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Translational read-through of a nonsense mutation in ATP7A impacts treatment outcome in Menkes disease

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Abstract

Protein translation ends when a stop codon in a gene's messenger RNA transcript enters the ribosomal A site. Mutations that create premature stop codons (nonsense mutations) typically cause premature translation termination. An alternative outcome, read-through translation (or nonsense suppression), is well known in prokaryotic, viral, and yeast genes but has not been clearly documented in humans except in the context of pharmacological manipulations. Here, we identify and characterize native read-through of a nonsense mutation (R201X) in the human copper transport gene, ATP7A. Western blotting, *in vitro* expression analyses, immunohistochemistry, and yeast complementation assays using cultured fibroblasts from a classical Menkes disease patient all indicated small amounts of native $ATP7A_{R201X}$ read-through and were associated with a dramatic clinical response to early copper treatment.

Introduction

In eukaryotic genes, stop codons, and mutations that produce them, signal proper or premature termination of protein translation, respectively1. Selenoproteins, in which the stop codon UGA encodes selenocysteine2, pose rare exceptions to this rule. Another alternative outcome, read-through translation, is well known in prokaryotic and viral genes3–6, mediated by tRNA mispairing, suppressor tRNAs, or ribosomal jumping but is seldom observed in eukaryotes7–9.

Menkes disease is a X-linked recessive disorder of copper transport caused by diverse mutations in a copper transporting ATPase, ATP7A10. Newborns with this typically fatal neurodegenerative condition may respond well to early copper treatment if their mutation does not completely abrogate ATP7A function11. We previously proposed that internal initiation or translation re-initiation could mediate favorable response to treatment in the context of a premature stop signal in the 5' region of this locus12, and *in vitro* evidence for re-initiation was recently reported in association with deletion of ATP7A exons 3 and 413.

In the current study, we evaluated the molecular mechanisms underlying an unexpectedly successful response to early copper treatment in a classical Menkes disease patient with a nonsense mutation in the amino terminal region of ATP7A.

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Subjects and Methods

Case Report

The patient was born at 37½ weeks gestation by normal spontaneous vaginal delivery with a birth weight of 3.1 kg. A cephalohematoma was noted over the left parieto-occipital scalp. There was a slight amount of hair present on the sides of the head and the hair was light in color with a fine texture. Low temperature, once on the first afternoon of life, and hyperbilirunemia (peak blood concentration 15.5 mg/dl) were noted and resolved. The family history was notable for a prior sibling affected with Menkes disease who was profoundly neurologically delayed at the time of his death at 6 months of age following a sudden and massive gastrointestinal hemorrhage14. Given that recurrence risk for subsequent male offspring was potentially as high as 50%, the subject of this report underwent careful biochemical evaluation in the immediate postnatal period and was diagnosed has having Menkes disease in the first week of life based on elevated placental copper level (J. Centeno, Ph.D., Armed Forces Institute of Pathology, Washington, DC), and abnormal plasma and cerebrospinal fluid catecholamine levels (C. Holmes, D. Goldstein, NIH). The infant began copper injections under an Institutional Review Board-approved protocol (ClinicalTrials.gov identifier NCT00001262), at eight days of age, with the informed consent of his parents. The study drug was copper histidine, FDA Investigational New Drug (#34,166) holder: SG Kaler, prepared by the NIH Pharmaceutical Development Service, as previously described15. The dose regimen was 250 μg by subcutaneous injection twice daily to age 1 year, and 250 μg once daily thereafter to age 3 years when it was stopped, as indicated by the protocol.

Tissue Culture

R201X and normal fibroblast cultures were established from explant cultures of full-thickness, skin punch biopsies. Cells were grown in MEM-Alpha medium (InVitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 2 mM l-glutamine, and were used before passage 10.

Mutation Analysis and DNA sequencing

Mutation analysis was performed as previously described16.

Antibody Production

Polyclonal antisera against ATP7A was obtained by immunizing rabbits with peptides containing amino acid residues 146–160 (N-terminal antibody) and 1483–1500 (C-terminal antibody).

Western blot analysis

Fibroblast microsomal protein fractions were obtained by ultracentrifugation (10,000g × 60m) following cell lysis and Western analyses were performed as previously described17. 100 μg microsomal protein was loaded in each lane.

Densitometric Quantitation

Western blots were scanned to create digital grey-scale images, and imported into ImageJ (http://rsbweb.nih.gov/ij/download.html) for densitometric analysis, as previously described17.

Confocal Microscopy

Immunohistochemical analyses were performed as previously described11.

Yeast complementation

Functional copper transport associated with normal, R201X, and Q1385X ATP7A alleles was assessed in a yeast complementation assay, as previously described11,17,18.

Timed Growth Assay

A timed yeast growth assay to estimate the percentage of copper transport by the R201X mutant allele relative to normal was performed as previously described18.

In vitro Expression

Reporter genes were constructed to mimic the *in vivo* molecular context, generating peptides 249 amino acids in length that spanned the region of the nonsense mutation. These cDNAs were cloned into pTR-UF11 to express them from the CMV enhancer/chicken ß-actin promoter. Reporter constructs were transfected into HEK-293 cells by standard calcium phosphate transfection. Cells were harvested 72 hours post-transfection and mechanically homogenized in CelLytic M Cell Lysis Reagent (Sigma). Western blots were performed with an ATP7A N-terminal antibody.

Results

Clinical Outcome

At 2, 8 and 13 months of age, the patient's serum copper levels were 79, 129, and 146 μg/dl (normal range 70–150 μg/dl), respectively. He had no seizures or electroencephalographic abnormalities, and walked independently at 16 months of age. Brain magnetic resonance imaging at 2 and 3 years of age showed appropriate myelination and no cerebral or cerebellar atrophy (Fig. 1a, top panel). Copper injection treatment was discontinued at 3 years of age, as called for by the clinical trial. On follow-up evaluation at 9 ½ years of age, the patient was a 4th grade student earning above average grades in regular (not special needs) classes and had no physical limitations. He was an intelligent conversationalist, and his neurological examination was non-focal. When seen at 11½ years of age, he continued to appear healthy and manifested no cognitive or neurological problems. Skull radiographs disclosed occipital exostoses (Fig. 1a, bottom panel), a phenotypic hallmark in long-surviving Menkes disease patients19.

Mutation Analysis and Characterization

We identified a novel C to T transition at nucleotide 746 in exon 3 of the ATP7A coding sequence, changing codon 201 from CGA to UGA (Fig. 1b) in two affected members of a classical Menkes disease family. In the context of an excellent neurologic outcome in the younger member whom we diagnosed at birth and treated with copper injections for the first three years of life, we suspected that translation re-initiation downstream of R201X produced a small quantity of a truncated but partially functional copper transporter.

We performed Western analyses of microsomal protein extracts from R201X patient fibroblasts using antibodies generated against amino- and carboxyl-terminal segments of human ATP7A (see model for antibody location, Fig. 2). We predicted detection of truncated forms with the latter anti-sera. Unexpectedly however, both amino- and carboxyl-terminal antibodies detected small amounts of the full-length (178 kDa) ATP7A protein in the R201X fibroblasts, and no products of size consistent with re-initiation closely downstream of R201X (Fig. 3a). Re-initiation at downstream AUG codons predicts that such smaller products would be detectable only with the C-terminal antibody, and the sole possible candidate on Western blots was approximately 64 kDa in size (Fig. 3a, right panel, right lane) which would lack many of the critical functional domains of the normal 178 kDa

ATP7A (Fig. 2). While these results render downstream re-initiation unlikely, our findings are consistent with translational read-through of R201X through which small quantities of full length ATP7A detectable with either antibody are generated. Reverse transcription-polymerase chain reaction of the relevant ATP7A segment using total RNA from R201X fibroblasts, and sequencing confirmed the C to T transition in cDNA, excluding RNA editing as an explanation for the presence of full length protein in these cells. Densitometric quantitation of the two Westerns, corrected for loading, indicated that R201X cells produced 5.7% (detected by the N-terminal antibody) and 6.4% (detected by the C-terminal antibody) of the wild type amount of full length ATP7A.

Immunohistochemical studies also detected trace amounts of an anti-ATP7A reacting material in R201X cells (Fig. 3b, lower panels). The perinuclear staining pattern in these cells was comparable in quality to that in wild type fibroblasts, and consistent with the known trans-*Golgi* localization of ATP7A20.

Next, we assessed whether functional copper transport was associated with the R201X ATP7A allele, utilizing a yeast complementation assay (Fig. 4a). The *S. cerevisiae* strain ccc2Δ is unable to grow on nutritionally restricted (low copper and low iron) media, however transformation with wild type ATP7A cDNA restores normal growth. Transformation with R201X cDNA complemented ccc2Δ sufficiently for partial restoration of growth in both solid and liquid media. Using a timed growth assay that we described previously15, we calculated the estimated residual copper transport capacity of the R201X allele to be 5–10% of wild type (Fig. 4b).

Finally, we performed *in vitro* expression experiments to express the initial 249 amino acids of ATP7A, using cDNA constructs containing either the wild type or mutant sequence for residue 201 in HEK-293 cells by transfection with the expression vector pTR-UF11 (Fig. 5a). Western analysis showed the predicted 28kD peptides in both, although the amount was much lower in the mutant (Fig. 5b, left panel), as expected. The mutant construct also showed a prominent 22 kDa species, corresponding to the truncated peptide produced when the R201X nonsense codon was not suppressed.

Discussion

Our patient's excellent treatment response, in conjunction with detection of full length ATP7A in R201X fibroblasts by Western blotting, discernible immunofluorescence on confocal microscopy of R201 cells, functional copper transport by the mutant allele in a yeast complementation assay, and the *in vitro* expression results all support the conclusion of $ATP7A_{R201X}$ read-through. Since Menkes disease is a X-linked recessive disorder, partial expression of the full length gene product by a second mutant allele (compound heterozygosity) is not possible and does not explain the finding.

In mammalian selenoproteins, such as glutathione peroxidase and iodothyronine 5' deiodinase, UGA encodes selenocysteine instead of causing translation termination2. Since *S. cerevisiae* does not contain this amino acid, the yeast complementation results presented here suggest that an alternative mechanism is active. In the rabbit β-globin gene, variable ribosomal hopping and tRNA mispairing are associated with normal stop codon read-through9. Amino acid sequencing of the 28 kDa peptide expressed by the R201X reporter construct will be required to clarify these or other mechanisms as the precise means of $ATP7A_{R201X}$ read-through. Our experiments suggest that the amount of nonsense suppression is not high, only about 6% compared to normal, and we were unable to isolate the R201X read-through product in a quantity and purity sufficient for this purpose.

Table 1A summarizes examples of UGA nonsense codon read-through in eukaryotes, with emphasis on the associated sequence contexts[7–9]. The $ATP7A_{R201X}$ context resembles that for native read-through in the yeast genes STE6 and ADE2, and in rabbit beta-globin, by possessing a cytosine/adenine dinucleotide upstream of the stop codon (−3 and −2 positions). For ADE2, this sequence was associated with read-through efficiency ranging from 10–16%[8]. Our findings lend support to the concept that unique 5' sequences have a critical role in nonsense suppression and that mRNA structure, through interaction with the ribosome, may modulate competition between eukaryotic release factors and suppressor tRNAs. We reviewed the 5' sequence context for eight ATP7A nonsense mutations that we identified in unrelated Menkes disease patients, and none shared the R201X sequence context (Table 1B).

Regardless of the precise mechanism, read-through of $ATP7A_{R201X}$ in this instance was directly relevant to a remarkable treatment response in a patient with Menkes disease, a neurogenetic disorder that is often lethal. Early diagnosis and treatment with copper injections, when associated with missense or splicing mutations that retain some copper transport capacity, may greatly improve clinical outcomes in this disease[11]. While the successful clinical outcome reported here involved an unusual example of native translational read-through, it highlights the distinct possibility that aminoglycosides or alternative agents that promote read-through[21–24] may be useful approaches for other Menkes disease patients with nonsense mutations.

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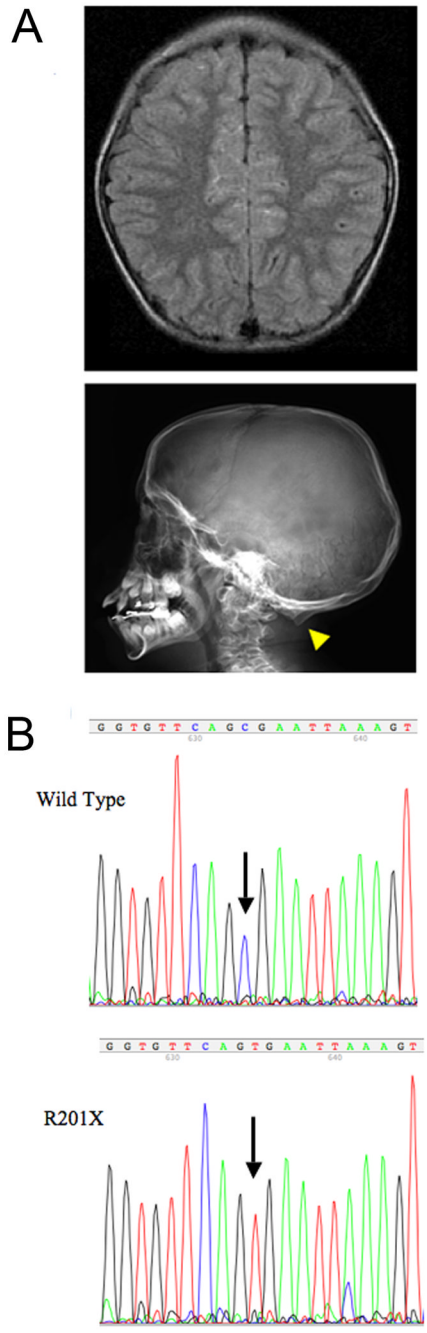

Figure 1. Molecular and Radiologic findings in Patient with Menkes disease

A. Upper panel: Axial flair magnetic resonance image of the patient's brain at 3 years of age shows normal volume and myelination (hypointense signal). (b) Lateral skull radiograph at age 11.5 years shows occipital exostoses (arrowhead). B. Sequence of cDNA illustrating a cytosine (C) to thymine (T) transition producing the R201X mutation in ATP7A.

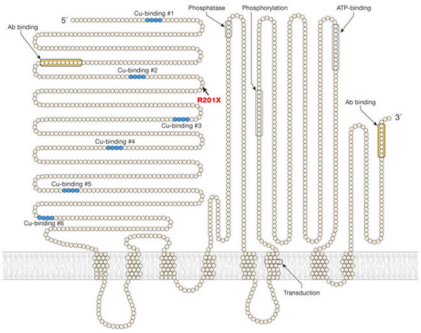

Figure 2. Model of the ATP7A Copper Transporter

The R201X mutation is located between the second and third copper-binding motifs. The amino- and carboxy-terminal antibody binding locations are noted (orange color).

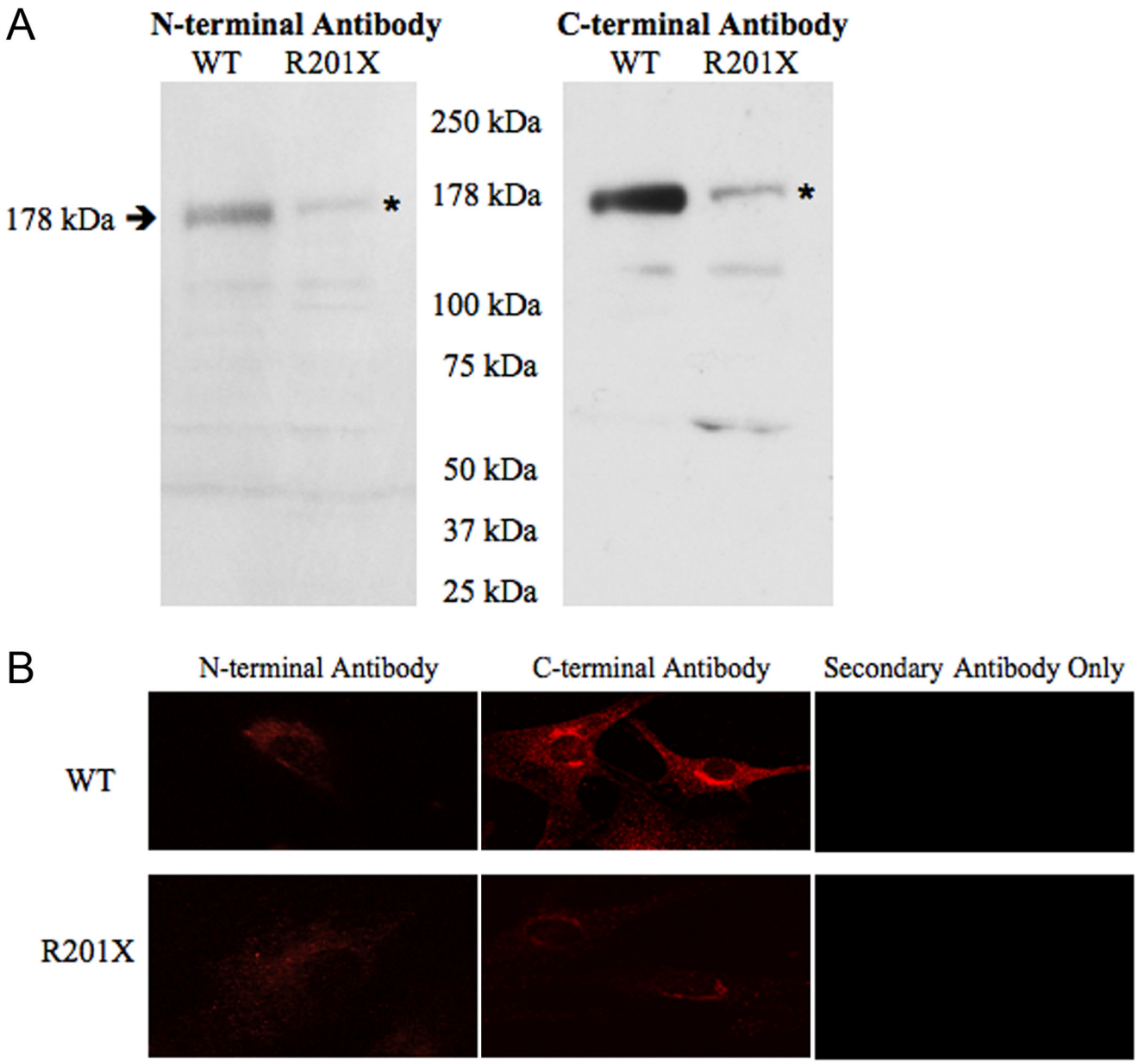

Figure 3. Evidence of Translational Read-through of R201X in ATP7A

A. Western blots of fibroblast microsomal protein from a normal (WT) control male and Menkes disease patient with the R201X mutation show that the full length (178 kDa) gene product is detected with an amino-terminal antibody generated against amino acids 146–160 of ATP7A (left hand panel), as well as with a carboxy-terminal antibody against residues 1479–1500 of ATP7A (right hand panel). The quantity is reduced in the R201X cells (note asterisks). The C-terminal antibody gives a stronger signal than the N-terminal antibody. B. Confocal microscopy of fibroblasts from normal (WT) control (top panels) and R201X patient (bottom panels) stained with N-terminal and C-terminal antibodies to ATP7A, and with Texas-Red labeled anti-rabbit IgG secondary antibody alone, as a negative control. The normal cells show perinuclear staining consistent with trans-*Golgi* localization, a pattern also seen in R201X cells at much lower intensity. The C-terminal antibody gives a stronger signal than the N-terminal antibody.

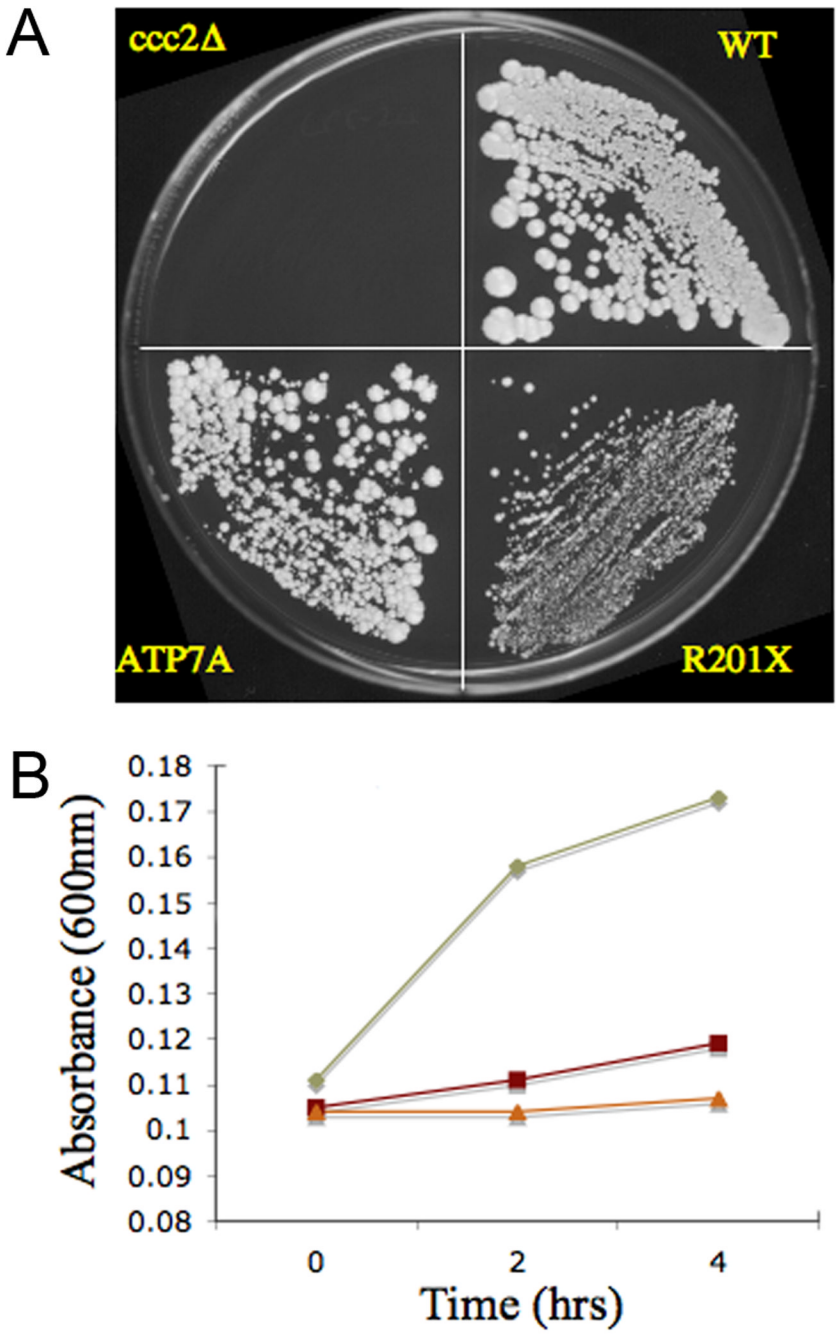

Figure 4. Yeast Complementation Documents Functional Copper Transport Associated with R201X

A. On copper/iron-deficient media, the copper transport mutant ccc2Δ fails to grow, whereas the WT strain grows normally. Transformation of ccc2Δ with the R201X mutant allele restores growth although less robustly than when transformed with the wild type ATP7A. B. Timed growth assays18 estimate R201X growth in the range of 5–10% compared to wild type (WT). Diamond = wild type, Square = R201X, Triangle = ccc2Δ.

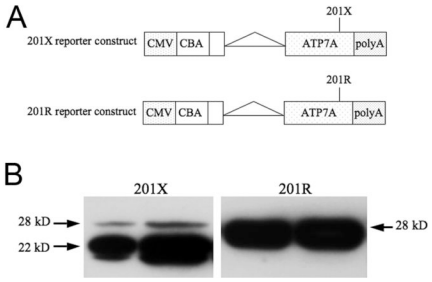

Figure 5. *In Vitro* Expression Analyses of 201X Indicate Full-length and Truncated ATP7A Peptides

A. Reporter constructs for 201X and 201R in the expression vector pTR-UF11. CMV = cytomegalovirus enhancer; CBA = chicken ß-actin promoter; ATP7A = cDNA sequence encoding the initial 249 amino acids of ATP7A; polyA = polyadenylation sequence. B. Western blots of HEK-293 protein following transient expression detect the full length peptide (28 kDa) using a N-terminal ATP7A antibody with both constructs, as well as a smaller form (22 kDa) corresponding in size to the prematurely terminated peptide (200 amino acids) from the mutant construct.

Table 1

Sequence Contexts Associated with Eukaryotic UGA Read-through

A. Sequence Contexts Associated with Eukaryotic UGA Read-through

Gene	Organism	Sequence
STE67	*S. cerevisiae*	GGG CAA **UGA** CAA AGA
ADE28	*S. cerevisiae*	ACC CAA **UGA** CAG UUA
Beta globin9	*Oryctolagus cuniculus*	UAC CAC **UGA** GAU CUU
$ATP7A_{R201X}$	*H. sapiens*	GUU CAG **UGA** AUU AAA

B. Sequence Contexts Associated with Other Nonsense Mutations in ATP7A

Gene	Mutation	Sequence
ATP7A	K155X	CUG GAG **UAA** AAG UCA
	Q168X	AUG GCU **UAA** GCU GGU
	R409X	UCC AUA **UGA** GUC UCC
	Y594X	AUC CUA **UAG** UGC UCC
	R645X	CAU AAA **UGA** GAA AUA
	R795X	CUA GGC **UGA** UGG CUG
	R980X	AUC UCC **UGA** ACA GAA
	Q1385X	GUU UUG **UAG** CCC UGG

All stop codons are shown in bold.

Conforming bases in the read-through sequence contexts are shown in red.