In Vivo Modeling of the Pathogenic Effect of Copper Transporter Mutations That Cause Menkes and Wilson Diseases, Motor Neuropathy, and Susceptibility to Alzheimer’s Disease*

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Stephen W. Mercer, Jianbin Wang, and Richard Burke
From the School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia

Edited by Paul E. Fraser

Copper is an essential biometal, and several inherited diseases are directly associated with a disruption to normal copper homeostasis. The best characterized are the copper deficiency and toxicity disorders Menkes and Wilson diseases caused by mutations in the p-type Cu-ATPase genes ATP7A and ATP7B, respectively. Missense mutations in the C-terminal portion of ATP7A have also been shown to cause distal motor neuropathy, whereas polymorphisms in ATP7B are associated with increased risk of Alzheimer’s disease. We have generated a single, in vivo model for studying multiple pathogenic mutations in ATP7 proteins using Drosophila melanogaster, which has a single orthologue of ATP7A and ATP7B. Four pathogenic ATP7A mutations and two ATP7B mutations were introduced into a genomic ATP7 rescue construct containing an in-frame C-terminal GFP tag. Analysis of the wild type ATP7-GFP transgene confirmed that ATP7 is expressed at the basolateral membrane of larval midgut copper cells and that the transgene can rescue a normally early lethal ATP7 deletion allele to adulthood. Analysis of the gATP7-GFP transgenes containing pathogenic mutations showed that the function of ATP7 was affected, to varying degrees, by all six of the mutations investigated in this study. Of particular interest, the ATP7B K832R Alzheimer’s disease susceptibility allele was found, for the first time, to be a loss of function allele. This in vivo system allows us to assess the severity of individual ATP7A/B mutations in an invariant genetic background and has the potential to be used to screen for therapeutic compounds able to restore function to faulty copper transport proteins.

Copper is an essential trace element required as a cofactor for enzymes involved in numerous important cellular functions and pathways (1, 2). The importance of copper, combined with the damaging nature of copper-induced free radical activity, has imposed evolutionary constraints on the mechanisms controlling copper homeostasis at the cellular level so that key copper regulatory proteins have been strongly conserved throughout animal evolution (3, 4).

The impacts of copper homeostasis defects are evidenced by two well studied hereditary disorders, Menkes and Wilson diseases. Menkes disease is an X-linked recessive disorder caused by mutations in the ATP7A gene (5–7), which encodes a transmembrane p-type Cu-ATPase that is expressed in numerous tissues and plays a central role in both the delivery of copper to copper-dependent enzymes and the efflux of excess copper from cells (8). Patients with Menkes disease lack functional ATP7A protein, leading to an inability to release copper from the intestinal enterocytes, which causes a severe copper deficiency that is often fatal in early childhood (9–12).

Although most mutations in the ATP7A gene cause Menkes disease, some missense mutations cause a milder disease called occipital horn syndrome (OHS).2 OHS patients suffer from the connective tissue abnormalities common to Menkes disease patients but do not usually present with neurological symptoms and have a considerably longer average lifespan (13, 14).

Recently, two novel missense mutations in the carboxyl portion of ATP7A not previously thought to be functionally important have been shown to cause distal motor neuropathy (DMN) in two affected families (15). DMN is the collective term for a group of genetically heterogeneous motor neuron disorders that share similar muscle atrophy symptoms. This finding highlighted the importance of copper homeostasis and proper ATP7A function for motor neuron health and function.

Wilson disease is an autosomal recessive disorder caused by mutations in the ATP7B gene, which, like ATP7A, encodes a transmembrane Cu-ATPase (16, 17). ATP7B is expressed primarily in the liver, residing at the trans-Golgi network of hepatocytes under normal conditions (11, 18). This protein plays an essential role in copper excretion from the liver, directing excess copper to the biliary duct where it is removed from the body. Wilson disease patients lacking fully functional ATP7B protein suffer from copper toxicity resulting from abnormal accumulation of copper in the liver and brain. Wilson disease...
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patients typically develop severe liver disease and may present with additional neurological symptoms as well (9). Recently, polymorphisms in the ATP7B gene have also been associated with increased risk of Alzheimer’s disease (AD) (19, 20).

The mechanisms regulating copper homeostasis are conserved between vertebrates and invertebrates (21, 22), making the vinegar fly Drosophila melanogaster a powerful experimental system in which to study copper metabolism. There is a single Drosophila orthologue of ATP7A and ATP7B, the ATP7 gene, which is critically required for copper efflux in the fly. A complete knock-out of ATP7 results in lethality at the first instar stage of larval development (21). ATP7 is expressed extensively in the larval and adult fly, including in the midgut and the central, enteric, and peripheral nervous systems (21, 23).

ATP7 displays high homology with both ATP7A and ATP7B (47.3 and 46.6% amino acid identity, respectively). Importantly, a survey of 46 pathogenic ATP7A/B mutations reviewed in de Bie et al. (14) found that in 44 cases the mutated amino acid was identical or similar in ATP7. Recently identified DMN-causing ATP7A mutations (15) and ATP7B AD susceptibility alleles (19, 20) are also variants of residues conserved in the fly transporter. Therefore we reasoned that the fly would be an excellent approach to disease treatment has been successfully adopted in the case of CFTR misfolding by expression of the apical membrane (24).

Here, we used a gene replacement system to characterize six mutations into Drosophila ATP7A/B proteins. There are multiple mechanisms by which a missense mutation can render a protein non-functional. The protein may be misfolded and degraded too rapidly, targeted to the incorrect cellular location, or, in the case of ion transporters, lose its ability to bind or translocate its substrate. A mutation-specific approach to disease treatment has been successfully adopted with cystic fibrosis where the G551D and R117H CFTR missense mutations, which affect CFTR channel gating and conductance, respectively, can be partially corrected by the CFTR potentiator drug ivacaftor, whereas the common ΔF508 mutation, which causes CFTR retention in the endoplasmic reticulum, requires an additional corrector drug, lumacaftor, which helps restoring function to defective ATP7A/B proteins.

A Full-length Genomic ATP7 Transgene Rescues ΔATP7 Flies to Adulthood—A null deletion allele of the Drosophila ATP7 gene, referred to herein as ΔATP7, results in late first instar larval lethality (21), which can be partially rescued to adult viability by expression of UAS-ATP7-FLAG under the control of an ATP7-GAL4 driver (23). To generate a novel rescue construct expressing endogenous levels of green fluorescent protein (GFP)-tagged ATP7, a GFP coding sequence was recombined into a P-element vector containing the entire genomic region of ATP7, replacing the gene’s stop codon at the C terminus. A minimal genomic rescue construct, gATP7-GFP, was amplified by PCR and subcloned into a Drosophila expression vector. Site-directed mutagenesis was used to introduce six different pathogenic ATP7A/B mutations into gATP7-GFP (Table 1) followed by the generation of stable wild type and mutant gATP7-GFP transgenic lines.

To determine whether the wild type or mutated gATP7-GFP transgenes were capable of rescuing the ΔATP7 deletion allele to adulthood, heterozygous XAATP7XFM7 females were crossed to males homozygous for gATP7-GFP transgenes on the third chromosome (X; Y; gATP7-GFP). gATP7-GFP where X is wild type or one of six mutant versions of ATP7, and adult progeny were scored. Numerous putative XAATP7Y(non-XFM7) adult male progeny were observed with the gATP7WT-GFP (WT), gATP7DMN-GFP (DMN), and gATP7WND-GFP (WND) transgenes, indicating that all three transgenes are capable of rescuing the ATP7 null mutation to adulthood. However, occasional non-XFM7 male progeny were also observed with the other four mutated gATP7 transgenes and even with a control cross with no transgene, leading to the suspicion that some or all of these males were false positives.

Single fly PCR was carried out on all putative XAATP7Y adult male progeny to determine whether they carried the ATP7 null deletion. Although the majority of these males from the WT (5 from 5), DMN (8 from 10), and WND (8 from 8) transgene crosses did indeed have the deletion, indicating that these were genuine rescues, none of the males from the gATP7MNK-GFP (MNK) (0 from 3), gATP7A-GFP (DEL) (0 from 6), gATP7OHS-GFP (OHS) (0 from 5), and gATP7ALZ-GFP (ALZ) (0 from 8) crosses still had the ΔATP7 deletion, proving these were in fact false positives.

The false positive male progeny from these rescue crosses could either be the result of double recombinant in the female parents despite the presence of the XFM7 balancer chromosome, which suppresses recombination, or could be XO males generated by non-disjunction in the female parents. Crosses of heterozygous XAATP7XFM7 females to males of various X chromosome genotypes (yellow, white; yellow, white; and yellow, white) all showed occasional aberrant non-XFM7 males; in each case, these males had the same phenotype as their male

### TABLE 1

| Mammalian mutation | ATP7 mutation | Disease              |
|--------------------|---------------|----------------------|
| hATP7ADEL1086  | G1036E = MNK  | Classical Menkes disease |
| hATP7ADEL1086  | 5536G = OH5  | Occipital horn syndrome |
| mATP7ADEL1086  | P1122S = DMN | X-linked DMN          |
| mATP7ADEL1086  | 5111-2 = DEL | Mouse mottled-brindled |
| hATP7ATP7OHS797 | K552R = ALZ  | Alzheimer’s disease susceptibility |
| hATP7DH778Q   | H778Q = WND  | Wilson disease        |
parents, indicating that non-disjunction was indeed the cause of the false rescues.

To quantify the adult rescue ability of each of the gATP7 transgenes, $X^{ΔATP7\gamma};gATP7^{\gamma\gamma}$-GFP male first instar larvae were selected and then raised under density-controlled conditions (20 larvae per vial) on basal diet (BD), and survival to adulthood was assessed (Fig. 1A). Only the WT transgene was able to consistently rescue the null mutation to adulthood under these conditions with the WND transgene also providing very weak rescue activity. The inability of the DMN and WND transgenes to rescue in this assay suggests that mutant larvae with these two transgenes are weakened and require the presence of their wild type siblings to occasionally survive to adulthood.

The adult survival experiments were repeated for all genotypes on copper-supplemented (250 $\mu$M CuSO$_4$) and copper-deficient (200 $\mu$M bathocuproine disulfonate (BCS)) media. No surviving adults were observed for any of the six mutant transgenes under these conditions (Fig. 1B and not shown). Survival of the $X^{ΔATP7\gamma\gamma};gATP7^{\gamma\gamma}$WT-GFP males decreased under both copper supplementation and copper deficiency (Fig. 1B). When $X^{ΔATP7\gamma};X^{ΔATP7\gamma}$ or $X^{ΔATP7\gamma};gATP7^{\gamma\gamma}$-GFP/gATP7$^{\gamma\gamma}$-GFP flies were generated, viable fertile adults were obtained with the WT, WND, and DMN versions of the gATP7 transgene, indicating that two copies of any of these three transgenes was sufficient to restore near-wild type ATP7 activity.

Mutated Versions of gATP7-GFP Show Variable Activity in a Larval Rescue Assay—The pathogenic missense mutations introduced into gATP7-GFP may result in ATP7 proteins with only partial function and therefore incomplete rescue ability. To better determine the functional level of each transgene, male $X^{ΔATP7\gamma\gamma};gATP7^{\gamma\gamma}$-GFP first instar larvae were monitored over 72 h with the number of surviving larvae quantified at 24-h intervals. Both basal and copper-supplemented diets were tested in this assay.

At 24 h post-egg hatching (PEH), only the WT gATP7-GFP transgene displayed strong rescue activity compared with the no-gATP7 transgene controls, whereas WND showed modest but not significant rescue ability (Fig. 2A). The presence of the ALZ transgene actually decreased the survival rate of $X^{ΔATP7\gamma\gamma}$ males at this early time point. Copper supplementation had no

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At 48 h PEH, there were no surviving $X^{\Delta gATP7}Y$ larvae with the MNK, OHS, or ALZ gATP7 transgene (not shown). The WT transgene still displayed strong rescue activity compared with the no-gATP7 transgene controls, whereas the WND transgene showed a modest but significant increase in survival (Fig. 2B). Only the WT and WND gATP7 transgenes were able to rescue the $X^{\Delta gATP7}Y$ mutants to 72 h PEH (Fig. 2C and not shown) and beyond.

$X^{\Delta gATP7}Y$ mutant larvae were previously reported to display minimal growth posthatching and underdeveloped and unpigmented mouthparts compared with age-matched wild type controls (21). As an additional measure of gATP7 transgene rescue ability, we assessed larval size and morphology at 24 h PEH. The WT, OHS, DMN, DEL, and WND transgenes all resulted in a significant size increase at this time point compared with the no-transgene controls (Fig. 3, A–F and I), whereas the MNK and ALZ transgenes had no positive impact on size (Fig. 3, G–I). Only the WT, DMN, and WND transgenes had an appreciable restorative effect on mouthpart development; therefore the midgut was the focus of initial localization studies.

Expression of Wild Type gATP7-GFP Is Expanded in Response to High Dietary Copper Levels—As the gATP7WT-GFP transgene was shown to be functional in vivo, we next examined the expression and cellular localization of both the wild type and mutant fusion proteins to determine whether the pathogenic mutations were affecting protein localization or stability. It has previously been documented that ATP7 is transcribed extensively in the copper cells of the third instar larval midgut (23), reflecting a key role in copper efflux and dietary copper absorption; therefore the midgut was the focus of initial localization studies.

gATP7WT-GFP expression was monitored in larvae raised on basal medium. Plasma membrane signal was observed in the copper cell region; this GFP signal co-localized with Discs Large (DLG) (Fig. 4, B and C), which is restricted to the apicalolateral membrane. ATP7WT-GFP was also seen in the cells directly anterior to the copper cells; however, this GFP signal appeared to be cytosolic and did not overlap with either DLG (Fig. 4A) or Spectrin (Fig. 4D).

$\alpha$-GFP Western blotting of protein lysates from adult males carrying each transgene (Fig. 4G) revealed considerable variation in the levels of ATP7-GFP produced by each transgene. The WT, DEL, DMN, and WND variants all displayed high molecular mass (>190 kDa) bands of roughly equivalent intensity, whereas the OHS variant produced a faint band of smaller size (~60 kDa), and no GFP fusion protein was detectable from the MNK or ALZ variants.

To determine whether ATP7-GFP localization was affected by dietary copper conditions, third instar larvae were cultured for 24 h on both low (200 μM BCS) and high copper (1 mM CuSO4) media and then dissected, and the midguts were analyzed by fluorescence microscopy. Expression of ATP7WT-GFP was clearly induced in response to increased copper levels. Under low copper conditions, ATP7-GFP is exclusively localized at the basolateral membrane of the copper cells (Fig. 5A). In contrast, when exposed to high copper levels, ATP7-GFP is enriched in the cytosol of cells in the region immediately anterior to the copper cells, referred to as the AC region, with

FIGURE 3. Rescue of $\Delta gATP7$ growth and mouthpart development defects. 24-h-old $X^{\Delta gATP7}Y$ larvae alone (A) or with WT (B), OHS (C), DMN (D), DEL (E), WND (F), MNK (G), or ALZ (H) gATP7 transgene. I, length (mm) of the larvae depicted in A–H (n = 5), including mean and S.D. (error bars) for each genotype. The WT, OHS, DMN, DEL, and WND gATP7 transgenes all restore growth to the $X^{\Delta gATP7}Y$ mutant males, whereas only the WT, DMN, and WND transgenes restore mouthpart development and pigmentation (black arrows in B, D, and F) (**, p value < 0.01; ****, p value < 0.0001; ns, not significant). No TG, no transgene.
weaker basolateral ATP7WT-GFP still observed in the copper cells (Fig. 5B).

Similar to the wild type fusion protein, ATP7\(^{MNK}\)-GFP (Fig. 5, C and D), ATP7\(^{DMN}\)-GFP (Fig. 5, G and H), ATP7\(^{WND}\)-GFP (Fig. 5, I and J), and ATP7\(^{DEL}\)-GFP (Fig. 5, K and L) all localized at the basolateral membrane of the copper cells under both low and high copper conditions (P). The ALZ GFP fusion is not visible at all on low (M) or high copper food (N).

outer basolateral membrane of these cells. This is evident when viewing the midgut both through the middle (transsect; B and E) and at the outer surface (C and F). Scale bar, 65 μm. G, Western blotting analysis using an α-GFP antibody revealed a high molecular mass band (>190 kDa) in adult males with the WT gATP7-GFP transgene that was absent in no-transgene (no TG) controls and present in flies with DEL, DMN, and WND variants. A faint band at ~60 kDa was detected with the OHS variant, and no band was visible with the MNK and ALZ variants. A background band of ~30 kDa was seen in all lanes, serving as a loading control. The blot shown is representative of three independent experiments.

FIGURE 4. Localization of wild type ATP7-GFP at the basolateral membrane of the larval copper cells. Dissected midguts from third instar larvae (anterior to the left) show wild type ATP7-GFP (green) together with α-DLG (red; A–C) or α-Spectrin (red; D–F) and nuclear DAPI stain (blue) in the merged image. In the cells just anterior to the copper cell region, ATP7-GFP does not co-localize with either DLG (A) or Spectrin (D) and appears restricted to the cytosol. In the copper cells, ATP7-GFP localizes with Spectrin (E and F; yellow in the merged image) but not DLG (B and C), indicating that it is restricted to the weaker basolateral ATP7\(^{WT}\)-GFP still observed in the copper cells (Fig. 5B).
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FIGURE 6. Whole mount imaging of ATP7-GFP in first instar larvae. First instar larvae with the WT (A and B), MNK (C and D), or OHS (E and F) gATP7-GFP transgenes at ~24 h PEH raised on low copper (A, C, and E; 200 μM BCS) or 1 mM CuSO4-supplemented (B, D, and F) medium. Minimal ATP7-GFP fluorescence (green) was observed with any of the three transgenes when first instar larvae were raised on a low copper diet. Induction of WT (B) and MNK (D) but not OHS (F) ATP7-GFP in the AC region was observed in response to overnight copper feeding (white asterisks). Visible red fluorescence is from the atp docking site for the transgene.

remained localized at the basolateral membrane of the copper cells under both low and high copper conditions with no induction in the AC region observed under high copper (Fig. 5, E and F). No GFP fluorescence was detectable from the AATP7MNK-GFP transgene on either low or high copper diets (Fig. 5, M and N).

Copper-induced Shift in ATP7 Expression Is Evident Early in Larval Development—To determine whether copper induction of AATP7WT-GFP was detectable earlier in larval development, copper-fed whole mount first instar larvae were compared with those raised on low copper medium. A strong induction of AATP7WT-GFP was observed in the anterior midgut of larvae fed on copper-supplemented medium (Fig. 6B) compared with larvae fed on low copper medium in which minimal AATP7-GFP was detectable (Fig. 6A). Similarly, AATP7MNK-GFP was also only detectable when larvae were exposed to high copper (Fig. 6, C and D). AATP7OHS-GFP was not detectable even on high copper food (Fig. 6, E and F), indicating that the GFP signal seen in the WT and MNK transgenics is from the cells just anterior to the copper cells where no ATP7OHS-GFP induction was observed previously in the third larval instar midgut.

Discussion

AATP7A and AATP7B mutations have previously been modeled in a number of different systems. For instance, mottled and brindled mouse mutants provide differing levels of AATP7a loss of function (25–27); however, these do not replicate human AATP7A disease mutations. Functional analysis of AATP7A/AATP7B mutant proteins has mainly been conducted using cell culture systems, such as cultured fibroblast cells isolated from disease patients (12, 28–32). Although useful, this analysis lacks the physiological and phenotypic data that are possible at the whole organism level. Complementation analysis in yeast has been useful in predicting the successful copper treatment of some milder AATP7A mutations (33) and temperature-sensitive variants (34). The in vivo system described in this study provides the ability to model a large array of AATP7A and AATP7B mutations in a complete multicellular animal.

The ability of gATP7WT-GFP to rescue ΔAATP7 male flies to adulthood demonstrated that this transgene is functional despite the presence of the C-terminal GFP fusion protein. The wild type appearance and fertility of the rescued adults reassures us that any functional deficiencies in this transgene are minor, and the transgene still provides an excellent system in which to examine the effect of pathogenic mutations.

The larval survival experiment enabled a finer discrimination between the rescue activities of the various gATP7 transgenes. ΔAATP7 larvae do not survive beyond 24–48 h after egg hatching (21), a result replicated and refined in this study. The inability of the gATP7MNK-GFP to provide any rescue activity was expected; the corresponding hAATP7A G1330E mutation is associated with classical Menkes disease and is known to disrupt the ATP binding domain of AATP7A, resulting in a complete loss of function (29). In our system, this mutation effectively acts as an AATP7 null mutation.

The ability of gATP7WND-GFP to rescue ΔAATP7 larvae to 72 h after egg hatching and indeed partially to adulthood shows that this transgene is at least semifunctional and that the hAATP7B H1106Q mutation does not abolish AATP7 copper efflux activity. This is consistent with previous results showing that this mutant protein possesses significant residual copper transporting activity (35, 36). Although hAATP7B H1106Q is the most common Wilson disease mutation across a broad range of ethnic populations, patients homozygous for this allele have a slower buildup of copper in the liver than compound heterozygotes and non-H1106Q Wilson disease patients (37), indicating that it is not the most severe Wilson disease mutation.

The fact that the DMN, OHS, and DEL gATP7 transgenes were able to partially rescue ΔAATP7 larvae with increased growth at 24 h after egg hatching indicates that these mutant forms of ATP7 also retain some copper efflux activity. The DMN and DEL transgenics were even able to restore some survival at 48 h PEH. In the case of the hAATP7A P1386S DMN mutation, such weak rescue ability was unexpected as this mutation is associated with late onset motor neuropathy in humans with the mutant protein shown to be stable and retaining 70% of wild type ATP7A copper efflux activity (15, 38). This result was also surprising given that our initial, crude rescue experiments showed that the DMN transgene was capable of rescuing the null mutant to adulthood. The major difference between the crude and quantified rescue experiments was the presence of many wild type sibling larvae in the crude experiment. Possibly, DMN-rescued larvae are very weak, and the presence of healthy siblings boosts their viability by, for instance, breaking up the food medium and making it easier to digest. Notably, two copies of the DMN transgene resulted in viable, fertile adults with no endogenous ATP7 activity.

The hAATP7A S933G mutation is associated with OHS, which is commonly referred to as a milder form of Menkes disease (13, 39). This mutation has been documented as activating a cryptic splice acceptor site in the downstream exon, resulting in aberrant splicing of the AATP7A transcript and a predicted mild loss of AATP7A activity (40). Although intron-exon structure is not conserved between AATP7 and mammalian AATP7A/B, aberrant splicing may still be the cause of the reduced activity of the OHS transgene demonstrated here as only a smaller molecular weight OHS GFP fusion protein was detected by Western blotting.
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The inability of dietary copper supplementation to boost the rescue activity of any of the six mutated ATP7 proteins tested here surprised us. The WND, DMN, OHS, and DEL variants all showed some rescue activity, indicating that these mutations result in ATP7 proteins that retain at least some copper efflux activity. We had predicted that this residual activity might be enhanced by an increase in copper availability but found no evidence for this. The CuSO₄ dose chosen (250 μM) is one previously shown to induce robust MtnB-GFP expression but to have no deleterious effect on the viability of wild type larvae; we previously shown to induce robust MtnB-GFP expression but to have no deleterious effect on the viability of wild type larvae; we wanted to avoid possible copper toxicity.

Future experiments could explore a wider range of CuSO₄ concentrations and also examine whether copper complexes such as Cu-ATSM (diacetylbis(N₄-methyl-3-thiosemicarbazone)coppper(II)) and Cu-GTSM (glyoxalbis(N₄-methyl-3-thiosemicarbazone)coppper(II)), which can bypass normal copper uptake mechanisms, have greater efficacy than CuSO₄. The fact that both copper supplementation and depletion reduced the rescue ability of the WT transgene indicates that achieving optimal copper absorption is difficult and that the gATP7 transgene is not expressed at identical levels to the endogenous ATP7 gene; it may be that excess ATP7 reduces viability under even mild copper supplementation but cannot compensate for severe copper depletion.

Expression analysis revealed that ATP7WT-GFP is found at the basolateral membrane of copper cells in the larval midgut under all copper conditions, whereas a cytosolic GFP distribution is observed in the region directly anterior to the copper cells only in normal and high copper conditions. Previously we have shown that ATP7 is transcriptionally up-regulated by copper in both these regions (23), indicating that ATP7 protein is in fact stabilized specifically in the copper cells in low copper conditions.

We found no evidence of copper-stimulated trafficking of ATP7WT-GFP; in the copper cells, it remains at the basolateral membrane regardless of dietary copper content. ATP7 also does not undergo copper-induced trafficking in Drosophila S2 cells but is located predominantly at the Golgi apparatus with minimal ATP7 detectable at the plasma membrane. ATP7 can, however, traffic from the Golgi apparatus to the basolateral membrane in mammalian cells (22) just as endogenous ATP7A has been shown to shift in mouse intestinal epithelial cells in response to a switch from a low to a high copper diet (41, 42). Whether we simply cannot detect trafficking of ATP7 or whether ATP7 trafficking is actually not required for intestinal copper absorption in flies remains an unresolved question. Possibly, the presence of the GFP tag inhibits the ability of ATP7 to traffic but does not affect its transport activity.

The cytoplasmic distribution of copper-induced ATP7 in the region of the midgut anterior to the copper cells has not been documented before and may represent a novel mechanism for storing excess copper; Drosophila have been shown to rapidly absorb high levels of copper, possibly as a strategy to survive subsequent periods of copper deficiency (43). Further investigation is required to properly determine the role of ATP7 in this region of the midgut. It is plausible that ATP7 may be pumping copper into storage vesicles or into organelles, such as lysosomes, in a role analogous to that found in ATP7B-mediated hepatic copper efflux (44). Our recent examination of midgut metal ion content by synchrotron X-ray fluorescence microscopy found that copper accumulates in this anterior region even in the absence of dietary supplementation (45), supporting the notion that these cells play an important physiological role in global copper homeostasis.

The expression/localization of ATP7OHS-GFP may provide a clue to the nature of the copper induction of ATP7 in the cells anterior to the copper cells. In contrast to wild type and most of the mutant versions, no anterior induction of the ATP7OHS-GFP fusion protein was observed. As the ATP7 transgenes differ only in specific coding region mutations, transcriptional regulation is unlikely to account for this difference. The lack of anterior induction of ATP7OHS may therefore be due to differential stability of the mutant proteins in these cells, although aberrant splicing as shown for the human ATP7A version of this mutation (40) may also be a factor because only a small, fainter band of ATP7OHS-GFP was detected by Western blotting.

The ATP7ALZ mutation was modeled on ATP7B⁸⁸⁴²R, one of the ATP7B alleles found recently to be more frequent in Alzheimer’s disease patients than in an age-matched healthy patient cohort (20, 46), a finding that led to the proposal that defective copper excretion may be a risk factor for AD. The gATP7ALZ-GFP transgene showed only minimal rescue ability, and strikingly no GFP signal was detectable in the larval midgut, indicating that the mutation may either destabilize ATP7 or cause the cleavage of the C terminus containing the GFP protein. This is the first time any functional analysis has been conducted on this mutation, highlighting the power of our experimental system to rapidly characterize the molecular pathology of numerous disease-related ATP7A and ATP7B mutations.

Western blotting revealed strong variation in the level of ATP7-GFP produced in the various transgenic strains. As each transgene was targeted to the same genomic location, this variation is probably due to differences in protein stability. The protein levels correspond in most cases with the rescue ability of each protein. Two interpretations of these data are possible. Loss of copper transport activity may lead to protein degradation, or a reduction in protein stability may contribute to the loss of function caused by a mutation. The levels of protein observed by Western blotting do not correlate with the strength of GFP signal seen in the midgut, suggesting that midgut ATP7 levels are tightly regulated and/or that the GFP signal intensity does not accurately represent protein levels.

The deaarth/absence of ATP7-GFP in low functioning variants such as ATP7ALZ and ATP7MNK indicates that these proteins may be prone to abnormal degradation by the proteasome or lysosome. If this were the case, inhibition of the appropriate degradation pathway could be a means to boost functional protein levels. Although indiscriminate blocking of protein degradation is likely to be detrimental, targeted inhibition of, for instance, a specific E3 ubiquitin ligase might have the potential to increase ATP7 levels while not affecting other critical cellular proteins.

The results presented here indicate that our in vivo gene replacement system is a powerful model to compare the effects of numerous ATP7A/B mutations and polymorphisms on the function of the ATP7 protein. In particular, the larval growth
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and survival assays allow the simple quantitation of different ATP7 protein activities in an invariant genetic background and under tightly controlled environmental conditions.

The ease and speed with which new transgenic Drosophila strains can be generated and the ability to target transgenes to the same genomic location, ensuring equivalent transcription levels, give the fly a significant advantage over comparable experimental systems, such as zebrafish. These benefits must, however, be weighed against the considerable physiological differences between vertebrates and invertebrates. Some of the unexpected findings highlighted here, such as the higher than predicted severity of the DMN mutation and the relatively mild effects of the WND mutation, could be attributed to such differences.

Although our system is restricted to amino acids that are conserved between ATP7A/ATP7B and ATP7, in practice the strong similarity between these proteins means this is a minor limitation. Importantly, the amenability of Drosophila larvae to medium throughput drug screening raises the possibility of using this system to search for compounds able to restore activity to these subfunctional mutant proteins, providing an exciting new way of looking for treatments for these diseases.

Experimental Procedures

Drosophila Stocks—The following fly stocks were used: w^1118 (BL, Bloomington Drosophila Stock Centre, Bloomington, IN; 3605) and double balancer (w; if/CyO; MKRS/TM6b; gift from G. Hime, University of Melbourne, Australia). The ATP7 null mutant line (ΔATP7) has been described previously (21). This X-linked mutation was maintained over an FM7 balancer X chromosome that contains Bar− and yellow− mutations as well as a ubiquitous GFP transgene that allows identification of larvae with/without this balancer chromosome; this GFP expression is much stronger than the gATP7-GFP expression, allowing easy differentiation between these two transgenes by fluorescence microscopy.

Drosophila Maintenance—All Drosophila stocks and crosses were maintained on standard medium at 25 °C unless stated otherwise. Standard medium was supplemented with either BCS (Sigma-Aldrich) to make copper-deficient food medium or copper sulfate (CuSO4·5H2O; Sigma-Aldrich) to make copper-supplemented medium.

Generation of a Genomic, GFP-labeled ATP7 Rescue Transgene—Recombineering was used to add an in-frame enhanced GFP tag to the C terminus of a full-length genomic fragment of ATP7 contained within a large bacterial artificial chromosome construct (Pacman clone CH3222-77E05), removing the gene’s endogenous stop codon. To facilitate in vitro mutagenesis of the ATP7 fragment, a minimal rescue construct was generated using the following primers to amplify the full-length genomic ATP7-GFP fragment: ATP7-GFPF (CCGAAGCTTGTACCCTGCGCGCTGA) and ATP7-GFPR (GGCCCGTACACAACCCTGTTAC). These primers contained 3′ HindIII and 5′ KpnI recognition sites, respectively, allowing the fragment to be cloned into a pUAST vector backbone while ablating the 5xUAS sequence.

Site-directed Mutagenesis—Six different missense mutations corresponding to ATP7A and ATP7B pathogenic mutations causing Menkes disease, OHS, DMN, and Wilson disease and a single AD susceptibility allele were chosen for site-directed mutagenesis of the gATP7-GFP transgene (Table. 1). The pUAST-gATP7-GFP-attb expression vector was found to be too large for efficient mutagenesis, so two gATP7-GFP subfragments were subcloned into pBluescript II SK. The QuikChange II-XL kit (Agilent Technologies) was utilized for site-directed mutagenesis following the method outlined in the manufacturer’s instructions and using primers designed for each specific mutation/deletion (primer sequences available on request). Each introduced mutation/deletion was confirmed by sequencing. ATP7 fragments containing the mutations of interest were then reintegrated into the pUAST-gATP7-GFP-attb vector backbone (Table 1).

Generation of Transgenic Flies—pUAST-gATP7-GFP-attb wild type and mutant constructs were injected into 86Fb-attP embryos with genomic integration occurring via the PhiC31 recombinase system. Microinjections utilized an Eppendorf Femtojet apparatus with Femtotips II prepulled glass needles. The 86Fb-attP site contains a 3xP3-RFP transgene, which is expressed in the brain and hindgut, allowing ready identification of larvae/adults containing this integration site.

Drosophila Survival Experiments—In all survival experiments, crosses were set up in cages with >50 virgin females and 20–25 males. To assess adult survival, replicates of 20 first instar larvae were placed onto basal medium or medium supplemented with CuSO4 or BCS. Successful adult survival was classified as the ability of the adults to eclose from their pupal cases.

To assess the survival rate of larvae, ΔATP7ΔF477♂ females were crossed to X^F477; gATP7-GFP males, and subsequent ΔATP7ΔF477♂; gATP7-GFP male first instar larvae were selected by 1) the absence of strong ubiquitous GFP from the FM7 balancer chromosome and 2) the presence of RFP from the gATP7 transgene attP insertion site. Replicates of 10 first instar larvae were transferred into wells of a DeepWell (Nunc) 96-well plate containing instant fly food with or without additional CuSO4. At 24 h PEH, each well was flooded with a 30% sucrose solution. At this time point, survival was assessed by manual observation of the larvae in each well; larvae showing independent movement were reported as alive. Survival in these same wells was assessed again at 48 and 72 h PEH. Wild type control larvae showed no decrease in survival for up to 96 h in the sucrose solution.

Microscopy—Whole midguts from wandering third instar larvae were dissected in phosphate-buffered saline (PBS) and then mounted onto glass slides in Permafluor (Thermo Scientific). Fluorescence imaging was performed using a Zeiss Axio Imager fluorescence microscope. Image processing was completed using Adobe Photoshop 6.0. For whole mount imaging, first instar larvae of the correct age were washed in PBS and then mounted in glycerol. Slides were left at 4 °C for 30–60 min to immobilize the larvae. Larvae were then imaged with a fluorescence dissecting microscope. Monoclonal α-Spectrin and α-DLG primary antibodies used for ATP7-GFP co-localization studies were obtained from the Developmental Studies Hybridoma Bank and used at 1:500 dilution followed by α-mouse Alexa Fluor 568 secondary antibody (Molecular
Probes). Imaging was performed using a spinning disk confocal microscope (Olympus CV1000) and a 20× dry objective.

Western Blotting—For Western blotting, 12 adult males/ genotype were lysed in 100 μl of 1% Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton-X, 10% glycerol, complete EDTA-free protease inhibitor mixture (Roche Applied Science)). Equal volumes of lysate were separated by SDS-PAGE (Any kDa TGX, Bio-Rad). ATP7-GFP mixture (Roche Applied Science)). Equal volumes of lysate were separated by SDS-PAGE (Any kDa TGX, Bio-Rad). ATP7-GFP was detected using rabbit α-GFP (Molecular Probes; 1:10,000) and HRP-conjugated α-rabbit secondary antibody (Southern Biotech; 1:10,000). Immunoblots were developed using ECL Prime (GE Healthcare) and imaged using a chemiluminescence microscope (Olympus CV1000) and a 20× dry objective.

Statistical Analysis—Quantitative data were collated and analyzed with Prism 6 (GraphPad Software) and are presented as the mean with S.E. For the adult survival assays on basal medium alone and for the larval length analysis, an initial one-way analysis of variance comparing the mean of each column with the mean of all other columns was carried out using the Dunnett test to correct for multiple comparisons. For the adult and larval survival assays where both basal and CuSO4-supplemented media were tested, a two-way analysis of variance comparing the mean of each column with the mean of all other columns was carried out using the Tukey test to correct for multiple comparisons. In each case, the p value for each pairwise comparison was corrected for multiple comparisons.

Author Contributions—S. W. M. carried out most of the experimental procedures and wrote the manuscript together with R. B. J. W. generated the initial gATP7WT-GFP construct by recombining. R. B. performed the final adult and larval survival assays.

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