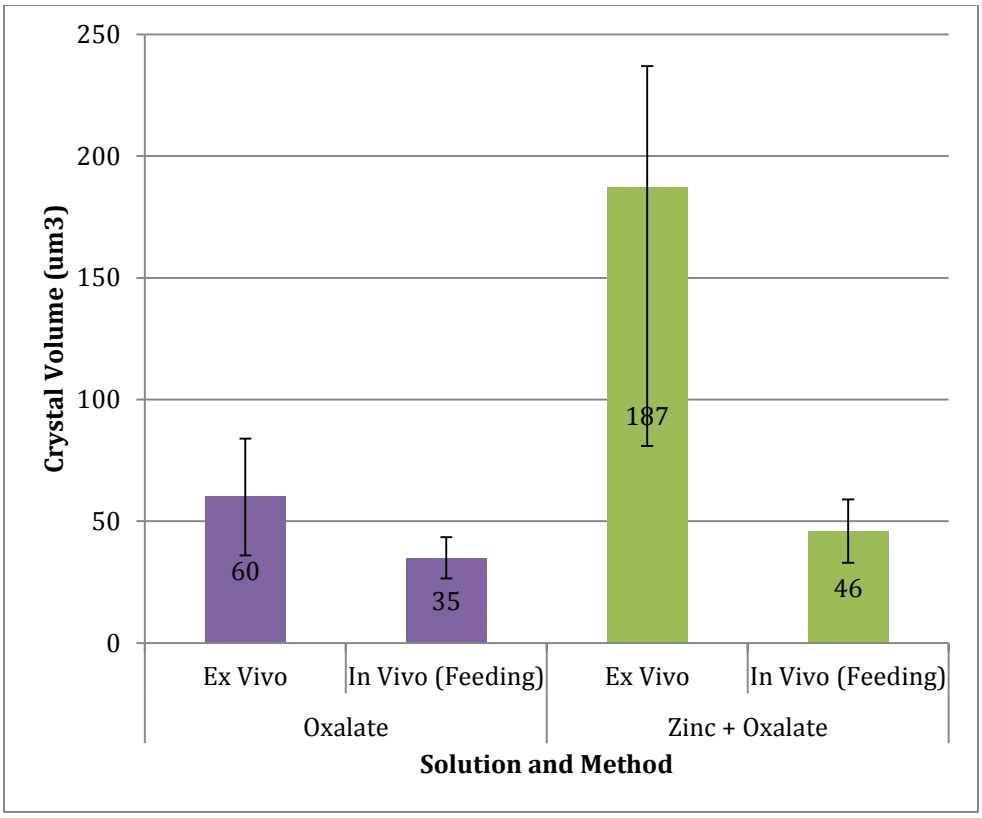

Figure 11: Comparison between *in vivo* feeding and *ex vivo* wild type fly tubules with oxalate (purple) and oxalate plus zinc solutions (green). The addition of zinc (green) to both ex vivo and in vivo solutions increased crystal volume in wild type fly tubules.

Discussion

$^{63}Zn^{2+}$ uptake experiments: Xenopus laevis oocytes injected with cRNA from human, canine and fruit fly

These results indicate that *Drosophila* Zip10 can be used as a model to study hZip10 and the contribution to human kidney stone disease by this transporter. Both clones (human and fly) transport $^{63}Zn^{2+}$, which supports the function of the ZIP family of trans-membrane transporters (Nam and Knutson, 2012). Both clones had highest transport of Zn^{2+} at a pH of 7.5, which roughly corresponds to the normal pH levels in human blood (pH 7.4). Although transport was decreased by an increase in pH, transport was not completely inhibited. Bicarbonate decreased Zn^{2+} uptake

significantly by human Zip10, but did not inhibit transport. Why this occurs remains unexplained, as neither increased HCO_3^- anion nor elevated pH (increased OH^-) increased uptake. Moreover, lower pH (6.5) did not stimulate Zn^{2+} uptake, suggesting that 7.5 may be an optimal pH. Additional studies on the mechanism of transport should be done, such as the addition of ions to uptake solutions, to determine stoichiometry, coupling, etc.

Ex Vivo Drosophila tubule dissections from tubules placed in experimental solutions

Crystal volume was increased in dZip10 KD mutant flies among all solutions and crystal count was increased in WT flies among all solutions. These results suggest that Zip10 plays a key role in CaOx stone formation. One explanation for lower crystal counts in WT flies is the decreased Zn^{2+} transport and possibly an inhibition of Randall's Plaque (initiation site for kidney stones) formation. Since we still do not know the specific direction or exact mechanism of transport, ideas on the effects of Zip10 on kidney stone formation remain speculative.

These results indicate that Cd^{2+} increases crystal volume in both WT and KD flies, suggesting that exposure to Cd^{2+} might also increase kidney crystal volume and formation in humans. Cd^{2+} has previously been linked to end stage renal disease, which allows us to delve deeper into the effects of cadmium on renal function and disease (Hellstrom et al., 2001). Since Cd^{2+} is a typical environmental toxin (i.e., heavy metal toxin), further research should be focused on kidney stone disease among populations living in areas with high levels of Cd^{2+} exposure.

Although *ex vivo Drosophila* studies can give us pertinent information about the effects of direct metal contact with tubules, we must consider how humans intake and absorb solutes. This was examined through feeding experiments.

In Vivo Drosophila tubule dissection from flies placed on experimental diets

In vivo experiments take into account gut absorption of solutes, which is important for studying the entire mechanism of stone formation and may explain the difference between *in vivo* and *ex vivo* results. In WT flies, Zn^{2+} intake increased crystal volume, which supports correlation between zinc intake and kidney stones in humans observed by Tang et al. (2012). An increase in crystal volume from an increase in Zn^{2+} availability may be correlated with Zn^{2+} being the most abundant metal in CaOx stones (Bazin et al., 2007). The relationship between Zn^{2+} and CaOx stone formation should continue to be studied, as the present study suggests a strong correlation between the two.

Knockdown flies formed larger crystals on control diets than wild type flies, and knockdown flies formed smaller crystals on diets including zinc. This suggests that the transporter does in fact have an effect on stone formation. It is possible that mutations in the transporter might also alter stone formation, prompting an additional research question on the high incidence of stones in Miniature Schnauzers.

Summary

Based on these experiments and the data gathered, I accept my hypothesis that a knockdown fly line of Zip10, as well as the addition of zinc to immersion solutions and diet, alter CaOx stone formation. Both Zn^{2+} and Zip10 have previously

been linked to the invasive behavior of breast cancer cells, so further research on this transporter and the effect of Zn^{2+} on renal disease should be performed, as they are likely linked as well (Kagara et al., 2007). Further studies should be performed on the composition of the crystals in both WT and KD flies, to determine if crystal composition differs between fly types. Additional research is needed to draw conclusions on the overall effects of a knockdown Zip10 model on renal disease and stone formation.

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