A role for the *Drosophila* zinc transporter *Zip88E* in protecting against dietary zinc toxicity

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Abstract

Zinc absorption in animals is thought to be regulated in a local, cell autonomous manner with intestinal cells responding to dietary zinc content. The *Drosophila* zinc transporter *Zip88E* shows strong sequence similarity to Zips 42C.1, 42C.2 and 89B as well as mammalian Zips 1, 2 and 3, suggesting that it may act in concert with the apically-localised *Drosophila* zinc uptake transporters to facilitate dietary zinc absorption by importing ions into the midgut enterocytes. However, the functional characterisation of *Zip88E* presented here indicates that *Zip88E* may instead play a role in detecting and responding to zinc toxicity. Larvae homozygous for a null *Zip88E* allele are viable yet display heightened sensitivity to elevated levels of dietary zinc. This decreased zinc tolerance is accompanied by an overall decrease in *Metallothionein B* transcription throughout the larval midgut. A *Zip88E* reporter gene is expressed only in the salivary glands, a handful of enteroendocrine cells at the boundary between the anterior and middle midgut regions, and in two parallel strips of sensory cell projections connecting to the larval ventral ganglion. *Zip88E* expression solely in this restricted subset of cells is sufficient to rescue the *Zip88E* mutant phenotype. Together, our data suggest that *Zip88E* may be functioning in a small subset of cells to detect excessive zinc levels and induce a systemic response to reduce dietary zinc absorption and hence protect against toxicity.

Introduction

Zinc is an essential dietary nutrient, required as a structural or enzymatic cofactor for potentially thousands of different proteins. It has been estimated that up to 10% of all human proteins are able to bind zinc [1]. There is also a growing body of evidence that unbound zinc ions may be able to act as signalling molecules to regulate cellular processes such as growth and neurotransmission [2].

Movement of zinc ions across cell membranes is facilitated by two large classes of proteins. Members of the Zip family have mostly been shown to transport zinc into the cytosol, either from outside the cell (cellular uptake) or from the lumen of cellular organelles in order to redistribute zinc within individual cells. ZnT proteins mostly function in the opposite
direction, removing zinc from the cell (cellular efflux) or supplying organelles such as the endoplasmic reticulum, Golgi and lysosome. Within such organelles, zinc may be loaded onto proteins that require zinc for their activity, stored for later use, or packaged for removal. The large number of Zip and ZnT proteins encoded by vertebrate and invertebrate genomes indicates that each protein has taken on a specialised role defined in part by its expression pattern, cellular localisation, zinc transport ability / specificity and post-translational regulation.

The relative simplicity of the zinc transport network in *Drosophila* (17 Zip and ZnT genes compared to 25 in vertebrates [3]) has facilitated the functional characterisation of a number of these genes. Focussing on the process of zinc absorption from the diet, two Zip proteins, Zip42C.1 and Zip42C.2, have been shown to play a major role in uptake of zinc from the intestinal lumen through a small cluster of cells in the larval midgut called the iron cells [4]. A third, closely related protein, Zip89B, is more widely expressed throughout the midgut and appears to play an ancillary role in zinc absorption [5]. Although Zip89B is non-essential, in its absence, Zip42C.1 and Zip42C.2 are upregulated, presumably to compensate for a reduction in zinc uptake [5]. Once inside the cells of the fly midgut, zinc must be released into the circulatory system for systemic supply, a function performed by ZnT63C with support from ZnT77C [4, 6].

Together with a fourth protein, Zip88E, Zips 89B, 42C.1 and 42C.2 form a highly-conserved clade with highest similarity to mammalian Zips 1, 2 and 3 [3]. Indeed, this is the only situation where there are more fly proteins than human proteins within a Zip or ZnT phylogenetic subgroup. The Zip1 to 3 mouse triple knockout shows no obvious defects in mice raised on a zinc replete diet [7]. The Zip1 and Zip3 single knockout mice do however show a high level of embryonic developmental abnormalities in pups of knockout mice raised on a zinc-deficient diet and in the Zip1/3 double knockout mice these defects are elevated in an additive fashion. The Zip1/2/3 triple knockout mouse has zinc deficiency phenotypes equivalent to those of the 1/3 double knockout suggesting that while Zip1 and Zip3 are playing overlapping roles, Zip2 functions differently. Indeed, expression of Zips 1 and 3 is broad and includes the intestinal stromal cells whereas Zip2 expression is limited to the pericentral hepatocytes, keratinocytes and immature dendritic cells [8].

Zip4 has been considered as the major zinc uptake gene in the mammalian intestine to date because a human zinc deficiency disease, Acrodermatitis enteropathica, is caused by Zip4 mutations [9]. However, intestinal-specific knockout of Zip4 actually results in a reprogramming of Paneth cells, accompanied by crypt dysplasia and reduced cell division in the small intestine [10]. Therefore the systemic zinc shortage caused by Zip4 mutations may in fact be due to general intestinal malfunction resulting from zinc deficiency specifically in the Paneth cells. In this scenario, Zips 1 and 3 may play an important role in general zinc absorption via the enterocytes, although clearly Zip4 and / or other uptake mechanisms must also be contributing since the Zip1/3 double knockout is relatively healthy.

While *Drosophila* Zips 89B, 42C.1 and 42C.2 have been well characterized, the closely-related Zip88E is yet to be examined in detail. Previous over-expression experiments have shown that Zip88E is localized both to the outer basolateral membrane and an endomembrane but does not overlap with endoplasmic reticulum or Golgi markers [11]. Zip88E overexpression alone has no effect on *Drosophila* viability or morphology but does modify over expression phenotypes of other fly Zip and ZnT genes. In these interaction experiments, Zip88E acts to increase cytosolic zinc levels, behaving similarly to Zips 89B, 42C.1 and 42C.1 but even more like the uncharacterised Zips 102B and 99C [11]. Double knockdown of Zips 42C.1 and 42C.2 in the fly larval midgut clearly causes zinc deficiency in animals raised on a zinc-poor diet yet has relatively little effect on a zinc-replete diet [4], indicating that additional enterocyte uptake mechanisms may be contributing to zinc
absorption in the fly. A similar phenomenon is observed in mouse Zip gene knockouts, suggesting that alternative zinc absorption mechanisms could be novel therapeutic targets for addressing zinc deficiency. Here we have generated a null mutation in Zip88E and examined its expression pattern to investigate whether this closely-related gene may be playing a supporting role in zinc absorption.

**Materials and methods**

**Drosophila stocks**

The following fly stocks were used: w^1118 (BL3605, Bloomington Stock Centre, Indiana USA); GMR-GAL4 (P[longGMR-GAL4]3, BL8121). RNA interference (RNAi) lines were obtained from the Vienna Drosophila RNAi Centre (VDRC). MtnB:EYFP was a gift from Walter Schaffner (University of Zurich, Switzerland). Microscopy utilized P[UAS-mCD8::GFP.L]2 and P[UAS-2xEYFP]AH2 to visualize reporter gene expression. A list containing the transgenic lines used in this study is provided in [11]. All transgenic Drosophila experiments carried out in this research were performed with the approval of the Monash University Institutional Biosafety Committee. No ethics approval is required for experiments involving insects in Australia.

**Cloning and generation of Zip88E:GAL4 reporter construct**

The predicted promoter/enhancer regions of Zip88E were PCR-amplified from genomic DNA extracted from w^1118 third instar larvae. The region directly upstream of the START codon of Zip88E until the STOP codon of the preceding gene (CG14864) was amplified and cloned into a modified pUAST-attB vector with the UAS sequence upstream of the multiple cloning site (MCS) removed. Full length GAL4 coding sequence was also cloned into the MCS. This construct was injected into PhiC31 attP 51C and 86Fb strains (provided by Konrad Basler). Microinjections utilized an Eppendorf Femtojet apparatus with Femtotips II pre-pulled glass needles (Eppendorf). Oligonucleotide sequences are provided in S1 Table.

**Drosophila maintenance and feeding experiments**

All Drosophila strains and crosses were maintained on standard (basal) medium at 25˚C unless stated otherwise. Standard medium was supplemented with 4–12 mmol l^-1 zinc chloride (ZnCl₂; Sigma Aldrich, St. Louis, MO, USA) to make zinc-supplemented medium, or 50–150 μmol l^-1 N,N,N’,N’-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN; Sigma Aldrich) to make zinc-deficient medium. For survival assays, Drosophila first instar larvae were transferred between 20–24 h post-emergence onto media supplemented with aqueous pre-diluted ZnCl₂ or TPEN (50 larvae per replicate). Adult survival was determined as the proportion of larvae that had emerged as adults after 15 days at 25˚C. Acute exposure to supplemented food was achieved by picking third instar larvae before the wandering stage onto treated food types and allowing development for 20–24 h before further analysis was conducted.

**Imprecise P-element excision**

Males with the P-element P[Epy2]Zip88E^EY11179 (BL20270) inserted just upstream of the Zip88E translation START codon were crossed to the Δ2,3 (99B) transposase stock to induce an imprecise excision event. Single male progeny with mosaic eyes were crossed to MRKS/TM6β females. Single white-eyed males in the subsequent generation were then tested for imprecise excision events, using PCR primers designed to span the majority of the Zip88E locus. Oligonucleotide sequences are provided in S1 Table.
Microscopy

Adult flies were partially dissected then mounted directly onto plasticine and monitored with a Leica MZ6 stereomicroscope. All eye images were recorded on a Leica DC300n digital camera and Leica Application Suite.

Midguts, salivary glands and the brain were dissected from third instar larvae in cold phosphate buffered saline (PBS) and mounted directly onto glass slides in Vectorshield (Vectorlabs) or fixed in 4% paraformaldehyde in PBS. Monoclonal α-Prospero and α–Fasciclin I primary antibodies used for Zip88E::mCD8::eGFP co-localization studies were obtained from the Developmental Studies Hybridoma Bank and used at 1:50 and 1:200 dilution respectively, followed by α-mouse AlexaFluor568 secondary antibody (Molecular Probes) used at 1:1000 dilution. MtnB:EYFP fluorescence in larval tissue was viewed on a Leica M165 FC dissecting microscope using a Leica DFC450 camera and Leica Application Suite. Higher magnification imaging was performed on either: 1) a Leica DMLB compound microscope using a Leica DC300 camera and Leica Application Suite at a magnification of 10x and 20x; or 2) an Olympus CV1000 spinning disk confocal microscope with a 10x dry objective lens or a 60x immersion objective lens.

Western blot analysis

Extraction of protein lysate was achieved by homogenising five whole third instar larvae in 2% SDS lysis buffer with a protease inhibitor cocktail (Sigma Aldrich). Protein samples were resolved on 4–12% NuPAGE® Bis-Tris gels (Invitrogen) and were transferred to a polyvinylidene di-fluoride membrane (Milipore) using the X Cell Surelock™ Mini Cell system (Invitrogen). Ponceau S (Sigma) staining was used to assess efficiency of the transfer and provide confirmation of equal sample loading between lanes. α-GFP (rabbit, Molecular Probes) primary antibody was used at 1:10,000 in 5% skim milk solution. Blots were viewed using the QUANTUM ST5 Gel Documentation System (Vilber Lourmat).

Semi-quantitative RT-PCR analysis

RNA extraction was performed by homogenising midguts in TRIsure RNA reagent (Bioline). Reverse transcription was performed using Tetro cDNA Synthesis Kit (Bioline). Semi-quantitative PCR analysis was performed using GoTaq green master mix with 1 μl of 100 ng/μl cDNA used per reaction. PCR products were separated by electrophoresis on a 2.5% agarose gel. Housekeeping gene RP49 was used as an endogenous control. Oligonucleotide sequences are provided in S1 Table.

Statistical analysis

Two-way ANOVA analysis followed by Tukey multiple comparisons test and multiple T-test analysis followed by the Holm-Sidak multiple comparison test were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical significance was deemed when the p-value ≤ 0.05. Quantification of western blot band intensity was achieved using Image J analysis software (1.47v).

Results

Previous, limited functional characterisation of Zip88E by targeted over expression and RNAi-mediated knockdown indicated that it acts to increase cytosolic zinc levels; over expression in the adult eye exacerbated zinc toxicity phenotypes caused by Zip71B::FLAG and ZnT86D over expression while Zip88E knockdown had the opposite effect, rescuing both these phenotypes.
back to wild type [11]. This analysis was extended by examining interactions between over expression of Zip88E::FLAG and manipulation of all other Drosophila Zip and ZnT' genes, using GMR-GAL4 to drive expression only in the eye. While over expression of Zip88E::FLAG alone had no impact on eye morphology (Fig 1B), co-expression with ZipFoi::FLAG (Fig 1D), Zip48C IR (Fig 1F), ZnT33D::FLAG (Fig 1H), and ZnT63C IR (Fig 1J) all caused mild but detectable disruptions to eye morphology. All other Zip / ZnT transgenes caused no appreciable phenotype when co-expressed with Zip88E::FLAG (S1 Fig).

To further characterise the systemic role of Zip88E during fly development, a putative null mutation was generated by imprecise P-element excision, creating a deletion removing the START codon and all of the first and second introns encoding the first 170 amino acids of the protein (S2 Fig). No Zip88E transcript could be amplified from cDNA extracted from Zip88EΔ/Δ homozygous larvae (S3 Fig), supporting the proposition that this deletion constitutes a null allele for Zip88E.

As Zip88EΔ/Δ animals survived to adulthood with no obvious morphological defects, the sensitivity of these mutants to alterations in dietary zinc content was tested. Early first instar larvae of various genotypes were transferred onto Drosophila media supplemented with either ZnCl₂ or the zinc chelator TPEN and survival to adulthood assessed. Compared to the w¹¹¹⁸ control strain, survival of the Zip88EΔ/Δ homozygous larvae was significantly reduced on both 4 and 8 mmol l⁻¹ ZnCl₂-supplemented food, but was unaffected on lower zinc concentrations and on zinc-chelated media (Fig 2).

To further assess the impact of loss of Zip88E on zinc levels in the fly, a reporter gene for the zinc-responsive Metallothionein B (MtNb) gene was employed to estimate zinc levels in the gastrointestinal tract. Mtns A to D have all been found to be transcriptionally activated by zinc, copper and cadmium in the fly midgut [12]. MtNb:EYFP has enhanced Yellow Fluorescent Protein (EYFP) expression driven by the MtNb regulatory region [13] and is strongly induced in the midgut by increased dietary zinc content [3, 6, 13]. While basal MtNb:EYFP expression was observed in control third instar larvae (Fig 3A), particularly in the crop / gastric caeca, middle midgut and posterior midgut regions, a strong overall reduction in MtNb:EYFP signal was seen in Zip88EΔ/Δ mutant larvae (Fig 3B). Quantification of MtNb:EYFP levels by α-GFP western blot from lysates extracted from whole third instar larvae confirmed a dramatic drop in MtNb:EYFP in the mutant larvae (Fig 3C and S4 Fig), particularly in the crop and posterior midgut regions. Induction by 24 hour exposure to 2 mmol l⁻¹ ZnCl₂-supplemented food greatly stimulated MtNb:EYFP in control larvae as expected (Fig 3D) but had considerably lower impact on the reporter in the Zip88EΔ/Δ mutant larvae (Fig 3E and 3F). Additional midgut images are provided in S5 Fig. Semi-quantitative RT-PCR carried out on other Zip genes showed that none of the tested genes were up or down regulated in the Zip88EΔ/Δ larval midgut (S3 Fig), suggesting that adequate zinc levels are retained in the mutant midgut cells.

To determine the endogenous expression pattern of Zip88E, a transgenic reporter line, Zip88E-GAL4, was generated by cloning the putative upstream enhancer sequences of Zip88E in front of the GALA coding sequence. Using UAS-mCD8::GFP (encoding membrane-bound GFP) in combination with Zip88E-GAL4, GFP expression was only observed in three larval tissues, the salivary glands (Fig 4A), in two parallel stripes down the ventral ganglion of the central nervous system (CNS, Fig 4C), and in a collection of enteroendocrine-like cells just anterior to the copper cells of the midgut (Fig 4E). Raising Zip88E>mCD8::GFP larvae on either ZnCl₂ or TPEN-supplemented food had no impact on GFP expression, indicating that this gene is not subjected to transcriptional regulation by dietary zinc content (S6 Fig).

To further explore the origin of the GFP expressed under Zip88E-GAL4 control, confocal microscopy was performed on larval brains and midguts. Co-staining of Zip88E>mCD8::GFP midguts with an α-Propero antibody revealed that all GFP +ve cells in the anterior larval
Fig 1. Zip88E over expression interacts with other zinc transport gene manipulations to disrupt eye development. GMR-GAL4 was used to drive ectopic Zip88E::FLAG expression in the developing Drosophila eye, alone and in combination with the over expression and RNAi (IR) knockdown of various Zip and ZnT genes. A) GMR-GAL4-only control. B) GMR>Zip88E::FLAG. C) GMR>ZipFoi::FLAG. D) GMR>Zip88E::FLAG + ZipFoi::FLAG. E) GMR>Zip48C IR. F) Zip88E::FLAG + Zip48C IR. G) GMR>ZipT33D::FLAG. H) GMR>Zip88E::FLAG + ZnT33D::FLAG. I) GMR>ZipT63C IR. J) GMR>Zip88E::FLAG + ZnT63C IR.

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midgut were also Prospero +ve, indicating these Zip88E-expressing cells are enteroendocrine cells (Fig 5A–5C). As no cell bodies were visible in the larval ventral ganglion (Fig 4C), the membrane-localised mCD8::GFP reporter was compared to a predominantly nuclear GFP reporter (nls::GFP, Fig 5D and 5E). Whereas the membrane-bound GFP highlighted the parallel stripes down the ventral ganglion as well as lateral projections emanating from these stripes (Fig 5D), the nuclear GFP was observed mainly in a small number of cells at the midline of the ventral ganglion (Fig 5E). While double-staining was not possible due to the presence of the 3xP3:DsRed transgene at the Zip88E-GAL4 docking site, α-Fasciclin I staining was carried out on control larval brains to provide a morphological landmark. The parallel stripes of Fasciclin I +ve cells appeared to be closer to the ventral ganglion midline than the Zip88E>mCD8::GFP staining (Fig 5F), therefore the Zip88E-expressing sensory neurons are unlikely to be associated with the Fasciclin-expressing dopaminergic neurons.

Previously we have reported that a Zip88E::eGFP fusion protein localises predominantly to an endomembrane, but not the Golgi or ER, when over expressed in larval salivary gland cells [11]. To investigate whether Zip88E is influenced by dietary zinc content, we examined Zip88E::eGFP localisation in the Zip88E-GAL4 expression domain. The same endomembrane localisation as previously reported was observed in salivary gland cells of larvae raised on...
normal, low or high-zinc diets (Fig 6A–6C). In the midgut enteroendocrine cells, Zip88E::eGFP was observed throughout the cytosol and on the outer membrane and this localisation did not differ in larvae raised on low or high zinc diets (Fig 6D–6K).

The highly restricted expression pattern of the Zip88E-GAL4 reporter gene came as a surprise given the strong zinc-sensitivity phenotype of the Zip88EΔ/Δ mutants. To examine whether Zip88E expression in just these tissues was sufficient to restore systemic Zip88E function, survival of Zip88EΔ/Δ mutants on a high zinc diet was compared with and without the presence of a Zip88E-GAL4>Zip88E::eGFP transgene combination. Expression of Zip88E...
Fig 4. Expression of a Zip88E reporter gene is highly restricted. Expression of membrane-bound mCD8::eGFP driven by Zip88E-GAL4 in third instar larval tissues. GFP expression was observed in the salivary glands (A, B = negative control), in the ventral ganglion of the central nervous system (C, D = negative control) and in a small number of enteroendocrine cells just anterior to the copper cell region of the midgut (E). All images were recorded on a compound fluorescence microscope at 10x (A, B, E) or 20x (C, D) magnification. In A-D), larvae were fixed and immune-stained with an α-GFP antibody followed by an FITC-conjugated secondary antibody. In E), native GFP signal without antibody staining was imaged.

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under the control of the Zip88E-GAL4 driver completely restored the tolerance of Zip88EΔ/Δ homozygotes back to wild type levels when larvae were raised on 4 mmol l⁻¹ ZnCl₂-supplemented media (Fig 7). All genotypes tested contained the same w¹¹¹⁸ X chromosomes and all but Zip88EΔ/+ had at least one autosomal mini-white transgene, abrogating the potential effect of white gene presence / absence on zinc content [14].

**Discussion**

The strong amino acid sequence conservation between mammalian Zips 1 to 3 and *Drosophila* Zips 42C.1, 42C.2, 89B and 88E suggests that these proteins may be playing similar, possibly overlapping roles in zinc transport. Expression data and functional analysis supports this notion for several of these transporters. Zips 42C.1 and 42C.2 have complementary roles in zinc absorption through the iron cells of the fly larval midgut and double knockdown of these transporters causes severe zinc deficiency under zinc-depleted conditions [4]. However, the double knockdown flies are viable under normal dietary conditions suggesting alternative
Fig 6. Zip88E protein localisation is not affected by altered zinc dietary content. Confocal microscopy showing salivary gland cells (A-C) and midgut enteroendocrine cells (D-K) with Zip88E-GAL4>Zip88E::eGFP (green) and nuclear DAPI staining (blue) from third instar larvae raised on basal media (A, D, G, J) or media supplemented with 100 μM TPEN (low zinc, B, E, H, K) or 4 mM ZnCl₂ (high zinc, C, F, I). In the enteroendocrine cells, Zip88E::eGFP is observed at both the outer cell surface (D-F) and surrounding the nucleus within the body of the cell (G-I). A lateral view of these cells (J, K) illustrates how they span the width of the midgut epithelial layer.

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absorption pathways are available. Zip89B is expressed both in the iron cells and extensively in the posterior midgut and in mutant larvae lacking this transporter, Zips 42C.1 and 42C.1 are upregulated [5], indicating that Zip89B may be providing additional zinc uptake capacity that is normally not essential.

This work investigated whether Zip88E may also be playing an auxiliary role in zinc absorption. First we confirmed that when over expressed, Zip88E acts in a manner consistent with a role in increasing cytosolic zinc levels, as seen previously [11]. Although ectopic Zip88E expression in the eye alone has no effect on eye morphology, when combined with ZipFoi over expression (increased zinc uptake) or ZnT63C RNAi knockdown (decreased zinc efflux), a
flattened eye with disrupted morphology results, suggesting a zinc toxicity leading to cell death. However, the interactions seen between Zip88E over expression and Zip48C knockdown / ZnT33D over expression were contrary to expectations since both these manipulations are predicted to decrease cytosolic zinc levels [11] yet both combine with Zip88E expression to cause an apparent zinc toxicity phenotype. Rather than a simple cellular zinc uptake role, Zip88E appears to provide a more complex contribution to cellular zinc distribution, consistent with its predominantly intracellular localisation [11]. One caveat in these over expression studies is that Zip88E is being expressed in tissues it may not normally be active in and therefore the interactions observed may not reflect endogenous interactions.

The sensitivity of the Zip88EΔ/Δ mutant larvae to normally sub-lethal levels of dietary ZnCl₂ argues against a function in dietary zinc absorption. If Zip88E were required normally for zinc uptake in the midgut, the mutants might be expected to show increased sensitivity to zinc depletion or higher tolerance to zinc toxicity, neither of which was observed. The expression pattern of our Zip88E-GAL4 reporter gene was also not consistent with a role for this gene in zinc absorption. The only midgut expression observed was in a highly restricted set of enteroendocrine cells at the anterior / middle midgut boundary. No expression was seen in the iron cells where Zips 42C.1, 42C.2 and 89B are thought to mediate most zinc absorption, nor in any other region of the midgut. While reporter genes do not necessarily capture the entire expression pattern of a gene, the ability of the Zip88E-GAL4:Zip88E:eGFP transgene combination to rescue the zinc sensitivity phenotype of the Zip88EΔ/Δ mutant larvae provides evidence that the Zip88E-GAL4 reporter is driving expression in the cell types responsible for the mutant phenotype. Previously we have reported that RNAi knockdown of Zip88E suppresses zinc toxicity phenotypes caused by eye-specific over expression of Zip71B or ZnT86D [11], implying that Zip88E plays an endogenous role in increasing cytosolic zinc levels in the eye. The absence of Zip88E-GAL4 expression in the eye may be due to an incomplete reporter gene or may indicate that the original RNAi result was in fact due to off-target knockdown of a similar Zip such as Zip42C.1.

The highly restricted expression pattern of the Zip88E reporter gene is strongly suggestive of an enteroendocrine role for this gene. In the midgut, we observed expression only in ~six Prospero +ve cells located at the boundary between the anterior and middle midgut segments. Previously, cells in this location have been show to express both allatostatin B / MIP and diuretic hormone DH31 [15]. Allatostatin B is a myo-inhibitory peptide that is able to suppress gut peristalsis in insects. DH31 signals through a G protein-coupled receptor encoded by CG17415 expressed in the Malpighian tubules [16] to regulate fluid secretion but is also able to increase peristaltic muscle contraction [17]. Additional membrane-bound GFP under Zip88E-GAL4 control was observed in two parallel stripes down the ventral nerve cord of the larval brain as well as occasional lateral projections. No GFP +ve cell bodies were observed in this region indicating that these may represent peripheral sensory neurons projecting to the central nervous system, a possibility supported by the absence of any nuclear signals when using a predominantly nuclear-localised GFP marker.

How might the zinc sensitivity phenotypes observed in the Zip88E mutant relate to endocrine signalling? A simple model would have Zip88E required for zinc detoxification specifically in the salivary glands and some enteroendocrine cells of the midgut. In the absence of Zip88E, these cell types would be more susceptible to zinc toxicity resulting in a decrease in functionality that is manifested as a general decline in larval survival rate. Alternatively, Zip88E may be playing a role in detecting elevated zinc levels and mounting a systemic response to this stress; in the absence of such a signal, the developing larvae would be less able to tolerate higher zinc in the diet. In support of this notion, a non-cell autonomous response to loss of Zip88E was observed in the decrease in MtnB:EYFP expression in the midguts of
Zip88E mutant larvae. Zip88E is not expressed in the many midgut cells MtnB is active in therefore this transcriptional response is presumably caused by a systemic signal. The decrease in MtnB:EYFP could be explained by an inappropriate release of zinc from the midgut cells into the haemolymph. While normally that zinc would be retained safely in the midgut cells, in the Zip88E mutants, the absence of an inhibitory signal would allow the influx of zinc to cause damage to internal organs, resulting in a higher mortality rate.

To date, evidence from mammalian and insect systems has mainly supported a model whereby regulation of dietary zinc absorption occurs cell-autonomously at the level of the intestinal enterocytes, so the concept of a mechanism for detecting systemic zinc levels and inducing a non-autonomous response to modify intestinal absorption must be treated as speculative. However precedence for such a mechanism can be found in both the copper and iron homeostasis systems. For instance, genetically-induced copper deficiency in the mouse heart triggers a non-autonomous release of copper from the liver and upregulation of absorption mechanisms in the intestine [18], while hepcidin, produced in the liver in response to iron loading, is able to inhibit iron absorption through enterocytes by inducing endocytosis of the ferroportin iron exporter [19].

It appears that Zip88E has diverged appreciably in function from its closest homologues, Zip89B, 42C.1 and 42C.2. First, its expression pattern is quite different, a situation similar to the comparison between mammalian Zip2 and its close homologues Zip1 and Zip3. However we do not seen any obvious parallels between the expression patterns of Zip88E and Zip2 and while Zip2 knockout mice reveal defects under zinc deficiency conditions, the Zip88E mutant flies are only affected by zinc toxicity.

Zip88E differs from its closest fly homologues at the cellular level as well. Unlike the zinc uptake Zips that are all found at the apical plasma membrane, Zip88E::eGFP was observed mainly on intracellular organelles when ectopically expressed [11]. Furthermore, genetic interaction data, looking at the effect of excess Zip expression on phenotypes caused by cellular zinc dysregulation, found that Zip88E behaved more like Drosophila Zips 102B and 99C than its closest homologues [11]. The mammalian homologue of Zip102B, Zip9, has recently been shown to act as a non-classical androgen receptor for testosterone [20–24], working together with an inhibitor G protein to activate MAP kinase signalling as well as releasing free zinc from mitochondria and the nucleus [23]. These endocrine links suggest that functionally, Zip88E may be more closely related to Zip102B / Zip9 and that an endocrine function for Zip88E may have arisen independently in the invertebrate lineage.

Zip88E plays an important role in protecting Drosophila larvae against dietary zinc toxicity and this protective action emanates from a small number of specialised cells. It will be of great interest to determine whether the production / activity of known peptides or their receptors is affected by the loss of Zip88E, and whether the Zip88E mutant zinc sensitivity phenotype can be replicated by inhibiting such peptide activity. A conclusive demonstration of a systemic zinc sensing / response mechanism would dramatically change our view of how this critical biometal is regulated in animals.

**Supporting information**

S1 Table. Oligonucleotide sequences of PCR primers used in this study.

(DOCX)

S1 Fig. Over expression of Zip88E shows no interaction with over expression or RNAi knockdown of most other Drosophila Zip and ZnT genes. GMR>Zip88E::FLAG in combination with the over expression and RNAi suppression (IR) of all remaining Zip / ZnT genes not shown in Fig 1. A) Zip88E::FLAG-only control. B) GMR-GAL4-only control. C)
S2 Fig. Generation of a putative null deletion allele of Zip88E by imprecise P-element excision. A) Annotated schematic of the Zip88E genomic region showing the 5’ and 3’ untranslated regions (UTRs), exons 1 to 4, the location of the original P(EPgy2) element in the 5’ UTR and the location of the oligonucleotide primers used to screen for internal deletions caused by mobilisation of the P(EPgy2) element. B) Schematic of the Zip88E region after a precise P (EPgy2) excision event. C) Schematic of the Zip88E region after an imprecise P(EPgy2) excision event that deleted all of the 5’UTR and exons 1 and 2 of Zip88E, resulting in a putative null allele. This allele, called Zip88E\(\Delta\Delta\), was used in all functional analyses presented here. D) Annotated alignment of the Zip88E genomic sequence from control (top line) and Zip88E\(\Delta\Delta\) (bottom line) flies, showing the full extent of the Zip88E deletion. E) Agarose gel showing PCR products generated using primers Zip88E PF1 and PR2 on gDNA extracted from single adult flies of genotypes Zip88E\(\Delta\Delta\) (1–5) or \(w^{1118}\) control (6–10). All mutant flies show a ~800 bp PCR product compared to the ~1500 bp product present in the control flies.

(TIF)

S3 Fig. Semi-quantitative PCR of Zip gene expression in larval midguts. A) 2.5% agarose gels showing PCR products from cDNA generated from mRNA extracted from dissected midguts of: \(w^{1118}\) control (1); Zip89B\(\Delta\Delta\) (2); and Zip88E\(\Delta\Delta\) (3) third instar larvae. Products for Zip88E, Zip89B, ZipFoi, Zip99C, ZipCatsup; Zip71B, Zip102B, Zip48C and RP49 are seen for each genotype. Zip42C.1 and Zip42C.2 did not produce bands of sufficient intensity for analysis. The lower molecular weight band seen for Zip71B is non-specific. Results shown are representative of two independent cDNA extractions / PCR analyses. B) Separated scatter plot showing quantification of PCR product band intensities from gels illustrated in A (n = 2). Band intensities for each Zip gene were determined using ImageJ then normalised to the control (RP49) band intensities for that particular cDNA sample. For each Zip gene, the normalised band intensity from the \(w^{1118}\) control cDNA sample was set at 1 then band intensities of the two mutant cDNA samples are expressed relative to the control. This semi-quantitative gene expression analysis indicates that no Zip88E expression was detectable in the Zip88E\(\Delta\Delta\) mutant larvae and no Zip89B expression was detectable in the Zip89B\(\Delta\Delta\) mutant larvae, confirming that these two mutations are most likely null mutations. While no Zip genes showed altered expression levels in Zip88E\(\Delta\Delta\) mutant midguts, Zip88E, ZipFoi, ZipCatsup, Zip102B and Zip48C all appeared to be down-regulated in Zip89B\(\Delta\Delta\) mutant midguts. Zip71B could not be analysed due to the presence of non-specific PCR products.

(TIF)

S4 Fig. Equivalent sample loading for westerns blots as shown by Ponceau S staining. A) \(\alpha\)-GFP western blot on lysates from either \(w^{1118}\) or Zip88E\(\Delta\Delta\) whole larvae both containing the MtnB:EYFP transgene, raised on either basal medium or medium supplemented with 2 mmol \(1^{-1}\) ZnCl\(_2\). Two replicates are shown for each condition. A strong GFP signal is observed at molecular mass of ~37kDa. The GFP signal is more intense with \(w^{1118}\) than Zip88E\(\Delta\Delta\) larvae and is induced by exposure to high dietary zinc. B) Ponceau S staining of the membrane
blotted in (A). Similar Ponceau S intensity is seen in each lane indicating that roughly equal amounts of protein are being loaded in each lane.

(TIF)

S5 Fig. Additional images of MtnB:EYFP expression in the larval midgut. MtnB:EYFP expression in third instar larval midguts from w^{1118} control (A and B) and Zip88EΔ/Δ homozygous larvae (C, D) on basal medium (A, C) and after exposure to 2 mmol l^{-1} ZnCl_2-supplemented medium (B, D). Variable MtnB:EYFP expression can be observed between individual flies but overall, decreased MtnB:EYFP expression is observed in Zip88EΔ/Δ midguts compared to control flies on both food types. Fluorescence was observed under dissecting microscope, images were taken with 3 second exposure.

(TIF)

S6 Fig. The Zip88E-GAL4 reporter gene does not respond to changes in dietary zinc content. Confocal microscopy showing dissected third instar salivary glands (A-C), midguts (D-F) and CNS (G-I) from larvae containing either Zip88E>nls::GFP (A-C) or Zip88E>mCD8::GFP (D-I) reporter gene combinations. Larvae were raised on basal medium (A, D, G) or media supplemented with 100 μmol l^{-1} TPEN (low zinc, B, E, H) or 4 mmol l^{-1} ZnCl_2 (high zinc, C, F, I). No changes in the overall Zip88E-GAL4 expression pattern were observed on either low or high zinc diets compared to basal medium. Native GFP signal (without α-GFP antibody staining) is shown in each case and images are representative of >10 individuals for each diet.

(TIF)

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