Interaction between the AAA ATPase p97/VCP and a concealed UBX domain in the copper transporter ATP7A is associated with motor neuron degeneration

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The copper-transporting ATPase ATP7A contains eight transmembrane domains and is required for normal human copper homeostasis. Mutations in the ATP7A gene may lead to infantile-onset cerebral degeneration (Menkes disease); occipital horn syndrome (OHS), a related but much milder illness; or an adult-onset isolated distal motor neuropathy. The ATP7A missense mutation T994I is located in the sixth transmembrane domain of ATP7A, represents one of the variants associated with the latter phenotype, and is associated with an abnormal interaction with p97/valosin-containing protein (VCP), a hexameric AAA ATPase (ATPase associated with diverse cellular activities) with multiple biological functions. In this study, we further characterized this interaction and discovered a concealed UBX domain in the third lumenal loop of ATP7A, between its fifth and sixth transmembrane domains. We show that the T994I substitution results in conformational exposure of the UBX domain, which then binds the N-terminal domain of p97/VCP. We also show that this abnormal interaction occurs at or near the cell plasma membrane. The UBX domain has a conserved hydrophobic FP (Phe-Pro) motif, and substitution with di-alanine abrogated the interaction and restored the proper interactions with multiple biological functions. In this study, we further characterized this interaction and discovered a concealed UBX domain in the third lumenal loop of ATP7A, between its fifth and sixth transmembrane domains. We show that the T994I substitution results in conformational exposure of the UBX domain, which then binds the N-terminal domain of p97/VCP. We also show that this abnormal interaction occurs at or near the cell plasma membrane. The UBX domain has a conserved hydrophobic FP (Phe-Pro) motif, and substitution with di-alanine abrogated the interaction and restored the proper interaction leading to motor neuron degeneration.

ATP7A is a copper transporter in the P1B-type ATPase category. This molecule and a closely related copper transporter, ATP7B, are crucially important for proper human copper metabolism. Together with Ctr1, Ctr2, and other copper chaperones, the two copper ATPases regulate intracellular copper homeostasis (1, 2). ATP7A is highly expressed in most tissues, except liver, in which organ ATP7B is dominantly expressed (1). ATP7A contains eight transmembrane segments with bulky N-terminal and short C-terminal tails located on the cytosolic aspect. The N terminus of ATP7A contains six copper-binding domains that receive copper from the chaperone ATOX1. A partial crystal structure of ATP7A based on the bacterial homolog CopA showed that the second transmembrane domain forms a “platform” to accept copper from the N-terminal copper-binding domains and transfer copper through the membrane channel (3).

ATP7A normally localizes to the trans-Golgi network under basal intracellular copper concentration (0.5 μM). When copper concentration increases, ATP7A traffics to the plasma membrane to mediate copper exodus, with subsequent retrieval to the trans-Golgi network (1, 2).

Mutations in ATP7A cause defects in normal copper metabolism. Three distinctive clinical phenotypes have been reported: classic Menkes disease, occipital horn syndrome (OHS), and isolated adult-onset distal motor neuropathy (1, 4, 5). Menkes disease and OHS patients have poor copper absorption, low copper levels in the blood and brain, and abnormal plasma and cerebrospinal fluid neurochemical ratios, reflecting partial deficiency of dopamine-β-hydroxylase, a copper-dependent enzyme. The distal motor neuropathy phenotype was reported in association with two distinct ATP7A missense mutations, T994I and P1386S. Affected patients showed no clinical or biochemical stigmata of Menkes disease or OHS and manifested normal blood copper levels and neurochemical ratios. In vitro biological assays of copper transport demonstrated near-normal function of the T994I and P1386S mutant alleles (5).

We subsequently reported that these unique ATP7A T994I and ATP7A P1386S alleles were associated with an exaggerated plasma membrane presence, connoting an abnormal shift in the steady-state equilibrium of ATP7A intracellular localization (6). We also connected the alteration caused by T994I with...
p97/VCP, a hexameric AAA-ATPase involved in multiple cellular functions (7, 8).

Valosin-containing protein (p97/VCP) is an 806-amino acid, 97-kDa protein with a large N-terminal domain and a short C-terminal tail plus two AAA ATPase domains, D1 and D2. It is among more than 1000 proteins in the Golgi proteome (9) and has multiple functions, including vesicular trafficking (fusion of transport vesicles with target membranes), protein degradation by the ubiquitin–proteasome system, endoplasmic reticulum–associated degradation, and autophagy (10, 11). Mutations in p97/VCP cause at least three autosomal dominant motor neuron diseases: inclusion body myopathy with early-onset Paget disease and frontotemporal dementia, familial ALS, and Charcot–Marie–Tooth disease type 2Y (12–15). The protein is considered to exert its diverse biological functions via binding to diverse co-factors via its N-terminal and C-terminal domains (16).

To date, more than 40 proteins have been reported as p97/VCP co-factors, including Ufd1/Npl4 (17), FAF1 (18), UBXD7 (19), p47 (20), and VCP135 (21); each interacts with p97/VCP at specific binding domains. Three categories of p97/VCP-binding domains have been described. One category includes folded domains; typical examples are UBX (ubiquitin regulatory X) or UBXL (UBX-like) domains, which have 80 amino acids similar in sequence to ubiquitin (22). A highly conserved FP (Phe-Pro) motif in UBX and UBXL domains was reported to play a key role in p97/VCP binding (23, 24). FAF1, Ufd1/Npl4, UBXD1, and UBXD2 belong to this group. UBX and UBXL domains interact with the N-terminal domain of p97/VCP.

Another group of p97/VCP-binding domains are PUG in Ubxd1 (25), PNGase (26, 27), and PUL in PLAP (28). The PUG and PLAP domains bind p97/VCP at its C-terminal domain. Linear motifs represent the third category of p97/VCP binding domains. This group includes VIM (VCP-interacting motif) in ANKZF1 (ankyrin repeat, zinc finger domain–containing 1) (29), VBM (VCP-binding motif) in ataxin 3 (30), and SHP box binding motif in Derlp (31). VIM, VBM, and SHP all bind at the N-terminal domain of p97/VCP.

Although our previous work revealed the interaction between ATP7A T994I and p97/VCP, here we explore the detailed molecular mechanism of this interaction. We discovered that ATP7A harbors a concealed UBX domain in its third luminal loop, which ATP7A T994I exposes for binding with the N-terminal domain of p97/VCP at the cell plasma membrane.

Results

p97/VCP–ATP7A T994I interaction is caused by a structural change induced by T994I

We previously reported the p97/VCP-ATP7A T994I interaction (6). Residue T994 is located in the sixth transmembrane segment of ATP7A. Because threonine is a hydrophilic amino acid, substitution with a bulky hydrophobic residue such as isoleucine is predicted to alter the position of this segment within the membrane. Such a change would presumably affect the conformation of the mutant protein, effecting structural changes that could explain the abnormal interaction with p97/VCP (6).

We speculated that substitution of leucine, another bulky hydrophobic amino acid, at position 994 could have similar effects and also provoke the abnormal interaction with p97/VCP. To test this hypothesis, we mutated T994 to three other amino acids, serine, leucine, and alanine. We co-expressed these individual mutant ATP7A alleles with p97/VCP and evaluated their interaction with p97/VCP via immunoprecipitation pulldown assays. Only T994L showed significant interaction with p97/VCP (Fig. 1, a–c).

We also studied the intracellular localization of these mutant ATP7A alleles (Fig. 1d). Correlating with the immunoprecipitation results, ATP7A T994S and ATP7A T994A localized mainly in the trans-Golgi network, similar to WT ATP7A, and ATP7A T994L localized on the plasma membrane, similar to ATP7A T994I.

The third loop region adjacent to the T994I mutation in ATP7A contains a p97/VCP-binding UBX domain

Co-factors that bind p97/VCP utilize diverse p97/VCP-binding domains such as UBX, UBXL, PUG, VIM, and VBM to do so. We performed sequence alignments to compare ATP7A with all known p97/VCP-binding domains (Fig. 2). This analysis revealed that the luminal loop of ATP7A immediately adjacent to the T994I mutation contains high sequence similarity to UBX domains. The conserved hydrophobic motif, FP, typically present in UBX domains, is within 22 residues of T994. This putative UBX domain is highly conserved among ATP7A homologs (Fig. 2c).

Based on our bioinformatics analysis, it appeared that the third luminal loop of ATP7A harbored a UBX domain normally concealed. Mutation of the hydrophilic threonine residue to a bulky hydrophobic like isoleucine presumably alters the conformation of the loop structure and leads to exposure of the UBX motif and provokes subsequent interaction with p97/VCP.

Mutation of the conserved FP motif in the p97/VCP-binding UBX domain abrogates physical interaction between ATP7A T994I and p97/VCP

Because the conserved FP motif is considered crucial for function of the UBXL domain (23, 24), we tested our hypothesis by mutating the Phe-971/Pro-972 motif in ATP7A T994I to dialanine. We tagged this mutant with Venus and co-expressed it with p97/VCP-FLAG in HEK293T cells together with p97/VCP-FLAG. This enabled study of the physical interaction with p97/VCP by immunoprecipitation. We first pulled down p97/VCP-FLAG with anti-FLAG M2 affinity gel (Sigma). As reported previously, p97/VCP pulled down ATP7A T994I (Fig. 3, a, second lane, and b) but not WT ATP7A (Fig. 3, a, first lane, and b). However, immunoprecipitation of ATP7A T994I/971A/972A T994I by p97/VCP was significantly diminished (Fig. 3, a, third lane, and b).

We also performed the converse immunoprecipitation experiments by pulling down Venus-tagged ATP7A and its mutant versions (Fig. 3, c and d). Again, ATP7A T994I significantly immunoprecipitated p97/VCP (Fig. 3, c, second lane, and d) whereas WT ATP7A did not (Fig. 3, c, first lane, and d).
and p97/VCP pulldown by ATP7A^{T994I} was significantly decreased (Fig. 3, c, third lane, and d). Confocal microscopy imaging (Fig. 3e) demonstrated that the ATP7A^{T994I} allele localized both in the trans-Golgi network and at the plasma membrane compared with preferential plasma membrane localization of ATP7A^{T994L}. Taken together, the immunoprecipitation and confocal imaging data suggest that removal of the FP motif impedes the interaction between p97/VCP and ATP7A^{T994I} and partially restores proper intracellular (trans-Golgi network) localization. These findings lend further support for the concealed UBX domain as crucial for the interaction between ATP7A^{T994I} and p97/VCP.

Overexpression of p47 decreases p97/VCP–T994I interaction

As noted, ATP7A harbors a previously unappreciated UBX domain that interacts with p97/VCP when exposed by mutations such as the T994I or T994L substitutions (Fig. 1). We hypothesized that co-expression of a different UBX domain–containing protein would compete with ATP7A^{T994I} for p97/VCP binding and, as a result, diminish the ATP7A^{T994I}–VCP interaction. To test this hypothesis, we co-expressed p47, one of the first reported p97/VCP binding proteins with a UBX domain. Its structure, function, and interaction with p97/VCP have been thoroughly studied (20, 22, 32, 33).

In a pilot immunoprecipitation assay, we co-expressed p97/VCP with either WT ATP7A or ATP7A^{T994I} and pulled down endogenous p47 with anti-p47 antibody. As predicted, p97/VCP was immunoprecipitated by p47 in the presence of either ATP7A or ATP7A^{T994I} (Fig. 4a, top row, third and fourth lanes). However, p47 itself did not bind ATP7A or ATP7A^{T994I} (Fig. 4a, bottom row, third and fourth lanes).

Next we cloned p47 and overexpressed Cerulean-tagged p47 (p47-Cerulean) together with WT ATP7A-Venus or ATP7A^{T994I}-Venus as well as p97/VCP-FLAG. An empty vector (pCerulean-N1) was used as a negative control. Cell lysates were pulled down with anti-FLAG M2 affinity gel (Sigma). As shown in Fig. 4b, all proteins were expressed in the total lysate. p47 was pulled down by p97/VCP (Fig. 4b, third row, second and fourth lanes). As expected, ATP7A^{T994I} was pulled down by p97/VCP (Fig. 4b, third row, second and fourth lanes). In a pilot immunoprecipitation assay, we co-expressed p97/VCP with either WT ATP7A or ATP7A^{T994I} and pulled down endogenous p47 with anti-p47 antibody. As predicted, p97/VCP was immunoprecipitated by p47 in the presence of either ATP7A or ATP7A^{T994I} (Fig. 4a, top row, third and fourth lanes). However, p47 itself did not bind ATP7A or ATP7A^{T994I} (Fig. 4a, bottom row, third and fourth lanes).
Figure 2. The third lumenal loop of ATP7A adjacent to the T994I mutation harbors a p97/VCP-binding UBX domain. a, diagram of ATP7A, with the yellow segment corresponding to the region of UBX homology, including the characteristic FP dipeptide (971–972), and the positions of two missense mutations (lavender circles) associated with isolated distal motor neuropathy. b, sequence alignment of ATP7A and known UBX domains from other proteins: ATP7A (entry ID Q04656), p47 (NSF1C, entry ID Q9UNZ2), p37 (UBX2B, entry ID Q14CS0), UBXD7 (UBXN7, entry ID O94888), and FAF1 (entry ID Q9UNN5). Shaded columns with an asterisk at the bottom indicate fully conserved residues. Shaded columns with a colon at the bottom indicate conservation of strongly similar residues. Periods indicate conservation of weakly similar residues. The arrow denotes residue Thr-994. c, sequence alignment of the putative UBX domain region in ATP7A homologs from other species, with the conserved FP motif highlighted in red. Amino acid conservation codes are as above. Sequence alignments were performed with the alignment program in UniProt.
p97/VCP binds to a concealed UBX domain in ATP7A

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The N-terminal domain of p97/VCP is the site of interaction with ATP7 \textsuperscript{9941}

We next investigated the topography accompanying the p97/VCP interaction with the UBX domain of ATP7 \textsuperscript{9941}. The functional form of p97/VCP is a homohexameric complex comprised of six 806-amino acid subunits with an N-terminal and C-terminal domain as well as two ATPase domains. It is known that the N-terminal domain of p97/VCP interacts with Fas-associated factor 1 (FAF1), a UBX domain-containing proapoptotic factor (18), although, in theory, either the N- or C-terminal domains of p97/VCP could bind to ATP7 \textsuperscript{9941}. To better evaluate the p97/VCP–ATP7 \textsuperscript{9941} interaction, we created constructs to express p97/VCP-FLAG with N-terminal or C-terminal domain deletions (VCP\textsuperscript{H9004N1–207-FLAG} and VCP\textsuperscript{H11005H11005N1–207-FLAG}).}

**Figure 3.** Mutation of the conserved FP sequence in the p97/VCP-binding UBX domain reduces physical interaction between ATP7 \textsuperscript{9941} and p97/VCP and partially restores proper intracellular (trans-Golgi network) localization. a, FLAG-tagged p97/VCP and Venus-tagged ATP7A or ATP7 \textsuperscript{9941} were co-expressed in HEK293T cells. Cell lysates were immunoprecipitated with anti-p47 antibody (Santa Cruz Biotechnology), denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal anti-p97/VCP (top row) or anti-GFP(Venus) antibodies (bottom row). Note that p47 interacts with p97/VCP but not with ATP7A or ATP7 \textsuperscript{9941}. b, FLAG-tagged p97/VCP, Venus-tagged ATP7A, or Venus-ATP7 \textsuperscript{9941} and Cerulean-tagged p47 or Cerulean alone were triple-expressed in HEK293T cells. The lysates were immunoprecipitated with anti-FLAG M2 affinity gel, denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal anti-p97/VCP (first row) and anti-GFP(Venus) antibody to detect both ATP7A (second row) and p47 (third row) because the latter antibody interacts with Cerulean as well as with Venus. The TL blot of ATP7A-Venus, p47-Cerulean, and Cerulean alone confirmed protein expression. c, densitometric summary of IP blots (n = 4, error bars = S.D.). Note the diminished interaction with p97/VCP with p47 co-expression. d, co-expression of p47 with ATP7 \textsuperscript{9941} in HEK293T cells (right panel) partially restores normal intracellular localization. Images shown are representative of ~100 cells for each co-transfection. Scale bars = 10 μm.

**Figure 4.** Overexpression of p47 decreases ATP7 \textsuperscript{9941} interaction with p97/VCP and restores proper intracellular (trans-Golgi network) localization. a, FLAG-tagged p97/VCP and Venus-tagged ATP7A or ATP7 \textsuperscript{9941} were co-expressed in HEK293T cells. Cell lysates were immunoprecipitated with anti-p47 antibody (Santa Cruz Biotechnology), denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal anti-p97/VCP (top row) or anti-GFP(Venus) antibodies (bottom row). Note that p47 interacts with p97/VCP but not with ATP7A or ATP7 \textsuperscript{9941}. b, FLAG-tagged p97/VCP, Venus-tagged ATP7A, or Venus-ATP7 \textsuperscript{9941} and Cerulean-tagged p47 or Cerulean alone were triple-expressed in HEK293T cells. The lysates were immunoprecipitated with anti-FLAG M2 affinity gel, denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal anti-p97/VCP (first row) and anti-GFP(Venus) antibody to detect both ATP7A (second row) and p47 (third row) because the latter antibody interacts with Cerulean as well as with Venus. The TL blot of ATP7A-Venus, p47-Cerulean, and Cerulean alone confirmed protein expression. c, densitometric summary of IP blots (n = 4, error bars = S.D.). Note the highly statistically significant reduction in p97/VCP binding. d, co-expression of p47 with ATP7 \textsuperscript{9941} in HEK293T cells (right panel) partially restores normal intracellular localization. Images shown are representative of ~100 cells for each co-transfection. Scale bars = 10 μm.
p97/VCP binds to a concealed UBX domain in ATP7A

![Diagram of full-length and N-terminal- and C-terminal domain-deleted versions of p97/VCP. These constructs were tagged with FLAG for IP studies.](image)

We co-expressed Venus-tagged ATP7A\(^{\text{T994I}}\) (Venus-ATP7A\(^{\text{T994I}}\)) with either VCP\(\Delta N1-207\)-FLAG or VCP\(\Delta C765-806\)-FLAG in HEK293T cells. Western blots showed expression of both truncated constructs (Fig. 5, b and c). VCP\(\Delta N1-207\)-FLAG was expressed as an \(\approx 75\)-kDa protein (Fig. 5b, bottom row, second lane), and the weaker, \(\approx 100\)-kDa band corresponded to endogenous full-length p97/VCP. VCP\(\Delta C765-806\)-FLAG was expressed as an \(\approx 90\)-kDa protein (Fig. 5b, bottom row, third lane). It was not possible to differentiate exogenous from endogenous p97/VCP because the molecular masses are similar (Fig. 5b, bottom row, first lane).

We used immunoprecipitation studies to evaluate the interaction of ATP7A\(^{\text{T994I}}\) with these p97/VCP deletion alleles. When we pulled down ATP7A\(^{\text{T994I}}\), full-length p97/VCP was detected in the pulldown pool (Fig. 5b, center row, first lane). VCP\(\Delta N1-207\)-FLAG was not detected in the pulldown pool (Fig. 5b, center row, second lane), indicating that the N-terminal domain is necessary for the interaction with ATP7A\(^{\text{T994I}}\). In contrast, the C-terminal domain deletion (VCP\(\Delta C765-806\)-FLAG) was pulled down by ATP7A\(^{\text{T994I}}\) (Fig. 5b, center row, third lane), indicating that the C-terminal domain is not required for the p97/VCP-ATP7A\(^{\text{T994I}}\) interaction. In fact, co-expression of VCP\(\Delta C765-806\)-FLAG resulted in relatively greater immunoprecipitation, suggesting an approximately 2.5-fold increased interaction between ATP7A\(^{\text{T994I}}\) and this construct (Fig. 5d, left).

We also performed the reverse immunoprecipitation experiments by pulling down p97/VCP-FLAG to confirm the above data (Fig. 5c). ATP7A\(^{\text{T994I}}\) was immunoprecipitated by full-

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Figure 5. The N-terminal domain of p97/VCP mediates the interaction with ATP7A\(^{\text{T994I}}\). a, diagram of full-length and N-terminal- and C-terminal domain-deleted versions of p97/VCP. These constructs were tagged with FLAG for IP studies. b, full-length p97/VCP-FLAG, VCP\(\Delta N1-207\)-FLAG, and VCP\(\Delta C765-806\)-FLAG were co-expressed with Venus-tagged ATP7A\(^{\text{T994I}}\) in HEK293T cells. Total lysates were immunoprecipitated with a rabbit polyclonal anti-GFP(Venus) antibody, denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with a mAb against GFP (top row) or p97/VCP (center row). The TL blot of p97/VCP-FLAG and the truncated proteins confirmed equivalent sample loading (bottom row). Note the presence of endogenous p97/VCP bands in all lanes. c, lysates were immunoprecipitated with anti-FLAG M2 affinity gel, denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal anti-p97/VCP (top row) or anti-GFP(Venus) antibodies (center row). The TL blot of ATP7A\(^{\text{T994I}}\)-Venus confirmed equivalent sample loading (bottom row). d, densitometric summary of ATP7A-Venus IP blots (\(n = 3\), error bars = S.D.) and FLAG-p97/VCP IP blots (\(n = 3\), error bars = S.D.). Note that deletion of the p97/VCP N-terminal segment significantly impairs interaction with ATP7A\(^{\text{T994I}}\), whereas C-terminal deletion enhances the interaction.
length p97/VCP (Fig. 5, *center row, first lane*), whereas the amount of ATP7A\textsuperscript{T994I} pulled down by VCPΔN1–207 was significantly reduced (~40%; *Fig. 5, c, center row, second lane, and d, right*). The amount of ATP7A\textsuperscript{T994I} pulled down by VCPΔC765–806 was increased ~1.5-fold (*Fig. 5, c, center row, third lane, and d, right*), recapitulating the previous result.

**Interaction between p97/VCP and ATP7A\textsuperscript{T994I} occurs at the cell plasma membrane**

Our data demonstrated that ATP7A contains a putative UBX domain in its third loop between the fifth and sixth transmembrane segment and that the T994I mutation in the adjacent (sixth) transmembrane segment appears to expose the UB domain for interaction with the N terminus of p97/VCP. We next explored the precise intracellular location where the abnormal binding occurs. Consistent with p97/VCP as a cytosolic protein, imaging of Cerulean-tagged p97/VCP documented high quantities in the cytosol (data not shown). However, because ATP7A\textsuperscript{T994I} preferentially localizes on the plasma membrane of cells (6), it seemed plausible that the T994I-VCP interaction may occur at or near the cell periphery. To evaluate this question, we performed total internal reflection fluorescence (TIRF) microscopy of HEK293T cells co-transfected with Cerulean-tagged p97/VCP and Venus-tagged ATP7A\textsuperscript{T994I} to distinguish ATP7A\textsuperscript{T994I}-bound p97/VCP from unbound p97/VCP. We permeabilized live cells with saponin to release unbound cytosolic p97/VCP and then fixed and stained the cells for p97/VCP. In the ATP7A\textsuperscript{T994I}- and p97/VCP- transfected cells, strong TIRF signals were evident for both proteins (*Fig. 6a, top row*). In contrast, WT ATP7A- and p97/VCP-transfected cells showed weak TIRF signals for both proteins (*Fig. 6a, bottom row*). These combined results suggested that the abnormal p97/VCP-ATP7A\textsuperscript{T994I} interaction occurs at the plasma membrane.

To clarify further, we characterized the p97/VCP-ATP7A\textsuperscript{T994I} interaction in co-transfected, saponin-permeabilized HEK293T cells by isolation of supernatant and ghost membrane fractions. As shown in *Fig. 6b*, more than 95% of ATP7A\textsuperscript{T994I} was recovered in the ghost membrane fraction (*Fig. 6, b, top panel, fourth lane, and c, fourth column*), and only small amounts of ATP7A\textsuperscript{T994I} were released by permeabilization (*Fig. 6, b, top panel, third lane, and c, third column*). In contrast, nearly 80% of intracellular p97/VCP was released to the supernatant buffer by saponin permeabilization (*Fig. 6, b, bottom panel, third lane, and c, sixth column*) compared with nonpermeabilized cells (*Fig. 6, b, bottom panel, second lane, and c, sixth column*). Approximately 20% was retained in the ghost membrane fraction (*Fig. 6, b, bottom panel, fourth lane, and c, eighth column*). WT ATP7A-p97/VCP–co-transfected cells were included as controls (*Fig. 6, b, top and bottom panels, first lane, and c, first and fifth columns*).

We also performed immunoprecipitation using 95% supernatant and ghost membrane collections to study the interaction between ATP7A\textsuperscript{T994I} and p97/VCP. Again, we immunoprecipitated in both contexts, pulling down p97/VCP to detect ATP7A\textsuperscript{T994I} and pulling down ATP7A\textsuperscript{T994I} to detect p97/VCP. As negative controls, WT ATP7A and p97/VCP showed no obvious binding to each other (*Fig. 6, d and e, fifth lanes*). For ATP7A\textsuperscript{T994I}-transfected cells, ~20% of p97/VCP remained in the ghost membrane fraction (*Fig. 6, bottom panels, fourth lane*); however, this 20% of protein pulled down even more ATP7A\textsuperscript{T994I} (*Fig. 6, eighth lane*). Similar results were obtained in the reverse immunoprecipitation assay (*Fig. 6e, fourth and eighth lanes*). Taken together, these data indicate that live-cell permeabilization removed ~80% of cytosolic p97/VCP unbound to ATP7A\textsuperscript{T994I}, and the ~20% of protein retained in the ghost membrane fraction interacted strongly with ATP7A\textsuperscript{T994I}.

**Mass spectrometry analysis of the p97/VCP–ATP7A\textsuperscript{T994I} complex**

We co-expressed p97/VCP and ATP7A\textsuperscript{T994I} in HEK293T cells and performed immunoprecipitation by pulling down p97/VCP and ATP7A\textsuperscript{T994I}, respectively. The pulldown samples were analyzed by MS to identify interacting proteins. We performed the same experiment with the cells co-transfected with p97/VCP and WT ATP7A for background comparison. The cumulative MS results identified 377 unique proteins specifically associated with both p97/VCP and ATP7A\textsuperscript{T994I}, representing a wide array of cellular functions (Table S1). Three known p97/VCP-binding proteins (CUL1, NSF1C (p47), and UFD1) were present exclusively (*Fig. 7*), suggesting their possible involvement in the p97/VCP complex with ATP7A\textsuperscript{T994I}. Six other known p97/VCP-binding partners were identified in the p97/VCP pulldown alone, two of which, YWHAZ and AMFR, were also found in the WT ATP7A and ATP7A\textsuperscript{T994I} pulldowns (*Fig. 7*).

**Discussion**

We previously reported that ATP7A\textsuperscript{T994I} induces an abnormal shift in the steady-state equilibrium of intracellular localization of ATP7A, resulting in exaggerated presence at the plasma membrane of cultured mammalian cells and neurons. We also identified an abnormal interaction between ATP7A\textsuperscript{T994I} and p97/VCP, a hexameric ATPase of the AAA category, mutations in which can cause ALS and other forms of motor neuron degeneration (6). Continuing our characterization of this abnormal interaction, we identified a UBX domain in the third luminal loop of ATP7A, between the fifth and sixth transmembrane segments, with which the p97/VCP N terminus interacts. Our findings suggest that conformational effects induced by the T994I mutation (located in the sixth transmembrane segment) expose this “hidden” UBX domain and allow interaction with p97/VCP.

p97/VCP is known to interact with ~40 different co-factors through a diversity of VCP-binding domains or motifs, such as p47, UBX, UBXL, PUG, VIM, and VBM (16, 17). To our knowledge, this is the first report of p97/VCP binding to a UB domain exposed via altered conformation of a mutant protein that results in motor neuron degeneration. Our data also suggest that other similar mutations, such as T994L (Fig. 1), alter ATP7A structure and expose the UBX domain. The interaction of ATP7A\textsuperscript{T994I} and p97/VCP was competitively blocked by overexpression of p47, a different UBX domain–containing p97/VCP binding partner (20, 21), lending further credence to our molecular findings. Our proteomics analysis (*Fig. 7* and...
Figure 6. The interaction between p97/VCP and ATP7A occurs at the cellular plasma membrane. a, Cerulean-tagged p97/VCP and Venus-tagged ATP7A were co-expressed in HEK293T cells, and 24 h later, cells were treated with 100 μg/ml saponin in permeabilization buffer for 3 min before 4% paraformaldehyde fixation and p97/VCP staining. TIRF imaging was performed to collect ATP7A and p97/VCP signals at the plasma membrane of the stained cells (top row). TIRF imaging in cells transfected with Venus-tagged WT ATP7A was used as a control (bottom row). Images are representative of 50–100 cells examined for each category. Scale bar = 10 μm. b, distribution of ATP7A and p97/VCP signals in live-cell permeabilized fractions. FLAG-tagged p97/VCP and either ATP7A-Venus or ATP7A-Venus were co-expressed in HEK293T cells. Western blotting of total lysates from nonpermeabilized samples reveal expression of both proteins (first and second lanes). The supernatant (Sup) and cell membrane ghost fractions of permeabilized cells were collected and blotted for ATP7A and p97/VCP in live-cell permeabilized fractions. FLAG-tagged p97/VCP and either ATP7A-Venus or ATP7A-Venus were co-expressed in HEK293T cells. Western blotting of total lysates from nonpermeabilized samples reveal expression of both proteins (first and second lanes). The supernatant (Sup) and cell membrane ghost fractions of permeabilized cells were collected and blotted for ATP7A and p97/VCP in live-cell permeabilized fractions. c, densitometric summary of b (n = 3–5, error bars = S.D.). d, IP studies of interaction between ATP7A and p97/VCP in supernatant and ghost membrane fractions. Total (first and second lanes) and fractionated lysates (third and fourth lanes) were immunoprecipitated with anti-FLAG M2 affinity gel, denatured and separated by electrophoresis, transferred to a PVDF membrane, and probed with monoclonal antibodies against GFP(Venus) (top row, fifth through eighth lanes) or p97/VCP (bottom row, fifth through eighth lanes). e, the same lysates were immunoprecipitated with a rabbit polyclonal anti-GFP(Venus) antibody and probed with monoclonal anti-p97/VCP (top row) or anti-GFP(Venus) antibodies (bottom row).
ATP7AT994I molecules fail to properly insert the fifth and sixth potential explanation would be that some proportion of ical interaction with cytosolic/intracellular p97/VCP. One the third lumenal loop so oriented would be accessible for phys-

Questions therefore remain regarding how the UBX domain of the UBX domain is expected to face the extracellular space. Table S1) implies that other coordinating components of the p97 complex may be relevant to the pathophysiology and clinical effects associated with ATP7A T994I. Further delineation of their potential roles may be fruitful topics for future investigation.

The UBX domain we identified in ATP7A spans the fifth transmembrane segment of ATP7A and its third lumenal loop (Fig. 2a, yellow). The loops of ATP7A presumably face the interior of intracellular compartments such as the ER, Golgi, and/or trafficking vesicles. At the plasma membrane, the FP region of the UBX domain is expected to face the extracellular space. Questions therefore remain regarding how the UBX domain of the third lumenal loop so oriented would be accessible for physical interaction with cytosolic/intracellular p97/VCP. One potential explanation would be that some proportion of ATP7A T994I molecules fail to properly insert the fifth and sixth transmembrane segments because of the conformational effects of the mutation. This would result in a topological change, with the UBX domain locating to the wrong side of membranes, as we documented previously for ATP7A P1386S (34). If active, this phenomenon would be intermittent and dynamic based on the relatively well-preserved copper transport of ATP7A T994I (~75% compared with the WT in yeast complementation assays (5, 6)) and the absence of abnormal copper metabolism in affected individuals (5). The CPC transduction domain of ATP7A, considered essential for transmembrane passage of copper, resides in the sixth transmembrane segment (1).

Alternatively, we hypothesize that p97/VCP may gain access to the UBX domain of ATP7A T994I at the lumenal aspect during endocytic retrieval. Co-localization of p97/VCP with EEA1, an early endosome marker, has been described (35), and p97/VCP associates with the protein clathrin, which normally participates in endocytosis (36). Exposure of the ATP7A T994I mutant allele to p97/VCP therefore may occur within early endosomes near the onset of endocytic retrieval and/or via clathrin com-
p97/VCP binds to a concealed UBX domain in ATP7A

complexes. These cumulative data may explain how newly synthesized ATP7A T994I can reach the trans-Golgi network normally and traffic properly to the periphery in response to increased copper while displaying preferential accumulation at or near the plasma membrane (6) as a consequence of binding p97/VCP.

In terms of this interaction, we show here, via p97/VCP domain deletion analysis (Fig. 5a), that the UBX domain in ATP7A T994I binds to the N-terminal domain of p97/VCP. We noted as well that this interaction appears considerably stronger when the p97/VCP C-terminal domain is deleted (Fig. 5, b–d). These data suggest that the 41-amino acid linear C-termi nal domain of p97/VCP may act in vivo to impede interactions at the N-terminal domain.

Mutations in p97/VCP, mostly occurring in the N-terminal domain, have been reported as the cause of other forms of motor neuron degeneration, including ALS, inclusion body myopathy with early-onset Paget disease and frontotemporal dementia, and Charcot–Marie–Tooth disease type 2Y (12–15). These illnesses are inherited in an autosomal dominant pattern and are typically associated with increased D2 ATPase activity (15, 33). The precise mechanism for the selective motor neuron degeneration remains uncertain, and it is unclear whether an overall gain or loss of p97/VCP function is associated with the increased ATPase activity. Based on our clinical, biochemical, and molecular findings for ATP7A T994I and interpretation of the literature, we speculate that it is loss of p97/VCP function, either via mutation in the gene or reduced availability because of consumption by an abnormal protein–protein interaction, that triggers the neurodegeneration.

The discovery of a cryptic UBX domain in ATP7A and its exposure by the T994I variant further clarifies the abnormal interaction of this allele with p97/VCP. Additional experimental analyses and assessment of individuals with this mutation and its associated distal hereditary motor neuropathy phenotype may shed light on the role of p97/VCP in age-associated neurodegenerative diseases.

**Experimental procedures**

**Cell culture**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, antibiotics, and l-glutamine under standard sterile culture conditions in a 5% CO₂ incubator at 37 °C.

**RT-PCR**

Total RNA in HEK293T cells was extracted using TriPure isolation reagent (Roche). First-strand cDNA synthesis was performed using the Enhanced Avian TR First Strand Synthesis Kit (Sigma, St. Louis, MO). Full-length ATP7A, human p97/ VCP, and p47 cDNA were obtained by reverse transcription and PCR using total RNA as template. Site-directed mutagenesis was used to generate the respective ATP7A mutant alleles. After DNA sequence fidelity was confirmed, ATP7A cDNA was inserted between the Sail and Apal sites of pVenus-C1. Human p97/VCP, the N-terminal deletion (VCPΔ1–207), and the C-terminal deletion (VCPΔ765–806) were inserted between the BglIII and KpnI sites of pFLAG-CMV-5.1 (Sigma). p47

Table S1) implies that other coordinating components of the p97 complex may be relevant to the pathophysiology and clinical effects associated with ATP7A T994I. Further delineation of their potential roles may be fruitful topics for future investigation.
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cDNA was inserted between the EcoRI and BamHI sites of pCerulean-N1.

Cell transfection, immunoprecipitation assays, and Western blotting

The constructs were introduced into HEK293T cells, with transfection mediated by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were collected and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and protein inhibitor mixture (Roche)) on ice for 20 min. The lysate was centrifuged at 12,000 × g for 10 min, and supernatants were used for immunoprecipitation with rabbit anti-GFP antibody (Clontech) or mouse p47 antibody (Santa Cruz Biotechnology, sc-365215) and protein G–agarose beads (Thermo Scientific) or anti-FLAG M2 affinity gel (Sigma). Immunoprecipitates were denatured with 2% SDS and electrophoresed through a 4–12% NOVEX Tris-glycerin SDS polyacrylamide gel (Invitrogen). Proteins were separated on polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Membranes were incubated at room temperature with Tris-buffered saline blocking buffer (20 mM Tris/HCl (pH 7.5), 0.9% (v/v) NaCl, and 0.1% Tween 20 v/v containing 5% (w/v) nonfat milk) and then incubated for 2 h at room temperature with a 1:100 dilution of anti-p97/VCP (BioLegend) or anti-GFP (Clontech) antibodies. After washing three times, membranes were incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate (1:2000, Santa Cruz Biotechnology) for 1 h at room temperature. Membranes were then washed and developed using SuperSignal West Pico Luminol/Enhancer Solution (Pierce) according to the manufacturer’s instructions.

Confocal imaging

For confocal experiments, HEK293T cells were transfected with either Venus-tagged ATP7A, ATP7AT994I, ATP7AT994L, ATP7AT994S, ATP7AT994A, or ATP7AF971A/P972A/T994I using Lipofectamine 2000 (Thermo Fisher Scientific). Co-transfections were performed with Cerulean-tagged p47 and Venus-tagged ATP7AT994I. 24 h post-transfection, HEK293T cells were examined by confocal microscopy (Zeiss 710), and the images were captured using Zen software.

TIRF

Transfected HEK293T cells were grown in 4-well or 2-well coverglass chambers, live-permeabilized with 100 μg/ml saponin (Sigma) in permeabilization buffer (20 mM HEPES (pH 7.4), 110 mM KCl, 5 mM MgCl2, 10 mM NaCl, 5 mM KH2PO4, and 2 mM EGTA (pH 7.0)) for 3 min, fixed with 4% paraformaldehyde at room temperature for 10 min, treated with 0.1% Triton X-100/PBS at room temperature for 10 min, blocked with 10% goat serum for 1 h, and probed with an anti-p97/VCP antibody (1:100) overnight. After washing twice with PBS, 10% goat serum in PBS containing Alexa Fluor 568-conjugated anti-mouse IgG was applied at room temperature for 30 min. The buffer was then removed, the cells were washed twice with PBS, and TIRF images were collected by Nikon Storm super-resolution microscopy.

Mass spectroscopy proteomics analysis

To identify protein binding partners by MS analysis, HEK293T cells were co-transfected with FLAG-tagged p97/VCP and Venus-tagged ATP7AT994I or ATP7A-Venus. Twenty-four hours post-transfection, the cells were rinsed three times with PBS and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and protein inhibitor mixture (Roche)) on ice for 20 min. The lysates were then centrifuged at 10,000 × g for 10 min, and the supernatants were collected for immunoprecipitation by rabbit anti-GFP antibody (Clontech) and protein G–agarose beads (Thermo Scientific) or anti-FLAG M2 affinity gel (Sigma). The eluted immunoprecipitates were then denatured with 2% SDS, electrophoresed through a 10% polyacrylamide gel, and stained with Coomassie Brilliant Blue. The whole gels were excised in three fragments based on molecular weight and analyzed by MS (Taplin Mass Spectrometry Facility, Harvard Medical School). Proteins were identified based on peptide amino acid sequence.

Densitometry

Relevant bands on Western blots from immunoprecipitation experiments were digitized and quantitated by densitometry using ImageJ with correction for background. Between four and seven blots were analyzed for each experiment.

Statistical analyses

Experimental data were analyzed by two-tailed Student’s t tests. p < 0.05 was considered statistically significant. GraphPad Prism software was used to generate scatterplots.

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