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The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity

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Abstract

The ubiquitin-proteasome pathway is critically involved in the pathology of neurodegenerative diseases characterized by protein misfolding and aggregation. Data in the present study suggest that the polyglutamine neurodegenerative disease protein, ataxin-3 (AT3), functions in the ubiquitin-proteasome pathway. AT3 contains an ubiquitin interaction motif (UIM) domain that binds polyubiquitylated proteins with a strong preference for chains containing four or more ubiquitins. Mutating the conserved leucine in the first UIM (L229A) almost totally eliminates binding to polyubiquitin chains while a similar mutation in the second UIM (L249A) also inhibits binding to polyubiquitin chains but to a lesser extent. Both wild-type and pathological AT3 increase cellular levels of a short-lived GFP that is degraded by the ubiquitin-proteasome pathway. AT3 has several properties characteristic of ubiquitin proteases including decreasing polyubiquitylation of ^{125}I-lysozyme by removing ubiquitin from polyubiquitin chains, cleaving a ubiquitin protease substrate, and binding the specific ubiquitin protease inhibitor, ubiquitin-aldehyde. Mutating the predicted catalytic cysteine in AT3 inhibits each of these ubiquitin protease activities. The ability to bind and cleave ubiquitylated proteins is consistent with AT3 playing a role in the ubiquitin-proteasome system. This raises the possibility that pathological AT3, which tends to misfold and aggregate, may be exposed to aggregate-prone misfolded/denatured proteins as part of its normal function.

Introduction

Spinocerebellar ataxia type-3 (SCA3) is the most common dominantly inherited cerebellar ataxia and a member of the polyglutamine neurodegenerative disease family (1-4). As is the case with other members of this family, the protein mutated in SCA3, ataxin-3 (AT3), causes pathology through an apparent gain of function associated with expansion of a CAG repeat that codes for a polyglutamine domain within the protein. Other diseases in this family include Huntington's, dentatorubral pallidoluysian atrophy, spinal and bulbar muscular atrophy, and SCA1, 2, 6, and 7. A prominent feature in these diseases is the presence of nuclear, and in some cases cytoplasmic inclusions of aggregated pathological protein (5-8). Typically, inclusions are highly ubiquitylated and in many cases proteasomes are associated with inclusions which suggests an attempt to degrade the aggregated proteins (5,6,9-11).

Targeting a protein for proteasome degradation is a multi-step iterative process involving an E1 activating enzyme, E2 ubiquitin carrier/conjugating enzymes, and E3 ubiquitin ligases which generate polyubiquitin chains linked to the ε-amino group of lysine(s) in the protein targeted for degradation (12). Editing and disassembly of polyubiquitin chains as well as recycling ubiquitin is critical for cellular homeostasis. These functions are carried out by cysteine proteases known as deubiquitinating enzymes (DUBs; 13,14). The two major families of DUBs are ubiquitin C-terminal hydrolases (UCHs) and ubiquitin specific proteases (USPs). UCHs are well-conserved proteases that prefer cleaving small leaving groups from the C-terminus of ubiquitin and primarily function in maintaining high levels of free ubiquitin in cells. USPs are a large highly divergent family of proteases with substrate specificities ranging from very general deubiquitinating properties to highly specific cleavage of ubiquitin from a single or small

number of protein targets; USPs primarily function in ubiquitin chain editing and disassembly (13,14).

DUBs as well as other regulators of the ubiquitin proteasome pathway (UPP) have been linked to neurodegenerative diseases that are characterized by protein misfolding and aggregation. In some cases mutations or polymorphisms in regulators of the UPP are responsible for inherited forms of the disease or modulate the disease phenotype. For instance, mutation of Parkin, an E3 ubiquitin ligase, results in early onset Parkinson's disease, and a polymorphism resulting in reduced ligase activity of the bifunctional DUB, UCH-L1 (15), is associated with decreased susceptibility to Parkinson's disease (16,17). This same polymorphism in UCH-L1 has also been linked to the age of onset in Huntington's disease (18). Mutations in UCH-L3, as well as mutations in ubiquitin and ubiquitin ligases enhance degeneration in a Drosophila model of SCA1 (19). These and other studies support the hypothesis that the UPP is intimately linked to neurodegenerative diseases characterized by protein misfolding and aggregation (20,21).

Although misfolded polyglutamine proteins may be exposed to the UPP as part of normal protein turnover there is little data to suggest that polyglutamine proteins are exposed to the UPP as part of their normal cellular functions. If a polyglutamine protein were part of this pathway it would create a particularly dangerous situation in which the pathological protein with its destabilizing expanded polyglutamine domain (22) and tendency to aggregate (23,24) is exposed to misfolded proteins which could increase the probability of protein aggregation.

Based on its sequence, AT3 may function in the UPP. Depending on the splice variant, AT3 has either 2 or 3 potential ubiquitin interaction motifs (UIMs; 25) that have recently been shown to

bind mono and/or polyubiquitylated proteins (26,27). During the course of our studies to determine if AT3 binds ubiquitylated species, we found that AT3 not only binds polyubiquitylated chains and proteins but it also exhibits ubiquitin protease activity that is inhibited by mutating the predicted active site cysteine.

Results

AT3 binds ubiquitin chains containing four or more ubiquitins through its UIM domain

The ubiquitin interaction motif (UIM) is a recently identified protein motif that binds mono and/or polyubiquitylated proteins (25-27). The major splice form of AT3 has two predicted UIMs located between its conserved N terminus and the polyglutamine domain (Fig. 1A). To determine if AT3 binds ubiquitylated proteins, cell lysates were incubated with MBP-AT3 and associated proteins pulled down with amylose beads. Both wild-type and pathological AT3 bind ubiquitylated cellular proteins equally whereas MBP does not bind ubiquitylated proteins (Fig. 1B). Monoubiquitylated as well as polyubiquitylated proteins are present in cell lysates; therefore, the preference of AT3 for ubiquitylated species was determined by the ability of AT3 to bind free ubiquitin, a monoubiquitin fusion protein, or polyubiquitin chains of varying lengths. Under assay conditions used, AT3 does not bind free ubiquitin, a monoubiquitin fusion protein or ubiquitin chains containing less than 4 ubiquitins (Fig. 1C). However, AT3 binds polyubiquitin chains containing 4 or more ubiquitins which is the chain length required to target protein substrates to the proteasome (28). No obvious difference in binding between wild-type and pathological AT3 was observed in these experiments.

Polyubiquitin chains were used to identify the domain of AT3 responsible for binding polyubiquitylated proteins. Full length AT3, AT3(1-291), and AT3 (191-291) containing the UIMs bind ubiquitin chains whereas AT3(1-191) or the polyglutamine containing C-terminus (C-29Q) do not (Fig. 1D). Mutating the conserved leucine in the first UIM (L229A) almost totally eliminates binding to polyubiquitin chains while a similar mutation in the second UIM (L249A) inhibits binding to polyubiquitin chains but to a lesser extent (Fig. 1E). Together these data indicate that the UIMs are responsible for AT3 binding to polyubiquitylated species containing chains of 4 or more ubiquitins.

AT3 increases levels of short-lived GFP-u

GFP-u is a short-lived GFP containing a destabilizing sequence that targets it for degradation by the ubiquitin proteasome pathway (29). Treating GFP-u cells with the proteasome inhibitor, MG132, increases the number of GFP positive cells (Fig. 2B). The number of GFP positive cells also increases when wild-type (Fig. 2C) or pathological (Fig. 2D) AT3 is co-transfected with GFP-u. Pathological AT3 increases the number of GFP positive cells to a greater extent than wild-type AT3. This observation is supported by western blot analysis showing that both wild-type and pathological AT3 increase the amount of GFP-u and that pathological AT3 increases levels of GFP-u to a greater extent (Fig. 2E). Both wild-type and pathological AT3 increase the amount of GFP-u in cells treated with MG132 (Fig. 2F); this suggests that AT3 may be affecting the ubiquitylation arm of the UPP rather than inhibiting proteasome activity.

AT3 decreases the level of ^{125}I-lysozyme polyubiquitylation

To determine if AT3 could directly alter ubiquitylation of proteins a standard ubiquitylation assay using ^{125}I-lysozyme was performed. Both wild-type and pathological AT3 decrease the

level of polyubiquitylation of ^{125}I-lysozyme while MBP has no effect on ubiquitylation (Fig. 3A). Lysozyme has six lysines that are potential targets for ubiquitylation; therefore, low molecular weight species of ubiquitylated lysozyme ("short chains") likely represent a composite of mono-, di- and tri-ubiquitylated lysines. Both wild-type and pathological AT3 decrease polyubiquitylated species of lysozyme about 90% compared to controls (Fig. 3B). The level of low molecular weight ubiquitylated species increases 2-3 fold suggesting that AT3 decreases polyubiquitin chain formation which results in a build-up of lysozyme containing short ubiquitin chains.

The effect of AT3 on ubiquitylation of proteins in reticulocyte lysates fraction II used in the assay and unlabeled lysozyme was determined by performing assays with unlabeled lysozyme and ubiquitylation measured by blotting for ubiquitylated proteins. Both wild-type and pathological AT3 decrease ubiquitylation of protein targets in fraction II lysates; lane1 in Fig. 3C shows control ubiquitylation of these substrates and lanes 5 and 6 show an absence of these ubiquitylated species in the presence of wild-type or pathological AT3. As expected, AT3 also decreases polyubiquitylated unlabeled lysozyme while MBP has not effect. Under conditions used in these assays, neither normal nor pathological AT3 is ubiquitylated (not shown; also note the absence of high molecular weight species in lanes 5 and 6 of Fig. 3C that should be present if AT3 is ubiquitylated).

One potential mechanism to account for AT3 decreasing polyubiquitin chains and increasing accumulation of short chains on lysozyme would be that the UIMs of AT3 bind to the growing ubiquitin chains and block polyubiquitin chain extension. To test this possibility the effect of AT3 with mutations in both UIMs (L229A/L249A) on ubiquitylation of ^{125}I-lysozyme was

determined. Surprisingly, the loss of polyubiquitin chain binding by AT3 does not increase formation of polyubiquitin chains on lysozyme but rather, decreases overall ubiquitylation (Fig. 4A,B).

AT3 has properties of a ubiquitin protease

Other potential mechanisms that could account for AT3 decreasing ubiquitylation would be that AT3 inhibits the ubiquitylation process itself or it could function as a deubiquitinating protease and remove ubiquitin from growing chains. If AT3 inhibits the enzymes that add ubiquitin to ^{125}I-lysozyme in a typical 6 hrs reaction, then adding AT3 to the ubiquitylation reaction after 4 hrs should result in levels of polyubiquitylation being maintained over the next 2 hours; however, if AT3 functions as a protease and removes ubiquitin then adding it to the reaction after 4 hrs should decrease ubiquitylated ^{125}I-lysozyme over the next 2 hours. Adding AT3 to the reaction after ^{125}I-lysozyme is ubiquitylated results in a loss of polyubiquitin chains from ^{125}I-lysozyme and an increase in lysozyme with short chains over the next 2 hrs (Fig. 5A). Adding the specific ubiquitin protease inhibitor, ubiquitin-aldehyde, decreases AT3-dependent loss of polyubiquitylated ^{125}I-lysozyme (Fig. 5B). These data suggest that AT3 decreases polyubiquitylation of ^{125}I-lysozyme by functioning as an ubiquitin protease. The sequence of AT3 shows little overall similarity to either the UCH or USP family of ubiquitin proteases (not shown); however, sequence similarity between these two families of ubiquitin proteases is restricted to modest similarity around the catalytic triad of cysteine, histidine, aspartate/asparagine. Homologues and orthologues of AT3 are present in a number species including *C. elegans* and *Arabidopsis thaliana* and the N-terminal 197 amino acids of AT3 has been designated “Josephin”/pfam 02099 and is present in more than 30 proteins of unknown function (Conserved Domain Database; http:www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtm).

Members of the Josephin family have one cysteine (cysteine-14 of AT3) and two histidines (histidines 17 and 119 of AT3) that are conserved in all family members. Comparing the sequence around cysteine-14 and histidine-119 of human AT3 (and the *C. elegans* and *Arabidopsis* homologues) with sequences around the catalytic cysteine, histidine, and aspartate/asparagine of members of the USP and UCH families indicate that AT3 shares sequence homology with both the UCH and USP families (Fig. 6A,B). Importantly, all 3 amino acids in the active site as well as additional amino acids shown to be involved in catalytic activity (marked by asterisks in Fig. 6) are conserved in the Josephin family. The only exception is one histidine shown to participate in USP catalytic activity is not conserved in Josephin family members; however, a second invariant histidine in the Josephin family (histidine 17 in AT3) is close to the predicted catalytic cysteine for Josephin proteins. A very recent bioinformatics study by Hoffmann's group provides a detailed analysis of the similarity of AT3 with ubiquitin proteases, including predicted catalytic residues (30). Based on sequence comparison to USP and UCH ubiquitin proteases, cysteine-14 of AT3 may function as part of a ubiquitin protease catalytic site. Mutating this cysteine to alanine (C14A) greatly decreases the ability of AT3 to remove polyubiquitin chains from ^{125}I-lysozyme (Fig. 7A). This suggests that AT3 has ubiquitin protease activity and cysteine-14 participates in its catalytic activity. Additional support for AT3 having ubiquitin protease activity are experiments showing that wild-type and pathological AT3 with or without mutations in the UIM domain cleave the ubiquitin protease substrate, ubiquitin-AMC (Fig. 7B). Mutating the predicted active site cysteine blocks its catalytic activity consistent with cysteine-14 being critical for ubiquitin protease activity. Although AT3 does not bind monomeric ubiquitin (Fig. 1C), if it is an ubiquitin protease it should bind monomeric ubiquitin aldehyde (ubiquitin with its C terminal glycine ending as an aldehyde rather than an acid) at its active site. Under the same conditions that ubiquitin does not bind to AT3, ubiquitin aldehyde

binds AT3; however, it does not bind to AT3 with the C14A mutation (Fig. 7C). Together these data indicate that AT3 has ubiquitin protease activity and that cysteine-14 is required for its activity.

Discussion

Data in the present study are consistent with AT3 functioning in the UPP. AT3 contains a UIM domain that binds polyubiquitylated proteins with a strong preference for binding chains containing 4 or more ubiquitins which is the chain length required for proteasome degradation. Additional support for AT3 functioning in this pathway is data showing that AT3 has ubiquitin protease properties including the ability to remove polyubiquitin chains from ^{125}I-lysozyme, cleave an ubiquitin protease substrate, and bind an active site inhibitor of ubiquitin proteases. Based on sequence similarity of ubiquitin proteases and the Josephin/pfam 02099 conserved domain present in AT3, AT3 homologues and orthologues are likely to function in the UPP in organisms ranging from humans to plants (30; present study). Key questions that need to be addressed experimentally and are the focus of the discussion include: 1) what is the function/role of AT3 in the UPP, 2) does expansion of the polyglutamine domain in pathological AT3 alter its functions in the UPP, and 3) is the UPP function of AT3 linked to disease.

It is likely that AT3 functions as an ubiquitin protease; however, it seems unlikely that AT3 will have the same ubiquitin protease functions as USP and UCH families. While there is a small amount of overlap in targets of the USP and UCH families in general these ubiquitin protease families appear to perform very different functions. UCH family members are responsible for cleaving ubiquitin from small peptides and adducts as well as UbCEP52 and 80 (31). Their primary function appears to be to maintain high levels of free ubiquitin in cells (13,14). USP

family members have a wide diversity of substrates but generally prefer cleaving monoubiquitin or ubiquitin chains from large proteins. Some USPs can also disassemble free ubiquitin chains not linked to proteins. A primary function of USP family members appears to be editing polyubiquitin chains; some USPs edit a wide range of substrates while others edit only a few substrates (13,14). In addition to editing, USPs also help maintain high levels of free ubiquitin. Based on sequence and initial characterization of AT3 protease activity, it is not possible to define a specific function or target preference. However, some potential functions might be editing functions different from general USPs such as maintaining proteins in a monoubiquitylated state, editing linkages different from K48, editing chains with mixed linkages, or preferentially editing ubiquitin-like proteins such as SUMO or Nedd8.

Several proteins have been identified that bind AT3; therefore, it is possible that AT3 may function at the interface of these interactions and the UPP. Our recent studies show that endogenous AT3 binds histones as well as transcriptional co-activators and can function as a transcriptional co-repressor (32). Therefore, at least one of its functions appears to be as a transcriptional regulator. Recent studies have shown a link between transcriptional regulation and the ubiquitin-proteasome pathway (33-35); however, currently there is no data to support a link between transcription and deubiquitylation effects of AT3.

AT3 interacts with two proteins, VCP/p97 (36) and Rad23 (37),that bind polyubiquitylated proteins and appear to link the proteasome to various cellular functions. AT3 binds VCP/p97 through its polyglutamine domain and pathological AT3 with an expanded polyglutamine domain appears to bind more effectively than wild-type AT3 (36). VCP is required for degradation of a large number of protein substrates and appears to be responsible for shuttling

polyubiquitylated proteins to proteasomes (38). Even though both AT3 and VCP may function in the UPP it is unlikely that an evolutionary conserved function of AT3 or other Josephin family members involves direct interaction with VCP because of the lack of a significant polyglutamine domain in any species other than humans. However, it is possible that in humans and in particular in the presence of a pathological expansion of the polyglutamine domain in AT3 that the presence of both proteins in the ubiquitin-proteasome pathway may result in encounters and abnormal interactions resulting in altered functions of both proteins. The Josephin domain of AT3 binds Rad23 (37). Rad23 along with XPC is important in recognizing altered DNA adducts and initiating global genome nucleotide excision repair (NER) in species from yeast to humans. Rad23 also binds the proteasome through its ubiquitin-like domain (39) as well as polyubiquitylated proteins through its ubiquitin-associated domain (40). Rad23 inhibits ubiquitylation in vitro and is proposed to be a critical link between NER and proteasome function (41-43). At the present it is not clear if interactions of AT3 with VCP or Rad23 have functional consequences; however, known functions for VCP and Rad23 in the UPP suggest that these interactions may be relevant for both normal and pathological functions of AT3.

Once a more defined protease function and targets have been identified for AT3 it will be critical to determine if expansion of the polyglutamine stretch alters or modulates this function. Data in the present study show that some properties such as binding polyubiquitin chains and proteins, decreasing polyubiquitylation of ^{125}I-lysozyme, or cleaving the simple substrate ubiquitin-AMC are the same for wild-type and pathological AT3; whereas, other properties such as increasing cellular levels of short-lived GFP-u and effects on short chain ubiquitylation in both the presence and absence of UIM mutations are differentially affected by wild-type and pathological proteins. Pathological protein increases cellular levels of GFP-u and decreases short-chain ubiquitylation

of ^{125}I-lysozyme to a greater extent than wild-type AT3. These initial studies raise the possibility that expansion of the polyglutamine domain in pathological AT3 modulates its protease activity and/or the range of its substrates.

A critical question is whether AT3's function in the UPP is linked to disease pathology. While it is possible that pathological AT3 acquires new properties that alter UPP functions and this results in dysfunction and disease, another obvious mechanism that would link the two is that pathological AT3 with its destabilizing polyglutamine domain (22) is exposed to misfolded and denatured proteins being degraded. A statistically small fraction of these encounters could result in stable interactions between pathological AT3 and these misfolded proteins. These tightly associated small aggregates may be degraded by the proteasome or act as a "seed" to create larger aggregates and eventually, inclusions. These larger aggregates/inclusions would sequester various proteins including proteasomes (9-11) and inhibit their function (29). This could create an insidious "feed forward" process in which continued encounters between pathological AT3 and misfolded denatured proteins coupled with decreased proteasome activity resulting from polyglutamine aggregates would increase the concentration of polyglutamine proteins and therefore the probability that misfolded polyglutamine proteins would aggregate. Over the course of decades this could result in inadequate degradation of many cellular regulators resulting in cellular dysfunction. If AT3 were exposed to denatured/misfolded proteins as part of its normal function then a prediction would be that AT3 might be a component of protein aggregates formed in other neurodegenerative diseases. While this can occur in cells co-transfected with two polyglutamine proteins (44) this possibility does not appear to have been examined in human disease. However, it is interesting that recent studies show that wild-type AT3 but not other polyglutamine proteins is a prominent component of the central core of ubiquitylated Marinesco

bodies, which are protein aggregates/inclusions found in human midbrain neurons and thought to be the result of cellular stress and aging (45,46).

Materials and Methods

Plasmids, proteins, and mutations

Bacterial expression constructs for MBP, MBP-AT3-29Q, MBP-AT3-72Q, MBP-AT3(1-291), and MBP-AT3(C-29Q) were described previously (32). MBP-AT3(1-191) and MBP-AT3(191-291) were generated by cloning PCR-amplified fragments into the MBP expression vector. Recombinant proteins were expressed in BL21 E. *coli* (Novagen) and MBP fusion proteins were purified using amylose beads (New England Biolabs). Leucine to alanine mutations in the UIMs and cysteine to alanine mutation of the active site were performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) as per the manufacturer's instructions. Forward and reverse primers contained the desired mutation. Reactions were performed in a total volume of 50 ul containing 100 ng ataxin-3 plasmid DNA, 125 ng of each forward and reverse primer, dNTPs, 7% dimethylsulfoxide, and 2.5 U Pfu Turbo DNA polymerase. Synthesis of mutated DNA was performed in a temperature cycler (Applied Biosystems, GeneAmp PCR System 2400). Aliquots of 50 ul Escherichia coli XL1 blue supercompetent cells were transformed with 1 ul DpnI-treated DNA and colonies selected on LB ampicillin agar plates. Plasmid DNA was isolated from random colonies and mutagenesis confirmed by DNA sequencing. Primers used were UIM(L229A): forward primer, 5'GAA GAT GAG GAG GAT GCG CAG AGG GCT CTG GCA C3', reverse primer, 5'GTG CCA GAG CCC TCT GCG CGT CCT CCT CAT CTT C3'; UIM(L249A): forward primer, 5'GAT GAG GAA GCA GAT GCC CGC AGG GCT ATT

CAG3', reverse primer, 5' CTG AAT AGC CCT GCG GGC ATC TGC TTC CTC ATC3'; C14A: forward primer, 5'CAA GAA GGC TCA CTT GCT CAA CAT TGC CTG3', reverse primer, 5'CAG GCA ATG TTG AGC AAC TGA GCC TTC TTG3'.

In vitro pull-down assays

MBP or MBP-AT3 fusion proteins bound to amylose beads were incubated at 4°C with lysates from CHO or 293T cells, free ubiquitin, ubiquitin aldehyde, polyubiquitin chains (Affiniti Research Products), or a fusion of monoubiquitin and the finger domain of tissue plasminogen activator. Binding reactions contained 15 mM HEPES-KOH, pH 7.9, 10% glycerol, 200 mM KCl, 2 mM $MgCl_2$, 1% NP40, 1 mM DTT, 20 ug/ml BSA, and protease inhibitor cocktail (Roche Molecular Biochemicals). Beads were washed extensively and bound proteins separated by SDS-PAGE and analyzed by western blotting using a polyclonal ubiquitin antibody (generated in our lab) or FK2 monoclonal ubiquitin antibody (Affiniti Research Products).

Cell transfection

293T cells were co-transfected with myc-tagged AT3 constructs and GFP-u construct. 48 hours after transfection, cells were either treated for 2 hours with 10 uM of the proteasome inhibitor MG132 or left untreated. Cells were harvested using lysis buffer containing 25 mM Tris –HCl, pH 8.0, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1% NP40, 1 mM DTT, and protease inhibitor cocktail and processed for western blots.

Ubiquitylation assay

Lysozyme was iodinated with $Na^{125}I$ using IodoBeads (Pierce) to a specific activity of 0.25 mCi/mg protein. ^{125}I-Lysozyme (100,000 cpm) was incubated at 30 ºC for 4 hours in a total

volume of 40 ul of reaction mixture containing 50 mM HEPES (pH 7.5), 4 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, an energy regenerating system (10 ug/ml creatine kinase, 5 mM creatine phosphate, 4 mM GTP), 20 ug ubiquitin conjugation enzyme fraction from rabbit reticulocyte (Fraction II; Sigma), 1 ug ubiquitin aldehyde (Sigma), 4 ug ubiquitin (Sigma), and 2.5 ug MBP or MBP-AT3. Reactions were stopped by adding SDS sample buffer, boiled for 5 minutes, and resolved on 12% SDS-PAGE gels. Phosphoimager analysis was used for quantitation. Ubiquitylation reactions with nonradioactive lysozyme were performed using 4 ug lysozyme per reaction and western blots probed with FK1 antibody (Affiniti Research Products).

Ubiquitin protease assays

Ubiquitin-AMC protease activity. The protease activity of AT3 was measured using the fluorogenic substrate ubiquitin-AMC (Boston Biochem). In short, 100 ul of assay buffer (50 mM HEPES, 0.5 mM EDTA, pH 7.5 containing 0.1 mg/ml ovalbumin and 1 mM DTT) was maintained at 37 °C in the presence of 100 nM MBP-AT3 for 30 minutes. The reaction was initiated by adding ubiquitin-AMC to a final concentration of 0.5 uM and the amount of fluorescence measured at 2.5 min intervals for 15 min using a Biotek Synergy HT fluorimeter.

Ubiquitylated ^{125}I-lysoszyme protease assay. Ubiquitylated ^{125}I-lysozyme was prepared as indicated for the ubiquitylation assay. After 4 hrs, the reaction mixture was divided into 25 ul fractions. Fractions received different additions as indicated in the figures including control buffer, 2.5 ug MBP or MBP-AT3 fusion proteins, 5 ug ubiquitin aldehyde, or SDS sample buffer (to stop the reaction at 4 hrs). The reaction was allowed to continue for another 2 hours and then stopped by adding SDS sample buffer. Samples were then boiled for 5 minutes, resolved on 12% SDS-PAGE gels and phosphoimager analysis used for quantitation.

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Legends to Figures

Figure 1. The UIM domain of AT3 binds ubiquitylated proteins and polyubquitin chains containing 4 or more ubiquitins. (A) Schematic of AT3. The UIMs (dark blocks) are located between the conserved N terminus Josephin domain (stippled) and the polyglutamine domain ("Q" block). Different lengths of the protein reflect different numbers of glutamines in the wild-type (29 glutamines) and pathological (72 glutamines) proteins used in the present study. Sequences of the two UIMs are shown in capital letters and the two conserved leucines in the UIMs that were mutated to alanine are underlined. (B) MBP, MBP-AT3-29Q, or MBP-AT3-72Q bound to amylose beads was incubated with cell lysates and bound proteins analyzed by western blot for ubiquitin. Normal and pathological AT3 interact similarly with ubiquitylated proteins in lysates. (C) MBP, MBP-AT3-29Q, or MBP-AT3-72Q bound to amylose beads was incubated with polyubiquitin chains (top panel), a monoubiquitin finger domain of tissue plasminogen activator (Ub-F, middle panel) or free ubiquitin (Ub, lower panel). Bound proteins were analyzed by western blot for ubiquitin. Both normal and pathological AT3 interact similarly with polyubiquitin chains containing 4 or more ubiquitins, while neither bind free ubiquitin, monoubiquitin finger domain, or ubiquitin chains containing less than 4 ubiquitins. (D) MBP-AT3-29Q, MBP-AT3(1-191), MBP-AT3(C-29Q), MBP-AT3(1-291), or MBP-AT3(191-291) bound to amylose beads was incubated with polyubiquitin chains and bound proteins analyzed

by western blot for ubiquitin. Full length AT3, AT3(1-291), and AT3(191-292) bind polyubiquitin chains containing 4 or more ubiquitins. (E) MBP, MBP-AT3-29Q, MBP-AT3-29Q(L229A), or MBP-AT3-29Q(L249A) bound to amylose beads was incubated with polyubiquitin chains and bound proteins analyzed by western blot for ubiquitin. Both mutations decrease binding to polyubiquitin chains with the L229A mutation being more effective. Experiments in this figure were performed 3-10 times with similar results.

Figure 2. AT3 increases cellular levels of short-lived GFP-u. 293T cells were co-transfected with GFP-u (29) and (A) pCDNA3 vector, (B) pCDNA 3 vector and cells treated with 10 uM MG132 for 2 hrs, (C) AT3-29Q, or (D) AT3-72Q. Photographs of GFP positive cells were taken 48 hrs after transfection. Both MG132 and AT3 increase the number of GFP positive cells. (E) Western blots of equal protein from 293T cells co-transfected with GFP-u and vector (control), AT3-29Q, or AT3-72Q; note that both wild-type and pathological AT3 increase levels of GFP-u. (F) Western blot of equal protein from 293T cells co-transfected with vector (control), AT3-29Q or AT3-72Q. One control culture and both AT3 cultures were treated with 10uM MG132 for 2 hrs prior to collecting cells; note that MG132 increases the level of GFP-u while AT3 + MG132 increases the level of GFP-u above that seen in the presence of MG132 alone. Experiments were performed 5 times with similar results

Figure 3. AT3 decreases polyubiquitylation of lysozyme and proteins in fraction II lysate. (A) In vitro polyubiquitylation of ^{125}I-lysozyme is decreased by MBP-AT3-29Q and MBP-AT3-72Q but not by MBP. Ubiquitylation in the absence of added ubiquitin is shown in lane 1. (B) Quantitation of short-chain ubiquitylaion and polyubiquitylation of ^{125}I-lysozyme by MBP, MBP-AT3-29Q or MBP-AT3-72Q relative to control ubiquitylation (data represent the mean $\pm$

sem of 4 experiments). (C) Decreased ubiquitylation of unlabeled lysozyme and protein substrates in fraction II lysate by MBP-AT3-29Q and MBP-AT3-72Q. Following SDS-PAGE the blot was probed with anti-ubiquitin FK1 monoclonal antibody. Lane 1 represents ubiquitylated protein substrates in fraction II lysate and lane 2 ubiquitylation in the absence of added ubiquitin. Note that ubiquitylation of endogenous protein substrates in fraction II (lane 1) is inhibited by wild-type and pathological AT3 (lanes 5 and 6). AT3 is not ubiquitylated under these conditions (note the absence of high MW ubiquitylated species in lanes 5 and 6).

Figure 4. Effect of mutating AT3 UIMs on ubiquitylation of ^{125}I-lysozyme. (A) MBP-AT3-29Q and MBP-AT3-72Q decrease polyubiquitylation of ^{125}I-lysozyme. Mutating the UIMs in both wild-type and pathological AT3 (L229/249A) decreases short chain ubiquitylation relative to MBP-AT3-29Q and 72Q. (B) Quantitation of effects of MBP, MBP-AT3-29Q, MBP-AT3-29Q(L229/249A), MBP-AT3-72Q, or MBP-AT3-72Q(L229/249A) on short chain ubiquitylation and polyubiquitylation relative to control ubiquitylation. Data represent mean $\pm$ sem from 5 experiments.

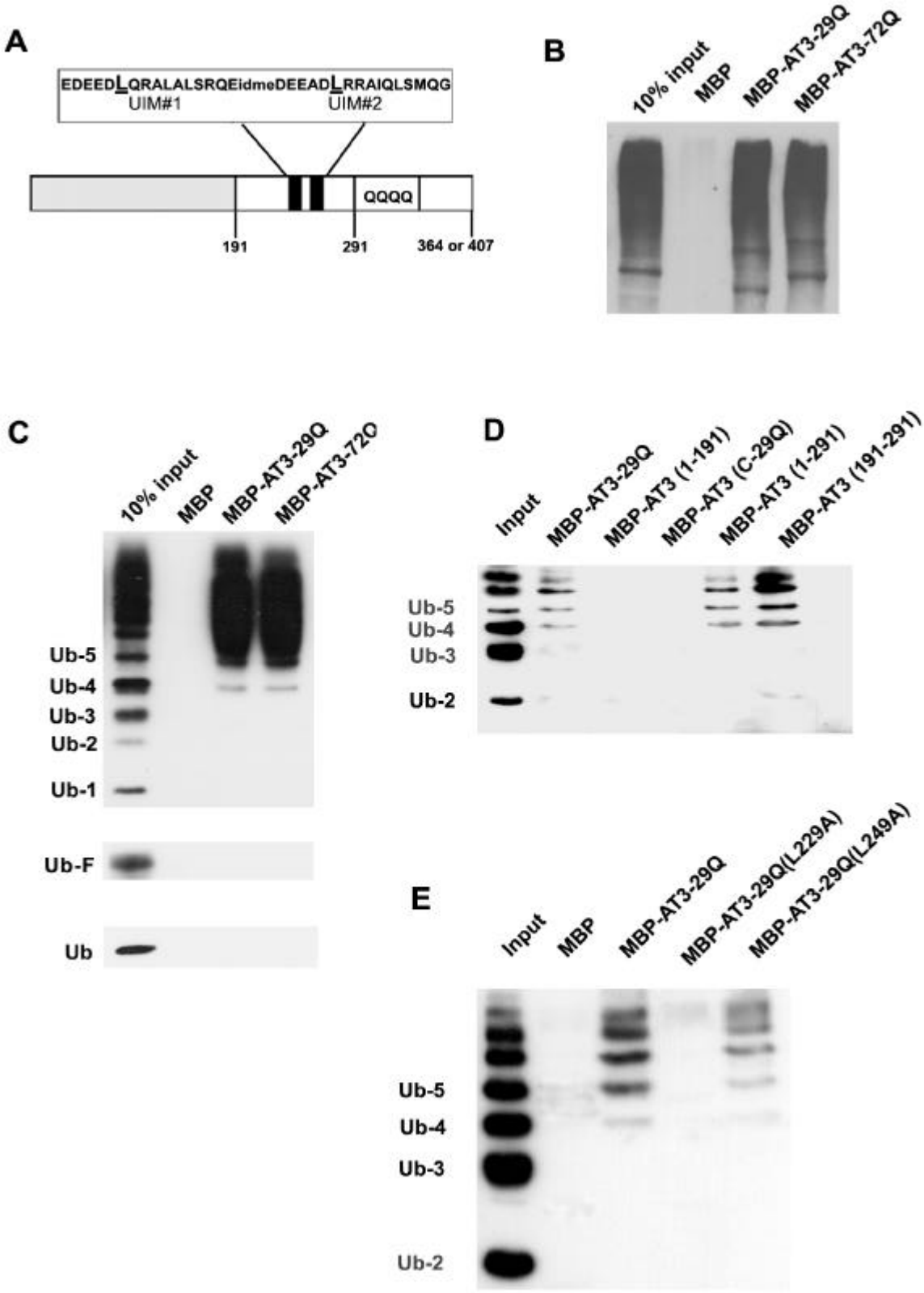
Figure 5. Addition of AT3 to polyubiquitylated ^{125}I-lysozyme results in loss of polyubiquitylation. (A) In vitro (poly)ubiquitylation of ^{125}I-lysozyme was carried out for 4 hrs and the reaction stopped by adding SDS sample buffer (lane 3) or the reaction allowed to proceed an additional 2 hrs in the absence of additions (lane 1) or with MBP (lane 2) or MBP-AT3-29Q (lane 4). Note the loss of polyubiquitylated ^{125}I-lysozyme and appearance of short chain ^{125}I-lysozyme following addition of MBP-AT3-29Q (compare lanes 3 and 4). (B) In vitro polyubiquitylation was carried out for 6 hrs (lane 1) or for 4 hrs and then MBP-AT3-29Q (lane 2) or MBP-AT3-29Q and the specific ubiquitin protease inhibitor, ubiquitin-aldehyde (lane 3)

added and the reaction allowed to proceed for an additional 2 hrs. Note that ubiquitin-aldehyde decreases the loss of polyubiquitylated ^{125}I-lysozyme in the presence of AT3 (compare lanes 2 and 3). Experiments were performed 3 times with similar results.

Figure 6. Sequence comparison of AT3/Josephin family members with ubiquitin proteases. (A) Alignment of AT3 and two other Josephin family members with the catalytic triad of three USP family members. Conservation of the catalytic triad (C,H,D/N) is shown by bold letters and dark boxes, asterisks (*) below the USP family represent amino acids involved in catalysis, and light boxes represent shared similarity/identity between multiple members of the USP and Josephin families. (Abbreviations: Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*). (B) Alignment of AT3 and other Josephin family members with the catalytic triad of three UCH family members. Conservation of the catalytic triad (C,H,D/N) is shown by bold letters and dark boxes, asterisks (*) below the UCH family represent amino acids involved in catalysis, and light boxes represent shared similarity/identity between multiple members of the UCH and Josephin families. Abbreviations: Dm, *Drosophila melanogaster*; other abbreviations are the same as in (A). See also ref. 30.

Figure 7. AT3 has properties of a ubiquitin protease. (A) Mutation of the predicted active site cysteine of AT3 decreases deubiquitylation of ^{125}I-lysozyme by AT3. In vitro polyubiquitylation of ^{125}I-lysozyme (lane 1) is decreased by MBP-AT3-29Q (lane 3) and this decrease (deubiquitylation) is blocked by mutating cysteine-14 of AT3 (lane 4). (B) Time course of cleavage of the ubiquitin protease substrate, ubiquitin-AMC by AT3 and various AT3 mutants. Ubiquitin-AMC and various AT3 fusions proteins were incubated for the indicated times and proteolytic activity quantitated by measuring release of AMC. Note that little or no cleavage

occurs with addition of control buffer, MBP, or active site mutant MBP-AT3-29Q(C14A) while MBP-AT3-29Q, MBP-AT3-72Q, and UIM mutations MBP-AT3-29Q(L229/249A) or MBP-AT3-72Q(L229/249A) show a time dependent cleavage of ubiquitin-AMC. (C) Binding of AT3 or AT3 containing an active site mutation to the specific ubiquitin protease inhibitor, ubiquitin-aldehyde. MBP, MBP-AT3-29Q, or MBP-AT3-29Q(C14A) bound to amylose beads were incubated with ubiquitin aldehyde and protein analyzed by western blot for ubiquitin. Note that MBP-AT3-29Q binds ubiquitin aldehyde (lane 3) but not ubiquitin lacking the C-terminal aldehyde (see Fig. 1C) and that AT3 with the C14A mutation does not bind ubiquitin-aldehyde. Experiments were performed 5 times with similar results.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UIM#2
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291
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MBP
MBP-AT3-29Q
MBP-AT3-72Q
C
10% input
MBP
MBP-AT3-29Q
MBP-AT3-72Q
Ub-5
Ub-4
Ub-3
Ub-2
Ub-1
Ub-F
Ub
D
Input
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MBP-AT3 (1-191)
MBP-AT3 (C-29Q)
MBP-AT3 (1-291)
MBP-AT3 (191-291)
Ub-5
Ub-4
Ub-3
Ub-2
E
Input
MBP
MBP-AT3-29Q
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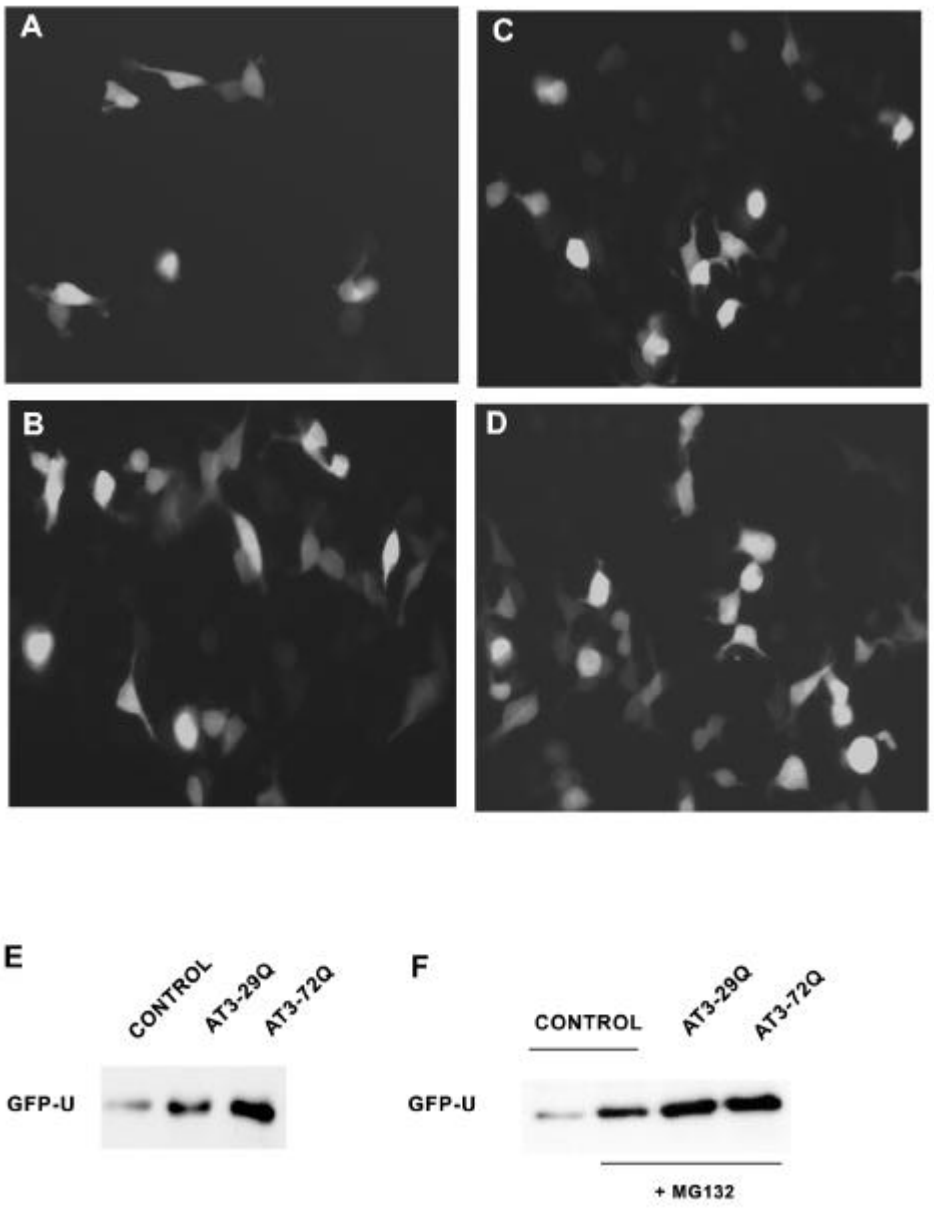
Ub-5
Ub-4
Ub-3
Ub-2

Figure 1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C
B
D
E
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AT3-72Q
GFP-U
F
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AT3-29Q
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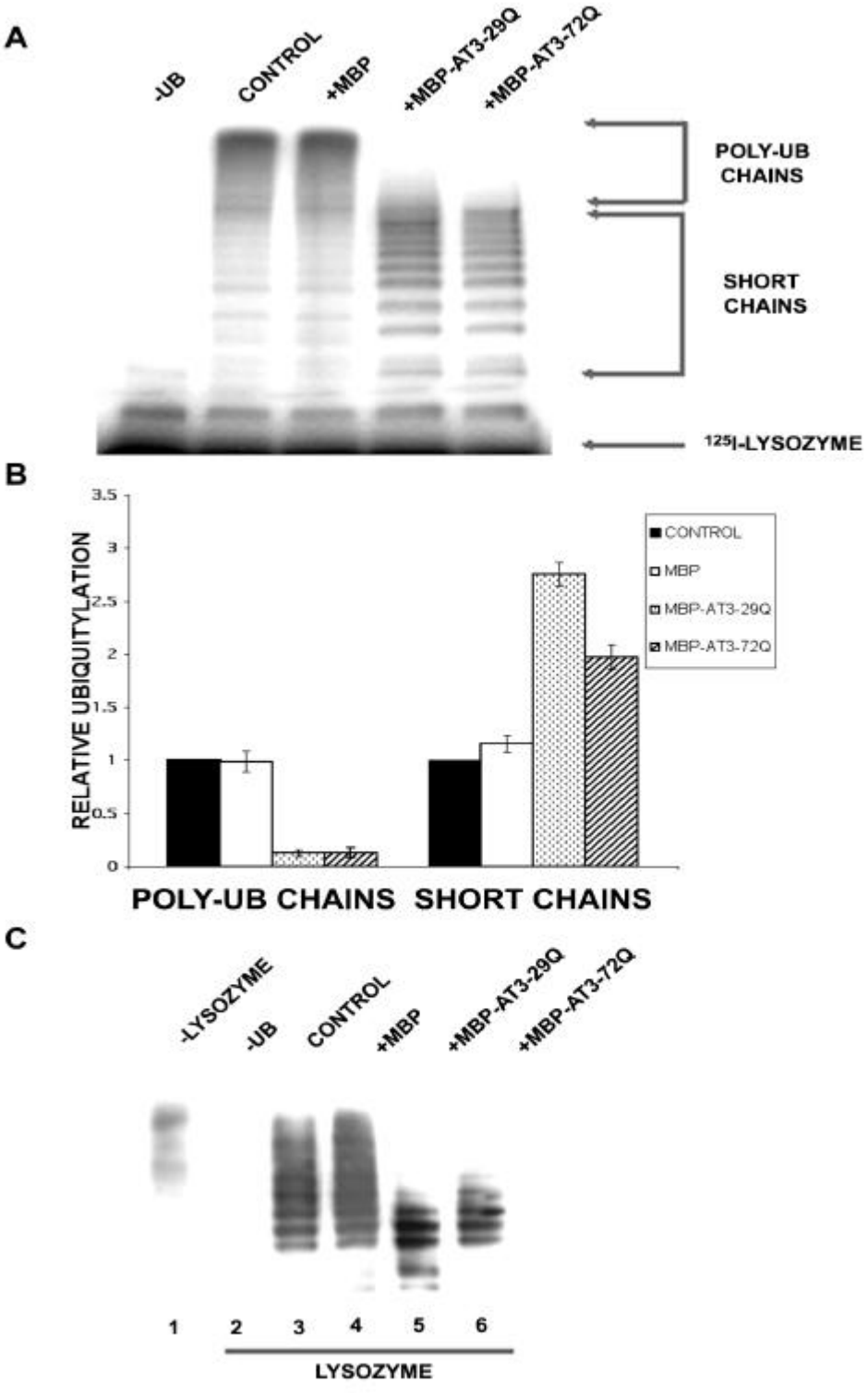
GFP-U
+ MG132

Figure 2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+MBP
+MBP-AT3-29Q
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POLY-UB CHAINS
SHORT CHAINS
125I-LYSOZYME
B
RELATIVE UBIQUITYLATION
3.5
3
2.5
2
1.5
1
0.5
0
CONTROL
MBP
MBP-AT3-29Q
MBP-AT3-72Q
POLY-UB CHAINS
SHORT CHAINS
C
-LYSOZYME
-UB
CONTROL
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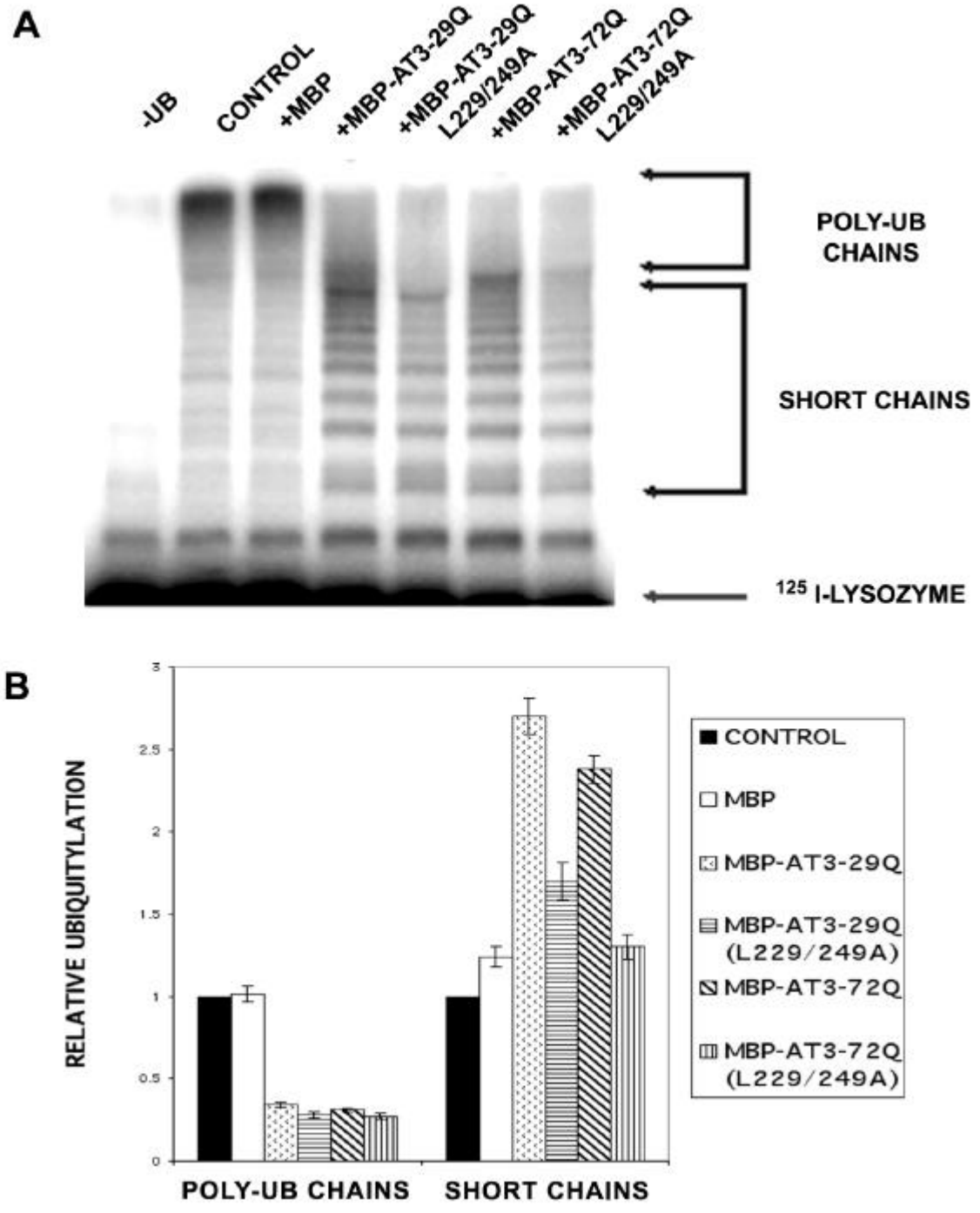
+MBP-AT3-72Q
1
2
3
4
5
6
LYSOZYME

Figure 3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+MBP-AT3-72Q L229/249A
POLY-UB CHAINS
SHORT CHAINS
125 I-LYSOZYME
B
RELATIVE UBIQUITYLATION
0
0.5
1
1.5
2
2.5
3
POLY-UB CHAINS
SHORT CHAINS
CONTROL
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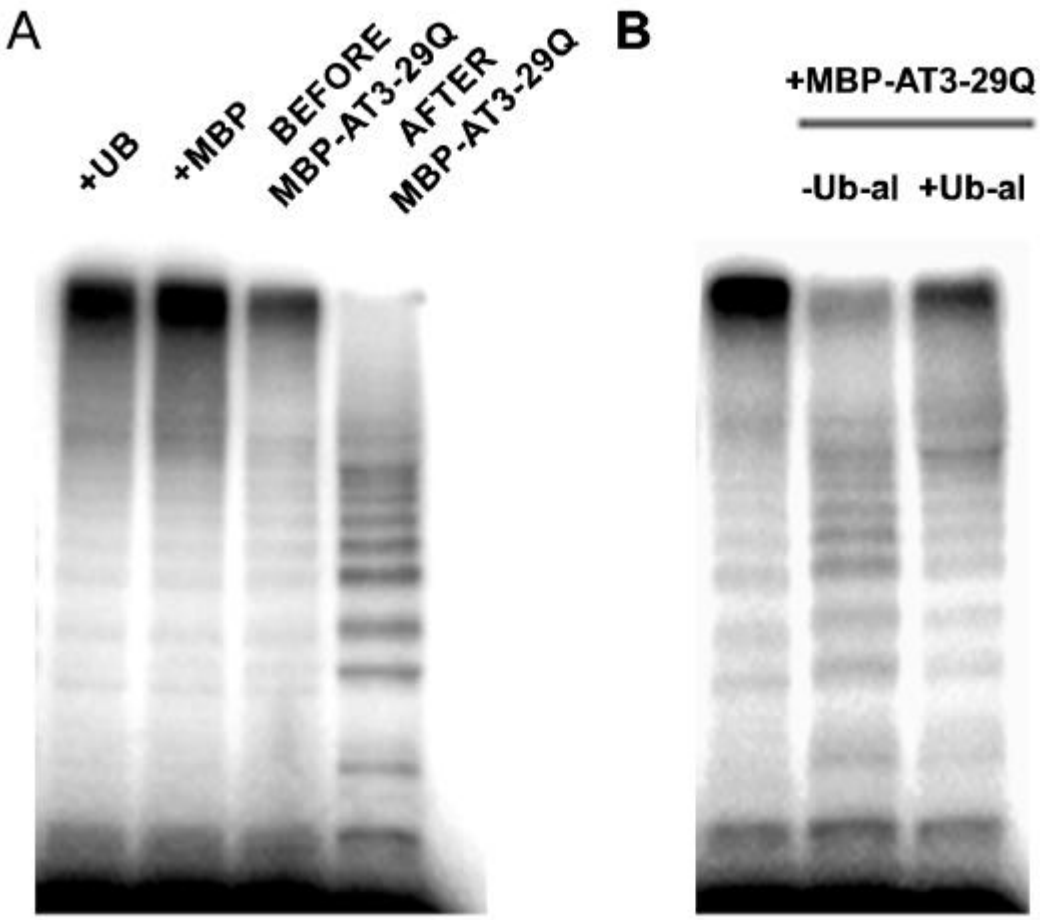
Figure 4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+UB
+MBP
BEFORE
MBP-AT3-29Q
AFTER
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B
+MBP-AT3-29Q
-Ub-al
+Ub-al

Figure 5

A

```
AT3  Hs    6  HEKQEGSLCAQHCLNNLLQ..... 110 RSFICNYKEHW.FTVR.KLGKQWFNLNS
AT3  Ce   12  FEHQEAALCAQHALNMLLQ..... 108 RAYICNLREHW.FVLR.KFGNQWFELNS
AT3  At   13  HEVQESNLCAVHCVNTVLQ..... 117 SAFICHLHDHW.FCIR.KVNGEWYNFDS
USP18 Hs  56  GLHNIGQTCC...LNSLLQ..... 309 AHVGMADSGHYCVYIRNAVDGKWFCFND
HAUSP Hs 215  GLKNQGATCY...MNSLLQ..... 455 VHSGDNHGGHYVVYLNPKGDGKWCKFDD
USP25 Hs 170  GLKNVGNTCW...FSAVIQ..... 598 VHEGQANAGHYWYAIFDHRESRWMKYND
                 *    *        *          *       *              *
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B

```
AT3 Hs     7 EKQ.EGSLCAQHCLNNLLQ..... 115 NYKEHWFTVRKLGKQWFNLNS
AT3 Ce    13 EHQ.EAALCAQHALNMLLQ..... 113 NLREHWFVLRKFGNQWFELNS
AT3 At    14 EVQ.ESNLCAVHCVNTVLQ..... 122 HLHDHWFCIRKVNGEWYNFDS
UCH-L1 Hs 81 MKQTIGNSCGTIGLIHAVA..... 155 KVNFHFILFNNVDGHLYELDD
UCH-L3 Hs 87 MKQTISNACGTIGLIHAIA..... 165 KVDLHFIALVHVDGHLYELDD
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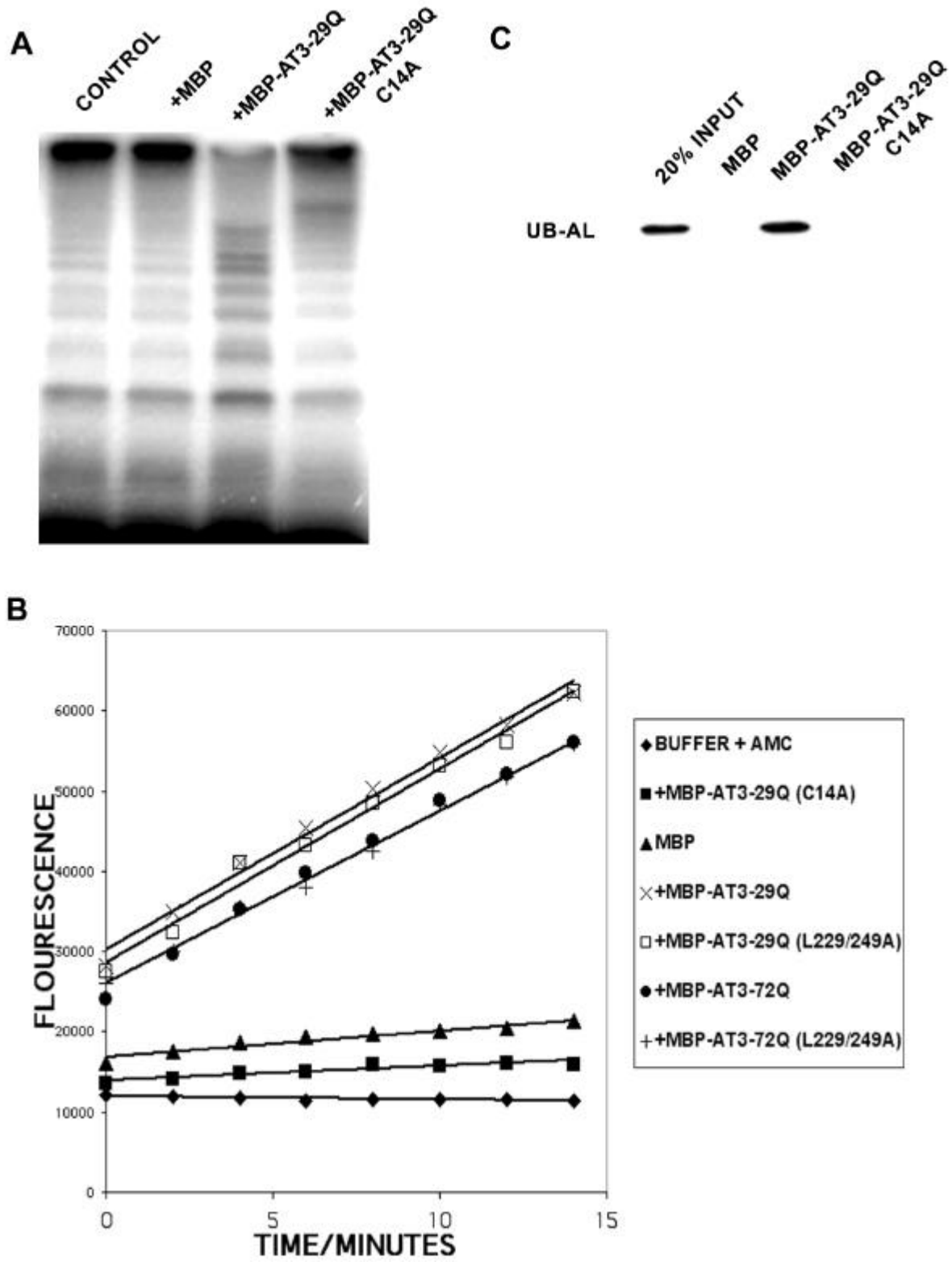
Figure 6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CONTROL
+MBP
+MBP-AT3-29Q
+MBP-AT3-29Q C14A
C
20% INPUT
MBP
MBP-AT3-29Q
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UB-AL
B
FLOURESCENCE
TIME/MINUTES
0
10000
20000
30000
40000
50000
60000
70000
0
5
10
15
BUFFER + AMC
+MBP-AT3-29Q (C14A)
MBP
+MBP-AT3-29Q
+MBP-AT3-29Q (L229/249A)
+MBP-AT3-72Q
+MBP-AT3-72Q (L229/249A)

Figure 7

Abbreviations

Abbreviations used are: AT3, ataxin-3; DUB, deubiquitinating enzyme; GFP, green fluorescent proteins, MBP, maltose binding protein; NER, nucleotide excision repair; SCA, spinocerebellar ataxia; UCH, ubiquitin C-terminal hydrolase; UPP, ubiquitin-proteasome pathway; USP, ubiquitin specific protease; UIM, ubiquitin interaction motif.