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Citation
Mohr, S. E., K. Rudd, Y. Hu, W. R. Song, Q. Gilly, M. Buckner, B. E. Housden, et al. 2018. "Zinc Detoxification: A Functional Genomics and Transcriptomics Analysis in Drosophila melanogaster Cultured Cells." G3: Genes|Genomes|Genetics 8 (2): 631-641. doi:10.1534/g3.117.300447.

Published Version
doi:10.1534/g3.117.300447

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Zinc Detoxification: A Functional Genomics and Transcriptomics Analysis in Drosophila melanogaster Cultured Cells

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ABSTRACT
Cells require some metals, such as zinc and manganese, but excess levels of these metals can be toxic. As a result, cells have evolved complex mechanisms for maintaining metal homeostasis and surviving metal intoxication. Here, we present the results of a large-scale functional genomic screen in Drosophila cultured cells for modifiers of zinc chloride toxicity, together with transcriptomics data for wild-type or genetically zinc-sensitized cells challenged with mild zinc chloride supplementation. Altogether, we identified 47 genes for which knockdown conferred sensitivity or resistance to toxic zinc or manganese chloride treatment, and >1800 putative zinc-responsive genes. Analysis of the ‘omics data points to the relevance of ion transporters, glutathione (GSH)-related factors, and conserved disease-associated genes in zinc detoxification. Specific genes identified in the zinc screen include orthologs of human disease-associated genes CTNS, PTPRN (also known as IA-2), and ATP13A2 (also known as PARK9). We show that knockdown of red dog mine (rdog; CG11897), a candidate zinc detoxification gene encoding an ABC-type transporter family protein related to yeast cadmium factor (YCF1), confers sensitivity to zinc intoxication in cultured cells, and that rdog is transcriptionally upregulated in response to zinc stress. As there are many links between the biology of zinc and other metals and human health, the ‘omics data sets presented here provide a resource that will allow researchers to explore metal biology in the context of diverse health-relevant processes.

KEYWORDS
metal detoxification, metal homeostasis, ABC transporters, glutathione, high-throughput screen

Whereas metals such as mercury or cadmium are solely toxic to cells, other metals, such as zinc and manganese, are essential for cell viability and are toxic only in excess. Zinc is a structural component of many proteins and is also thought to act as a signaling molecule (Fukada et al. 2011). Adding to the complexity of cellular zinc regulation, zinc is maintained at different levels in different organelles. Cells have evolved complex mechanisms for surviving zinc insufficiency, maintaining cellular and subcellular zinc homeostasis, and surviving exposure to toxic levels of zinc. The molecular mechanisms underlying regulation of zinc homeostasis and detoxification are, in some cases, zinc-specific, and in other cases relevant to other metals. Methods used by cells to maintain zinc levels and/or survive metal toxicity include the regulation of proteins required for metal influx (e.g., ZIP family importers of zinc), metal efflux (e.g., ZnT family exporters of zinc), or metal chelation (e.g., by metallothioneins), as well as sequestration of zinc and/or biomolecules damaged by zinc in membrane-bound organelles, such as the yeast vacuole or mammalian lysosome (Kambe et al. 2015). In addition to involving transporters and chelators, metal detoxification also involves more general detoxification strategies. GSH has long

Copyright © 2018 Mohr et al. doi: https://doi.org/10.1534/g3.117.300447
Manuscript received November 17, 2017; accepted for publication December 6, 2017; published Early Online December 9, 2017.
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Supplemental material is available online at www.g3journal.org/lookup/suppl/doi:10.1534/g3.117.300447/-/DC1.
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been known to have the ability to form a complex with zinc or cadmium (Perrin and Watt 1971). Data from plants, yeasts, tunicates, fish, and other organisms suggest the relevance of GSH levels, conjugation, and/or transport to metal detoxification (Perego and Howell 1997; Penninckx 2002; Gharieb and Gadd 2004; Franchi et al. 2012; Seth et al. 2012). Genetic evidence provides further support for a connection between GSH and metal detoxification. The ABCC-family transporter YCF1 is one example. YCF1 was originally identified based on a cadmium sensitivity phenotype and has been implicated in GSH-mediated detoxification of cadmium. The YCF1 protein is thought to be localized to the vacuole and to mediate transport of bis(glutathionato)cadmium into the vacuole (Nagy et al. 2006). Evidence suggests that the related protein YOR1, which is localized to the plasma membrane, can transport GSH-conjugated cadmium out of the cell. Consistent with this idea, ycf1, yor1 double mutant yeast strains are reportedly more sensitive to cadmium intoxication than either single mutant strain (Nagy et al. 2006).

The biology of zinc and other metals has many connections with human diseases. For example, genetic disruption of genes encoding metal transporters can lead to diseases of metal insufficiency or excess (Clayton 2017). In addition, accumulation of high levels of metals, which can occur following consumption of contaminated drinking water or through occupational exposure, can negatively impact human development and cause disease. Further, because metals are used by cells as “weapons” in their defense against pathogens (Weiss and Carver 2017), metal insufficiency can impact immune function. Moreover, metals or metal-related genes have been implicated in, or levels correlated with, diseases such as diabetes, Parkinson’s disease, and Alzheimer’s disease (Chasapis et al. 2012). The zinc transporter ZNT8 is a common autoantigen in type 1 diabetes (Arvan et al. 2012), for example, and ATP13A2 (PARK9), a Parkinson’s disease gene, has been implicated in zinc homeostasis (Kong et al. 2014; Park et al. 2014; Tsunemi and Krainc 2014). Furthermore, adaptation of insect vectors of disease such as mosquitoes to metals might confer concomitant resistance to insecticides (Poupardin et al. 2008), such that understanding metal detoxification in insects might impact our understanding of disease vector control and the impact of polluted environments on the spread of insect-borne diseases (Poupardin et al. 2012).

Although yeast provides an excellent genetic platform for study of the cell biology of metal detoxification, using a single-celled organism has limited potential to model multicellular systems such as humans or insect vectors of disease. Drosophila presents many advantages as a genetic model system, including the study of metal homeostasis and detoxification at the cellular and whole-organism levels, study of the effects of genetic or environmental perturbation of metal levels in the context models of human diseases (e.g., in Drosophila models of Parkinson’s or Alzheimer’s disease), and as a model of adaptation to metals and/or insecticides by insect vectors of disease. Work by several laboratories has established Drosophila as an in vivo model for study of zinc biology in a multicellular system (Richards and Burke 2016; Xiao and Zhou 2016), as well as for evolutionary studies of metal-related genes (Sadrne and Missirlis 2011; Rempoulakis et al. 2014). In particular, R. Burke and colleagues have performed a comprehensive genetic survey of in vivo ZIP and ZnT family zinc transporter functions using combined knockdown and overexpression approaches (Ly et al. 2012, 2013). Moreover, R. Burke, B. Zhou, and others have established the fly gut as a system for the study of zinc and other metals (Wang et al. 2009; Wang and Zhou 2010; Jones et al. 2015). Studies in Drosophila have also identified a role for zinc in kidney stone disease (Chi et al. 2015), and Drosophila is an established model in which to study the effects of metal-containing nanomaterials (Aidarby et al. 2016).

Altogether, the existing literature suggests that Drosophila provides an excellent system in which to study the cellular and organismal biology of metal homeostasis and detoxification. However, despite the growing body of work in Drosophila on zinc biology and other metal-related studies, there has remained a need for the application of high-throughput functional genomic methods for the study of zinc and other metals in Drosophila. Here, we describe the results obtained by applying two complementary ‘omics approaches to the identification of genes relevant to metal homeostasis and detoxification. Specifically, we performed large-scale Drosophila cell-based RNA interference (RNAi) screens to identify genes relevant to zinc or manganese detoxification, and performed a transcriptome-wide analysis of genes regulated in response to mild metal supplementation of wild-type or genetically zinc-sensitized cells. The results point to conserved genes and functions, and provide a resource for further study.

**MATERIALS AND METHODS**

**Cultured cell lines**

The screen was performed using the Drosophila RNAi Screening Center (DRSC) isolate of the S2R+ Drosophila cell line. Derivatives of this cell line newly generated in this work are available from the Drosophila Genome Resource Center (DGRC) cultured cell repository in Bloomington, IN (DGRC cell IDs 1000 and 1001; see below and Supplementary Material, File S4 [Reagent Table]).

**Cell RNAi screening**

In total, we screened four double-stranded RNA (dsRNA) reagent libraries for Drosophila cell-based RNAi screening from our DRSC collection (Hu et al. 2017b): the TM library targeting genes encoding transmembrane domain-containing proteins (17 unique 384-well assay plates), the AUTGY library targeting genes encoding autophagy-related factors (three plates), the MBO1 library targeting genes encoding proteins associated with membrane-bound organelles (two plates), and a custom-designed plate with candidate metal-related factors that we refer to as the “Megadeath” plate (one plate). In all cases, experimental dsRNAs were excluded from the outermost two wells of the final 384-well assay plate design to limit edge effects. Three replicates of each unique plate in the library (metal-supplemented conditions) or two replicates of each unique plate (control) were screened. To perform the screens, we added S2R+ cultured cells to dsRNA-containing assay plates as described previously (Echeverri and Perrimon 2006). We then incubated the plates in a 25°C incubator with humidity control for 4 d. Next, freshly prepared ZnCl2 or MnCl2 (Sigma Aldrich) in solution or a control treatment (water) was added to the assay plates using a Multiatrix Mantis liquid handling robot to a final concentration of 15 mM. Twenty-four hours following metal supplementation or control treatment, cells were lysed, and total ATP levels per well were determined using Promega Cell Titer Glo and a Molecular Devices SpectraMax Paradigm luminometer. The step-by-step screen and assay protocols that we used are available online at https://fgr.hms.harvard.edu/flu-cell-rnai-384-well-format and https://fgr.hms.harvard.edu/flu-cell-total-atp-readout. Relative luciferase values for each plate were normalized to the plate average, replicates were averaged, and average normalized relative luciferase values were then compared across plates by calculating Z-scores.

**Generation of CRISPR knockout cell lines**

The sgRNA sequence used to target ZnT63C was 5’-TGTGAC CAATTCCATGGGCTG-3’ and the sgRNA sequence used to target IA-2 was 5’-CGGCTGTTCCGCTGCTCTGG-3’ (see also File...
enrichment analysis of the RNAi screen and RNAseq data sets

The gene hits from the RNAi screen and RNAseq profiling were analyzed for overrepresented gene sets using an in-house JAVA program based on hypergeometric distribution. The gene sets we queried were assembled using gene ontology (GO) annotation, pathway annotation from GLAD (Hu et al., 2015), and the Drosophila protein complex annotation from COMPLETE (Vinayagam et al., 2013). Human pathway annotation of Reactome (Crot et al., 2011) and KEGG (Kanehisa et al., 2017) were mapped to Drosophila gene sets using DIOPT (Hu et al., 2011), and included in enrichment analyses.

Data availability
High-confidence gene-level hits from the RNAi screens are shown in Figure 1 and listed with additional details (human orthologs, Z-scores, etc.) in Table 1, Table 2, and Table 3. In addition, a complete list of gene-level high-, moderate-, and low-confidence RNAi screen hits, as well as enrichment analysis results, is provided in File S1. Reagent-level RNAi screen data are available from the FlyRNAi database of the DRSC (see “Screen Summary” for a full list of screen data sets or “Gene Lookup” to query by gene or reagent) (Hu et al., 2017b). The RNAi screens were assigned DRSC Project IDs 177 through 185 and 193. To view the full data set for a screen, replace “X” with the three-digit DRSC Project ID in the URL http://www.flyrnai.org/cgi-bin/RNAi_public_screen.pl/project_id=X. The RNAi screen data sets are also available at NCBI PubChem BioAssay (Wang et al., 2014). The screens were assigned PubChem BioAssay IDs 1259314–1259316 and 1259326–1259331. To view a data set at PubChem BioAssay, replace “X” with the seven-digit PubChem ID in the URL https://pubchem.ncbi.nlm.nih.gov/bioassay/X. Analyzed results of the transcriptomics study are summarized in Figure 2 and Table 4, and provided in full in File S2 (gene-level data and enrichment analysis results). FPKM values for genes listed in Figure 2 or discussed are presented in File S3 (each of two replicates, all genotypes and conditions). In addition, the RNAseq data sets are available from the NCBI Gene Expression Omnibus, GEO accession ID GSE99332 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99332).

results and discussion

Drosophila cell-based screen for modifiers of metal chloride toxicity

We reasoned that the application of high-throughput ‘omics approaches in Drosophila cultured cells would provide a robust data set that can inform future in vivo studies. To get a genome-scale view of genes relevant to zinc detoxification, we performed three large-scale RNAi screens in parallel using Drosophila S2R+ cultured cells. We used total ATP levels as an indirect assay readout of cell viability and number, and screened under three conditions: control conditions, toxic zinc conditions (i.e., supplementation to a concentration of 15 mM ZnCl2), and toxic manganese conditions (15 mM MnCl2). Although our main goal was to identify genes relevant to zinc, we chose to screen in parallel under toxic manganese conditions for two reasons. First, we reasoned that screening with an equimolar concentration of a different metal chloride salt would help exclude the possibility that the genes we identify in the zinc intoxication screen are relevant to the chloride ion, changes in osmolality, or other nonspecific effects. Second, we reasoned that the data would provide a helpful high-throughput data set relevant to the biology of manganese, a metal that, like zinc, is essential for cell viability and toxic in excess, but underexplored in the literature.

In order to quickly identify high-confidence screen hits (positive results) at the gene level, we screened RNAi reagent libraries from the
conditions and in the same direction. We define this set of genes for which two unique reagents scored in the same direction in a given condition as the set of high-confidence hits (Figure 1, Table 1, Table 2, and Table 3). Third, the set of high-confidence hits in any given condition and direction include multiple members of a protein family (e.g., several tetraspanin family proteins are high-confidence hits in the same direction in the manganese screen), multiple members of a protein complex (e.g., several nuclear pore components are high-confidence hits in the same direction in the zinc screen), or multiple components of a given organelle (e.g., for both control and manganese conditions, mitochondrial proteins are among the high-confidence hits with lower ATP levels vs. the internal control; Figure 1A, blue or green text).

Altogether, we identified 30 high-confidence hits in the control treatment group, 29 in the zinc toxicity treatment group, and 36 in the manganese toxicity treatment group (Figure 1, Table 1, Table 2, and Table 3). Hits were identified from all four libraries, with 23% of genes in the candidate metal-related gene library identified as high-confidence hits and 4–8% of genes in the other three libraries identified as high-confidence hits. There is limited overlap among genes that scored as high-confidence hits from each of the three screens. Three genes (CG5805, Ctr1A, and RFeSP) are in common between high-confidence hits for control conditions, decreased ATP (“down”) direction and MnCl₂ conditions, down direction (Figure 1A). Two genes (αCOP, hay) are in common between high-confidence hits for control conditions, increased ATP (“up”) direction and ZnCl₂-supplemented conditions, up direction; one gene (Pvr) is in common between high-confidence hits for control conditions, up direction and MnCl₂-supplemented conditions, up direction; and one gene (CG17119) is in common between high-confidence hits for ZnCl₂-supplemented conditions, up direction and MnCl₂-supplemented conditions, up direction (Figure 1B). For the zinc toxicity screen, there was a clear bias in detection of high-confidence hits conferring higher ATP levels (28 high-confidence hits) as compared with lower ATP levels (one high-confidence hit). We suspect that the zinc treatment conditions were so toxic that it was difficult to detect a significant further reduction in total ATP levels vs. the internal control. In addition, some genes identified in the screens are “frequent hitters” (Figure 1, gray or green text), which we define here as hits scoring in >50% of Drosophila RNAi screens in the GenomeRNAi database (Schmidt et al. 2013).

False discovery analysis
We used two approaches to address false positive and false negative discovery in the RNAi screens. As a first approach, we used RNAseq data from the modENCODE project (modEncode Consortium et al. 2010; Hu et al. 2017a) and this study (below) to determine the fraction of hits for which there is no evidence of expression in S2R+ cells (FPKM < 1), suggesting false positive discovery. This was true for 16 of 86 high-confidence hits (19%), 33 of 164 moderate-confidence hits (20%), and 246 of 548 low-confidence hits (45%), suggesting a false positive discovery rate of ~20% for high- and moderate-confidence hits in the screens. Notably, the zinc screen appears to contribute the least to false positive discovery: only 2 of 29 high-confidence hits in the ZnCl₂-supplemented conditions screen (7%) have FPKM < 1. Moreover, these two genes are annotated as having zinc or chloride ion-related functions. Thus, the false positive discovery rate for the zinc screen appears to be low.

Our second approach to false discovery analysis was to compare the results of the control screen to a previously published RNAi screen reported by Boutros et al. (2004) for genes essential in Drosophila cultured cells (Boutros et al. 2004). Among the genes identified as essential in that study, 11 met the criteria that they were (a) identified...
using dsRNA designs that meet current cutoffs for quality and (b) included in our control conditions screen. For 2 of 11, the FPKM values for cultured cell lines are $<1$, suggesting that these are false positive results in the Boutros et al. (2004) study. Of the nine genes with evidence for expression in cultured cells, three of nine scored in the down direction in our control screen (Nup53 and Desat1, low-confidence hits and Fts2Ket, high-confidence hit), consistent with the idea that these are essential genes in S2R+ cells. We next asked if human cancer cell essential gene data support the idea that the six of nine genes identified in the Boutros screen are indeed essential genes. We identified a set of human cancer cell essential gene data support the idea that the six of nine genes identified in the Boutros screen are indeed essential genes. We identified a set of human cancer cell essential genes based on two studies that together initially categorized as low-confidence hits (Table 2). The single high-confidence hit in the lower ATP levels direction in the zinc screen hits in the up direction also include CG32000, a putative ortholog of human ATP13A2 (PARK9); evidence from mammalian cells suggests a role for ATP13A2 in lysosomal zinc transport (Kong et al. 2014; Tsunemi and Krainc 2014). The zinc screen hits in this direction further include IA-2 protein tyrosine phosphatase (IA-2), an ortholog of human PTPRN (better known as IA2), which, like the human zinc transporter ZNT8, is a common autoantigen associated with type 1 diabetes (Arvan et al. 2012).

### Table 1: High-confidence RNAi screen results for control cells

| FlyBase ID | Gene Symbol | Human Ortholog$^a$ | Treatment | Direction$^b$ | dsRNAs$^c$ | Avg Z-Score |
|------------|-------------|--------------------|-----------|--------------|-------------|-------------|
| FBgn0039830 | ATPsynC     | ATP5G2             | Control   | Down         | 2           | −2.17       |
| FBgn0026872 | CG14777     | MPV17L             | Control   | Down         | 2           | −1.82       |
| FBgn0051663 | CG31663     | –                  | Control   | Down         | 2           | −1.58       |
| FBgn0052512 | CG32512     | TMEM205            | Control   | Down         | 2           | −2.49       |
| FBgn0039223 | CG8505      | SLC25A4            | Control   | Down         | 2           | −1.97       |
| FBgn0032833 | COX4        | COX41,2            | Control   | Down         | 2           | −1.76       |
| FBgn0040529 | COX7A       | COX7A2             | Control   | Down         | 2           | −2.02       |
| FBgn0062413 | Cr1A        | SLC31A             | Control   | Down         | 2           | −2.06       |
| FBgn00262743 | Fts2Ket     | KPNB1              | Control   | Down         | 2           | −3.91       |
| FBgn0024319 | Nach        | SCNN1B,G           | Control   | Down         | 2           | −1.89       |
| FBgn0035382 | Or63a       | –                  | Control   | Down         | 2           | −1.73       |
| FBgn0021906 | RFeSP       | UQCRFS1            | Control   | Down         | 2           | −2.93       |
| FBgn00266098 | rg          | NBEA               | Control   | Down         | 2           | −1.82       |
| FBgn0003360 | sesB        | SLC25A4            | Control   | Down         | 2           | −1.83       |
| FBgn0025725 | aCOP        | COPA               | Control   | Up           | 2           | 2.11        |
| FBgn0038415 | CG17929     | –                  | Control   | Up           | 2           | 2.17        |
| FBgn00264907 | CG44098     | –                  | Control   | Up           | 2           | 1.76        |
| FBgn0027556 | CG4928      | UNC93A             | Control   | Up           | 2           | 1.99        |
| FBgn0036742 | CG7497      | PTGER1,3,4         | Control   | Up           | 2           | 1.71        |
| FBgn0034438 | CG9416      | ERMP1              | Control   | Up           | 2           | 1.85        |
| FBgn0043903 | dome        | –                  | Control   | Up           | 2           | 1.82        |
| FBgn0000636 | Fas3        | –                  | Control   | Up           | 2           | 2.97        |
| FBgn0001179 | hay         | ERCC3              | Control   | Up           | 2           | 3.19        |
| FBgn00263782 | Hmgcr       | HMGCR              | Control   | Up           | 2           | 2.59        |
| FBgn0051072 | Lerp        | IGF2R              | Control   | Up           | 2           | 1.69        |
| FBgn00259214 | PMCA        | ATP2B1,2,3,4       | Control   | Up           | 2           | 2.29        |
| FBgn0032006 | Pvr         | FLT1               | Control   | Up           | 2           | 5.12        |
| FBgn0086357 | Sec61a      | SEC61A1,2          | Control   | Up           | 2           | 2.48        |
| FBgn0011708 | Syst5       | STX5               | Control   | Up           | 3           | 2.87        |
| FBgn0021796 | Tor         | MTOR               | Control   | Up           | 2           | 2.51        |

$^a$Best match orthologs are shown (DIOPT score cutoff ≥ 2) (Hu et al. 2011).

$^b$Down, decreased total ATP levels following plate-based normalization within a treatment group; up, increased total ATP levels following plate-based normalization within a treatment group.

$^c$Number of unique dsRNAs in the library that target the gene. For all high-confidence hits as shown here, each of the designs resulted in a Z-score > 1.5 or < −1.5 and in the same direction as what was found for other designs targeting the same gene.

### Analysis of zinc screen results

We found a number of zinc-related genes among the high-confidence hits in the zinc screen. For example, fear of intimacy (foi), which encodes a ZIP family zinc influx protein (Mathews et al. 2005), was identified as a high-confidence hit for increased total ATP levels (up hits) in the zinc screen but not in the other screens (Table 2). The zinc screen hits in the up direction also include CG32000, a putative ortholog of human ATP13A2 (PARK9); evidence from mammalian cells suggests a role for ATP13A2 in lysosomal zinc transport (Kong et al. 2014; Park et al. 2014; Tsunemi and Krainc 2014). The zinc screen hits in this direction further include IA-2 protein tyrosine phosphatase (IA-2), an ortholog of human PTPRN (better known as IA2), which, like the human zinc transporter ZNT8, is a common autoantigen associated with type 1 diabetes (Arvan et al. 2012).
ATP levels (down) category: CG7627, CG3790, COX7AL, CR43469, and mhd1 (File S1). Notably, like rdog, CG7627 also encodes an ABCC family member, further supporting the idea that ABCC-type transporters are relevant to zinc chloride detoxification. Based on enrichment analysis (below), CG7627 was promoted to a moderate-confidence hit (File S1).

We performed enrichment analysis for GO “cellular compartment” or “biological process” terms, pathways as annotated in Reactome (Croft et al., 2011), and protein complexes as annotated by COMPLETE (Vinayagam et al., 2013). In all cases, we used the full set of hits in the analysis (i.e., both low- and high-confidence hits). We reannotated low-confidence hits as moderate confidence if they were members of a significant enrichment group (File S1). Overall, the results of the enrichment analysis further suggest the quality and specificity of the screens. For the zinc toxicity screen, enrichment is driven by the presence of multiple components of the nuclear pore complex (e.g., enrichment for GO cellular compartment “nuclear pore,” p-value 7.15e-45, suggesting the possible involvement of nuclear transport in zinc-induced cell death or another relevant process. We also note that there are related genes in the human genome for all of the high-confidence hits identified in the zinc screen (human orthologs are included in Table 2).

Transcriptomics analysis of wild-type and zinc-sensitized cells
The use of genetically zinc-sensitized strains of Drosophila has helped uncover mechanisms of zinc homeostasis in vivo (Lye et al., 2012, 2013). We reasoned that production of mutant cell lines lacking activity of the zinc exporter ZnT63C would similarly result in cultured cells genetically sensitized to zinc supplementation and allow for detection of zinc-related genes. Such an approach would allow us to capitalize on the advantages of performing transcriptomics studies in a relatively homogeneous cultured cell line, allowing for robust detection of down- or upregulated genes, as well as minimize the need to treat cells with high-ionic strength solutions. We used a CRISPR-Cas9 strategy to target ZnT63C, which encodes a zinc efflux protein, and also targeted IA-2, which encodes the Drosophila ortholog of human PTPRN/IA2, which, like the human zinc transporter ZNT8, is a common autoantigen in type 1 diabetes (Arvan et al., 2012). Following transfection of CRISPR reagents, single-cell isolation, and identification of candidate knockout cell lines (see Materials and Methods), we confirmed by Sanger sequencing the presence of frameshift mutations in all copies of the genes in clonal cell lines. We will refer to the cell lines hereafter as ZnT63C-KO and IA2-KO.

We next treated wild-type S2R+, IA2-KO, or ZnT63C-KO cells with mild zinc or manganese chloride supplementation, and isolated RNA from the samples for next-generation transcriptome sequencing (RNAseq). In total, we performed RNAseq analysis on two replicates each of nine combinations of genetic and treatment conditions: wild-type, IA2-KO, or ZnT63C-KO cells under control, mild zinc supplementation (1 mM ZnCl2), or mild manganese supplementation conditions (1 mM MnCl2). Following sequencing, we obtained analyzed FPKM

Table 2 High-confidence RNAi screen results for zinc chloride-treated cells

| FlyBase ID | Gene Symbol | Human Ortholog(s) | Treatment | Direction | dsRNAs | Avg Z-Score |
|------------|-------------|-------------------|-----------|-----------|---------|-------------|
| FBgn0039644 | rdog        | ABCC family       | ZnCl2     | Down      | 2       | −1.81       |
| FBgn0025725 | αCOP        | COPA              | ZnCl2     | Up        | 3       | 2.47        |
| FBgn0270926 | AsnS        | ASNS              | ZnCl2     | Up        | 2       | 1.85        |
| FBgn0052672 | Atg8a       | GABARAP           | ZnCl2     | Up        | 2       | 2.20        |
| FBgn0025724 | βCOP        | COPB2             | ZnCl2     | Up        | 3       | 2.54        |
| FBgn0263979 | Cafl-SS     | RBBP4,7           | ZnCl2     | Up        | 2       | 2.09        |
| FBgn0030996 | CG14194     | TMEM185A,B        | ZnCl2     | Up        | 2       | 3.22        |
| FBgn0039045 | CG17119     | CTNS              | ZnCl2     | Up        | 3       | 4.88        |
| FBgn00352000 | CG32000   | ATP13A2,3,4,5     | ZnCl2     | Up        | 3       | 4.14        |
| FBgn0051116 | Ci/C-a      | CLCN1,2           | ZnCl2     | Up        | 2       | 3.04        |
| FBgn0002183 | dreh        | SUPT16H           | ZnCl2     | Up        | 3       | 3.30        |
| FBgn00373249 | elf3-S10   | EIF3A             | ZnCl2     | Up        | 3       | 2.22        |
| FBgn0020443 | Elf         | GSPT1,2           | ZnCl2     | Up        | 2       | 1.72        |
| FBgn0024236 | foi         | SLCO39A family    | ZnCl2     | Up        | 2       | 3.42        |
| FBgn0001179 | hay         | ERCC3             | ZnCl2     | Up        | 2       | 3.21        |
| FBgn0014189 | Hel25E      | DDX39A,B          | ZnCl2     | Up        | 3       | 7.74        |
| FBgn0031294 | IA-2        | PTPRN,N2          | ZnCl2     | Up        | 2       | 2.54        |
| FBgn00284253 | LeuRS      | LARS, LARS2       | ZnCl2     | Up        | 2       | 1.73        |
| FBgn0034641 | mahj        | DCAF1             | ZnCl2     | Up        | 3       | 1.87        |
| FBgn00259111 | Ndae1      | SLCO4A family     | ZnCl2     | Up        | 2       | 1.60        |
| FBgn0039125 | Ndc1        | NDC1              | ZnCl2     | Up        | 2       | 1.87        |
| FBgn0039004 | Nup133      | NUP133            | ZnCl2     | Up        | 2       | 3.01        |
| FBgn0021761 | Nup154      | NUP155            | ZnCl2     | Up        | 3       | 2.69        |
| FBgn0039302 | Nup358      | RANBP2            | ZnCl2     | Up        | 2       | 3.17        |
| FBgn0027537 | Nup93-1     | NUP93             | ZnCl2     | Up        | 2       | 5.60        |
| FBgn0039120 | Nup98-96    | NUP98             | ZnCl2     | Up        | 2       | 6.02        |
| FBgn00260962 | pic         | DDB1              | ZnCl2     | Up        | 3       | 3.22        |
| FBgn00264798 | Slih       | SCFD1             | ZnCl2     | Up        | 2       | 1.58        |
| FBgn00283469 | Vps4       | VPS4A,B           | ZnCl2     | Up        | 2       | 4.60        |

ID, identifier; dsRNAs, double-stranded RNAs; Avg, average.

Best match orthologs or paralog families are shown (DIOPT score cutoff > 2) (Hu et al., 2011).

Down, decreased total ATP levels following plate-based normalization within a treatment group; up, increased total ATP levels following plate-based normalization within a treatment group.

Number of unique dsRNAs in the library that target the gene. For all high-confidence hits as shown here, all of the designs resulted in a hit as defined by a Z-score > 1.5 or or < −1.5, and in the same direction as what was found for other designs targeting the same gene.

We reasoned that production of mutant cell lines lacking activity of the zinc exporter ZnT63C would similarly result in cultured cells genetically sensitized to zinc supplementation and allow for detection of zinc-related genes. Such an approach would allow us to capitalize on the advantages of performing transcriptomics studies in a relatively homogeneous cultured cell line, allowing for robust detection of down- or upregulated genes, as well as minimize the need to treat cells with high-ionic strength solutions. We used a CRISPR-Cas9 strategy to target ZnT63C, which encodes a zinc efflux protein, and also targeted IA-2, which encodes the Drosophila ortholog of human PTPRN/IA2, which, like the human zinc transporter ZNT8, is a common autoantigen in type 1 diabetes (Arvan et al., 2012). Following transfection of CRISPR reagents, single-cell isolation, and identification of candidate knockout cell lines (see Materials and Methods), we confirmed by Sanger sequencing the presence of frameshift mutations in all copies of the genes in clonal cell lines. We will refer to the cell lines hereafter as ZnT63C-KO and IA2-KO.

We next treated wild-type S2R+, IA2-KO, or ZnT63C-KO cells with mild zinc or manganese chloride supplementation, and isolated RNA from the samples for next-generation transcriptome sequencing (RNAseq). In total, we performed RNAseq analysis on two replicates each of nine combinations of genetic and treatment conditions: wild-type, IA2-KO, or ZnT63C-KO cells under control, mild zinc supplementation (1 mM ZnCl2), or mild manganese supplementation conditions (1 mM MnCl2). Following sequencing, we obtained analyzed FPKM
### Table 3: High-confidence RNAi screen results for manganese chloride-treated cells

| FlyBase ID | Gene Symbol | Human Ortholog | Treatment | Direction | dsRNAs | Avg Z-Score |
|------------|-------------|----------------|-----------|-----------|---------|-------------|
| FBgn0010217 | ATPsynB | ATP5B | MnCl2 | Down | 2 | –2.45 |
| FBgn0030263 | CG2076 | GHITM | MnCl2 | Down | 2 | –1.63 |
| FBgn0039223 | CG5805 | SLC25A44 | MnCl2 | Down | 2 | –1.80 |
| FBgn0030768 | CG9723 | NEMP1,2 | MnCl2 | Down | 2 | –1.79 |
| FBgn0062413 | Ctr1A | SLC31A1 | MnCl2 | Down | 3 | –1.90 |
| FBgn00264000 | GluRIB | GRIA1,2,3,4 | MnCl2 | Down | 2 | –1.57 |
| FBgn0029970 | Marf | MFN2 | MnCl2 | Down | 2 | –2.81 |
| FBgn0039302 | Nup358 | RANBP2 | MnCl2 | Down | 2 | –2.17 |
| FBgn00336770 | Prestin | SLC26A5 | MnCl2 | Down | 2 | –2.46 |
| FBgn0021906 | RFeSP | UQCRFS1 | MnCl2 | Down | 2 | –2.74 |
| FBgn00366260 | Rh7 | OPN4,3 | MnCl2 | Down | 2 | –2.09 |
| FBgn0027603 | Ulp1 | SENP1,2,3,5 | MnCl2 | Down | 2 | –1.83 |
| FBgn0031937 | CG13795 | – | MnCl2 | Up | 2 | 3.32 |
| FBgn0030030 | CG1636 | – | MnCl2 | Up | 2 | 3.20 |
| FBgn0039045 | CG17119 | CTNS | MnCl2 | Up | 2 | 2.38 |
| FBgn00250757 | CG42235 | SLC5A family | MnCl2 | Up | 3 | 2.92 |
| FBgn0027556 | CG4928 | UNC93A | MnCl2 | Up | 2 | 1.71 |
| FBgn0034275 | CG5002 | SLC26A11 | MnCl2 | Up | 2 | 1.81 |
| FBgn0037764 | CG9459 | ELOVL7 | MnCl2 | Up | 2 | 2.36 |
| FBgn0042701 | CR12628 | – | MnCl2 | Up | 2 | 3.10 |
| FBgn0061492 | lao | TMED6 | MnCl2 | Up | 2 | 1.95 |
| FBgn0025814 | Mgstl | MGST1 | MnCl2 | Up | 2 | 3.10 |
| FBgn0052475 | mthl8 | – | MnCl2 | Up | 2 | 2.38 |
| FBgn0032006 | Pvr | FLT1 | MnCl2 | Up | 2 | 3.46 |
| FBgn0031760 | Tsp26A | TSPAN5 | MnCl2 | Up | 2 | 1.99 |
| FBgn0029508 | Tsp42Ea | CD63 | MnCl2 | Up | 2 | 2.92 |
| FBgn0033136 | Tsp42Eo | – | MnCl2 | Up | 2 | 2.59 |
| FBgn0033137 | Tsp42Ep | – | MnCl2 | Up | 2 | 2.37 |
| FBgn0033139 | Tsp42Er | – | MnCl2 | Up | 2 | 1.98 |
| FBgn0022097 | Vha36-1 | ATP6V1D | MnCl2 | Up | 2 | 1.64 |
| FBgn0040377 | Vha36-3 | ATP6V1D | MnCl2 | Up | 2 | 2.21 |
| FBgn00262511 | Vha44 | ATP6V1C1 | MnCl2 | Up | 2 | 2.05 |
| FBgn00263598 | Vha68-2 | ATP6V1A | MnCl2 | Up | 2 | 2.57 |
| FBgn0028662 | VhaPPA1-1 | ATP6V0B | MnCl2 | Up | 2 | 1.99 |
| FBgn0027779 | VhaSFD | ATP6V1H | MnCl2 | Up | 3 | 2.37 |
| FBgn0035432 | ZnT63C | SLC30A1 | MnCl2 | Up | 2 | 2.27 |

**Notes:**

- **ID:** identifier; dsRNAs, double-stranded RNAs; Avg, average.
- **Human Ortholog:** matches human orthologs (if available).
- **Z-Score:** calculated as described in (Hu et al. 2011). 
- **Direction:** Down, decreased total ATP levels following plate-based normalization within a treatment group; up, increased total ATP levels following plate-based normalization within a treatment group.
- **dsRNAs:** Number of unique dsRNAs in the library that target the gene.

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Values and used these as the data set for all subsequent analyses. The number of down- or upregulated genes for mild zinc and manganese chloride supplementation conditions, relative to values for untreated cells of the same genotype, is summarized in Table 4. We observed a larger overall transcripational response in ZnT63C-KO cells treated with ZnCl2 than in the other genotypes and conditions (i.e., a larger total number of genes with significant log2 values as compared to the control and a large fold-change among the top hits), consistent with the idea that ZnT63C-KO are more sensitive to zinc supplementation than wild-type S2R+ cells. File S2 includes a list of the genes the data suggest are down- or upregulated in each genotype and condition. File S3 displays the read counts for each replicate for genes listed in Figure 2 or discussed. How to access the RNAseq data is outlined in the Data availability section of the Materials and Methods.

As summarized in Table 4, we identified >1800 putative zinc-responsive genes. Several indicators suggest that the strategy of genetically sensitizing cells to zinc supplementation was successful in identifying high-confidence zinc-responsive genes, including the observation that several genes were down- or upregulated in two or more zinc-supplemented S2R+, IA2-KO, and/or ZnT63C-KO genotypes (Figure 2). Not surprisingly, the set of zinc-responsive genes includes metallothioneins, which act as metal chelators, as well as a number of heat-shock proteins (Figure 2B and Table S1). Interestingly, at least for some genes, the level of upregulation induced by zinc in IA2-KO cells was intermediate as compared with wild-type and ZnT63C-KO zinc-treated cells (File S2 and Table S1). As compared with zinc, supplementation with low levels of MnCl2 of any genotype resulted in a small number of genes showing significant down- or upregulation as compared to wild-type untreated cells (Figure 3, File S2, and Table 4), consistent with the idea that knockout of ZnT63C sensitizes cells to zinc but not manganese. However, despite the smaller numbers, some genes are in common between the zinc and manganese data sets (Figure 2 and File S2), suggesting that these genes might be chloride-responsive or general factors. The knockout cell production method that we used includes a single-cell cloning step. Single-cell cloning alone could, in principle, result in changes that affect the transcriptional profile. For this reason, we consider the highest confidence zinc-responsive genes to be those found in all three zinc-treated genotypes. These are listed in Figure 2.
Enrichment analysis of transcriptomics data from zinc-treated cells

As we did with the RNAi screen data, we performed enrichment analysis of the transcriptomics data to detect GO terms, molecular pathways, or protein complexes that were significantly enriched in these data sets (File S2). Enrichment among genes downregulated in response to ZnCl2 supplementation includes processes or protein complexes related to ribosomes (e.g., Reactome “translation,” p-value $6.88 \times 10^{-15}$ in zinc-treated wild-type cells), and pathways or cellular components of respiratory electron transport. Enrichment among genes upregulated in response to ZnCl2 supplementation includes processes or complexes involving heat-shock proteins and starvin (stro), an ortholog of the human “BCL2-associated athanogene 3” or BAG3 gene. Significant enrichment was also seen in zinc-treated wild-type cells for the GO molecular function heme-copper terminal oxidase activity; 3 of the 21 genes in this group that result in enrichment are COX4L and COX7A1, which encode subunits of cytochrome c oxidase, and CG42376, which encodes an ortholog of human “cytochrome c oxidase assembly factor 6” or COA6. For zinc-treated ZnT63C-KO cells, upregulated genes are also enriched for GSH-related activities (e.g., KEGG “glutathione metabolism,” p-value $3.62 \times 10^{-9}$ in ZnT63C-KO cells). Indeed, 9 of 11 GstD subfamily genes, other GSH S-transferase genes, and Glutamate-cysteine ligase catalytic subunit (Gclc), a rate-limiting enzyme in the GSH synthesis pathway, are upregulated in zinc-treated ZnT63C-KO cells.

Altogether, the transcriptomics data show that zinc stress results in upregulation of metal chelators and heat-shock proteins, and suggests that zinc stress has specific impacts on mitochondrial function that elicit compensatory transcriptional responses. Moreover, the data obtained using a zinc-sensitized genotype suggest that, under high zinc stress conditions, there is a significant need for conjugation of substrates to GSH. This is consistent with a recent report that GSH S-transferase activity is relevant to methyl mercury toxicity in Drosophila (Vorojeikina et al. 2017), and with results obtained for other species that associate metal detoxification with GSH conjugation and/or flux (Perego and Howell 1997; Femminick et al. 2002; Gharieb and Gadd 2004; Nagy et al. 2006; Franchi et al. 2012; Seth et al. 2012).

Comparison of functional screen and transcriptomics data

We reasoned that genes encoding proteins normally involved in zinc influx would be expected to score in the up direction in the screen (higher ATP values as compared with the internal control, consistent with resistance to zinc treatment) and down in response to zinc supplementation in the transcriptomics analysis. Consistent with this, we found that the high-confidence screen hit foi was downregulated in zinc-treated S2R+ and zinc-treated IA2-KO cells as compared with untreated cells of the same genotype, and downregulated in zinc-treated ZnT63C-KO cells as compared with wild-type untreated cells. In addition, CG3000 was downregulated in zinc-treated ZnT63C-KO cells as compared with wild-type untreated cells. We also compared the data for components of the nuclear pore. The low-confidence screen hit Nup107 was downregulated in zinc-treated wild-type and zinc-treated ZnT63C-KO cells as compared with genotype controls, and in zinc-treated IA2-KO cells as compared with untreated cells of the same genotype. In addition, the high-confidence hit Nup93-1 was downregulated in zinc-treated ZnT63C-KO cells as compared with the wild-type untreated control, and the additional nuclear pore component-encoding genes Nup43, Nup44A, Nup50, Nup54, and Nup160 were downregulated in ZnT63C-KO cells as compared with untreated cells of the same genotype.

We next explored the converse prediction: that genes encoding proteins protective against zinc intoxication would be expected to score in the down direction in the screen and to be upregulated in response to zinc supplementation. Despite the relatively small number of down

Table 4 Summary of transcriptomics analysis of wild-type and zinc-sensitized cells under control or metal-supplemented conditions

| Condition                  | Wild-Type S2R+ | IA2-KO | ZnT63C-KO |
|----------------------------|----------------|--------|-----------|
| + 1 mM ZnCl₂               | 319 down       | 66 down| 998 down  |
|                            | 121 up         | 128 up | 835 up    |
| + 1 mM MnCl₂               | 23 down        | 26 down| 68 down   |
|                            | 33 up          | 26 up  | 16 up     |

Down, downregulated as compared with untreated cells of the same genotype; up, upregulated as compared with untreated cells of the same genotype.
Materials and Methods

Metal treatment samples were normalized to the 0 mM control (see Materials and Methods). RNA levels from metal treatment samples were normalized to the control and rdog transcript levels are upregulated in response to zinc sulfate supplementation with untreated wild-type cells. In addition, the one high-concentration hit in the zinc screen (Figure 1, File S1, and Table 1), we did find overlap between the RNAi down direction hits and zinc-responsive gene lists. The low-confidence hit COX7AL was significantly upregulated in zinc-treated S2R+ and zinc-treated ZnT63C-KO cells as compared with untreated genotype controls, and in all three as compared with untreated wild-type cells. In addition, the one high-confidence down direction RNAi screen hit, rdog, scored as significantly upregulated in zinc-treated ZnT63C-KO cells; the log2 values for rdog were 1.21 for zinc-treated IA2-KO cells and 3.53 for zinc-treated ZnT63C-KO cells as compared with genotype controls (FPKM values for each of two replicates in all genotypes and conditions are provided in File S3).

dog is upregulated in response to zinc in Drosophila S2R+ cells

We further confirmed that rdog is upregulated in response to zinc supplementation using a graded series of ZnCl2 to supplement the culture media of wild-type S2R+ cells followed by qPCR, as shown in Figure 3. As expected, under ZnCl2-supplemented conditions, levels of the metallothionein-encoding gene MinA are upregulated and levels of the zinc importer-encoding gene foi are downregulated. Under the same conditions, the levels of rdog are upregulated (Figure 3A). Based on the identification of rdog in the ZnCl2-supplemented screen and transcriptomics data sets, we suspected that the effect was zinc-specific, rather than being attributable to the chloride ion. To further test this experimentally, we performed qPCR analysis on wild-type S2R+ cells supplemented with ZnSO4. The trends for control and rdog transcript levels were similar to those found for ZnCl2 (Figure 3B), demonstrating that rdog expression is upregulated by zinc in Drosophila S2R+ cells.

Analysis of parallel studies using manganese chloride

As mentioned, we performed the RNAi screens and transcriptomics studies with MnCl2 in parallel to help distinguish zinc-specific factors from general factors, and to provide an additional metal intoxication-related data resource. For the MnCl2 toxicity screen, enrichment among genes conferring higher ATP levels upon knockdown is driven by the presence of multiple components of the vacuolar H+ ATP transport machinery (Figure 1B and Table 2). This suggests the possible relevance of proton transport to manganese-induced cell death or another related process. Several tetraspanin family proteins are also hits in the manganese screen. This is intriguing, as three tetraspanin family proteins were detected as coregulated by trans-eQTLs following feeding of flies with lead (Pb) (Ruden et al. 2009), suggesting the possibility of a general role for tetraspanin family proteins in detection of, or responses to, metals or metal-induced stress. Enrichment analysis of genes conferring lower ATP levels in the MnCl2 screen points to the relevance of mitochondria.

Conclusions

The functional genomics and transcriptomics data sets described here provide a genome-scale resource for the study of zinc biology in Drosophila. Despite the fact that we performed the RNAi screens under high-metal supplementation conditions, we were able to identify factors known to be relevant to zinc homeostasis at physiological levels (i.e., foi and CG32000). This is consistent with known overlap between metal homeostasis and detoxification genes, and suggests the validity of the approach. In addition, despite assay bias, we were able to detect one high-confidence gene, rdog, for which knockdown results in lower ATP values as compared with the internal control. We further found that rdog, an ortholog of yeast YCF1, is upregulated in genetically zinc-sensitized cells following mild zinc supplementation. Identification of rdog in the cell-based screen, as well as identification in the transcriptomics data of rdog, Gclc, and genes encoding GSH S-transferase family proteins (Saisawang et al. 2012), supports the idea that GSH is relevant to zinc detoxification in Drosophila. Moreover, the observation that another gene encoding ABCC family member CG7627 was also identified as a zinc sensitivity screen hit in this work, together with the fact
that a third Drosophila ABCC family member, dMRP, has previously been implicated in methylmercury toxicity (Prince et al. 2014), suggest a general role for ABCG-type transporters in metal detoxification in Drosophila. Altogether, we expect that the ‘omics data presented here will guide further research into the mechanisms underlying metal homeostasis and detoxification in Drosophila and other systems. For example, the data provide a focused set of candidates for in vivo analyses of wild-type and genetically metal-sensitized flies under normal or metal-supplemented conditions, as well as for in vivo analyses in fly models of human diseases such as diabetes or neurodegeneration.

ACKNOWLEDGMENTS
We thank Richard Burke (Monash University, Melbourne, Australia), Juan Antonio Navarro Langa (Universitat Regensburg, Regensburg, Germany), Fanis Missirlis (Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico City, Mexico), and Daniela Zarnescu (University of Arizona) for helpful conversations. In addition, we thank Chiao-Lin Chen, Kevin Kim, Afroditi Petsakou, Donghui Yang-Zhou, and other members of the Drosophila RNAi Screening Center (DRSC) and Transgenic RNAi Project, and the Perrimon laboratory, for helpful input on the project. We also thank the New York University RNAi Core Facility for collaboration on production of the TM RNAi library. The DRSC is supported by National Institutes of Health (NIH) National Institute of General Medical Sciences grant R01 GM 067761 (to N.P.). This work was also supported in part by NIH National Institute of Environmental Health Science grant R21 ES-025615 (to N.P.). In addition, S.E.M. is supported in part by the Dana-Farber/Harvard Cancer Center, which is supported in part by National Cancer Institute Cancer Center Support grant number NIH 5 P30 CA-06516. N.P. is an investigator of the Howard Hughes Medical Institute.

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Communicating editor: M. Boutros