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# The UCH-L1 Gene Encodes Two Opposing Enzymatic Activities that Affect α-Synuclein Degradation and Parkinson's Disease Susceptibility

**Yichin Liu, Lara Fallon,[2] Hilal A. Lashuel, Zhihua Liu, and Peter T. Lansbury, Jr.[1]**
Center for Neurologic Diseases
Brigham and Women's Hospital
Department of Neurology
Harvard Medical School
65 Landsdowne Street
Cambridge, Massachusetts 02139

## Summary

**The assumption that each enzyme expresses a single enzymatic activity in vivo is challenged by the linkage of the neuronal enzyme ubiquitin C-terminal hydrolase-L1 (UCH-L1) to Parkinson's disease (PD). UCH-L1, especially those variants linked to higher susceptibility to PD, causes the accumulation of α-synuclein in cultured cells, an effect that cannot be explained by its recognized hydrolase activity. UCH-L1 is shown here to exhibit a second, dimerization-dependent, ubiquityl ligase activity. A polymorphic variant of UCH-L1 that is associated with decreased PD risk (S18Y) has reduced ligase activity but comparable hydrolase activity as the wild-type enzyme. Thus, the ligase activity as well as the hydrolase activity of UCH-L1 may play a role in proteasomal protein degradation, a critical process for neuronal health.**

## Introduction

UCH-L1 is an abundant neuronal enzyme (1%–2% of brain protein [Wilkinson et al., 1989]) of unknown function. In vitro, UCH-L1 catalyzes hydrolysis of C-terminal ubiquityl esters and amides; peptide-ubiquityl amides are the preferred substrates (Larsen et al., 1996, 1998). This activity is presumed to be critical for cytoplasmic protein degradation, recycling free ubiquitin by cleaving ubiquitylated peptides that are the products of the proteasomal degradation of polyubiquitylated proteins (Larsen et al., 1996, 1998). The importance of the cytoplasmic protein degradation pathway in Parkinson's disease (PD) is suggested by the fact that two gene products that are linked to familial PD are sensitive to or involved in this pathway: α-synuclein forms potentially pathogenic aggregates (protofibrils) when its concentration exceeds a critical threshold, and parkin is an E3 ligase that ubiquitylates α-synuclein and other protein substrates, tagging them for degradation and preventing this threshold from being reached (Lansbury and Brice, 2002).

UCH-L1 was first linked to PD by an autosomal dominant point mutation (I93M) that was identified in two siblings with a strong family history of PD (Leroy et al., 1998). Since I93M decreases the in vitro hydrolytic activity of UCH-L1, this form of PD was proposed to result from a partial loss of UCH-L1 hydrolytic function. However, several pieces of evidence suggest that simple loss of hydrolytic function does not completely explain the PD phenotype in this family. First, the mutation is not 100% penetrant; the father of the two affected individuals and the presumed carrier of I93M did not develop PD. Second, mice lacking functional UCH-L1 (the gracile axonal dystrophy [GAD] mouse) do not develop a Parkinsonian phenotype (Kurihara et al., 2001; Saigoh et al., 1999; Miura et al., 1993; Mukoyama et al., 1989; Oda et al., 1992; Wu et al., 1995). Neuronal loss in the GAD mice occurs in the gracile tract as opposed to the *substantia nigra* in PD and no Parkinsonian movement disorder is observed.

In the course of a failed search for additional I93M mutants, a previously unrecognized polymorphism in the UCH-L1 gene (S18Y) was discovered and subsequently found to be linked to a decreased susceptibility to PD (Levecque et al., 2001; Maraganore et al., 1999; Momose et al., 2002; Satoh and Kuroda, 2001; Wang et al., 2002). The S18Y polymorphism is relatively rare in the European population (allele frequency is 14%–20%) but common in the Japanese (39%–54%) and Chinese (~50%) populations. Protection is dependent on the S18Y allele dosage; that is, homozygotes are at significantly lower risk (relative risk of 0.31) than are heterozygotes (relative risk between 0.55 and 0.81) (Levecque et al., 2001; Maraganore et al., 1999; Satoh and Kuroda, 2001). The simplest explanation for these findings, that S18Y is protective because it has the opposite effect on UCH-L1 hydrolytic activity as I93M, is inconsistent with the predicted location of residue 18 on the protein surface, distal from the active site and the ubiquitin binding site (Figure 1; Johnston et al., 1997, 1999). Furthermore, the fact that position 18 is one of only a few residues that are *not* conserved between human and other mammals (horse, mouse, and rat have Ala at position 18) suggests that residue 18 is not involved in the normal "biological" activity of UCH-L1 and suggests the existence of a distinct pathological UCH-L1 activity. We set out to compare the properties of the high- and low-risk human polymorphic UCH-L1 variants, using α-synuclein as a model substrate. In doing so, we discovered a novel ubiquitin-ubiquitin ligase activity of UCH-L1 that may also be important in PD pathogenesis. The existence of this activity is inconsistent with the one gene-one enzyme paradigm and has important implications regarding targeting UCH-L1 with anti-Parkinson's disease drugs.

## Results

### UCH-L1 and α-Synuclein Colocalize with Synaptic Vesicles and Can Be Coimmunoprecipitated from Mammalian Brain

In an effort to localize UCH-L1 and identify potential substrates, synaptic vesicles were isolated from rabbit brain using a standard method (Hell and Jahn, 1998).

[1]Correspondence: plansbury@rics.bwh.harvard.edu
[2]Present address: Montreal Neurological Institute, 3801 University St., Montreal, Quebec H3A 2B4, Canada.

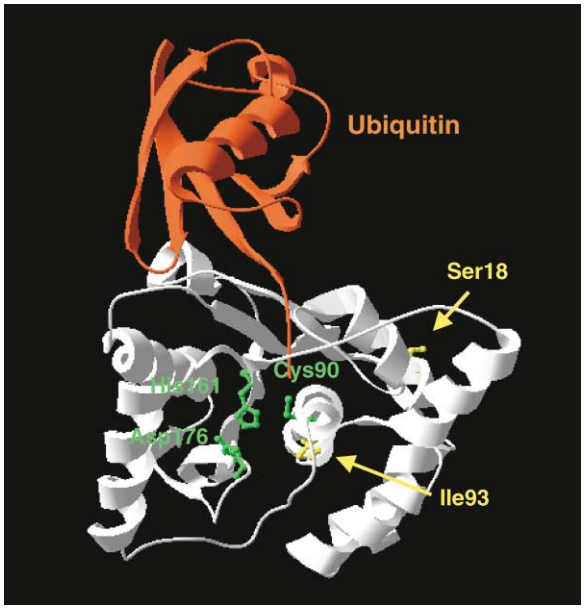


Figure 1. A Structural Model for the UCH-L1-Ubiquitin Complex Does Not Explain the Protective Effect of the S18Y Polymorphism

The sequence of UCH-L1 is shown, mapped onto the determined structure of the highly homologous (52% identity) UCH-L3 complexed to the inhibitor ubiquitin aldehyde (SwissPdbViewer V. 3.7b2) (Johnston et al., 1997, 1999). Residue 93 is proximal to the active site nucleophile (C90), while S18 is distal from the active site and from the ubiquitin binding site (Johnston et al., 1997, 1999).

A significant portion (~2%–5%) of both UCH-L1 and α-synuclein were found in the synaptic vesicle fraction, together with the synaptic vesicle markers synapsin, synaptotagmin I, and synaptophysin (Figure 2A). The synaptic vesicle localization of UCH-L1 is consistent with the fact that it is O-glycosylated in nerve terminals (Cole and Hart, 2001). UCH-L1 and α-synuclein were coimmunoprecipitated from the synaptic vesicle fraction using a UCH-L1 antibody (Chemicon, pig polyclonal, Figure 2B). In a separate experiment, UCH-L1 was isolated from rat brain lysate using an anion exchange column. A small fraction of the total α-synuclein coeluted with UCH-L1. Fractions containing both UCH-L1 and α-synuclein were treated with a UCH-L1 antibody (Chemicon, rabbit polyclonal), an α-synuclein antibody (Transduction Lab), or buffer (as a control), and the antibody complexes were precipitated. The precipitated complexes were dissociated and analyzed. The immunoprecipitation with UCH-L1 antibody revealed α-synuclein monomer and a ~34 kDa species that was immunoreactive to both α-synuclein (Figure 2C, eluent fractions) and ubiquitin (data not shown) antibodies. The MW of this species is consistent with diubiquitylated α-synuclein (see below). The fact that UCH-L1 coimmunoprecipitates with free and ubiquitylated α-synuclein suggests that either one may be a substrate of UCH-L1. This possibility contrasts with expectations based on the optimal in vitro substrates, which are ubiquitylated peptides and amino acids, not ubiquitylated proteins (Larsen et al., 1996, 1998).

## Overexpression of UCH-L1 Variants in COS-7 Cells Leads to a Buildup of α-Synuclein

Having demonstrated that UCH-L1 and α-synuclein colocalize and interact in brain, we sought to determine the potential effect of this interaction on the cytoplasmic degradation of α-synuclein. COS-7 cells, which do not normally express detectable levels of α-synuclein or UCH-L1, were cotransfected with α-synuclein and histidine-tagged ubiquitin. Treatment with the proteasome inhibitor lactacystin (Fenteany et al., 1995) increased, by 5-fold, the level of α-synuclein, consistent with previous reports (Figure 3A, lane 1 versus lane 2; Bennett et al., 1999). Cotransfection of these cells with wt or I93M UCH-L1 variants increased α-synuclein levels by ~2.5-

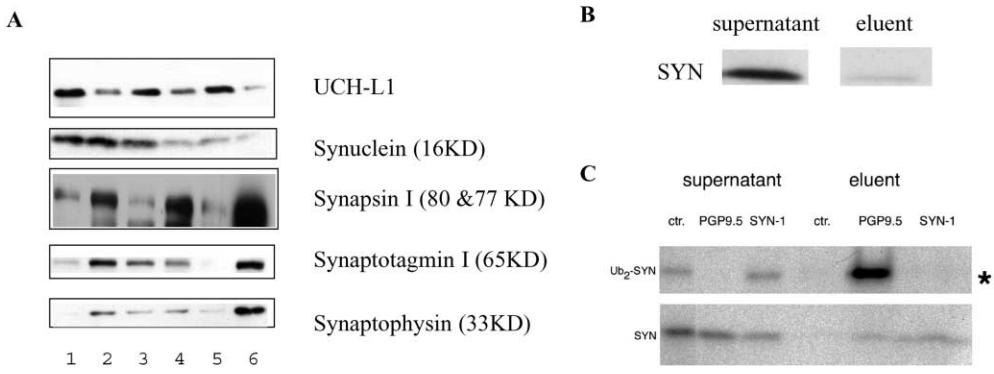


Figure 2. A Fraction of UCH-L1 Is Localized to Synaptic Vesicles and Interacts with α-Synuclein and Ubiquitylated α-Synuclein

(A) UCH-L1 associates with synaptic vesicle. Different fractions from synaptic vesicle (S3, P3, LS1, LP1, LS2, and LP2) were subjected to Western blotting with antibodies against UCH-L1, synuclein, synapsin I, synaptotagmin I, and synaptophysin. LP2 was the fraction of synaptic vesicle.

(B) α-synuclein coimmunoprecipitates with UCH-L1 in SV fraction. The supernatant after immunoprecipitation with UCH-L1 antibody was compared to the precipitated fraction (eluent). Western blotting was performed with SYN-1 antibody.

(C) Immunoprecipitation of α-synuclein with UCH-L1 or synuclein antibodies. The supernatant after immunoprecipitation was compared to the precipitated complexes, after dissociation (eluent). Western blotting was performed with the α-synuclein antibody SYN-1. The 34 kDa species marked with an asterisk in (C) may be equivalent to the major substrate in Figure 4 (the former lacks a His tag). The control lane showed immunoprecipitation without any antibody.

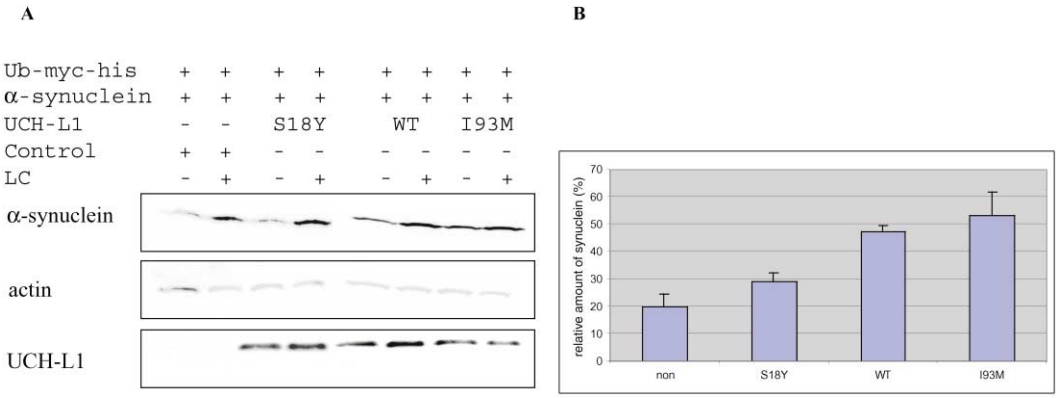


Figure 3. Expression of UCH-L1 or I93M Promotes Buildup of α-Synuclein in Transfected Cells

(A) COS-7 cells were transfected with plasmids as indicated. The proteasome inhibitor lactacystin (LC) was added 48 hr after transfection. Total cell lysates were analyzed by Western blotting.

(B) The relative amount of 16 kDa α-synuclein (syn-1) was quantified by NIH Image program and normalized against the amount of actin. The *y* axis (relative amount of undegraded α-synuclein) reports the amount of α-synuclein as compared to the LC-treated samples.

fold (Figure 3B), but cotransfection with S18Y had little, if any effect (≤50% increase). In every case, treatment of the UCH-L1-transfected cells with lactacystin added to the UCH-L1 effect, to produce comparable levels of α-synuclein as in the LC-treated untransfected cells. This result indicates that UCH-L1 transfection does not increase α-synuclein expression and that accumulation of α-synuclein resulted from inhibition of its degradation. Under similar conditions, overexpression of wt and treatment with a different proteasome inhibitor, MG132, also synergistically increased the accumulation of α-synuclein-ubiquitin conjugates (see Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1). Accumulation of α-synuclein is unlikely to result from the hydrolytic activity of UCH-L1, since (1) hydrolase activity is expected to *promote* protein degradation by recycling free ubiquitin (Larsen et al., 1996, 1998), (2) the effects of I93M and wt were indistinguishable, whereas I93M is a much less active hydrolase (Leroy et al., 1998), and (3) S18Y, which has comparable hydrolytic activity as wt in vitro (see below), does not cause significant accumulation. Regardless of the molecular mechanism for the effect, it is consistent with a model in which S18Y reduces a toxic function of UCH-L1, since increased accumulation of α-synuclein is likely to be toxic, and the protective variant S18Y causes less accumulation than wt UCH-L1.

### UCH-L1 Seems to Ubiquitylate α-Synuclein-Ubiquitin Conjugates

The unexpected outcome of the cell culture experiments (Figure 3) suggested to us that UCH-L1 could have an unreported activity that is responsible for the inhibition of α-synuclein degradation. To determine the nature of this activity, α-synuclein was conjugated to histidine-tagged ubiquitin using a crude cell-free system (Figure 4A; Ciechanover et al., 1978). The crude products of the cell-free ubiquitylation were fractionated by nickel affinity chromatography, which was expected to isolate all covalent ubiquitin conjugates and, possibly, other proteins that tightly bind to ubiquitin. The predominant α-synuclein-containing conjugate was a ~34 kDa species, probably the diubiquitylated species (see asterisk, Figure 4A). Addition of UCH-L3 to the mixture of ubiquitylated species had little effect, though a small amount of free α-synuclein was produced after a 2 hr incubation (data not shown). In contrast, UCH-L1 rapidly consumed the α-synuclein-ubiquitin conjugates, especially the 34 kDa species (this species approximately comigrates with the coimmunoprecipitating species in Figure 2C), producing a trace amount of free α-synuclein in the process. Surprisingly, the major products of UCH-L1 treatment were high-molecular-weight α-synuclein-ubiquitin conjugates, presumably formed by the net *addition* of ubiquitin to mono- and diubiquitylated α-synuclein (free α-synuclein did not appear to be a substrate for chain extension [see below]). The possibility that UCH-L1, as opposed to a contaminating ligase, was directly responsible for polyubiquitin chain extension (red arrow in Figure 4A), was strengthened by the observation that UCH-L3 treatment of the same crude mixture did not result in chain extension.

The possibility that UCH-L1 itself possesses "ubiquityl ligase" activity is mechanistically reasonable (Figure 4B). Rather than reacting with water, the ubiquityl-enzyme thioester (or acyl enzyme), an intermediate in the hydrolysis reaction, may be able to react directly with a nucleophilic lysine of another ubiquitin to produce a ubiquitin-ubiquitin amide bond. In contrast to the well-characterized pathway of "biological" ubiquitylation (using the E1, E2, and E3 ligases), which requires activation of free ubiquitin as an AMP ester in order to form the acyl enzyme (Pickart, 2001; Weissman, 2001), UCH-L1 ligase activity does not require ATP, since the acyl enzyme can be derived directly from cleavage of a ubiquitin C-terminal amide (Figure 4B). The proposed ligation mechanism is reminiscent of other hydrolytic enzymes (e.g., proteases) that can also catalyze amide bond formation under extreme in vitro conditions. However, this may be the first example of catalysis of amide bond formation under physiologically reasonable conditions, suggesting that it could occur in vivo.

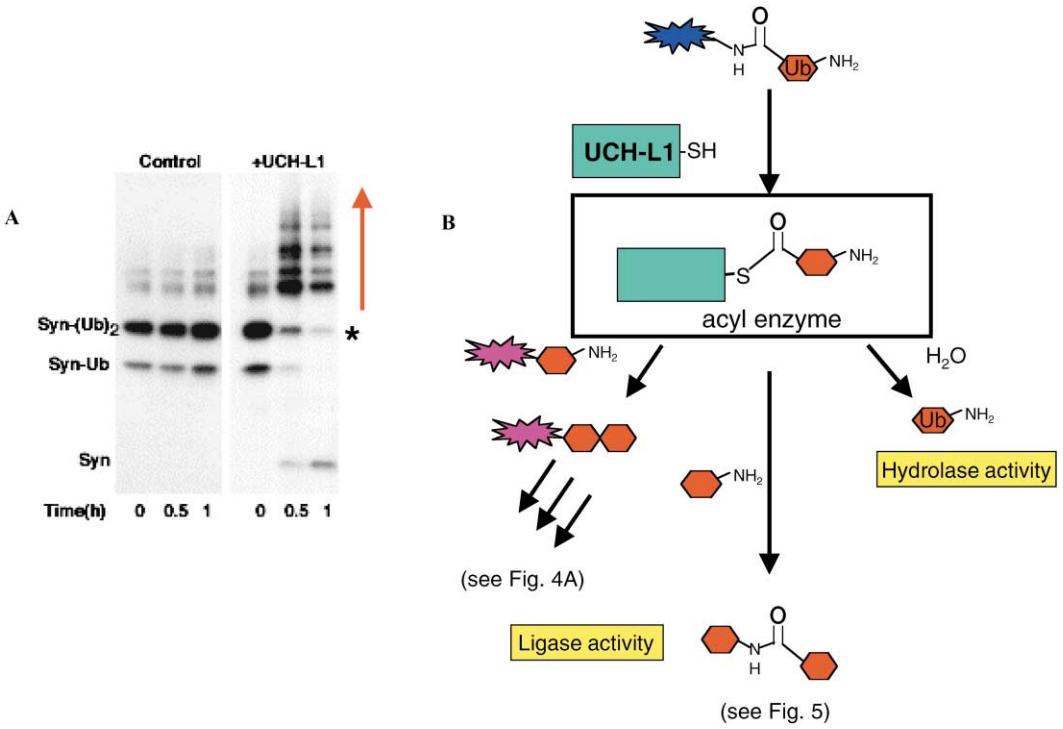


Figure 4. UCH-L1 Seems to Extend Polyubiquitin Chains, Specifically Those Ligated to α-Synuclein

(A) Reaction of wt UCH-L1 with ubiquitylated α-synuclein (produced by treatment of α-synuclein with $His_6$-tagged ubiquitin in Rabbit reticulocyte lysate). Control, under identical conditions, showed little change.

(B) Model to explain the mechanisms of UCH-L1 ligase and hydrolase activities.

### UCH-L1 Has Concentration-Dependent Ubiquityl Ligase Activity That Is Reduced in the Case of S18Y, the "Protective" Variant

Recombinant human UCH-L1 proteins corresponding to the three characterized UCH-L1 genes (S18/I93 = wild-type [wt], S18/M93 = I93M [Leroy et al., 1998], and Y18/I93 = S18Y); a point mutant of wt containing the mouse/rat/horse amino acid, Ala, at position 18 (S18A); and human UCH-L3, the highly homologous (55% identical) systemic variant, were expressed in *E. coli* and purified to apparent homogeneity (Larsen et al., 1996). All proteins had hydrolytic activity toward a fluorogenic ubiquitin C-terminal amide, with the $V_{max}$ for UCH-L3 being ~200-fold greater than that of the four UCH-L1 variants (see Experimental Procedures and Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1). Of the four UCH-L1 variants, only I93M had significantly different hydrolytic activity, ~50% of the three others (Leroy, et al., 1998).

To test the possibility that UCH-L1 could be a ubiquityl ligase (see Figure 4B), we incubated the enzyme (0.4–3 μM) with ubiquitin AMC amide (3 μM) in the presence of 50 μM free ubiquitin (this concentration is physiologically reasonable [Chain et al., 1995; Mastrandrea et al., 1999]). After 2 hr, at which time ubiquitin AMC amide had been completely consumed (data not shown), the incubation was analyzed by SDS-PAGE, with detection by Coomassie blue staining (Figure 5A). In the case of wt, but *not* UCH-L3, a significant amount of diubiquitin was produced (Figure 5B). I93M produced approximately 60% as much dimer as wt, consistent with its reduced ability to form the acyl enzyme. Amino acid changes at residue 18 had a significant effect on ligase activity, with wt and S18A having ~5-fold greater activity than S18Y (Figure 5C). Moreover, the ratio of ligase activity to the total acyl enzyme formation decreased on dilution of wt and S18A, indicating that this activity is *not* derived from the monomeric enzymes. This result can be explained by a model in which the ligase activity is derived from a dimeric (and/or oligomeric) form of UCH-L1 that is increasingly populated at higher enzyme concentrations (see below). No ligation was observed when free ubiquitin was replaced with free α-synuclein (data not shown), indicating that the ligase recognizes ubiquitin as the amine component of the reaction (Figure 4B). To determine if a specific Lys residue was preferentially ubiquitylated, wt ubiquitin was replaced in the incubation with K63R ubiquitin, drastically reducing the amount of ubiquitin dimer product (Figure 5D). This result indicated that Lys63 may be the primary ubiquityl acceptor residue, in contrast to the E2/E3 ligases involved in proteasomal degradation, which ubiquitylate Lys48 of the "nucleophilic" ubiquitin.

### The Ubiquityl Ligase Activity of the S18 Variants Correlates to Their Tendency to Dimerize

Analytical ultracentrifugation (AU) was employed to measure the oligomerization of each UCH variant (Figure 6 and Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1). This sensitive technique allows assessment of the distribution of oligomeric species in solution and determination of their molecular size

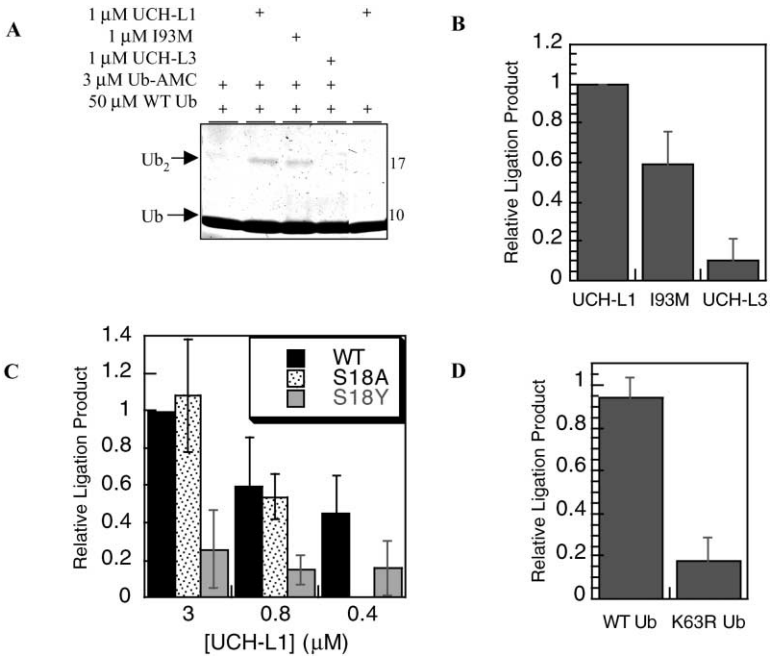


Figure 5. ATP-Independent Ubiquitin Ligation Is Catalyzed by UCH-L1 Variants

(A) 3 μM ubiquitin-AMC and 50 μM wt ubiquitin were combined with 1 μM of UCH-L1, I93M, or UCH-L3. Fluorescence emission intensity progress curve (not shown) indicated that the Ub-AMC was completely consumed within 1 hr. After incubation at 37°C for 2 hr, reaction products were resolved on 14% or 16% Tris-Glycine SDS gel stained with Coomassie brilliant blue. Only lanes that contained UCH-L1, Ub-AMC, and wt Ub (also see Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1) yielded dimeric ubiquitin.

(B) Intensity of $Ub_2$ band in each reaction, normalized to the $Ub_2$ band intensity in the wt UCH-L1 incubation.

(C) Intensity of $Ub_2$ band in each reaction, normalized to the $Ub_2$ band intensity in the 3 μM wt UCH-L1 reaction: wt UCH-L1 (black), S18A (dot), and S18Y (gray).

(D) Use of the K63R variant of ubiquitin resulted in 80% reduction of ubiquitin dimer product.

and does not require a reporter group. UCH-L3 was completely monomeric (~2.3S, MW = 24 [± 1] kDa) at a concentration of 27 μM (far in excess of the concentration at which ligase activity was detected). In contrast, wt UCH-L1, an active ligase, populated a dimer (red curve in Figure 6, ~15% of total wt, 4S, calc. MW = 47 [± 2] kDa) and a higher-order oligomer (green curve, ~5%, 8S) at 7 μM. The population of both oligomers increased with protein concentration, and UCH dimer became the predominant species at 70 μM in the case of wt (see Supplemental Data). The protective polymorphic variant, S18Y, which did not show significant ligase activity, was completely monomeric at 10 μM, although dimer was detected at 28 μM (see Supplemental Data). As a complement to AU, higher oligomers were also detected by native gel electrophoresis; they were much more prominent in the case of wt, as compared to S18Y at concentration of 25 μM (see Supplemental Data). Like wt, the S18A variant also contained a significant amount of dimer at 13 μM. Thus, the tendency for UCH-L1 to dimerize correlated with the ligase activity. The I93M protein, which showed reduced ligase activity as compared to wt, also showed a reduced population of dimer as compared to wt.

### The Dose-Dependent Effect of the Protective Allele Is Mirrored by the Effect of S18Y on the In Vitro Ligase Activity of Wild-Type

The suggested relationship between UCH-L1 ligase activity and enzyme dimerization prompted us to examine the in vitro enzymatic activities of 1:1 mixtures of UCH-L1 variants. Three incubations were set up to mimic the common human genotypes with respect to the residue 18 polymorphism: S18Y/S18Y, wt/S18Y, and wt/wt (Figure 7A). Within this series, hydrolytic activity was constant (data not shown), but ligase activity increased, correlating to increasing risk (the analogous S18A/wt series showed no differences in ligase or hydrolase activity [see Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1]). This finding strengthens the case that UCH-L1 ligase activity partly determines PD susceptibility.

### S18Y Inhibits the In Vitro Ligase Activity of I93M in *Trans*, Suggesting a Possible Explanation for the Incomplete Penetrance of the I93M Mutation

The possibility of an indirect interaction between the polymorphism at position 18 and the I93M mutation is suggested by the incomplete penetrance of the mutation (Figure 7B). Specifically, the father of the two effected individuals (asterisk in Figure 7B), who is the presumed carrier of I93M, did not develop PD. The genotype of the two characterized I93M-carrying individuals with respect to position 18 was not reported (Leroy et al., 1998). We set out to test the proposition that the incomplete penetrance may result from a modifying effect of one S18Y allele, and that the mechanism of modification could be reduced ligase activity.

The 1:1 wt/I93M mixture, a possible model of the effected heterozygotes (Leroy et al., 1998), showed a hydrolase activity (blue bars in Figure 7C) that was intermediate between that of the two enzymes, comparable to the sum of the monomeric hydrolases. The ligase activity of this mixture was also intermediate between wt and I93M (gray bar, Figure 7C). The 1:1 S18Y/I93M mixture had comparable hydrolytic activity (blue) but significantly reduced ligase activity (gray) relative to the wt/I93M mixture. If ligase activity confers susceptibility, then the S18Y polymorphism could protect against I93M in *trans*. This effect may explain the incomplete penetrance of I93M. Furthermore, the nonadditivity of ligase activity is consistent with our proposal that ligase activity is a property of the dimer, and not the UCH-L1 monomer.

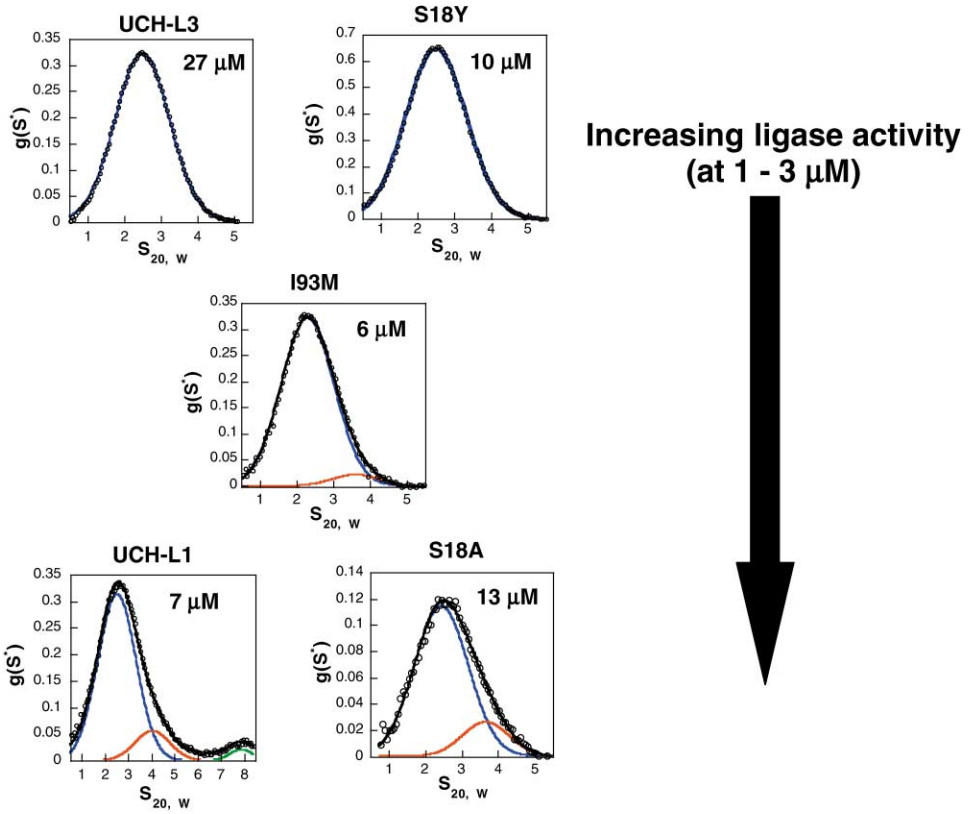


Figure 6. Analytical Ultracentrifugation Demonstrates that UCH-L1 Variants Differ with Respect to Their Oligomerization Behavior

The apparent distributions of the sedimentation coefficient (g(s*)) for UCH-L3, UCH-L1 wt, S18Y, S18A, and I93M and obtained from the time-derivative analysis (Stafford, 1992) of the sedimentation velocity data for each protein. The black line reflects the best least squares fit of the data (O) to a single (UCH monomer, blue), two (monomer-dimer), or three species (monomer-dimer-oligomer) model using a Gaussian function. Attempts to fit the wt, I93M, and S18A data to a single species model failed to yield a good fit.

## Discussion

An important line of defense against neurotoxic protein aggregation is a fully functional protein degradation pathway, which includes UCH-L1 (Lam et al., 2000; Layfield et al., 2001). In addition to Parkinson's disease, UCH has been implicated in two other neurodegenerative diseases that may also be triggered by protein aggregation (Rochet and Lansbury, 2000): spinocerebellar ataxia (SCA), in which a UCH mutant is a genetic enhancer of degeneration in SCA transgenic flies (Fernandez-Funez et al., 2000), and Huntington's disease, in which the UCH-L1 S18Y polymorphism is linked to age at onset (Naze et al., 2002). Other gene products in the protein degradation pathway have also been implicated in PD: an active site deletion in the parkin gene product, an E3 ligase critical in preparing proteasome substrates, results in juvenile-onset Parkinsonism (Kitada et al., 1998). Biochemical studies of PD brain demonstrate impaired proteasome activity (McNaught et al., 2001). Inhibition of the proteasomal pathway in cultured neurons and rat brains by lactacystin (a proteasome inhibitor) (McNaught et al., 2002a) or by ubiquitin aldehyde (a UCH inhibitor) (McNaught et al., 2002b) causes inclusion formation and cell death (Rideout et al., 2001; Tofaris et al., 2001). The fact that these effects are selective for dopaminergic neurons, as is PD cell loss, strengthens the case that they model the PD pathogenic pathway.

We report here a novel in vitro ligase activity of the UCH-L1 dimer that offers a simple mechanistic explanation for the fact that the S18Y polymorphism reduces susceptibility to PD. There is precedent for a "dimeric" ubiquityl ligase: the crystallographically characterized Mms2/Ubc13 heterodimer (Moraes et al., 2001; VanDemark et al., 2001). Mms2 and Ubc13 are E2 homologs, but the former lacks an active site cysteine and is thus an inactive hydrolase. The Mms2-Ubc13 heterodimer has ligase activity and its cocrystal appears to be capable of binding two ubiquitin molecules in an orientation that would allow ubiquityl transfer from the Ubc13 thioester to K63 of a second ubiquitin, which is the preferred ligation chemistry. It is interesting that the UCH-L1 dimer also ubiquitylates Lys63. Whether this ligation chemistry is important to PD pathogenesis is the subject of the speculation below.

Although our cell culture studies demonstrate that the UCH-L1 ligase activity could be pathogenic for PD, it is possible that there may be a biological role for this activity. Given the high hydrolase activity of UCH-L3, it is attractive to imagine that UCH-L1 may not be produced solely for its relatively weak hydrolase activity and that its ligase activity could have evolved to be regulated

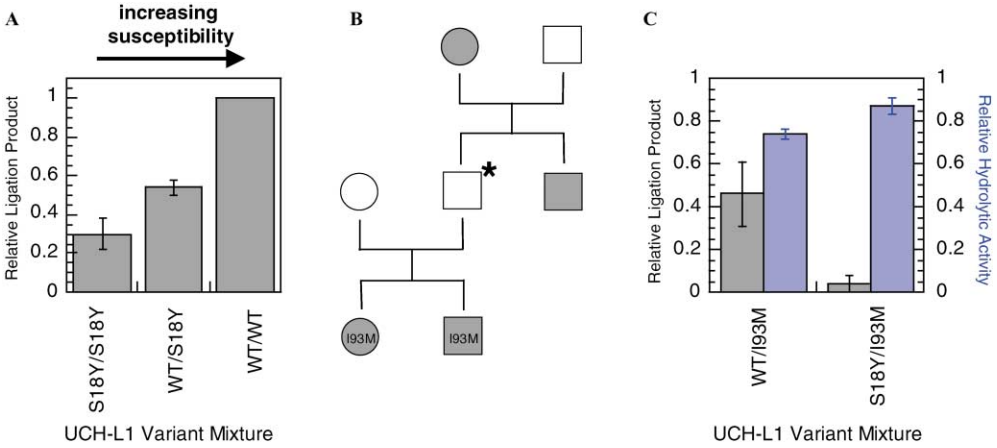


Figure 7. Ubiquityl Ligase Activity of Mixtures of UCH-L1 Variants Supports a Correlation between This Activity and PD Susceptibility

1:1 mixtures of two variants, models of heterozygotes, were compared to pure variants of equal total [UCH-L1], models of homozygotes. Ligation (gray columns) and hydolysis (blue columns) are reported as in Figure 3.

(A) Increasing ligase activity correlates to increasing risk of PD.

(B) The family in which the I93M mutation was discovered (taken from Leroy et al., 1998), demonstrating that I93M mutation is not 100% penetrant (squares = males, shaded = PD). Note the unaffected father, presumed to be the carrier of I93M. Genotypes are available only for the most recent generation, with the results shown (the polymorphism at position 18 was not recognized at the time that this information was reported).

(C) The S18Y variant effects ligase (but not hydrolase) activity in *trans*, suggesting an explanation for the incomplete penetrance of I93M.

posttranslationally. Dimerization-dependent ligase activity (Figure 8A) could be regulated by any number of cytoplasmic events, such as membrane (synaptic vesicle) binding, which would be expected to promote dimerization. The loss of control of the ligase activity could be pathogenic.

The mechanism by which UCH-L1 ligase activity promotes PD pathogenesis is not clear, but several models should be considered. Although the models discussed below are, in principal, experimentally distinguishable, determination of the precise mechanism will be difficult since it is likely that a combination of these (and other) models is operative. We assume that, in order to avoid PD, α-synuclein levels are normally kept under the critical concentration for oligomerization (dashed line in Figure 8B; Rochet and Lansbury, 2000) by proteasome-dependent degradation. Degradation is initiated by elaboration of a K48-linked polyubiquityl chain in an ATP-dependent process that requires E1, E2, and E3 ligases (Figure 8B, to the left). UCH-L1 may be able to elaborate K63-linked polyubiquityl chains (note different pattern in Figure 8B) on α-synuclein in a dimerization-dependent, ATP-independent process. The UCH-L1 ligase activity could produce an elevation of the cyto-

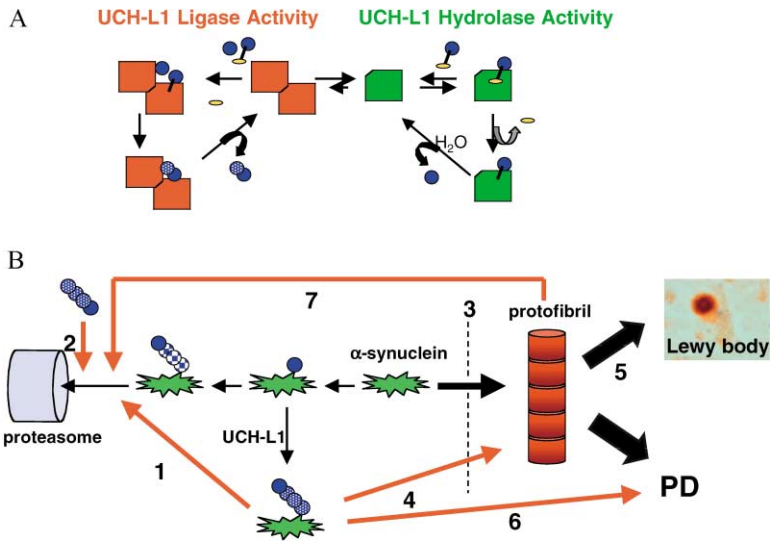


Figure 8. Several Speculative Models Could Explain the Apparent Pathogenicity of UCH-L1 Ligase Activity

(A) A model for the two activities of UCH-L1. The hydrolytic activity (green) may be beneficial, while the ligase activity (red), which is more sensitive to increases in UCH-L1 expression, could be harmful. Thus, both loss of UCH-L1 hydrolytic activity and gain of UCH-L1 ligase activity could deplete the pool of free ubiquitin and compromise the ubiquitin-dependent degradation pathway. The ubiquityl donor can be a peptide or protein conjugate, and thus no "activation" of its C terminus, by ATP, for example, is required. UCH-L1 ligase activity could produce undegradable, K63-linked (indicated by dotted background) polyubiquitin chains that could inhibit proteasomal activity.

(B) Evolution strives to divert the flux of α-synuclein away from protofibrillar aggregates and the "PD" pathway. However, these models, discussed thoroughly in the text, suggest several mechanisms whereby PD can be promoted by UCH-L1 ligase activity. The shading of the blue spheres, which represent ubiquitin molecules, was chosen to emphasize differences in interubiquitin amide linkages: the checked pattern indicates K48 linkage, whereas the dotted background indicates K63 linkage.

plasmic concentration of α-synuclein by inhibiting its "normal" degradation. Inhibition of the proteasome could be achieved by either the K63-linked polyubiquitylated α-synuclein (**1** in Figure 8B) or K63 polyubiquitin chains formed by repeated rounds of ubiquitin ligation as shown in Figure 8 (**2** in Figure 8B). There is precedent for the later mechanism (Hofmann and Pickart, 2001). The magnitude of inhibition needed to produce a significant effect need not be great, since it is possible to promote the aggregation of α-synuclein by "molecular crowding" (Shtilerman et al., 2002), that is, by increasing the total cytoplasmic protein concentration by a small amount. Crowding will promote processes that have a negative volume of reaction, for example, α-synuclein fibrillization (Shtilerman et al., 2002), and will effectively decrease the α-synuclein concentration threshold (**3** in Figure 8B). It is also possible that K63 polyubiquitylation of α-synuclein (like both PD-linked mutations) may promote formation of pathogenic protofibrils (**4**) and/or inhibit the protofibril-to-fibril/Lewy body transformation, which may be detoxifying (**5**). Mechanism **5** is precedented in the case of another α-synuclein adduct: the dopamine-α-synuclein adduct inhibits the protofibril-to-fibril transformation, leading to accumulation of protofibrils (Conway et al., 2001). Finally, one must consider the possibility that the K63-linked polyubiquitylated α-synuclein species could itself be neurotoxic (**6**). This mechanism, while unprecedented, is consistent with the fact that elevation of ligase activity and elevation of [α-synuclein] are synergistically pathogenic, since both would boost the concentration of the toxic species (this is also true of mechanisms **1–5**). Of these six possible mechanisms (the list is not exhaustive), **1** and **2** are clearly consistent with the effect of UCH-L1 variants in cell culture reported here. It is also possible that α-synuclein protofibrils, which would accumulate in scenarios **4** and **5**, could inhibit the proteasome (**7** in Figure 8B), as has been demonstrated for other protein aggregates (Bence et al., 2001). Overexpression of a PD-linked mutant from of α-synuclein in PC12 cells causes a decrease in proteasome activity (Engelender et al., 2001). Thus, it is clear that α-synuclein aggregation and α-synuclein degradation are linked and that small perturbations of either process by UCH-L1 could amplify and produce a significant overall effect.

The traditional loss-of-function versus gain-of-function analysis of a disease-associated mutation is based on a one gene = one enzymatic activity model. In the case of UCH-L1, we have demonstrated that one gene can encode two related enzyme activities. UCH-L1 possesses a beneficial hydrolase activity and a dimerization-dependent ligase activity that is at least partly pathogenic. Decreasing UCH-L1 expression could have a therapeutic benefit, since the hydrolase activity would decrease less rapidly than ligase activity (the former would decrease linearly as a function of [UCH-L1], while the latter would decrease as a function of $[\text{UCH-L1}]^2$). An ideal PD therapeutic would, like the protective S18Y polymorphism, inhibit the ligase activity and, in addition, promote the hydrolase activity. This could be accomplished by inhibiting UCH-L1 dimerization, thus increasing the concentration of monomeric UCH-L1 (Figure 8). This strategy is being tested through the identification of drug-like small-molecule UCH-L1 ligase inhibitors. Ultimately, trials of such inhibitors in animal models of PD will test the hypothesis proposed here.

## Experimental Procedures

### Proteins and Antibodies

Plasmids for prokaryotic expression of UCH-L1 and I93M were a gift from K. Wilkinson, and the plasmid for expression of S18Y was generated using QuickChange Mutagenesis Kit (Strategene). Antibodies used for immunoblotting were monoclonal anti-α-synuclein (15–123, SYN-1, Transduction Labs, 1:2000) and monoclonal anti-ubiquitin (mUb, Chemicon Intl., 1:15,000). Recombinant α-synuclein and wt UCH-L1, S18Y, and I93M were expressed and purified as reported. Concentrations of UCH enzymes were determined by amino acid analysis. Recombinant UCH-L3 was both a gift from H.L. Ploegh and purchased from Boston Biochem (Cambridge, MA).

### Isolation of Synaptic Vesicles

Synaptic vesicle preparation was performed according to the published protocol (Hell and Jahn, 1998). S3, P3, LS1, LP1, LS2, and LP2 fractions were subjected to Western blotting using UCH-L1 antibody (Chemicon), α-synuclein (Transduction Lab), synaptophysin (Transduction Lab), synaptotagmin I (Transduction Lab), and synaptin I (Chemicon). In the cases for synaptotagmin I and synaptophysin I, rat cerebrum lysate purchased from Transduction Lab was added as the positive control.

### Rat Brain Preparation and Immunoprecipitation

Whole brains from rats (*Sprague-Dawley*) in equal volume of UCH buffer were homogenized using 1.0 mm glass beads (Biospec Products) with Mini-Beadbeater (Biospec Products). The lysate was centrifuged at 14,000 rpm for 10 min and the supernatant was manually loaded onto a 1 ml HiTrap Q XL column. The column was then installed onto FPLC system and washed with 5 column volume of UCH buffer. Proteins were eluted with a gradient of 0 to 1 M NaCl in UCH buffer over 60 column volume. A small fraction of synuclein was retained in the column and they were coeluted with UCH-L1 (from 200 to 300 mM NaCl gradient). The fractions that contained UCH-L1 and synuclein were determined by Western blotting. They were combined and dialyzed in UCH buffer at 4°C overnight. For each immunoprecipitation assay, 0.5 ml of combined UCH-L1/synuclein fractions was added with 1 μl of SYN-1, Anti PGP9.5, or buffer. Binding reactions were sat at RT for 1 hr prior to addition of protein G agarose (Boehringer Mannheim). The mixtures were rotated using Labquake Shaker (Barnstead) for half an hour. The agarose was isolated by centrifugation and washed with UCH buffer three times. Proteins were eluted with SDS loading buffer and result was analyzed using Western blot.

### Transfection of COS-7 Cells with UCH-L1, Ubiquitin, and α-Synuclein

The plasmid for eukaryotic expression of α-synuclein (pcDNA) was constructed by insertion of the cDNA for human α-synuclein into pcDNA3.1(+) (Invitrogen). UCH-L1 and its variants were also cloned into pcDNA3.1 with restrict enzymes Xho I and Hind III. UB-HIS pWC7 was a generous gift from D. Finley. COS-7 cells were grown in DMEM +10% FBS and were transfected with plasmids using FuGene6 (Roche Molecular Biochemicals). Transfected cells were cultured at 37°C for 48 hr before being treated with 35 μM lactacystin, MG132, or DMSO. After 24 hr of incubation, the cells were lysed using 8 M Urea, 100 mM $NaH_2PO_4$, 10 mM Tris-HCl (pH 8.0), and 0.5% Triton X-100. Cell lysates were resolved on SDS-PAGE and analyzed using Western blot.

### Ubiquitylation of α-Synuclein in Rabbit Reticulocyte Lysate

Crude rabbit reticulocyte-rich blood was obtained from Pel-Freez Biologicals. Rabbit reticulocyte lysate (RRL) was prepared according to Ciechanover et al. (1978). In a total volume of 700 μl, 5 μM α-synuclein and 100 μM $His_6$-ubiquitin (Affiniti Research Products, Ltd., Exeter, UK) were incubated in RRL in the presence of 2 mM ATP, 50 μM LC, 2 μM Ubal, 2 mM DTT, 5 mM $MgCl_2$, and an ATP regenerating system (10 mM creatine phosphate/1 mg/ml creatine phosphokinase). The reaction was allowed to proceed for

2.5 hr at 37°C before being placed on ice and quenched by 50 μl of protease inhibitor cocktail (Sigma) and 1 mM N-ethylmaleimide (Sigma). An equal volume of binding buffer (50 mM $NaH_2PO_4$ [pH 8.0], 300 mM NaCl, 10 mM imidazole) and 500 μl of a 20% slurry of Ni-nta magnetic agarose beads (Qiagen) were added to the lysate. This mixture was incubated at 4°C overnight, then washed with binding buffer (2 × 1 ml) and washing buffer (2 × 1 ml, 50 mM $NaH_2PO_4$ [pH 8.0], 300 mM NaCl, 20 mM imidazole). The $His_6$-ubiquitin conjugates of α-synuclein were eluted from the agarose beads using 2 × 90 μl of elution buffer (50 mM $NaH_2PO_4$ [pH 8.0], 300 mM NaCl, 250 mM imidazole) and dialyzed into PBS overnight at 4°C using a Slide-A-Lyzer mini dialysis unit with 10,000 MWCO (Pierce). Protein samples were resolved using 14% Tris-glycine (TG) or 4%–20% TG for high-molecular-weight proteins. The proteins were transferred to polyvinylidine difluoride (PVDF), and all membranes were blocked overnight at 4°C with 5% nonfat milk/TBST (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Tween 20). They were then incubated with primary antibody for 1 hr in 1% BSA/TBST, washed three times with TBST, and incubated in horseradish peroxidase-conjugated secondary antibody for 1 hr (Promega). Bound antibodies were detected using enhanced chemiluminescence (Amersham or NEN).

### UCH Enzyme Reactions with Ubiquitylated α-Synuclein

The dialyzed ubiquitin conjugates (2–15 μl) were taken up in 50 μl of buffer containing 50 mM Tris [pH 7.4], 5 mM DTT, and 50 μg/ml ovalbumin and then treated with either UCH-L1 or an equal volume of buffer. The ubiquitin conjugates of α-synuclein were electroblotted onto PVDF and immunoblotted using SYN-1 for all UCH-L1 experiments.

### UCH Hydrolytic Activity Measurements

Ubiquitin C-terminal hydrolysis was initiated by sequentially adding 10 μl of 2 μM UCH-L1 enzymes or 10 nM UCH-L3 and 2 μl of various concentrations of ubiquitin-AMC (Boston Biochem, Cambridge, MA) (both stored on ice) into 2 ml of UCH buffer (50 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 5 mM DTT). The reactions were monitored at 25°C using Hitachi F4500 fluorescence spectrophotometer. The AMC fluorophore was excited at 380 nm and the rates of release of free AMC were measured by watching the increase in fluorescence emission at 460 nm. $K_M$ values were impossible to accurately determine with titration of Ub-AMC because the experimental concentration of UCH-L1 was 10 nM. For mixture experiment, 5 μl of 2 μM of each enzyme were added. All five enzymes hydrolyzed the model fluorogenic amide substrate, ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC, see Supplemental Data at http://www.cell.com/cgi/content/full/111/2/209/DC1; Dang et al., 1998). Consistent with previous reports, UCH-L3 had much greater hydrolytic activity ($V_{max}$ = 13 ± 1.3 $s^{-1}$, $K_M$ = 41 ± 16 nM) than wt UCH-L1 ($V_{max}$ = 0.046 ± 0.0014 $s^{-1}$, $K_M$ = 38 ± 5.5 nM). The I93M mutant had approximately 50% of wt activity ($V_{max}$ = 0.027 ± 0.0014 $s^{-1}$, $K_M$ = 46 ± 11 nM) (Larsen et al., 1998; Leroy et al., 1998; see Supplemental Data). The S18Y ($V_{max}$ = 0.047 ± 0.001 $s^{-1}$, $K_M$ = 19 ± 2.6 nM) and S18A ($V_{max}$ = 0.054 ± 0.0019 $s^{-1}$, $K_M$ = 49 ± 6.1 nM) variants had comparable maximal rates to wt, consistent with the remoteness of residue 18 from the enzyme active site (Figure 1).

### UCH-L1 Ligation Reactions

Ligation was performed with 3 μM ubiquitin-AMC and 50 μM wt ubiquitin (Sigma) in presence of 1 μM of UCH-L1, I93M, UCH-L3 or in the absence of any enzyme in UCH buffer (50 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 1 mM DTT). For the enzyme concentration-dependent ubiquitin ligation reactions with UCH-L1 and S18Y, the reaction was performed with 3 μM ubiquitin-AMC and 50 μM wt ubiquitin in presence of 3, 1.5, 0.8, and 0.4 μM of UCH-L1, S18A, or S18Y. Reactions containing mixtures of UCH-L1 variants were performed using total of 3 μM enzyme, 4.5 μM Ub-AMC, and 57 μM wt ubiquitin. K63R was a gift from C. Pickart. Reactions were incubated at 25°C for 2 hr and reaction products were resolved on 14% or 16% Tris-Glycine SDS gel stained with Coomassie brilliant blue. Gels were dried and scanned. Band intensity was quantified using NIH Image 1.61/ppc program.

### Sedimentation Analysis by Analytical Ultracentrifugation

Sedimentation velocity experiments were performed in a temperature-controlled Beckman XL-I. 400–420 μl of protein (UCH-L3, UCH-L1 wt, I93M, S18A, and S18Y) at the desired concentration in UCH buffer was loaded into two-channel 1.2 cm path cell. The data were recorded at rotor speeds of 3,000–60,000 rpm in continuous mode with a step size of 0.005 cm at 20°C–25°C. The sedimentation velocity absorbance profiles were then analyzed to obtain the apparent distribution of sedimentation coefficients g(s*) for all the quaternary structures in solution using the DCDT software provided by Philo (Stafford, 1992). The observed sedimentation coefficient, s, was corrected to standard conditions (water at 20°C). The partial specific volume of UCH-L1 (0.735 $cm^3/g$) was estimated based on the partial specific volumes of its component amino acid residues.

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