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RESEARCH ARTICLE

Biogenesis of zinc storage granules in *Drosophila melanogaster*

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ABSTRACT

Membrane transporters and sequestration mechanisms concentrate metal ions differentially into discrete subcellular microenvironments for use in protein cofactors, signalling, storage or excretion. Here we identify zinc storage granules as the insect's major zinc reservoir in principal Malpighian tubule epithelial cells of *Drosophila melanogaster*. The concerted action of Adaptor Protein-3, Rab32, HOPS and BLOC complexes as well as of the white-scarlet (ABCG2-like) and ZnT35C (ZnT2/ZnT3/ZnT8-like) transporters is required for zinc storage granule biogenesis. Due to lysosome-related organelle defects caused by mutations in the homologous human genes, patients with Hermansky–Pudlak syndrome may lack zinc granules in beta pancreatic cells, intestinal paneth cells and presynaptic vesicles of hippocampal mossy fibers.

KEY WORDS: AP-3 complex, Eye color mutants, ICP-OES, Malpighian tubules, Synchrotron, Zincosomes

INTRODUCTION

Metal ions are cofactors of enzymes (Warner and Finnerty, 1981; Kirby et al., 2008; Gonzalez-Morales et al., 2015; Llorens et al., 2015; Dow, 2017). Iron and copper are required for mitochondrial respiration and cuticle formation (Villee, 1948; Anderson et al., 2005; Binks et al., 2010; Kroll et al., 2014), manganese for superoxide and arginine (nitrogen) turnover (Duttaroy et al., 1997; Samson, 2000), and molybdenum for cysteine and methionine (sulfur) metabolism, and purine and aldehyde catabolism (Bogaart and Bernini, 1981; Marelja et al., 2014). The shared chemical property that turns iron, copper, manganese and molybdenum into essential cofactors of enzymes is the physicochemical stability of their ions in different oxidation states. In contrast, zinc ions do not readily change their valence and are therefore preferentially used as structural binding elements in zinc-finger transcription factors (Schuh et al., 1986; Redemann et al., 1988). Alternatively, the strong Lewis acid activity of the zinc cation is utilized in proteolytic enzymes and carbonic anhydrase (Wessing et al., 1997; Llano et al., 2000).

The development, growth and reproduction of all animals depend on the physiological regulation of metal ions: specific protein metallation is achieved in specialized cellular compartments, facilitated by metal chaperones (Lye et al., 2013; Qin et al., 2013; Southon et al., 2013). Such physiological regulation takes place both systemically through circulating factors secreted from specialized organs and at the cellular level through metal sensing coupled to gene and protein responses (Cyert and Philpott, 2013; Bird, 2015) and is highly relevant in disease (Esposito et al., 2013; Xiao et al., 2013; Zhu et al., 2014; Chi et al., 2015; Ott et al., 2015; Calap-Quintana et al., 2017; Mercer et al., 2017).

These systems are well understood in human iron physiology. At the systemic level, the liver senses transferrin iron saturation (i.e. sufficient iron availability), stores excess iron in ferritin, and secretes hepcidin as a response; hepcidin binds to and internalizes the iron exporter ferroportin from cell membranes, reducing iron efflux at intestinal basolateral membranes, and spleen macrophages recycle iron from senescent red blood cell hemoglobin (Drakesmith et al., 2015; Camaschella et al., 2016; Muckenthaler et al., 2017). At the cellular level, cytosolic iron deficiency results in the stabilization of the transferrin receptor for iron uptake from circulation and in the translational inhibition of ferritin for iron storage through the action of iron regulatory proteins; the opposite effects occur under cytosolic iron overload, and these processes can be viewed as a balancing act (Hentze et al., 2004; Zhang et al., 2014; Kühn, 2015). The similarities and differences between iron regulation in *Drosophila melanogaster* and mammals have been reviewed (Mandilaras et al., 2013; Tang and Zhou, 2013). In *Drosophila*, iron availability is linked to key developmental signals, such as ecdysone synthesis (Llorens et al., 2015; Palandri et al., 2015), and to processes such as the formation of epithelial septate junctions (Tiklová et al., 2010), the functionality of the circadian clock (Mandilaras and Missirlis, 2012), and the induction of mitotic events (Li, 2010). So far, a single iron transporter moving iron into the cytosol has been identified in flies (Orgad et al., 1998; Bettedi et al., 2011) and a single iron exporter has been suggested to traffic iron from the cytosol into the endoplasmic reticulum and Golgi apparatus (Xiao et al., 2014), where insect ferritin resides (Missirlis et al., 2007; Rosas-Arellano et al., 2016).

In contrast to iron, the systemic regulation of zinc homeostasis is not well understood in either human or insect biology. There is a growing appreciation of the specific, directional membrane transport functions provided by the Zrt- and Irt-like proteins (ZIPs) and Zn transporters (ZnTs) and of the metal sequestration properties of the cytosolic metallothioneins (Plum et al., 2010; Babula et al., 2012; Kimura and Kambe, 2016). Zinc-responsive gene regulation is largely mediated through Metal Transcription Factor-1 (MTF-1) (Günther et al., 2012). The metallothionein genes are major targets of MTF-1 because the encoded proteins sequester zinc and other metals such as copper or cadmium. Nevertheless, no humoral factor has been described responding to zinc deficiency, or to zinc overload. Nor is a tissue reserve known from which zinc is mobilized to meet functional requirements under conditions of dietary deprivation. The same considerations apply to *Drosophila*

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zinc physiology (Richards and Burke, 2016; Xiao and Zhou, 2016; Navarro and Schneuwly, 2017). *Drosophila* ZIPs and ZnTs are phylogenetically conserved, with different members of each family localizing to separate subcellular compartments (Lye et al., 2012; Dechen et al., 2015), enabling zinc absorption at the intestine (Wang et al., 2009; Qin et al., 2013; Richards et al., 2015) and zinc excretion from the Malpighian tubules (Yepiskoposyan et al., 2006; Chi et al., 2015; Yin et al., 2017). Cellular responses to zinc are coordinated via MTF-1 and metallothioneins (Egli et al., 2003, 2006; Atanesyan et al., 2011; Sims et al., 2012; Merritt and Bewick, 2017; Mohr et al., 2017; Qiang et al., 2017). MTF-1 also regulates the ferritin subunit genes, for reasons that are unclear (Yepiskoposyan et al., 2006; Gutiérrez et al., 2010, 2013). Little is known about the mechanism of zinc homeostasis in the organism as a whole (Richards et al., 2017), particularly how zinc is stored in *Drosophila* (Schofield et al., 1997).

We came to the question of physiological zinc storage by studying *poco-zinc*, a previously identified recessive X-linked mutation that causes a threefold reduction of total body zinc accumulation in laboratory strains of *D. melanogaster* (Afshar et al., 2013). By genetic mapping, we show that mutants in the *white* gene (Morgan, 1910) have a threefold reduction in zinc content. The white protein encodes an ATP-binding cassette sub-family G2 (ABCG2) transporter that is best known for its function in the transport of two types of pigment precursors in the pigment granules of the eye, functioning as a dimer with either of two other members of the *Drosophila* ABCG2 protein family, brown and scarlet (Dreesen et al., 1988; Mackenzie et al., 2000). Two types of pigment granules have been identified in wild-type animals on the basis of ultrastructure morphology (Nolte, 1961; Shoup, 1966). Many eye color mutants affect enzymes of biosynthetic pathways for the brown ommochromes (Wiley and Forrest, 1981) and bright red drosopterins (Kim et al., 2013), but a subset, known as transport mutants (Sullivan and Sullivan, 1975), affect the formation of the pigment granules *per se*. Amongst these transport mutants, we have also analysed total body zinc accumulation in the adaptin protein complex-3 (AP-3) mutants *garnet* (*g*) (Ooi et al., 1997), *carmine* (*cm*) (Mullins et al., 1999), *ruby* (*rb*) (Kretzschmar et al., 2000) and *orange* (*or*) (Mullins et al., 2000), in *lightoid* (*ltd*) and *claret* (*ca*) that encode for Rab32 and its Guanine Exchange Factor (Ma et al., 2004), in *pink* (*p*), which encodes for the Hermansky–Pudlak syndrome 5 homologue (Falcón-Pérez et al., 2007; Syrzycka et al., 2007) and in *light* (*lt*), which encodes for the VPS41 HOPS complex homologue (Warner et al., 1998). Collectively, all these proteins are required for the biogenesis of lysosome-related organelles (LROs) – specialized low-pH subcellular compartments that accumulate a variety of complex metabolites (Lloyd et al., 1998; Krämer, 2002; Dell'Angelica, 2009; Cheli et al., 2010; Harris et al., 2011). Here we describe a LRO in the Malpighian tubules of *Drosophila* that forms the major physiological zinc storage site in this animal. This zinc storage granule concentrates the entire chelatable pool of total body zinc in flies, and is distinct from the previously described riboflavin-containing granules that give the wild-type tubule its characteristic yellow-orange color (Nickla, 1972; van Breugel, 1987).

MATERIALS AND METHODS

Drosophila melanogaster stocks

In this study, w^* and w^+ refer to isogenic stocks generated in the laboratory using the w^* mutant and the *Tan3* wild-type strains, respectively (Afshar et al., 2013). First, single crosses between w^* siblings were set for 20 generations. A single *Tan3* male was then crossed to a w^* isogenic female. For 20 further generations, a w^* male (always taken from the w^* isogenic stock) was backcrossed to a w^*/w^+ female. Finally, a w^+ male from the heterozygous mothers was backcrossed to a w^*/w^+ female to re-establish the isogenic w^+ stock.

A new allele of st^{e01330} resulting from a piggy-Bac insertion into the open reading frame of the *st* gene (Thibault et al., 2004) was crossed into the w^+ background and used in this study. All strains were obtained from the Bloomington Drosophila Stock Center and are listed along with the respective stock numbers (Table 1) except for X-chromosome meiotic recombination mapping stocks cm^1, m^{74f}, sd^1, os^s and w^a, cv^1, t^1 corresponding to #1282 and #121, respectively, and $y^1,w^{67c23};ZnT35C^{MI07746-GFSTF.1}/SM6a$, a GFP protein-trap line (Nagarkar-Jaiswal et al., 2015), corresponding to #59419. The latter chromosome was also introduced into the w^+ background. All flies were fed on molasses and yeast and kept at 25°C (Rempoulakis et al., 2014).

Metal measurements

Both flame atomic absorption spectrometry (AAS) and inductively coupled plasma optic emission spectrometry (ICP-OES) were used for metal determinations. Adult fruit flies, 4–8 days old, of mixed sex were collected and stored at –80°C. They were freeze-dried for 8 h to remove water. For the experiments with AAS, 200 mg of dry sample was digested with metal-free nitric acid (Fluka, Hampshire, IL, USA) at 60°C for 48 h, whereas for ICP-OES, 20 mg of dry sample was digested at 200°C for 15 min in closed vessels of MARS6 microwave digestion system (CEM Corporation, Matthews, NC, USA). Metal determinations in individual tissues were performed with ICP-OES; five pairs of Malpighian tubules (anterior and posterior), five pairs of ovaries, five pairs of testes, five heads and five intestines were dissected in phosphate-buffered saline (PBS), transferred to 400 µl water in MARS6 vessels, where 400 µl nitric acid was added for the digestion step and samples were diluted to 1.2 ml final volume with water. Total Zn, Fe, Mn and Cu concentrations were measured against calibration curves and a digestion blank in the Avanta M System 300 GF 3000 AAS (Dandenong, Victoria, Australia) and the PerkinElmer Optima 8300 ICP-OES (Shelton, CT, USA) instruments, respectively.

Confocal fluorescence microscopy

Malpighian tubules were dissected from adult female flies in PBS (130 mmol l^{-1} NaCl, 7 mmol l^{-1} Na_2HPO_4, 3 mmol l^{-1} NaH_2PO_4; pH 7.0). The tissue was fixed with ice-cold methanol for 5 min and rinsed three times for 3 min with PBS. Fluozin-3AM (Invitrogen, Carlsbad, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) at 5 µmol l^{-1}, stored in frozen aliquots (Groth et al., 2013) and protected against exposure to direct light at all times. For each experiment, a fresh aliquot was diluted at 2.5 µmol l^{-1} in PBS containing 0.02% Triton X-100 and 0.001% Tween 20. The fixed tissues were incubated with the Fluozin-3AM solution for 45 min at 38°C in a humid heat chamber. After three washes with PBS, the tissues were carefully mounted in Vecta Shield with DAPI (Vector H-1200, Burlingame, CA, USA) and observed without delay under a TCS SP8 Leica confocal system coupled to a DMI6000 inverted microscope (Leica Microsystems, Wetzlar, Germany). Methanol-fixation procedure and corresponding mounting was also used for direct visualization of the $ZnT35C^{GFP}$ construct.

Synchrotron X-ray fluorescence microscopy

Malpighian tubules were dissected from adult female flies in PBS, washed three times with 0.1 mol l^{-1} ammonium acetate (Jones

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Table 1. Metal determinations by AAS and ICP-OES in different strains of *Drosophila melanogaster*

			Zn (mg g^{-1})		Fe (mg g^{-1})		Cu (mg g^{-1})		Mn (mg g^{-1})	
Stock	Genotype	*N*	AAS	ICP-OES	AAS	ICP-OES	AAS	ICP-OES	AAS	ICP-OES
	Tan3	3|4	0.19±0.03	0.20±0.01	0.12±0.01	0.12±0.01	0.017±0.003	0.013±0.002	0.026±0.012	0.022±0.004
#1	*Canton S*	3|3	0.18±0.01	0.19±0.01	0.14±0.01	0.15±0.01	0.021±0.001	0.017±0.002	0.025±0.005	0.015±0.001
#5	*Oregon RC*	3|3	0.21±0.04	0.19±0.00	0.15±0.01	0.14±0.00	0.016±0.005	0.018±0.000	0.022±0.009	0.018±0.002
iso	w^+	3|6	0.21±0.03	0.18±0.01	0.13±0.03	0.12±0.01	0.015±0.004	0.012±0.001	0.028±0.009	0.017±0.001
iso	***w****	3|7	**0.07±0.00**	**0.05±0.01**	0.13±0.00	0.15±0.02	0.017±0.008	0.015±0.003	0.027±0.007	0.020±0.005
#3605	w^{1118}	3|3	**0.05±0.01**	**0.04±0.01**	0.12±0.02	0.16±0.03	0.021±0.005	0.022±0.004	0.018±0.006	0.013±0.001
#145	w^1	0|6	–	**0.05±0.00**	–	0.17±0.01	–	0.017±0.001	–	0.021±0.001
#148	w^a	0|6	–	**0.07±0.02**	–	0.14±0.00	–	0.016±0.003	–	0.024±0.001
#164	w^{sat}	3|4	0.16±0.01	0.12±0.01	0.13±0.04	0.14±0.00	0.017±0.001	0.015±0.001	0.016±0.010	0.014±0.001
#245	bw^1	5|4	0.16±0.03	0.12±0.01	0.18±0.03	0.17±0.01	0.025±0.008	0.019±0.004	0.019±0.011	0.013±0.000
#9474	bw^{19}	4|4	0.13±0.01	0.10±0.00	0.10±0.01	0.15±0.00	0.026±0.002	0.017±0.003	0.012±0.000	0.014±0.000
#605	st^1	4|4	**0.07±0.01**	**0.05±0.00**	0.11±0.01	0.11±0.00	0.021±0.004	0.014±0.001	0.028±0.009	0.020±0.001
17944	w^+;*PBac* st^{e01330}	0|7	–	**0.06±0.00**	–	0.12±0.01	–	0.016±0.007	–	0.009±0.003
#67	bw^1;st^1/*TM3*,st^{24}	0|7	–	**0.05±0.02**	–	0.10±0.01	–	0.019±0.004	–	0.023±0.007
#169	y^1	3|6	0.17±0.03	0.21±0.02	0.12±0.01	0.16±0.02	0.023±0.007	0.017±0.002	0.016±0.006	0.019±0.008
#21	***cm*^{1}**	3|6	**0.05±0.01**	**0.05±0.00**	0.13±0.01	0.12±0.03	0.019±0.005	0.013±0.001	0.021±0.007	0.014±0.001
#88	***rb*^{1}**	3|3	**0.05±0.02**	**0.06±0.00**	0.12±0.01	0.11±0.00	0.020±0.004	0.015±0.001	0.018±0.007	0.015±0.000
#3958	***g*^{1}**	3|3	**0.06±0.01**	**0.06±0.00**	0.12±0.01	0.09±0.00	0.018±0.004	0.013±0.001	0.036±0.013	0.025±0.002
#1554	***a*^{1}, *px*^{1}, *or*^{1}**	4|3	**0.07±0.01**	**0.06±0.00**	0.13±0.05	0.11±0.00	0.022±0.004	0.013±0.000	0.017±0.005	0.024±0.001
#2385	***or*^{49h}**	4|3	**0.08±0.01**	**0.05±0.01**	0.13±0.03	0.10±0.01	0.021±0.006	0.013±0.002	0.023±0.011	0.013±0.000
#19	car^1	4|3	0.14±0.03	0.15±0.00	0.11±0.02	0.11±0.00	0.016±0.003	0.014±0.001	0.018±0.006	0.013±0.001
#1051	***lt*^{1}, *rl*^{1}**	3|3	**0.06±0.02**	**0.07±0.00**	0.12±0.02	0.12±0.00	0.027±0.008	0.019±0.002	0.019±0.009	0.013±0.000
#338	***ltd*^{1}**	3|3	**0.07±0.01**	**0.05±0.01**	0.14±0.03	0.15±0.02	0.019±0.010	0.012±0.002	0.030±0.017	0.017±0.003
#459	***ca*^{1}**	3|3	**0.06±0.01**	**0.05±0.00**	0.17±0.01	0.15±0.00	0.026±0.005	0.016±0.001	0.024±0.011	0.020±0.001
#553	***p*^{p}**	3|3	**0.05±0.01**	**0.05±0.00**	0.17±0.01	0.12±0.01	0.020±0.003	0.013±0.000	0.025±0.010	0.018±0.002

Values are means±s.d. in mg g^{-1} dry mass. Bloomington stock center numbers are provided in the first column. Low zinc genotypes are shown in bold; genotypes with intermediate zinc are shown in gray. *N* is the number of biological replicates determined by AAS and ICP-OES (*n*|*n*), respectively.

et al., 2015), placed on microscope slide coverslips (Thermo Scientific Nunc Thermanox) and air-dried at 4°C. X-ray fluorescence images were collected at the Stanford Synchrotron Radiation Lightsource using beam line 2–3. The incident X-ray energy was set to 11 keV using a Si (111) double crystal monochromator with a storage ring (Stanford Positron Electron Accelerating Ring) containing 500 mA at 3.0 GeV. The fluorescence lines of the elements of interest, as well as the intensity of the total scattered X-rays, were monitored using a silicon drift Vortex detector (SII NanoTechnology USA, Northridge, CA, USA) mounted at 90 deg to the incident beam. Photon processing was accomplished with Xpress3 signal processing electronics (Quantum Detectors, Chilton, Oxfordshire, UK). In addition to these regions of interest, the entire fluorescence spectrum was also collected at each data point. The microfocused beam of 3×3 microns was provided by an Rh-coated Kirkpatrick-Baez mirror pair (Xradia, Pleasanton, CA, USA). The incident and transmitted X-ray intensities were measured with nitrogen-filled ion chambers. Samples were mounted at 45 deg to the incident X-ray beam and were spatially rastered in the microbeam using a Newport VP-25XA-XYZ stage. Beam exposure was 100 ms per pixel. Fluorescence signals were normalized against the incident X-ray beam intensity to take into account its fluctuations. Data analysis was performed using the MicroAnalysis Toolkit computer program (Webb, 2011). No smoothing or related data manipulations were performed.

RESULTS

Mapping the mutant that caused threefold reduction in total body zinc

We refer to the X-linked recessive mutant with 3-fold reduction in total zinc accumulation as *poco-zinc* (Afshar et al., 2013). The X-chromosome meiotic recombination mapping stock cm^1, m^{74f}, sd^1, os^s accumulated 0.07 mg Zn g^{-1} dry mass, suggesting that it also carried the *poco-zinc* allele. Flies from this stock were crossed to counterparts from wild-type *Tan3*, accumulating 0.20 mg Zn g^{-1} dry mass. A total of 121 recombinants were established arising from single or double crossovers between the parental chromosomes and were screened for the presence of *poco-zinc*. The *cm* mutant was present in 48 recombinants, all of which also carried *poco-zinc*, whereas in the remaining 73 recombinants neither *poco-zinc* nor *cm* was present (Fig. 1A). To estimate the distance between *poco-zinc* and *cm*, we generated a new recombinant y^1, cm^1 stock (low in zinc) and outcrossed it with wild-type flies. We were unable to dissociate *poco-zinc* from *cm*: 259 single cm^1 mutants derived from this cross segregated with *poco-zinc*, whereas 221 single y^1 mutants were normal (Fig. 1B). To confirm the proximity between *poco-zinc* and *cm*, we also used the strain w^a, cv^1, t^1. Surprisingly, all recombinants carrying the w^a allele (irrespectively of whether they carried either cv^1, or t^1, or neither of the two) were associated with *poco-zinc*, and, conversely, all w^+ flies were normal. As cv^1 lies between *w* and *cm*, the findings pointed to a new chromosomal location for *poco-zinc*, this time in the vicinity of *w* and distant from *cm* (Fig. 1C).

The *cm* gene encodes for the µ3 subunit of the AP-3 complex (Mullins et al., 1999; Rodriguez-Fernandez and Dell'Angelica, 2015). In mammalian cells, the AP-3 complex is required for the formation of LROs, organelles known to accumulate zinc (Kantheti et al., 1998; Salazar et al., 2004; McAllister and Dyck, 2017). Furthermore, and despite the generally held idea that *w* mutants lack pigment because of defective transport of 3-hydroxy-kynurenine and 6-pyruvoyl tetrahydropterin into pigment granules (Sullivan et al., 1979; Evans et al., 2008; Green et al., 2012; Hersh, 2016; Navrotskaya and Oxenkrug, 2016), earlier studies had demonstrated physical absence of these organelles in the *w* mutants (Nolte, 1961; Shoup, 1966; Nickla, 1972). Could it be that all *Drosophila* mutants in the LRO-biogenesis pathway (Lloyd et al., 1998; Krämer, 2002;

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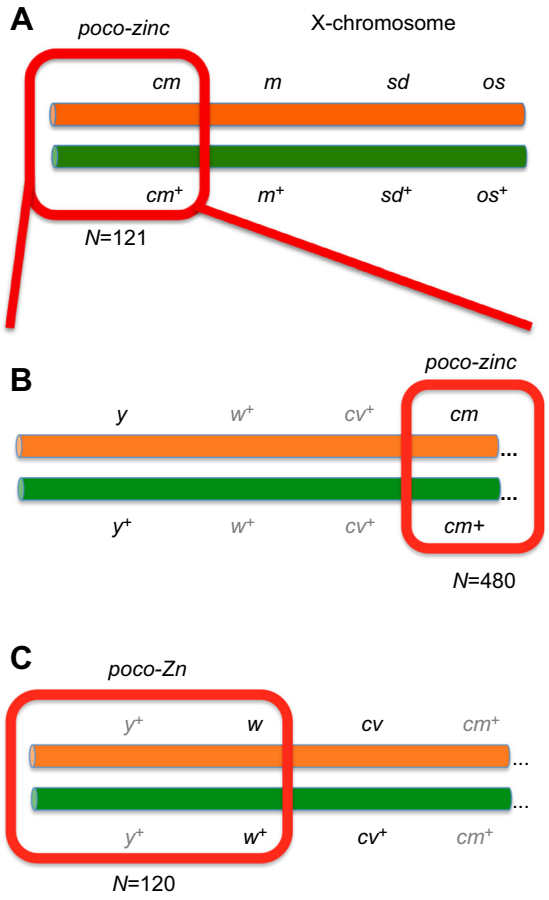

Fig. 1. Meiotic recombination mapping strategy for *poco-zinc* in *Drosophila melanogaster*. The wild-type *Tan3* chromosome is represented in green, whereas the mapping stock chromosome, carrying recessive alleles with visible phenotypes, is orange. (A) The first set of recombinant analysis situated the *poco-zinc* allele on the left part of the X-chromosome as it segregated 100% together with the *cm* gene. (B) Efforts to dissociate *poco-zinc* from *cm* were not successful, suggesting that *poco-zinc* is tightly linked (or identical) to the *cm* gene. (C) Efforts to map *poco-zinc* using a different mapping stock resulted in joint segregation of *poco-zinc* together with the *w* gene and far away from the *cm* gene. *N* is the total number of recombinants analysed.

Dell'Angelica, 2009; Cheli et al., 2010; Harris et al., 2011), including *w*, lacked zinc storage? To test this idea, total body zinc content was determined in the corresponding mutants.

LRO-biogenesis mutants lack body zinc stores

Metal measurements by AAS and ICP-OES produced a good correlation between the two sets of data (Table 1). The analysed genotypes readily separated into three groups according to their zinc content (Fig. 2A). Null mutants in the *w* gene were all low in zinc; the reduction in metal content was specific for zinc and not seen for iron, copper and manganese measured in parallel (Table 1 and Fig. 2B). In contrast, a w^+ strain derived after 20 consecutive generations of single-pair backcrossing into *w* showed normal zinc accumulation.

We calculated the zinc-to-iron ratio from every measurement obtained per genotype irrespective of the technique used, and plotted the mean values and standard deviations from the mean (Fig. 2C). Given the 3-fold lower zinc-to-iron ratio in *w* mutants, we also tested bw^1 and bw^{19}, which showed a minor reduction in zinc accumulation, whereas st^1 and st^{e01330} were low in zinc, similar to *w* and to the double mutant $bw^1;st^{1/24}$. These results implicated the white-scarlet dimer (Mackenzie et al., 2000) in *Drosophila* body zinc accumulation. Moreover, the AP-3 complex related mutants cm^1, g^1, rb^1 and or^{49h} also had low body zinc as was true for the other LRO-biogenesis mutants ltd^1, ca^1, p^p and lt^1.

The response to dietary zinc chelation or supplementation was compared between *Tan3* wild-type flies and y^1 mutants (used as an additional laboratory strain control) and *w** and cm^1 mutants (Fig. 3). The control genotypes responded as expected to both treatments, reducing body zinc content when feeding on a diet supplemented with 200 µmol l^{-1} *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; a zinc-specific chelator) and increasing body zinc content on a diet supplemented with 1 mmol l^{-1} zinc sulfate. In contrast, zinc chelation with TPEN had no effect on the body zinc content of *w** and cm^1 mutants, whereas zinc supplementation resulted in a small increase, barely reaching the body zinc content of *Tan3* wild-type flies and y^1 mutants fed on 200 µmol l^{-1} TPEN (Fig. 3). These results suggest that *w* and cm^1 mutants are defective in zinc storage, lacking the part of wild-type zinc that is chelatable with dietary TPEN.

Malpighian tubule LROs are a major site for physiological zinc storage

A common feature of all LRO-biogenesis mutants is a reduction of pigment granules in their eyes. Null mutants in the *w* gene completely lack these organelles (Nolte, 1961; Shoup, 1966). Eye pigment granules are commonly rescued with the *mini-white* transgene (Pirrotta et al., 1985), but the resulting stocks often remain low in body zinc (Bettedi et al., 2011; Gutiérrez et al., 2013). Thus eye pigment cells are an unlikely location for the LROs mediating body zinc storage. Previous reports have documented zinc storage granules in the Malpighian tubules of *Musca domestica* (Sohal et al., 1976), *Drosophila hydei* (Zierold and Wessing, 1990) and of *Tumulitermes tumuli*, a termite species (Stewart et al., 2011). Another common feature of all LRO-biogenesis mutants is a reduction of riboflavin-containing pigment granules in their Malpighian tubules (Beadle, 1937; Brehme and Demerec, 1942; Nickla, 1972; van Breugel, 1987; Yagi and Ogawa, 1996) and, moreover, using a penetrative ion microprobe technique, high zinc concentrations were detected in the Malpighian tubules of *Drosophila melanogaster* (Schofield et al., 1997). Generally, yellow Malpighian tubules correlated with normal zinc content, whereas loss of coloration correlated with low body zinc (Fig. 4). Note, however, that *bw* and *st* mutants did not follow this rule, an exception to which we shall return later. For the remaining mutants presented in this study, our hypothesis was that disruption of LROs in the Malpighian tubules (as evidenced by loss of riboflavin granules) resulted in loss of zinc storage granules.

To directly visualize zinc, synchrotron X-ray fluorescence imaging was performed (Korbas et al., 2008; Popescu et al., 2009; Bourassa et al., 2014; Jones et al., 2015). Zinc was the major metal element detected in Malpighian tubules from w^+ female flies (Fig. 5A). In contrast, only trace amounts of zinc were detectable in Malpighian tubules from *w** flies (Fig. 5B). Variation in the spectral emissions from other elements was minimal between the two samples, suggesting once again that the *w* gene affects zinc accumulation specifically in this tissue.

To visualize zinc storage granules, Malpighian tubules were incubated with the zinc indicator Fluozin-3AM and examined by confocal microscopy. Whereas the Malpighian tubules from adult female *w** flies showed only a diffuse background signal (Fig. 6A), in Malpighian tubules from adult female w^+ flies multiple, distinct

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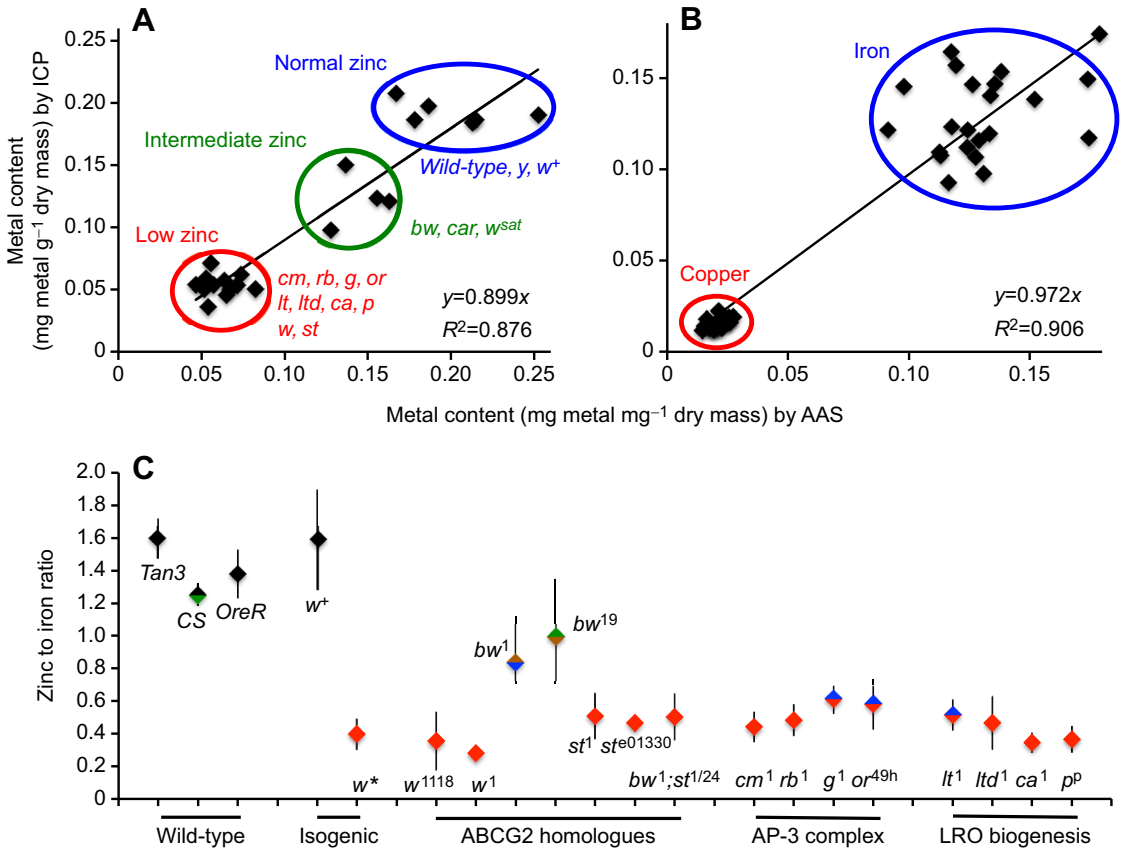

Fig. 2. Mutants in the LRO-biogenesis pathway have low body zinc content. (A) Linear regression between zinc determinations by AAS versus ICP-OES. The mean value determined for each genotype (Table 1), measured by both methods in biological replicates, is plotted. Stocks with normal, intermediate and low levels of zinc are readily identifiable. (B) Values for iron and copper are shown; here genotypes do not segregate. (C) Zinc (mg) to iron (mg) ratio was calculated for every independent measurement made (by either AAS or ICP-OES). Mean values and standard deviations are plotted; the different colors indicate statistically significant differences as revealed by Tukey's *post hoc* test following one-way ANOVA.

accumulations of fluorescence with a diameter of approximately 1 µm were observed (Fig. 6B). These fluorescent structures, which we suggest are zinc storage granules, were present only in principal tubule cells and not in the supporting stellate cells (Halberg et al., 2015).

Zinc accumulation in vesicles normally depends on specialized transporters. ZnT8, for example, is responsible for zinc entry into pancreatic insulin-granules (Pound et al., 2009), while ZnT3 takes over this function in glutamatergic vesicles of the mossy fibers (Cole et al., 1999; McAllister and Dyck, 2017). We hypothesized

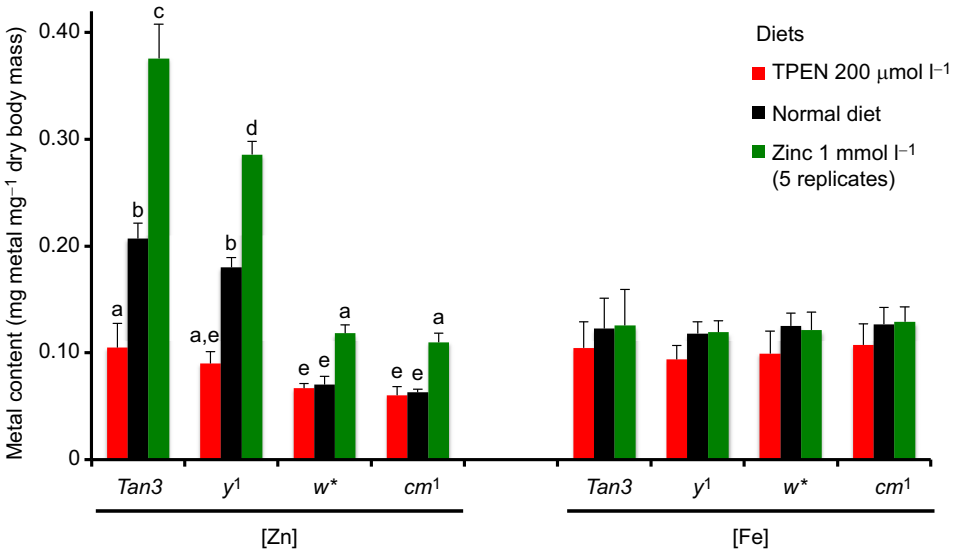

Fig. 3. Zinc storage is affected in *w* and *cm* mutants. Two control strains (wild-type *Tan3* and y^1) and two low-zinc mutants (w^* and cm^1) were grown on media with the zinc chelator TPEN (red bars) or supplemented with zinc sulfate (green bars) prior to measuring zinc and iron by AAS in whole flies from these populations. The control strains respond to the zinc treatments by changing body zinc stores, whereas this response is impaired in low-zinc mutants. Iron was unaffected. Two-way ANOVA showed differences by diet and by genotype; groups not different from each other in a Tukey's *post hoc* analysis are marked by the same lower case letter.

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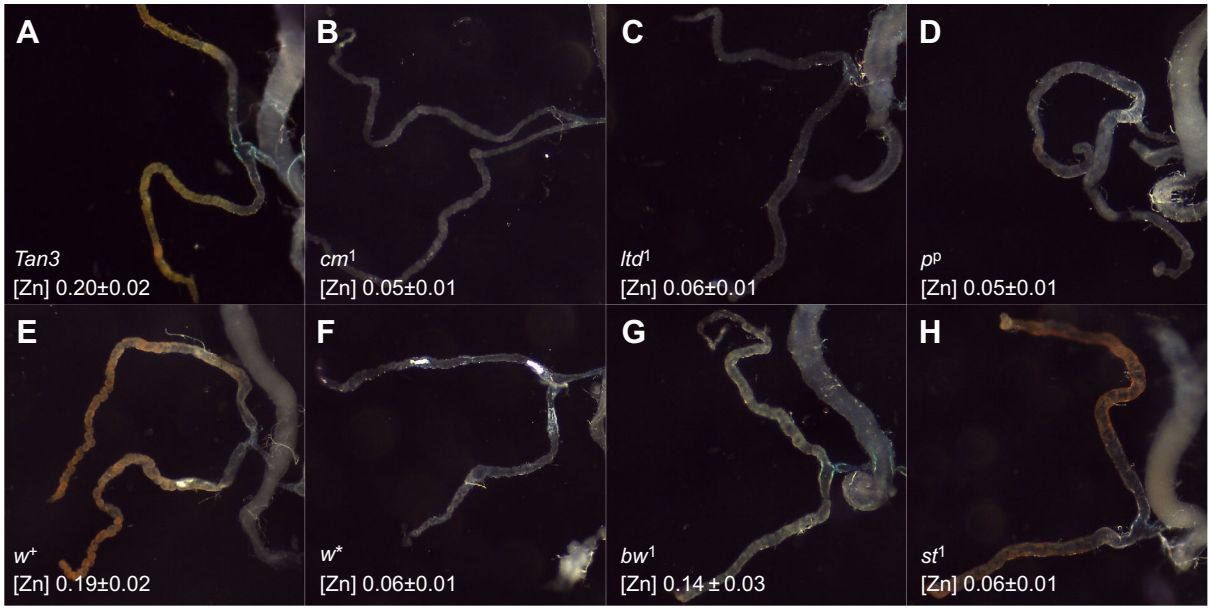

Fig. 4. Mutants in the LRO-biogenesis pathway fail to accumulate riboflavin in the Malpighian tubules. The Malpighian tubules of adult females are shown for the indicated genotypes along with the concentration of total body zinc in mg Zn g^{-1} dry mass. (A) Riboflavin in wild-type Malpighian tubules gives them their characteristic yellow-orange color (Nickla, 1972). (B–D) Three representative LRO-biogenesis mutants are all colorless and low in zinc. A comparison between isogenic (E) *w*$^{+}$ and (F) *w** suggests that the *w* gene is required for the accumulation of both riboflavin and zinc. (G) The *bw* mutants are severely reduced in their coloration and less so in their zinc content. (H) The *st* mutants show riboflavin coloration, but severely reduced zinc concentration.

that the best candidate *Drosophila* zinc transporter to mediate this function was ZnT35C, which is phylogenetically related to human ZnT3 and ZnT8 (Lye et al., 2012) and highly expressed in the Malpighian tubules (Yepiskoposyan et al., 2006; Chi et al., 2015; Yin et al., 2017). We used a strain that inserts GFP into the endogenous ZnT35C open reading frame (Nagarkar-Jaiswal et al., 2015) and observed the subcellular localization of the tagged transporter in Malpighian tubules of adult female flies grown on a zinc-supplemented diet. In the *w* mutant that lacks LROs, ZnT35C was localized in the proximity of the plasma membrane (Fig. 6C), consistent with previous observations in Malpighian tubules from the larvae (Yepiskoposyan et al., 2006; Yin et al., 2017). In contrast,

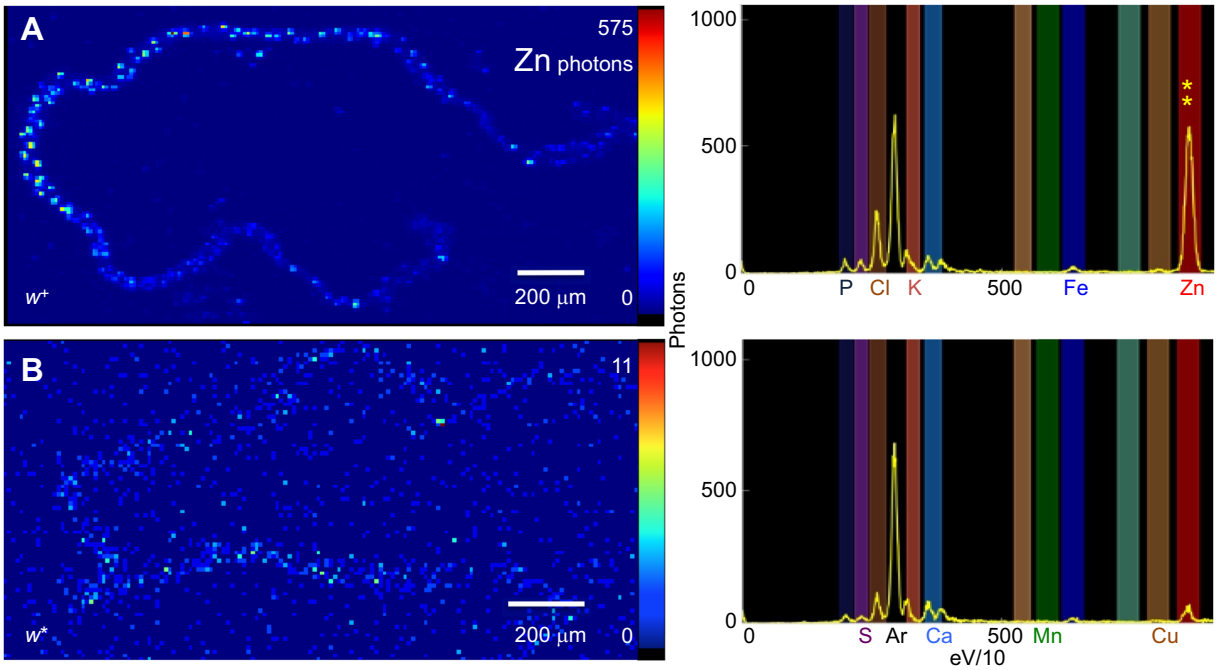

Fig. 5. Synchrotron X-ray fluorescence microscopy demonstrates the presence of zinc in Malpighian tubules from adult female flies. Pixel resolution was 10 µm^{2}. (A) Representative heat-map image for the zinc signal is shown for *w*$^{+}$ Malpighian tubules. The spectral maximum for zinc emission is 575 photons per 100 ms (also indicated by the two yellow asterisks along with the full spectrum in the right-hand panel). (B) Almost no zinc is detectable in Malpighian tubules from the *w* mutant.

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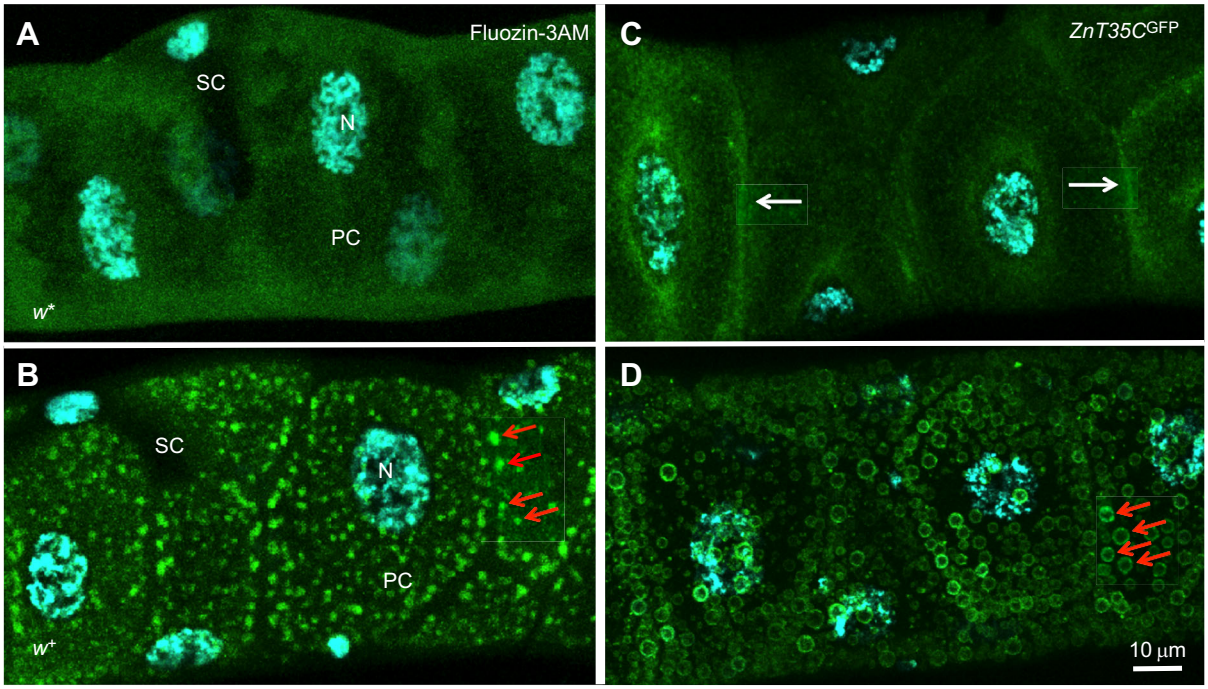

Fig. 6. Zinc storage granules are present in the Malpighian tubules. Confocal images of Fluozin-3AM fluorescence, indicative of labile zinc in the Malpighian tubule of (A) a *w** female adult or (B) an isogenic w^+ female adult. Red arrows point to a subset of zinc storage granules. (C) The protein trap line *y,w;ZnT35C*^{GFP} was used to monitor the subcellular localization of ZnT35C, confirming previous observations that it associates with the plasma membrane (white arrows). (D) The same reporter in the w^+ background clearly marks the zinc storage granule membrane (red arrows). All flies were grown on a diet supplemented with 5 mmol l^{-1} zinc sulfate. SC, stellate cell; PC, principal cell; N, nucleus.

ZnT35C^{GFP} clearly marked a subset of LROs in w^+ flies, which correspond to zinc storage granules (Fig. 6D).

Lastly, to determine if the Malpighian tubules are the main tissue where zinc accumulates and to directly assess if zinc accumulation varies in a sex-dependent manner, we used ICP-OES in samples prepared from females, males and dissected body parts, including the Malpighian tubules, intestines, ovaries, testes and heads (Table 2 and Fig. 7). Malpighian tubules from both sexes accumulate zinc in w^+ individuals. Zinc was also present at high concentrations in three out of nine w^+ testes from this genotype. These preliminary observations require further study given that riboflavin granules are also present in the epithelial sheath of the testes (Nickla, 1972; van Breugel, 1987).

Table 2. Metal determinations by ICP-OES in different tissues of *Drosophila melanogaster*

Sample	*N*	Zn (mg)	Fe (mg)	Cu (mg)	Mn (mg)
w^+					
Female adult	6	**0.059±0.013**	0.046±0.024	0.006±0.002	0.0081±0.0066
Malpighian tubules	6	**0.031±0.005**	0.002±0.001	0.001±0.001	0.0007±0.0005
Female head	6	0.001±0.001	0.004±0.001	0.001±0.001	0.0004±0.0001
Intestine	6	0.001±0.001	0.006±0.003	0.002±0.002	0.0008±0.0011
Ovaries	6	0.003±0.002	0.005±0.003	0.001±0.001	0.0001±0.0001
Male adult	6	**0.034±0.005**	0.030±0.012	0.004±0.001	0.0050±0.0043
Malpighian tubules	6	**0.029±0.003**	0.006±0.006	0.002±0.002	0.0012±0.0009
Male head	6	0.002±0.003	0.006±0.003	0.001±0.001	0.0002±0.0002
Intestine	6	0.004±0.002	0.004±0.001	0.002±0.002	0.0001±0.0001
Testes	9	0.009±0.010	0.002±0.002	0.001±0.001	0.0000±0.0001
*w**					
Female adult	6	**0.016±0.006**	0.044±0.022	0.006±0.002	0.0059±0.0040
Malpighian tubules	6	**0.001±0.001**	0.002±0.001	0.001±0.000	0.0005±0.0002
Female head	6	0.001±0.001	0.003±0.001	0.000±0.001	0.0003±0.0002
Intestine	6	0.001±0.002	0.003±0.001	0.001±0.001	0.0002±0.0002
Ovaries	6	0.001±0.002	0.002±0.001	0.001±0.000	0.0001±0.0001
Male adult	6	**0.012±0.005**	0.033±0.016	0.004±0.001	0.0051±0.0046
Malpighian tubules	6	**0.003±0.002**	0.003±0.004	0.001±0.001	0.0009±0.0001
Male head	6	0.002±0.001	0.004±0.002	0.001±0.000	0.0002±0.0003
Intestine	6	0.001±0.001	0.003±0.003	0.001±0.000	0.0001±0.0001
Testes	9	0.001±0.002	0.003±0.002	0.002±0.001	0.0000±0.0001

Values are means±s.d. in mg per whole tissue (given for clarity simply as 'mg' above). Genotypes w^+ and *w** were used; tissues with marked differences in zinc accumulation between genotypes are indicated in bold. *N* is the number of biological replicates.

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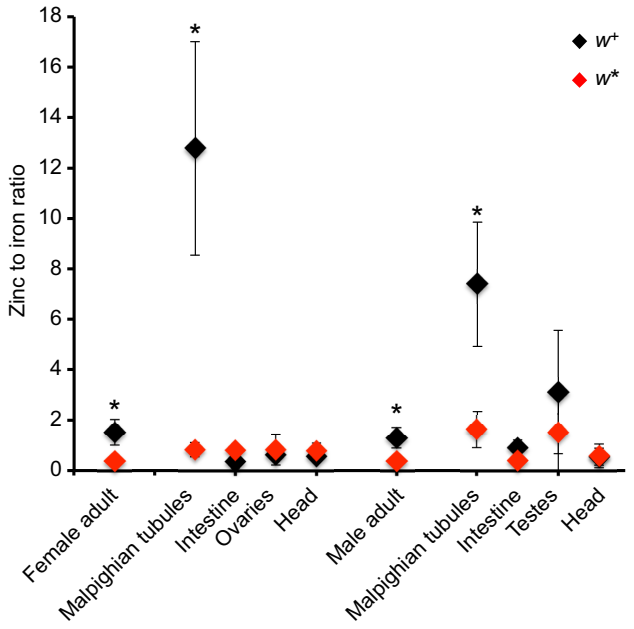

Fig. 7. Zinc accumulates in the Malpighian tubules of both sexes and in testes. Zinc (mg) to iron (mg) ratio was calculated for every independent measurement made by ICP-OES. Mean values and standard deviations are plotted (w^+ in black and w^* in red). Two-way ANOVA indicated statistically significant differences both by tissue and by genotype; asterisks denote difference between genotypes for any given tissue from a Tukey's *post hoc* analysis.

DISCUSSION

Zinc storage in *D. melanogaster* and other animals

We propose that the zinc storage granules in principal cells of the *Drosophila* Malpighian tubules (Fig. 6) have a similar function to ferritin-containing Golgi-related vesicles in iron cells of the middle midgut (Locke and Leung, 1984; Missirlis et al., 2007). The latter serve for iron storage as a physiological parallel of liver ferritin (Mehta et al., 2009), and the former could do the same for zinc storage. As there is no generally accepted site for body zinc storage in humans or other mammals, it is worth investigating if zinc-containing granules such as those present in the pancreas (Scott and Fisher, 1938; Timm and Neth, 1958; Kawanishi, 1966; Rutter et al., 2016; Maret, 2017) or in intestinal paneth cells (Okamoto, 1942; Giblin et al., 2006) serve as a reservoir for this metal, as may be the case, alternatively or additionally, for bone depositions of zinc (Berg and Kollmer, 1988; Huang et al., 2007). The only animal where a zinc storage site has been proposed is the nematode *Caenorhabditis elegans* (Roh et al., 2012; Warnhoff et al., 2017). Upon feeding on a diet with high content of zinc, *C. elegans* generates new granules within intestinal cells to store the excess metal. Thus zinc storage granules, or zincosomes as they have been also called (Beyersmann and Haase, 2001; Colvin et al., 2016), appear to be a conserved LRO in animal biology.

Insect zinc storage granules were first described in *M. domestica* (Sohal et al., 1976), *D. hydei* (Zierold and Wessing, 1990) and have also been observed in termites (Stewart et al., 2011). Twenty-four species of flies (from the Drosophilidae and the Tephritidae families) have similar zinc content, suggesting that the function of Malpighian tubules in zinc storage is evolutionarily conserved (Sadraie and Missirlis, 2011; Rempoulakis et al., 2014). Nevertheless, the findings we report show that zinc storage granules are not required for the viability of flies, raising the question of what physiological function(s) they might serve. As the Malpighian tubules are typically involved in excretion (Chi et al., 2015; Halberg et al., 2015; Yin et al., 2017), one intriguing possibility is that zinc storage granules provide a source of zinc for the intestinal lumen. In this view, the Malpighian tubule principal cells would have a similar physiological purpose as the intestinal paneth cells in mammals, where the secreted zinc has been implicated in the maintenance of the stem cell niche and epithelial integrity (Geiser et al., 2012; Ohashi et al., 2016; Podany et al., 2016; Sunuwar et al., 2016, 2017a, 2017b). Another possibility is that luminal zinc may function as a regulator for intestinal microbiota and endosymbionts, or for the control of a commonly occurring natural pathogen (Brownlie et al., 2009; Bonfini et al., 2016; Mistry et al., 2016; Martino et al., 2017; Martinson et al., 2017; Clark and Walker, 2018). Finally, it will be of interest to investigate if secreted zinc is reabsorbed by the hindgut, as is the case for the alkali metal ions (O'Donnell and Maddrell, 1995).

Different types of LROs defined by *bw* and *st*

The Malpighian tubules are also known to store riboflavin into LROs (Nickla, 1972; van Breugel, 1987). Our results suggest that the brown-white dimer is primarily required for the formation of the riboflavin LRO (Fig. 4G), a finding also true in the silkworm *Bombyx mori* (Zhang et al., 2018), whereas the scarlet-white dimer is required for the zinc storage granule (Fig. 4H). All other genes tested and known to be involved in the biogenesis of LROs affected both riboflavin accumulation and zinc storage (Fig. 4). This poses an interesting cell biology question, as the function of the Rab32, AP3, HOPS and BLOC complexes is understood as enabling the trafficking (segregation) of transporters such as ZnT35C to the LRO, defining in this way the identity of the organelle (Lloyd et al., 1998; Dell'Angelica et al., 2000; Mackenzie et al., 2000; Bultema et al., 2012; Gerondopoulos et al., 2012; Bonifacino and Neefjes, 2017). Our description of two types of LROs in the Malpighian tubule principal cells (Fig. 8) requires an explanation of how the brown-white dimer is segregated away from the scarlet-white dimer to give rise to different types of LROs.

On the function of the ABCG2 transporters

The human ABCG2 has been studied extensively, because of early reports associating ABCG2 over-expression with the development of cancer resistance to drugs inhibiting the cell cycle by intercalating into DNA (Chen et al., 1990). The consensus for the mechanism of action of ABCG2 is its direct activity in exporting drugs from cells, but the apparent lack of specificity in the transported molecules is puzzling: more than 200 substrates have been described for ABCG2 (Goler-Baron and Assaraf, 2011; Horsey et al., 2016; Taylor et al., 2017). Amongst these substrates, one is riboflavin (van Herwaarden et al., 2007), another is cGMP (de Wolf et al., 2007; Evans et al., 2008). Does the function of ABCG2 relate more to the failure of formation of a LRO (Lloyd et al., 2002; Goler-Baron et al., 2012) in the cells or animal models where it has been silenced, and less to the specific transport of the various substrates it has been claimed to move across membranes? At least in *Drosophila*, the *w* gene is required for the process of pigment granule formation *per se* (Yagi and Ogawa, 1996). How the white protein functions in the biogenesis of the LROs remains unclear. Based on the results described here, we cannot formally exclude a direct implication of the white-scarlet dimer in zinc transport. Transporters of the ZnT family have been proposed to function through a Zn^{2+}/H^+ mechanism (Ohana et al., 2009; Shusterman et al., 2014). If the proton gradient sustained by V-ATPase (Bouché et al., 2016;

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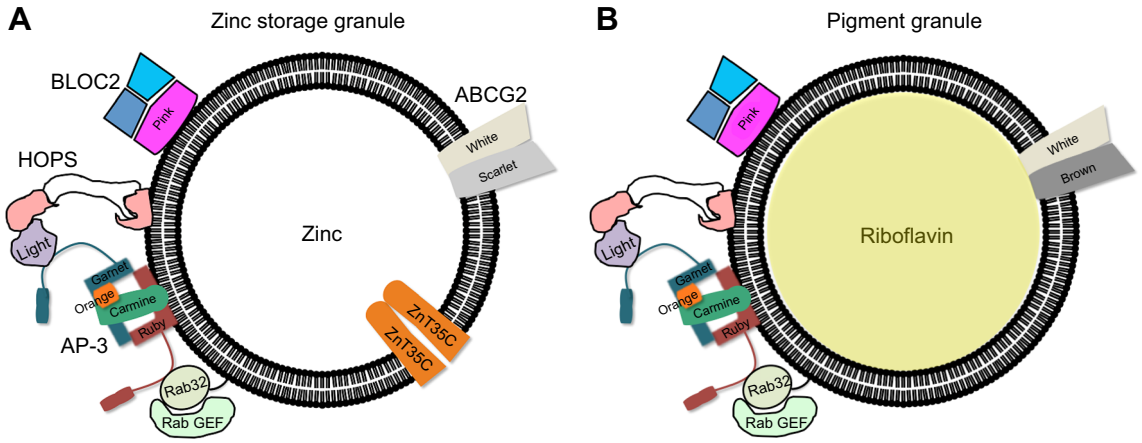

Fig. 8. Schematic representation of a zinc storage granule and a riboflavin pigment granule, in principal cells of the Malpighian tubules. (A) Zinc storage granule. (B) Riboflavin pigment granule. On the left of each vesicle known players in protein trafficking are shown, common for the biogenesis of both types of LROs. Transporters are on the right-hand side. It is unclear how cells differentially give rise to the two LROs.

Overend et al., 2016; Tognon et al., 2016) does not provide a thermodynamic explanation how ZnT35C accumulates zinc in storage granules as would be the current thinking in the field (Jeong and Eide, 2013; Kambe et al., 2017; Lee et al., 2017b), the alternative hypothesis would be that the ATPase activity of the ABCG2 homologues white and scarlet are implicated in zinc transport, perhaps working in a similar way to K_{ATP} channels (Lee et al., 2017a), i.e. by forming a pump in complex with ZnT35C. No evidence for an association between ZnTs and ABCG2 exists to date. Further experiments are required to distinguish between the above possibilities.

On the use of the *w* mutant as a control in *Drosophila* experiments

Many authors warn against the possible alterations of normal cell physiology in the *w* mutant and consider implications of using it as the major control strain in experiments (Campbell and Nash, 2001; Borycz et al., 2008; Chetverina et al., 2008; Krstic et al., 2013; Chan et al., 2014; Xiao and Robertson, 2016; Ferreiro et al., 2018). Direct implications for the field of *Drosophila* zinc biology, almost entirely based on transgenes carrying the *mini-white* marker, have been raised before (Afshar et al., 2013; Richards and Burke, 2016).

A potential role for zinc in human Hermansky–Pudlak syndrome

Mutations in nine different genes can cause Hermansky–Pudlak syndrome (HPS) in humans. HPS is characterized by oculocutaneous albinism, a platelet storage pool deficiency and lysosomal accumulation of ceroid lipofuscin (Seward and Gahl, 2013). Patients with the genotypes HPS-1, HPS-2 or HPS-4 are predisposed to interstitial lung disease and may develop granulomatous colitis. Hypopigmentation is the prominent feature of HPS, attributable to the disrupted biogenesis of LROs (Wei et al., 2013). Is zinc homeostasis altered in human patients diagnosed with HPS? To our knowledge this question has not been addressed. We conclude by pointing out that fly mutants lacking zinc storage granules correspond to known genetic alterations in HPS patients. Besides the identification of *p* as the homologue of the HPS type 5 gene (Falcón-Pérez et al., 2007; Syrzycka et al., 2007), *rb* encodes for the type 2 syndrome protein (Gochuico et al., 2012), and a second neuronal-specific human homologue of the β-subunit of AP-3 was recently related to an early-onset epileptic encephalopathy with optic atrophy (Assoum et al., 2016), and *g* encodes for the δ-subunit of the AP-3 complex, recently shown to define a new type of the HPS (Ammann et al., 2016). Likewise, the corresponding mouse mutants are known to lack zinc granules (Kantheti et al., 1998). Thus the regulation of zinc homeostasis and trafficking in HPS patients deserves further investigation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.T.-G., A.R.-A., B.O., F.M.; Methodology: C.T.-G., A.R.-A., T.K., S.M.W., M.B.-A., B.O., F.M.; Software: S.M.W.; Validation: C.T.-G., A.R.-A., M.B., B.O., F.M.; Formal analysis: C.T.-G., F.M.; Investigation: C.T.-G., A.R.-A., B.O., F.M.; Resources: T.K., F.M.; Data curation: C.T.-G., T.K., F.M.; Writing - original draft: F.M.; Writing - review & editing: C.T.-G., A.R.-A, T.K., S.M.W., M.B., B.O., F.M.; Visualization: A.R.-A, T.K., F.M.; Supervision: A.R.-A, T.K., S.M.W., B.O., F.M.; Project administration: T.K., B.O., F.M.; Funding acquisition: T.K., F.M.

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Data availability

Raw data for metal determinations and the synchrotron spectra are available from the corresponding author upon request. The same applies to fly strains not available from the Bloomington Drosophila Stock Center.

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