A Drosophila model of Menkes disease reveals a role for DmATP7 in copper absorption and neurodevelopment

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SUMMARY

Human Menkes disease is a lethal neurodegenerative disorder of copper metabolism that is caused by mutations in the ATP7A copper-transporting gene. In the present study, we attempted to construct a Drosophila model of Menkes disease by RNA interference (RNAi)-induced silencing of DmATP7, the Drosophila orthologue of mammalian ATP7A, in the digestive tract. Here, we show that a lowered level of DmATP7 mRNA in the digestive tract results in a reduced copper content in the head and the rest of the body of surviving adults, presumably owing to copper entrapment in the gut. Similar to Menkes patients, a majority of flies exhibit an impaired neurological development during metamorphosis and die before eclosion. In addition, we show that survival to the adult stage is highly dependent on the copper content of the food and that overexpression of the copper homeostasis gene, metal-responsive transcription factor-1 (MTF-1), enhances survival to the adulthood stage. Taken together, these results highlight the role of DmATP7-mediated copper uptake in the neurodevelopment of Drosophila melanogaster and provide a framework for the analysis of potential gene interactions influencing Menkes disease.

INTRODUCTION

The trace metal copper is an essential nutrient that is absorbed mainly by the small intestine into the bloodstream and other tissues, where it acts as a cofactor for a number of key metabolic enzymes (Nelson, 1999; Phillips and Hilliker, 1990). Although essential for life, copper can be very toxic at high concentrations and can promote the production of highly reactive hydroxyl radicals through the Haber-Weiss/Fenton reaction (Phillips and Hilliker, 1990). Therefore, heavy metal homeostasis needs to be regulated tightly to ensure an adequate supply of metal ions for normal cellular metabolism while protecting cells from their toxic effects (Williams et al., 2000). In humans, the primary copper exporters are the ATP7B and ATP7A copper-transporting ATPases. In Wilson’s disease, mutations in the ATP7B gene are associated with copper accumulation in the liver and the brain, with subsequent development of progressive hepatic and neurological abnormalities (Lutsenko et al., 2007; Pfeiffer, 2007). Menkes disease and occipital horn syndrome (OHS) are allelic X-linked recessive disorders of copper deficiency that are caused by mutations in the ATP7A copper-transporting gene (Cox and Moore, 2002; de Bie et al., 2007; Kaler et al., 1994; Levinson et al., 1996). The ATP7A copper-transporting protein is located in the trans-Golgi network, where it delivers copper to the cellular secretory compartments for incorporation into various copper-dependent enzymes; in the presence of excess copper, ATP7A traffics to the plasma membrane and regulates copper homeostasis by exporting excess copper across the cellular membrane (Harris, 2000; Lutsenko et al., 2007; Kim and Petris, 2007; Koskoboinik and Camakaris, 2002). These properties of ATP7A are required for copper efflux from intestinal cells and, as a consequence, mutations in the ATP7A gene can disrupt copper release from the intestinal tract into the blood and restrict its supply to other tissues, particularly the brain (Harris, 2000; Kodama et al., 1999; Lutsenko et al., 2007). Thus, Wilson’s and Menkes disorders are opposite pathologies, in that the former disease is associated with copper accumulation, whereas the latter is associated with profound copper deficiency.

Menkes disease patients display severe developmental, neurological and connective tissue abnormalities along with various other symptoms that are associated with a decreased function of copper-dependent enzymes (Lutsenko et al., 2007; Tomita et al., 1992). Patients with OHS, a milder form of Menkes disease, also exhibit connective tissue impairments but are spared of severe neurological abnormalities, presumably owing to residual expression of functional ATP7A (Das et al., 1995; Mercier, 1998; Möller et al., 2000; Tang et al., 2006; Tsukahara et al., 1994). The mottled mutant mice are a series of copper-deficient mice that possess mutations in the murine homologue of the Menkes disease gene; these mutants display a diversity of phenotypic severities ranging from prenatal lethality to an adult viable phenotype (Kim and Petris, 2007; La Fontaine et al., 1999; Llanos et al., 2006). The mottled-brindled (MoB) mutant mouse is the closest animal model to human Menkes disease and has been used widely for copper therapy and gene interaction studies (Grimes et al., 1997; Kelly and Palmeter, 1996). Similar to Menkes patients, the MoB copper-deficient mice exhibit profound neurological abnormalities and die early after birth. However, blotchy (MoB) is a less severe allele of mottled that is characterized mainly by connective tissue defects, akin to human OHS (Mercier, 1998).

During the last decade, studies on mottled mutant mice have greatly advanced our understanding of the mechanisms of Menkes disease and OHS pathogenesis. Studies in mice, however, can be costly and time consuming. In the present study, we have constructed a Drosophila model of Menkes disease by conditional silencing of DmATP7 (Southon et al., 2004), the sole Drosophila orthologue of the mammalian ATP7A and ATP7B genes, in the gut. An earlier study has shown that DmATP7 is required for normal development and adult pigmentation, presumably by facilitating...
copper uptake from the diet (Norgate et al., 2006). Here, we show that RNA interference (RNAi)-induced silencing of DmATP7 in the gut alone inhibits copper absorption from the diet and, consequently, induces abnormal phenotypes that are similar to those of Menkes patients and Mobm mice. This invertebrate model provides a genetic complement to Mobm mice and, given that genetic studies in flies are simpler and less time consuming than in mice, should facilitate our understanding of gene interactions in Menkes disease.

RESULTS

RNAi suppresses DmATP7 in the larval and adult gut, and increases pre-adult mortality

In an attempt to construct a fruit fly model of Menkes disease and to study the role of DmATP7 in copper absorption, we silenced the expression of this gene specifically in the gut using the yeast GAL4-UAS expression system (Brand and Perrimon, 1993) and the Sym-pLIAST-DmATP7 RNAi-silencing construct (Giordano et al., 2002). The Sym-pLIAST-DmATP7 transgene that we used in these experiments was constructed by cloning a segment of DmATP7 cDNA into the Sym-pUAST transformation vector, which consists of two convergent UAS enhancers for GAL4-dependent production of sense-antisense double-stranded RNA and consequent activation of RNAi machinery (Fig. 1A).

Quantitative real-time PCR shows that GAL4-induced expression of Sym-pUAST-DmATP7 in the gut lowers DmATP7 mRNA levels in both adults and larvae (Fig. 1B). To determine the lethal effects of DmATP7 silencing in the gut, we measured survival into adulthood by dividing the number of eclosing flies by the total number of pupae in each container. As Fig. 2A illustrates, silencing of DmATP7 in the digestive tract enhanced lethality throughout pupation, mainly at about stage P12 of metamorphosis (Bainbridge and Bownes, 1981). Given that about 50% of the affected flies survive to adulthood, it would appear that the RNAi silencing results in an expression level that is very near the threshold for normal development.

Suppression of DmATP7 in the gut lowers whole-body copper content

A previous study on DmATP7 loss-of-function mutant larvae suggested that DmATP7 knockout can enhance copper

![Fig. 1. RNAi-mediated silencing of DmATP7 in the digestive tract.](image)

(A) Schematic diagram of the Sym-pUAST-DmATP7 RNAi construct regulated by two convergent UAS enhancers; GAL4-induced activation of the UAS enhancers results in production of sense-antisense double-stranded RNA and subsequent activation of the RNAi pathway. (B) Quantitative real-time PCR of RNA isolated from the digestive tract; expression of the Sym-pUAST-DmATP7 transgene under the control of the gut-specific 2020-GAL4 driver dramatically reduced DmATP7 mRNA levels in both adults and larvae. Bars represent mean ± standard error of the mean (S.E.M.) for duplicate samples (two independent samples of 50 adult guts and two independent samples of 50 larval guts for each genotype). Genotypes were as follows: 2020-GAL4/+; +/+ (Control), 2020-GAL4/++; Sym-UAS-DmATP7/++; Sym-UAS-DmATP7/++; (Gut RNAi).

![Fig. 2. Reduced copper absorption is associated with enhanced lethality at the pupal stage.](image)

(A) Percentage survival to adulthood on normal food (NF) and ammonium tetrathiomolybdate (TTM)-supplemented medium; RNAi-mediated silencing of DmATP7 in the gut increases mortality at the pupal stage. (B) Partial silencing of DmATP7 in the gut reduces the copper content of the head and body of adult flies. Bars represent mean ± S.E.M. for a minimum of four samples. The significance of the difference between means (‘Control, NF’ versus ‘Menkes, NF’, ‘Control, TTM’ versus ‘Menkes, TTM’) was analyzed using the one-way analysis of variance (ANOVA) statistical test (*P<0.05). Genotypes were as follows: 2020-GAL4/++; +/+ (Control), 2020-GAL4/++; Sym-UAS-DmATP7/++; Sym-UAS-DmATP7/++; (Menkes).
accumulation in the gut, which may consequently interfere with copper uptake (supplementary material Fig. S1) (Norgate et al., 2006). To determine the consequences of DmATP7 silencing on copper absorption, we measured the copper content of newly eclosed flies using an atomic absorption spectrophotometer. Previous studies on Menkes patients and mottled-brindled mutant mice demonstrated that mutations in the ATP7A gene interfere with copper transport to the brain (Madsen and Gitlin, 2007; Nicu et al., 2007; Zatta and Frank, 2007). Here we show that, analogous to Menkes patients and brindled mice, silencing of DmATP7 in the gut interferes with copper delivery to the head and the rest of the body of the adult flies (Fig. 2B). In addition, supplementation of the copper chelator TTM to the culture medium further reduced copper levels in the body of flies but had no significant effect on the copper content of the head (Fig. 2B). These findings indicate that the phenotypes of Menkes disease may not be simply recapitulated by addition of a copper chelator into the culture medium, despite the consequent reductions in whole-body copper content (but not the head). Given that the pupal lethal phenotype was not observed in wild-type flies raised on TTM-supplemented medium, despite the consequent reductions in whole-body copper content, it appears that the cause of lethality in our fly model is mainly the result of reductions in the copper content of the head.

Copper supplementation and overexpression of MTF-1 enhances pupal survival into the adult stage

Reductions in whole-body copper content suggest that the cause of mortality in our Menkes flies may be the result of decreased copper absorption. To further clarify the role of copper absorption in our fly model, we tested survival into the adult stage on copper-supplemented medium. As Fig. 3A illustrates, supplementation of copper into fly food enhances pupal survival into the adult stage, indicating that a reduced copper pool is the cause of mortality in these flies.

The gene metal-responsive transcription factor-1 (MTF-1) is a copper homeostasis gene that helps Drosophila cope with both copper load and copper starvation by inducing expression of metallothioneins and the copper importer Ctr1B, respectively (Selvaraj et al., 2005). An earlier study demonstrated that MTF-1 overexpression enhances Drosophila survival to the adult stage on both copper-supplemented and copper-depleted media (Bahadorani et al., 2008b). Here, we hypothesized that ubiquitous overexpression of MTF-1 in our fly model may enhance pupal survival to the adulthood stage. As Fig. 3B illustrates, ubiquitous overexpression of MTF-1 in Menkes flies significantly enhances pupal survival, suggesting that MTF-1 induction, perhaps through zinc therapy, may be used as a supplementary approach for the treatment of Menkes disease.

Fig. 3. Survival of Menkes flies is dependent on copper content of the food and expression of copper homeostasis genes. (A) Copper supplementation and (B) overexpression of MTF-1 enhances survival of Menkes flies into the adult stage. Bars are expressed as mean±S.E.M. The significance of the difference between means was analyzed using the one-way ANOVA (*P<0.05). Genotypes were as follows: 2020-GAL4/+; +; + (Control), 2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+ (Menkes), 2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/Tub-MTF-1 (MTF-1).

DmATP7 silencing and the consequent reductions in the copper pool are associated with severe neurodevelopmental defects

Prominent clinical features of Menkes disease patients and brindled mice include severe neurodevelopmental and growth defects (Mercer, 1998; Rossi et al., 2001). To investigate the biological effects of DmATP7 partial silencing and the consequent reductions of the copper pool on neurodevelopment, we analyzed brain sections of stage P12 pupae in both normal and Menkes flies (Fig. 4). Here, we demonstrate that our Menkes model (DmATP7 gut-RNAi) has severe neurodevelopmental defects compared with control flies, where the brain size in Menkes pupae was severely reduced to approximately half the size of normal flies (Fig. 4C). Furthermore, Menkes brains contained less stainable material than the control brains, presumably owing to hypointensity of the brain matter. These data are in accord with the essential role of copper in normal brain development.

Fig. 4. DmATP7 suppression interferes with pupal brain development. (A,B) Control pupae (A) have a larger and more intensely stained brain than the Menkes pupae (B). (C) Menkes pupae have a significantly smaller brain size when compared with the control pupae. Paraffin sections (7 µm) were stained in Mallory’s stain and the brain size was measured using Scion Image software. Data are expressed as mean±S.E.M. for a minimum of six samples; the significance of the difference between means was analyzed using the one-way ANOVA statistical test (F1, 11=27.6; *P<0.0003). Genotypes were as follows: 2020-GAL4/+; +; + (Control), 2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+ (Menkes), 2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/Tub-MTF-1 (MTF-1).
Eclosing adults have a normal morphology and live to a normal age, but are sensitized to oxidative stress

Despite the severe consequences of DmATP7 silencing in some flies, there are adult survivors that have a normal morphology (Fig. 5). Unlike Menkes patients and brindled mice, which exhibit hypopigmentation and an abnormal hair phenotype (Mercer, 1998), our surviving flies have normal cuticle pigmentation and bristle formation. Given that the copper content in these flies was significantly lower than the control flies, we hypothesized that the surviving adults would be short-lived, similar to OHS patients. However, to our surprise, the life span of Menkes flies was only slightly shorter than the control flies (Fig. 6A). These observations highlight the physiological difference for copper demand between mammals and invertebrates, where a reduced copper supply during adulthood causes early death in humans, whereas in flies, copper deficiency mainly causes mortality throughout the developmental stages. However, surviving flies were sensitized to oxidative stress (Fig. 6B), suggesting that the activity of the copper-dependent antioxidant enzyme, Cu/Zn superoxide dismutase (SOD), may be compromised in these flies.

DISCUSSION

In Menkes disease, mutations in the ATP7A gene are characterized by hypopigmentation, kinky hair, neurological deterioration and early death in childhood owing to inhibition of copper absorption in the intestine, which results in a systemic copper deficiency (Lutsenko et al., 2007; Tomita et al., 1992). Menkes disease patients die within their first few years of life, whereas OHS patients can remain alive until adulthood (Kodama et al., 1999). Because of the blockage of copper absorption in the intestine, the current approaches to the treatment of Menkes disease are subcutaneous or intravenous injection of a copper-histidine complex. The copper treatment may inhibit neurodegeneration in some patients and prolong survival only if the treatment is initiated prenatally or soon after birth. However, the early treatment is not effective in improving non-neurological impairments such as the connective tissue abnormalities that are associated with reduced activity of lysyl oxidase, a copper-dependent enzyme; as a consequence, early copper therapy usually leads to a milder OHS-like phenotype (Cox, 1999; George and Casey, 2001; Gu et al., 2002; Kodama et al., 2001; Kodama et al., 1999; Royce et al., 1980; Sarkar et al., 1993).

Given that the current treatments for Menkes disease only partially ameliorate the copper-deficiency phenotypes, it would be interesting to find other genetic pathways that are involved in the pathogenesis of this disorder, with the hope of developing more effective treatments. Here, we have successfully constructed a Drosophila model of Menkes disease which provides an economic and expeditious system for studying biological and genetic factors influencing the disease.
In contrast to mammals, *Drosophila melanogaster* carries only one copper-transporting ATPase known as *DmATP7*, which is expressed in various tissues, such as the gut and the nervous system (Burke et al., 2008; Norgate et al., 2006) (S.B. and A.J.H., unpublished). As a consequence, loss-of-function mutants of *DmATP7* generate larvae with phenotypes that are similar to both Wilson’s and Menkes diseases (Norgate et al., 2006). For instance, *DmATP7/Y* larvae have hypopigmented mouth hooks and are short-lived (similar to Menkes disease symptoms), although they are extremely lethargic (akin to the movement disorder in Wilson’s disease) (Cox, 1999; Norgate et al., 2006; Tomita et al., 1992). Our results demonstrate that conditional silencing of *DmATP7* specifically in the gut recapitulates major features of Menkes disease, such as the reduced copper pool, severe neurodevelopmental defects and early mortality (Figs 2-4). These observations indicate that, similar to humans and mice, the *DmATP7* copper-transporting gene is required for copper absorption from the digestive tract and that disruption of this gene can reduce the copper pool and, consequently, enhance neurodevelopmental defects (Fig. 4). A reduction in the activity of the mitochondrial copper-dependent cytochrome c oxidase, and the consequent impairment of energy metabolism, is thought to be the primary cause of neurodegeneration in Menkes disease (Mercer, 1998; Rossi et al., 2001). In a case report study, a 7-month-old male patient with the classical form of severe Menkes disease presented with marked atrophy and hypointensity of the brain (Agertt et al., 2007). Similarly, our fly model exhibits a severely atrophied brain with a faint staining intensity, presumably owing to hypointensity of the brain matter. The fact that these phenotypes match closely with those of Menkes patients and brindled mice suggests that an evolutionary conserved mechanism is responsible for normal intestinal copper absorption and neurodevelopment.

Considering that copper is essential for the structural and catalytic properties of various enzymes, copper deficiency in Menkes disease can lead to the inactivation of copper-dependent enzymes such as tyrosinase and enhance the hypopigmentation of the hair and the skin (Cox, 1999; Kamolsilp, 2005; Petris et al., 2000). In *Drosophila melanogaster*, overexpression of *DmATP7* confers the hypopigmentation of the skin and the loss of thoracic bristles, whereas simultaneous expression of the copper uptake gene *Ctr1A* rescues the phenotypes (Norgate et al., 2006). These observations suggest that, similar to humans, Drosophila demands a sufficient supply of copper for proper pigmentation and bristle formation. However, our *DmATP7* gut-RNAi adult flies have a normal morphology (Fig. 5) despite the significant reductions in their whole-body copper pool (Fig. 2B). From these observations, we conclude that the residual expression of *DmATP7* in the gut is sufficient for normal development, pigmentation and bristle formation in the surviving flies. It is also worth noting that, despite the significant mortality that was observed throughout the developmental stages upon *DmATP7* silencing in the gut, surviving adults were only slightly shorter-lived than the control flies (Fig. 6A). These observations suggest that the fruit fly has high demands for copper absorption throughout the developmental stages but not in the adult stage. This conclusion is further supported by the observation that dietary supplementation of the copper chelator TTM significantly induces mortality during developmental stages, whereas its lethal effects during adulthood are far less significant (data not shown). Nevertheless, surviving adults are still sensitized to the oxidative stress of hyperoxia (Fig. 6B), presumably owing to reduced activity of the copper-dependent antioxidant enzyme Cu/ZnSOD. Indeed, an earlier study revealed that a sufficient copper supply is essential for normal Cu/ZnSOD activity in flies, by showing that supplementation of 500 μM of the copper chelator bathocuproine disulfonate (BCS) significantly reduces Cu/ZnSOD activity (Egli et al., 2006). Therefore, a slight reduction in the normal adult life span may be attributed to potential reductions in Cu/ZnSOD activity and the consequent increases in the oxidative injury.

Finally, we tested the effects of the copper homeostasis gene *MTF-1* on our fly model. Substantial evidence suggests that *MTF-1* and its target genes that encode metallothioneins may play a role in the pathogenesis of the disease; increased metallothionein synthesis occurs in the mutated brindled mice (Prins and Van den Hamer, 1980), presumably to mediate enhanced protection against copper toxicity (Kelly and Palmiter, 1996). Our results demonstrate that expression of *MTF-1* in *DmATP7* gut-RNAi larvae enhances their survival to the adulthood stage, suggesting that MTF-1 may play a protective role in Menkes disease. There could be at least two potential mechanisms through which MTF-1 protects our fly model: (1) MTF-1 induces the expression of metallothioneins, which bind to excess copper that has accumulated in the gut and prevent its toxic effect; (2) MTF-1 induces the expression of the copper importer *Ctr1B* and, consequently, additional copper is supplied to the tissues. In either case, this finding suggests that therapeutic approaches aimed at inducing MTF-1 may help in the treatment of Menkes disease.

In summary, we have created a partially lethal fly model of Menkes disease by RNAi-mediated silencing of the copper transporter *DmATP7* in the gut. Similar to Menkes patients and brindled mutant mice, disruption of intestinal copper absorption through silencing of *Drosophila DmATP7* results in a systemic copper deficiency, neurological abnormalities and early death at the developmental stages. This model allows for the identification of genetic modifiers of *DmATP7* in future studies by enhancing or decreasing pre-adult mortality. Our model, however, mimics a phenotype that would be associated with a hypomorphic allele. Thus, reduced levels of copper transport are deleterious to the probability of survival at developmental stages, and yet are not as significant to survival in the adulthood stage. These observations suggest that hypomorphic alleles of *DmATP7* are likely to have different effects at different stages of the life cycle.

**METHODS**

**Construction of transgenic flies**

The *DmATP7* insert (nucleotides 2000-2900) for the RNAi construct was amplified by PCR and cloned into the EcoRI-BgIII sites of the transformation vector *Sym-pLIAST* (Giordano et al., 2002). The *Sym-pLIAST-DmATP7* transgene construct and the *p*Δ2-3 helper plasmid were injected into w1118 embryos by the Genetic Services (Cambridge, MA) microinjection service, using the standard P element microinjection procedures (Spradling and Rubin, 1982). Transgenic lines were isolated on the basis of orange/red eye color. The gut-specific 2020-GALA driver was obtained from the Kyoto Stock Center. This driver was tested for tissue specificity using the green fluorescent protein (GFP) reporter, and expression was found to be confined to the gut (Bahadorani et al., 2002). The *DmATP7* insert (nucleotides 2000-2900) for the RNAi construct was amplified by PCR and cloned into the EcoRI-BgIII sites of the transformation vector *Sym-pLIAST* (Giordano et al., 2002). The *Sym-pLIAST-DmATP7* transgene construct and the *p*Δ2-3 helper plasmid were injected into w1118 embryos by the Genetic Services (Cambridge, MA) microinjection service, using the standard P element microinjection procedures (Spradling and Rubin, 1982). Transgenic lines were isolated on the basis of orange/red eye color. The gut-specific 2020-GALA driver was obtained from the Kyoto Stock Center. This driver was tested for tissue specificity using the green fluorescent protein (GFP) reporter, and expression was found to be confined to the gut (Bahadorani et al., 2002).
et al., 2008b). To increase the efficiency of DmATP7 silencing, a transgenic strain carrying RNAi constructs on both the second and the third chromosome was constructed and crossed to a strain carrying a gut-specific 2020-GAL4 driver. The Tub-MTF-1 transgene expressing MTF-1 has been described previously (Bahadorani et al., 2008b).

**Quantitative real-time PCR**

Total RNA was isolated from the gut of adult flies or larvae using Trizol reagent (Invitrogen, Carlsbad, CA). For each extraction, a total of 50 larval or adult guts were dissected and homogenized in 500 μl of Trizol reagent. Duplicate or triplicate samples were used for each genotype. After RNA isolation, cDNA was synthesized from 1 μl of Trizol reagent. Duplicate or triplicate samples were used for each primer set. Gene expression levels were analyzed by quantitative real-time PCR in duplicate using the Platinum SYBR green qPCR SuperMix-UDG (Invitrogen), and normalized to the housekeeping gene, Act57B. All protocols were performed according to the manufacturer’s instructions. Primers for real-time PCR were designed using Invitrogen’s OligoPerfect Designer, available at www.invitrogen.com/oligos. Forward and reverse primers for the target were as follows: DmATP7: 5’-ATATCGACGACAATGGGGCTTC-3’, 5’-TGCGAAAGCATTTGTCAG-3’; Act57B: 5’-GTGCATATGGGTACGAC-3’, 5’-GCTGGAAGGTGACAGAGAG-3’.

**Survival assays**

Throughout all survivorship assays, flies were maintained on a 12:12 light:dark cycle at 25°C on culture medium, as described previously (Bahadorani et al., 2008a). Copper-depleted medium was prepared by supplying the copper chelator TTM into the normal culture medium at a final concentration of 10 μM. Copper-supplemented medium was prepared by the addition of copper (II) sulfate into the normal culture medium at a final concentration of 1 mM. Survival to adulthood was determined by quantifying the percentage of pupae (n>500) that eclosed. For each survival assay, a minimum of four samples were used. The difference between means was analyzed using a one-way ANOVA statistical test. A total of 100-200 males (initially 20 per vial) were tested for each longevity assay, with survivors transferred into fresh medium every 2-3 days. For the hyperoxia assay, we used a similar protocol to that described previously (Bahadorani et al., 2008a); for each genotype, a total of 200 flies (initially 20 per vial) were aged on normal food for 10 days and then transferred into a sealed chamber with a steady flow of 100% oxygen bubbled into water. Survivors were transferred into fresh food vials every 2 days. The significance of the difference between survival curves was analyzed using the Kaplan-Meier log-rank test.

**Copper content measurements**

Copper content was measured using a similar protocol to that described previously (Bahadorani et al., 2008a; Massie et al., 1985). Newly eclosed males were dried at 65°C overnight and, thereafter, heads were separated from the bodies using razor blades. Samples containing a minimum of 100 heads or 40 bodies were digested in 200 μl of 65% nitric acid for 10 days and diluted (1:30) in distilled water. The copper content in each sample was measured at a wavelength of 324.75 nm using an atomic absorption spectrophotometer (AAnalyst 200, Perkin Elmer, CT). A minimum of four samples (from a separate 100 sets of heads or 40 sets of bodies) were used for each analysis. The significance of the difference between means was analyzed using the one-way ANOVA test.

**Histological analysis**

RNAi-induced silencing of DmATP7 in the digestive tract arrested pupal development mainly at stage P12 of metamorphosis (Bainbridge and Bownes, 1981). Thus, prior to the experiment, bottles were cleared of all stage P12 pupae and, 24 hours later, pupae that had newly reached stage P12 were collected for analysis. To analyze the brain morphology, the pupal case surrounding the head was removed and, afterward, samples were fixed in 4% paraformaldehyde, dehydrated through a graded ethanol series, embedded in paraffin and sectioned at 7 μm. Paraffin-embedded sections were de-waxed in xylene, rehydrated through a graded ethanol series to water, and stained in Mallory’s stain for 5 minutes. Finally, samples were dehydrated, treated with xylene, and mounted in Permount for microscopic examination.

**Scanning electron microscopy**

For scanning electron microscopy, 2–3-day-old flies were killed by overexposure to diethyl ether, then air-dried at room temperature for a few days, and subsequently coated in gold for photography at approximately 110× magnification.
Statistical analysis
Bars are expressed as mean±S.E.M., with the significance of the difference between means analyzed using one-way ANOVA (*P<0.05). For the longevity assay, the significance of the difference between survival curves was analyzed using the Kaplan-Meier log-rank statistical test (*P<0.0001), using codes provided by Fox (Fox, 1993). All statistical analyses were performed using SAS software (version 9.1.3, SAS Institute, Cary, NC). Brain size (n=6 or 7 for each genotype) was measured using Scion Image software (Scion Corporation, Frederick, MD).

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
S.B. designed, performed and analyzed all of the experiments; P.B. and E.M. assisted S.B. with longevity and real-time PCR assays; A.J.H. supervised S.B. and contributed to the reagents; S.B. wrote the manuscript, with A.J.H. and D.W.W. providing useful comments on the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.002642/-/DC1

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