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The orphan pentameric ligand-gated ion channel *pHCl-2* is gated by pH and regulates fluid secretion in *Drosophila* Malpighian tubules.

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Statement Summary

A novel role for pentameric ligand-gated ion channels (pLGICs) beyond neurotransmission. A pLGIC regulates fluid secretion in *Drosophila* Malpighian (renal) tubules by modulating chloride transport in response to luminal pH.

Abstract

Pentameric ligand-gated ion channels (pLGICs) constitute a large protein superfamily in metazoa whose role as neurotransmitter receptors mediating rapid, ionotropic synaptic transmission has been extensively studied. Although the vast majority of pLGICs appear to be neurotransmitter receptors, the identification of pLGICs in non-neuronal tissues and homologous pLGIC-like proteins in prokaryotes points to biological functions, possibly ancestral, that are independent of neuronal signaling. Here we report the molecular and physiological characterization of a highly divergent, orphan pLGIC subunit, *pHCl-2* (*CG11340*), in *Drosophila melanogaster*. We show that *pHCl-2* forms a channel that is insensitive to a wide array of neurotransmitters, but is instead gated by changes in extracellular pH. *pHCl-2* is expressed in the Malpighian tubules, which are non-innervated renal-type secretory tissues. We demonstrate that *pHCl-2* is localized to the apical membrane of the epithelial principal cells of the tubules and that loss of *pHCl-2* reduces urine production during diuresis. Our data implicate *pHCl-2* as an important source of chloride conductance required for proper urine production, highlighting a novel role for pLGICs in epithelial tissues regulating fluid secretion and osmotic homeostasis.

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Introduction

Pentameric ligand-gated ion channels (pLGICs) constitute a superfamily of ionotropic neurotransmitter receptors that includes vertebrate cys-loop nicotinic acetylcholine, GABA, glycine and 5HT3 receptors (Jones and Sattelle, 2006; Karlin, 2002; Littleton and Ganetzky, 2000; Putrenko et al., 2005). pLGICs play a central role in mediating rapid ionotropic neurotransmission and are expressed in all characterized bilateria (Dent, 2006). These channels typically reside on post-synaptic membranes of excitable cells and open in response to binding of neurotransmitter released from presynaptic axon terminals. Ligand binding induces allosteric changes to protein conformation that result in the opening of a transmembrane, ion-selective pore that initiates the flow of specific ions down their electro-chemical gradients, altering the membrane potential of the post-synaptic cell (Lummis et al., 2005; Purohit et al., 2007). The subunits of pLGICs have a stereotypical tertiary structure that consists of three general domains: an amino terminal extracellular ligand binding domain, four transmembrane domains (M1-M4), which collectively form the ion permeable channel pore, and an intracellular loop between M3 and M4 (Dent, 2006; Hille, 2001; Sine and Engel, 2006; Tasneem et al., 2005). Functional channels can exist as homomers, or as heteromers, containing as many as five distinct channel subunits (Boulin et al., 2008).

Sequencing of invertebrate genomes has led to the recognition that the pLGIC subunit superfamily is much larger and more diverse than was previously realized based on work in vertebrate nervous systems. In vertebrates, there are five main classes of pLGICs that have been defined based on ligand specificity: the cation-selective nicotinic acetylcholine receptors, serotonin 5HT3 receptors and zinc activated receptors and the anion-selective GABA and glycine receptors (Davies et al., 2003; Le Novère et al., 2002; Ortells and Lunt, 1995). Invertebrate genomes, on the other hand, encode a greater assortment of channel types with a wider range of ligand specificities, and ligand-ion combinations than those found in vertebrates (Dent, 2006). In addition to the nicotinic acetylcholine and GABA receptors found in vertebrates, invertebrate genomes encode anion-selective acetylcholine (Putrenko et al., 2005; van Nierop et al., 2005), histamine (Gengs et al., 2002; Zheng et al., 2002), glutamate (Dent et al., 1997; Dufour et al., 2013; Kehoe et al., 2009), serotonin (Ranganathan et al., 2000), dopamine (Ringstad et al., 2009), tyramine (Ringstad et al., 2009) and pH (Mounsey et al., 2007; Schnizler et al., 2005) channels, as well as cation-selective GABA (Gisselmann et al., 2004) and proton

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(Beg et al., 2008) channels. Moreover, multiple putative invertebrate pLGICs have been identified that cannot be assigned to any neurotransmitter family based on sequence homology.

The biological functions of pLGICs are also likely to be much more diverse than has generally been appreciated. For instance, the cation-selective, proton-activated *pbo-5,-6* channel in *Caenorhabditis elegans* mediates an intercellular pH signal that stimulates muscle contraction (Beg et al., 2008). The proton signal is generated by a proton pump in the intestine rather than by synaptic release from neurons. The function of the *Drosophila melanogaster pHCl* channel, which is open under alkaline conditions, is not known but its expression in the nervous system and the hindgut suggests non-canonical roles in signaling and/or ion regulation (Schnizler et al., 2005). Finally the discovery of the proton-gated channel from cyanobacterium *Gloeobacter violaceus* suggests that pLGICs originally evolved to regulate ion homeostasis in response to environmental changes (Bocquet et al., 2009; Tasneem et al., 2005).

Here we show that CG11340, a putative pLGIC subunit in *Drosophila melanogaster* which we have named *pHCl-2*, forms a pH-gated chloride channel that is expressed in the Malpighian tubules, which are non-innervated secretory tissues. *pHCl-2* channels are localized to the apical (lumen-facing) membrane of Malpighian tubule principal cells, precluding a role in responding to humoral signals originating in the haemolymph. We present evidence that, instead, *pHCl-2* regulates fluid secretion by the Malpighian tubules in response to the pH of urine by controlling chloride counter-ion availability. Based on these data we propose a new role for pLGICs in ion homeostasis and implicate *pHCl-2* in a previously unrecognized mechanism regulating urine secretion, a mechanism that will enrich current models of insect secretion.

Materials and Methods

Cloning *pHCl-2* cDNA

To isolate the cDNA of *CG11340* - which we refer to hereafter as *pHCl-2* based on its electropysiological properties and the prior existence of a homologous *Drosophila* gene, *pHCl* (see below) - whole RNA was purified from adult Oregon-R flies. First strand cDNA was synthesized using oligo (dT) primers and AMV (avian myeloblastosis virus) reverse transcriptase

(Invitrogen). The *pHCl-2* open reading frame was amplified by PCR using the primers 5'-CGAATTCCATATGGATACACTTGGGATTTTCGTA-3' and 5'-TGCTCTAGATCAAAGGCAGTAGACCAGGGT-3'. The resulting PCR product was cloned into the pDONR201 vector by ligation using the NdeI and XbaI sites encoded in the primers. Using the published *Drosophila* genome sequence as a reference (www.flybase.org), two non-synonymous polymorphisms in the 527 amino acid protein predicted by this cDNA were identified: Thr^{57}-Ala and Pro^{82}-Ser. These polymorphisms were also present in the *pHCl-2* genomic DNA from the Oregon-R fly strain used to amplify the cDNA, suggesting that the polymorphisms represent naturally occurring variations in the coding sequence.

Sequence analysis

Using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/), the predicted *pHCl-2* amino acid sequence was aligned to that of the previously characterized *Drosophila* RDL GABA channel subunit to identify the presence of the primary functional domains common to pLGICs. The annotated amino acid sequence of the *Drosophila* RDL channel from Jones and Sattelle, 2006 was used as a reference to identify the extracellular ligand binding domain, the four transmembrane domains and the intracellular loop between the third and fourth transmembrane domain.

pHCl-2 expression in *Xenopus* oocytes and electrophysiology

The *pHCl-2* cDNA was subcloned into a modified pT7 *Xenopus* expression vector (Cary et al., 1994) using NdeI and XbaI cloning sites (described above). The resulting *pT7-pHCl-2* construct was linearized with BamH1 and capped RNA (cRNA) was synthesized using the mMESSAGE mMACHINE T7 *in vitro* transcription kit (Ambion). *pHCl-2* cRNA was then dissolved in RNAse-free water.

Oocytes were harvested from mature *Xenopus laevis* according to standard procedures (Goldin, 1992) approved by the McGill University animal safety committee. Oocytes were maintained at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES) and injected with 40nl of cRNA at varying concentrations (60

ngµl^{-1} - 1 µgµl^{-1}) using a Nanoject microinjection system (Drummond Scientific). Oocytes injected with 40nl water were used as controls. Oocytes were incubated at 15°C for 12-24 hours before recordings were initiated.

Injected oocytes were analyzed by two-electrode voltage clamp (TEVC) using a Maltese Cross chamber perfusion system (ALA Scientific Instruments). Oocytes were voltage-clamped at -80mV and then exposed to test compounds. Data were recorded at 1kHz using Clampex 8.1 digital oscilloscope software (Axon Instruments). The neurotransmitters tested were acetylcholine, GABA, glutamate, glycine, histamine, serotonin, dopamine, nicotine, tyramine and octopamine (Sigma-Aldrich). All neurotransmitters were dissolved in ND96 solution at pH 7.5. HCl or NaOH was added to ND96 to make solutions of varying pH. pH response curves were obtained by exposing oocytes to increasing pH (pH6 -pH9.5) separated by 30s-1 min washes at pH6. pH response curves were fit to the Hill equation using Igor Pro software (Wave Metrics) and EC_{50} values calculated.

Current-voltage (I-V) curves were generated using voltage ramps at 4mV/s. For each oocyte a voltage ramp was performed at pH 6 (closed channel) and pH 7.75 (open channel). Current (I) values at pH 6 were subtracted from those at pH 7.75 to filter out the activity of endogenous *Xenopus* channels. For ion substitution experiments, the sodium and chloride content in regular ND96 (96 mM and 103.6 mM respectively) were reduced to final concentrations of 6 mM and 13.6 mM respectively by replacing sodium chloride with either choline chloride (sodium replacement), or sodium acetate (chloride replacement).

Fly strains and maintenance

All *Drosophila* strains were maintained at room temperature and raised on a standard diet consisting of dry yeast, corn flour and glucose. *w* served as the wildtype strain. Strains bearing three independent mutant alleles for *pHCl-2* were also used: (i) w^{1118}*;Df(3R)Exel16218/TM6B,Tb* (Bloomington Drosophila Stock Center [BDSC] # 7696), referred to as pHCl-2^{Df} , contains a deletion that removes multiple genes including the entire *pHCl-2* locus, (ii) w^{1118}*; Mi{ET1}CG11340*^{MB03564} (BDSC # 23854), referred to as *pHCl-2*^{Mi} , contains a *Minos* transposable element inserted in the second exon of *pHCl-2*, and (iii) a deletion in *pHCl-2*,

referred to as *pHCl-2*$^{\Delta}$, was generated in our lab using the FRT-derived deletion technique (Parks et al., 2004; Thibault et al., 2004).

To generate *pHCl-2*$^{\Delta}$, FRT-mediated recombination was induced in flies that were trans-heterozygous for two *Piggybac* elements that flanked the *pHCl-2* locus, thereby removing the intervening DNA. The fly strains containing the relevant *Piggybac* elements were *w*^{1118}*; PBac{RB}CG11340*^{e00225} (BDSC # 17827) and *w***; PBac{WH}CG11340*^{f00507} (Exelixis Collection, Harvard Medical School # f00507). The deletion was confirmed by two-sided PCR (Parks et al., 2004) using the following primers: 5'-TCCAAGCGGCGACTGAGATG-3' is specific to the FRT bearing transgene that remains following the deletion of *pHCl-2*, and was used in both PCR reactions, and 5'-CCAGAGAATGTTCGAGTGACTACAGA- 3' and (iii) 5'-AAACCTGGGTGGGTGGAAAGTG-3', which anneal to genomic DNA proximal to the *e00225* and *f00507 Piggybac* insertion sites respectively. The resulting deletion spans 13980 bp and is predicted to remove all but the first 30 amino acids of the 527 amino acid protein. *pHCl-2* is the only coding DNA predicted to be affected by the resulting deletion.

Generation of *pHCl-2* antiserum:

The putative intracellular loop domain was chosen as an antigen for the generation of a *pHCl-2*-specific antibody because the loop displays the lowest conservation among Cys-loop LGICs (Jones and Sattelle, 2006; Talwar and Lynch, 2014). Using the primers 5'TCGCATATGGCCATGGAAACATTTGTCCCACATCTGAAG-3'and 5'-TGATGGTGATGAATTCTGGCGAAGGTCTGCGC-3', we amplified a PCR product from *pT7-pHCl-2* and cloned it into a pGEX2T vector modified to include a 6X His tag (gift from P. Lasko) that was predigested with NcoI and EcoRI using the In-Fusion cloning kit (Clontech). The resulting construct was expressed in BL21 cells and the recombinant protein was purified using His-affinity binding columns (Talon kit, Clontech). The purified *pHCl-2* antigen was used to immunize rats.

Immunohistochemistry

pHCl-2 antiserum was pre-absorbed by incubation with fixed homozygous *pHCl-2*$^{\Delta}$ wandering third instar larvae and used at a final concentration of 1:100. Anti-Rat-Alexa Fluor 488 secondary antibody (Invitrogen) was pre-absorbed by incubation with fixed wildtype (Oregon-R) embryos and used at a final concentration of 1:1000. All tissues used for immunostaining were dissected from adult females 3-7 days post eclosion. Tissues were fixed in 4% (volvol^{-1}) formaldehyde/PBS for 20 minutes, followed by two 5 minute washes in PBS with 0.3% (volvol^{-1}) Triton X-100 and one 10 minute wash in PBS with 0.1% Triton X-100 (0.1% PBT). Tissues were then blocked in PBS containing 0.1% (volvol^{-1}) tween-20 and 1% (wtvol^{-1}) bovine serum albumin (PBSt-BSA) for 30-45 minutes, incubated overnight at 4°C with primary antibody diluted in PBSt-BSA, then washed three times for 20 minutes in 0.1% PBT, and incubated overnight again at 4°C with secondary antibody diluted in PBSt-BSA. After three additional 20 minute washes in 0.1% PBT, tissues were incubated with DAPI at 0.5ngµl^{-1} in 1x PBS for 5 minutes followed by a final wash for 20 minutes in 1x PBS. Unless stated otherwise, all procedures were carried out at room temperature. Tissues were then mounted in a standard mounting medium.

Malpighian tubule dissection and Ramsay fluid secretion assays

All experiments were conducted with adult females 3-10 days post eclosion. Malpighian tubule dissections and Ramsay fluid secretion assays were carried out as described previously (Chahine et al., 2012; Dow et al., 1994; O'Donnell and Rheault, 2005). Briefly, pairs of explanted Malphigian tubules were arranged such that one tubule was bathed in a droplet of *Drosophila* saline while the other was positioned outside the droplet to allow for the collection and measurement of secreted fluid at 40 minute intervals. Fluid secretion rate (FSR) (nlmin^{-1}) was calculated by dividing the secreted droplet volume by the amount of time it took for the droplet to form. For the application of cAMP (Sigma-Aldrich), 1.5 minutes prior to the end of the first 40 minute interval, the bathing droplet (18 µl) was spiked with 2 µl of 2 mM cAMP in saline to produce a final concentration of 0.2 mM cAMP. The secreted droplet isolated after the first interval was used to calculate baseline FSRs and the secreted droplet isolated from the

second interval was used to calculate FSRs after the addition of cAMP. Mock treatments using *Drosophila* saline alone were done in parallel on separate Malpighian tubules to determine how FSRs changed between the two intervals in the absence of drug. In both conditions (mock and cAMP) the second interval FSR was normalized to the first interval FSR to quantify the relative change in secretion after treatment. The *actual* response to cAMP (presented in Fig. 5C) was calculated by dividing all the normalized FSR values in the cAMP trials by the average normalized FSR in mock conditions.

Statistical analysis

All data are presented as the mean ± SEM. Where required, data were analyzed by the Mann-Whitney rank sum test with P = 0.05 as the threshold for significance. Comparisons of the cAMP response between genotypes presented in Fig. 5B and 5C were analyzed by one-way ANOVA followed by a Tukey's post-hoc test.

Results

pHCl-2 forms a homomeric pH sensitive chloride channel

The cDNA of *pHCl*-2 was isolated from wildtype (Oregon-R) adult flies. Analysis of the corresponding protein sequence confirmed the presence of all the functional domains typical of a pLGIC subunit (Fig. 1). To determine if *pHCl-2* forms a functional ion channel, we expressed it in *Xenopus* oocytes and looked for changes in membrane conductance in response to putative ligands. Oocytes expressing *pHCl-2* did not respond to 1 mM acetylcholine, GABA, glutamate, glycine, histamine, serotonin, dopamine, nicotine, tyramine and octopamine (data not shown). Based on the homology of *pHCl-2* to a class of pH-gated Cys-loop LGICs in *Drosophila melanogaster* (*pHCl*) and *Sarcoptes scabiei* (*SsCl*) (Mounsey et al., 2007; Schnizler et al., 2005), we also tested the effects of pH on the activity of the putative *pHCl-2* channel. Similar to the previously characterized pH-gated channels, oocytes expressing *pHCl-2* and clamped at -80 mV displayed a pH-dependent inward current that was not detected in water injected oocytes, suggesting that *pHCl-2* forms a functional homomeric, pH-sensitive channel (Fig. 2A). The

pHCl-2 channel was closed at pH 6 and displayed a maximal saturating response at pH 8.75 with an EC_{50} pH of 7.84 ± 0.01 and a Hill coefficient of 2.32 ± 0.15 (Fig. 2B).

pHCl-2 contains a proline-alanine-arginine motif just upstream of the M2 domain, followed by a threonine 12 amino acids downstream in the M2 (see Fig. 1) that collectively have been shown to be highly predictive of anion selectivity (Galzi et al., 1992; Gunthorpe and Lummis, 2001). To confirm the ion selectivity of the *pHCl-2* channel directly, we generated I-V curves. In regular ND96 medium (98 mM Na^+ and 103.6 mM Cl^-), the reversal potential of the current generated by *pHCl-2* was -44.02 mV ± 0.94 mV (Fig. 2C). When the extracellular chloride was reduced to 16 mM, including 2.6 mM Cl^- from the HCl used to titrate pH), the reversal potential was shifted positively to -2.83 mV ± 0.82 mV, which is consistent with a 47 mV shift predicted by the Nernst equation (Fig. 2C). When the extracellular sodium was reduced to 6 mM, the reversal potential was -43.31 mV ± 1.07 mV, representing a negligible shift compared to the regular ND96 condition (Fig. 2C). Collectively these results demonstrate that *pHCl-2* is a chloride channel.

pHCl-2 is expressed in secretory tissues

Although pLGICs are typically expressed throughout the nervous system and in muscle cells where they mediate fast, ionotropic synaptic transmission (Sine and Engel, 2006), previously generated gene expression assays have not detected *pHCl-2* expression in the nervous system, but instead identified expression enriched in the midgut and Malpighian tubules (Chintapalli et al., 2007; Graveley et al., 2011; Remnant et al., 2016). Using polyclonal antibodies raised against the poorly conserved M3-M4 intracellular loop of *pHCl-2*, we confirmed that the *pHCl-2* protein is expressed in the Malpighian tubules. *pHCl-2* immunostaining was detected in the primary cell-type, the principal cells (Fig. 3A, arrows), but not in the smaller, less abundant stellate cells (Fig. 3A, arrow-heads). Within the principal cells, *pHCl-2* displayed a polarized distribution with immunostaining localized to the apical (lumen facing) plasma membranes (Fig. 3B, arrows). Moreover, the principal cell-specific staining observed in wildtype was absent from the Malpighian tubules of flies lacking a wildtype copy of *pHCl*-2 (*pHCl-2*$^{\Delta}$/*pHCl-2*$^{\Delta}$, *pHCl-2*$^{\Delta}$/ *pHCl-2*^{Mi} and *pHCl-2*^{Mi}/ *pHCl-2*^{Mi}), thus confirming that this signal corresponds to *pHCl-2* protein (Fig. 3C-E). We also saw primary antibody-dependent

punctate staining in the mutant Malpighian tubules that we interpret as non- *pHCl-2* staining. We could not confirm *pHCl-2* expression in the midgut with this antiserum, because we were unable to distinguish between *pHCl-2* staining in wildtype and *pHCl-2$^{\Delta}$/pHCl-2$^{\Delta}$* tissue (data not shown).

pHCl-2 regulates fluid secretion in the Malpighian tubules:

Insect Malpighian tubules are functionally similar to the vertebrate kidney and serve to maintain osmotic homeostasis in various insect orders (Phillips, 1981)**.** In *Drosophila,* the Malpighian tubules consist of two pairs of blind-ended tubes that filter the haemolymph and form the primary urine. The Malpighian tubules are made up of two main cell types, the principal cells, which mediate the transcellular secretion of Na^+ and K^+, and the stellate cells, which contribute to Cl^- secretion (Blumenthal, 2003; Cabrero et al., 2014; O'Donnell et al., 1996; O'Donnell et al., 1998). In *Drosophila,* as in other insects, transepithelial secretion is an active process powered by an electrogenic V-type proton ATPase located in the apical membrane of the principal cells (Beyenbach, 1995; Du et al., 2006; Maddrell and O'Donnell, 1992; Torrie et al., 2004; Weng et al., 2003). The ATPase extrudes H^+ into the tubule lumen generating a net lumen positive transepithelial potential (TEP). Protons are returned to the cytoplasm via apically localized proton/alkali metal antiporters in exchange for Na^+ and K^+ which are, as a result, exported into the tubule lumen. Chloride and water enter the lumen passively, flowing down their respective electrochemical and osmotic gradients (see Fig. 4). Chloride secretion is critical for the production of urine because it serves as the main negative counter-ion that reduces the magnitude of the positive TEP, thus allowing continued activity of the electrogenic proton ATPase and further development of an osmotic gradient (Beyenbach et al., 1993; O'Donnell et al., 1996).

Given its expression in the Malpighian tubules and the importance of transepithelial chloride transport to urine production we asked whether disrupting *pHCl-2* function alters fluid secretion rates (FSRs) in the Malpighian tubules. We used the Ramsey assay, which directly measures urine production in explanted Malpighian tubules (Dow et al., 1994) to compare FSRs from wildtype and three different *pHCl-2* heteroallelic combinations: *pHCl-2$^{\Delta}$/pHCl-2$^{\Delta}$*, *pHCl-2$^{\Delta}$/pHCl-2^{Df}* and *pHCl-2$^{\Delta}$/pHCl-2^{Mi}*. In standard *Drosophila* saline, wildtype Malpighian tubules

spontaneously secreted fluid at 0.45 ± 0.01 nlmin^{-1} and *pHCl-2$^{\Delta}$/pHCl-2$^{\Delta}$*, *pHCl-2$^{\Delta}$/pHCl-2^{Df}* and *pHCl-2$^{\Delta}$/pHCl-2^{Mi}* Malpighian tubules secreted at 0.41 ± 0.01 nlmin^{-1}, 0.56 ± 0.02 nlmin^{-1} and 0.48 ± 0.01 nlmin^{-1} respectively (Fig. 5A). While the FSRs from *pHCl-2$^{\Delta}$/pHCl-2$^{\Delta}$* Malpighian tubules appeared to be slightly reduced compared to wildtype, this difference was not significant ($p = 0.138$, Mann-Whitney rank sum test). FSRs between *pHCl-2$^{\Delta}$/pHCl-2^{Mi}* Malpighian tubules and wildtype were also not significantly different ($p = 0.061$), and in fact, FSRs from *pHCl-2$^{\Delta}$/pHCl-2^{Df}* Malpighian tubules were significantly higher than wildtype ($p < 0.001$) (Fig. 5A). Since Malpighian tubules lacking a functional *pHCl-2* gene show no consistent change in spontaneous FSRs, *pHCl-2* is not a primary source of chloride conductance for basal secretion, consistent with the presence of additional sources of chloride conductance. Indeed, the major route of chloride entry into the lumen of *Drosophila* Malpighian tubules has been proposed to be through chloride channels in the apical membranes of stellate cells, not the principal cells where *pHCl-2* is expressed (Cabrero et al., 2014; O'Donnell et al., 1998). Chloride may also enter the tubule lumen paracellularly, via septate junctions, as has been demonstrated in the Malpighian tubules of *Aedes aegypti* (Beyenbach and Piermarini, 2011; Beyenbach et al., 2009; Miyauchi et al., 2013). More subtle effects of loss of *pHCl-2* on secretion rate may also be obscured by variability among strains, possibly resulting from variations in genetic background.

Mutations in *pHCl-2* reduce the diuretic effect of cAMP

Given the apparently normal levels of fluid secretion produced by *pHCl-2* mutant Malpighian tubules, we reasoned that increasing the rate of cation transport, which would increase the need for chloride transport to the lumen, might reveal more subtle deficits in chloride conductance in *pHCl-2* mutant tissues. Transepithelial cation transport in the Malpighian tubules can be stimulated with the second messenger cAMP, which increases fluid secretion by enhancing the output of the V-type H^+ ATPase (Cabrero et al., 2002; Coast et al., 2001; Johnson et al., 2005). Measuring FSR in the same tubule before and after cAMP treatment also allowed us to normalize the variability among tubules.

To test the effect of disrupting *pHCl-2* on Malpighian tubule output when cation transport is stimulated, we compared the FSRs of wildtype and *pHCl-2* mutant Malpighian tubules treated

with cAMP. Addition of 0.2 mM cAMP significantly raised the mean FSRs in wildtype as well as in *pHCl-2*$^{\Delta}$/*pHCl-2*$^{\Delta}$, *pHCl-2*$^{\Delta}$/*pHCl-2*^{Df} and *pHCl-2*$^{\Delta}$/*pHCl-2*^{Mi} (Fig. 5B) ($P < 0.001$, Mann Whitney rank sum test), suggesting that the H^+ ATPase activity can be enhanced in both wildtype and *pHCl-2* mutant Malpighian tubules. However, the *pHCl-2* mutant Malpighian tubules displayed a significantly smaller normalized diuretic response to cAMP compared to wildtype. FSRs in wildtype increased by 55.1 ± 3.97%, while *pHCl-2*$^{\Delta}$/*pHCl-2*$^{\Delta}$, *pHCl-2*$^{\Delta}$/*pHCl-2*^{Df} and *pHCl-2*$^{\Delta}$/*pHCl-2*^{Mi} FSRs only increased by 31.1 ± 3.43%, 28.3 ± 3.84% and 31.5 ± 6.48% respectively (Fig. 5C) ($P < 0.05$, one-way ANOVA followed by Tuckey's post-hoc test). These results, therefore, are consistent with the presence of a *pHCl-2*-mediated chloride conductance that is capable of influencing FSRs in the Malpighian tubules.

Discussion

pLGICs constitute a large and diverse superfamily of structurally related subunit proteins in metazoans. The vast majority of pLGICs are involved in rapid ionotropic synaptic transmission, opening in response to small molecule neurotransmitters. In this study, however, we demonstrate that *pHCl-2* functions as a pH-gated ion channel, and propose that it serves a non-canonical biological function: regulation of fluid secretion in epithelial tissues by modulating the chloride permeability of apical membranes in response to extracellular pH. The role of *pHCl-2* in the Malpighian tubules thus represents a new biological context under-which pLGICs function to regulate membrane ion permeability.

pHCl-2 belongs to a highly divergent clade of pH-gated pLGICs common to arthopods

Previous phylogenetic analysis identified *pHCl-2* as a member of an arthropod-specific clade of divergent orphan Cys-loop pLGICs. In *Drosophila*, *pHCl-2* groups with two other orphan pLGIC subunits, *CG7589* and *CG6927* (Dent, 2006; Jones and Sattelle, 2006; Remnant et al., 2016), which together most closely resemble the *Drosophila* pH-sensitive chloride channel (*pHCl*) and the pH-sensitive chloride channel in *Sarcoptes scabiei* (*SsCl*) (Mounsey et al., 2007; Schnizler et al., 2005). Clades of channel subunits orthologous to the clade of subunits defined by *pHCl-2*, *CG7589* and *CG6927* have been reported in other insects such as *Apis mellifera*, *Aedes egypti*, *Nasonia vitripennis* and *Tribolium cataneums* (Jones and Sattelle, 2006; Jones and

Sattelle, 2007; Jones et al., 2010; Remnant et al., 2016); www.flybase.organd in non-insect arthropods such as the deer tick *Ixodes scapularis* (www.flybase.org), but not in nematodes, molluscs, annelids or chordates (Dent, 2006). The pH response of *pHCl-2* closely resembles that of the two other characterized pH-sensitive arthropod pLGICs, *Drosophila* pHCl and *Sarcoptes* SsCl; both pHCl and SsCl are inhibited by protons and are increasingly activated by a rise in alkalinity, exhibiting half-maximal activity at pH 7.33 $\pm$ 0.16 and 7.55 $\pm$ 0.06 respectively (Mounsey et al., 2007; Schnizler et al., 2005). In contrast, the other well-characterized pH-gated pLGICs identified to date, the *pbo-5,-6* heteromeric cation channel in *C. elegans,* and GLIC from cyanobacterium *Gloeobacter violaceus* are inhibited by alkaline conditions, and instead, are increasingly activated by a rise in proton concentration, displaying half-effector responses at pH6.83 $\pm$ 0.01 and pH 5.1 $\pm$ 0.1 respectively (Beg et al., 2008; Bocquet et al., 2007). While we have shown that standard neurotransmitters do not gate *pHCl-2* channels expressed in oocytes, we cannot rule out that *pHCl-2* channels are sensitive to other ligands that might be found in gastric fluid or urine.

pHCl-2 -mediated chloride conductance regulates fluid secretion in the Malpighian tubules during diuresis

Fluid secretion in the Malpighian tubules is mediated by active transpithelial ion transport which establishes the osmotic gradient necessary for the formation of the primary urine. This active transport is powered by an apically localized, electrogenic H^+ ATPase that pumps protons into the tubule lumen and generates a net positive apical membrane potential. The proton gradient is used to drive alkali-metal cation/H^+ antiporters, which replace luminal protons with sodium and potassium. Chloride enters the lumen passively, following its electrochemical gradient and is a critical regulator of secretion because, in the absence of this negative counter-ion, cation transport into the lumen would result in an increasingly positive apical membrane potential which would oppose transport by the ATPase before a significant osmotic gradient has formed.

A role for *pHCl-2* as an important source of chloride conductance in the Malpighian tubules is supported by our observation that urine production is affected in *pHCl*-2 mutants. Loss of *pHCl*-2 did not obviously impair fluid secretion in unstimulated Malpighian tubules,

consistent with alternative routes of chloride flow into the lumen, either via known channels in the stellate cells or through a putative paracellular route via septate junctions (Beyenbach and Piermarini, 2011; Beyenbach et al., 2009; Cabrero et al., 2014; O'Donnell et al., 1998). However, upon stimulation of the Malpighian tubules with cAMP, a second messenger that enhances the output of the H^+ ATPase, *pHCl*-2 mutant Malpighian tubules showed a significantly reduced diuretic response compared to wildtype. These data suggest that under conditions of enhanced cation transport into the lumen *pHCl-2* provides a necessary source of chloride conductance in the principal cells, without which maximal secretion rates are not achieved.

A possible role for pH as a regulator for chloride permeability in the Malpighian tubules

The expression of *pHCl-2* in the apical membrane of principal cells, together with our electrophysiology data demonstrating that *pHCl-2* forms a pH-sensitive channel, suggests that the *pHCl-2*-mediated chloride conductance may be regulated by the pH of the luminal environment. Luminal pH is strongly influenced by the relative activities of the proton ATPase and the cation/H^+ antiporter, which transport protons into and out of the tubule lumen respectively (Day et al., 2008; Dow et al., 1994; Linton and O'Donnell, 1999; Maddrell and O'Donnell, 1992; Nicolson, 1993; O'Donnell and Maddrell, 1995; Petzel et al., 1999) and we propose that the gating of *pHCl-2* by pH may reveal a homeostatic mechanism that maintains an appropriate balance between these two cation transporters. Under conditions where the activity of the ATPase is high relative to the antiporter, the pH of the lumen would drop because the rate of proton transport into the lumen would exceed their removal by the antiporter. Since secondary-active transport of sodium and potassium into the tubule lumen by the antiporter is coupled to active proton transport, an accumulation of excess protons in the tubule lumen would reflect a decrease in the energy efficiency of sodium and potassium secretion. We propose that the presence of a pH sensitive chloride channel like *pHCl-2* would counteract such an imbalance. If the pH of the lumen was too acidic, *pHCl-2* would be antagonized, chloride permeability would become rate limiting for secretion and the increasingly positive apical membrane potential generated by the electrogenic ATPase would oppose further transport of protons into the lumen. The proton gradient, however, would continue to drive the antiporter, increasing luminal pH,

which would, in turn, increase *pHCl-2* conductance, and decrease the electrical barrier opposing the ATPase, thus re-establishing homeostasis. *pHCl-2* would therefore serve as a "brake" on the H^+ ATPase, providing an upper limit to which the ATPase can operate relative to the antiporter, thus minimizing the expenditure of ATP under conditions where protons are not being put to metabolically efficient use.

If *pHCl-2* is in fact inhibited by acidic luminal pH, then one might predict that the stimulatory effects of cAMP on the H^+ ATPase would lead to inhibition of *pHCl-2*-mediated chloride conductance. cAMP signaling in the Malpighian tubules leads to the secretion of a more acidic urine, with pH decreasing from 7.8 to 7.4, consistent with an increase in proton transport by the ATPase (Coast et al., 2001; O'Donnell et al., 1996). Based on its pH-sensitivity in oocytes, the chloride conductance of *pHCl-2* should decrease ~80% in response to cAMP, and the normalized chloride conductance-limited secretion rate increase should be significantly smaller in the presence of a pH-sensitive channel (i.e. wildtype) than in its absence (i.e. *pHCl*-2 knockout). Yet we observed the opposite – the normalized response of secretion to cAMP in the *pHCl*-2 knockout was smaller than in wildtype, indicating that a simple physiological model does not account for the *pHCl*-2 phenotype. Instead, the *pHCl*-2 knockout may have indirect feedback effects on lumen pH or membrane potential, for example by directly affecting the ability of the H^+ ATPase to respond to cAMP.

Another effect of down-regulating *pHCl-2* may be to increase the relative chloride current through stellate cell chloride channels. Decreased luminal pH that inhibits *pHCl-2* resulting in a rise in V_{ap} should increase the driving force for chloride through apically localized chloride channels in stellate cells, thus increasing the relative contribution of stellate cells to apical chloride current. The increased activity of stellate cell chloride channels could in turn drive anion exchange via the basal membrane-localized Cl^-/HCO_3^- transporter in stellate cells, thereby alkalinizing the haemolymph. Interestingly, pharmacological block of the Cl^-/HCO_3^- transporter in *Aedes aegypti* has little effect on resting secretion rate but inhibits stimulated secretion, similar to the mutant phenotype of *pHCl-2* (Piermarini et al., 2010). Since HCO_3^- is thought to be produced by carbonic anhydrase in the principal cells and enter the stellate cells through intracellular junctions, the effect of the *pHCl-2* mutant on stimulated transport may be an indirect effect on the Cl^-/HCO_3^- balance in principal cells feeding back and affecting chloride transport through stellate cells.

Recent work by Remnant et al. 2016 demonstrated that *pHCl-2* is expressed in the copper cells of the midgut and influences sensitivity to dietary copper: flies deficient for *pHCl-2* display increased copper-tolerance, whereas the opposite is observed when *pHCl-2* is over-expressed. Copper cells are the principal site of acid-secretion in the midgut, and like the Malpighian tubules, are thought to transport protons via apically localized V-type H^+ ATPases (Dubreuil, 2004; Shanbhag and Tripathi, 2005). While a clear relationship between H^+ ATPase output and copper uptake has yet to be elucidated, it has been shown that copper uptake is impaired in flies whose copper cells are deficient in acid secretion, and that the copper cell midgut region is less acidic following copper feeding (Dubreuil, 2004; McNulty et al., 2001). Similar to our proposed model in the Malpighian tubules, *pHCl-2* may influence ATPase output in copper cells by regulating chloride counter-ion availability, which in turn, could affect the rate of copper uptake. Curiously, the role of *pHCl-2* both in the Malpighian tubules and copper cells points to a somewhat counter-intuitive physiological model in which the activity of an alkaline-gated chloride channel provides the necessary counter-current for an acid-secreting transporter.

pHCl-2 function in the Malpighian tubules points to an expanded role for Cys-loop pLGICs:

Our demonstration that *pHCl*-2 regulates secretion in the Malpighian tubules, a polarized epithelial tissue that is not directly associated with the nervous system, underscores the ability of pLGICs to function in a wide array of biological contexts beyond their canonical function in the nervous system. Previous work, for instance the discovery of pLGIC-like proteins in bacteria (Tasneem et al., 2005), has also hinted at possible roles for this ion channel superfamily that are entirely independent of neuronal signaling. There are also examples of pLGICs with well-characterized roles in the nervous system that appear to function in non-neuronal tissues. For example, the mammalian immune system is rich in pLGICs, including nicotinic acetylcholine (nACh), $GABA_A$ and glycine receptors (Barragan et al., 2015; Borovikova et al., 2000; Dionisio et al.; Froh et al., 2002; Prud'homme et al., 2015; Rosas-Ballina et al., 2011; Tracey, 2002). While such non-neuronal roles of the GABA and glycine receptors are poorly understood, the alpha7nAChR is expressed in macrophages where it regulates tumor necrosis factor-α in response to acetylcholine released from spleen lymphocytes (Rosas-Ballina et al., 2011). nAChR function has also been reported in bronchial epithelia, where nAChRs expressed on the apical

membrane (Maus et al., 1998) respond to non-neuronal autocrine/paracrine ACh release to regulate chloride permeability through the CFTR channel (Maouche et al., 2013). Non-neural roles for nAChRs have also been identified in vascular endothelia (reviewed in (Egleton et al., 2009)), in urothelia (Zarghooni et al., 2007), and in keratinocytes (Grando et al., 1995).

pHCl-2 represents an extreme in the evolution of pLGIC functions. Like epithelial nAChRs, it is expressed in non-innervated tissues, but *pHCl-2* is unique in that it is not obviously responding to an autocrine/paracrine signal. Whether *pHCl*-2 has an additional function in the nervous system remains unclear; microarray data suggest that it is not expressed in the head, brain and eyes of adult flies, or in the larval central nervous system (Chintapalli et al., 2007), but this broad survey would not necessarily detect expression in a small subset of neurons. Additionally, although we see *pHCl-2* localized to the apical membrane of principal cells, we cannot rule out the possibility that it functions in apically enriched endosomal vesicles to regulate secretion, similar to *cup-4* in *C. elegans*, which is localized to endosomes and is necessary for endosomal trafficking, although its activating ligand, if any, is unknown (Patton 2005). Nevertheless, whether it acts in endosomes or the apical membrane, our characterization of *pHCl-2* illustrates the remarkable ability of the pLGICs to evolve diverse physiological functions.

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Competing Interests

The authors declare no financial or competing interests.

Author Contributions

D.F., J.A.D., and L.N. conceptualized experiments and wrote the manuscript. D.F., and T.S. performed experiments. J.A.D., and L.N. provided supervision, and J.A.D. secured funding. M.J.O. provided expertise and feedback on experiments and the manuscript.

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Figures

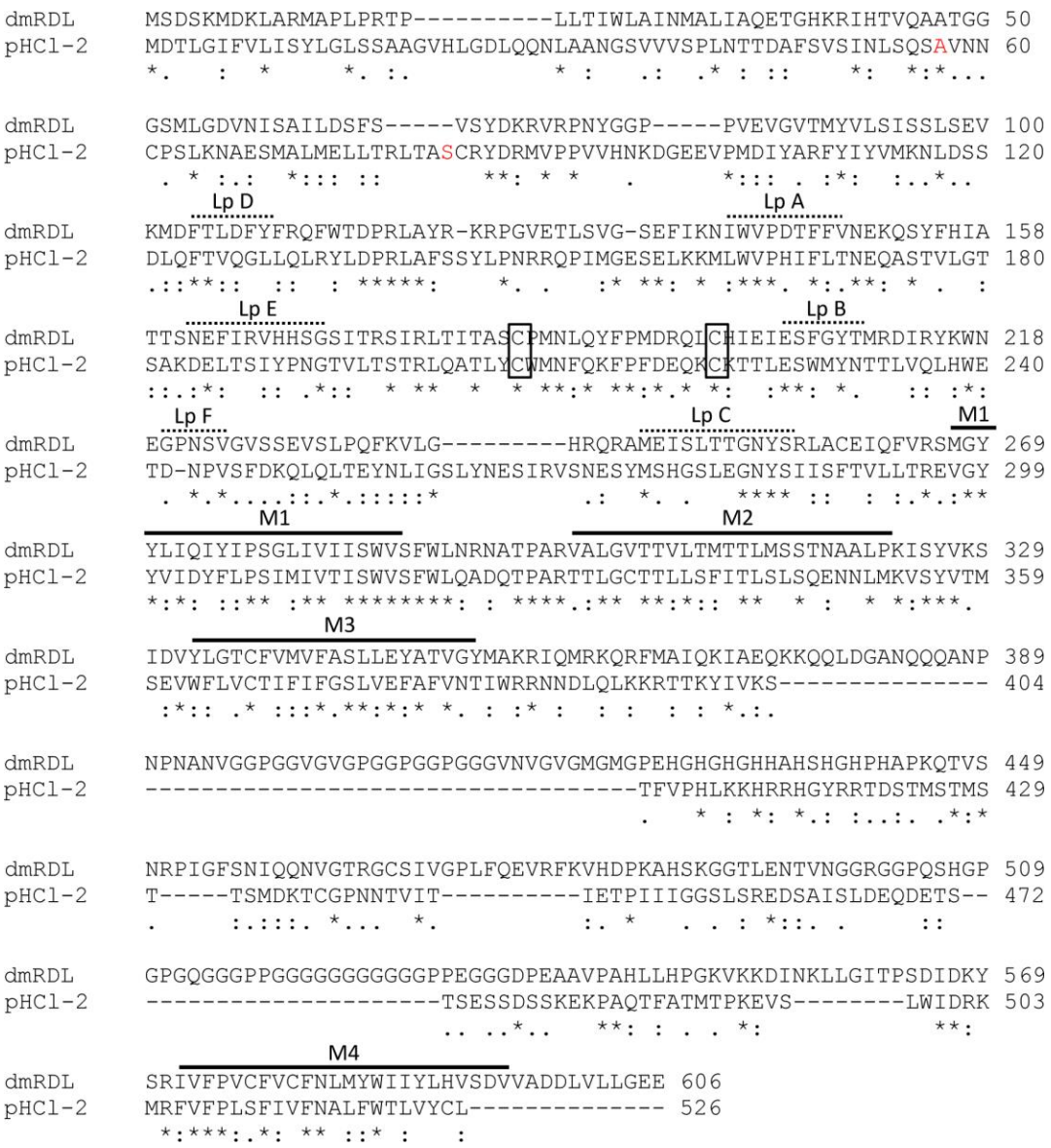

Figure 1: *pHCl-2* encodes a protein that contains the features of a typical Cys-loop LGIC subunit.

Amino acid alignment of *pHCl-2* with the Drosophila RDL Cys-loop LGIC subunit. The two cysteines that form the cys-loop are indicated by rectangles. M1-M4 are marked with solid lines. The six loops (Lp A-F) associated with ligand binding are indicated with a dashed line. The two polymorphisms identified for *pHCl-2* are indicated in red. Notations "* : ." represent degree of conservation in descending order.

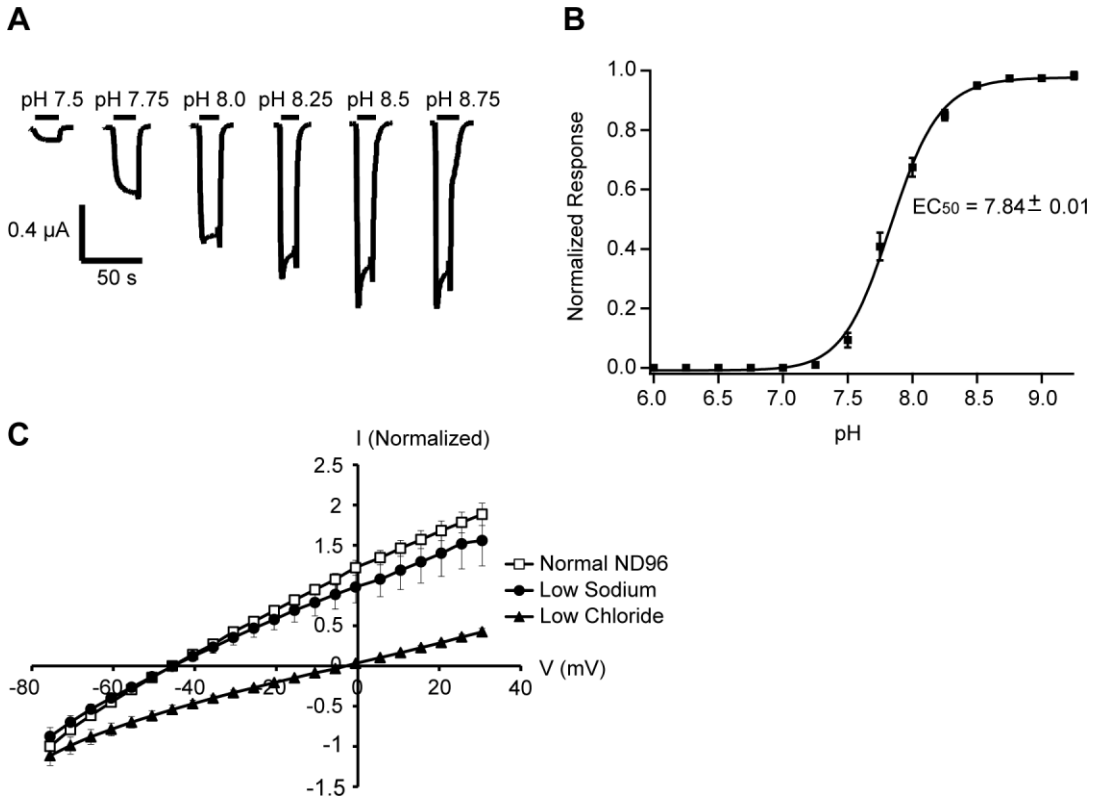

Figure 2: *pHCl-2* forms a homomeric pH-sensitive chloride channel.

A. Representative trace from oocytes expressing *pHCl-2* and clamped at -80mV, showing responses to changes in pH. Occytes were washed in ND96 at pH 6 to close the channel between successive pH treatments. B. Trace showing the response profile of the channel to changes in pH. The responses were normalized to the maximum current generated by each individual oocyte. The curve represents a fit to the Hill equation (n=7). C. Representative trace of the current (I) - voltage (V) relationship under "normal" (96mM Na^+ and 103.6mM Cl^-) (n=8), "low extracellular sodium" (6mM Na^+ and 103.6 Cl^-) (n=4) and "low extracellular chloride" (96mM Na^+ and 16mM Cl^-) (n=6). In B and C, error bars represent $\pm$ SEM.

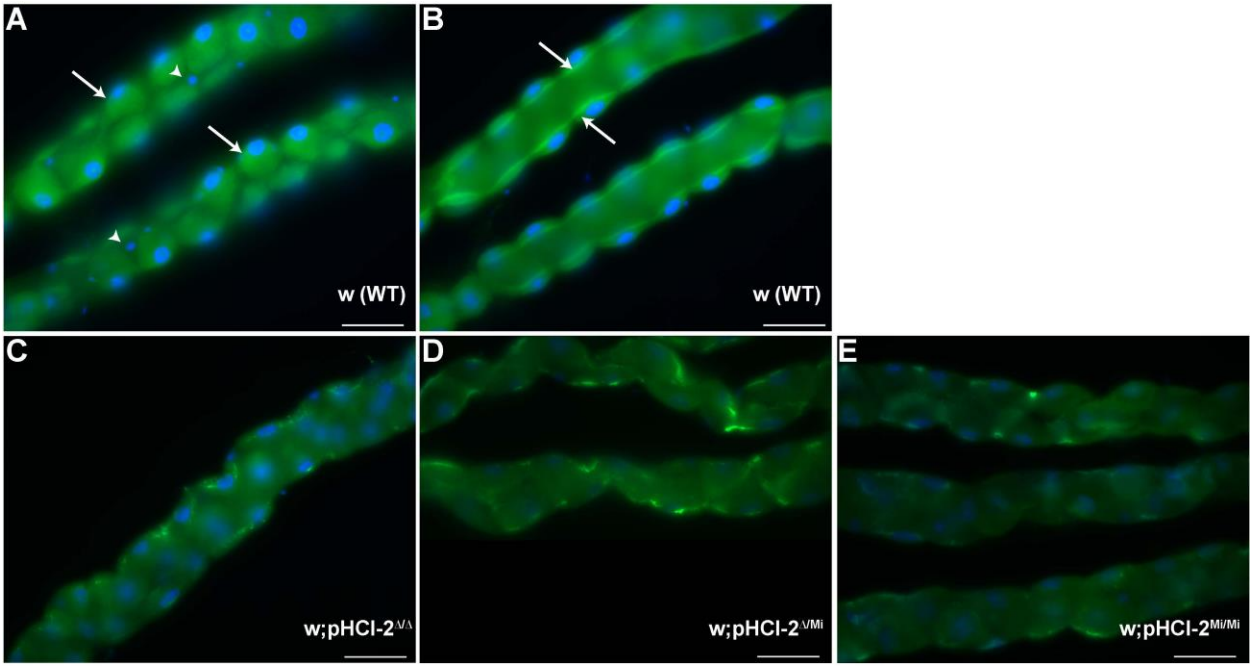

Figure 3: *pHCl-2* expression patterns.

A-E. Immunostaining of adult Malpighian tubules using anti- *pHCl-2* (green). Nuclei are labelled with DAPI (blue). A. Surface view of a wildtype Malpighian tubule showing *pHCl-2* expression in principal cells (arrows), but not stellate cells (arrow-heads). B. Cross section view of the same tissue as in A, showing expression of *pHCl-2* localized to the apical membrane of principal cells (arrows). C. The apical membrane-specific signal observed in the principal cells of wildtype Malpighian tubules is absent in homozygous $pHCl\text{-}2^{\Delta}$ Malpighian tubules as well as in the heteroallelic $pHCl\text{-}2^{\Delta}/pHCl\text{-}2^{Mi}$ (D) and homozygous $pHCl\text{-}2^{Mi}$ mutant Malpighian tubules (E). Scale bars for A-E are 50μm.

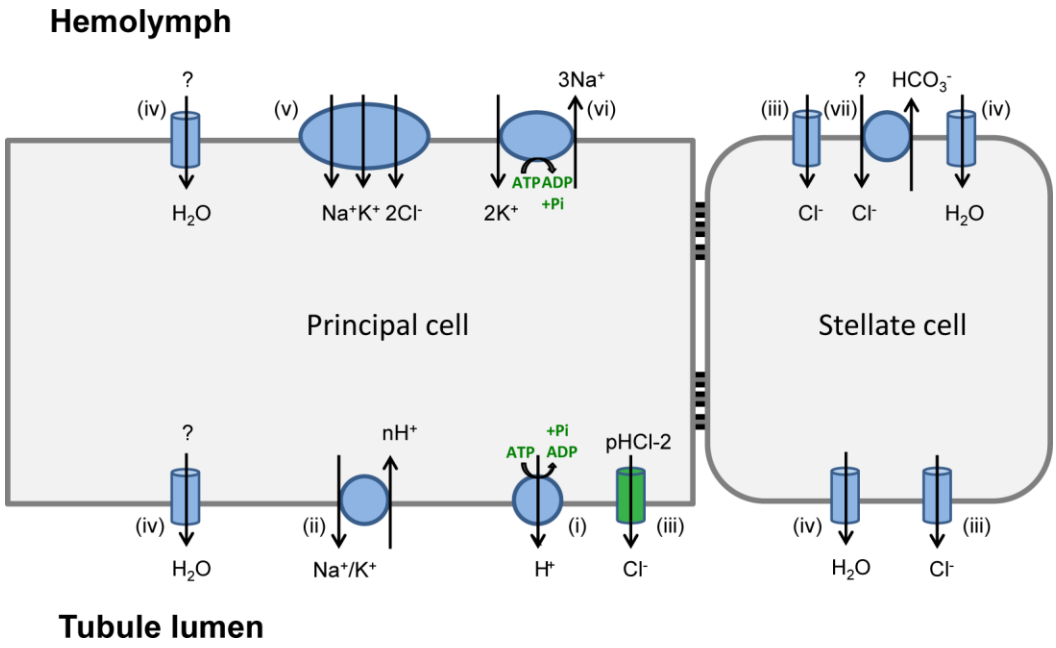

Figure 4: General model of transepithelial secretion in the Malpighian tubules.

Transepithelial secretion is powered by an electrogenic vacuolar-type proton ATPase (H^+ ATPase) (i) located in the apical membrane of the principal cells. The H^+ ATPase extrudes H^+ into the tubule lumen generating a net lumen positive transepithelial potential. H^+ are returned to the cytoplasm via apically localized Na^+- K^+/H^+ antiporters (ii) in exchange for the export of Na^+ and K^+. Chloride enters the lumen passively, following a favorable electrochemical gradient, via apically localized chloride channels (iii) and water enters the lumen through aquaporins (iv), following the osmotic gradient generated as a consequence of net ion transport. At the basolateral membrane of principal cells, Na^+, K^+ and Cl^- enter the cell via Na^+: K^+: $2Cl^-$ co-transporters (v). The activity of a Na^+/ K^+ ATPase (vi) recycles most of the Na^+ that enters the cell back to the hemolymph. Chloride also enters stellate cells from the hemolymph via basolaterally localized chloride channels (iii) and may also enter stellate cells via a Cl^-/HCO_3^- antiporter (vii).

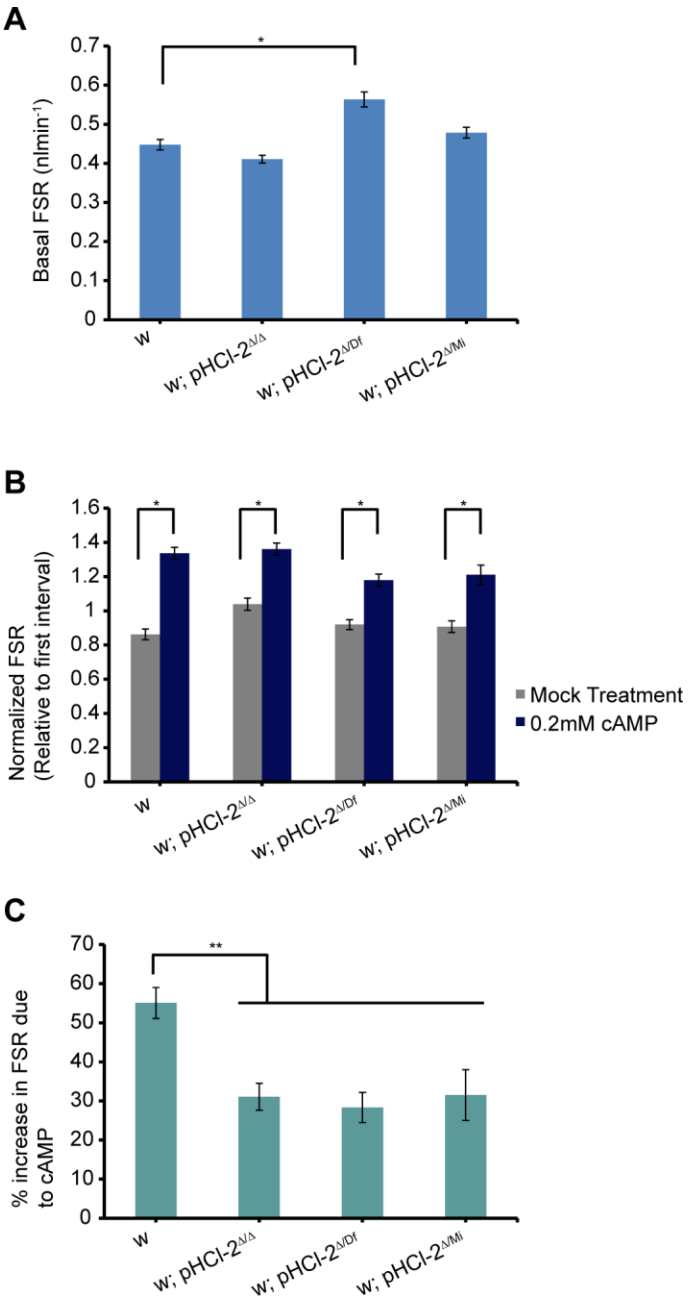

Figure 5: Loss of *pHCl-2* reduces the cAMP-mediated diuretic response.

A-C. Fluid secretion assays conducted on *w*, *w;pHCl-2$^{\Delta}$/ pHCl-2$^{\Delta}$*, *w; pHCl-2$^{\Delta}$/ pHCl-2^{Df}* and *w; pHCl-2/pHCl-2^{Mi}*. A. Basal fluid secretion rate (FSR) over 40 minutes in regular saline: *w* (n=149), *w; pHCl-2$^{\Delta}$/ pHCl-2$^{\Delta}$* (n =116), *w; pHCl-2$^{\Delta}$/ pHCl-2^{Df}* (n=41), *w; pHCl-2$^{\Delta}$/ pHCl-2^{Mi}*

(n=56). B. Change in FSR in response to 0.2mM cAMP or regular saline (mock treatment) relative to the first interval (see methods). Sample size (mock/cAMP treatments): *w* (n=67/82), *w; pHCl-2*$^{\Delta}$*/ pHCl-2*$^{\Delta}$ (n=58/58), *w; pHCl-2*$^{\Delta}$*/ pHCl-2*^{Df} (n=19/22), *w; pHCl-2/ pHCl-2* (n=27/29). C. Percent increase in FSR following treatment with 0.2mM cAMP calculated as the ratio of the mock and cAMP responses indicated in B (see methods). In all panels, error bars represent $\pm$ SEM. *P < 0.001 (Mann Whitney rank sum test) and ** P < 0.05 (one-way ANOVA Tuckey's post-hoc test).

List of abbreviations

Pentameric ligand-gated ion channels (pLGICs), capped RNA (cRNA), two electrode voltage clamp (TEVC), transepithelial potential (TEP), nicotinic acetylcholine receptor (nAChR), fluid secretion rate (FSR).