Divergent N-terminal Sequences of a Deubiquitinating Enzyme Modulate Substrate Specificity*

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Ubiquitin-specific processing proteases (UBPs) are characterized by a conserved core domain with surrounding divergent sequences, particularly at the N-terminal end. We previously cloned two isoforms of a testis UBP, UBP-t1 and UBP-t2, which contain identical core regions but distinct N termini that target the two isoforms to different subcellular locations (Lin, H., Ke- riel, A., Morales, C. R., Bedard, N., Zhao, Q., Hingamp, P., Lefrancois, S., Combaret, L., and Wing, S. S. (2000) Mol. Cell. Biol. 20, 6568–6578). To determine whether the N termini also influence the biochemical functions of the UBP, we expressed UBP-t1, UBP-t2, and the common core domain, UBP core, in Escherichia coli. The three isoforms cleaved branched triubiquitin at >20-fold faster rates than linear diubiquitin, suggesting that UBP-testis functions as an isopeptidase. Both N-terminal extensions inhibited the ability of UBP-core to generate free ubiquitin when linked in a peptide bond with itself, another peptide, or to small adducts. The N-terminal extension of UBP-t2 increased the ability of UBP-core to cleave branched triubiquitin. UBP-core removed ubiquitin from testis ubiquitinated proteins more rapidly than UBP-t2 and UBP-t1. Thus, UBP enzymes appear to contain a catalytic core domain, the activities and specificities of which can be modulated by N-terminal extensions. These divergent N termini can alter localization and confer multiple functions to the various members of the large UBP family.

The ubiquitin-proteosome pathway of protein degradation is a major mechanism for intracellular protein catabolism (reviewed in Refs. 1–3). Proteins destined to be degraded through the ubiquitin-proteasome pathway are first covalently ligated with ubiquitin, a 76-amino acid peptide. This reaction involves the sequential action of three enzymes. Ubiquitin is first activated by ubiquitin-activating enzyme (E1) (4) and is then transferred to a specific cysteine residue of one of a family of ubiquitin-conjugating enzymes (E2s) (5). Although some E2s can transfer ubiquitin to substrates directly in vitro, most E2s support ubiquitin conjugation to substrates by interaction with one of the many ubiquitin protein ligases (E3s) (6–8). These E2/E3 enzymes form an isopeptide bond between the C terminus of ubiquitin and the ε-amino group of the side chain of lysine residues of the target protein. A branched polyubiquitin chain is then formed on the protein through the ligation of additional monomers of ubiquitin to the side chain of a lysine residue of the previous ubiquitin in successive rounds of ubiquitination (5). The covalent attachment of a polyubiquitin chain to proteins generally acts as a signal for their degradation by a multisubunit protease, the 26 S proteasome (9, 10).

In addition to the families of enzymes involved in conjugation of ubiquitin, a very large family of deubiquitinating enzymes has recently been identified from various organisms (reviewed in Refs. 11–13). These enzymes have several possible functions. First, they may have peptidase activity and cleave the products of ubiquitin genes. Ubiquitin is encoded by two distinct classes of genes. One is a polyubiquitin gene, which encodes a linear polymer of ubiquitins linked through peptide bonds between the C-terminal Gly and N-terminal Met of contiguous ubiquitin molecules (14). Each copy of ubiquitin must be released by precise cleavage of the peptide bond between Gly-76–Met-1 of successive ubiquitin moieties (15). The other class of ubiquitin genes encodes ubiquitin C-terminal extension proteins, which are peptide bond fusions between the C-terminal Gly of ubiquitin and N-terminal Met of the extension protein (16–18). To date, the extensions described are ribosomal proteins consisting of 52 or 76–80 amino acids (19, 20). These ubiquitin fusion proteins are processed to yield ubiquitin and the corresponding C-terminal extension proteins (21). Second, deubiquitinating enzymes may have isopeptidase activities. When a target protein is degraded, deubiquitinating enzymes can cleave the polyubiquitin chain from the target protein or its remnants (22–24). The polyubiquitin chain must also be disassembled by deubiquitinating enzymes during or after proteolysis by the 26 S proteasome, regenerating free monomeric ubiquitin (25, 26). In this way, deubiquitinating enzymes can facilitate the ability of the 26 S proteasome to degrade ubiquitinated proteins. Third, deubiquitinating enzymes may hydrolyze ester, thiolester, and amide linkages to the carboxyl group of Gly-76 of ubiquitin (27–29). Such nonfunctional linkages may arise from reactions between small intracellular compounds such as glutathione and the E1-, E2-, or E3-ubiquitin thiolester intermediates. Fourth, deubiquitinating enzymes

ubiquitin-specific processing protease; UCH, ubiquitin C-terminal hydrolase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; Ub, ubiquitin; Ubα, diubiquitin; Ubβ, triubiquitin; AMC, amidomethyl-coumarin; Mes, 4-morpholineethanesulfonic acid.

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20357
may compete with the conjugating system by removing ubiquitin from protein substrates, thereby rescuing them from degradation or any other function mediated by ubiquitination. Thus generation of ubiquitin by deubiquitinating enzymes from the linear polyubiquitin and ubiquitin fusion proteins and from the branched polyubiquitin ligated to proteins should be essential for maintaining a sufficient pool of free ubiquitin. Many deubiquitinating enzymes exist, suggesting that these deubiquitinating enzymes recognize distinct substrates and are therefore involved in specific cellular processes (11–13). Although there is recent evidence to support such specificity of these deubiquitinating enzymes (30–32), the structure-function relationships of these enzymes remain poorly studied.

Deubiquitinating enzymes can be divided broadly on the basis of sequence homology into two classes, the ubiquitin-specific processing protease (UBP or USP, also known as type 2 ubiquitin C-terminal hydrolase (type 2 UCH) and the UCH, also known as type 1 UCH) (12, 13). UCH (type 1 UCH) enzymes hydrolyze primarily C-terminal esters and amides of ubiquitin (27) but may also cleave ubiquitin gene products and disassemble polyubiquitin chains (30). They have in common a 210-amino acid catalytic domain, with four highly conserved blocks of sequences that identify these enzymes. They contain two very conserved motifs, the CYS and HIS boxes. Mutagenesis studies revealed that the boxes play important roles in catalysis (33, 34). Some UCH enzymes have significant C-terminal extensions (12, 32). The functions of the C-terminal extensions are still unknown but appear to be involved in proper localization of the enzyme (24, 32). The active site of these UCH enzymes contains a catalytic triad consisting of cysteine, histidine, and aspartate and utilizes a chemical mechanism similar to that of papain (33, 34).

UBP (type 2 UCH) enzymes are capable of cleaving the ubiquitin gene products (21) and disassembling polyubiquitin chains after hydrolysis (15). It appears that there is a core region of about 450 amino acids delimited by CYS and HIS boxes. Many of these isoforms have N-terminal extensions and a few have C-terminal extensions (12). In addition, there are variable sequences in the core region of many of the isoforms. The functions of these divergent sequences remain poorly characterized. Recently, we identified UBP-t, an UBP enzyme that is primarily expressed in the testis as two isoforms with the same core region (347 residues) but distinct N termini. The N-terminal extension of UBP-t1 has 49 residues and that of UBP-t2 has 271 residues. The divergent N termini were found to target distinct subcellular compartments (35). UBP-t1 is located primarily in the nucleus, whereas UBP-t2 is found primarily in a perinuclear location and can be associated with the centrosome. To evaluate whether in addition these N termini have functions in substrate specificity, we have tested the abilities of the two isoforms as well as the common core domain to cleave natural and semi-synthetic ubiquitin substrates.

MATERIALS AND METHODS

Preparation of Recombinant UBP-core, UBP-t1, and UBP-t2—To express full-length UBP-t1 and UBP-t2 and only the common core region of UBP-t1 and UBP-t2 (UBP-core), DNA fragments encoding the proteins were amplified by polymerase chain reaction and subcloned into the bacterial expression vector pETT11-d (Novagen). Plasmids were sequenced to confirm accurate amplification and cloning and then transformed into Escherichia coli BL21 (DE3). After induction of expression with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 28 °C, cells were harvested from 800 ml of culture and rinsed with PBS, and the cell pellets were frozen at −20 °C. Subsequent manipulations were on ice or at 4 °C. The frozen pellets were resuspended in 1⁄5 of the original culture volume of 50 mM Tris, pH 7.5, 1 mM DTT, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin A and lysed by sonication. The lysate was clarified by centrifugation at 100,000 × g for 1 h, and then proteins were differentially precipitated by ammonium sulfate (ICN Ultra Pure). The UBP-core was enriched in the 30–50% ammonium sulfate fraction. This fraction was dialyzed against 20 mM Mes, pH 6.2, 1 mM DTT overnight before being applied to a 5 × 100-mm propyleneglycol acetonitril column (Waters SP/HR15) equilibrated in the same buffer. Bound proteins were eluted with 0.15 M NaCl and in most fractions was >99% pure as evaluated by Coomassie Blue-stained acrylamide gels. The 30–40% ammonium sulfate fraction containing the UBP-t1 was dialyzed against 50 mM Tris, pH 7.5, 1 mM DTT overnight before being applied to a quaternary amine anion exchange column (Amersham Pharmacia Biotech MonoQ) equilibrated in the same buffer. Full-length UBP-t1 was eluted at 0.028 M NaCl. The 35–45% ammonium sulfate fraction containing the UBP-t2 was dialyzed against 50 mM Tris, pH 7.5, 1 mM DTT overnight before being applied to the quaternary amine anion exchange column equilibrated in the same buffer. Full-length UBP-t2 eluted at ~0.31 M NaCl. The fractions of each step were screened by both Western blots with anti-UBP-core-specific antibody and activity assays. The activities of the enzymes in different fractions were monitored by determining their abilities to hydrolyze 125I-labeled Ub-PESTc (ubiquitin extended at the PESTc peptide bearing the iodinated tyrosine). For the linear diubiquitin (Ub2) or branched Ub3 substrates, the reaction was stopped by boiling and labeling with Na125I using Iodobeads (Pierce). Unincorporated 125I was removed by passing the reaction products over a Sephadex G-25 column.

Ubiquitin Peptidase and Isopeptidase Assays—To measure the abilities of UBP-core, UBP-t1, and UBP-t2 to cleave some natural substrates and synthetic ubiquitin derivatives, the different enzymes were incubated with a total volume of 20 μl as follows: 100 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mg/ml bovine serum albumin or ovalbumin, 2 μM Ub2-labeled substrate. After incubating at 37 °C for various times, the reactions were quenched as indicated below. For the substrate Ub-PESTc, the reaction was terminated by adding 100 μl of 2.5% bovine serum albumin and 1 ml of 20% (w/v) trichloroacetic acid. After incubation on ice for 30 min, the samples were centrifuged, and the resulting supernatants were counted for their radioactivities to detect the PESTc peptide bearing the iodinated tyrosine. For the linear diubiquitin (Ub2) or branched Ub3 substrates, the reaction was stopped with Laemmli sample buffer containing 2-mercaptoethanol, and the products were resolved by SDS-PAGE on 20% acrylamide gels and detected by autoradiography. After detection by autoradiography, the monoubiquitin (Ub) band and Ub3 band in the assay with Ub3 was excised from the dried gel and counted. The rate of cleavage of peptide bonds for linear Ub3 substrate was calculated as half of the Ub produced in this reaction. The rate of cleavage of isopeptide bonds for branched Ub3 substrate was calculated as (2/3 (Ub – Ub(i)) + Ub3) produced in this reaction. To test the abilities of these UBP enzymes to cleave ubiquitin ester and ubiquitin linked to lysine by the a or ε amino groups, they were incubated with these substrates (16 μM) in a total volume of 50 μl of Mes, pH 7.6, 3 mM DTT, 5 mM MgCl2 at 37 °C for 20 or 30 min. The reactions were quenched with 0.1 M HCl and then injected onto a 4.6 × 250-mm C8 column equilibrated and eluted with 60% acetonitrile in 25 mM phosphate to resolve the free ubiquitin product from the substrate (29). Fluorometric assays used ubiquitin-AMC as the substrate (37). In a typical assay, 80 μl of assay buffer (50 mM Tris, pH 7.8,
fitting analysis using fitting function dimers. The dissociation constant was calculated by a non-linear curve-ubiquitin-AMC as substrate at five or more different concentrations of core by nonhydrolyzable diubiquitin, reactions were conducted using velocities.

UBP-t1 and UBP-t2 consist of identical core regions containing the conserved motifs of the UBP family (CYS and HIS boxes are indicated) but distinct N termini.

UBP-core was analyzed by SDS-PAGE followed by staining with Coomassie Blue.

100 μg/ml ovalbumin, 10 mM DTT containing 6.6 μM enzyme were added to a 100-μl cuvette. When present, diubiquitin analogs were added, and after a 5-min preincubation at 37 °C to achieve thermal equilibrium, ubiquitin-AMC was added to a final concentration of 40 nM. Reaction progress was monitored by the increase in fluorescence emission at 450 nm (λex = 355 nm) that resulted from the cleavage of AMC from the substrate.

To compare the abilities of these enzymes to cleave the substrates Ub-PESTc, linear Ub2, and branched Ub3, equivalent amounts of these enzymes (38 nM) were incubated with 2 μM substrate. The initial rates obtained with the different enzymes were compared. To compare the abilities of UBP-core, UBP-t1, and UBP-t2 to interact with the Ub-PESTc substrate, the apparent $K_m$ values for this substrate were determined. Variable concentrations of the substrate were assayed in reactions as described above. The products were monitored by removing aliquots from the reaction at various times. Initial velocities were calculated from the time courses and used in double-reciprocal plots (Lineweaver-Burk) to determine apparent $K_m$ values and maximal velocities.

To determine the dissociation constants for the inhibition of UBP-core by nonhydrolyzable diubiquitin, reactions were conducted using ubiquitin-AMC as substrate at five or more different concentrations of dimers. The dissociation constant was calculated by a non-linear curve-fitting analysis using fitting function $v = v_i + (v_o - v_i)/(1 + [I]/K_i)$ where $v_o$ is the uninhibited rate, $v_i$ is the rate at high concentration of inhibitor, and $K_i$ is the inhibition constant. In all cases inhibition was fully competitive, and $v_i = 0$.

To evaluate the abilities of these enzymes to remove ubiquitin from testis ubiquitinated proteins, rat testis extracts were clarified by centrifugation at 3000 × g for 20 min at 4 °C and then frozen at −80 °C until analysis. Before assaying, the excess N-ethylmaleimide in the samples was neutralized by adding DTT to a final concentration of 5 mM. Aliquots (50 μg) of the protein were then incubated with equal amounts of each of the UBP enzymes (38 nM) for various times at 37 °C. The reaction was stopped with Laemmli sample buffer containing 2-mercaptoethanol, and the proteins were resolved by SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose membranes. The ubiquitinated proteins were detected by Western blotting with anti-ubiquitin antibodies (Sigma) followed by protein A coupled to horseradish peroxidase and a chemiluminescent detection method (ECL, Amersham Pharmacia Biotech).

**RESULTS AND DISCUSSION**

**Preparation of UBP-core, UBP-t1, and UBP-t2**—To characterize the biochemical properties of UBP-t1 and UBP-t2 as well as their common core region, UBP-core (Fig. 1A), the proteins were expressed in *E. coli*. Since expressed proteins were susceptible to degradation, purification of the various enzymes was undertaken to remove degradation products (Fig. 1B). UBP-core was purified to apparent homogeneity, as estimated by Coomassie Blue-staining of the protein on a polyacrylamide gel. UBP-t1 and UBP-t2 were enriched to levels of specific activity that were 17- and 27-fold greater, respectively, than in the crude bacterial lysates. Control lysates not expressing enzymes did not show activity in these assays, and the activities of all isoforms were found to be highly susceptible to inhibition by ubiquitin aldehyde, and therefore, they were quantitated by titration with this inhibitor.

**N-terminal Extensions Inhibit the Peptidase Activity of UBP-core**—Since a key function of many deubiquitinating enzymes is to process the products of ubiquitin genes, we tested whether UBP-t1 and UBP-t2 can generate free ubiquitin from a fusion protein with another peptide, as in the case of the model substrate Ub-PESTc. UBP-core readily cleaved the N-terminal Extensions Inhibit the Peptidase Activity of UBP-core (Fig. 2). Thus the N-terminal extensions inhibited the hydrolytic activity inherent in the core domain. To test whether the N-terminal extensions of UBP exert this inhibitory effect by decreasing the abilities of the UBP core to interact with Ub-PESTc, we measured apparent $K_m$ values of Ub-
substrate than did UBP-core (Table I). However, the $V_{\text{max}}$ value of UBP-t2 was similar to that of the UBP-core enzyme, indicating that the N-terminal extensions of UBP can negatively influence binding of the core domain to the substrate without significantly affecting catalytic function.

To test whether these enzymes may be involved in processing the linear polyubiquitin, we tested the activities of these enzymes against linear Ub$_2$. UBP-t1, UBP-t2, and UBP-core all had low abilities to generate free ubiquitin from ubiquitin and further supports the role of these extensions in imposing substrate selectivity.

The indicated substrates (16 $\mu$m) were incubated with equal concentrations of the indicated enzymes (76 nM). Products of the reaction were resolved from substrate and quantitated by HPLC as indicated under "Materials and Methods."

### Table I

| Enzyme    | $K_m$ (mM) | $V_{\text{max}}$ (mol/min/mol enzyme) |
|-----------|------------|---------------------------------------|
| UBP-core  | 1.7 ± 0.7  | 0.49 ± 0.16                           |
| UBP-t1    | >20        | ND                                    |
| UBP-t2    | 18.2 ± 3.8 | 0.59 ± 0.08                           |

This is similar to UCH enzymes tested to date that also do not appear to discriminate between these two model substrates (30).

### Branched Ub$_2$ Is Preferentially Cleaved by UBP-t1 or UBP-t2 Isofoms—Most biological functions of ubiquitin are mediated by the linkage of the C-terminal glycine of ubiquitin in an isopeptide bond with the $\epsilon$-amino group of the side chain of lysine residues of the protein substrate. Recognition of ubiquitinates proteins for degradation by the 26 S proteasome generally requires the presence of a polyubiquitin chain on the protein substrate in which each ubiquitin is linked to each other via these isopeptide linkages. To evaluate whether UBP-core, UBP-t1, and UBP-t2 can potentially disassemble such a polyubiquitin chain, Ub$_2$, a branched triubiquitin chain linked via isopeptide bonds between the $\epsilon$-amino group of lysine 48 in one ubiquitin molecule and the C-terminal of another ubiquitin molecule, was used as a substrate. All three enzymes had activity against this substrate, and the rates of cleavage were similar to that for Ub-PESTc but 1–2 orders of magnitude higher than that seen for linear Ub$_2$ (Fig. 4). Thus, the UBP-core domain appears to prefer branched rather than linear ubiquitin polymers as substrate. The higher rate of cleavage of triubiquitin compared with diubiquitin was not due to the extra moity of ubiquitin in the former, as diubiquitin did not accumulate over time in the reactions with triubiquitin as substrate (data not shown). In addition, UBP-t2 had higher activity than UBP-core. This indicates that the N-terminal extension of UBP-t2 not only suppresses peptidase activity but enhances isopeptidase activity. In contrast, UBP-t1 had lower activity.

### Table II

| Substrate                | Rate of hydrolysis |
|--------------------------|--------------------|
|                          | UBP-core | UBP-t1 | UBP-t2 |
| Ubiquitin ethyl ester    | 4.3      | 1.2    | 0.72   |
| Nε-ubiquitin-l-lysine    | 2.6      | 0.18   | 0.98   |
| Nε-ubiquitin-t-lysine    | 3.5      | 0.31   | 0.63   |
than UBP-core in cleaving branched Ub₃ (Fig. 4). This indicates that the N-terminal extensions can modulate the intrinsic activity of the core domain both positively and negatively.

Although ubiquitin moieties joined by Gly-76–Lys-48 linkages appear to be most efficient at targeting proteasome-mediated degradation, linkages of Gly-76 to other lysine residues of ubiquitin have been observed. To evaluate whether these enzymes show preference for specific linkages, the ability of nonhydrolyzable ubiquitin dimer analogs joined in various linkages (38) to inhibit these enzymes was tested. These dimer analogs were synthesized by using dichloroacetone to cross-link ubiquitin containing a terminal cysteine to another ubiquitin in which individual lysines have been mutated to cysteine. To determine inhibitory constants easily and accurately, a sensitive fluorometric assay using ubiquitin-AMC as substrate was employed (Table III). At the low concentrations of ubiquitin-AMC used, only UBP-core and UBP-t₂ had significant activities, and so UBP-t₁ was not tested. The apparent $K_m$ values of monoubiquitin and the dimers for UBP-core was similar to that of Ub-PESTc. However, in contrast to Ub-PESTc, where the affinity for UBP-t₂ decreased 1 order of magnitude compared with UBP-core, the affinity of ubiquitin-AMC actually increased due to any contaminating bacterial proteases.

In summary, the UBP core region containing the conserved elements of the UBP family of enzymes and delimited by the CYS and HIS boxes contains ubiquitin-specific protease activity. Thus we provide the first direct evidence that this core domain appears to preferentially cleave isopeptide rather than peptide linkages (compare rates in Figs. 3 and 4), thus suggesting that the UBP family functions primarily in either the regeneration of ubiquitin from polyubiquitin chains produced after the action of the 26 S proteasome or in the editing or rescuing of ubiquitinated protein substrates before the action of the 26 S proteasome. Indeed, the isopeptides were capable of removing ubiquitin from endogenous testis proteins (Fig. 5). The lower $K_i$ values for the ubiquitin dimers compared with ubiquitin alone (Table III) would also be consistent with preferential binding of branched polyubiquitin to the core region of the enzyme.

We have previously shown that the N-terminal extensions of UBP-t₁ and UBP-t₂ serve to localize the enzyme to different compartments of the cell (35). It should be noted that UBP-t₂ is localized in a perinuclear pattern resembling that of the proteasome and is also the most active on branched polyubiquitin conjugates. It is intriguing to speculate that these indicators

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**TABLE III**

| Inhibitor | $K_i$ (μM) | $V_{max}$ (nM) |
|-----------|------------|---------------|
| Ubiquitin | 2.4        | 3.82          |
| 11-76Ub₂ | 2.4        | 3.82          |
| 29-76Ub₂ | 1.0        | 1.05          |

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**Fig. 4.** Hydrolysis of ¹²⁵I-labeled branched Ub₃ by UBP-t₁, UBP-t₂, and UBP-core enzymes. UBP-t₁ (filled circles), UBP-t₂ (open circles), and UBP-core (filled triangles) (all at 38 nm) were incubated in the presence of 2 μM ¹²⁵I-Ub₃. Aliquots of the reaction mixtures were removed at the indicated times and resolved by SDS-PAGE on 20% acrylamide gels. After detection by autoradiography, the Ub and Ub₃ bands were excised from the gel and counted separately. The concentration of isopeptide bonds cleaved by the enzymes was calculated as 2/3([Ub] − [Ub₃]) + [Ub₃].
may suggest a role for UBP-t2 in metabolism of branched polyubiquitin at or near the proteasome. Our data demonstrate clearly and for the first time that these divergent N termini can also modulate the activity of the core domain. Interestingly, the activity can be both positively and negatively affected by the extension depending on the particular substrate. For the substrates for which measures of affinity could be determined, the parameters obtained were relatively high (in the micromolar range). Rates of cleavage were also relatively low. Thus, there are probably specific substrates for these enzymes, and the N-terminal extensions may play roles in recognizing these specific ubiquitinated proteins. Indeed, without N-terminal extensions, UBP-core efficiently removed ubiquitin from endogenous proteins. However, with N-terminal extensions, UBP-t2 did this at a slower rate, and UBP-t1, hardly at all (Fig. 5). Thus, it is quite possible that the N-terminal extensions are positioned near the S1' site, and the different effects of the UBP-t2 extension on affinity for ubiquitin-AMC and ubiquitin-PESTc would support this model (Tables I and III). In addition, given their precise localization in the cell, the enzymes may be co-localized with their substrates in specific compartments. Such co-localization would permit privileged delivery of substrates to the enzymes and diminish dependence of access to the enzymes on factors such as affinity constants and diffusion rates. Finally, the observations on UBP-testis in this and our previous work (35) indicate that the numerous distinct N-terminal extensions of UBP enzymes could permit specific spatial, temporal, and kinetic modulation of UBP function and allow these enzymes to mediate quite precise functions in the ubiquitin-dependent proteolytic pathway.

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Function of N Termini of a UBP Enzyme

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