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ATP7A trafficking and mechanisms underlying the distal motor neuropathy induced by mutations in ATP7A

Ling Yi and Stephen Kaler

Section on Translational Neuroscience, Molecular Medicine Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

Abstract

Diverse mutations in the gene encoding the copper transporter ATP7A lead to X-linked recessive Menkes disease or occipital horn syndrome. Recently, two unique ATP7A mutations, T994I and P1386S, were shown to cause isolated adult-onset distal motor neuropathy. These mutations induce subtle defects in ATP7A intracellular trafficking resulting in preferential accumulation at the plasma membrane compared to wild-type ATP7A. Immunoprecipitation assays revealed abnormal interaction between ATP7A^{T994I} and p97/VCP, a protein mutated in two autosomal dominant forms of motor neuron disease. Small-interfering RNA knockdown of p97/VCP corrected ATP7A^{T994I} mislocalization. For ATP7A^{P1386S}, flow cytometry documented that non-permeabilized fibroblasts bound a C-terminal ATP7A antibody, suggesting unstable insertion of the 8th transmembrane segment due to a helix-breaker effect of the amino acid substitution. This could sabotage interaction of ATP7A^{P1386S} with adaptor protein complexes. These molecular events appear to selectively disturb normal motor neuron function and lead to neurologic illness that takes years and sometimes decades to develop.

Keywords

ATP7A; distal motor neuropathy; p97/VCP; adaptor protein complexes

Copper homeostasis and ATP7A

As a redox-active metal that is essential for oxidation/reduction reactions, copper plays an important role in normal biological function, mainly through the activities of enzymes that require copper as a cofactor for normal function. These cuproenzymes include cytochrome c oxidase (CCO), dopamine β-monooxygenase (DBM), superoxide dismutase (SOD), and peptidylglycine α-amidating monooxygenase (PAM). Regulated copper homeostasis is necessary for normal heart development and CNS and liver function, as well as lipid metabolism, inflammation, and resistance to chemotherapeutic drugs.¹

Address for correspondence: Ling Yi, Ph.D., Section on Translational Neuroscience, Molecular Medicine Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Building 10; Room 10N313, 10 Center Drive MSC 1853, Bethesda, Maryland 20892-1853. yil@mail.nih.gov.

Copper is taken up into cells through high-affinity copper transporter 1 (CTR-1) or divalent metal transporter 1 (DMT-1) on the plasma membrane. Inside cells, copper is bound by chaperone proteins such as CCS, Cox17, Cox11 and Atox1. The copper ATPase ATP7A plays an important role in intracellular copper homeostasis both by pumping copper into the Golgi compartments of cells, and by removing excess copper via relocation to the plasma membrane. The *ATP7A* gene is located on the X chromosome, and mutations in it lead to Menkes Disease, occipital horn syndrome (OHS), or isolated distal motor neuropathy.²⁻⁴

ATP7A is a member of the P1B-type ATPase family. Most P1B ATPases are heavy metal transporters and play important roles in regulating the intracellular metabolism related with heavy metals such as Cu, Zn, Co, Cd, and Pb. ATP7A has eight transmembrane domains, and both its bulky N-terminal and short C-terminal segments locate to the cytosol. The N-terminus of ATP7A harbors six copper-binding domains that receive copper from the copper chaperone ATOX1.⁵ A protein model based on the crystal structure of a bacterial homolog, CopA, suggests that the second transmembrane domain forms a platform via a glycine-glycine kink (Fig. 1) that accepts copper from the copper-binding domains.⁶ Subsequently, ATP7A transfers copper through a transmembrane channel, pumping it across to the other side.

ATP7A trafficking

The copper efflux function of ATP7A correlates with its intracellular trafficking. ATP7A continuously recycles between the Golgi and the plasma membrane. At basal copper concentrations ($\approx 0.5 \mu\text{M}$), ATP7A localizes to the *trans*-Golgi network (TGN). When intracellular copper concentration increases, ATP7A exits the TGN and migrates to the plasma membrane to remove excess copper from the cell. After releasing the copper, ATP7A is returned to the TGN.^{5,7}

Previous work has focused on the roles of specific motifs and domains in ATP7A. It was reported that (1) a 38 amino acid segment in transmembrane domain 3 is associated with movement from the endoplasmic reticulum to the TGN;⁸ (2) the metal-binding sites in the N-terminus mediate movement from the TGN to the plasma membrane;⁹⁻¹¹ (3) the C-terminal dileucine motif (amino acid residues 1487–1488) regulates endosomal retrieval of ATP7A from the plasma membrane;^{8,12} (4) the CPC motif in the 6th transmembrane domain (residues 1000–1002) enables copper-induced relocation from post-Golgi vesicles to the plasma membrane;¹³ (5) a phosphorylation motif (DKTG; residues 1044–1047) regulates copper-induced relocation from post-Golgi vesicles to the plasma membrane, whereas a phosphatase motif (LITGEA; residues 873–878) regulates endosomal retrieval of ATP7A from the plasma membrane;¹³ and (6) a C-terminal PDZ motif (DTAL; amino acids 1497–1500) mediates basolateral (instead of apical) localization of ATP7A in polarized cells.¹⁴

More recent work has begun to explore the components and regulators in the trafficking machinery. Pascale *et al.* reported that endosomal trafficking of ATP7A was mediated by vesicles containing the Rab7 and Rab5 GTPases.¹⁵ Holloway *et al.* described the role of ADP-ribosylation factor (Arf1) in the biogenesis of Cu-induced ATP7A trafficking.¹⁶ Subsequent work by the latter group suggested involvement of Rab11 as well as adaptor

protein complexes 1 and 2 in ATP7A trafficking.¹⁷ Although this previous work has contributed to clarification of ATP7A trafficking, the precise details remain unclear.

ATP7A and distal motor neuropathy

Mutations in ATP7A lead either to Menkes Disease, a milder variant called occipital horn syndrome, or a phenotypic variant more recently identified by Marina Kennerson and James Garbern that involves a later-onset isolated X-linked distal motor neuropathy (DMN).² This latter condition is referred to by some as X-linked spinal muscular atrophy type 3 (SMAX-3). Affected subjects with the latter phenotype were identified from two large families. One, from North America and Europe, carries the P1386S mutation in *ATP7A*, whereas the other family, from Brazil, harbors the T994I mutation. The two families each demonstrated X-linked recessive inheritance (only males affected) of a distal motor neuropathy phenotype defined electrophysiologically by decreased motor action potentials, with normal nerve-conduction velocities and limited or no sensory involvement. Affected family members showed late onset of symptoms between 5 and 50 years of age with progressive weakness in their feet and legs and wasting of hand muscles.

Compared to patients with Menkes disease and occipital horn syndrome, these distal motor neuropathy patients had normal serum copper levels and normal plasma neurochemical ratios, reflecting normal DBH activity. Western blot analyses with the patients' fibroblasts shown that ATP7A^{T994I} and ATP7A^{P1386S} mutant proteins were the normal size and that their expression quantity was equivalent to the wild-type protein in normal fibroblasts. A yeast complementation assay was also used to evaluate the copper-transport function of the mutants. In *Saccharomyces cerevisiae*, Ccc2p, an ATP7A homolog, delivers copper to the multi-copper oxidase Fet3p that is required for high-affinity iron uptake. Thus Ccc2p plays a role in copper and iron homeostasis, and the Ccc2p protein knockout strain *ccc2*⁻ does not grow under iron-limited conditions.¹⁸ When Ccc2p function was complemented by transforming the *ccc2*⁻ strain with the human motor neuropathy *ATP7A* alleles, the data revealed complementation of $\approx 80\%$ of wild-type function for ATP7A^{P1386S} and $\approx 73\%$ of wild-type function for ATP7A^{T994I}. These results correlated with the clinical observation that distal motor neuropathy patients have a normal biochemical phenotype in terms of copper metabolism.¹⁹

Taken together, these clinical and biological findings suggested that ATP7A^{P1386S} and ATP7A^{T994I} do not affect global copper metabolism as do other ATP7A molecular defects, and that mechanisms apart from copper deficiency underlie this unique phenotype.

DMN-related ATP7A alleles demonstrate abnormal intracellular localization and trafficking

The ATP7A^{T994I} mutation affects the sixth transmembrane segment, whereas ATP7A^{P1386S} affects the eighth transmembrane segment (Fig. 1). In the ATP7A structural model based on the crystal structure of CopA, these two residues are closely aligned.^{6,19}

As an initial approach to dissect the underlying mechanism(s) of DMN related to ATP7A^{T994I} and ATP7A^{P1386S}, we studied the localization of these alleles. As mentioned previously, ATP7A normally resides in the TGN under basal copper conditions, and migrates to the plasma membrane only when intracellular copper concentration rises. However, immunofluorescence staining of ATP7A mutant alleles in fibroblasts from the ATP7A-related neuropathy patients showed that ATP7A^{T994I} and ATP7A^{P1386S} did not fully localize to the TGN under basal copper conditions (Fig. 2). Total internal reflection fluorescence microscopy (TIRFM) revealed the steady-state shift of the mutant alleles to the plasma membrane (Fig. 2). In HEK293T cells, overexpressed Venus-tagged ATP7A^{T994I} and ATP7A^{P1386S} were clearly found on the plasma membrane; statistical analysis demonstrated that only $\approx 30\%$ of ATP7A^{T994I} and $\approx 20\%$ of ATP7A^{P1386S} localized to the TGN. The localization of ATP7A^{T994I} and ATP7A^{P1386S} was further studied in trafficking assays. When transfected HEK293T cells were treated with 100 μM CuCl₂ for 3 hours followed by careful washing and 4 hours incubation with cycloheximide and the copper chelator bathocuproinedisulfonic acid (BCS), we documented impaired endocytic retrieval of the mutant alleles from the plasma membrane to the TGN.¹⁹

ATP7A^{T994I} interacts with valosin-containing protein (p97/VCP)

Immunoprecipitation followed by mass spectroscopy revealed that a 97 kD protein named valosin-containing protein (p97/VCP) is a specific binding partner for ATP7A^{T994I}.¹⁹ p97/VCP belongs to the AAA+ ATPase family, is ubiquitously expressed, and performs multiple functions. p97/VCP is known to work in endoplasmic reticulum-associated degradation (ERAD) to remove improperly folded proteins from the ER to the cytosol for degradation. It associates with polyubiquitinated proteins such as 26S proteasome proteins, ubiquitin ligase (gp78, Ufd2), and deubiquitinating enzymes (VCIP135, ataxin 3). p97/VCP may help coordinate the activity of these different factors and participate in the delivery of ubiquitin-conjugated proteins to the proteasome.²⁰ Notably, mutations in p97/VCP cause two different autosomal dominant motor neuron diseases, inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD), and familial amyotrophic lateral sclerosis (ALS).²¹ ALS is a motor neuropathy with lower motor neuron symptoms similar to those seen in the ATP7A distal motor neuropathy patients.

As described earlier, ATP7A^{T994I} patient fibroblasts express equal amounts of ATP7A protein as normal fibroblasts. ATP7A^{T994I}-transfected Hek293T cells did not manifest increased expression of BiP or the XBP-1 splice variant, hallmarks of the mammalian misfolded protein response. These facts suggested that the interaction between ATP7A^{T994I} and p97/VCP was not related to misfolding of the mutant protein.

The abnormal interaction of ATP7A^{T994I} with p97/VCP may reduce the pool of active p97/VCP available for its normal cellular functions in ATP7A^{T994I} patient fibroblasts or transfected Hek293T cells. To evaluate the possible role of aberrant allosteric p97/VCP interactions in ATP7A^{T994I} intracellular mislocalization, small interfering RNA (siRNA) knockdown assays were performed to deplete the expression of p97/VCP in transfected Hek293T cells. The results demonstrated that knock-down of p97/VCP could restore the proper localization of ATP7A^{T994I} at the TGN.¹⁹ While further investigations are needed

and in are progress, the discovery of abnormal interaction between p97/VCP and ATP7A^{T994I} opens the gate to clarification of the precise mechanism(s) of ATP7A-related distal motor neuropathy.

ATP7A^{P1386S} and distal motor neuropathy

The other mutation (P1386S) in *ATP7A* that causes distal motor neuropathy is located near the last transmembrane segment that connects to the short C-terminal tail. The ATP7A C-terminal tail is essential for normal ATP7A trafficking, since it contains a dileucine motif (LL, positions 1477–1478) required for copper-dependent ATP7A trafficking;¹² it also harbors a PDZ motif (DTAL; positions 1497–1500) that was reported to be important for basolateral (instead of apical) localization of ATP7A in polarized epithelial cells.¹⁴ As the P1386S mutation is located very close to the C-terminal tail, we speculated that the proline to serine mutation might have a helix-breaker effect and destabilize insertion of the eighth transmembrane domain into the plasma membrane. A consequence would be partial mislocalization of the C-terminal tail from its normal cytosolic location. To test this hypothesis, we performed fluorescence-activated cell sorting (FACS) tests with a C-terminal tail antibody. Non-permeabilized normal and ATP7A^{P1386S} fibroblasts were stained with an antibody targeting the final 15 amino acids of the C-terminus that include the dileucine signal. Our results indicated that mean fluorescence intensity was significantly higher in ATP7A^{P1386S} patient fibroblasts.¹⁹ This phenomenon could partially explain preferential accumulation of ATP7A^{P1386S} at the PM, since it would result in relocation of the dileucine signal known to mediate ATP7A endocytic retrieval from the cytosolic to non-cytosolic protein face. The result refocused our attention of the important role of the C-terminal dileucine motif in proper ATP7A trafficking.

Dileucine motifs are well documented as sorting signals in the C-terminal tail of transmembrane proteins (e.g., DKHSL in ATP7A), and typically denote a capacity to bind adaptor protein complexes. Adaptor protein complexes play important roles in the regulation of intracellular vesicular trafficking, especially clathrin-coated vesicle (CCV) trafficking.^{22,23} Five adaptor protein complexes have been discovered so far, namely AP-1, AP-2, AP-3, AP-4, and AP-5. All of these adaptor complexes have four subunits, including one small subunit named $\sigma 1$ – $\sigma 5$, correspondingly; one medium subunit, named $\mu 1$ – $\mu 5$; two large subunits, $\beta 1$ and γ for AP-1, $\beta 2$ and α for AP-2, $\beta 3$ and δ for AP-3, $\beta 4$ and ϵ for AP-4, and $\beta 5$ and ζ for AP-5.

Recent studies suggest the involvement of adaptor protein complexes in the ATP7A trafficking. Hirst *et al.* reported the colocalization of ATP7A and AP-1 in CCVs, and copper treatment disrupted this co-localization.²⁴ Martinelli *et al.* demonstrated reduced TGN localization of ATP7A in MEDNIK syndrome patients who have mutations in the *AP1S1* gene encoding the $\sigma 1$ A subunit of AP-1.²⁵ Holloway *et al.* also suggested an involvement of AP-1 and AP-2 in ATP7A trafficking.¹⁷

Conclusions

Two mutations in the copper transporter ATP7A (ATP7A^{T994I} and ATP7A^{P1386S}) lead to isolated distal motor neuropathy (DMN). Both ATP7A^{T994I} and ATP7A^{P1386S} show a cellular phenotype of excessive plasma membrane localization compared to wild-type ATP7A. ATP7A^{T994I} interacts strongly with p97/VCP, linking the new ATP7A motor neuron phenotype with autosomal dominant forms of motor neuron degeneration, specifically IBMPFD and ALS, which are induced by mutations in p97/VCP. The abnormal interaction of ATP7A^{T994I} with p97/VCP may reduce the pool of active p97/VCP available for its normal function. Alternatively, aberrant p97/VCP-mediated vesicular trafficking or endosomal sorting of ATP7A^{T994I} may be a crucial consequence. ATP7A^{P1386S} alters the stability of the ATP7A C-terminal tail, which may sabotage physical interaction with adaptor protein complexes involved in intracellular trafficking and disturb neuronal events, resulting distal motor neuropathy. While further details are under investigation in our laboratory, findings to date help illuminate the distinctive mechanisms underlying ATP7A-related distal motor neuropathy.

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Figure 1.

Altered intracellular localization of mutant *ATP7A* alleles causing motor neuropathy. (A) Distal motor neuropathy related mutant proteins *ATP7A*^{T994I} and *ATP7A*^{P1386S} (lavender) locate to transmembrane segments 6 and 8, respectively. Examples of missense mutations causing more typical phenotypes are also indicated, as are certain functional domains of *ATP7A*. Red asterisks denote potential p97/VCP binding sites with *ATP7A*^{T994I}.

Figure 2. Confocal and total internal reflection (TIRF) microscopy indicates a shift in the steady-state equilibrium of ATP7A^{T994I} and ATP7A^{P1386S}. The mutations result in less *trans*-Golgi accumulation represented by less intense colocalization of immunohistochemical markers for TGN46 and ATP7A (panel A), and increased plasma membrane localization on TIRF microscopy. Reproduced with permission from Ref. 19.