RESEARCH ARTICLE

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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; Voskoboinik 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; Mercer, 1998; Møller 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 (Mo^{br}) 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 Palmiter, 1996). Similar to Menkes patients, the Mo^{br} copper-deficient mice exhibit profound neurological abnormalities and die early after birth. However, blotchy (Mo^{blo}) is a less severe allele of mottled that is characterized mainly by connective tissue defects, akin to human OHS (Mercer, 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

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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 Mo^{br} mice. This invertebrate model provides a genetic complement to Mo^{br} 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-pUAST-DmATP7* RNAi-silencing construct (Giordano et al., 2002). The *Sym-pUAST-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).

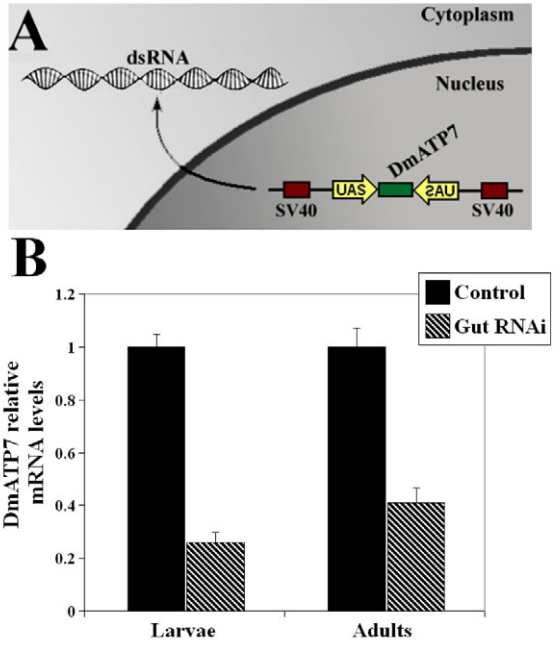

Fig. 1. RNAi-mediated silencing of *DmATP7* in the digestive tract. (A) Schematic diagram of the *Sym-pUAST-DmATP7* RNAi construct regulated by two convergent UAS enhancers; GAL4-induced activation of the UAS enhancers results in production of sense-antisense double-stranded RNA and subsequent activation 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).

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

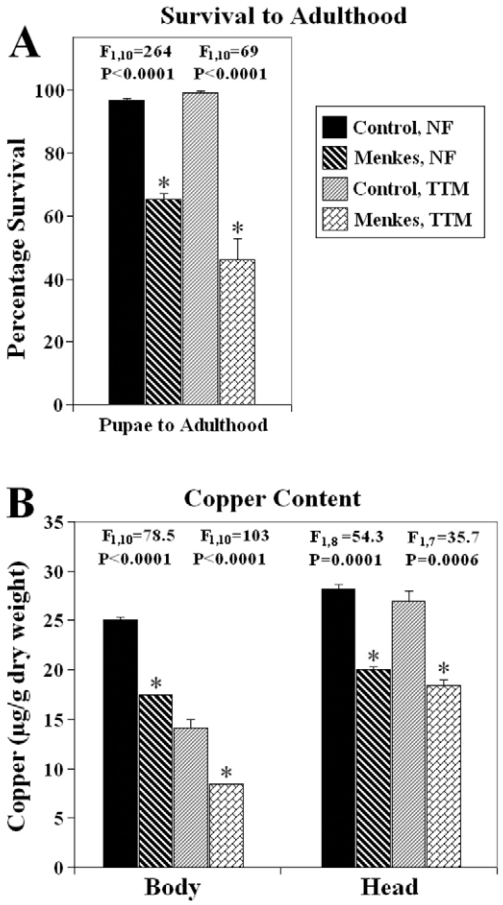

Fig. 2. Reduced copper absorption is associated with enhanced lethality at the pupal stage. (A) Percentage survival to adulthood on normal food (NF) and ammonium tetrathiomolybdate (TTM)-supplemented medium; RNAi-mediated silencing of *DmATP7* in the gut increases mortality at the pupal stage. (B) Partial 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; Niciu 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 significant reductions in the copper pool in the body), 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.

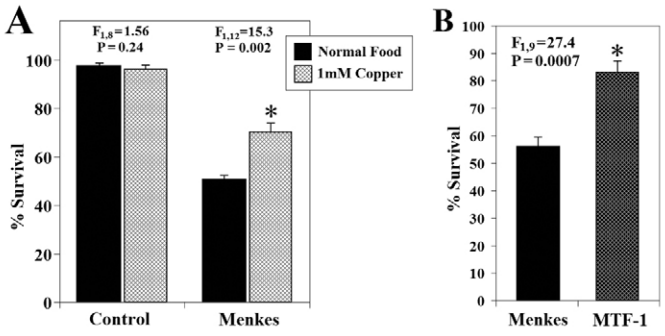

Fig. 3. Survival of Menkes flies is dependent on copper content of the food and expression of copper homeostasis genes. (A) Copper supplementation and (B) overexpression of *MTF-1* enhances survival of Menkes flies into the adult stage. Bars are expressed as mean±S.E.M. The significance of the difference 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.

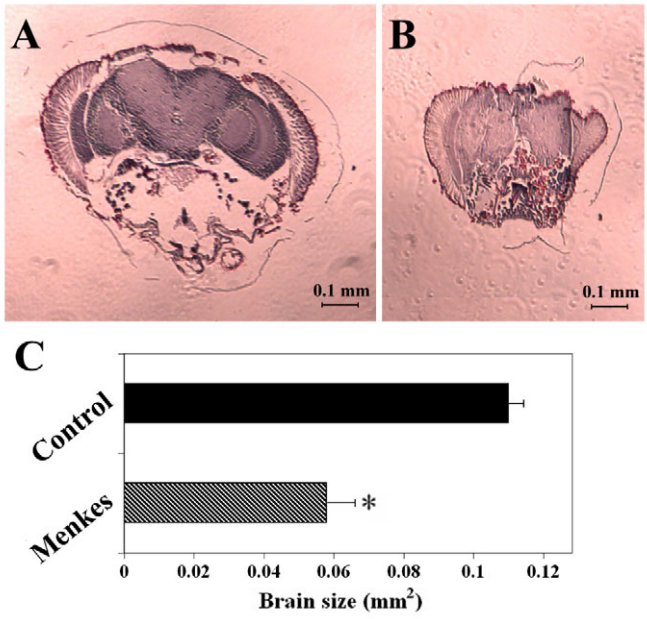

Fig. 4. *DmATP7* suppression interferes with pupal brain development. (A,B) Control pupae (A) have a larger and more intensely stained brain than the Menkes pupae (B). (C) Menkes pupae have a significantly smaller brain size when compared with the control pupae. Paraffin sections (7 µm) were stained 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 ($F_{1,11}$=27.6; *P=0.0003). Genotypes were as follows: *2020-GAL4/+; +; +* (Control), *2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+* (Menkes).

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.

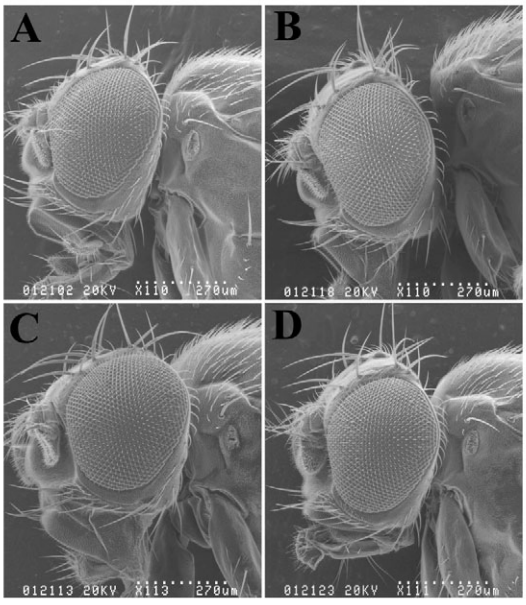

Fig. 5. Silencing of *DmATP7* in the gut and the associated reductions in the copper pool has no effect on the external morphology of surviving adults. Panels A-D represent the scanning electron microscopy images of: (A) *2020-GAL4/+; +; +* flies fed on normal food, (B) *2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+* flies fed on normal food, (C) *2020-GAL4/+; +; +* flies fed on TTM-supplemented medium, (D) *2020-GAL4/+; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+* flies fed on TTM-supplemented medium.

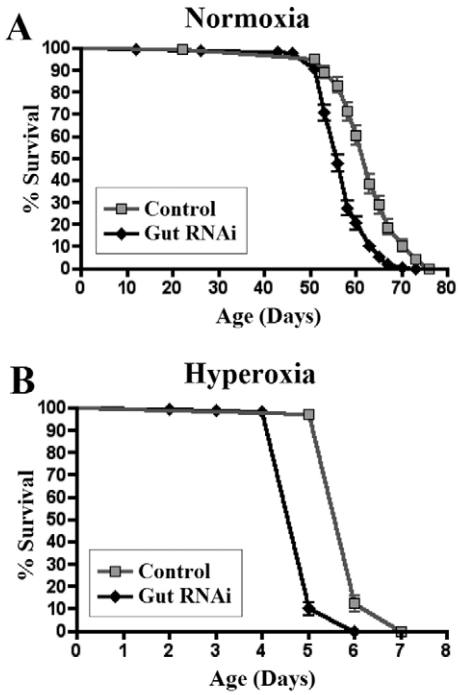

Fig. 6. DmATP7 silencing in the gut has minor effects on the physiology of surviving adults. (A) Partial silencing of *DmATP7* in the digestive tract slightly shortens the normal adult life span and (B) sensitizes flies to the acute oxidative stress of hyperoxia. Throughout the entire experiment, adult males were maintained at 25°C on normal food. Under both normoxia and hyperoxia, control flies lived significantly longer than the *DmATP7* gut RNAi flies (log-rank statistical test, **P*<0.0001). For the hyperoxia assays, flies were aged for 10 days before the experiment. *DmATP7* gut-RNAi males (*2020-GAL4/Y; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+*) were obtained from the cross between *2020-GAL4* females and *+/Y; Sym-UAS-DmATP7; Sym-UAS-DmATP7* males. For collection of the control males (*+/Y; Sym-UAS-DmATP7/+; Sym-UAS-DmATP7/+*), we performed a reciprocal cross, crossing *+; Sym-UAS-DmATP7; Sym-UAS-DmATP7* females to *2020-GAL4* males.

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 *Eco*RI-*Bgl*II sites of the transformation vector *Sym-pUAST* (Giordano et al., 2002). The *Sym-pUAST-DmATP7* transgene construct and the *p(Δ2-3)* helper plasmid were injected into w^{1118} 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-GAL4* 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., 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 µg of total RNA using the SuperScript III First-Strand SuperMix (Invitrogen) for reverse transcriptase PCR (RT-PCR) kit. Levels of gene expression in each cDNA sample 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 primer sequences were as follows: *DmATP7*: 5′-ATATCGACGACATGGGCTTC-3′, 5′-TGCAGAAAGCATTTGGTCAG-3′; *Act57B*: 5′-GTGCTATGTTGCCCTGGACT-3′, 5′-GCTGGAAGGTGGACAGAGAG-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.

TRANSLATIONAL IMPACT

Clinical issue

Menkes disease is a recessive X-linked disorder that causes lethal copper deficiency. It results from mutations in a copper exporter gene, *ATP7A*, expressed in the gut. As a consequence of these mutations, copper is trapped in the gut of Menkes patients and cannot be delivered to the brain, resulting in severe neurological abnormalities, seizures and early death. Patients are also characterized by coarse thin hair and growth defects. Current disease treatments supply copper by subcutaneous or intravenous injection, but are not very effective. If treatment is started prenatally or perinatally, some neurodegeneration is prevented and survival can be enhanced, but non-neurological abnormalities are still present. To develop better treatments, more needs to be known about the pathogenesis of the disorder, and the complex nature of the disease requires whole-organism approaches. Although mouse models have been developed, a simpler, faster and cheaper model system such as Drosophila would also be of use.

Results

This paper describes a new Drosophila model of Menkes disease. The authors silence the Drosophila *DmATP7* copper exporter gene in the digestive tract to mimic the human disease. Copper is trapped in the gut of the mutant, preventing copper delivery to other parts of the fly's body, including the head. Mutant flies exhibit severe neurological abnormalities and die at an early age, similar to human patients. Expression of the copper homeostasis gene *MTF-1*, which is thought to play a role in Menkes disease, rescues the lethal phenotype, suggesting that MTF-1 induction may be used as a supplementary approach for the treatment of Menkes disease.

Implications and future directions

This fly model recapitulates major features of human Menkes disease. The powerful genetic tools that are available for manipulation of Drosophila make it a cost-effective and simple system for the identification and validation of other genes involved in the Menkes disease phenotype, and for screening of future therapeutics.

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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
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REFERENCES

Agertt, F., Crippa, A. C., Lorenzoni, P. J., Scola, R. H., Bruck, I., Paola, L., Silvado, C. E. and Werneck, L. C. (2007). Menkes' disease: case report. *Arq. Neuropsiquiatr.* **65**, 157-160.

Bahadorani, S., Bahadorani, P., Phillips, J. P. and Hilliker, A. J. (2008a). The effects of vitamin supplementation on *Drosophila* life span under normoxia and under oxidative stress. *J. Gerontol. A. Biol. Sci. Med. Sci.* **63**, 35-42.

Bahadorani, S., Mukai, S., Egli, D. and Hilliker, A. J. (2008b). Overexpression of metal-responsive transcription factor-1 (MTF-1) in Drosophila melanogaster ameliorates life-span reductions associated with oxidative stress and metal toxicity. *Neurobiol. Aging* Sep 3 [Epub ahead of print] [doi:10.1016/j.neurobiolaging.208.08.001].

Bainbridge, S. P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **66**, 57-80.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Burke, R., Commons, E. and Camakaris, J. (2008). Expression and localisation of the essential copper transporter DmATP7 in Drosophila neuronal and intestinal tissues. *Int. J. Biochem. Cell Biol.* **40**, 1850-1860.

Cox, D. W. (1999). Disorders of copper transport. *Br. Med. Bull.* **55**, 544-555.

Cox, D. W. and Moore, S. D. (2002). Copper transporting P-type ATPases and human disease. *J. Bioenerg. Biomembr.* **34**, 333-338.

Das, S., Levinson, B., Vulpe, C., Whitney, S., Gitschier, J. and Packman, S. (1995). Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse. *Am. J. Hum. Genet.* **56**, 570-576.

de Bie, P., Muller, P., Wijmenga, C. and Klomp, L. W. (2007). Molecular pathogenesis of Wilson and Menkes disease: correlation of mutations with molecular defects and disease phenotypes. *J. Med. Genet.* **44**, 673-688.

Egli, D., Yepiskoposyan, H., Selvaraj, A., Balamurugan, K., Rajaram, R., Simons, A., Multhaup, G., Mettler, S., Vardanyan, A., Georgiev, O. et al. (2006). A family knockout of all four Drosophila metallothioneins reveals a central role in copper homeostasis and detoxification. *Mol. Cell. Biol.* **26**, 2286-2296.

Fox, G. A. (1993). Failure-time analysis: emergence, flowering, survivorship, and other waiting times. In *Design and analysis of Ecological Experiments* (ed. S. M. Scheiner and J. Gurevitch), pp. 253-289. New York: Chapman & Hall.

George, D. H. and Casey, R. E. (2001). Menkes disease after copper histidine replacement therapy: case report. *Pediatr. Dev. Pathol.* **4**, 281-288.

Giordano, E., Rendina, R., Peluso, I. and Furia, M. (2002). RNAi triggered by symmetrically transcribed transgenes in Drosophila melanogaster. *Genetics* **160**, 637-648.

Grimes, A., Hearn, C. J., Lockhart, P., Newgreen, D. F. and Mercer, J. F. (1997). Molecular basis of the brindled mouse mutant (Mo(br)): a murine model of Menkes disease. *Hum. Mol. Genet.* **6**, 1037-1042.

Gu, Y. H., Kodama, H., Sato, E., Mochizuki, D., Yanagawa, Y., Takayanagi, M., Sato, K., Ogawa, A., Ushijima, H. and Lee, C. C. (2002). Prenatal diagnosis of Menkes disease by genetic analysis and copper measurement. *Brain Dev.* **24**, 715-718.

Harris, E. D. (2000). Cellular copper transport and metabolism. *Annu. Rev. Nutr.* **20**, 291-310.

Kaler, S. G., Gallo, L. K., Proud, V. K., Percy, A. K., Mark, Y., Segal, N. A., Goldstein, D. S., Holmes, C. S. and Gahl, W. A. (1994). Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. *Nat. Genet.* **8**, 195-202.

Kamolsilp, M. (2005). Menkes syndrome: a case report. *J. Med. Assoc. Thai.* **88**, 290-294.

Kelly, E. J. and Palmiter, R. D. (1996). A murine model of Menkes disease reveals a physiological function of metallothionein. *Nat. Genet.* **13**, 219-222.

Kim, B. E. and Petris, M. J. (2007). Phenotypic diversity of Menkes disease in mottled mice is associated with defects in localisation and trafficking of the ATP7A protein. *J. Med. Genet.* **44**, 641-646.

Kodama, H., Murata, Y. and Kobayashi, M. (1999). Clinical manifestations and treatment of Menkes disease and its variants. *Pediatr. Int.* **41**, 423-429.

Kodama, H., Gu, Y. H. and Mizunuma, M. (2001). Drug targets in Menkes disease-prospective developments. *Expert Opin. Ther. Targets* **5**, 625-635.

La Fontaine, S., Firth, S. D., Lockhart, P. J., Brooks, H., Camakaris, J. and Mercer, J. F. (1999). Intracellular localization and loss of copper responsiveness of Mnk, the murine homologue of the Menkes protein, in cells from blotchy (Mo blo) and brindled (Mo br) mouse mutants. *Hum. Mol. Genet.* **8**, 1069-1075.

Levinson, B., Conant, R., Schnur, R., Das, S., Packman, S. and Gitschier, J. (1996). A repeated element in the regulatory region of the MNK gene and its deletion in a patient with occipital horn syndrome. *Hum. Mol. Genet.* **5**, 1737-1742.

Llanos, R. M., Ke, B. X., Wright, M., Deal, Y., Monty, F., Kramer, D. R. and Mercer, J. F. (2006). Correction of a mouse model of Menkes disease by the human Menkes gene. *Biochim. Biophys. Acta.* **1762**, 485-493.

Lutsenko, S., Barnes, N. L., Bartee, M. Y. and Dmitriev, O. Y. (2007). Function and regulation of human copper-transporting ATPases. *Physiol. Rev.* **87**, 1011-1046.

Madsen, E. and Gitlin, J. D. (2007). Copper and iron disorders of the brain. *Annu. Rev. Neurosci.* **30**, 317-337.

Massie, H. R., Aiello, V. R. and Williams, T. R. (1985). Iron accumulation during development and ageing of Drosophila. *Mech. Ageing Dev.* **29**, 215-220.

Mercer, J. F. (1998). Menkes syndrome and animal models. *Am. J. Clin. Nutr.* **67**, 1022S-1028S.

Møller, L. B., Tümer, Z., Lund, C., Petersen, C., Cole, T., Hanusch, R., Seidel, J., Jensen, L. R. and Horn, N. (2000). Similar splice-site mutations of the ATP7A gene lead to different phenotypes: classical Menkes disease or occipital horn syndrome. *Am. J. Hum. Genet.* **66**, 1211-1220.

Nelson, N. (1999). Metal ion transporters and homeostasis. *EMBO J.* **18**, 4361-4371.

Niciu, M. J., Ma, X. M., El Meskini, R., Pachter, J. S., Mains, R. E. and Eipper, B. A. (2007). Altered ATP7A expression and other compensatory responses in a murine model of Menkes disease. *Neurobiol. Dis.* **27**, 278-291.

Norgate, M., Lee, E., Southon, A., Farlow, A., Batterham, P., Camakaris, J. and Burke, R. (2006). Essential roles in development and pigmentation for the Drosophila copper transporter DmATP7. *Mol. Biol. Cell* **17**, 475-484.

Petris, M. J., Strausak, D. and Mercer, J. F. (2000). The Menkes copper transporter is required for the activation of tyrosinase. *Hum. Mol. Genet.* **9**, 2845-2851.

Pfeiffer, R. F. (2007). Wilson's Disease. *Semin. Neurol.* **27**, 123-132.

Phillips, J. P. and Hilliker, A. J. (1990). Genetic analysis of oxygen defense mechanisms in *Drosophila melanogaster*. *Adv. Genet.* **38**, 43-71.

Prins, H. W. and Van den Hamer, J. A. (1980). Abnormal copper-thionein synthesis and impaired copper utilization in mutated brindled mice: model for Menkes' disease. *J. Nutr.* **110**, 151-157.

Rossi, L., De Martino, A., Marchese, E., Piccirilli, S., Rotilio, G. and Ciriolo, M. R. (2001). Neurodegeneration in the animal model of Menkes' disease involves Bcl-2-linked apoptosis. *Neuroscience* **103**, 181-188.

Royce, P. M., Camakaris, J. and Danks, D. M. (1980). Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome. *Biochem. J.* **192**, 579-586.

Sarkar, B., Lingertat-Walsh, K. and Clarke, J. T. (1993). Copper-histidine therapy for Menkes disease. *J. Pediatr.* **123**, 828-830.

Selvaraj, A., Balamurugan, K., Yepiskoposyan, H., Zhou, H., Egli, D., Georgiev, O., Thiele, D. J. and Schaffner, W. (2005). Metal-responsive transcription factor (MTF-1) handles both extremes, copper load and copper starvation, by activating different genes. *Genes Dev.* **19**, 891-896.

Southon, A., Burke, R., Norgate, M., Batterham, P. and Camakaris, J. (2004). Copper homoeostasis in Drosophila melanogaster S2 cells. *Biochem. J.* **383**, 303-309.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* **218**, 341-347.

Tang, J., Robertson, S., Lem, K. E., Godwin, S. C. and Kaler, S. G. (2006). Functional copper transport explains neurologic sparing in occipital horn syndrome. *Genet. Med.* **8**, 711-718.

Tomita, Y., Kondo, Y., Ito, S., Hara, M., Yoshimura, T., Igarashi, H. and Tagami, H. (1992). Menkes' disease: report of a case and determination of eumelanin and pheomelanin in hypopigmented hair. *Dermatology* **185**, 66-68.

Tsukahara, M., Imaizumi, K., Kawai, S. and Kajii, T. (1994). Occipital horn syndrome: report of a patient and review of the literature. *Clin. Genet.* **45**, 32-35.

Voskoboinik, I. and Camakaris, J. (2002). Menkes copper-translocating P-type ATPase (ATP7A): biochemical and cell biology properties, and role in Menkes disease. *J. Bioenerg. Biomembr.* **34**, 363-371.

Williams, L. E., Pittman, J. K. and Hall, J. L. (2000). Emerging mechanisms for heavy metal transport in plants. *Biochim. Biophys. Acta* **1465**, 104-126.

Zatta, P. and Frank, A. (2007). Copper deficiency and neurological disorders in man and animals. *Brain. Res. Rev.* **54**, 19-33.