The Journal of Experimental Biology 215, 3254-3265

doi:10.1242/jeb.069260

RESEARCH ARTICLE

Systematic functional characterization of putative zinc transport genes and identification of zinc toxicosis phenotypes in *Drosophila melanogaster*

Jessica C. Lye[1], Christopher D. Richards[1], Kesang Dechen[1], David Paterson[2], Martin D. de Jonge[2], Daryl L. Howard[2], Coral G. Warr[1] and Richard Burke[1,*]

[1]School of Biological Sciences, Monash University, Melbourne, Victoria, Australia and [2]X-ray Fluorescence Microscopy, Australian Synchrotron, Melbourne, Victoria, Australia

*Author for correspondence (richard.burke@monash.edu)

SUMMARY

The heavy metal zinc is an essential component of the human diet and is incorporated as a structural component in up to 10% of all mammalian proteins. The physiological importance of zinc homeostasis at the cellular level and the molecular mechanisms involved in this process have become topics of increasing interest in recent years. We have performed a systematic functional characterization of the majority of the predicted *Drosophila Zip* (*zinc/iron regulated transporter-related protein*) and *ZnT* genes, using the Gal4-UAS system to carry out both ubiquitous and targeted over-expression and suppression studies for 13 of the 17 putative zinc transport genes identified to date. We found that six of these 13 genes may be essential for fly viability and that three of the remaining seven demonstrate over-expression phenotypes. Our findings reaffirm the previously proposed function of *dZnT63C* (CG17723: FBgn005432) as an important zinc efflux protein and indicate that the fly homolog of *hZip1*, *dZip42C.1* (*CG9428:* FBgn0033096), is a strong zinc importer in *Drosophila*. By combining over-expression of *dZip42C.1* with suppression of *dZnT63C* we were able to produce easily identifiable zinc toxicosis phenotypes, which can be rescued or worsened by modifying dietary zinc content. Our findings show that a genetically based zinc toxicosis situation can be therapeutically treated or exacerbated by modifications to the diet, providing a sensitized background for future, more detailed studies of Zip/ZnT function.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/18/3254/DC1

Key words: zinc, zinc transport, ZnT, Zip, *Drosophila melanogaster*, *dZnT63C*, *dZip42C.1*.

Received 5 January 2012; Accepted 6 June 2012

INTRODUCTION

The heavy metal zinc is an essential component of the human diet. Adequate, bioavailable zinc is crucial for correct functioning of the immune response, protein and DNA synthesis, retinal development, liver function, blood clotting, metalloenzyme function and olfaction (Berg and Shi, 1996; Tubek et al., 2008). In biological systems, zinc, a group IIB transition metal, is redox inert and remains in the Zn^{2+} ionic state (Maret, 2010). Sequence data mining projects have estimated that up to 10% of human proteins bind zinc (Andreini et al., 2006). The ability of Zn^{2+} ions to form structure-stabilizing cross-links makes the metal an ideal structural cofactor for small proteins and the flexible coordination geometry of zinc ions allows zinc binding proteins to shift conformations quickly in order to carry out complex biological reactions (Berg and Shi, 1996; Brown et al., 2002; Wellinghausen et al., 1997). As such, zinc is an essential catalytic cofactor of some key metabolic proteins such as carbonic anhydrase and alkaline phosphatase (Vallee and Auld, 1990a; Vallee and Auld, 1990b). Symptoms of zinc deficiency in humans include the impairment of eyesight, taste (hypogeusia) and smell (anosmia), a decreased immune response, skin lesions, hyperkeratinization of the epidermis and testicular atrophy as well as decreased cognitive and motor functions in children (Brown et al., 2002; Hambidge, 2000; Tubek, 2006; Tubek, 2007).

Symptoms of zinc toxicosis include manifestation of the immune disorder neutropenia, a haematological disorder characterized by an abnormally low number of neutrophil granulocytes and anaemia (Willis et al., 2005). In addition, inhalation of zinc salts has been shown to cause metal fume fever, which manifests as an acute respiratory tract infection and can lead to chronic asthma (Fuortes and Schenck, 2000). The link between zinc homeostasis and widespread or debilitating diseases such as diabetes mellitus, Alzheimer's disease, asthma, acrodermatitis enteropathica and Crohn's disease has come under intense scrutiny in recent years (Chen et al., 2000; Frederickson et al., 2005; Friedlich et al., 2004; Gyulkhandanyan et al., 2008; Smidt et al., 2009; Sturniolo et al., 2001).

At the cellular level, zinc homeostasis is achieved through the interplay of proteins involved in zinc uptake and export. These processes have been shown to be regulated by two gene families that are common across many phyla; the *Zrt/IRT-like* (*Zip*) gene family (*solute carrier family 39*, *SLC39A*) and the *cation diffusion facilitator* (*CDF*) family (*SLC30A*) – commonly described as *ZnT* genes (Kambe et al., 2008). *In vivo* studies have produced a large amount of data regarding zinc transporter function (Falcón-Pérez, Dell'Angelica, 2007; Mao et al., 2007; Murakami and Hirano, 2008; Ohana et al., 2009). The *lethal milk* (*lm*) mutant mice carry a loss of function mutation in the *ZnT4* gene which results in reduced zinc secretion into the milk produced in epithelial mammary gland cells (Dufner-Beattie et al., 2006). As a result, homozygous *lm/lm* pups suckling from *lm/lm* dams do not survive past the first 2 weeks of neonatal development (Murgia et al., 2006). Mice with impaired ZIP1, ZIP2 or ZIP3 function display a decreased ability to absorb

zinc from the diet, particularly during pregnancy when zinc absorption is normally increased (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). The *ZnT8* knockout (KO) mouse model, which displays decreased insulin secretion, has provided insights into the endogenous function of ZnT8 as an importer of zinc into insulin-containing granules in pancreatic β-cell islets (Hardy et al., 2011). Additionally, *ZnT5* KO mice have defects in mast cell delayed-type allergic reactions, illustrating the potentially important role of zinc transport proteins in immune response pathways (Nishida et al., 2009).

KO mouse models have also provided evidence for important signalling roles for some *Zip* genes, suggesting that there is an even broader range of functions carried out by zinc transport proteins than previously thought. *Zip13* KO mice display impaired connective tissue development – this transporter has been suggested to play a role in localizing SMAD to the nucleus (Fukada et al., 2008) – whilst *Zip14* KO mice display impaired gluconeogenesis, presumably due to the disruption of endogenous ZIP14 function as a regulator of G protein-coupled receptor signalling (Hojyo et al., 2011). Furthermore, it has recently been shown that proteins other than ZIP and ZnT may be able to transport zinc. Mammalian TRPM3 has been demonstrated to mediate the influx of zinc into pancreatic β-cells (Wagner et al., 2010) and loss of function of the *Drosophila* TRPM channel gene, *dTRPM*, resulted in larval growth inhibition and cell autonomous growth defects associated with defects in mitochondrial structure and function (Georgiev et al., 2010). All of the *dTRPM* mutant phenotypes could be phenocopied by zinc depletion and partially rescued by zinc supplementation (Georgiev et al., 2010), indicating a key role for dTRPM in regulating zinc homeostasis in a specific cellular organelle, although the cellular localization of this channel is not yet known.

In addition to transport and channel genes, several other gene classes are known to play an integral role in regulating cellular and systemic zinc levels. The *metallothionein* (*mtn*) genes encode small metallophilic proteins whose production is stimulated upon an increase in cellular levels of metals such as zinc, copper or cadmium. Mtns are thought to sequester and detoxify these metals in the cytosol while also providing a storage option. Genetic ablation of all four *Mtn* genes (A–D) in the vinegar fly *Drosophila* results in viable flies that are exquisitely sensitive to elevated dietary metal levels (Egli et al., 2006). Transcriptional induction of the *Mtn* genes requires the activity of metal transcription factor 1 (Mtf-1), which is also essential for viability under metal stress in both *Drosophila* and mouse (Egli et al., 2003; Wang et al., 2004b).

In *Drosophila*, disruption of metal transport gene expression has previously been shown to result in severe and easily recognizable phenotypes. Copper-related phenotypes that result from over-expression or suppression of important copper transport genes, such as *DmATP7* and *Ctr1a*, commonly result in abnormal pigment phenotypes, neuronal defects and impaired survival when larvae and adults are reared on modulated copper diets (Binks et al., 2010; Burke et al., 2008; Norgate et al., 2006). In addition to the previously characterized *Drosophila* zinc transport genes *ZnT35c* (Yepiskoposyan et al., 2006), *dZnT63C* (Wang et al., 2009), *fear of intimacy* (*foi*) (Mathews et al., 2006; Mathews et al., 2005) and *Catchecholamines up* (*Catsup*) (Stathakis et al., 1999), nine more putative zinc transport genes were identified for analysis during this study with an additional four being recently identified by homology to mammalian proteins. Thus, with 17 predicted zinc transport genes compared with the 25 in mammals, the *Drosophila* system is clearly still complex yet has reduced scope for redundancy compared with higher organisms and, more importantly, provides rapid and flexible gene manipulation tools for sophisticated *in vivo* functional analyses.

Here, we used the UAS-Gal4 system to analyse *Drosophila* zinc transporter function *in vivo*. In particular, we focused on characterizing the functions of zinc transporter genes in two distinct tissue types, the developing eye and midline epithelial tissue. We generated tissue-specific zinc toxicosis situations after genetically modifying the expression levels of two zinc transporter genes in a combinatorial manner and are able modify these toxicosis phenotypes by controlling dietary zinc uptake during development.

MATERIALS AND METHODS

Drosophila stocks

The following fly stocks were used: w^{1118} (BL3605, Bloomington Stock Centre, IN, USA). *gmr-Gal4*, *P{GMR-GAL4.w⁻}2* (BL9146). *pannier-Gal4*, *P{GawB}pnr*^{MD237} (BL3039). *Tubulin-Gal4*, *P{tubP-GAL4}LL7/TM3*, Sb^1 (BL5138). RNA interference (RNAi) lines (Dietzl et al., 2007) obtained from the Vienna *Drosophila* RNAi Centre (VDRC) include: v13311 (*dZnT41F*), v7461 (*dZnT63C*), v105145 (*dZnT63C*), v7688 (*dZnT33D*), v103398 (*dZnT33D*), v3836 (*dZnT35C*), v103263 (*dZnT35C*), v5390 (*dZnT77C*), v12132 (*dZnT86D*), v107388 (*dZnT86D*), v49329 (*dZip88E*), v106785 (*dZip88E*), v7338 (*dZip42C.2*), v3986 (*dZip42C.1*), v37358 (*dZip89B*), v10102 (*fear of intimacy*), v1362 (*dZip99C*) and v10095 (*Catchecholamines up*). CG numbers for zinc transport genes are listed in supplementary material Table S1. Over-expression lines generated for this study were: *pUAST dZnT41F*^{flag}, *pUAST dZnT63C*^{flag}, *pUAST dZnT33DPB*^{flag}, *pUAST dZnT35C*^{flag}, *pUAST dZnT77C*^{flag}, *pUAST dZnT86D*^{flag}, *pUAST dZip88E*^{flag}, *pUAST dZip42C.2*^{flag}, *pUAST dZip42C.1*^{flag}, *dZip42C.1*^{eGFP}, *pUAST dZip89B*^{flag}, *pUAST fear of intimacy* (*FOI*)^{flag}, *pUAST dZip99C*^{flag} and *pUAST Catchecholamines up* (*Catsup*)^{flag}.

Cloning and generation of transgenic *Drosophila*

Drosophila full-length open reading frames for all putative zinc transport genes were PCR amplified from cDNA extracted from w1118 third instar larvae using primer pairs listed in supplementary material Table S2. In each case the termination codon was omitted to allow C-terminal tagging with either a FLAG epitope or enhanced green fluorescent protein (eGFP tag). Amplified gene fragments were subcloned in-frame with FLAG and eGFP tags into the pUAST-attB vector. This construct was injected into PhiC31 attP 51C and 96E or 86Fb strains (provided by Konrad Basler, Institute of Molecular Life Sciences, University of Zurich). Microinjections utilized an Eppendorf Femtojet apparatus with Femtotips II pre-pulled glass needles (Eppendorf, Hamburg, Germany). The C-terminus was chosen in each case so that expression of full-length cDNA *in vivo* could be confirmed by western blotting or immunohistochemical analysis. Addition of C-terminal epitope tags or fluorescent protein can interfere with the function of the tagged protein; therefore, caution must be taken when interpreting over-expression results using such transgenic constructs.

Bioinformatics

The online source MUSCLE (MUltiple Sequence Comparison by Log-Expectation) was used to align zinc transporter amino acid sequences with default settings. Highly divergent alignments were manually edited using BioEdit Sequence Alignment Editor V.7.0.9.0. Phylogenies were estimated using PhyML 3.0 by maximum likelihood. Phylogenetic trees were annotated using FigTree v1.3.1. Similarity and identity values were calculated using

EMBOSS Matcher (Bill Pearson's *lalign* application, version 2.0u4) (Guindon et al., 2010).

Drosophila maintenance and feeding experiments

All *Drosophila* strains and crosses were maintained on standard medium at 25°C unless stated otherwise. Experiments were performed under the same conditions with zinc- or chelator-supplemented media as described. Standard medium was supplemented with 3–8 mmol l^{-1} zinc chloride ($ZnCl_2$; Sigma-Aldrich, St Louis, MO, USA) to make zinc-supplemented medium, or with *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl)-ethylenedidiamine (TPEN; Sigma-Aldrich) or histidine (Ralph et al., 2010) to make zinc-deficient medium. *Drosophila* embryos were transferred between stages 7 and 16 onto medium supplemented with aqueous prediluted $ZnCl_2$ or TPEN. Feeding experiments were density controlled with 100 embryos transferred per vial in triplicate.

Microscopy

Adult flies were partially dissected then mounted directly onto Plasticine and monitored with a Leica MZ6 stereomicroscope. All images were recorded with a Leica DC300 digital camera using Leica Application Suite (LAS) software. Salivary glands used for localization analyses were freshly dissected in 0.1% phosphate-buffered saline (PBS)-Tween 20 and mounted onto glass slides in 70% glycerol. Fluorescence was detected using a Nikon C1 Upright microscope and an oil-emersion objective lens (×40), using excitation energies of 405, 488 and 561 nm.

Sample preparation for X-ray fluorescence analysis

Wandering third instar larvae were dissected in cold PBS then fixed in 4% paraformaldehyde in PBS for 30 min. Fixed tissues were washed in PBS then rinsed briefly in H_2O prior to two final 2 min washes in 0.1 mol l^{-1} ammonium acetate. Imaginal discs were transferred to a small drop of ammonium acetate on a silicon nitride window. Remaining liquid was allowed to air dry and tissues were left to air dry overnight at room temperature. Silicon nitride windows (product code SiRN-7.5-200-3.0-500; Silson, Northampton, UK) were used: frame size 7.5×7.5 mm; frame thickness 200 μm; membrane size 3.0×3.0 mm; membrane thickness 500 nm.

X-ray fluorescence microscopy analysis

X-ray fluorescence (XRF) microscopy was carried out as per a previously published protocol (Lye et al., 2011), with the following changes: excitation of K-shell fluorescence emission from first row transition metals was carried out using X-rays of 12.73 keV energy. Secondary X-ray fluorescence and scattering was collected using a 384-element Maia detector, which was oriented in back scatter geometry to the beam.

RESULTS

Bioinformatic search reveals a total of 17 putative *Drosophila* zinc transport genes based on homology to mammalian *Zip* and *ZnT* genes

A series of Blastp searches identified 17 genes in the *Drosophila melanogaster* genome whose encoded proteins share amino acid sequence homology to mammalian *Zip* and *ZnT* genes (supplementary material Table S1). Four of these genes, *catsup*, *foi*, *dZnT63C* (also known as *dZnT1*) and *dZnT35C* have previously been characterized and the last three have been confirmed as functional zinc transporters. DZnT35C and DZnT63C have been shown to transport zinc through cells lining the lumen of the Malphigian tubules and midgut, respectively (Wang et al., 2009; Yepiskoposyan et al., 2006), whilst FOI has been shown to take up zinc through the outer plasma membrane in yeast and mammalian cell culture assays (Mathews et al., 2005). Note that while we have retained the long-held names for *Catsup* and *foi*, for the remaining 15 genes we have adopted the nomenclature of Yepiskoposyan (Yepiskoposyan et al., 2006), calling each gene *dZnT* or *dZip* followed by the gene's cytological location. We have avoided assigning a number to each gene to avoid the risk of assuming orthology to mammalian genes that may not prove accurate.

Our phylogenetic analysis shows that individual putative *Drosophila* ZnT and ZIP transporters often bear greater sequence similarity to related human zinc transporters than to each other (supplementary material Figs S1, S2). *Drosophila* and human ZnTs cluster into four distinct clades containing: (1) hZnT2–4, hZnT8, dZnT35C, dZnT33D and dZnT41F; (2) hZnT5–7 and dZnT86D; (3) hZnT1, hZnT10, dZnT63C and dZnT77C; and (4) hZnT9 and dZnT49B. The *Drosophila* and human ZIPs cluster into five distinct clades containing: (1) hZIP1–3, dZip42C.1, dZip42C.2, dZip89B and dZip88E; (2) hZIP9 and dZip102B; (3) hZIP5–6, hZIP10, FOI and dZip71B; (4) hZIP7, hZIP13, dZip99C and CATSUP; and (5) hZIP11 and dZip48C.

Ubiquitous suppression and over-expression analysis indicates that a subset of putative zinc transport genes are essential for viability

It has previously been shown that *dZnT63C* is required for viability, particularly under conditions of zinc stress (Wang at al., 2009). We therefore hypothesized that viability would be challenged by the ubiquitous suppression or ectopic expression of zinc transport genes that play major roles in zinc homeostasis and have little or no functional redundancy with alternative transporters. Therefore a small-scale screen was carried out, which utilized over-expression lines and suppression lines (VDRC RNAi lines) for 13 of the 17 putative zinc transport genes in *Drosophila*. These lines were initially crossed to *tubulin-Gal4*, which drives expression ubiquitously throughout the larval and adult stages, in order to identify zinc transport genes that are important for viability in *Drosophila*.

At least six zinc transport genes appear to be important for viability (Table 1), although possible off-target effects cannot be discounted without rescue experiments with RNAi-resistant over-expression constructs. *foi* has previously been shown to play an important role during development (Van Doren et al., 2003). Suppression of *foi* using *tubulin-Gal4* resulted in lethality at the early larval stage. Ubiquitous suppression of *Catsup*, *dZnT33D* and *dZip42C.2* or enhanced expression of *dZip42C.1* also resulted in lethality during the early larval stages. Ubiquitous suppression of *dZip42C.1* generated a phenotype whereby adult flies display moderate to severe melanotic defects (J.C.L., unpublished) and flies carrying two copies of the $dZip42C.1^{RNAi}$ suppression construct under *tubulin-Gal4* control did not advance beyond the L1 larval stage. Suppression of *dZnT86D* resulted in an extended larval second instar period (up to several weeks) and an inability to progress through the pupal stage of development, resulting in eventual lethality at the wandering third instar or early pupal stage. These larvae displayed distinct reductions in body size in comparison to wild-type larvae and pupae (J.C.L., unpublished). Similar delays in larval development due to disrupted zinc homeostasis by suppression of *dZnT63C* (Wang et al., 2009) or mutation of the ion channel gene *dTRPM* (Georgiev et al., 2010) have previously been reported, with melanotic accumulations attributed to prolonged larval growth phases rather than a specific disruption to zinc levels (Georgiev et al., 2010).

Table 1. Summary of zinc transporter gene knockdown and over-expression phenotypes

Zinc transporter	Over-expression (tubulin-GAL4)	Suppression (*tubulin-Gal4*)	Over-expression (*gmr*† and *pnr*‡-*Gal4*)	Suppression (*gmr*† and *pnr*‡-*Gal4*)
dZnT41F	Viable	Viable	No phenotype	No phenotype
dZnT63C	Viable – melanotic masses	Viable	No phenotype	No phenotype
dZnT33D	Stunted humeral bristles	Early larval lethal*	Stunted humeral bristles‡	Mild rough eye†; thorax cleft‡, pigment loss‡, late pupal lethality‡ *
dZnT35c	Viable – melanotic masses	Viable	No phenotype	No phenotype
dZnT77C	Viable	Viable	No phenotype	No phenotype
dZnT86D	Viable	Developmental delay; no progression to pupal stage*	No phenotype	Moderate rough eye†, pigment loss‡ *
dZip88E	Viable	Viable	No phenotype	No phenotype
dZip42C.2	Viable	Early pupal lethal	No phenotype	No phenotype
dZip42C.1	Lethal at early larval stage	Melanotic masses; early larval lethal (×2)	Very mild rough eye†, larval lethal‡	No phenotype
dZip89B	Delayed development	Viable	No phenotype	No phenotype
foi	Viable	Early larval lethal	No phenotype	No phenotype
dZip99C	Viable	Viable	No phenotype	No phenotype
Catsup	Viable	Early larval lethal	No phenotype	Thorax cleft‡, pigment loss‡, late pupal lethality‡

Ubiquitous and tissue-specific suppression or over-expression of 13 putative *Drosophila* zinc transport genes using *tubulin-Gal4, gmr-Gal4*† and *pannier-Gal4*‡. Correct functioning of six zinc transport genes – *dZnT33D*, *dZnT86D*, *dZip42C.1*, *dZip42C.2*, *Catsup* and *fear of intimacy* (*foi*) – appears to be necessary in order to retain fly viability. In cases in which more than one RNAi line was tested, only stronger phenotypes are listed (asterisk). Progeny displayed few abnormal phenotypes following targeted over-expression or suppression of putative zinc transport genes. Over-expression crosses were performed with transgenic flag-tagged lines integrated at the 51C attP docking site.

This suppression/over-expression screen was repeated using two different *Gal4* driver lines. *gmr-Gal4* drives expression in all cells of the eye disc posterior to the morphogenetic furrow, as well as the larval Bolwigs organ and the corpus cardiaca neurons of the larval ring gland (J.C.L., unpublished). The *pannier-Gal4* expression domain encompasses the dorsal midline epidermal cells from the occipital head region to the end of the abdomen but excludes the terminalia. It is also expressed in the dorsal region of the larval wing disc, which eventually will become the notum of the adult fly. The *gmr-Gal4* driver commonly produces morphological phenotypes whilst still retaining larval and adult viability. The *pannier-Gal4* driver has been shown to present phenotypes ranging from abnormalities in pigment, bristle and cuticular arrangements to a loss of viability (Mummery-Widmer et al., 2009). The results from these experiments are summarized in Table 1. Whilst suppression of *dZip42C.1* produced no phenotypes, enhanced expression of *dZip42C.1* using *pannier-Gal4* resulted in a lethal phenotype but had no effect on eye development using *gmr-Gal4* under normal conditions.

Over-expression of *dZip42C.1* in the eye leads to disrupted eye development under high zinc conditions

We attempted to gauge the sensitivity of eye tissue to dietary zinc in flies over-expressing *gmr>dZip42C.1*^{flag}. The effect of raising these flies on zinc-supplemented diets was noticeable. Severe reductions in total eye tissue, as well as disorganized ommatidial and sensory bristle arrangements were observed in a zinc dose-dependent manner (Fig. 1). When raised on 3 mmol l^{-1} zinc-supplemented media, *gmr>dZip42C.1*^{flag} adults displayed a mild rough eye phenotype (Fig. 1F) compared with untreated media (NF,

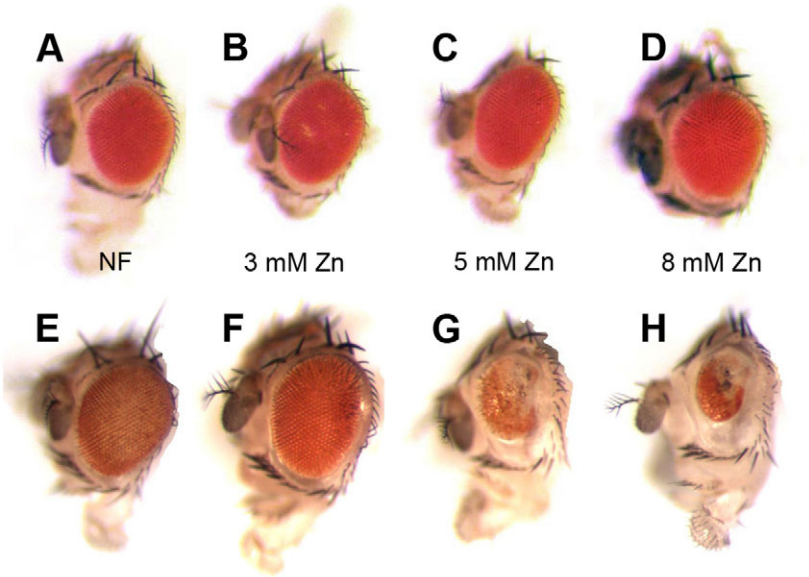

Fig. 1. *Drosophila* over-expressing *dZip42C.1*^{flag} in the developing eye display a zinc-sensitive phenotype. (A–D) *gmr*>wild-type controls and (E–H) *gmr>dZip42C.1*^{flag} over-expression phenotype raised on (A,E) untreated media (NF), (B,F) 3 mmol l^{-1} zinc media, (C,G) 5 mmol l^{-1} zinc media and (D,H) 8 mmol l^{-1} zinc media. Eye phenotypes are progressively worsened in a zinc dose-dependent manner.

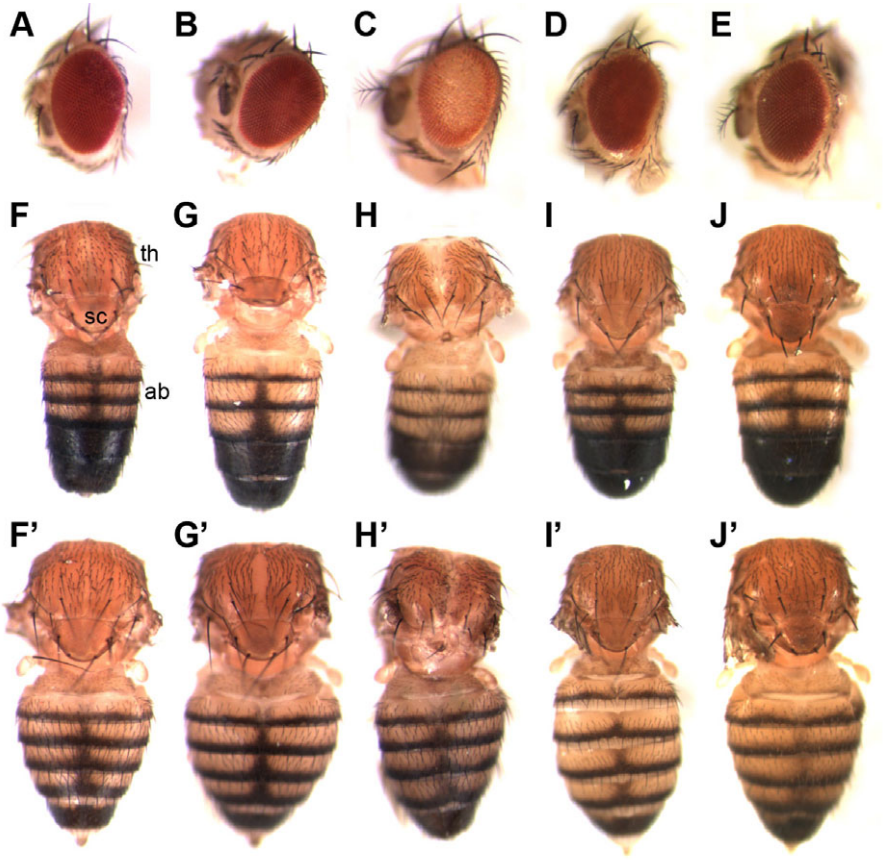

Fig. 2. *dZip42C.1*^{eGFP} over-expression phenotypes are exacerbated by co-suppression of *dZnT63C*. (A–E) *gmr-Gal4*. (F–J) *pannier-Gal4* males. (F′–J′) *pannier-Gal4* females. (A,F,F′) Suppression of *dZnT63C* in the *gmr* and *pannier* domains results in a wild-type phenotype. (B,G,G′) Over-expression of *dZip42C.1*^{eGFP} in the *gmr* and *pannier* domains results in a wild-type phenotype. (C,H,H′) Combinatorial over-expression of *dZip42C.1*^{eGFP} and suppression of *dZnT63C* with *gmr-Gal4* and *pannier-Gal4* results in morphological abnormalities and eye hypo-pigmentation, which is rescued to wild-type by additional suppression of *dZip42C.1* (D,I,I′) or over-expression of *dZnT63C* (E,J,J′). In F: th, thorax; sc, scuttellum; ab, abdomen.

Fig. 1E). *gmr>dZip42C.1*^{flag} flies raised on 5 mmol l^{-1} zinc-supplemented media displayed a moderate rough eye (Fig. 1G) whilst development on 8 mmol l^{-1} zinc-supplemented media resulted in highly irregular adult eye morphology and an obvious decrease in total eye tissue (Fig. 1H). At all zinc doses, wild-type control eyes were unaffected (Fig. 1A–D). Because of sequence homology between dZip42C.1 and hZIP1, we predict that this protein is involved in cellular zinc uptake and that enhanced expression may lead to increased uptake and subsequent cellular toxicity when dietary zinc concentrations are increased.

Generation of a modifiable zinc toxicosis phenotype in two distinct tissue types

We aimed to produce an obvious but non-lethal zinc toxicosis phenotype within the fly, which was morphologically evident under normal dietary conditions but could be modified by altering dietary zinc load. As our zinc feeding experiment results suggested that *dZip42C.1* is a strong zinc transporter, we used *dZip42C.1* enhanced expression as a baseline for achieving a modifiable zinc toxicosis phenotype. We hypothesized that the phenotype gained from an increased zinc supply due to *dZip42C.1*^{flag} over-expression could

Table 2. The effect of zinc transporter gene knockdown and over-expression in combination with *dZip42.1*^{eGFP} over-expression

	gmr-Gal4	*pannier-Gal4*
Zinc transporter suppression		
dZnT41F	Wild-type	Wild-type
dZnT63C	Rough eye, loss of pigment	Scutellum loss, thorax cleft, pigment loss, bristles misaligned
dZnT33D	Mild rough eye	Larval lethal (no change from control)
dZnT35c	Wild-type	Wild-type
dZnT77C	Wild-type	Sporadic abdominal cuticle defects
dZnT86D	Wild-type	Wild-type
Zinc transporter over-expression		
dZip88E	Wild-type	Wild-type
dZip42C.2	Mild rough eye	Reduced scutellum, mild cleft, abnormal thoracic bristle alignment
dZip42C.1	Mild rough eye	Embryonic/early larval lethal
dZip89B	Flattened eye	Abnormal thoracic bristle alignment
foi	Wild-type	Wild-type
dZip99C	Wild-type	Abnormal thoracic bristle alignment
Catsup	Wild-type	Wild-type

Over-expression of *dZip42C.1*^{eGFP} in combination with *ZnT* suppression or *Zip* over-expression. In comparison with crosses in which single zinc transport genes were over-expressed or suppressed, more abnormal phenotypes were produced by over-expression of *dZip42C.1*^{eGFP} in combination with *ZnT* suppression or *Zip* over-expression. Over-expression of *dZip42C.1*^{eGFP} with *dZnT63C* suppression produced strong, highly penetrant phenotypes in both tissue types.

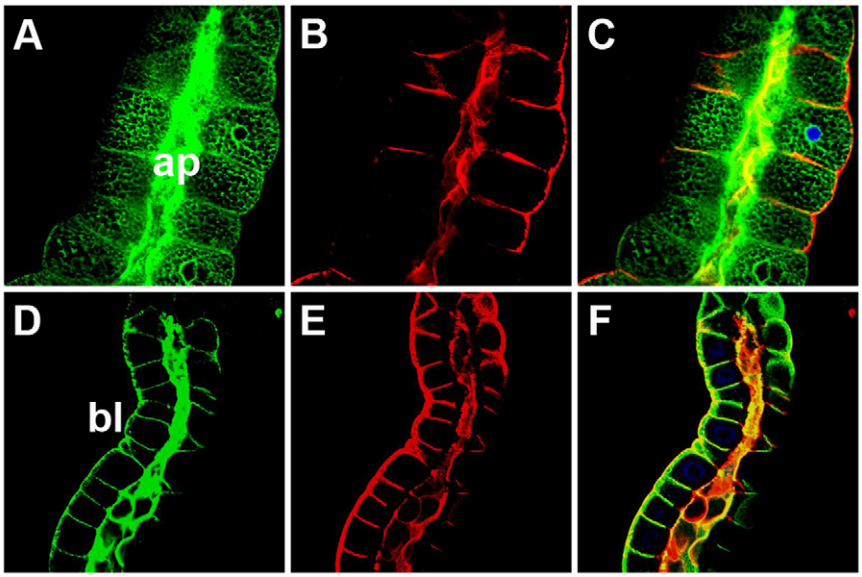

Fig. 3. Localization of dZIP42C.1^{eGFP} and dZNT63C^{eGFP} in larval salivary gland cells. (A–C) The dZIP42C.1^{eGFP} fusion protein (green) localizes to the inner apical (ap) membrane and some intracellular compartments in larval salivary gland cells. In some cells dZIP42C.1^{eGFP} also displays a mild perinuclear localization. (D–F) dZNT63C^{eGFP} localizes strongly at the outer basolateral (bl) plasma membrane as well as to the apical membrane. Both fusion constructs are driven by *elav-GAL4*. (B,E) F-actin is stained with phalloidin (red). (C,F) Merged image showing additional DNA staining with DAPI (blue).

be emulated *in vivo via* co-expression of other *Zip* genes or suppression of *ZnT* genes. As *pannier>dZip42C.1^{flag}* produced a lethal phenotype, we used the attenuated *dZip42C.1^{eGFP}* line for this screen as it produced only a mild thoracic phenotype when over-expressed in the midline and did not compromise viability.

The screen yielded several interesting phenotypes, shown in Fig. 2 and summarized in Table 2. Suppression of *dZnT63C* coupled with over-expression of *dZip42C.1* produced the strongest phenotype. In the eye, sensory bristles are visibly misaligned and the entire eye, particularly in the centre, appears highly de-pigmented when the *gmr-Gal4* driver was used (Fig. 2C). When crossed to *pannier-Gal4*, this combination produced severe thoracic clefts, loss of cuticle pigmentation, thoracic bristle misalignment and loss of scutellum (Fig. 2H). The stronger *dZip42C.1^{flag}* line was also crossed to the *dZnT63C^{RNAi}* suppression line in the eye. The result was a pupal lethal phenotype and almost a complete lack of eye development under *gmr-Gal4* control. Co-over-expression of the related *Zip* genes *dZip89B* or *dZip42C.2* with *dZip42C.1^{eGFP}* also resulted in a mild abnormal eye and midline phenotype (J.C.L., unpublished), further suggesting that *dZip42C.1* over-expression is promoting increases in cellular zinc uptake that can be boosted by co-expression of related zinc importers. Phenotypes caused by the combination of *dZip42C.1^{eGFP}* expression and *dZnT63C^{RNAi}* suppression in either the eye or the midline could be rescued completely by the additional suppression of *dZip42C.1* (Fig. 2D,H) or over-expression of *dZnT63C^{flag}* (Fig. 2E,I). This indicates that the RNAi lines for both these genes are highly specific and unlikely to be exhibiting off-target effects and that the over-expression lines for both genes are indeed active despite the presence of the C-terminal FLAG epitope.

Ectopic expression and visualization of the dZIP42C.1^{eGFP} fusion protein verified that this line was expressing the *dZip42C.1^{eGFP}* transgene. This fusion protein localized to the inner apical (lumenal) membrane and some intracellular compartments of third instar larval salivary gland cells (Fig. 3A–C), consistent with the previously reported apical localization of mammalian ZIP1 (Wang et al., 2004a). Additionally, ectopic expression of a dZNT63C^{eGFP} fusion protein in the larval salivary gland indicated that this protein localized to the outer basolateral plasma membrane of gland cells (Fig. 3D–F) consistent with dZNT63C localization at the outer membrane of larval midgut enterocyte cells (Wang et al., 2009). Some dZNT63C was also observed at the apical membrane. In the light of a previous report that identified *dZnT63C* as a zinc transport gene (Wang et al., 2009), and our own *dZip42C.1^{eGFP}/dZnT63C^{RNAi}* phenotypes, we then combined the *dZnT63C^{RNAi}* suppression line with all *Zip^{flag}* over-expression lines in an attempt to produce another zinc toxicosis condition; however, no additional phenotypes were observed.

Zinc toxicosis eye phenotypes are modified by modulating dietary zinc intake in a dose-dependent manner

Verification of the zinc-specific nature of the eye and midline phenotypes was achieved by raising these crosses on zinc- or TPEN-supplemented media. Raising control *w^{1118}* flies on media supplemented with 1–14 mmol l^{-1} zinc chloride from the embryo stage does not affect morphology post-eclosion (J.C.L., unpublished). In contrast, expression of *dZip42C.1^{eGFP}* coupled with *dZnT63C* suppression under *gmr-Gal4* control resulted in increasingly acute ommatidial degradation and loss of sensory bristles upon dietary zinc supplementation (Fig. 4A–D). Raising these flies on TPEN-supplemented media rescued the rough eye/loss of pigment phenotype in a dose-dependent manner (Fig. 4E–G). The therapeutic effects of dietary TPEN intake at 50 µmol l^{-1} was minimal (Fig. 4E); however, flies raised on 100 µmol l^{-1} TPEN-supplemented media displayed partial rescue of eye pigmentation (Fig. 4F). When these flies were raised on 150 µmol l^{-1} TPEN-supplemented media, eye morphology and pigmentation appeared almost wild-type (Fig. 4G). The pupal lethal/loss of eye phenotype that resulted from *gmr>dZip42C.1^{flag}/dZnT63C^{RNAi}* was also rescued with dietary TPEN treatment as well as by treatment with 1 mmol l^{-1} dietary histidine or 300 µmol l^{-1} EDTA – two other zinc chelators (Fig. 4I–L). The rescued adults displayed eyes that were a stronger variation of the phenotype observed in *gmr>dZip42C.1^{eGFP}/dZnT63C^{RNAi}* flies.

The zinc toxicosis midline phenotype is modified in a dose-dependent manner by modulating dietary zinc intake

Raising *pannier>dZip42C.1^{eGFP}/dZnT63C^{RNAi}* suppression flies on zinc-supplemented media resulted in an exacerbation of the previously observed phenotype (Fig. 5). When raised on zinc-supplemented diets, adult flies from this cross began to display the formation of large melanotic masses (Fig. 5B–D). These masses were observed primarily in the abdominal regions of both male and female

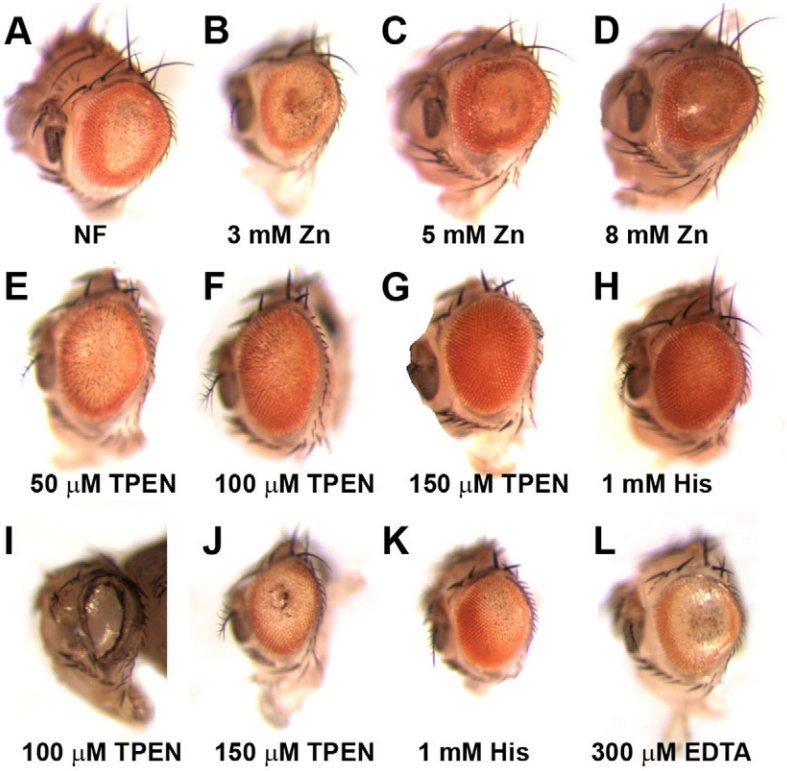

Fig. 4. Modulation of the *dZip42C.1*^{eGFP};*dZnT63C*^{RNAi} phenotype in eye tissue by dietary zinc levels. (A–H) *gmr>dZip42.1*^{eGFP};*dZnT63c*^{RNAi} phenotype raised on (A) untreated media, (B) 3 mmol l^{-1} zinc media, (C) 5 mmol l^{-1} zinc media, (D) 8 mmol l^{-1} zinc media, (E) 50 μmol l^{-1} TPEN media, (F) 100 μmol l^{-1} TPEN media, (G) 150 μmol l^{-1} TPEN media and (H) 1 mmol l^{-1} histidine media. Eye phenotypes are progressively worsened by zinc treatment and rescued by zinc chelator treatment in a dose-dependent manner. (I–L) *gmr>dZip42C.1*^{flag};*dZnT63c*^{RNAi} flies raised on (I) 100 μmol l^{-1} TPEN media, (J) 150 μmol l^{-1} TPEN media, (K) 1 mmol l^{-1} histidine media and (L) 300 μmol l^{-1} EDTA media. The lethality of this transgene combination is rescued by zinc chelation.

adult flies and became more numerous when flies were raised on increasing concentrations of zinc (5 and 8 mmol l^{-1}). At 5 mmol l^{-1} supplemented zinc, partially penetrant lethality at the pupal stage became evident. When flies were raised on 8 mmol l^{-1} zinc-supplemented media, almost complete lethality was observed. Dissection of partially developed pupae from these cases revealed an extremely high occurrence of melanotic masses in pupal tissue and a complete absence of notal/scutellar structures in the *pannier* domain (Fig. 5D).

Decreased dietary zinc uptake resulted in a reversion of the abnormal thoracic phenotype to wild-type (Fig. 5E–H). This occurred in a TPEN dose-dependent manner. Development on 50 μmol l^{-1} TPEN-supplemented food had a minimal effect on the phenotype in the form of partially corrected bristle alignment and occasional evidence of pigment production in the thoracic midline (Fig. 5E). However, raising these flies on 100 μmol l^{-1} TPEN-supplemented food resulted in a reduced thoracic cleft, slight development of the scutellum and scutellar bristles, as well as almost wild-type thoracic

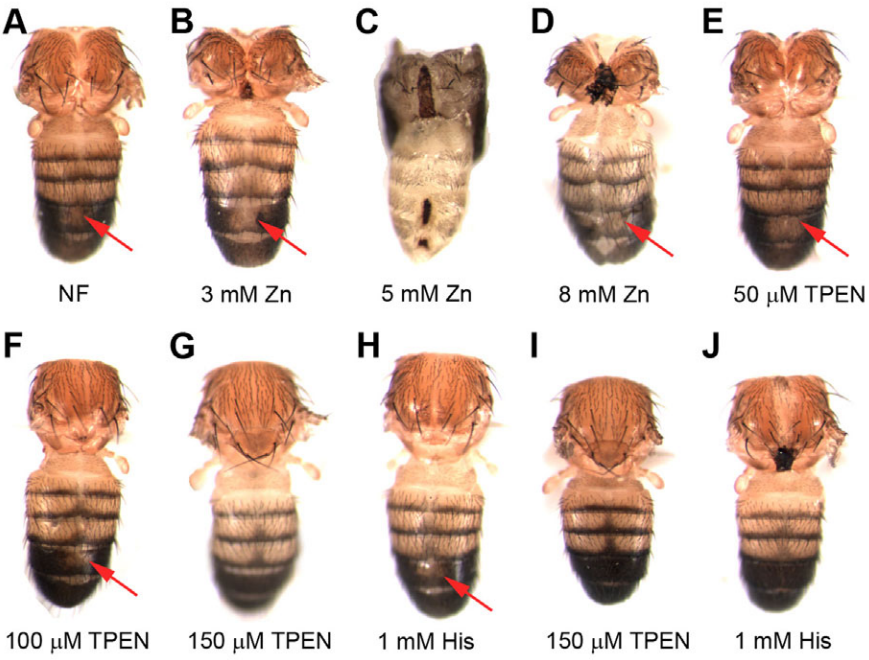

Fig. 5. Modulation of the *dZip42C.1*^{eGFP};*dZnT63C*^{RNAi} phenotype in midline epithelial tissue by dietary zinc levels. (A–D) Adult male *pannier>dZip42C.1*^{eGFP};*dZnT63C*^{RNAi} phenotypes raised on (A) untreated media, (B) 3 mmol l^{-1} zinc media, (C) 5 mmol l^{-1} zinc media, (D) 8 mmol l^{-1} zinc media, (E) 50 μmol l^{-1} TPEN media, (F) 100 μmol l^{-1} TPEN media, (G) 150 μmol l^{-1} TPEN media and (H) 1 mmol l^{-1} histidine media. (I–J) Rescue of *pannier>dZip42C.1*^{flag};*dZnT63C*^{RNAi}-induced lethality on (I) 150 μmol l^{-1} TPEN media and (J) 1 mmol l^{-1} histidine media. Phenotypes are progressively worsened by zinc treatment and rescued by zinc chelator treatment in a dose-dependent manner. Areas of abdominal hypo-pigmentation due to the zinc toxicosis phenotype are indicated by red arrows.

bristle structure and alignment (Fig. 5F). These flies also displayed reduced pigment loss in the midline region of the thorax. A partial-to-full rescue of the abnormal zinc toxicosis phenotype was observed when these flies were raised on 150 μmol l^{-1} TPEN supplemented food (Fig. 5G). Adults displayed an almost wild-type appearance, the only evidence of any phenotypic abnormality being a slight loss of pigment in the midline thoracic region and occasionally a reduction in scutellum size. Additionally, despite the severity of the *pannier>dZip42C.1*^{flag}*/dZnT63C* suppression phenotype, viability could be restored when flies were raised on 150 μmol l^{-1} TPEN-supplemented media (Fig. 5I). These flies displayed only subtle thoracic pigmentation defects and morphologically normal sensory bristle patterns and scutellae.

Inhibition of apoptosis in midline cells exacerbates the zinc toxicosis phenotype

The cleft phenotype caused by *pannier>dZip42C.1*^{eGFP}*/dZnT63C*^{RNAi} and the rough eye phenotype caused by the *gmr>dZip42C.1*^{eGFP}*/dZnT63C*^{RNAi} combination may be due to increased apoptosis within targeted tissues during larval–pupal development. To test this possibility, we inhibited apoptosis by crossing flies displaying zinc toxicosis phenotypes to a UAS p35 line. While expression of p35 alone had no phenotypic effect (Fig. 6A,D), we found that suppressing apoptosis in the *gmr* domain mildly exacerbated the original zinc toxicosis phenotype (Fig. 6B,C). Additionally, suppressing apoptosis in the *pannier* domain noticeably exacerbated the midline phenotype (Fig. 6E,F). This combination caused lethality at the late pupal stage. Dissection of these flies from pupal cases revealed an almost total abolition of thoracic tissue.

A zinc-sensitive reporter line reveals subtle changes in cellular zinc levels in the fly midgut under genetic manipulation of zinc transport genes

Expression of the *MtnB* gene in the fly midgut enterocytes is induced by increases in cellular zinc (and copper and cadmium) levels and a reporter construct driving enhanced yellow fluorescent protein (eYFP) under the control of the *MtnB* regulatory region (*MtnB-eYFP*) can be used as an *in vivo* reporter of zinc levels (Selvaraj et al., 2005). *MtnB-eYFP* displays moderate expression levels in specific sections of the midgut (Fig. 7A). This expression is significantly increased by increasing dietary zinc levels (Fig. 7C) and is suppressed almost to background fluorescence levels in larvae raised on TPEN food (Fig. 7B). To establish the effect of manipulation of zinc transport genes on enterocyte zinc levels, we drove transgene expression using the pan-midgut driver *HR-Gal4* (Chung et al., 2007) in the presence of the *MtnB-eYFP* reporter. Significant increases in *MtnB-eYFP* expression were observed under the suppression of *dZnT63C* (Fig. 7D) and *Catsup* (Fig. 7E) and over-expression of *dZip89B*^{flag} (Fig. 7F), whereas decreases were observed with the suppression of *dZip89B* (Fig. 7G), suppression of *dZnT33D* (Fig. 7H) and over-expression of *dZnT63C*^{flag} (Fig. 7I). The *dZnT63C* and *dZip89B* results are consistent with genes involved in zinc efflux and uptake, respectively, whereas the *Catsup* and *dZnT33D* results indicate a role in intracellular zinc distribution. *dZip89B*^{flag} over-expression could even induce *MtnB-eYFP* expression in TPEN-fed larvae (Fig. 7J). Interestingly, midgut-specific expression of *dZnT86D*^{flag}, *dZip42C.1*^{flag} and *dZip42C.2*^{flag} all resulted in larval death.

The effect of the zinc toxicosis combination on zinc levels was harder to determine. The *dZip42C.1*^{eGFP} construct could not be used as the eGFP would interfere with the *MtnB-eYFP* reporter and the

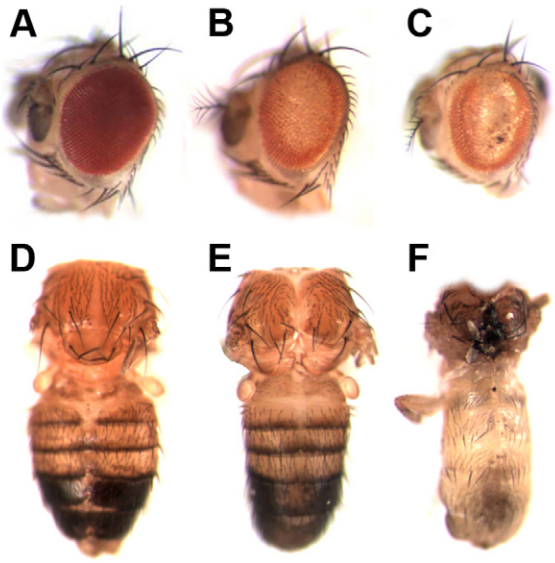

Fig. 6. Inhibition of apoptosis exacerbates the zinc toxicosis phenotypes. *gmr>p35* (A) and *pannier>p35* (D) flies show no abnormal phenotypes. The *gmr>dZip42C.1*^{eGFP}*;dZnT63C*^{RNAi} phenotype (B) is exacerbated by additional expression of *p35* (C). The cleft phenotype displayed by adult flies expressing *pannier>dZip42C.1*^{eGFP}*;dZnT63C*^{RNAi} (E) is exacerbated by additional expression of *p35* (F), resulting in lethality pre-eclosion.

dZip42C.1^{flag} construct caused early lethality under *HR-Gal4* control, both alone and in combination with *dZnT63C* suppression. However, continuous, high-level zinc chelation (150 μmol l^{-1} TPEN) rescues both these genotypic combinations to small but viable larvae. *HR-gal4>dZip42C.1*^{flag} larvae displayed minimal *MtnB-eYFP* activity under these high-TPEN conditions (Fig. 7K) but the addition of *dZnT63C* suppression resulted in increased *MtnB-eYFP* signal (Fig. 7L) indicating that even in these low zinc-availability conditions, a section of the midgut is accumulating high zinc levels.

XRF microscopy analysis of wing imaginal discs indicates a difference in spatial metal distribution

To further examine the effect of zinc transport gene manipulation on cellular zinc content, the spatial distribution of zinc was analysed using larval wing imaginal discs (Fig. 8) by Synchrotron XRF microscopy. The *pannier-Gal4* driver expresses strongly in the dorsal-most region of the wing disc, making this an ideal tissue in which to observe changes in metal distribution when zinc transport gene expression is modified (Lye et al., 2011). In control wing discs, zinc was distributed ubiquitously throughout the entire wing disc, whilst copper was distributed primarily at the ventral margins (Fig. 8A). Calcium was widespread throughout the dorsal region of the disc. Over-expression of *dZip42C.1*^{flag} resulted in a reduction of copper in the ventral margins of the disc and redistribution of zinc and calcium to the dorsal *pannier* region (Fig. 8B). This trend was observed in three out of four wing discs. The effect of *dZnT63C* suppression in the *pannier* domain on zinc distribution appeared to be negligible in all five wing discs observed (Fig. 8C), suggesting there may be an ancillary efflux mechanism at play in response to suppression of *dZnT63C*. Interestingly, *pannier>dZnT63C*^{RNAi} wing discs did display a redistribution of copper to the *pannier* domain and a less polarized distribution of calcium in three wing discs. One out of three wing discs of flies expressing *pannier>dZip42C.1*^{eGFP}*/dZnT63C*^{RNAi} displayed similar zinc,

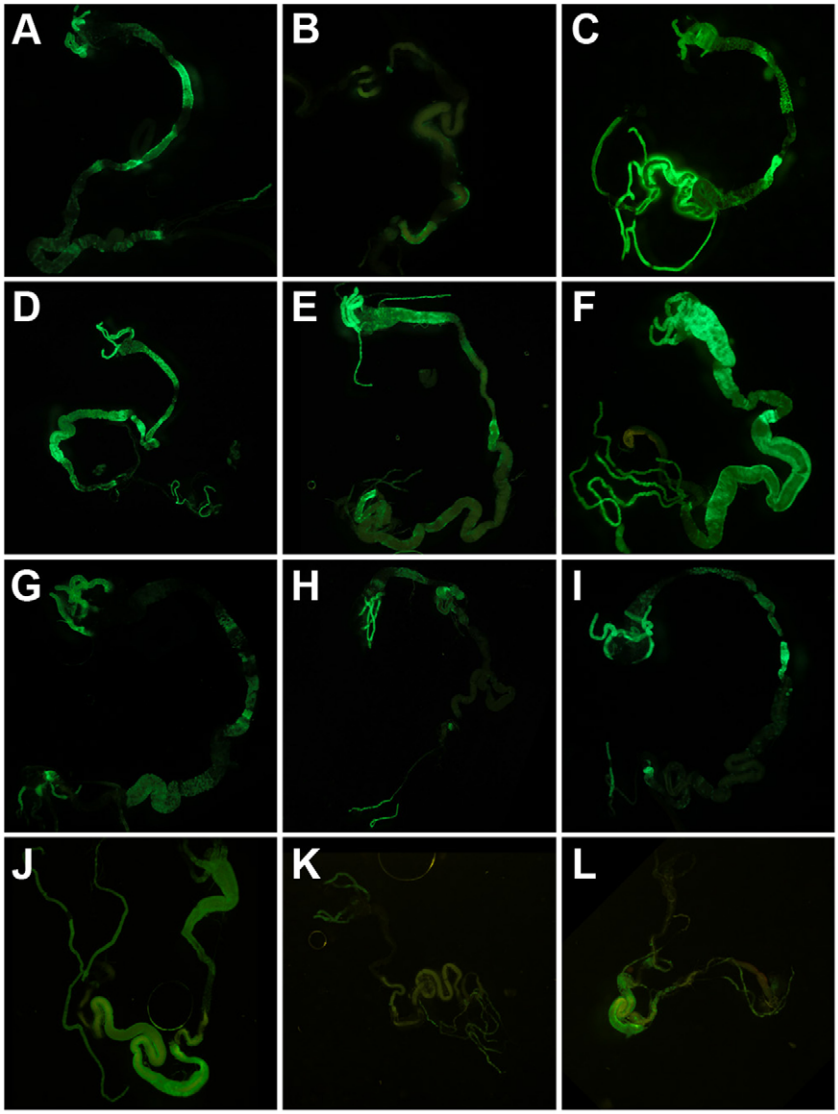

Fig. 7. Manipulation of zinc transporter gene expression alters expression of the zinc-sensitive *MtnB-eYFP* reporter. The *MtnB-eYFP* reporter line shows moderate expression in the midgut of third instar control larvae raised on untreated media (A). *MtnB-eYFP* expression is suppressed in larvae grown on 50 μmol l^{-1} TPEN media (B) and enhanced in larvae grown on 1 mmol l^{-1} zinc-supplemented media (C). Using the pan-neuronal *HR-Gal4* driver, zinc transporter gene expression was manipulated in an *MtnB-eYFP* background (D–L). *MtnB-eYFP* increased under the suppression of *dZnT63C* (D) and *Catsup* (E) and over-expression of $dZip89B^{flag}$ (F), whereas decreases in *MtnB-eYFP* were observed with the suppression of *dZip89B* (G), suppression of *dZnT33D* (H) and over-expression of $dZnT63C^{flag}$ (I). $dZip89B^{flag}$ also induced *MtnB-eYFP* under 50 μmol l^{-1} TPEN conditions (J). Under *HR-Gal4* expression, expression of $dZip42C.1^{flag}$ (K) or $dZip42C.1^{flag}$;$dZnT63C^{RNAi}$ (L) caused lethality unless raised on 150 μmol l^{-1} TPEN. Even under these extreme low-zinc conditions, the zinc toxicosis combination showed significant *MtnB-eYFP* expression (L).

copper and calcium distributions to those observed in *pannier>dZnT42C.1*^{flag} discs; however, copper was still observed along the ventral margins (Fig. 8D). Additional images for each genotype are shown in supplementary material Fig. S3.

DISCUSSION

While *foi* has previously been identified as an important factor during *Drosophila* development (Mathews et al., 2006) and *Catsup* has been implicated in pigment formation by acting as a negative regulator of tyrosine hydroxylase activity (Stathakis et al., 1999), very few putative *Drosophila* ZIP and ZnT transporters have so far been characterized *in vivo*, and focus on the potential zinc-transporting nature of these proteins has been minimal. However, work conducted by Gutiérrez and colleagues (Gutiérrez et al., 2010) does suggest that *foi* is mildly upregulated in zinc-stressed *fumble* mutants. Our findings suggest that *dZip42C.1* is a strong *Drosophila* zinc uptake transporter. Additionally, our data reaffirm the previously proposed function of *dZnT63C* as an important zinc efflux protein. *dZnT63C* expression in enterocyte cells lining the midgut lumen is particularly important in the maintenance of correct zinc homeostasis (Wang et al., 2009). Ubiquitous suppression of *dZnT63C* resulted in decreased lifespan, presumably as a result of insufficient systemic supplies of zinc. Our data have added to the proposed function of *dZnT63C* by extending the known functional requirements of this gene to the eye and dorsal epithelial cells, as suppression of *dZnT63C* in these cells results in strong zinc toxicosis phenotypes when combined with *dZip42C.1* over-expression.

Six of the 13 zinc transport genes characterized here appear to be essential for fly viability, as seen by the lethality caused by ubiquitous RNAi suppression, highlighting the broad requirement for zinc homeostasis in numerous tissues. Of the remaining seven genes, three demonstrated over-expression phenotypes indicating that tight regulation of their activity is needed for animal health. The lack of suppression phenotypes for these seven genes could be due to either redundancy between the *Drosophila* transporters or a conditional requirement for these genes under zinc deficiency or excess conditions. It must be noted that RNAi constructs rarely generate complete suppression of gene activity and therefore residual transcripts could also be responsible for the absence of phenotype. Conclusive loss-of-function analysis will require the generation of null alleles of each of these genes.

Our targeted genetic interaction results indicate that multiple zinc transport genes must be suppressed or enhanced in a combinatorial

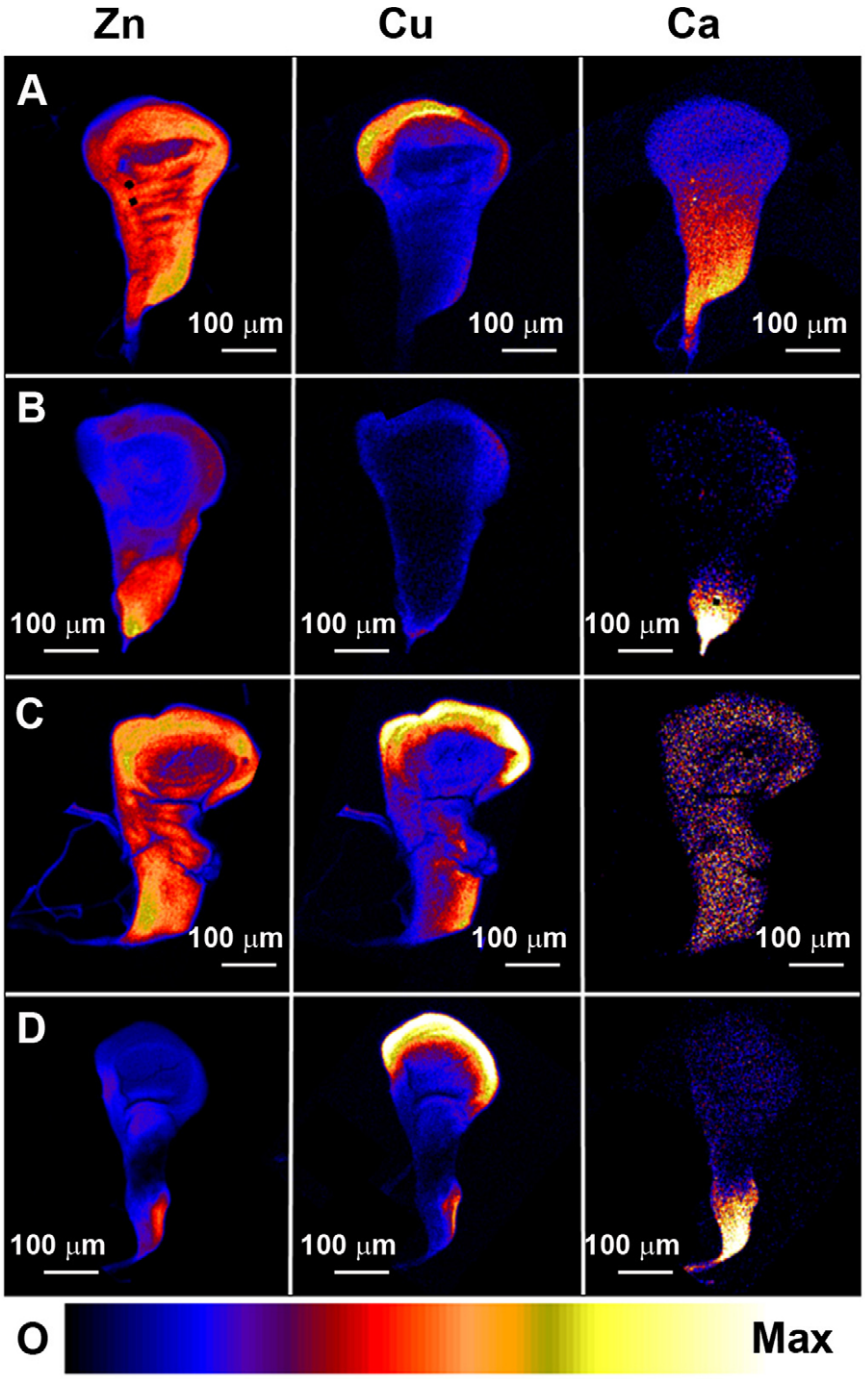

Fig. 8. Manipulation of zinc transporter gene expression alters the spatial distribution of metals *in vivo*. X-ray fluorescence analysis of third instar wing imaginal discs, showing the distribution of zinc (left panel), copper (middle panel) and calcium (right panel). Distribution is displayed as a heat map with the relative concentration shown at the bottom of the figure. For imaginal discs (A–D), element visualization is normalized so that maximum values are as follows: zinc 264 ng cm^{-2}, copper 59 ng cm^{-2}, calcium 264 ng cm^{-2}. Genotypes were: (A) wild-type control disc; (B) *pannier>dZip42C.1^{flag}*; (C) *pannier>dZnT63C^{RNAi}*; and (D) *pannier>dZip42C.1^{eGFP};dZnT63C^{RNAi}*.

manner before target tissues are visibly affected by zinc toxicosis. This buffering effect may be a result of one or a combination of cellular mechanisms. Transcriptional up- or down-regulation of alternative zinc transport genes within the same tissue could offset potential zinc toxic effects, until a certain concentration of cytosolic zinc is reached. In particular, increased activity of ZnT proteins at vesicular zinc storage sites such as zincosomes may confer protective benefits. However, *Drosophila* expression studies have found little evidence that zinc transport genes are strongly up- or down-regulated in response to changes in environmental zinc availability. Indeed, *Drosophila* micro-array analyses have identified only two zinc transport genes that were marginally induced by feeding larvae 5 mmol l^{-1} zinc (Yepiskoposyan et al., 2006).

Our findings suggest that a genetically based zinc toxicosis situation can be therapeutically treated or exacerbated by modifications to the larval diet. Variations in dietary zinc availability had noticeable effects on the zinc toxicosis phenotypes in both eye and midline tissue. Raising wild-type larvae on a diet supplemented with 5 mmol l^{-1} $ZnCl_2$ has previously been shown to increase adult total body zinc content 2.4-fold (Gutiérrez et al., 2010). In the same experiment, dietary supplementation with 100 μmol l^{-1} TPEN was shown to decrease total body zinc in adults by almost 60%. Studies by Yepiskoposyan and colleagues also showed a strong correlation between zinc feeding and significant increases in total body zinc (Yepiskoposyan et al., 2006). These results, coupled with our own, suggest that mechanisms for trafficking zinc from the diet, through

enterocyte cells lining the midgut lumen and into the haemolymph are extremely efficient. The sudden rescue of the more severe *pannier>dZip42C.1*^{flag} phenotype on 150 μmol l^{-1} TPEN-supplemented media, in contrast to the continued lethality observed when these flies are raised on 100 μmol l^{-1} TPEN-supplemented media, further suggests that there is a sensitive threshold effect at play. This threshold effect is also evidenced by the wild-type phenotypes of flies over-expressing *dZip42C.1*^{flag} in contrast to the highly irregular phenotypes observed when *dZnT63C* is additionally suppressed.

The lethality observed when *dZip42C.1*^{flag} over-expression was combined with *dZnT63C* suppression under *gmr-Gal4* control was unexpected as genetic ablation of eye tissue in *gmr>reaper* flies has previously been shown to have no effect on viability (Ditzel et al., 2008). However, we have also found *gmr-Gal4* to drive expression in the corpus cardiacae neuroendocrine cells of the larval ring gland (J.C.L., unpublished), which regulate secretion of the eclosion hormone ecdysone. Lethality at the late pupal stage is therefore likely to be the result of disrupted hormone signalling in the ring gland rather than loss of eye tissue. While we did not observe lethality using this combination under control of a *CC-Gal4* driver (J.C.L., unpublished), which expresses specifically in the corpus cardiacae cells (Kim and Rulifson, 2004), this might be attributable to lower expression levels compared with the *gmr-Gal4* driver.

The zinc toxicosis phenotypes described here have strong potential as sensitive zinc sensors, providing a quick and effective *in vivo* model system for the further characterization of mammalian zinc transport genes. Additionally, treatment of the zinc toxicosis lines with various therapeutics could help to validate the chelating nature of these drugs. Our results suggest that *Drosophila* can be used as an effective model for investigating zinc homeostasis *in vivo*. The relative ease with which combinatorial genetic manipulations can be carried out in targeted tissues will allow a comprehensive analysis of the functional interactions between the various transporter proteins that control cellular zinc levels. In addition, *Drosophila*, and the zinc toxicosis lines in particular, may prove an effective model, which may facilitate the discovery of novel genes that regulate zinc homeostasis. *Drosophila* could also provide an exceptional model for testing therapeutic treatments for diseases that commonly display zinc dys-homeostasis. The implications of these findings to the treatment of zinc disorders such as acrodermatitis enteropathica, a disease characterized by decreased absorption of ingested zinc as a result of a null mutation in the human zinc transport protein ZIP4 (Dufner-Beattie et al., 2003), could be far reaching with respect to the further study of zinc-related diseases as well as to the development of therapeutic agents to control the symptoms of these diseases.

ACKNOWLEDGEMENTS

Drosophila stocks were provided by The Australian *Drosophila* Research Support Facility www.ozdros.com. All *Drosophila* RNAi lines were provided by the Vienna *Drosophila* RNAi Centre. This research was undertaken on the XFM beamline at the Australian Synchrotron, Victoria, Australia.

FUNDING

This research was supported by a Project Grant [grant number 606609] from the Australian National Health and Medical Research Council.

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