

A Copper-regulated Transporter Required for Copper Acquisition, Pigmentation, and Specific Stages of Development in *Drosophila melanogaster**

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The trace element copper is required for normal growth and development, serving as an essential catalytic co-factor for enzymes involved in energy generation, oxidative stress protection, neuropeptide maturation, and other fundamental processes. In yeast and mammals copper acquisition occurs through the action of the Ctr1 family of high affinity copper transporters. Here we describe studies using *Drosophila melanogaster* to investigate the role of copper acquisition through Ctr1 in normal growth and development. Three distinct *Drosophila* Ctr1 genes (Ctr1A, Ctr1B, and Ctr1C) have been identified, which have unique expression patterns over the course of development. Interestingly, Ctr1B, which is expressed exclusively during the late embryonic and larval stages of development, is transcriptionally activated in response to nutritionally induced copper deprivation and down-regulated in response to copper adequacy. The generation of Ctr1B mutant flies results in decreased larval copper accumulation, marked body pigmentation defects that parallel defects in tyrosinase activity, and specific developmental arrest under conditions of both nutritional copper limitation and excess. These studies establish that copper acquisition through the *Drosophila* Ctr1B transporter is crucial for normal growth and in early and specific stages of metazoan development.

The normal growth and development of eukaryotic cells and organisms require appropriate spatial and temporal patterns of gene expression, signal transduction, and biosynthetic and metabolic reactions. Key to these important biochemical reactions is the availability of many enzymatic co-factors that are acquired dietarily. One such co-factor is copper, a trace metal that is essential for normal growth and development (1–3). Indeed, copper serves as a catalytic co-factor for a wide variety of enzymes that play general roles in cell growth and protection including cytochrome oxidase (energy generation), ceruloplas-

min (iron mobilization), and Cu,Zn-superoxide dismutase (oxidative stress protection), as well as enzymes with specialized roles in development such as tyrosinase (pigmentation) and peptidylglycyl- α amidating monooxygenase (neuropeptide processing) (4–7). Consistent with its role as a critical enzymatic co-factor, dietary copper deprivation, or genetic diseases of copper maldistribution, result in severe developmental defects in animal models and in humans (3, 8, 9). Although copper plays many essential biochemical roles that may be crucial to normal growth and development, copper can also be a potent cytotoxin when allowed to accumulate in excess of cellular needs (10).

Cells have evolved sophisticated mechanisms to control copper homeostasis at the level of uptake, distribution, sequestration, and export (1, 4, 11). The Ctr1 proteins are a family of high affinity copper transporters, characterized in fungi, plants, amphibians, and mammals, that are predominantly localized to the plasma membrane and that are thought to transport reduced copper (Cu(I)) (1, 12–15). Ctr1 proteins are structurally conserved, harboring three membrane-spanning domains, a hydrophilic methionine-rich amino terminus, several conserved and functionally important amino acid residues, and a Met-X₃-Met motif found in the second transmembrane domain (16–18). Upon delivery of copper by Ctr1 proteins to the interior of cells, it is transferred to copper-dependent enzymes, or the compartments where these enzymes are assembled, via the action of three copper-binding chaperones: CCS for delivery to Cu,Zn-superoxide dismutase, Atx1/Atox1 for delivery to secretory compartments, and Cox17 for delivery to mitochondrial cytochrome oxidase (11, 19). Loss of function of the yeast high affinity copper transport machinery results in copper delivery defects to target cuproproteins downstream of each of the three known copper chaperones (20). The importance of copper and the Ctr1 high affinity copper transporter in development is demonstrated by the observations that mice bearing a targeted deletion of the Ctr1 gene exhibit profound developmental defects and die *in utero* approximately midway through gestation (21, 22). Furthermore, clear tissue-specific defects in copper accumulation, and in the activities of copper-dependent enzymes, are manifest in heterozygous Ctr1 knockout mice and in Ctr1^{-/-} embryonic fibroblasts (21, 23). However, because of the early lethality of Ctr1^{-/-} mice and the failure to rescue this phenotype by dietary copper, the precise roles that copper and Ctr1 play in mammalian development are not well understood.

Drosophila melanogaster is a powerful model system for dissecting the identity and function of genes that play crucial roles in gene expression, signal transduction, and other important events in metazoan development. Although little is known about the mechanisms for copper accumulation and homeostasis in flies, previous studies have demonstrated the existence of

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specialized cells, denoted copper cells (24), that line the lumen of the middle mid-gut and that accumulate high levels of copper in lysosomes (25). Furthermore, the activities of highly specialized copper-dependent enzymes such as peptidylglycyl α -amidating monooxygenase, involved in neuropeptide maturation (26, 27), and tyrosinase, involved in pigmentation and cuticle sclerotization (6), are likely to have important developmental functions in flies. In this work we have identified three *Drosophila* genes encoding high affinity copper transporters in the Ctr1 family that exhibit distinct expression profiles in fly development. One copper transporter, Ctr1B, is exclusively expressed in the late embryonic and larval stages of development, and Ctr1B mRNA levels are induced under conditions of copper limitation. The generation of Ctr1B mutant flies results in markedly decreased larval copper accumulation, abdominal pigmentation defects that parallel defects in tyrosinase activity, and a terminal developmental arrest at the second instar larval stage under conditions of nutritional copper limitation. These studies demonstrate the importance of copper delivery for the activation of specific copper-dependent enzymes, via Ctr1, and for early and defined stages of normal metazoan development.

EXPERIMENTAL PROCEDURES

Cloning and the Expression of *Drosophila* Ctr1 Genes in *Saccharomyces cerevisiae*—Three potential copper transporter (Ctr1) genes were identified from the *D. melanogaster* genome by a BLAST search at the Flybase web site (flybase.bio.indiana.edu/) using human Ctr1 and *Schizosaccharomyces pombe* Ctr5 protein sequences as probes; the three genes were designated Ctr1A, Ctr1B, and Ctr1C. Ctr1A (Flybase no. CG3977) was directly cloned from a *Drosophila* embryo cDNA library by PCR methods with gene-specific primers (28). Ctr1B (Flybase no. CG7459) was cloned from a genetic screen in the *S. cerevisiae* *ctr1Δctr3Δ* strain MPY17, which is deficient in high affinity copper transport and, consequently, cannot grow on nonfermentable carbon sources (29). This strain was transformed with a *Drosophila* cDNA library expressed from the constitutive yeast *ADH1* promoter (30), and transformants were identified that complement the respiratory growth defect of *ctr1Δctr3Δ* cells. The full sequence of the Ctr1B cDNA was recovered twice from the screen (data not shown). Ctr1C (Flybase no. CG15551) was cloned by reverse transcriptase-PCR from third instar larval and S2 cell RNA. The PCR products were subcloned into the SpeI/EcoRI sites of the *S. cerevisiae* expression vector p413GPD (31). The plasmids were transformed into the strain MPY17, and growth on SC-His and YPEG was assayed.

Copper Transport Assays—The Ctr1A, Ctr1B, and Ctr1C open reading frames were subcloned into the expression vector pAC5.1A (Clontech). S2 cells were independently transfected with these plasmids or the empty vector using FuGENE 6 (Roche) and cultured in Schneider medium (Invitrogen) with 10% fetal bovine serum. After 48 h, cells were washed with fresh medium and incubated with 1 μ M 64 CuCl₂ for 10 min at room temperature before washing with cold 20 μ M EDTA to terminate 64 Cu uptake, followed by phosphate-buffered saline, and counted with a Cobra II γ counter (Packard).

Fly Stocks and Genetics—To generate a *Drosophila* Ctr1B null allele, an imprecise P element excision strategy was used (32). The EP(3)833 line, which harbors a single P element ~800 bp upstream of the Ctr1B transcription start site, was obtained from the Bloomington Stock Center (Bloomington, IN). This strain was crossed to a transposase-expressing $\Delta 2-3$ Sb/TM6 line, and the Sb/p[w⁺] male progeny were crossed to TM3/TM6 females. The male w^X; Sb⁺ progeny were pooled into groups of 30–40, crossed to TM3/TM6 females, and genomic DNA was extracted from the eggs laid by these pools. PCR reactions with primers OHZ115 and OHZ146 were performed using genomic DNA as template. Males in the pools that gave shorter products than predicted precise excision were separated and individually crossed to TM3/TM6 females; their progeny were again characterized by the PCR method described above, and the DNA sequence in the Ctr1B region determined. Among ~1,500 independent excision events, 12 imprecise excision events, carrying different deletions in the Ctr1B genomic region, were identified. Several precise excision events, with 8-bp sequence differences from the wild type, were also recovered. The deficiency line Df(3R) p40, which removes the entire Ctr1B genomic region, was acquired from the Bloomington Stock Center. Flies were raised on standard glucose/yeast

fly food defined as 10% D-dextrose, 10% yeast peptone, 1.5% agar, and 0.3% Tegosept (33).

RNA Extraction and Copper Accumulation Studies—Flies were allowed to lay eggs on normal yeast/glucose food or with either copper- or bathocuproine disulfonate (BCS)-supplemented food, and were allowed to develop into late third instar larvae before wandering, when they were collected and total RNA was extracted using TRIzol reagent (Invitrogen). RNA blots were performed as described (28), and the membranes were scanned by PhosphorImager (Amersham Biosciences). Larvae collected in the same manner were digested with 0.1 M hot nitric acid and total body copper accumulation determined by ICP-MS in triplicate (34).

Fly Development Assays—*ctr1B³⁻⁴*/TM6 and the +/TM6 precise excision flies were self-crossed and allowed to lay eggs on food supplemented with different metals or metal chelators. The density of the eggs was controlled for each vial such that 80–150 adult progeny emerged. The progeny with different genotypes were counted as F1 adults, and the frequency of development to adulthood of the homozygous flies was calculated by dividing the number of homozygous progeny with the expected number according to Mendel's law. For data presentation we used the relative development index to adulthood to reflect the survival of *ctr1B³⁻⁴* homozygous adult flies on different types of food. The development rate on each food type was normalized with the rate on normal food to give the relative development index, and the difference between wild type and *ctr1B³⁻⁴* flies was compared. The hatching rate of *ctr1B³⁻⁴* homozygotes was normal, as 100% of the eggs laid by *ctr1B³⁻⁴*/TM6 flies hatched into first instar larvae, as counted on grape juice plates.

Body Pigmentation and Tyrosinase Assays—*ctr1B³⁻⁴*/TM6 and +/TM6 flies were grown on food supplemented with 100 μ M AgNO₃ and 100 μ M BCS and adults photographed within 4 h after emergence. For tyrosinase assays, total protein was extracted from late pupae or young adults and the in-gel tyrosinase assay was performed as previously described (23).

RESULTS

The *Drosophila* Genome Encodes Three Ctr1 Family Copper Transporters—We have initiated studies of copper homeostasis in *D. melanogaster* to begin to understand the roles of copper and the Ctr1 family of copper transporters in metazoan development. The completion of the *Drosophila* genome DNA sequence allowed a genome-wide search for potential Ctr1 family members. Using sequences from mammalian and yeast Ctr1 family members (14, 15, 29, 35) in BLAST searches, we identified three Ctr1 homologs, encoded by distinct genes, in the *Drosophila* genome. All three share common structural features with other Ctr1 family members that include the presence of three predicted transmembrane domains with conserved hydrophobic sequences, the MX₃M motif in the second transmembrane domain, and a conserved methionine residue preceding the first transmembrane domain that is essential for Ctr1 function in yeast and mammals (17) (Fig. 1). We predicted that the three genes encode putative Ctr1 family copper transporters, and denoted these genes and their encoded proteins Ctr1A, Ctr1B, and Ctr1C. We also detected two apparent splicing isoforms of Ctr1A that differed by 10 amino acids (data not shown), and therefore designated these as Ctr1A-L (Ctr1A long, 217 amino acid residues) and Ctr1A-S (Ctr1A short, 207 amino acid residues).

As an initial test of whether the putative *Drosophila* Ctr1 family members function as high affinity copper transporters, we expressed each cDNA in an *S. cerevisiae* mutant strain to test whether they can suppress the respiratory deficiency that results from defects in high affinity copper uptake (12). As shown in Fig. 2A, the expression of each of the three *Drosophila* Ctr1 cDNAs in yeast can suppress this respiratory deficiency, as indicated by their ability to restore growth on the non-fermentable carbon sources glycerol and ethanol in these cells. To test whether these Ctr1 family members stimulate copper

¹ The abbreviations used are: BCS, bathocuproine disulfonate; ICP-MS, inductively coupled plasma-mass spectrometry; TTM, tetrathiomolybdate; PHM, peptidylglycyl α -amidating monooxygenase.

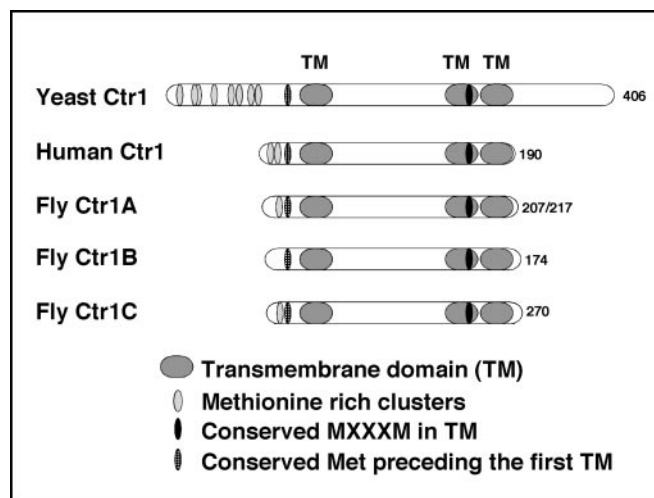


FIG. 1. Overall structure of Ctr1 high affinity copper transport proteins. The structures of the *S. cerevisiae* (Yeast) Ctr1, and human Ctr1, and three Ctr1 family members from *D. melanogaster* (Fly) are shown with amino acid chain lengths indicated. Conserved transmembrane domains (TM) and methionine-rich sequences are indicated.

transport activity in *Drosophila* cells, we expressed them under actin promoter by transfection in S2 cells and ^{64}Cu uptake studies were carried out. As shown in Fig. 2B, expression of either Ctr1A or Ctr1B strongly stimulated copper uptake from the medium. Although no stimulation of copper uptake was observed with a Ctr1C expression vector in S2 cells, it is currently unclear whether the encoded protein is poorly expressed, improperly localized, or naturally resides on the membrane of an intracellular compartment. Taken together, the structural features of the *Drosophila* Ctr1A, Ctr1B, and Ctr1C proteins; their function in yeast; and their ability to stimulate ^{64}Cu uptake in cultured S2 cells strongly suggest that these proteins function in copper transport in *Drosophila*.

The *Drosophila* Ctr1 Family Is Differentially Expressed during Development—To gain insight into the potential roles of the three distinct Ctr1 family members, the expression profile of these genes was assessed during *Drosophila* development. Fertilized eggs from wild type flies were collected and allowed to mature for 4-h intervals before total RNA was extracted. RNA was also extracted from wandering third instar larvae, adults, and cultured S2 cells, and the expression profile for Ctr1A, Ctr1B, and Ctr1C was assessed by RNA blotting. As shown in Fig. 3, the three Ctr1 genes displayed very distinct expression patterns during development. Ctr1A was constitutively expressed at all stages represented in the experiment, with relatively minor variations in early and late embryos, as well as slightly elevated levels in females as compared with male adults. S2 cells, which have an embryonic origin (36), also express high levels of Ctr1A mRNA. Ctr1B was robustly expressed in late embryonic stages and larval stages, but was poorly expressed in early embryonic stages and adults, and not detectable in S2 cells. Ctr1C expression was also found in late larval stages, and interestingly, in adult males, but was not detected at other stages of development or in S2 cells. These observations suggest that the three Ctr1 genes might have distinct roles in copper homeostasis during *Drosophila* development.

Ctr1B Expression Is Regulated by Dietary Copper Availability—Because Ctr1B was most strongly expressed in the larval stages of development, when flies absorb most of their nutrients and build body mass, we reasoned that Ctr1B might play a major role in copper uptake in early stages of the *Drosophila* life cycle and therefore further characterized this gene in de-

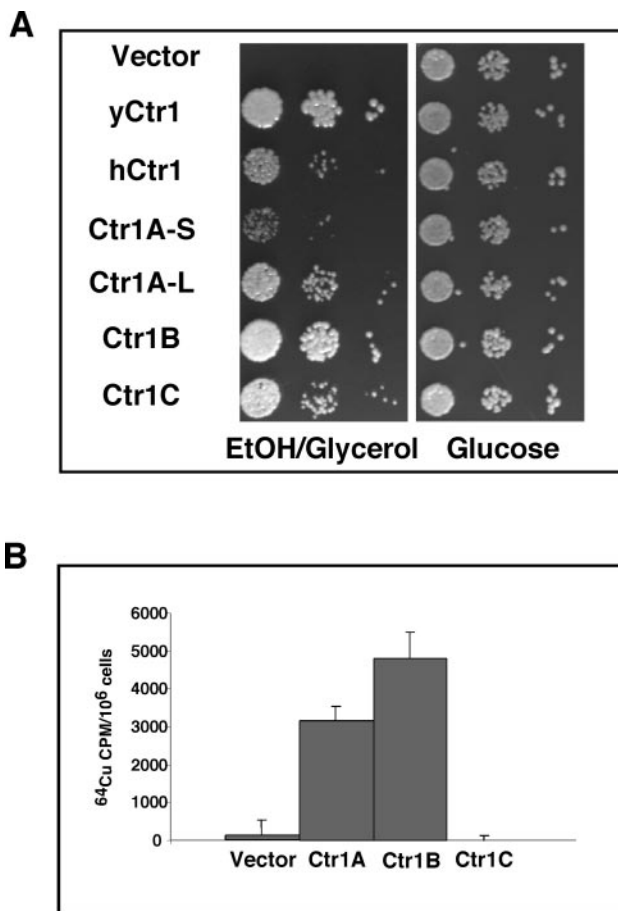


FIG. 2. *Drosophila* Ctr1 proteins function in copper accumulation. A, complementation of *S. cerevisiae* mutants defective in copper transport by *Drosophila* Ctr1 family members. The *Drosophila* Ctr1 cDNAs were subcloned into the yeast expression vector p413GPD and transformed into strain MPY17 (*ctr1Δctr3Δ*), with *S. cerevisiae* Ctr1 (*yCtr1*) and human Ctr1 (*hCtr1*) as positive controls and the p413GPD vector as a negative control. Transformants were diluted in 10-fold serial dilutions and spotted onto either SC-His (Glucose) or YPEG (EtOH/Glycerol) plates. Pictures were taken after incubation for 2 days on SC-His or 4 days on YPEG at 30 °C. B, ^{64}Cu uptake by *Drosophila* S2 cells transfected with Ctr1 family cDNA expression vectors. S2 cells were transfected with the empty vector pAC5.1A (vector) or with the same vector in which the Ctr1A, Ctr1B, or Ctr1C cDNAs had been inserted. Transfected cells were incubated with 10 μM $^{64}\text{CuCl}_2$ for 10 min at room temperature, and ^{64}Cu uptake was quantitated. The error bars indicate standard deviation from two independent experiments.

tail. Although Ctr1 family members share common structural and functional features across species, there is an important difference among organisms in the regulation of Ctr1 gene expression. Genes encoding Ctr1 family members in bakers' yeast and fission yeast are transcriptionally regulated by copper availability and by copper metalloregulatory transcription factors; expression of Ctr1 mRNA is high during copper scarcity and low under conditions of copper adequacy (29, 35, 37, 38). In contrast, studies in cultured mammalian cells or whole animals thus far indicate no significant changes in Ctr1 mRNA as a function of copper status, but rather copper levels regulate changes in Ctr1 trafficking and stability (15, 39). To determine whether *Drosophila* Ctr1A, Ctr1B or Ctr1C mRNA expression is regulated by dietary copper availability, wild type larvae were raised on food supplemented with either 100 μM CuSO_4 or 10 μM of the Cu(I)-specific chelator BCS, RNA was extracted and analyzed by RNA blotting. Ctr1A and Ctr1C mRNA levels were not detectably altered in response to the changes in copper availability imposed by these conditions (data not shown). However, as shown in Fig. 4A (lanes +/+), *Drosophila* Ctr1B

FIG. 3. The expression pattern of *Drosophila* Ctr1 gene family members during development. Eggs from wild type flies were collected at 4-h intervals and allowed to develop for the indicated times (h) before total RNA was extracted. RNA from wandering third instar larvae, female (F) and male (M) adults, and cultured S2 cells was extracted, and RNA blotting experiments were performed using the Ctr1A, Ctr1B, and Ctr1C cDNAs as ^{32}P -radiolabeled probes. Ribosomal RNA (rRNA) was stained with ethidium bromide. mRNA species corresponding to the Ctr1 and rRNA species are indicated with arrows.

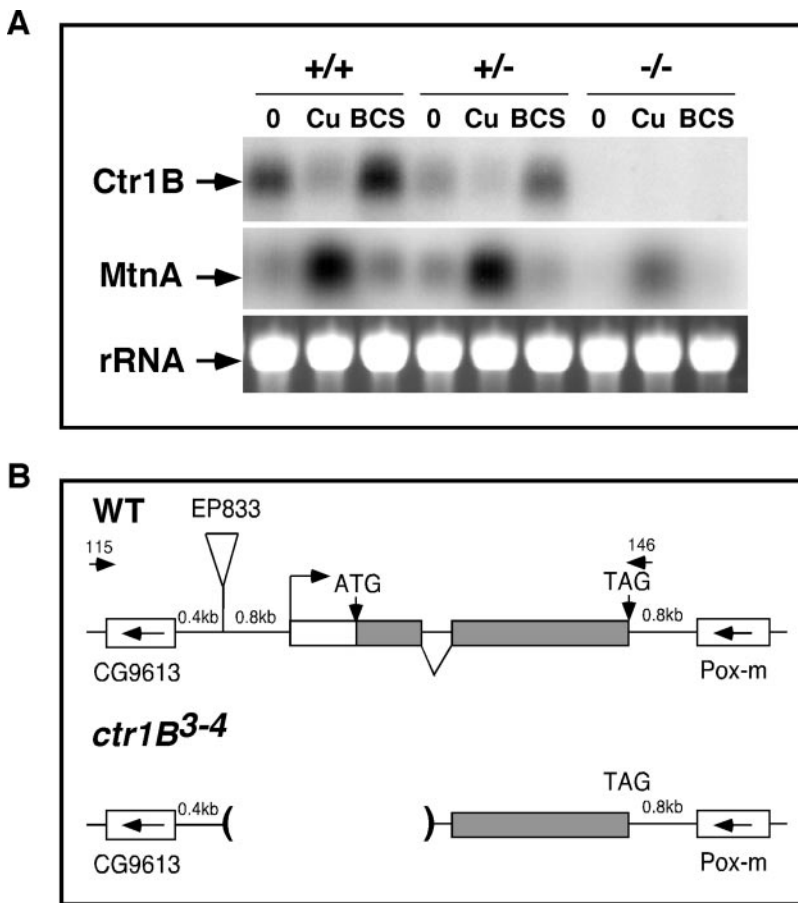
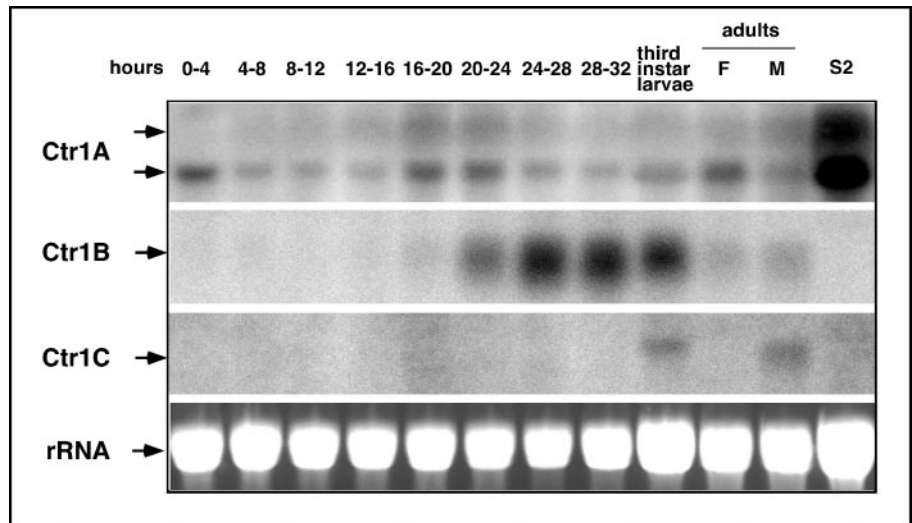


FIG. 4. Generation and analysis of *Drosophila* Ctr1B knock outs. A, the regulation of Ctr1B mRNA levels by copper limitation or excess. Third instar larvae before wandering were collected from animals reared on normal food (0), food supplemented with $100\ \mu\text{M}$ CuSO_4 or with $10\ \mu\text{M}$ BCS, and total RNA was extracted. RNA blotting experiments were performed using the Ctr1B and MtnA cDNAs as ^{32}P -labeled probes. The position of the Ctr1B, MtnA, and ribosomal RNA (rRNA, stained with ethidium bromide) are indicated with arrows. B, Ctr1B genomic deletion allele generated by imprecise excision of the P-element EP(3)833. The exons of Ctr1B and nearby genes are shown as boxes, with the open reading frame of Ctr1B shaded. The Ctr1B transcription start site (arrow line), translation start codon (ATG), and stop codon (TAG) and the position of the EP(3)833 P-element, prior to excision, are also shown. The locations of PCR primers used to screen for imprecise excision events are shown with small arrows. The deleted sequence in the *ctr1B*³⁻⁴ allele is shown. The Ctr1B alleles are not drawn to scale. WT, wild type.

steady state mRNA levels were strongly regulated by dietary copper availability. When wild type larvae were grown on food supplemented with $100\ \mu\text{M}$ CuSO_4 Ctr1B mRNA levels were significantly reduced as compared with larvae raised on normal food. Conversely, the inclusion of BCS in fly food, to limit copper availability by chelation, resulted in a significant elevation in Ctr1B steady state mRNA levels. As a control for the imposed changes in copper availability in food, steady state mRNA levels for a metallothionein isoform encoded by the MtnA gene were assessed. Metallothionein A (MtnA) is one of four metallothionein isoform genes in flies that bind copper and other metals and protect flies from metal toxicity (40, 41). These four genes are transcriptionally induced by elevated

copper levels and the metalloregulatory transcription factor MTF1 (41, 42). As shown in Fig. 4A, MtnA mRNA expression follows a pattern that is opposite to that of Ctr1B, with MtnA being strongly induced by exogenous copper, but not induced by copper limitation. Taken together, these results demonstrate that the Ctr1B member of the high affinity copper transporter family is regulated by dietary copper availability in a manner consistent with a predicted role in copper acquisition in *Drosophila*.

Generation of Ctr1B Mutants—To investigate the physiological function of Ctr1B, a Ctr1B loss-of-function mutation was generated in flies by imprecise excision of a P element transposon adjacent to the Ctr1B locus. We performed 5' rapid

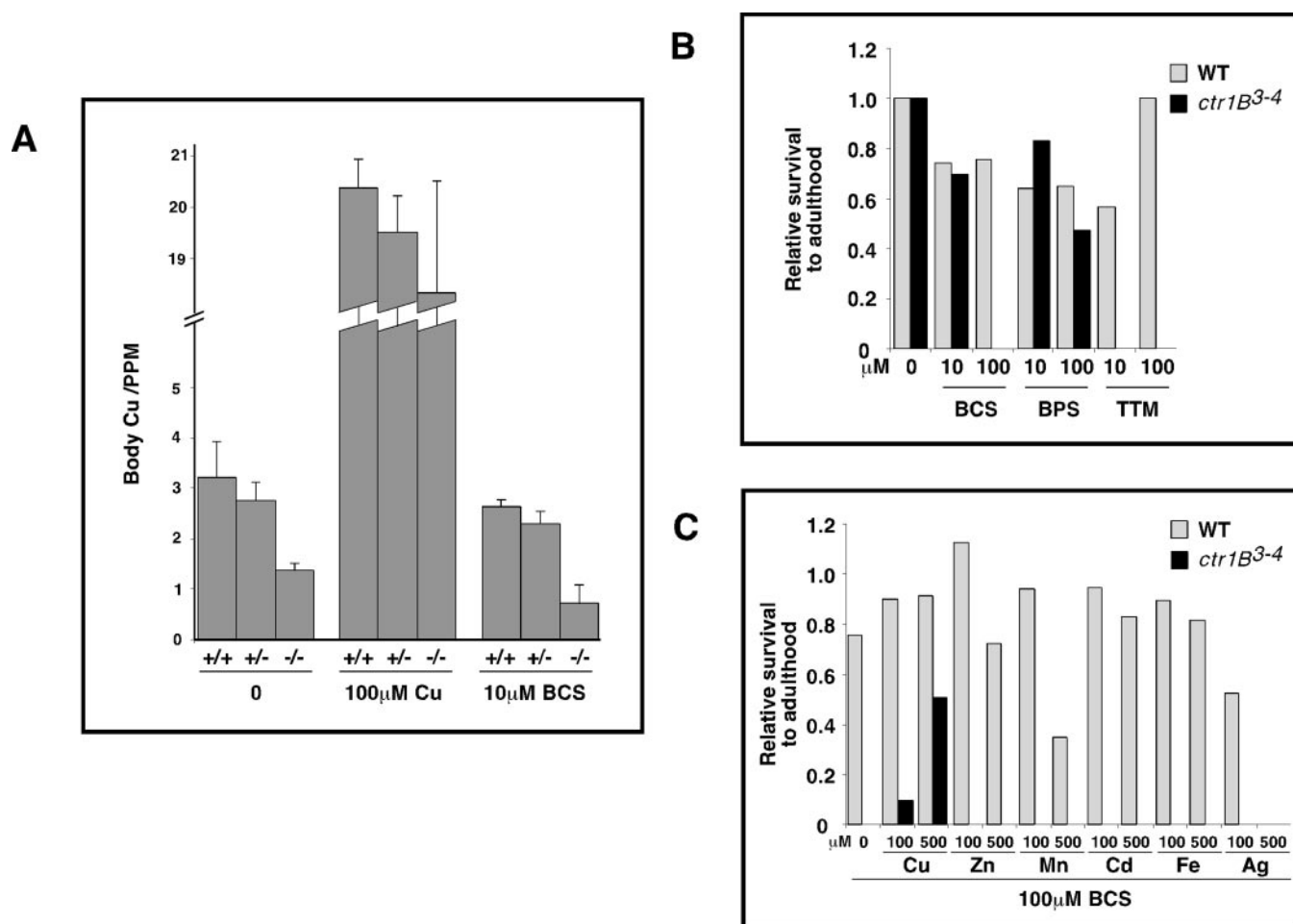


FIG. 5. Copper accumulation and adult development in wild type *Drosophila* and *Ctr1B* mutants. A, *Ctr1B* null flies exhibit copper accumulation defects. *Drosophila* third instar larvae were reared on the indicated growth medium and collected, and total body copper levels were measured by ICP-MS and normalized to body weight. The error bars indicate the standard deviation of three independent measurements. B, defective development of *ctr1B*³⁻⁴ flies under conditions of copper limitation. *ctr1B*³⁻⁴/TM6 and +/TM6 flies were self-crossed, and progeny were propagated on food supplemented with the indicated concentrations (in μ M) of BCS, bathophenanthroline disulfonate, or TTM. Relative development to adulthood was calculated as described under "Experimental Procedures." C, specific limitation of copper availability underlies the *ctr1B*³⁻⁴ terminal development phenotype. Fly crosses and relative development calculations were carried out as described in B. The indicated concentrations (in μ M) of copper, zinc, manganese, cadmium, iron, or silver were added in the presence of 100 μ M BCS. These data are representative of two independent experiments. WT, wild type.

amplification of cDNA ends to determine the *Ctr1B* transcription start site and found that *Ctr1B* transcription initiates ~20 bp further upstream than was predicted at www.flybase.org (data not shown). Using a fly strain harboring a P-element upstream of the *Ctr1B* initiation site (32), flies were generated and selected in which imprecise excision events eliminated the P-element and variable lengths of downstream flanking sequences, without perturbation of sequences upstream of the P-element. One such allele, *ctr1B*³⁻⁴, suffered a deletion of ~1.2 kb of chromosomal sequence, which removed the entire first exon of the *Ctr1B* gene, including the transcription start site and the ATG start codon (Fig. 4B). To verify the loss of *Ctr1B* expression in this background, RNA blotting experiments were carried out on total RNA isolated from homozygous *ctr1B*³⁻⁴ third instar larvae. As shown in Fig. 4A, homozygous *ctr1B*³⁻⁴ larvae (-/-) expressed no detectable *Ctr1B* RNA under any growth condition, whereas *ctr1B*³⁻⁴ heterozygous larvae (+/-) expressed less than the wild type under all conditions of growth. Isogenic flies harboring an allele with a precise P-element excision expressed *Ctr1B* mRNA at levels that were indistinguishable from the wild type under all conditions analyzed (data not shown). Because the precise excision line is isogenic to the *ctr1B*³⁻⁴ mutant, we used this line as the wild type control for further analysis.

Defective Copper Homeostasis in *Ctr1B* Mutant Larvae—The analysis of *MtnA* expression, as a control for *Ctr1B* regulation by copper, demonstrated that the expression level of *MtnA* in homozygous *ctr1B*³⁻⁴ flies was significantly lower under all conditions (Fig. 4A). Because *MtnA* expression is tightly regulated by the *Drosophila* MTF1 metalloregulatory transcription factor and copper availability (41, 42), the failure to robustly induce *MtnA* by copper in *ctr1B*³⁻⁴ larvae would be consistent with a copper acquisition defect. To test this hypothesis, total larval-associated copper was measured for wild type, *ctr1B*³⁻⁴ heterozygous, and *ctr1B*³⁻⁴ homozygous larvae that were reared on standard fly food, or food supplemented with 100 μ M copper or 10 μ M BCS. As shown in Fig. 5A, when reared on standard fly food, *ctr1B*³⁻⁴ homozygous larvae accumulated ~40% of the copper that was associated with either wild type or *ctr1B*³⁻⁴ heterozygous flies. When *ctr1B*³⁻⁴ homozygous larvae were propagated on food with 10 μ M BCS, conditions under which wild type larvae have induced *Ctr1B* mRNA levels, the difference was exacerbated, with wild type larvae possessing nearly 4 times the total copper as *ctr1B*³⁻⁴ homozygous larvae. Heterozygous *ctr1B*³⁻⁴ larvae displayed only a modest defect in copper accumulation under these conditions, and there were no significant differences in copper accumulation for either of the three *Ctr1B* genotypes when 100 μ M copper was added to the

fly food. These observations support the hypothesis that Ctr1B is a physiologically significant copper transporter that plays an important role in dietary copper acquisition by *Drosophila* larvae during development when copper is present at limiting concentrations.

To ascertain whether deletion of Ctr1B, and the resultant larval copper accumulation defect would affect the progression of flies through development, *ctr1B³⁻⁴* heterozygous flies were self-crossed and allowed to lay eggs on standard growth medium, or in the presence of metal chelators, metal supplements or both. Relative frequencies of development to adulthood were determined in parallel with same measurements for isogenic homozygous wild type F1 adults on the same growth medium. As shown in Fig. 5B, the frequency of development to adulthood of *ctr1B³⁻⁴* homozygous flies decreased dramatically as the copper availability in food decreased as a result of the presence of the copper chelator BCS. The addition of 100 μM BCS to the medium eliminated the emergence of *ctr1B³⁻⁴* homozygous adults, whereas wild type flies under the same conditions developed to adults with a frequency comparable to those on normal food. The same arrest of development was also observed with the heterozygous animals between *ctr1B³⁻⁴* and a deficiency line Df(3R) p40, which removes the entire Ctr1B genomic region, indicating that the effect was not the result of genetic background. Visual inspection of their terminal developmental phenotype demonstrated that the *ctr1B³⁻⁴* homozygotes were arrested as second instar larvae, as determined by the appearance of diagnostic larval mouth hook features (data not shown) (33). Furthermore, these second instar larvae failed to burrow into the food, in contrast to what was observed for wild type larvae or for the *ctr1B³⁻⁴* homozygous larvae on normal food. Whether the inability to burrow as a result of motility defects was the cause of arrested development, or this was simply a general sign of immobility or weakness caused by copper deprivation, is not yet clear. To test the specificity of this BCS effect, another copper-specific chelator tetrathiomolybdate (TTM), which is membrane-permeable, was used. The inclusion of TTM had a more potent effect on the developmental arrest of *ctr1B³⁻⁴* homozygotes, as no homozygous *ctr1B³⁻⁴* adults were found on food supplemented with only 10 μM TTM (Fig. 5B). In contrast, an Fe^{2+} chelator, bathophenanthroline disulfonate, had no effect on the arrest of *ctr1B³⁻⁴* homozygotes when added to medium at a concentration of 10 μM and only a slight decrease in the development of *ctr1B³⁻⁴* homozygotes at 100 μM . The sensitivity of *ctr1B³⁻⁴* homozygotes to two different copper chelators, but not to an iron chelator, strongly suggests that copper limitation during development was the cause of lethality.

We next examined whether the addition of copper could rescue the developmental defect of *ctr1B³⁻⁴* homozygotes on food supplemented with BCS. Indeed, when grown on food supplemented with 100 μM BCS and either 100 or 500 μM copper, ~10 and 50% of the *ctr1B³⁻⁴* homozygotes reached adulthood, respectively (Fig. 5C). Although these concentrations of exogenous copper did not completely rescue the developmental arrest for all larvae, the majority of the *ctr1B³⁻⁴* homozygotes on the 100 μM BCS and 500 μM copper food progressed beyond the early lethality stage of second instar larvae that was observed as the terminal phenotype on the 100 μM BCS-only food. Indeed, most of the *ctr1B³⁻⁴* homozygotes propagated in 100 μM BCS plus 500 μM copper medium reached at least to late pupal stages. The failure to uniformly develop to adulthood could reflect toxicity by high concentrations of copper (see below). To further ascertain the specificity of copper for remediation of this phenotype, the same concentrations of other metals were added to the BCS-supplemented food and

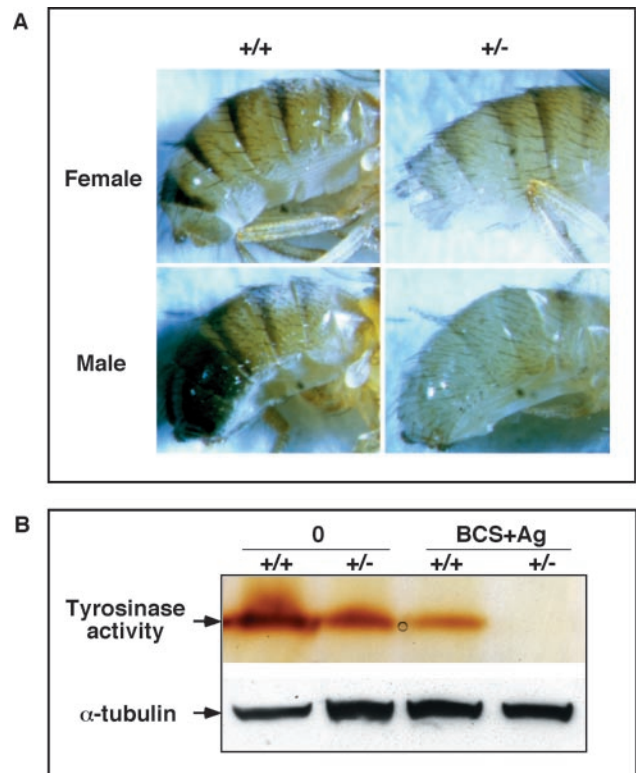


FIG. 6. *Drosophila* Ctr1B mutants exhibit body pigmentation and tyrosinase activity defects. A, male and female *ctr1B³⁻⁴* heterozygous flies (+/-) exhibit body pigmentation defects when propagated on food supplemented with 100 μM BCS and 100 μM AgNO_3 to impose copper limiting conditions. Wild type (+/+) and *ctr1B³⁻⁴* heterozygous (+/-) flies are shown. B, *ctr1B³⁻⁴* heterozygous flies possess lower tyrosinase activity under conditions of copper limitation. In-gel tyrosinase assays were performed on total protein extracts from late pupae derived from wild type and *ctr1B³⁻⁴* heterozygotes, reared on food in the absence or presence of 100 μM BCS and AgNO_3 . Tyrosinase activity stains are indicated and immunoblots blots with anti- α -tubulin antibody are shown for loading controls.

development to adulthood ascertained as above. As shown in Fig. 5C, none of the other metals tested, including zinc, manganese, cadmium, iron, and silver, at either concentration, could rescue the developmental arrest of *ctr1B³⁻⁴* homozygotes on 100 μM BCS-supplemented food. Taken together these data strongly support the conclusion that the phenotype of *ctr1B³⁻⁴* homozygous flies is the result of a copper-specific deficiency that has early and severe consequences for normal development.

ctr1B³⁻⁴ Flies Exhibit Defects in Body Pigmentation and Tyrosinase Activity—*ctr1B³⁻⁴* heterozygous flies had normal viability, morphology and copper accumulation under all of the conditions tested above, and their viability, fertility, and morphology remained normal after three generations on food supplemented with 100 μM BCS (data not shown). However, when these larvae were propagated on normal food supplemented with 100 μM BCS and 100 μM AgNO_3 , dramatic defects in body pigmentation were evident in newly emerged *ctr1B³⁻⁴* heterozygous adults, but not in wild type adults propagated on the same medium (Fig. 6A). Ag(I) is isoelectronic to and a known competitor of Cu(I) , which can compete for copper transport by Ctr1 from yeast and mammals, as well as copper uptake by the P-type ATPases of prokaryotes (43, 44). The inclusion of both silver and BCS is predicted to severely reduce dietary copper availability. Although *ctr1B³⁻⁴* homozygous flies exhibited a developmental arrest on this food (Fig. 5C), the *ctr1B³⁻⁴* heterozygotes reached adulthood but possessed pigmentation defects after emergence from the pupal case, which was most

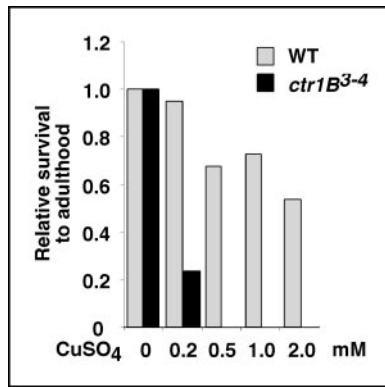


FIG. 7. **Ctr1B mutants are sensitive to elevated copper levels.** *ctr1B³⁻⁴/TM6* and *+TM6* flies were self-crossed, and progeny were propagated on food supplemented with the indicated concentrations of CuSO₄ in mM. Relative development to adulthood was calculated as described under "Experimental Procedures."

obvious in the abdominal region. However, within a day after emergence on this media, the *ctr1B³⁻⁴* heterozygous flies were virtually indistinguishable from wild type flies with respect to body pigmentation (data not shown).

Drosophila cuticle melanization is a multistep process, in which the copper-requiring enzyme tyrosinase is involved in early steps of tyrosine hydroxylation (6). We examined tyrosinase activity from *ctr1B³⁻⁴* heterozygotes under normal or copper-deficient conditions, to test whether the delayed pigmentation phenotype paralleled defects in tyrosinase activity. As shown in Fig. 6B, although there was little apparent difference in tyrosinase activity under normal conditions between wild type and *ctr1B³⁻⁴* heterozygotes, the tyrosinase activity of *ctr1B³⁻⁴* heterozygotes was undetectable by this assay in extracts from pupae grown on food supplemented with 100 μ M silver plus 100 μ M BCS, whereas the tyrosinase activity of wild type pupae remained strong. Tyrosinase activity was also assayed in adults and no significant differences between wild type and *ctr1B³⁻⁴* heterozygotes was observed for flies grown on food supplemented with 100 μ M silver plus 100 μ M BCS (data not shown).

ctr1B³⁻⁴ Mutants Are Hypersensitive to Copper Toxicity—Consistent with the developmental expression pattern for Ctr1B, the data presented here support an important role for Ctr1B in larval copper uptake and in development. Because yeast cells defective for high affinity copper uptake are also more resistant to challenge with environmental copper (20), we tested whether deletion of Ctr1B would result in changes in resistance to high dietary copper concentrations. As shown in Fig. 7, wild type larvae develop to adulthood on food supplemented with as high as 2 mM copper, albeit with a lower overall frequency as compared with normal medium. Surprisingly, *ctr1B³⁻⁴* homozygotes exhibited dramatically lower tolerance to copper, with \sim 25% adult development at 100 μ M CuSO₄ and no adults emerging with 500 μ M copper added to the food. Although *ctr1B³⁻⁴* homozygotes exhibited a developmental arrest under both low copper (Fig. 5B) and high copper conditions (Fig. 7), the developmental stages at which the terminal phenotype was evident were different for these distinct conditions. When propagated and maintained on food supplemented with BCS to limit copper availability, *ctr1B³⁻⁴* homozygous larvae terminated development at an early second instar larval stage. On food supplemented with additional copper, *ctr1B³⁻⁴* homozygous larvae survived through pupation, with most arresting at a late pupal stage with visible folded wings. These observations suggest that the arrest as a result of high copper might be a consequence of stage-specific targets of copper tox-

icity or to accumulated copper toxicity. The observations that Ctr1B mutants are sensitive to both low and high copper concentrations suggest a complex role for Ctr1B in *Drosophila* copper homeostasis and perhaps multiple modes of Ctr1B function or regulation.

DISCUSSION

Studies in yeast have demonstrated that the Ctr1 protein is required for delivery of copper to well characterized cuproenzymes in the cytosol, mitochondria, and the secretory pathway, and that defects in these enzymes lead to characteristic and predictable growth phenotypes in this eukaryotic microbe (1). Mice bearing a homozygous deletion of the Ctr1 gene suffer severe growth and developmental defects and fail to survive beyond mid-gestational development (21, 22). Given this dramatic phenotype, and the inability to rescue these mice by providing supplemental dietary copper, it is difficult to assess the precise biochemical mechanisms of copper delivery by Ctr1 in development. We identified and isolated the cDNAs for three Ctr1 family members from the *Drosophila* genome, with all three sharing common conserved features with other Ctr1 family members. Furthermore, all three Ctr1 family members functionally complement the respiratory deficiency growth phenotypes associated with yeast mutants defective in high affinity copper transport. Moreover, the expression of Ctr1A or Ctr1B, but not Ctr1C in *Drosophila* S2 cells, stimulated ⁶⁴Cu uptake under high affinity transport conditions. These structural and functional similarities, taken together, suggest that *Drosophila*, like *Arabidopsis* (45), is distinct from mammals in their possession of multiple Ctr1 family members (1).

The results of gene expression and gene inactivation experiments presented here demonstrate that the three *Drosophila* Ctr1 family members are not functionally redundant. Although Ctr1A was found to be expressed at essentially all stages of development and in adulthood, Ctr1B was specifically expressed in late embryonic and in larval stages, during which flies take up most of their nutrients (33). Furthermore, Ctr1B expression in larvae is strongly regulated by copper availability. However, although Ctr1B null mutants were viable and possessed no obvious morphological defects when propagated under standard conditions, these flies suffered severe developmental defects when copper was present in limiting amounts or in excess of needs. How can we reconcile the need for Ctr1B only under defined sets of conditions during development? As demonstrated here, the *Drosophila* genome encodes two other distinct copper transporters, Ctr1A and Ctr1C. The transport activity of these two Ctr1 proteins could provide the copper needed for fly development under conditions of copper sufficiency and might also account for the residual copper accumulation observed in Ctr1B null larvae. Based on the constitutive expression pattern of Ctr1A during development, and the larval specific and copper-regulated expression pattern of Ctr1B, the two copper transporters could play different roles in *Drosophila* copper uptake. Ctr1A may function as a key copper transporter for *Drosophila* under conditions where copper is not scarce and where the organism is not engaged in rapid growth and development. When there is an increased need for copper, such as during the rapid growth phase in the larval stages, or when environmental copper availability is low, the expression of Ctr1B may be induced and serve as the major copper transporter. Whether Ctr1A and Ctr1B provide copper to the same targets in the same manner, or whether they provide copper to distinct targets during fly development, is currently unclear.

Importantly, the conditional progression through development of Ctr1B null flies allows us to further explore the role of Ctr1 in copper acquisition and homeostasis in development.

Although the embryonic lethality of Ctr1 knock-out mice could not be rescued by dietary copper supplementation, we were able to rescue the developmental arrest of Ctr1B null flies that was manifest under conditions of copper limitation by the addition of copper, but not other metals. This strongly supports the notion that the developmental defect is the result of the copper transporting activity of Ctr1B, rather than an as yet uncharacterized function of the protein. We also observed a dramatic defect in the abdominal melanization for heterozygous *ctr1B³⁻⁴* flies, which parallels decreased tyrosinase activity, providing an opportunity for future studies aimed at a more detailed understanding of the importance of this, and other copper-dependent enzymes during metazoan development.

Interestingly, *Drosophila* shares copper transporter gene regulation with both yeasts and mammals. Like mammalian Ctr1, the *Drosophila* Ctr1A gene is constitutively expressed (15). Ctr1B, mRNA levels of which are regulated by copper availability, is more similar to the bakers' yeast and fission yeast Ctr1 family copper transporters, which are regulated by copper and the Mac1/Cuf1 transcription factors, respectively (29, 35, 37, 38). The regulation of Ctr1B mRNA levels by copper also prompts the question of whether this regulation occurs at the transcriptional or post-transcriptional level and if the former, which transcription factor senses and responds to changes in copper levels. Although there is no obvious Mac1/Cuf1 homolog found in the *Drosophila* genome, the activation of metallothionein gene transcription in flies is highly responsive to copper and cadmium (also see Fig. 4B), and dependent on the MTF-1 transcription factor (41, 42). Interestingly, several copies of metal-responsive elements, which are bound by MTF-1 for MtnA transcription induction, are found in the Ctr1B promoter upstream of the Ctr1B transcription start site (data not shown). Moreover, recent studies have shown that MTF-1 knock-out flies exhibit very similar phenotypes to Ctr1B null flies including sensitivity to both high and low copper in food (41). Based on the similarity between MTF1 and Ctr1B null flies, and the regulation of Ctr1B expression by copper, it is possible that MTF-1 is the copper-responsive transcription factor that regulates Ctr1B expression, and the sensitivity of MTF-1 knock-out flies to copper deprivation is caused, at least partially, by the loss of regulation of Ctr1B. Alternatively, the changes in Ctr1B mRNA levels in response to copper could also be caused by a copper-modulated change in mRNA stability.

A puzzling question remains as to why Ctr1B null flies exhibit profound developmental sensitivity to both copper scarcity and copper excess. Neuropeptide maturation, a copper-dependent process carried out by peptidylglycyl α -amidating monooxygenase (PHM), is essential for the activity of the majority of neuropeptides in *Drosophila* and for the normal function of the nervous, endocrine, and other systems (7, 46). Mutation of the *Drosophila* PHM gene has been shown to be a lethal event that results in death as very late embryos or early larvae and, moreover, that PHM is required for peptide amidation throughout the lifespan of flies (26, 27). Furthermore, pigmentation, and the melanization and sclerotization of the cuticle, are important processes for *Drosophila* development that are carried out in part by the copper-dependent tyrosinase enzymes (6). Although it is currently unclear which of these or other copper-dependent processes may lead to the specific developmental arrest observed for Ctr1B null mutants under conditions of copper deprivation, a clear diminution of tyrosinase activity, demonstrated here, is likely to play a role in defective pigmentation and perhaps development.

It is currently unclear why Ctr1B null flies are also sensitive to high copper levels. One clue may lie in the observation that, although Ctr1B null homozygotes arrest early in the second

instar larvae stage on low copper, on high copper-containing food they progress to a late pupal stage. Perhaps this is the result of the accumulation of copper to levels that are ultimately toxic, thereby invoking a role for Ctr1B in both copper acquisition and copper detoxification. This would be reminiscent of the Menkes (ATP7A) and Wilson (ATP7B) copper-transporting P-type ATPases in humans, which play roles both in copper loading within the secretory pathway and in copper excretion across the basolateral membrane of the intestinal epithelium or into the portal circulation, respectively (47). Mutations in either gene result in both copper deficiency defects, and in copper overload in specific tissues. Under conditions of low copper these proteins are localized to membranes of the secretory compartment, whereas under conditions of high copper, ATP7A and ATP7B are trafficked to the plasma membrane and intracellular vesicles, respectively (48, 49). How could Ctr1B function in this regard? Several models could be envisioned. First, upon high copper challenge, Ctr1B could change its localization from the plasma membrane to an intracellular vesicle, examples of which have been shown to store and detoxify copper, as in the case of vacuoles in yeast, or lysosomes in flies (25, 50). Thus, Ctr1B could serve both as a plasma membrane copper uptake transporter and an intracellular copper detoxification protein that functions in compartmentalization. Consistent with this possibility, studies of both bakers' yeast Ctr1 and human Ctr1 demonstrate that high copper levels stimulate the endocytosis of these proteins from the plasma membrane (39, 51). Alternatively, Ctr1B might function within a copper detoxification organ in flies to internalize and therefore sequester excess copper. Early studies in flies demonstrated that the mesenteron and Malpighian tubule epithelial cells accumulate high levels of copper when flies were chronically intoxicated with the antifungal agent Bordeaux mixture, which contains high concentrations of CuSO_4 (52). It is also possible that, because we have shown that Ctr1B null larvae mount a weak transcriptional induction of the MtnA metallothionein, an important copper detoxification gene, this could lead to a copper toxicity phenotype. Perhaps copper imported by Ctr1B is directed to a regulatory pool for the activation of MTF1. Our future investigations will explore the specific copper-dependent targets required for *Drosophila* development, the specific roles of the Ctr1 family members, and potential interactions between MTF1 and Ctr1B in copper-responsive gene expression and in fly copper homeostasis.

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