

# Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3

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Misfolded proteins of the endoplasmic reticulum undergo retrotranslocation to enter the cytosol where they are degraded by the proteasome. Retrotranslocation of many substrates requires an ATPase complex consisting of the p97 ATPase and a dimeric cofactor, Ufd1-Npl4. We report that efficient elimination of misfolded ER proteins also involves ataxin-3 (*atx3*), a p97-associated deubiquitinating enzyme mutated in type-3 spinocerebellar ataxia. Overexpression of an *atx3* mutant defective in deubiquitination inhibits the degradation of misfolded ER proteins and triggers ER stress. Misfolded

polypeptides stabilized by mutant *atx3* are accumulated in part as polyubiquitinated form, suggesting an involvement of its deubiquitinating activity in ER-associated protein degradation regulation. We demonstrate that *atx3* transiently associates with the ER membrane via p97 and the recently identified Derlin-VIMP complex, and its release from the membrane appears to be governed by both the p97 ATPase cycle and its own deubiquitinating activity. We present evidence that *atx3* may promote p97-associated deubiquitination to facilitate the transfer of polypeptides from p97 to the proteasome.

## Introduction

The endoplasmic reticulum (ER) is a major site of protein folding and assembly in eukaryotes. Polypeptides entering the ER often encounter various folding problems, resulting in aggregated or misfolded proteins. To preserve ER homeostasis, eukaryotes have evolved a conserved quality control pathway termed retrotranslocation, dislocation, or ER-associated protein degradation (ERAD), which efficiently eliminates misfolded ER proteins by exporting them into the cytosol for degradation by the ubiquitin-proteasome system (Meusser et al., 2005).

Retrotranslocation is initiated when misfolded polypeptides are selectively targeted to the site of translocation at the ER membrane from where they are subsequently dislocated into the cytosol. The export of a subset of substrates requires the Derlin proteins, members of a highly conserved multi-spanning membrane protein family postulated to form a channel (Lilley and Ploegh, 2004; Ye et al., 2004; Oda et al., 2006). Other substrates might use different routes to exit the ER. Most substrates undergoing retrotranslocation are modified by polyubiquitination, which is achieved by the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Meusser et al., 2005). Polyubiquitination

occurs at the cytosolic side of the ER membrane after a portion of the substrate has emerged into the cytosol. This modification is required for the subsequent dislocation of polyubiquitinated substrates from the ER membrane, a process that is mediated by the p97-Ufd1-Npl4 ATPase complex (Bays and Hampton, 2002). During Derlin-dependent retrotranslocation, the ATPase complex is recruited to the Derlins together with a single spanning membrane protein called VIMP and certain ER-specific ubiquitin ligases (Ye et al., 2004, 2005; Lilley and Ploegh, 2005). The assembly of a retrotranslocation complex containing both the “pulling” ATPase p97 and the ubiquitination machinery ensures that substrate ubiquitination and dislocation are tightly coupled. For misfolded glycoproteins, their dislocation is also associated with the removal of the attached N-linked glycan by a cytosolic N-glycanase (Kim et al., 2006).

After entering the cytosol, polypeptides need to be transferred to the proteasome for degradation. This process is poorly defined. In *Saccharomyces cerevisiae*, the p97 homologue *cdc48p* does not seem to form a stable complex with the 26S proteasome (Verma et al., 2000). Substrates bound by p97 thus need to be shuttled to the proteasome, which likely involves interactions between ubiquitin conjugates and various ubiquitin binding proteins (Richly et al., 2005). Several proteasome-associated proteins such as *rad23p* in yeast contain ubiquitin binding motifs (Elsasser and Finley, 2005), and can serve as receptors to collect ubiquitinated substrates (Verma et al., 2004).

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Abbreviations used in this paper: *atx3*, ataxin-3; DUB, deubiquitinating enzymes; ERAD, ER-associated protein degradation; PAD, p97-associated deubiquitination; U/N, Ufd1-Npl4; wt, wild-type.

The online version of this article contains supplemental material.

However, little is known about the “shuttling factors” that deliver substrates to these receptors. One candidate for a shuttling factor is ataxin-3 (atx3) (Kuhlbrodt et al., 2005), a poly-glutamine (poly-Q) containing deubiquitinating enzyme (DUB) that interacts with both p97, and HHR23A and B, mammalian homologues of rad23p (Wang et al. 2000; Doss-Pepe et al., 2003; Matsumoto et al., 2004; Boeddrich et al., 2006). Expansion of the poly-Q segment in atx3 has been linked to a dominantly inherited form of spinocerebellar ataxia, a member of the poly-Q-induced family of neurodegenerative diseases (Kawaguchi et al., 1994), but how such poly-Q expansion causes the disease is unclear. In addition, although the enzymatic activity of atx3 has been extensively characterized (Burnett et al., 2003; Chow et al., 2004; Berke et al., 2005; Mao et al., 2005), its physiological function remains elusive. This study was initiated to explore the potential involvement of atx3 in ERAD. We conclude that atx3 acts in conjunction with p97 to regulate the degradation of misfolded ER proteins.

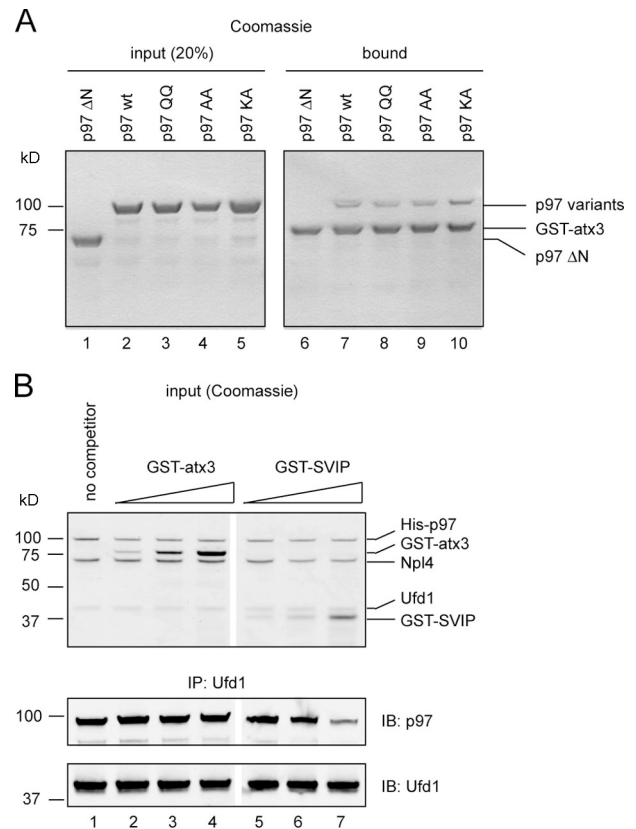
## Results

### Interaction of ataxin-3 with the p97 complex

To test the potential involvement of atx3 in ERAD, we first characterized its interaction with p97 using purified components. Indeed, recombinant protein containing the GST fused to atx3 (GST-atx3) bound purified wild-type (wt) p97 as well as several p97 variants that were defective either in ATP binding (AA and KA) or in ATP hydrolysis (QQ) (Ye et al., 2003). In contrast, a p97 mutant lacking its N-terminal domain (N-domain) failed to bind GST-atx3 (Fig. 1 A). These results suggest that atx3 binds p97 via its N-domain. Because many cofactors bind the p97 N-domain in a mutually exclusive manner (Meyer et al., 2000), we tested whether atx3 would influence the interaction of p97 with Ufd1–Npl4 (U/N), a binary cofactor complex required for ERAD. Recombinant p97 was incubated with purified U/N in the presence of increased concentrations of either GST-atx3 or of a recombinant protein containing the GST fused to SVIP, a small p97 cofactor of unknown function (Nagahama et al., 2003). The p97–Ufd1–Npl4 complex immunoprecipitated with anti-Ufd1 antibodies was analyzed by immunoblotting. As previously reported (Nagahama et al., 2003), addition of SVIP resulted in reduced association of p97 with U/N, presumably because these cofactors compete for the same binding site on p97 (Fig. 1 B, lanes 5–7). In contrast, GST-atx3 did not affect the assembly of the p97–Ufd1–Npl4 complex (Fig. 1 B, lanes 1–4), suggesting that atx3 either uses a different mode to interact with the p97 N-domain or binds p97 with an affinity significantly lower than other cofactors. The lack of competition between atx3 and U/N raises the possibility that these cofactors may act in conjunction with p97 to regulate ERAD.

### Interaction of ataxin-3 with components of the ERAD pathway

Therefore, we tested whether atx3 also interacts with other known components of the ERAD pathway, such as the recently identified Derlin–VIMP complex. Detergent extracts of 293T cells expressing FLAG-tagged atx3 were subjected to immuno-

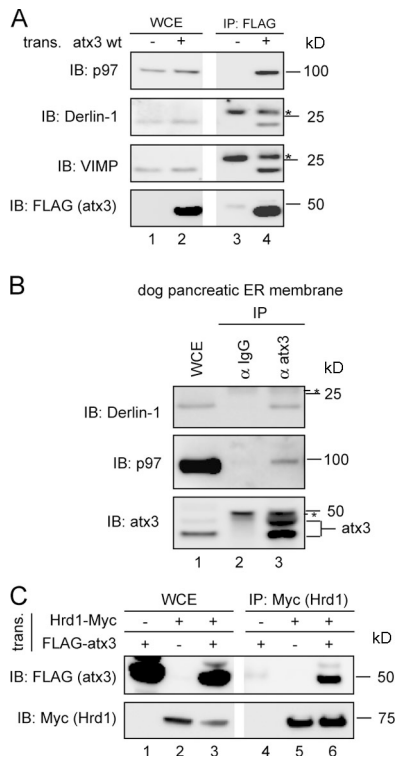


**Figure 1. Interaction of atx3 with the p97 complex.** (A) atx3 binds p97 via its N-domain. A GST fusion protein containing wt atx3 was immobilized on glutathione beads and incubated with either wild-type p97 (p97 wt) or various p97 mutants as indicated. Bound proteins were separated by SDS-PAGE and stained with Coomassie blue. A fraction of the binding reaction was analyzed directly by SDS-PAGE (input). (B) atx3 and Ufd1–Npl4 do not compete for binding to p97. p97 was incubated with purified Ufd1–Npl4 in the presence of increased concentration of either GST-atx3 or GST-SVIP. The p97 complex was immunoprecipitated with anti-Ufd1 antibodies and analyzed by immunoblotting.

precipitation with anti-FLAG antibodies. Immunoblotting with specific antibodies showed that a fraction of Derlin-1, VIMP, and p97 was coprecipitated with atx3 (Fig. 2 A, lane 4). Endogenous atx3 could be coimmunoprecipitated with Derlin-1 and p97 from detergent extract of dog pancreatic ER membranes (Fig. 2 B, lane 3). When FLAG-tagged atx3 was coexpressed with Myc-tagged Hrd1, an ER-specific ubiquitin ligase (Bays et al., 2001), both proteins could be coimmunoprecipitated (Fig. 2 C). Together, these data suggest that a fraction of atx3 is associated with the ER membranes via interactions with components of the retrotranslocation machinery, including Derlin-1, VIMP, p97, and an ER-specific ubiquitin ligase. Because only a small fraction of the Derlin complex could be coprecipitated with atx3, and immunoprecipitation using the VIMP antibody pulled down little atx3 (see Fig. 5 A), atx3 appears to transiently interact with the ER membrane (see Discussion).

### Dominant-negative atx3 interferes with ER degradation

Because knock-down of atx3 by ~80% using atx3-specific siRNA was not sufficient to cause an apparent defect in



**Figure 2. atx3 interacts with the components of the retrotranslocation pathway at the ER membrane.** (A) atx3 binds Derlin-1, VIMP, and p97. Detergent extracts of 293T cells transfected (trans.) with indicated plasmids were subjected to immunoprecipitation (IP) with anti-FLAG antibodies to enrich atx3 and its associated proteins. A fraction of the extracts (WCE, whole cell extract, 15%) was analyzed directly by immunoblotting (IB). Asterisks, IgG. (B) Interaction of endogenous atx3 with Derlin-1 and p97. As in A, except that dog pancreatic ER membranes were used. Bracket indicates different atx3 isoforms. (C) Interaction of atx3 with Hrd1. As in A, except that cells transfected with the indicated plasmids were used, and that immunoprecipitation was performed with anti-Myc antibody. Asterisks, IgG.

retrotranslocation (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200605100/DC1>), we attempted to interfere with the function of endogenous atx3 with a catalytically inactive atx3 mutant (atx3 C14A). We reasoned that this atx3 variant was likely to exert a dominant-negative effect because it binds ubiquitinated substrates with high affinity, but cannot process them (Burnett et al., 2003). We first tested whether the expression of atx3 C14A inhibits the p97-dependent degradation of misfolded TCR $\alpha$  (Fig. S2) (DeLaBarre et al., 2006), a type I transmembrane protein targeted for retrotranslocation due to the lack of its assembly partners (Huppa and Ploegh, 1997). To this end, we expressed TCR $\alpha$  together with either FLAG-tagged wt atx3 or the atx3 C14A mutant in 293T cells. Quantitative immunoblotting using fluorescence-labeled secondary antibodies showed that the FLAG-tagged atx3 variants were expressed  $\sim$ 12-fold higher than the endogenous protein (unpublished data). When the level of TCR $\alpha$  was determined, it was indeed elevated in cells expressing atx3 C14A (Fig. 3 A, lane 2 vs. lane 1; Fig. 3 B, lane 2 vs. lane 1). A pulse-chase experiment confirmed that TCR $\alpha$  was more stable in atx3 C14A-expressing cells (Fig. S3,  $t_{1/2} \sim$ 60 min in atx3 C14A cells vs.  $\sim$ 30 min in control cells). Because a significant fraction of the stabilized TCR $\alpha$  appeared to carry polyubiquitin conjugates as determined by

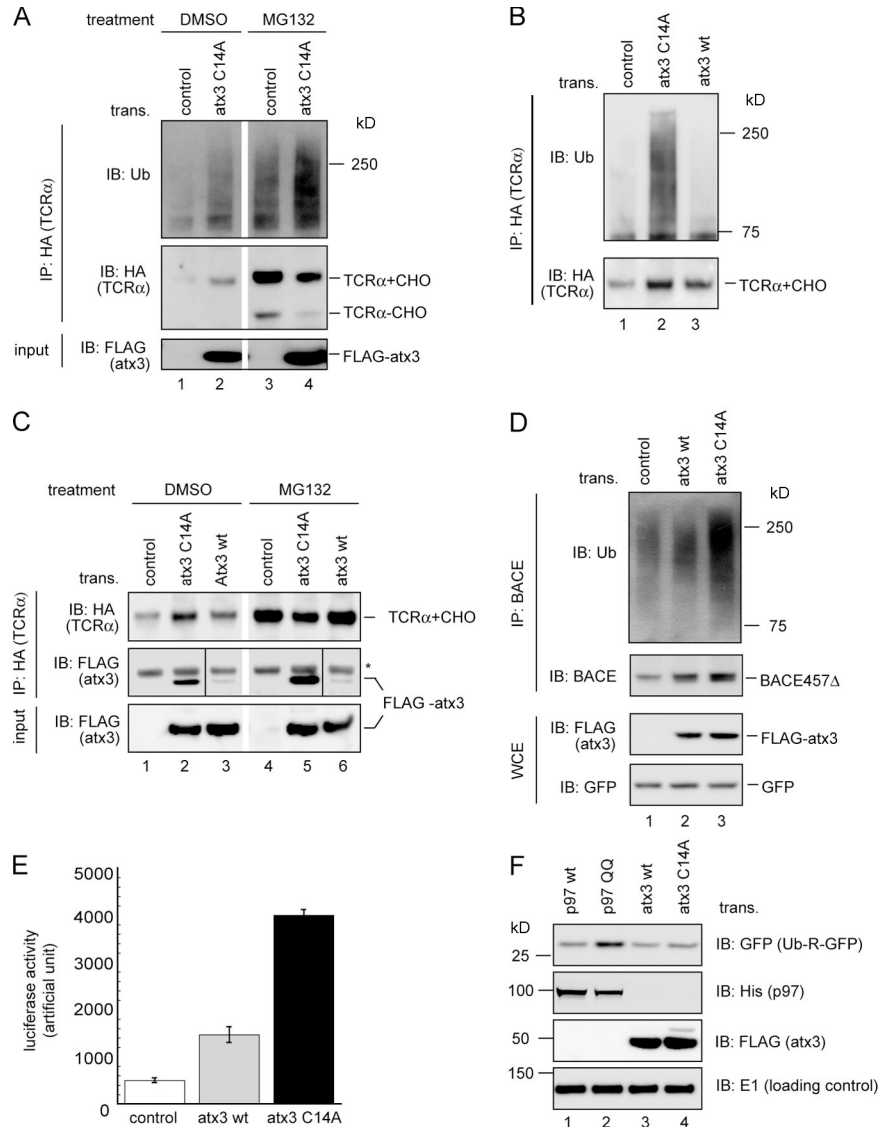
immunoblotting the precipitated TCR $\alpha$  with ubiquitin antibodies (Fig. 3 A, lane 2; Fig. 3 B, lane 2), the stabilization effect by atx3 C14A was likely underestimated in the pulse-chase experiment because only the nonubiquitinated TCR $\alpha$  species could be analyzed. In contrast to atx3 C14A, overexpression of wt atx3 only moderately stabilized TCR $\alpha$  (Fig. S3), and did not increase the level of ubiquitinated TCR $\alpha$  (Fig. 3 B, lane 3). atx3 C14A-induced accumulation of ubiquitinated TCR $\alpha$  was more apparent when cells were treated with the proteasome inhibitor MG132 (Fig. 3 A, lane 4). Although MG132-treated control cells also contained a significant amount of the ubiquitinated TCR $\alpha$ , most substrates in these cells remained nonubiquitinated, which included both the glycosylated retrotranslocation precursor molecules and the dislocated deglycosylated degradation intermediates (Fig. 3 A, lane 3). In contrast, atx3 C14A-expressing cells treated with MG132 accumulated more ubiquitinated TCR $\alpha$  molecules at a compensatory loss of the nonubiquitinated species (Fig. 3 A, lane 4 vs. lane 3). These data suggest that the deubiquitination of TCR $\alpha$  is inhibited in atx3 C14A-expressing cells, leading to the stabilization of TCR $\alpha$  in part as polyubiquitinated species.

To test for a direct involvement of atx3 in ERAD, we examined its interaction with TCR $\alpha$ . Detergent extracts of cells expressing HA-tagged TCR $\alpha$  together with either wt atx3 or atx3 C14A were subjected to immunoprecipitation with HA antibodies. Immunoblotting analysis showed that only a small fraction of wt atx3 was coprecipitated with TCR $\alpha$  (Fig. 3 C, lane 3), whereas significantly more atx3 C14A was bound to TCR $\alpha$  (Fig. 3 C, lane 2). These results are consistent with the concept that the atx3 C14A mutant is able to sequester its substrates in a stable complex. Treatment of cells with MG132 stabilized TCR $\alpha$  (Fig. 3 C, lanes 4–6 vs. lanes 1–3), and further enhanced the interaction between TCR $\alpha$  and atx3 C14A (Fig. 3 C, lane 5 vs. lane 2). In contrast, the association of TCR $\alpha$  with wt atx3 remained weak (Fig. 3 C, lane 6). These data suggest that atx3 transiently binds TCR $\alpha$  to directly regulate its degradation.

To see whether atx3 also plays a role in degradation of soluble ERAD substrates, we examined the degradation of misfolded  $\beta$ -site amyloid precursor protein cleaving enzyme (BACE457 $\Delta$ ) (Molinari et al., 2003) in cells expressing either wt atx3 or atx3 C14A. Similar to TCR $\alpha$ , BACE457 $\Delta$  was stabilized in atx3 C13A-expressing cells, and a significant fraction of the accumulated BACE457 $\Delta$  contained polyubiquitin conjugates (Fig. 3 D, lane 3). In contrast, expression of wt atx3 only moderately increased the level of nonubiquitinated BACE457 $\Delta$  (Fig. 3 D, lane 2 vs. lane 1). Thus, atx3 is also involved in the degradation of luminal ERAD substrates.

Because a defect in ERAD is usually associated with the accumulation of misfolded proteins in the ER, which triggers ER stress, we monitored the ER stress level in cells expressing atx3 C14A to further confirm its involvement in ERAD. Indeed, expression of atx3 C14A caused strong induction of an ER stress reporter gene, pGL3-GRP78 (-132)-luciferase (Fig. 3 E). Expression of wt atx3 also induced ER stress, albeit to a much lesser degree. This is consistent with the observation that wt atx3 caused a moderate stabilization of TCR $\alpha$  and BACE457 $\Delta$ . Together, these results further support the notion that the

**Figure 3. atx3 C14A interferes with ERAD and triggers ER stress.** (A) Expression of atx3 C14A stabilizes TCR $\alpha$ . HA-tagged TCR $\alpha$  from cells transfected (trans.) with indicated plasmids was immunoprecipitated with HA antibodies and analyzed by immunoblotting (IB) (TCR $\alpha$ +CHO, glycosylated TCR $\alpha$ ; TCR $\alpha$ -CHO, deglycosylated TCR $\alpha$ ). Where indicated, cells were treated with MG132 (10  $\mu$ M) for 14 h. (B) atx3 C14A inhibits deubiquitination of TCR $\alpha$ . Cell extracts were denatured before being subjected to immunoprecipitation as in A. (C) Interaction of atx3 with TCR $\alpha$ . HA-TCR $\alpha$  was immunoprecipitated with HA antibodies from detergent extracts of cells transfected with indicated plasmids. Asterisk, IgG. (D) atx3 C14A inhibits the degradation of BACE457 $\Delta$ . As in B, except that cells expressing BACE457 $\Delta$  together with the indicated atx3 variants were analyzed. In addition, a GFP-expressing plasmid was included to control the transfection efficiency, and a ubiquitin-expressing plasmid was cotransfected to increase the level of ubiquitinated BACE457 $\Delta$ . WCE, whole cell extract. (E) atx3 C14A induces ER stress. 293T cells were transfected with an ER stress reporter plasmid pGL3-GRP78 (-132) luciferase (Yoshida et al., 2001) together with indicated constructs. A GFP-expressing construct was also included to monitor the transfection efficiency. Luciferase activity in cell extracts was determined 24 h after transfection. The graph represents three independent experiments normalized to the GFP level. (F) atx3 C14A does not affect the degradation of Ub-R-GFP. Detergent extracts of cells expressing Ub-R-GFP together with indicated proteins were analyzed by immunoblotting.



deubiquitinating activity of atx3 may be essential for efficient elimination of misfolded ER proteins.

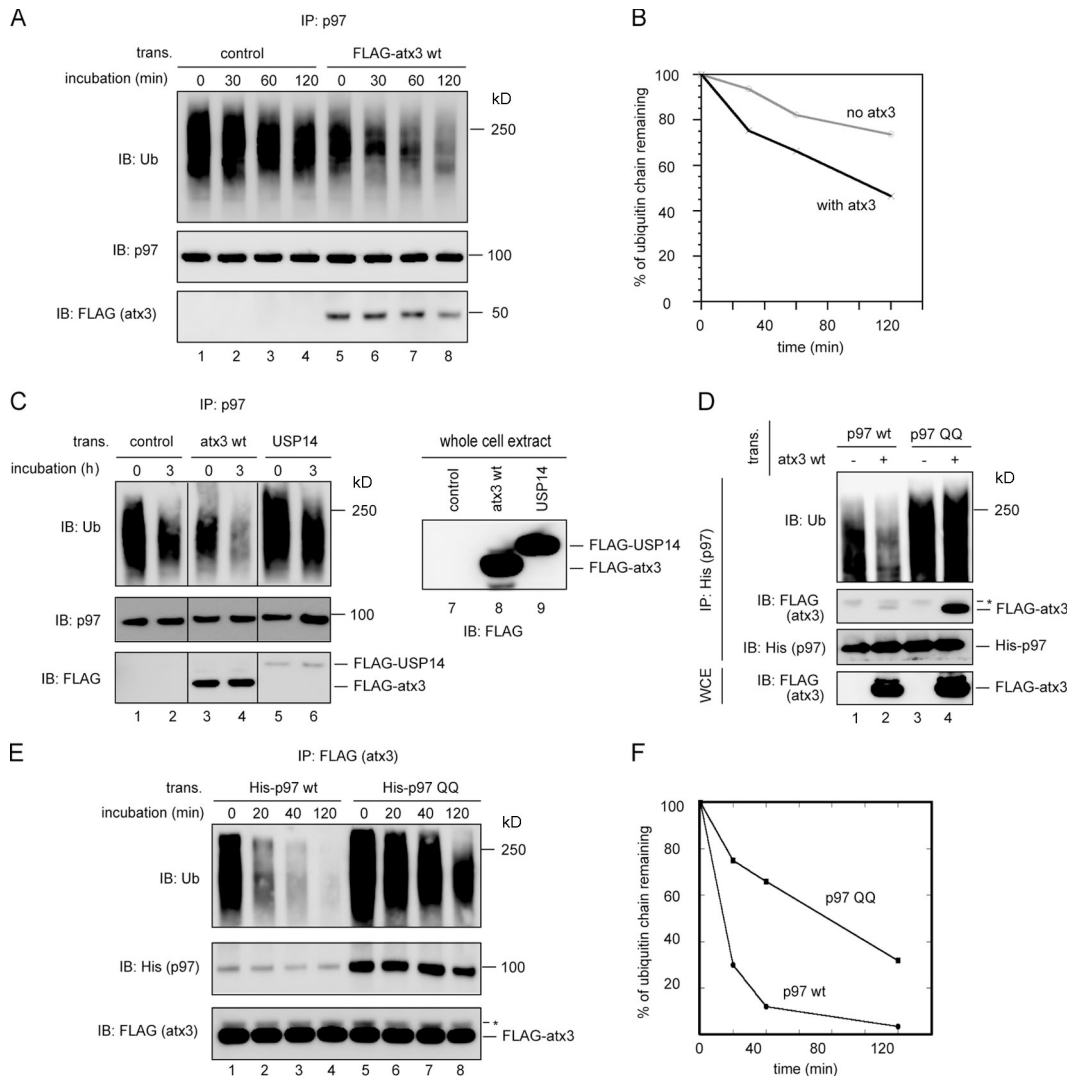
The inhibition of ERAD by atx3 C14A cannot be attributed to general perturbation of the ubiquitin proteasome system by the overexpressed defective DUB, because the proteasome-dependent degradation of an N-end rule substrate Ub-R-GFP (Dantuma et al., 2000) was not affected (Fig. 3 F). Interestingly, the degradation of Ub-R-GFP could be inhibited by a p97 mutant that was defective in ATP hydrolysis (QQ). Thus, the inhibitory effect of atx3 C14A on protein degradation cannot be simply due to nonselective disruption of p97 function by the overexpressed mutant protein.

#### atx3 promotes p97-associated deubiquitination

Because atx3 is a p97-associated DUB (Burnett et al., 2003), it may facilitate ERAD by promoting deubiquitination of substrates bound by p97. To examine p97-associated deubiquitination (PAD), p97 and its associated proteins were immunoprecipitated from cell extracts and analyzed by immunoblotting.

A fraction of the precipitated material carried polyubiquitin conjugates. Because these ubiquitinated proteins were not detected when immunoprecipitation was performed under denaturing condition, they were likely substrates bound to p97 (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200605100/DC1>). Next, we tested whether these ubiquitinated proteins could be subjected to deubiquitination in vitro by DUBs coprecipitated with p97. Indeed, the amount of p97-bound ubiquitinated material was slowly reduced during incubation (Fig. 4 A, lanes 1–4), a process that could be blocked by *N*-ethylmaleimide, an inhibitor of deubiquitination (unpublished data). To test whether atx3 can facilitate this process, p97 and its associated substrates purified from cells expressing either wt atx3 or atx3 C14A mutant were tested for in vitro deubiquitination. Expression of wild type, but not the catalytically inactive atx3 mutant, accelerated the decay of these ubiquitin conjugates (Fig. 4 A, lanes 5–8 vs. lanes 1–4; Fig. 4 B; Fig. S5). These data indicate that atx3 indeed facilitates PAD. Notably, a smaller amount of ubiquitinated proteins was coprecipitated with p97 from cells expressing wt atx3 (Fig. 4 A, lane 5 vs. lane 1;





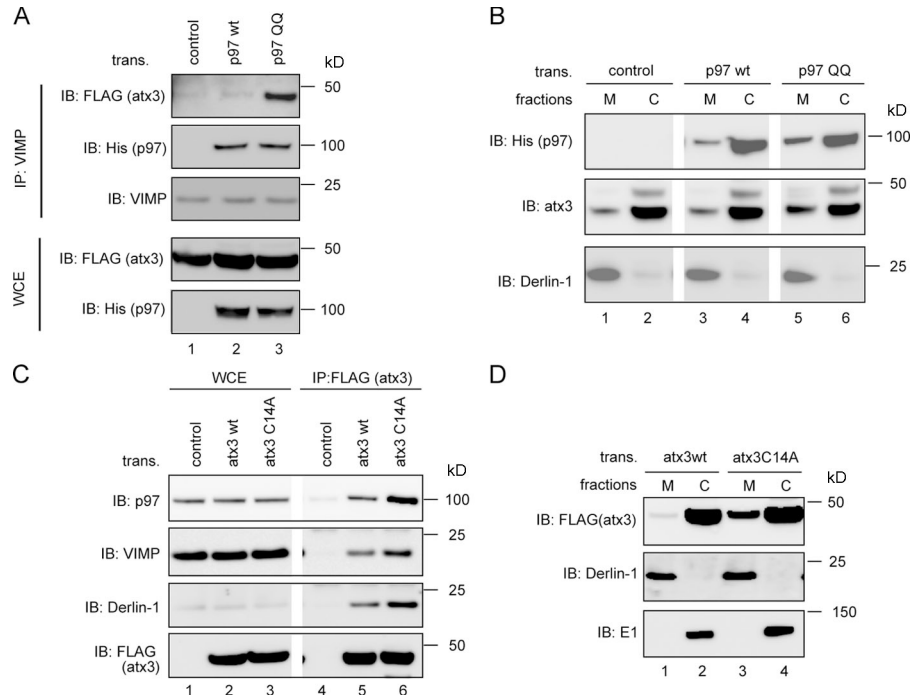
**Figure 4. atx3 acts downstream of p97 to promote PAD.** (A) atx3 promotes deubiquitination associated with p97. 293T cells were transfected (trans.) with the indicated plasmids. p97 and its associated proteins were immunoprecipitated (IP) and subjected to in vitro deubiquitination. (B) Quantification of experiment in A. (C) USP14 has no effect on p97-associated deubiquitination. Cells transfected with either an empty vector (control), FLAG-tagged atx3, or FLAG-tagged USP14 were analyzed as in A. A fraction of the cell extract (whole cell extract) was directly analyzed by immunoblotting. (D) A p97 mutant defective in ATP hydrolysis (QQ) blocks atx3-mediated PAD. His-tagged wt p97 or p97 QQ mutant was immunoprecipitated from detergent extracts of cells transfected with either a control vector (–) or a FLAG-tagged atx3 construct (+). Asterisk, IgG (E) p97 QQ inhibits atx3-dependent deubiquitination in vitro. FLAG-tagged atx3 and its associated proteins were immunoprecipitated from detergent extracts of cells expressing either wt p97 or p97 QQ, and subjected to in vitro deubiquitination. (F) Quantification of the experiment in E.

Fig. 4 C, lane 3 vs. lane 1), suggesting that atx3-mediated PAD may also occur in intact cells. Expression of USP14, a DUB primarily associated with the proteasome (Borodovsky et al., 2001; Leggett et al., 2002), did not decrease the amount of ubiquitinated proteins bound to p97 (Fig. 4 C, lane 5 vs. lane 1), and did not increase the rate of PAD in vitro (Fig. 4 C, lanes 5 and 6). Together, these data suggest that atx3 can promote PAD both in intact cells and in vitro.

To understand how atx3 cooperates with p97 to promote deubiquitination, we wished to determine the order between atx3-mediated deubiquitination and p97-dependent ATP hydrolysis. We reasoned if atx3 acts subsequent to the ATPase cycle of p97, inhibition of p97 ATPase activity would likely prevent ubiquitinated substrates from reaching the enzymatic site of atx3 because substrates cannot be released from p97 under this

condition (Ye et al., 2001). To test this idea, FLAG-tagged wt atx3 was coexpressed together with either His-tagged wt p97 or a p97 mutant defective in ATP hydrolysis (QQ) (Ye et al., 2001). The amount of ubiquitinated substrates associated with His-tagged p97 was determined. The expression of atx3 reduced the amount of ubiquitinated proteins associated with wt p97 (Fig. 4 D, lane 2 vs. lane 1), but not of those bound by p97 QQ (lane 4 vs. lane 3), although more atx3 was bound by p97 QQ (lane 4 vs. lane 2). Moreover, the expression of p97 QQ resulted in more ubiquitinated proteins in association with atx3 and p97 (Fig. 4 E, lane 5 vs. lane 1), and these ubiquitinated proteins were more resistant to deubiquitination in vitro (Fig. 4 E, lanes 5–8 vs. lanes 1–4; Fig. 4 F). These data indicate that ubiquitinated substrates accumulated upon p97 QQ expression cannot be efficiently deubiquitinated by atx3. Although these

**Figure 5. Regulated association of atx3 with the ER membrane.** (A) A dominant-negative p97 mutant (QQ) enhances the association between VIMP and atx3. Detergent extracts of 293T cells transfected (trans.) with FLAG-tagged atx3 together with the indicated plasmids were subjected to immunoprecipitation (IP) with VIMP antibodies. A fraction of the extract (WCE) was analyzed directly by immunoblotting (IB). (B) p97 QQ increases the level of membrane-bound atx3. Cells transfected with the indicated plasmids were homogenized in a low salt buffer. The post-nuclear extracts were fractionated into a membrane fraction (M) and a cytosolic fraction (C) before being analyzed by immunoblotting. (C) atx3 C14A exhibits increased affinity to p97, Derlin-1, and VIMP. As in A, except that cells transfected with the indicated plasmids were analyzed, and that anti-FLAG antibodies were used for immunoprecipitation. (D) Enhanced association of atx3 C14A with the ER membrane. As in B, except that cells transfected with the indicated plasmids were used.



substrates can be coimmunoprecipitated with atx3, they are likely still bound by the p97 QQ mutant, which is also present in the complex. Thus, ATP hydrolysis by p97 appears to precede atx3-mediated deubiquitination. Perhaps p97-dependent ATP hydrolysis is coupled to the transfer of substrates to the enzymatic site of atx3. Because the p97 ATPase cycle is required for the extraction of misfolded proteins from the ER membranes (Ye et al., 2001), atx3 likely acts at a step downstream of dislocation.

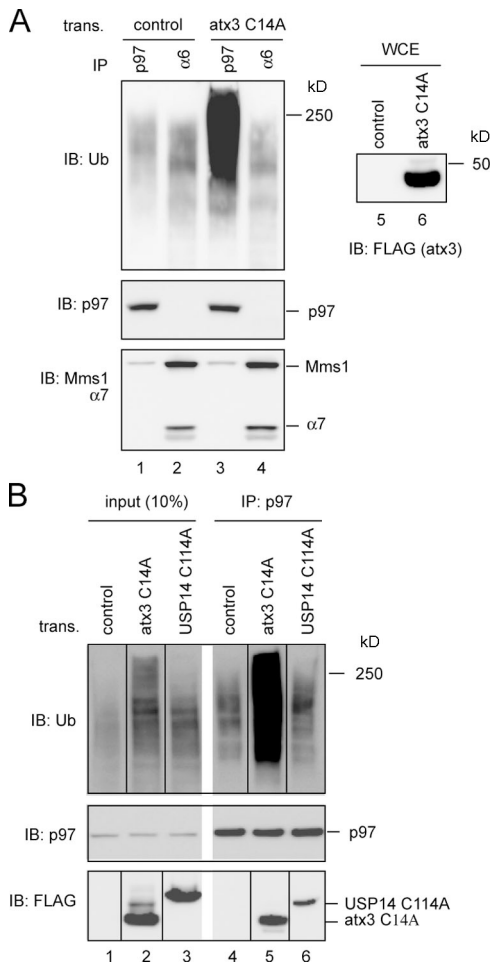
#### Regulated association of atx3 with the ER membrane

We noticed that more atx3 was bound to the p97 QQ than to wt p97 (Fig. 4 D, lane 4 vs. 2; Fig. 4 E). In addition, an increased association between atx3 and VIMP was observed in cells expressing p97 QQ (Fig. 5 A, lane 3 vs. lanes 1 and 2). Consequently, more atx3 was found in association with the ER membrane in these cells (Fig. 5 B, lane 5 vs. lanes 1 and 3). These data indicate that the association of atx3 with p97 and VIMP at the ER membrane may be regulated by the p97 ATPase cycle, a conclusion that is also supported by the recent observation that the interaction of p97 with atx3 in brain extracts is enhanced when ATP or ATP $\gamma$ S is present (Boeddrich et al., 2006). Because atx3 bound purified wt p97 and the p97 QQ mutant with similar affinity in vitro (Fig. 1 A), the enhanced interaction between atx3 and p97 QQ mutant may require additional factors present in cells. Interestingly, mutant atx3 lacking its deubiquitinating activity (C14A) also exhibited an increased affinity to p97, VIMP, and Derlin-1 (Fig. S5; Fig. 5 C). Consistent with this finding, atx3 C14A also accumulated at the ER membrane to a much higher level than wt atx3 (Fig. 5 D, lane 3 vs. lane 1). The simplest explanation of these observations is that a small fraction of atx3 is transiently associated with the ER membrane

via interactions with p97 and VIMP. atx3 may shuttle on and off p97 to promote PAD, a process that is linked to the ATPase cycle of p97 and to the subsequent delivery of substrates to the proteasome. Mutations affecting the enzymatic activity of either p97 or atx3 appear to interfere with the release of atx3 from p97, which may also prevent substrates from leaving p97 (see following paragraph).

#### atx3 C14A blocks the transfer of substrates from p97 to the proteasome

To test whether atx3 C14A inhibits ERAD by interfering with the transfer of substrates from p97 to the proteasome, we determined the relative amount of ubiquitinated substrates bound to p97 versus those bound by the proteasome. We reasoned that a defect in substrate transfer would lead to an accumulation of ubiquitinated substrates in complex with p97. Detergent extracts of 293T cells were subjected to immunoprecipitation with antibodies to either p97 or  $\alpha$ 6, a subunit of the 20S proteasome. Immunoblotting showed that the  $\alpha$ 6 antibodies precipitated  $\alpha$ 7, another subunit of the 20S proteasome, together with Mms1, a subunit of the 19S particle, but not p97. Likewise, antibodies against p97 precipitated the ATPase itself, some Mms1, and no  $\alpha$ 7 (Fig. 6 A). These data confirm that p97 and the 26S proteasome do not form a stable complex. When ubiquitinated proteins were examined, control cells contained little polyubiquitinated proteins, and they were evenly distributed between the proteasome and p97 (Fig. 6 A, lane 2 vs. lane 1). In contrast, atx3 C14A-expressing cells contained more ubiquitinated proteins, and they were almost entirely associated with p97 (Fig. 6 A, lanes 3 and 4; Fig. 6 B). The increased association of p97 with ubiquitinated proteins cannot be simply due to the accumulation of ubiquitinated proteins in cells expressing a defective DUB, because overexpression of the catalytically



**Figure 6. atx3 is involved in transferring ubiquitinated proteins from p97 to the proteasome.** (A) atx3 C14A causes the accumulation of ubiquitinated proteins in association with p97. Ubiquitinated proteins associated with p97 and the proteasome were immunoprecipitated (IP) with anti-p97 and anti- $\alpha 6$  antibodies, respectively. A fraction of the extracts (WCE) was analyzed directly by immunoblotting (IB) (B) USP14 C114A does not increase the level of p97-associated ubiquitinated proteins. Ubiquitinated proteins bound to p97 in cells transfected with the indicated plasmids were immunoprecipitated and analyzed by immunoblotting.

inactive USP14 mutant (USP14 C114A) also increased the level of ubiquitinated proteins, but did not cause an apparent accumulation of ubiquitinated proteins in complex with p97 (Fig. 6 B). These data indicate that atx3 C14A likely blocks the transfer of substrates from p97 to the proteasome, leading to their stabilization.

## Discussion

We have investigated the role of atx3 in retrotranslocation of misfolded ER proteins. We show that atx3 interacts with the recently identified retrotranslocation complex consisting of Derlin-1, VIMP, certain ER-specific ubiquitin ligases, and the pulling ATPase p97 in the ER membrane. A direct involvement of atx3 in ERAD is suggested by its binding to model ERAD substrates en route to the proteasome. Moreover, expression of a mutant atx3 defective in deubiquitination (atx3 C14A) dramatically inhibits the degradation of both membrane and

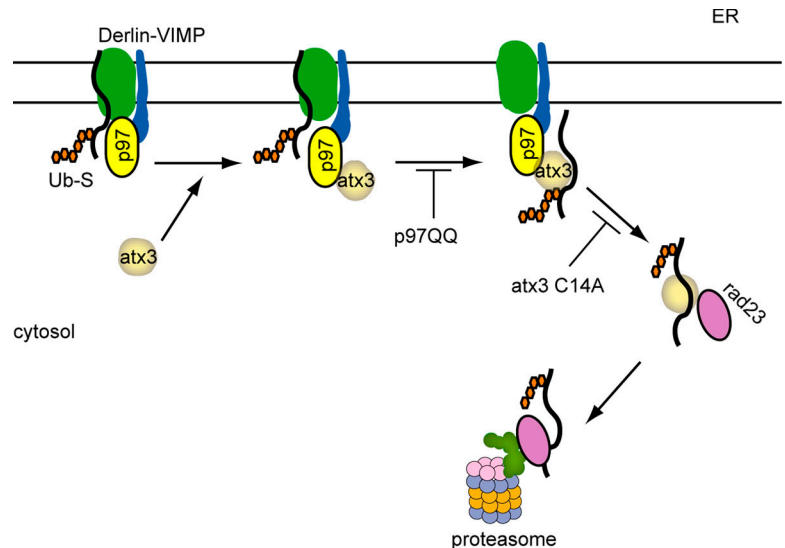
luminal ERAD substrates. Consequently, ER stress is induced in these cells. ERAD substrates stabilized by mutant atx3 are accumulated in part as polyubiquitinated degradation intermediates, suggesting that atx3 acts on these substrates to remove their ubiquitin conjugates. Intriguingly, ubiquitinated proteins accumulated upon mutant atx3 expression are sequestered in complex with p97, raising the possibility that atx3-mediated deubiquitination may be coupled to the transfer of substrates from p97 to the proteasome.

The observation that overexpression of wt atx3 moderately inhibits ERAD is unexpected, but is not without precedence. In fact, overexpression of rad23, an atx3-interacting partner that facilitates the delivery of ubiquitinated proteins to the proteasome (Elsasser and Finley, 2005), also inhibits protein turn-over. Recent studies suggest that the effect of rad23 on protein degradation is highly dependent on its concentration, with low concentrations being stimulatory and high concentrations inhibitory (Verma et al., 2004). The inhibition of ERAD by wt atx3 may be similarly caused by its overexpression, which may titrate out certain factors essential for ERAD. Alternatively, the deubiquitinating activity of atx3 may need to be tightly regulated in conjunction with other reactions to mediate the degradation of ERAD substrates (see following paragraph). The presence of excessive atx3 may uncouple deubiquitination from these processes, which may cause a defect in ERAD.

We propose that atx3 may promote deubiquitination of p97-bound substrates to facilitate their transfer to the proteasome during retrotranslocation (Fig. 7). In this model, atx3 would first interact with p97, which is prebound to its substrates together with Derlin-1 and VIMP at the ER membrane. In the next step, substrates may be transferred from p97 to atx3, a process that is likely associated with the p97 ATPase cycle. Next, atx3 would act on the ubiquitinated substrates, which might be subsequently transferred to a downstream ubiquitin receptor such as rad23. Because ubiquitin chains consisting of at least four ubiquitin molecules are required for efficient targeting of modified substrates to the proteasome, deubiquitination of substrates en route to the proteasome by atx3 is likely tightly coupled to the transfer process to ensure that the targeting signal is not prematurely erased. In *S. cerevisiae*, ubiquitin chains on substrates of cdc48p appear to be progressively shortened while the substrates are in transit to the proteasome (Richly et al., 2005). In light of this finding, we speculate that atx3 may function as an editing enzyme to trim ubiquitin conjugates on substrates to guide them to the proteasome. Although atx3 immunoprecipitated from mammalian cells appears to deubiquitinate its substrates completely, recombinant atx3 purified from bacteria indeed exhibits chain trimming activity, leading to a shortening of ubiquitin chains rather than to their complete removal (Burnett, et al., 2003). Because atx3 binds the ubiquitin-like domain of rad23, which also mediates the interaction between rad23 and the proteasome (Doss-Pepe et al., 2003), it is likely that rad23 may not interact with atx3 and the proteasome simultaneously.

Our analysis demonstrates a physiological function for atx3. We postulate that the expression of the poly-Q-expanded

**Figure 7. A working model for the role of atx3 in ERAD.** atx3 transiently associates with p97 together with its substrates at the ER membrane. For certain substrates, the complex likely also contains the Derlin-VIMP complex. After the p97-dependent dislocation, substrates may be transferred to atx3, which will shorten the ubiquitin chains on the substrates. This process may be coupled to the delivery of the substrates to an ubiquitin receptor such as rad23 on the proteasome. Mutations affecting either the ATPase cycle of p97 or the deubiquitinating activity of atx3 would prevent substrates from reaching the proteasome.



atx3 mutants may also impair ERAD and trigger ER stress, which may contribute to the pathogenesis of spinocerebellar ataxia. Further analysis of these mutants will help to clarify the long-suspected connection between ER stress and neurodegenerative diseases.

## Materials and methods

### Constructs

The plasmids pcDNA3.1 His-p97 wt and pcDNA3.1 His-p97 QQ were described previously (Ye et al., 2004). The plasmid pRK-FLAG atx3 wt (consisting of 20 CAG repeat) was constructed by cloning the human atx3 cDNA (a gift from R. Pittman, University of Pennsylvania, Philadelphia, PA; Burnett et al., 2005) into the Sall and NotI sites of the pRK-FLAG vector (a gift from H. Shu, the National Jewish Medical Center, Denver, CO). pRK-FLAG-USP14 was constructed by cloning the human USP14 cDNA into the Sall and NotI sites of the pRK-FLAG vector. pRK-FLAG-atx3 C14A and pRK-FLAG-USP14 C114A were constructed by site-directed mutagenesis using the QuikChange mutagenesis kit from Stratagene. All plasmids were sequenced. HA-tagged TCR $\alpha$  clone was a gift from R. Kopito (Stanford University, Stanford, CA; Yu et al., 1997). pGL-3-GRP78 (-132) luciferase plasmid was provided by K. Mori (Kyoto University, Kyoto, Japan). Plasmids expressing Ub-R-GFP, GST-SVIP, and BACE457 $\Delta$  were provided by N. Dantuma (Karolinska Institute, Stockholm, Sweden), M. Nagahama (Tokyo University of Pharmacy and Life Science, Tokyo, Japan), and M. Molinari (Institute for Research in Biomedicine, Bellinzona, Switzerland), respectively.

### Antibodies and chemicals

Anti-BACE antibody was a gift from P. Paganetti (Novartis Pharma AG, Basel, Switzerland). Antibodies to ubiquitin, p97, Ufd1, and VIMP were described previously (Ye et al., 2004, 2005). Rabbit polyclonal antibodies to Derlin-1 were raised against a peptide corresponding to residues 238–251 of the human Derlin-1. All antibodies used in this study were affinity purified using the corresponding antigenic peptides. FLAG, HA, and GFP antibodies were purchased from Sigma-Aldrich, Roche, and Invitrogen, respectively. MG132 was purchased from Calbiochem.

### Protein purification and in vitro binding experiments

The purification of His-tagged p97 variants and the Ufd1-Npl4 complex has been described previously (Ye et al., 2003). GST-atx3 was purified according to standard procedure using glutathione beads. GST pull-down experiments were performed as described previously (Ye et al., 2005). In brief, GST fusion proteins (5  $\mu$ g) bound to glutathione beads were incubated with various purified recombinant proteins in 50 mM Hepes (pH 7.3), 150 mM potassium chloride, 2.5 mM magnesium chloride, 5% glycerol, and 0.1% Triton X-100. Bound material was analyzed by SDS/PAGE and stained with Coomassie blue.

### Mammalian cell culture, transient gene expression, fractionation, and coimmunoprecipitation

293T cells were maintained according to standard procedures. Transfections in 293T cells were done with Trans IT (Mirus). For fractionation experiments, cells were homogenized by passing through a 25G needle in a low salt buffer (50 mM Hepes, pH 7.4, 10 mM potassium chloride, 1 mM DTT, and a protease inhibitor). Cell extracts were then subjected to sequential centrifugation at different speeds. For coimmunoprecipitation experiments, tissue culture cells or dog pancreatic microsomes were extracted with buffer N (30 mM Tris/HCl, pH 7.4, 150 mM potassium acetate, 4 mM magnesium acetate, 1% DeoxyBigCHAP, and a protease inhibitor cocktail). To detect ubiquitinated TCR $\alpha$ , cell extracts were adjusted to contain 0.2% SDS, 0.5 mM DTT, and 5 mM *N*-ethylmaleimide. Samples were heated at 95°C before being subjected to immunoprecipitation. To isolate the 26S proteasome, 2 mM ATP was included in the extraction buffer to preserve the interaction between the 19S and the 20S proteasome particles. All cell extracts were cleared by centrifugation (20,000 g) and subjected to immunoprecipitation with various antibodies. Immunoblots were visualized with a cooled CCD digital camera system (LAS-3000; Fuji). Results were quantified using MultiGauge v3.0 software (Fuji).

### In vitro deubiquitination experiments

To detect p97 or atx3-associated deubiquitination, cells were extracted with buffer N. Cell extracts were subjected to immunoprecipitation with either p97 antibodies or anti-FLAG antibody to isolate p97 or FLAG-tagged atx3. Proteins bound to the beads were incubated in a deubiquitinating buffer (50mM Tris/HCl, pH 7.4, 20mM potassium chloride, 5mM magnesium chloride, 1mM DTT, 2.5% BSA) at 37°C. Deubiquitination was stopped by addition of SDS sample buffer. Samples were analyzed by immunoblotting.

### Online supplemental material

Fig. S1 shows that knock-down of atx3 by  $\sim$ 80% by siRNA has little effect on the degradation of TCR $\alpha$ . Fig. S2 shows that the degradation of TCR $\alpha$  requires p97. Fig. S3 provides evidence that the accumulation of TCR $\alpha$  in atx3 C14A-expressing cells is due to its stabilization. Fig. S4 shows the association of p97 with ubiquitinated proteins. Fig. S5 demonstrates that an atx3 mutant defective in deubiquitination fails to promote PAD. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200605100/DC1>.

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