

Drosophila Ctr1A Functions as a Copper Transporter Essential for Development*

Received for publication, May 8, 2007, and in revised form, June 14, 2007. Published, JBC Papers in Press, June 15, 2007, DOI 10.1074/jbc.M703792200

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Copper is an essential trace element required by all aerobic organisms as a cofactor for enzymes involved in normal growth, development, and physiology. Ctr1 proteins are members of a highly conserved family of copper importers responsible for copper uptake across the plasma membrane. Mice lacking Ctr1 die during embryogenesis from widespread developmental defects, demonstrating the need for adequate copper acquisition in the development of metazoan organisms via as yet uncharacterized mechanisms. Whereas the fruit fly, *Drosophila melanogaster*, expresses three Ctr1 genes, *ctr1A*, *ctr1B*, and *ctr1C*, little is known about their protein isoform-specific roles. Previous studies demonstrated that Ctr1B localizes to the plasma membrane and is not essential for development unless flies are severely copper-deficient or are subjected to copper toxicity. Here we demonstrate that Ctr1A also resides on the plasma membrane and is the primary *Drosophila* copper transporter. Loss of Ctr1A results in copper-remedial developmental arrest at early larval stages. Ctr1A mutants are deficient in the activity of copper-dependent enzymes, including cytochrome *c* oxidase and tyrosinase. Amidation of Phe-Met-Arg-Phe-amides, a group of cardiomodulatory neuropeptide hormones that are matured via the action of peptidylglycine α -hydroxylating monooxygenase, is defective in neuroendocrine cells of Ctr1A mutant larvae. Moreover, both the Phe-Met-Arg-Phe-amide maturation and heart beat rate defects observed in Ctr1A mutant larvae can be partially rescued by exogenous copper. These studies establish clear physiological distinctions between two *Drosophila* plasma membrane copper transport proteins and demonstrate that copper import by Ctr1A is required to drive neuropeptide maturation during normal growth and development.

Enzymes are required to carry out a complex orchestration of biochemical reactions, and many of these enzymes require cofactors obtained through the diet. One such cofactor is copper, an essential trace element involved in such diverse biological and developmental processes as energy generation, peptide maturation, free radical detoxification, blood vessel formation,

and melanin production (1–3). In addition to the canonical role of copper as a cofactor, data support novel roles for copper in the secretion of growth factors and in modulating neuronal function (4–6). Its utility in biological systems, however, is tempered by its ability to catalyze the formation of free radicals that are damaging to cells (7). Thus cells have evolved a homeostatic pathway for the uptake, distribution, and sequestration of copper such that the essential cellular requirements for copper can be achieved while minimizing its toxic potential. Studies in bakers' yeast, *Saccharomyces cerevisiae*, facilitated the discovery of many of the components of the copper homeostatic pathway, and these proteins are strongly conserved among species extending all the way to mammals (3, 8, 9).

The Ctr proteins, a family of conserved, high affinity copper transporters, function as a primary means through which individual cells obtain copper (10–12). Inside cells, copper is escorted to intracellular compartments or proteins by copper chaperone proteins as follows: CCS incorporates copper into Cu,Zn superoxide dismutase; Atx1 delivers copper to the Golgi network where it is then pumped into the lumen of the Golgi via the action of ATP7A and ATP7B and from within the Golgi lumen can be incorporated into copper-dependent proteins, and Cox17 facilitates copper insertion into mitochondrial cytochrome *c* oxidase (14–18).

The consequence of improper copper balance is illustrated in two hereditary disorders of copper homeostasis. Menkes disease, an X-linked copper deficiency disorder caused by mutations in *ATP7A* that leads to the hyper-accumulation of copper in intestinal epithelial cells and subsequent hypo-accumulation of copper in peripheral tissues, results in early childhood lethality. Wilson disease is an autosomal recessive copper toxicosis disorder caused by mutations in *ATP7B* that leads to massive hyper-accumulation of copper in the liver and subsequent copper overload in peripheral tissues and can result in liver disease and/or neurological conditions (19, 20). Additionally, mutations in other components of the copper homeostatic pathway can result in either early embryonic lethality, perinatal lethality, or pathologic conditions. Mutations in *Cox17* (21) and Ctr1 (22, 23) result in embryonic lethality in mouse models, whereas Atox1-deficient mice fail to thrive with a large number of pups dying perinatally (24). Whereas systemic Ctr1 knock-out mice die early in embryogenesis accompanied by severe and widespread developmental defects, including poorly developed mesoderm and neural ectoderm layers and impaired neural tube closure, intestinal epithelial cell-specific Ctr1 knock-out mice exhibit marked growth retardation, cardiac hypertrophy, and overall viability defects resulting in postnatal lethality by

* This work was supported by National Institutes of Health Grant 5P42ES010356. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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~3 weeks of age (25). Additionally, mutations in *Sco2*, a protein involved in the assembly of copper into cytochrome *c* oxidase, have been associated with fatal infantile cardiomyopathy (26, 27), whereas mutations in *Sco1*, also involved in cytochrome *c* oxidase assembly with copper, have been associated with neonatal-onset hepatic failure and encephalopathy (28). Taken together, these observations illustrate the importance of copper homeostasis in normal development and physiology.

Previously, three *Ctr1* genes were identified in the *Drosophila* genome based on sequence homology of the encoded proteins to mammalian and yeast Ctr1 proteins. *Drosophila* Ctr1A, Ctr1B, and Ctr1C each possess the following three structural features characteristic of all known Ctr family members: three transmembrane domains, a methionine residue preceding the first transmembrane domain, and an MXXXM motif within the second transmembrane domain. Additionally, expression of each *Drosophila* Ctr1 protein can rescue a yeast strain deficient in high affinity copper uptake, and expression of Ctr1A and Ctr1B stimulates copper uptake in S2 cells (29).

Previously, we demonstrated that *Drosophila* Ctr1B is necessary only under growth conditions where copper is limiting or, paradoxically, when copper is in excess (29). The focus of this study was to characterize *Drosophila* Ctr1A. Here we show that *ctr1A* is an essential gene for *Drosophila* development. Furthermore, Ctr1A mutant flies exhibit deficiencies in copper-dependent enzyme activities, deficient amidation of a peptide known to have cardiomodulatory properties and copper-remedial defects in heart function.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics—To generate a Ctr1A mutant, an imprecise P-excision strategy was used (30). Line G788 was obtained from GenExel, Inc., and the location of the P-insertion was confirmed via inverse PCR. To screen for a potential excision within Ctr1A, a PCR-based strategy was used. Primer A1, which anneals to an intergenic region ~40 bp upstream of the Ctr1A locus, was used as the forward primer in all test PCRs, and reverse primers were designed ~500 bp apart that spanned the entire Ctr1A locus as well as an additional 1 kb downstream of the Ctr1A locus. Lines that yielded a PCR product with a smaller than expected size were marked as positive and examined further.

Excision line 25 was selected for further analysis; sequencing of the PCR product spanning the excision was done to determine the exact molecular nature of the excision. Generating female germ line Ctr1A mutants was accomplished using the Flip recombinase-dominant female sterile technique (31).

Developmental Progression Analyses—For hatching efficiencies, embryos were dechorionated in 50% bleach. The number of fertilized embryos placed on a grape juice plate were counted, and the number of unhatched embryos remaining after 24 h and again after 48 h were counted. For both embryo and larval cuticle preparation images, embryos were mounted onto slides containing Hoyer's solution, and a coverslip was placed on top of them. Mechanical devitellinization for the embryos was accomplished by applying gentle pressure onto the coverslip until the vitelline membrane burst. Slides were then baked at 60 °C for 2–3 days. Staging of larvae was based on mouth hook

structure, and the presence and appearance of anterior spiracles were as described by Demerec (32). For the images of tyrosinase-deficient larvae, live larvae were mounted in 70% glycerol; a coverslip was gently placed on top of the animals, and images were taken immediately using a Leica MZFL III fluorescence stereomicroscope mounted with a QImaging Retiga EXi digital camera.

Ctr1A Antibody—The rabbit anti-Ctr1A antibody was made by Bethyl Laboratories, Inc.

Immunoblot—Embryo lysates were prepared by homogenizing dechorionated embryos in a mild lysis buffer (20 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). Protein concentration for this and all subsequent experiments was determined using the BCA method (Pierce). Samples were run on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with rabbit anti-Ctr1A (1:200 for deficiency immunoblot; 1:1000 for line 25 immunoblot) and mouse JLA20 (Developmental Studies Hybridomas Bank)(1:100). Secondary antibodies were donkey anti-rabbit and donkey anti-mouse conjugated with horseradish peroxidase (Amersham Biosciences) (1:5000).

Embryo Staining Protocol—W1118 adult females were allowed to lay eggs overnight. Embryos were fixed according to standard procedures (33). Primary antibodies used were rabbit anti-Ctr1A (1:500) and mouse anti-neurotactin (1:10) (BP106; Developmental Studies Hybridomas Bank). Secondary antibodies used were donkey anti-rabbit conjugated with rhodamine and donkey anti-mouse conjugated with fluorescein isothiocyanate (1:1000) (Jackson ImmunoResearch). Embryos were mounted in Prolong antifade kit (Molecular Probes). Images were taken using a Zeiss LSM 410 confocal microscope.

Larval Tissue Staining Protocol—Wandering 3rd instar W1118 larvae were dissected and fixed according to standard procedures (33). Primary and secondary antibody incubations and mounting were the same as described for the embryo staining. Mouse anti-actin (JLA20; Developmental Studies Hybridomas Bank) was used at 1:500.

Immunohistochemistry Protocol for Dissected Larval Brain—1st instar larvae were dissected in 1× PBS.³ Fixation and staining were performed according to methods published previously (34). Signal was detected using the metal-enhanced DAB substrate kit (Pierce ImmunoPure) per the manufacturer's instructions. Tissue was mounted onto a slide in 70% glycerol, 1× PBS. Images were taken with a Leica MZFL III fluorescence stereomicroscope mounted with a QImaging Retiga EXi digital camera.

Cox Enzyme Activity Assay—To detect cytochrome *c* oxidase activity, the cytochrome *c* oxidase assay kit from Sigma (CTYOC-OX1) was used. Preparation of reagents and assay procedure were followed as described in kit protocol. Lysates were prepared by washing larvae in 1× PBS for 10 min and then by homogenizing larvae in Phosphate Buffer, pH 6.8, 0.5% Tween. 20 μg of lysate was used for each sample in the assay.

³ The abbreviations used are: PBS, phosphate-buffered saline; PHM, peptidyl-glycine α -hydroxylating monooxygenase; FMRF-amide, Phe-Met-Arg-Phe-amide; ICP-MS, inductively coupled plasma mass spectrometry.

Enzyme activity was calculated using the formula presented in kit's protocol.

Heart Rate Assay—Glass 8-well multitest slides were pre-coated with polylysine. 1st instar larvae were briefly rinsed in 1× PBS and then placed onto the slide such that the dorsal side of the larvae was facing up. Larvae were given 5–10 min to adjust to the slide prior to heart rate analyses. Slides were viewed using a Zeiss Axio Imager wide field epifluorescence microscope. Heart movement was captured using a Hamamatsu Orca ER monochrome cooled CCD camera with IEEE and MetaMorph software. Heart movement was captured in 5-s intervals with each animal recorded for a total of 15 s (three 5-s intervals). Files were subsequently played back, and the number of contractions was counted. Heart rate was calculated by dividing the number of contractions counted in one 5-s interval by 5 s, and the three 5-s intervals were subsequently averaged to obtain final heart rate for the animal. Statistical significance was determined using single factor analysis of variance analyses.

ICP-MS—Larvae were collected in 1× PBS and then washed once with 1× PBS. Wash was removed, and larvae were homogenized in a lysis buffer (20 mM HEPES, 50 mM NaCl, 1 mM EGTA). 50 μg of total protein was sent for ICP-MS analysis at a final concentration of 1 μg of total protein per μl. Measurements were done in quadruplicate with the final data representing an average of the measurements. ICP-MS analysis was performed by Dr. Ted Huston, University of Michigan.

RESULTS

Ctr1A Localizes to the Plasma Membrane and to Intracellular Vesicles—Earlier work established that the *Drosophila* Ctr1B copper transporter is inducibly expressed in response to copper deficiency, localizes to the plasma membrane, and has a physiologically important role in copper accumulation and in development under conditions of either copper limitation or copper excess. mRNA blotting experiments demonstrated that in contrast to Ctr1B, Ctr1A mRNA is expressed during all major stages of development and in adult flies (29). To begin to understand the relative contributions of Ctr1A and Ctr1B in copper acquisition and homeostasis in *Drosophila*, Ctr1A subcellular localization was examined in cultured *Drosophila* cells and in tissues from distinct developmental stages. An anti-Ctr1A peptide antibody was developed specific to a region between the first and second transmembrane domains, but which does not recognize Ctr1B or Ctr1C (Fig. 1A). To ascertain the specificity of the antibody for Ctr1A protein, an immunoblot was carried out using total protein extracts from wild type embryos (homozygous wild type for Ctr1A), deficiency heterozygote embryos possessing only one copy of the Ctr1A locus, and deficiency hemizygous embryos (Ctr1A is on the X chromosome) that had no copies of the Ctr1A locus and thus represents a Ctr1A null mutant. As shown in Fig. 1B, the anti-Ctr1A antibody predominantly recognizes three electrophoretic species of ~37, ~66, and ~85 kDa. Given that Ctr1 proteins from yeast and mammals homotrimerize to form a functional, copper-transporting unit (10, 11, 35, 36), these three species detected likely represent the monomeric, dimeric, and trimeric forms of Ctr1A, respectively. Whereas the primary

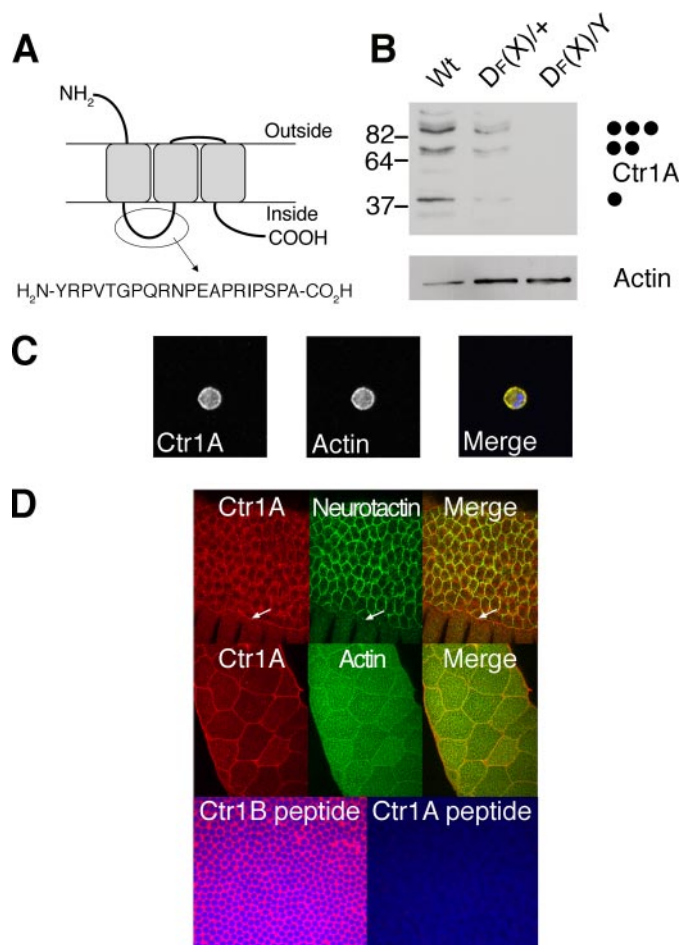


FIGURE 1. Ctr1A is predominantly localized to the plasma membrane. A, Ctr1A antibody was raised against a 20-amino acid epitope located in the cytosolic loop between transmembrane domains one and two. The amino acid sequence of the epitope is shown. B, to test the specificity of the anti-Ctr1A antibody, an immunoblot was performed on embryo lysates from Ctr1A homozygous wild type (*Wt*) levels, Ctr1A heterozygous lines, and Ctr1A hemizygote lines. W1118 flies were used as the source for Ctr1A homozygous wild type flies. A deficiency uncovering Ctr1A was used as the source for heterozygous and hemizygous Ctr1A protein. Actin was used as a loading control. Monomeric, dimeric, and trimeric forms of the protein are indicated by one, two, or three black circles, respectively. C, localization of Ctr1A in S2 cells. Ctr1A largely colocalizes with rhodamine phalloidin, a stain for cortical actin. D, Ctr1A localization in fixed *Drosophila* tissue. The upper panel images are of a wild type *Drosophila* embryo undergoing dorsal closure; arrows show the concentrated localization of Ctr1A to the apical membrane in polarized epithelial cells. Middle panels are images of wild type 3rd instar *Drosophila* larval salivary glands. Lower left panel is a wild type *Drosophila* embryo incubated with anti-Ctr1A antibody and a Ctr1B peptide, and the lower right panel is a wild type *Drosophila* embryo incubated with anti-Ctr1A antibody and the cognate Ctr1A antigen peptide; red channel is Ctr1A and blue channel is 4,6-diamidino-2-phenylindole staining.

translation product of Ctr1A is predicted to have a mass of ~25.6 kDa, Ctr1 from yeast and mammals is glycosylated, and this modification could explain the deviation from the *Drosophila* Ctr1A predicted electrophoretic mobility (13, 37, 38). The anti-Ctr1A antibody shows high specificity for Ctr1A; protein extracts from Ctr1A heterozygous embryos exhibit an approximately half-reduction in Ctr1A protein levels, and no protein bands were detected in the Ctr1A hemizygous mutant embryos. Additionally, peptide competition experiments on fixed, wild type *Drosophila* embryos demonstrate the specificity of the Ctr1A antibody; incubation of embryos in a primary

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mixture consisting of the anti-Ctr1A antibody and a peptide specific for Ctr1B does not affect localization or expression of Ctr1A (Fig. 1D, lower left panel), whereas a primary mixture consisting of the anti-Ctr1A antibody and a peptide specific for Ctr1A abolishes the detection of Ctr1A (Fig. 1D, lower right panel).

Ctr1 proteins are predominantly localized to the plasma membrane in yeast and in some mammalian cells and tissues and have been shown in intestinal epithelial cells to localize to the apical membrane (22, 25, 37). In other cells and conditions, mammalian Ctr1 is found in intracellular vesicular compartments (38, 39). The subcellular localization of Ctr1A in cultured *Drosophila* S2 cells and in fly tissues was examined using the affinity-purified anti-Ctr1A antibody. In cultured S2 cells, Ctr1A was predominantly localized to the plasma membrane (Fig. 1C), and this localization was not altered by incubation of these cells in the presence of 400 μM copper (data not shown). Ctr1A localization was evaluated in whole embryos and 3rd instar larval salivary glands and found to co-localize with plasma membrane markers (Fig. 1D) in addition to showing punctate intracellular staining. Moreover, Ctr1A steady state protein levels were not responsive to a 24-h incubation with the Cu(I)-specific chelator, bathocuproine disulfonate, or with 100 μM copper (data not shown). One interesting observation is that in embryonic amnioserosal cells, a nonpolarized cell type Ctr1A co-localizes with neurotactin. However, in the embryonic epithelia, a polarized cell type Ctr1A does not co-localize with neurotactin, which is found at the basolateral membrane, but instead is preferentially localized to the apical membrane (arrow in Fig. 1D). Taken together, these results demonstrate that the *Drosophila* Ctr1A copper transporter, like Ctr1B (29, 40, 41), is largely localized to the plasma membrane.

Ctr1A Is Essential for *Drosophila* Development—Ctr1A and Ctr1B are plasma membrane proteins that stimulate copper uptake when overexpressed in cultured *Drosophila* S2 cells and can substitute for loss of the high affinity copper uptake system in baker's yeast (29). Flies lacking Ctr1B grow and develop normally but arrest at distinct developmental stages in response to both copper deprivation (1st instar larvae) and copper toxicity (pupae). Additional studies suggest that Ctr1B is the primary larval intestinal copper importer that functions on the apical membrane (41). Whereas Ctr1B is strongly regulated at the level of transcription by copper availability and the MTF-1 transcription factor and clearly plays important roles in response to copper deprivation, little is known about the physiological role of *Drosophila* Ctr1A.

We evaluated the consequences of specific loss of Ctr1A in *Drosophila* utilizing P-element-mediated imprecise-excision mutagenesis (30). Inverse PCR and sequence analysis demonstrated that the P-element insertion line G788 harbors a P-element inserted 90 bp into the first noncoding exon of the *ctr1A* gene (Fig. 2A). As shown in Fig. 2A, one of the excision lines generated from the P-element mutagenesis, line 25, yielded a 5,148-bp deletion that removed most of the coding sequence of Ctr1A but left the upstream gene, CG3224, intact. PCR analysis and RT-PCR confirmed that CG3224 genomic integrity and mRNA expression levels were unaffected by the line 25 excision (data not shown).

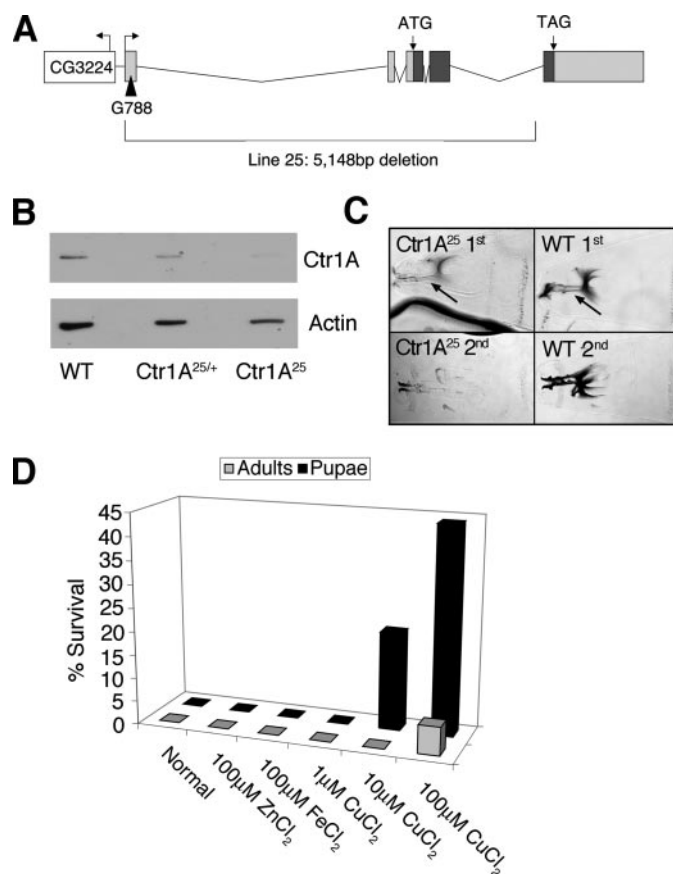


FIGURE 2. Ctr1A is essential for development. A, schematic diagram of the Ctr1A locus. The entire locus is 6,621 nucleotides in length and is organized into five exons. The beginning of the Ctr1A coding sequence is indicated (ATG), as is the end of coding sequence (TAG). The *ctr1A* gene encodes for a 231-amino acid protein with an estimated molecular mass of 25.6 kDa. Neighboring gene CG3224, located 200 bp upstream of the Ctr1A locus, is also shown. The location of P-element G788 used to generate Ctr1A mutant lines is indicated. Mutant line 25 yielded a 5,148-bp deletion that removes most of Ctr1A coding sequence. B, immunoblot analysis of line 25. W1118 embryo lysates were used to detect homozygous wild type (WT) Ctr1A protein levels, and embryo lysates were made using line 25 heterozygotes and line 25 hemizygotes. Ctr1A was detected with anti-Ctr1A affinity-purified antibody. Actin was used as a loading control. C, cuticle preparations of wild type larvae and *ctr1A*²⁵ hemizygote mutant larvae at 1st and 2nd instar larval stages. Arrows point to mouth hook structures. D, Ctr1A mutants were allowed to develop on either normal food or normal food supplemented with either 100 μM ZnCl₂, 100 μM FeCl₂, 1 μM CuCl₂, 10 μM CuCl₂, or 100 μM CuCl₂. Dead animals were removed, and the stage at which the animals arrested was determined by inspection. Data are plotted as percent of animals that survived to either pupal stages (black bars) or to adult stages (gray bars). $n = 125$ animals for each food condition except for the 100 μM CuCl₂ condition where $n = 200$.

Analysis of the mutant line showed that hemizygous Ctr1A line 25 mutants, hereafter referred to as *ctr1A*²⁵, arrest as either 1st instar larvae (~40%) or as 2nd instar larvae (~60%). Line 25 can be rescued both by a Y chromosome carrying a fragment of translocated X chromosome, which includes the Ctr1A locus, and by transgenic flies that express Ctr1A expressed under the control of the yeast GAL4 upstream-activating sequence promoter (data not shown). Rescue by the transgenic flies could occur without the use of a Gal4 driver line suggesting that low levels of Ctr1A protein, because of leaky expression from the upstream activating sequence promoter, are sufficient to restore viability to Ctr1A mutants. We have used line 25 as a loss-of-function mutation in *Ctr1A* for the experiments described here.

As shown in Fig. 2B, immunoblot analyses of Ctr1A levels are consistent with the excision of Ctr1A in line 25. Ctr1A protein levels in embryonic lysates show ~50% the level of Ctr1A protein in line 25 heterozygous embryos as compared with wild type embryos. Very low levels of Ctr1A are present in *ctr1A*²⁵ hemizygous embryos, suggesting the possibility that Ctr1A may be maternally loaded in oocytes. Cuticle preparations of wild type 1st and 2nd instar larvae (Fig. 2C) demonstrate the strong pigmentation of mouth hook structures and spiracles because of the copper-dependent enzyme tyrosinase. In contrast, 1st and 2nd instar larvae from *ctr1A*²⁵ hemizygotes (Fig. 2C) demonstrate significant pigmentation defects in these structures.

Studies have shown that yeast Ctr1 mutant growth and viability on nonfermentable carbon sources can be rescued by adding exogenous copper to growth media, demonstrating that the growth phenotype is because of a copper deficiency (11, 37). Previous studies characterizing *Drosophila* Ctr1B mutants, which conditionally arrest on copper-depleted media, show that the developmental arrest of Ctr1B homozygous mutants can also be rescued by copper supplementation (29). Given these observations, we tested whether the 1st and 2nd instar larval arrest of *ctr1A*²⁵ mutants could be rescued by dietary copper supplementation. *ctr1A*²⁵ hemizygous mutants were generated and allowed to develop on food supplemented with increasing concentrations of copper or other essential metals. As shown in Fig. 2D, at 10 μM CuCl_2 , ~20% of *ctr1A*²⁵ hemizygous mutants pupariate without successful eclosion, and at 100 μM CuCl_2 , ~45% of *ctr1A*²⁵ hemizygous mutants pupariate and ~5% eclose as viable adults. In contrast, the developmental arrest of *ctr1A*²⁵ hemizygous mutants could not be rescued with either 100 μM ZnCl_2 or 100 μM FeCl_2 . These data support the hypothesis that *ctr1A*²⁵ mutants developmentally arrest because of a copper-specific deficiency. Interestingly, the dietarily rescued *ctr1A*²⁵ mutant males that do eclose still exhibit outward signs of copper deficiency, including hypo-pigmented abdomen and thoracic bristles (data not shown), suggesting that although copper levels permit some level of viability and development to adult flies, these levels are still insufficient to meet the demands to drive all of the copper-dependent processes.

The Ctr1A loss of function experiments indicate this protein plays an important role in normal embryonic development that can be partially rescued by added copper. To evaluate whether *ctr1A*²⁵ mutant embryos exhibit global changes in total copper accumulation, wild type larvae and heterozygous and hemizygous *ctr1A*²⁵ mutant larvae were collected, washed, and subjected to ICP-MS analysis of total copper. As shown in Fig. 3A, compared with wild type larvae, *ctr1A*²⁵ heterozygous mutant larvae showed an ~20% reduction in copper levels, and *ctr1A*²⁵ hemizygous mutant larvae showed an ~30% reduction in copper levels. The modest reduction in copper levels in both the heterozygous and hemizygous *ctr1A*²⁵ mutant larvae can potentially be explained by the copper transporting activity of Ctr1B, which is expressed in 1st instar larvae, the stage at which metal content analysis was performed.

To ascertain whether *ctr1A*²⁵ mutants exhibit an intracellular copper deficiency, the activity of cytochrome *c* oxidase, a mitochondrial enzyme involved in oxidative phosphorylation,

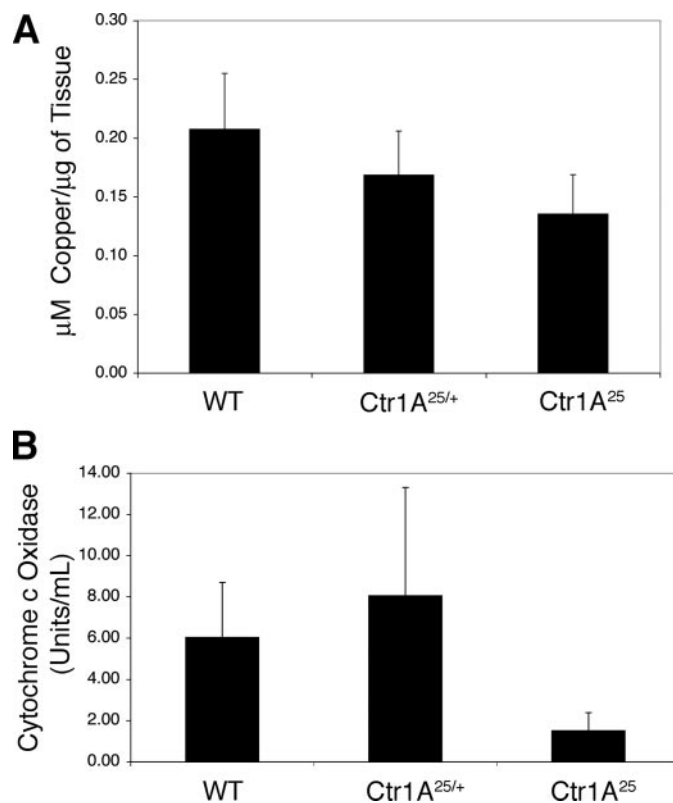


FIGURE 3. Ctr1A mutants are copper-deficient. A, ICP-MS analysis of copper levels in wild type (WT), *ctr1A*^{25/+} heterozygote, and *ctr1A*²⁵ hemizygote 1st instar mutant larvae. *ctr1A*²⁵ hemizygote 1st instar mutant larvae have significantly lower copper levels as compared with wild type 1st instar larvae (p value <0.01) and as compared with *ctr1A*^{25/+} heterozygote larvae (p value <0.05). Copper levels between wild type larvae and *ctr1A*^{25/+} heterozygote larvae are not significantly different. B, cytochrome oxidase enzyme activity was assessed in wild type 1st instar larval lysates, Ctr1A heterozygous 1st instar larval lysates (*ctr1A*^{25/+}), and in Ctr1A hemizygous 1st instar larval lysates (*ctr1A*²⁵). Activity is expressed as units of cytochrome *c* oxidase activity/ml. Ctr1A hemizygous 1st instar larvae have a significant reduction in cytochrome *c* oxidase activity as compared with wild type larvae (p value <0.05).

was assayed. As shown in Fig. 3B, *ctr1A*²⁵ hemizygous mutants show a nearly 3-fold reduction in cytochrome oxidase enzyme activity as compared with wild type larvae. Interestingly, *ctr1A*²⁵ heterozygous animals exhibit cytochrome oxidase enzyme activity higher than that measured in wild type flies, an observation noted for copper-dependent enzymatic assays. One possible explanation for this result is that *ctr1A*²⁵ heterozygous animals sense a partial copper deficiency imbued by loss of one copy of Ctr1A and as a result enhance the expression or function of proteins involved in intracellular copper homeostasis, which has been observed in mammals with the stabilization of the CCS copper chaperone for Cu,Zn superoxide dismutase (42–45). In some experiments we observed enhanced levels of Ctr1B expression (data not shown), which is induced under conditions of copper deficiency by the MTF-1 transcription factor (29, 40).

Previous experiments support the notion that both Ctr1A and copper are maternally loaded into oocytes (Fig. 2B)⁴ (41). Furthermore, developmental RNA blotting experiments as well as *in situ* hybridization experiments suggest Ctr1A is expressed

⁴ M. L. Turski and D. J. Thiele, unpublished data.

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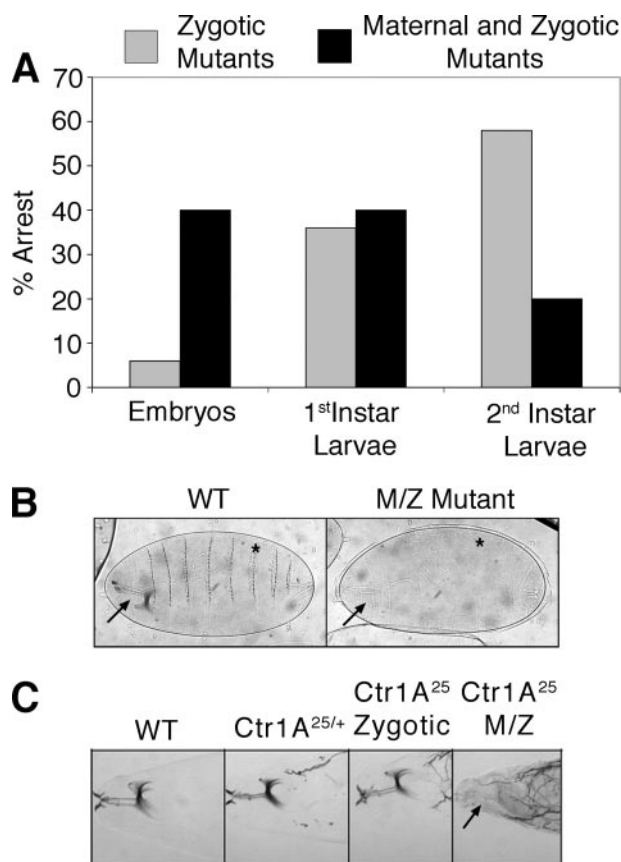


FIGURE 4. Ctr1A maternal and zygotic mutants arrest at earlier stages of development and yield a qualitative defect in tyrosinase activity. *A*, histogram comparing the developmental arrest of Ctr1A zygotic mutants (gray bars) to Ctr1A maternal and zygotic mutants (black bars). For the *ctr1A*²⁵ zygotic experiments, ~200 embryos were used for hatching efficiency studies, and ~50 hatched larvae were tracked for larval developmental progression studies. For the *ctr1A*²⁵ maternal and zygotic experiments, ~400 embryos were used for hatching efficiency studies, and ~100 hatched larvae were tracked for larval developmental progression studies. *B*, cuticle preparations of a stage 17 wild type (WT) embryo (left) and a stage 17 Ctr1A maternal and zygotic embryo (M/Z, right). Anterior is to the left, and posterior is to the right within each panel. Arrows point to the mouth hook structures, and the asterisk marks one of eight ventral denticle belts. *ctr1A*²⁵ maternal and zygotic embryos exhibit a severe deficiency in tyrosinase activity. *C*, images of 1st instar larvae. Anterior is to the left. Arrow in the right panel indicates the severely hypo-pigmented mouth hook structures of a Ctr1A maternal and zygotic mutant.

during early stages of development (29).⁴ To explore the potential role of maternally inherited Ctr1A in fly developmental progression, we generated *ctr1A*²⁵ maternal and zygotic mutants (31). One prediction for maternal and zygotic mutants versus zygotic mutants is that *ctr1A*²⁵ maternal and zygotic mutants should show an earlier developmental arrest because of the loss of a maternal load of Ctr1A and copper. The analysis of *ctr1A*²⁵ maternal and zygotic mutants supports this prediction and shows a shift in the severity of their developmental arrest as compared with *ctr1A*²⁵ zygotic mutants (Fig. 4A). Approximately 40% of *ctr1A*²⁵ maternal and zygotic mutants arrest during embryogenesis, as compared with ~5% of zygotic *ctr1A*²⁵ mutants arresting at the embryonic stage. Another ~40% of the *ctr1A*²⁵ maternal and zygotic mutants arrested as 1st instar larvae, with ~20% arresting as 2nd instar larvae as compared with 60% of *ctr1A*²⁵ zygotic mutants arresting as 2nd instar larvae. Furthermore, *ctr1A*²⁵ maternal and zygotic

mutants visibly demonstrate a more severe tyrosinase-dependent pigmentation deficiency as compared with wild type embryos of the same stage; mouth hook apparatus and denticle belts are barely perceptible, although present, because of the lack of pigmentation in the *ctr1A*²⁵ maternal and zygotic mutants (Fig. 4B). This severe compromise in pigmentation extends to larval stages as shown in Fig. 4C. Quantitative *in vitro* experiments have also verified the deficiency in tyrosinase activity in *ctr1A*²⁵ zygotic mutants and *ctr1A*²⁵ maternal and zygotic mutants, and the enzymatic activity could be restored to wild type levels by the addition of copper to the mutant lysates (data not shown).

Copper Uptake via Ctr1A Drives Neuropeptide Maturation—The developmental arrest observed in *ctr1A*²⁵ mutants can most likely be attributed to pleiotropic effects of copper deficiency. However, both zygotic and maternal and zygotic Ctr1A mutants develop to larval stages without gross morphological aberrations, suggesting that body plan organization and early organogenesis proceeds relatively normally. One important component of the developmental arrest could be due to Ctr1A mutants being unable to initiate or regulate later developmentally important behaviors, transitions, or physiological processes. Neuropeptides serve as modulators within the nervous system and as regulatory hormones involved in many physiological processes (46, 47). Neuropeptides are synthesized as biologically inactive precursor proteins (pre-propeptides), which undergo processing and modification within the secretory pathway into mature bioactive peptides (48). One such modification is peptide amidation, a process conserved in metazoans that is catalyzed by the copper-dependent enzyme, peptidylglycine α -hydroxylating monooxygenase (PHM) (46). It has been estimated that ~90% of all *Drosophila* neuropeptides undergo amidation via PHM (34, 49).

To evaluate PHM activity in Ctr1A mutant flies, we utilized *in situ* analysis for the presence of prepro-FMRF peptide and its processed and matured amidated neuropeptides, FMRF-amide-related peptides (50), using an antibody that recognizes the FMRF pre-propeptide (34) and one specific to the amidated form of FMRF-amide-related peptides (34, 51). As shown Fig. 5, dissected wild type 1st instar larval brains have a stereotypical pattern of neuroendocrine cells in the ventral ganglion of the central nervous system that produce the FMRF pre-propeptide (52). Dissected 1st instar larval brains from *ctr1A*²⁵ maternal and zygotic mutants also show a similar pattern of FMRF pre-propeptide producing neuroendocrine cells within the ventral ganglion without qualitative differences in abundance (Fig. 5). Although neuroendocrine cells from wild type 1st instar larvae produce easily detectable levels of FMRF-amide, very low levels of FMRF-amide were detected in neuroendocrine cells from Ctr1A maternal and zygotic mutants.

To ascertain whether the reduction in FMRF-amide production in Ctr1A mutant neuroendocrine cells is because of a loss of PHM enzyme or a copper deficiency as a result of systemic loss of *ctr1A*²⁵, copper remediation experiments were carried out. As shown in Fig. 5, *ctr1A*²⁵ maternal and zygotic mutants reared on 100 μ M CuCl₂ accumulated mature FMRF-amide peptide within neuroendocrine cells that was qualitatively similar to that observed in wild type flies under the same condi-

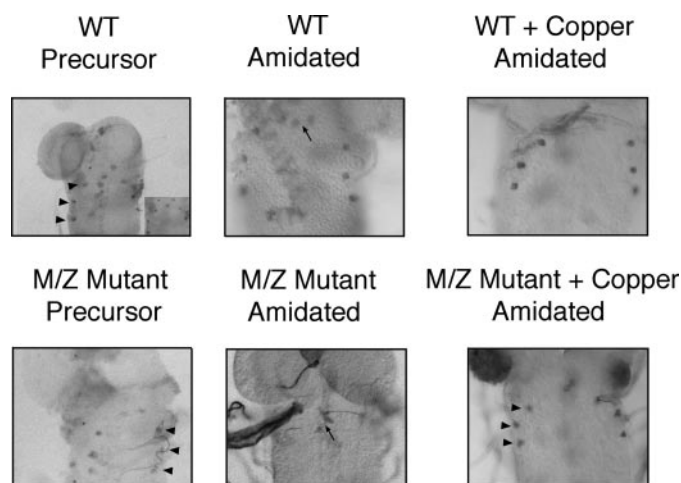


FIGURE 5. Ctr1A maternal and zygotic mutants are defective in production of amidated FMRF-amide-related peptides. Wild type 1st instar or Ctr1A maternal and zygotic mutant 1st instar larval brains were dissected and stained with either an antibody that recognizes the FMRF-amide prepeptide or an antibody that recognizes the mature, FMRF-amidated-related peptides. Arrowheads indicate the Tv neuroendocrine cells, and arrows indicate the SE neuroendocrine cells, which produce FMRF-amide-related peptides in the ventral ganglion of the nervous system.

tions. These observations strongly suggest that *ctr1A*²⁵ maternal and zygotic mutants are deficient in neuroendocrine cell-derived PHM activity, and this deficiency is reversible by dietary copper supplementation, suggesting the deficiency is attributable to reduced copper availability because of loss of Ctr1A.

FMRF-amides represent a family of neuropeptides, the FMRF-amide-related peptides, which share an RF-amide carboxyl terminus but have unique amino-terminal extensions (50). The first family member identified, FMRF-amide, was shown to have cardioexcitatory effects in the clam *Macrocaltista nimbosa* (53). Since then, this family of FMRF-amide related peptides has been shown to act as neuromodulators of several physiological processes, including heart rate (50). Given that *ctr1A*²⁵ maternal and zygotic mutants exhibit a strong defect in the production of FMRF-amide-related peptides in neuroendocrine cells of the ventral ganglion and FMRF-amide related peptides can modulate heart rate, we tested whether *ctr1A*²⁵ maternal and zygotic mutant larvae exhibit an alteration in heart beat rate. Heart beat rates were measured for 1st instar larvae from wild type and *ctr1A*²⁵ maternal and zygotic mutants reared on normal food and, independently, on fly food supplemented with 100 μ M CuCl₂. As shown in both Fig. 6, A and B, wild type 1st instar larvae have an average heart rate of 3.17 beats/s (S.D. = \pm 0.69), whereas *ctr1A*²⁵ maternal and zygotic 1st instar larvae exhibit an average heart rate of \sim 2.00 beats/s (S.D. = \pm 0.36). This difference in heart rate between wild type and *ctr1A*²⁵ maternal and zygotic mutant animals is statistically significant, with a *p* value $<$ 0.01. Although copper supplementation only modestly altered wild type 1st instar larval heart rate (3.17 \pm 0.69 beats/s versus 3.01 \pm 0.86 beats/s on normal versus copper-supplemented food, respectively; *p* value $<$ 0.5), copper supplementation significantly elevated the heart rate of *ctr1A*²⁵ maternal and zygotic 1st instar larvae (2.00 \pm 0.36 beats/s versus 2.49 \pm 0.63 beats/s on normal versus copper-

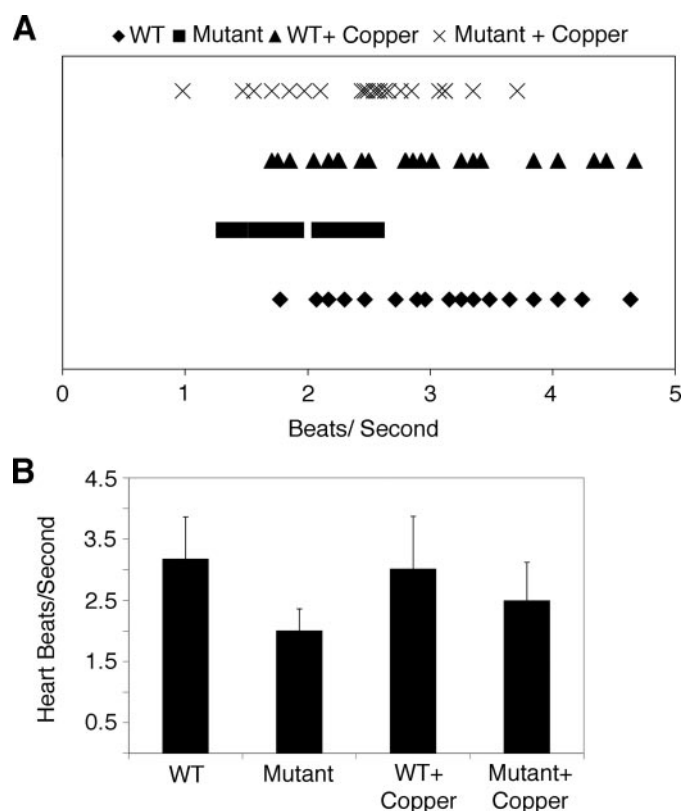


FIGURE 6. Ctr1A maternal and zygotic mutants exhibit a lower heart beat rate. A, heart rate analyses of wild type (WT) 1st instar larvae and Ctr1A maternal and zygotic 1st instar mutant larvae reared on either normal food or food supplemented with 100 mM CuCl₂. Heart rates are given as beats/s along the x axis. Each point on the chart represents the average heart rate of a single animal of a given genotype (*n* = 25 for each genotype). Black diamonds = wild type, normal food. Black squares = Ctr1A maternal and zygotic mutants, normal food. Black triangles = wild type, copper-supplemented food. \times = Ctr1A maternal and zygotic mutants, copper-supplemented food. B, representation of the heart rate data given as histogram. Overall averages and standard deviations for each group are given.

supplemented food, respectively; *p* value $<$ 0.02). Although the defect in heart beat rate is likely to be the consequence of a deficiency of multiple copper-dependent activities, the correlation between reduced FMRF-amide formation, reduced heart beat rate/ and partial amelioration of both by copper supplementation suggests that copper delivery by Ctr1A is important for PHM activity and amidated neuropeptide physiology.

DISCUSSION

In this work we have characterized *Drosophila* Ctr1A and have shown it to be an essential gene in the development of *Drosophila melanogaster*. *Drosophila* encodes three Ctr1-related proteins in its genome (29), and each presumably contributes to total body copper homeostasis. Data reported here strongly suggest that Ctr1A is the primary copper transporter in *Drosophila* given its ubiquitous expression pattern throughout development and into adulthood, the fact that it shares the highest degree of homology to mammalian Ctr1 proteins, and its lethal loss of function phenotype. Within this model where Ctr1A functions as the primary copper transporter, Ctr1B contributes to copper uptake in some fly tissues during development and in adult flies but is only essential for development and viability under growth conditions where copper is either

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extremely limiting or abnormally abundant (29). At present, little is known regarding the function of Ctr1C in *Drosophila* although one possibility, based on the observation that overexpression of Ctr1C in S2 cells does not mediate copper uptake (29), is that Ctr1C may function on an intracellular compartment and in that regard may be more similar to *S. cerevisiae* Ctr2 (54). However, further experiments will be necessary to ascertain the contributions of *Drosophila* Ctr1C to total body copper homeostasis and to determine whether it is an ortholog of *S. cerevisiae* Ctr2.

Through P-element-mediated mutagenesis we generated a Ctr1A loss-of-function mutation that results in early larval lethality for hemizygous mutants. Despite the striking phenotype of the maternal and zygotic *ctr1A*²⁵ mutants, maternal and zygotic mutants are still able to complete earlier stages of embryonic development during which gastrulation, pattern formation, and organogenesis occurs (55). This is in contrast to mouse Ctr1 mutants that die in mid-embryonic stages and have severe developmental defects, including a poorly developed mesoderm and neural ectoderm (22, 23). This difference in mutant phenotype between mouse and fly might be attributable to the considerable maternal load of copper itself present in *Drosophila* oocytes. Although there may also be maternally derived copper in mouse oocytes, the maternal stores of copper in *Drosophila* oocytes may be adequate to support embryonic development, a process that only takes 24 h, whereas mouse embryonic development, a much longer process requiring ~20 days, may require additional copper and thus may be more dependent upon Ctr1 function. It would be interesting to test if rearing *Drosophila* females on copper-depleted food over successive generations might result in an earlier lethality for *ctr1A*²⁵ mutants. Alternatively, the presence of the Ctr1B isoform in *Drosophila*, but not in mammals, could partially suppress an otherwise more severe embryonic phenotype.

In addition to traditional genetic rescue experiments, Ctr1A zygotic mutants could be partially rescued by food supplemented with copper. This dietary rescue was specific for copper, as supplementation with other metals such as iron or zinc, at comparable concentrations, did not result in developmental rescue. Copper-rescued *ctr1A*²⁵ flies still exhibited signs of a copper deficiency with hypo-pigmented bristles and abdomen. This suggests that copper levels, although adequate to support viability, were insufficient to meet all of the copper-dependent processes and that perhaps copper-dependent processes are prioritized for copper delivery. Indeed experiments in Zebrafish suggest that a hierarchy of copper metabolism exists such that when copper is limiting, copper-dependent cellular processes most essential for viability are prioritized over less essential processes. Specifically within the Zebrafish model, pigmentation mediated by tyrosinase activity was typically one of the first copper-dependent processes to be affected by copper limitation, whereas notochord development, potentially mediated by lysyl oxidase activity and perhaps other copper-dependent processes, was one of the last processes to be affected (56). Thus tyrosinase activity and pigmentation in flies might also be lower on the copper metabolism hierarchy and more dispensable under conditions of copper deprivation such that

enzymes and processes higher up within the hierarchy can be metallated with limited amounts of copper available.

We also observed copper-dependent enzyme activity deficiencies in *ctr1A*²⁵ mutants, indicative of an intracellular/organismal copper deficit, and for several of the copper-dependent enzymes, such as tyrosinase and PHM, enzyme activity defects were partially reversed through addition of copper to the lysate, as was the case for tyrosinase, or through rearing the mutants on a high copper diet, as was the case for PHM. This brings up the interesting question of how both viability and *in vivo* PHM activity can be rescued in *ctr1A*²⁵ mutants via high copper supplementation in the diet. It is known that in *S. cerevisiae* Fet4 is able to transport both iron and copper although at a low affinity (K_m of ~35 μM compared with Ctr1 proteins that have a K_m for copper of ~1–5 μM) (11, 57), and Smf1 can transport a variety of divalent metal ions, including copper (58). Both mammals and flies have a homolog of Smf1, termed DMT1 (59, 60), and malvolio (61), respectively, and both proteins have been shown to be broad spectrum metal transporters. Thus one possible explanation for the dietary rescue of *ctr1A*²⁵ mutants is that at a particular concentration of copper in the food, the K_m value for copper of malvolio or an as yet unidentified metal transporter is met or exceeded, and this permits an adequate uptake of copper into cells such that intracellular requirements of copper for cell survival are partially met.

Another striking observation was the significant deficiency of *ctr1A*²⁵ maternal and zygotic mutants in producing the mature, amidated form of FMRF-amide-related peptides despite being able to synthesize the pre-propeptide, suggesting that these animals are deficient in PHM activity at least in some cell or tissue types. Although Menkes protein has been shown to contribute to the function of peptidylglycine α -amidating monooxygenase (mammalian PHM) by transporting copper into the secretory pathway where it can then be loaded onto the enzyme (62), this is the first study of which we are aware that demonstrates a deficiency in PHM enzyme activity because of loss of a plasma membrane copper importer.

Within the *Drosophila* genome there are at least 32 genes that encode neuropeptides with the potential processing of the precursors to generate even more bioactive neuropeptides (63). The majority of these neuropeptides is either known or predicted to be α -amidated (49) and as such could be substrates for PHM. It is therefore reasonable to suggest that although in this work we demonstrated that Ctr1A mutants are deficient in the production of one family of amidated peptides, FMRF-amide-related peptides, Ctr1A mutants may be deficient in the production of other amidated neuropeptides as well. Given that neuropeptides play important and diverse roles in the regulation of developmental processes as well as homeostatic processes, organization of behaviors, and in the modulation of neural and motor activity (46), Ctr1A and copper are likely to function upstream of these processes as well.

We specifically examined one physiological readout of the FMRF-amide-related peptide function, heart beat rate, and found *ctr1A*²⁵ maternal and zygotic mutants have a significantly lower heart beat rate than wild type animals. At present, we can correlate but not establish a causal relationship with the low heart beat rate and the reduction in abundance of FMRF-

amide-related peptides. Given that the low heart beat rate observed in *ctr1A*²⁵ maternal and zygotic mutants could not completely be restored to a wild type rate on food supplemented with copper suggests that there are multiple factors contributing to the low heart beat rate observed in *ctr1A*²⁵ maternal and zygotic mutants. Previous studies have shown that impaired copper homeostasis within cardiac tissue can lead to cardiomyopathy. Animals fed a low copper diet developed cardiomyopathy, including histological and ultrastructural alterations in cardiac tissue as well as functional alterations in cardiac performance (64–66). Mutations in *SCO2*, a gene that encodes a metallochaperone involved in mitochondrial copper delivery and in cytochrome oxidase assembly, result in fatal infantile cardiomyopathy (27, 67). Most recently, we have shown that intestinal epithelial cell-specific *Ctr1* knock-out mice also exhibit cardiac hypertrophy (25). At present, the mechanisms explaining how copper deficiency results in cardiomyopathy are unknown. However, it has been thought that reduced activity of the copper-dependent enzymes lysyl oxidase and cytochrome *c* oxidase contribute to the pathology (68). Amidated neuropeptides are also important in cardiovascular function (69), and the data in this paper would now suggest that deficient PHM activity could also be added as a potential contributing factor in copper deficiency-induced cardiomyopathy. Although with respect to cardiac function, *Drosophila* has predominantly been used as a model system to address questions of heart development, and recently it has become an emerging model to address questions of heart disease (70, 71). These and future studies may provide additional insights into the roles of copper in normal heart function and how copper imbalance can result in cardiovascular pathology.

Acknowledgments—We thank members of the Thiele laboratory for helpful suggestions, technical assistance, and critical reading of this manuscript. We thank Dr. Eric P. Spana (Duke University) for technical assistance and expertise in *Drosophila* genetics and cell biology as well as for discussions regarding the work. The anti-PT2 and anti-CT-FMRF antibodies were generously provided by Dr. Paul H. Taghert (Washington University).

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