Published in final edited form as:

Anal Chem. 2018 March 20; 90(6): 4032–4038. doi:10.1021/acs.analchem.7b05234.

Top-down analysis of branched proteins using mass spectrometry

Dapeng Chen^{#1}, Fabio Gomes^{#1}, Dulith Abeykoon¹, Betsegaw Lemma¹, Yan Wang¹, David Fushman¹, and Catherine Fenselau¹

¹University of Maryland, College Park, Maryland 20742, United States

[#] These authors contributed equally to this work.

Abstract

Post-translational modification by covalent attachment of Rub1 (NEDD8), ubiquitin, SUMO and other small signaling proteins has a profound impact on the function and fate of cellular proteins. Investigations of the relationship of these bioactive structures and their functions are limited by analytical methods that are scarce and tedious. A novel strategy is reported here for analysis of branched proteins by top-down mass spectrometry and illustrated by application to four recombinant proteins and one synthetic peptide modified by covalent bonds with ubiquitin or Rub1. The approach allows an analyte to be recognized as a branched protein, participating proteins to be identified, the site of conjugation to be defined, and chemical, native and recombinant modifications to be characterized. In addition to high resolution provided by the mass spectrometer, success is based on sample fragmentation by electron transfer dissociation assisted by collisional activation and software designed for graphic interpretation adapted for branched proteins. This strategy allows for the first time structures of unknown two component branched proteins to be elucidated directly.

Graphical abstract

Fenselau@umd.edu.

Notes. The authors declare no conflict of interest.

Recent advances in instrumentation and bioinformatics have facilitated mass spectrometry-based analysis of intact proteins. This top-down exploration has generated new appreciation of the broad range of post-translational modifications that occur, and of the abundance of proteoforms present in cells.¹ A number of areas have benefitted significantly from top-down workflows, including quality control in the production of therapeutic antibodies² and recombinant therapeutic proteins.³ Basic research in biochemistry has also bloomed, with studies of the coincidence, kinetics and interactions of post-translational modifications. Among the most challenging and least studied modifications are the branched proteins formed when ubiquitin and other ubiquitin-like modifiers (e.g., SUMO, Rub1/NEDD8, FAT10, ISG15) or their mixed or homo-polymers form covalent bonds with cellular proteins.^{4,5} Such modifications have profound effects on their protein substrates, including regulating subcellular localization, modulating the inflammatory response, DNA repair and transcription, and controlling protein degradation.^{6,7} Clinical disorders are associated with malfunctions in these modifications, such as neurodegenerative disorders, cardiovascular diseases, inflammation and cancer.^{8,9} Consequently, deciphering the code--the relationship of structures of protein modifiers to their functions--is a major objective in cell biology. Although the use of GG-tags in bottom up proteomic strategies has expanded understanding of how widespread ubiquitination is,¹⁰ critical information about the length and connectivity of the polyubiquitin modification is lost. Even less is known about the occurrence and functions of SUMO and Rub/NEDD8.¹¹ Top-down analysis of branched proteins is expected to characterize their complete structures, recognize concurrent and interacting modifications and support the direct association of structures with functions.

Top-down MS/MS spectra of homo polymers of ubiquitin,¹² isomeric ubiquitin trimers¹³ and isomeric ubiquitin tetramers¹⁴ have been reported recently, in which UVPD or CID-

assisted ETD was used to activate the heavy ions. However, examples of top-down analysis of branched proteins, proteins modified by ubiquitin or Rub1/NEDD8 have not been reported. This is in large part because synthetic methods are not well developed and isolation of these modified proteins from cells in sub microgram amounts is challenging.¹⁵ We report here progress in sensitivity, acquisition, and interpretation of top-down spectra exemplified here by the analysis of four synthetic conjugates of ubiquitin and Rub1, and one fluorescent peptide. We demonstrate a strategy that allows recognition that a branched protein may be present, identification of the proteins involved, localization of the sites of attachment, and analysis of chemical, engineered, or native modifications.

Experimental Section

Expression and purification of Ubiquitin variants, UBE1 and Ubch5b

Wild type ubiquitin (Ub) and all its variants were expressed using *E. coli* BL-21(DE3) Rosetta cells. Ubiquitin variants used in this study include K0 ubiquitin (UbK0), in which all seven lysines are mutated to arginines, and UbD77, where an extra aspartate residue (D77) is added at the C-terminus of wild type ubiquitin. These modifications were introduced in order to control the length and composition of the resulting conjugates and to prevent these ubiquitin variants from making homo-conjugates.^{16,17} After expression, cells were lysed by sonication, and the lysate was centrifuged at 22,000 rpm to obtain a clear supernatant. The supernatant was precipitated by heating at 65°C for 15 minutes followed by plunging into an ice bath for 10 minutes and cleared via centrifugation. The final supernatant was dialyzed against 2 L of 50 mM ammonium acetate, pH 4.5 buffer using a 3 kDa molecular weight cutoff membrane. WT Ub and UbD77 were purified using perchloric acid precipitation as detailed elsewhere.¹⁶ In case of Ub K0, the supernatant was precipitated by heating at 65°C for 15 minutes followed by plunging into an ice bath for 10 minutes and cleared via centrifugation. The final supernatant containing UbK0 was dialyzed against 2 L of 50 mM ammonium acetate, pH 4.5 buffer using a 3 kDa molecular weight cutoff membrane. Final solution was loaded onto a 5 mL cation exchange column (GE Healthcare Life Sciences) and eluted over a 36 column volume gradient from 0–40% solvent B (50 mM ammonium acetate, 1 M NaCl, pH 4.5). Ub-containing fractions were collected and the purity was confirmed using 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and AccuTOF-ESI mass spectrometer.

His₆-tagged enzymes UBE1 (ubiquitin activating enzyme E1) and Ubch5b were expressed using *E. coli* cells. Both enzymes were purified using a 5 mL His Trap column (GE Healthcare Life Sciences).

Rub1 Expression and purification

The Rub1 (related to ubiquitin protein 1) protein from *Saccharomyces cerevisiae* used in this study contained a T72R mutation (Rub1T72R). The protein was expressed in *E. coli* BL-21(DE3) cells as a fusion with intein tag containing a chitin-binding domain. Cells were lysed by sonication, and the lysate was centrifuged at 22,000 rpm to obtain clear supernatant. Supernatant was loaded on a chitin bead column, and Rub1 was eluted by adding the on-column cleavage buffer (20 mM Hepes, 200 mM NaCl, 1 mM EDTA, and 50

mM DTT, pH 8.0). Fractions with purified Rub1 T72R were collected and buffer exchanged into a 20 mM sodium phosphate, pH 6.0 buffer. Purity was confirmed using SDS-PAGE and ESI mass spectrometry.

Ubiquitination of Ubch5b

Conjugation of His₆-tagged E2 enzyme, Ubch5b, with Ub K0 was carried out with 90 μ M Ubch5b, 500 nM UBE1 and 2.2 mM UbK0 (10 mg) in a 2 mL volume containing 5 mM ATP, 5 mM MgCl₂, creatine phosphate, and creatine phosphokinase in 50 mM Tris, pH 8.0 buffer as detailed elsewhere.¹⁸ After 15 hours of reaction, the mixture was diluted with 10 mL of 20 mM sodium phosphate, 130 mM NaCl, pH 6.8 buffer and concentrated to a final volume of 1 mL. This mixture was fractionated by SEC, and different states of ubiquitinated Ubch5b were detected using 15% SDS-PAGE.

Assembly and purification of Rub1-Ub dimers

10 mg each of purified Rub1T72R and UbD77 were incubated with 500 nM of ubiquitin-activating enzyme E1 (UBE1), 20 μ M of ubiquitin-conjugating enzyme E2-25K (UBE2K), 5 mM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, and creatine phosphokinase in 50 mM Tris-HCl buffer (pH 8) for ~16 h. See also Singh et al.¹⁹ The reaction was stopped by addition of 7 mL cation buffer A (50 mM ammonium acetate, pH 4.5). The solution was spun at 13,000 rpm to remove precipitated E1 and E2 enzymes and then slowly injected onto a 5 mL cation-exchange column at 1 mL/min using FPLC (GE Healthcare Life Sciences). The Rub1/Ub-containing species were eluted with cation buffer B (50 mM ammonium acetate containing 1 M NaCl, pH 4.5).

Ubiquitination of PTEN peptide.

A chemically synthesized peptide (PTEN), Ac-I[Lys(Alloc)EIVSRNKRRYQEDGF[Lys(FITC)]], comprising residues 5–21 of human PTEN protein and an additional C-terminal lysine with a fluoresceine isothiocyanate (FITC) attached to its side chain was generously provided by Dr. Suresh Kumar (Progenra Inc.). Ubiquitin was conjugated to the lysine at position 9 of the peptide using non-enzymatic ubiquitination approach.²⁰ To direct the ubiquitination reaction to K9 exclusively, all other amines of the PTEN peptide were protected: the N-terminal amine was acetylated and the ϵ -amine of K2 was modified with a removable allyloxycarbonyl (Alloc) group. In addition, all amines on Ub were protected with Alloc by reaction with N-(allyloxycarbonyloxy) succinimide. Ubiquitin's C-terminus was converted into a reactive thioester by UBE1, followed by chemical protection of all amines with Alloc by reaction with N-(allyloxycarbonyloxy) succinimide. Complete protection of all amines on Ub and the presence of the thioester were confirmed by ESI-MS. Formation of the Ub-PTEN product was monitored by both SDS-PAGE gel and UV illumination. The Alloc protecting groups were subsequently removed as previously described.²⁰

Liquid chromatography-tandem mass spectrometry.—The approach was adapted from methodologies previously described by this laboratory.^{13,14} Briefly, intact proteins were separated using an Ultimate 3000 ultrahigh performance liquid chromatograph (Thermo Fisher, San Jose CA, USA). Approximately 100 ng of rubylated ubiquitin or the

UbK0-UbcH5b product mixture was desalted and concentrated in a PepSwift Monolithic trap (200 $\mu\text{m} \times 5\text{ mm}$) and subsequently separated on a ProSwift RP-4H column (100 $\mu\text{m} \times 25\text{ cm}$) (Thermo Fisher) at a flow rate of 1.5 $\mu\text{L}/\text{min}$ using a linear gradient of 5% – 55% solvent B (75% acetonitrile, 25% water and 0.1% formic acid) for 20 min. UbK0-UbcH5b was handled similarly and eluted at a flow rate of 1.0 $\mu\text{L}/\text{min}$ using a linear gradient of 5% – 55% solvent B for 120 min. Solvent A was 97.5% water, 2.5% acetonitrile and 0.1% formic acid. The column oven and auto-sampler temperatures were set to 35°C and 4°C, respectively. Mass spectra were acquired on an orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) in the “intact protein mode” with an ion routing multipole pressure of 3 mtorr. The potential for in-source fragmentation was maintained at 10V. A resolving power of 120,000 was used to acquire both precursor and fragment ions. Automatic Gain Control (AGC) target was defined as 10^6 ions during both precursor and fragmentation ion acquisition.

All MS/MS spectra were produced in data dependent mode, using electron transfer dissociation (ETD) supported by high energy collision induced dissociation (ETHCD) with a 6 msec ETD reaction time and supplemental activation with 10% normalized high energy collisions.

Bioinformatics.—The software ProSightPD 1.1 (integrated in Proteome Discoverer 2.1) was used in “Absolute mass search” mode for analysis of the conjugate of Rub1 and UbD77. A database was constructed using ProSight PC 4.0 with a FASTA file containing 7965 yeast protein entries from Uniprot, and sequences of mono-ubiquitin, synthetic Rub1_T72R and synthetic Ub_D77. Database search parameters were set to provide precursor mass tolerance of 9000 Da and fragment mass tolerance of 4 ppm, peptide length over 60 amino acids. From the proteins identified, spectra of the protein conjugate were analyzed using a combination of fragmentation templates provided by ProSight Lite software (Website: prosightlite.northwestern.edu)²¹ and manual interpretation. The strategy for determining the linkage site is discussed in the Results and Discussion section.

Top-down analysis of Ub-UbcH5b and Ub-PTEN protein conjugate samples were also conducted using ProSight Lite software. Product ion spectra were deconvoluted using the Xtract protein in Xcalibur 3.1 Qual Browser (Thermo Fisher). Since ETD supported by HCD produces c/z and b/y ions, the fragmentation method was defined as “ETHcd”. Fragmentation patterns were observed by adding selected masses to certain amino acids in the template sequence. The linkage determination based on different fragmentation patterns is discussed in Results and Discussion section.

Results and Discussion

Characterization of branched proteins in an unknown LC peak requires recognition that the peak corresponds to a branched protein, identification of components present in the branched protein, and, finally, analysis of the branching sites(s) and other modifications by tandem mass spectrometry. One such strategy is illustrated here with rubylated ubiquitin, i.e., ubiquitin covalently modified with Rub1, predicted in this case to be Rub1^{T72R}—⁴⁸Ub^{D77} (see the chain/linkage nomenclature in Nakasone et al²²). An automated search of

the tandem spectrum of this LC eluent against a custom database (see Experimental) identified five candidate proteins (Table 1). In the general case, the presence of ubiquitin, Rub1 (NEDD8) or SUMO among multiple candidates would suggest that the protein may be branched, since they universally modify cellular proteins.

If the experimental context or presence of common modifiers suggests a branched protein, the mass spectrum should be interrogated in that context. In Table 2 the molecular masses that would result from various combinations of the candidate proteins are compared to the observed molecular mass. In this case the combination of Rub1-T72R and Ub-D77 provides the observed mass.

ProSight Lite is then used to evaluate which protein is the anchor and which is the modifier. Figure 1A shows the foundation maps for each partner. In foundation maps the fragment ions in the complete spectrum are mapped to the sequences of both unmodified proteins.^{13,14} Only a series of b/c ions can be mapped onto the modifier-protein whose C-terminal is conjugated, while series of both b/c and y/z ions are usually recognized in the anchor protein. The patterns assigned to fragment ions in Figure 1A support the conclusion that this branched protein is a rubylated ubiquitin and not a ubiquitinated Rub1. In the two partner branched proteins whose fragmentation is discussed here, a gap or window usually occurs between the b/c and y/z series when they are mapped onto the sequence, and this window contains the site of the modification. This window is outlined in green in Figure 1A, and contains K48. Additional template-based interpretation (Fig. 1B) of the spectrum indicates that the site of attachment is at K48 of ubiquitin and also confirms the T72R mutation expected in Rub1. Graphic analysis was applied to isomers with the modification on each lysine in the anchor protein to further test the assignment. Modification at K48 provided the most bond cleavages, including both b/c and y/z series on both the anchor and modifier proteins (Supplemental Figure 1 A-F). Automated scoring is not yet available for branched proteins. The fit between MS/MS spectrum and structure can be manually scored by counting the product ions indicated on the map. Many bond cleavages produce two ionized fragments. In the ideal case every peptide bond should be cleaved in order to provide the location of modified residues with amino acid resolution.

Ubiquitination of enzyme UbcH5b.

The autocatalytic E2 ubiquitin-conjugating enzyme UbcH5b is itself a substrate for ubiquitination and provided the opportunity to characterize isomeric products with unknown sites of modification.²³ The enzyme (tagged with six histidines at the carboxyl terminus) was conjugated by ubiquitin-K0 (UbK0), in which all lysine residues had been mutated to arginines to prevent formation of polyubiquitin chains. Because the synthetic reaction was designed with the E2 enzyme as the anchor protein, UbcH5b-H₆ is used as the template for foundation maps in Figures 2–4. Three isomeric products were identified (A–C) in our LC-MS/MS experiments. Windows in the sequences are visible in all three maps, and the sites of modification or branching were assigned within these gaps after consideration of other chemically feasible possibilities by graphic interpretation. (See Supplemental Figure 2.) The three final structures proposed are presented in Figures 2B, 3B and 4B.

In isomer C, the top-down analysis reveals that Ubi-K0 is covalently attached to Cys 85 (Fig 4B). This is especially interesting, and is independently confirmed, because this conjugation site has previously been identified as the active site in the enzyme.²⁴ *In vitro*, UbcH5b is known to auto-conjugate promiscuously, in a concentration- and time-dependent manner, and other positional conjugates have also been reported.¹⁸

Alloc derivatization in ubiquitinated peptide PTEN.

A final example is summarized in Figure 5, in which a sequence of fragmentation maps demonstrates the capability of top-down analyses to characterize and locate chemical modifications on branched proteins. In this case WT ubiquitin has been attached to a synthetic octadecapeptide PTEN that carries a fluorescent moiety. The conjugation procedure required that some lysines be blocked by reaction with N-(allyloxycarbonyloxy) succinimide. The sequential removal of the resulting Alloc blocking groups was monitored by tandem mass spectrometry. The spectra (Figure 5) confirm that the octadecapeptide PTEN retains the fluorescent probe on the carboxyl terminus, as well as other modifications on both termini. Two Alloc groups were found to remain on the ubiquitin moiety and to be specifically localized. One of several strategies for graphic interpretation of this MS/MS spectrum and location of the Allocs is illustrated in Figure 5. The spectrum is mapped onto the sequence of the ubiquitinated peptide with modifications in place on the peptide (top). The window between the b/c and y/z ion series contains three reaction sites (lysines). Addition of the mass of an Alloc to K29 or K48 redefines the window to eliminate K33. Both b/c and y/z ions are assigned across the ubiquitin chain and location of the Alloc only when it is fully derivatized (bottom map). Successful characterization of chemical and engineered modifications in this and the other examples in this paper indicates that the strategy can also be applied to locate and characterize native post-translational modifications.

Conclusion.

A quasi-interpretive strategy based on tandem mass spectrometry is introduced and demonstrated to work well to recognize and characterize branched systems comprising two proteins. The strategy is facilitated by recognition of a window in the sequence between b/c and y/z ions, and is potentially automatable. The opportunity exists to develop automated scoring for branched proteins, where the presence of multiple proteins requires novel considerations. Cellular proteins are post-translationally modified by single molecules of ubiquitin, SUMO or Rub/NEDD8, by multiple single modifiers, and also by large homo- and mixed-polymeric chains. Previously we have applied an interpretive approach supported by graphic display programs^{21,25} to characterize isomeric trimers¹³ and tetramers¹⁴ of polyubiquitin, and we are currently testing strategies to characterize branched proteins that carry these more complex modifiers. We observe that the limiting factor for extending this research using current tandem instrumentation is no longer resolution, but is adequate activation. We agree with others^{26–28} that cleavage of a high number of the bonds in a protein is required to locate modifications reliably, and that activation becomes more challenging as the mass of the precursor ion increases. These general observations also apply

to analysis of branched proteins, however in our experience no additional instrumental requirements are introduced by the presence of branching itself.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

This research was supported by NIH grants GM021248, GM065334 and OD019938. We thank Dr. Sitara Chauhan for technical assistance as the project began, and Dr. Suresh Kumar (Progenra Inc.) for a sample of P-TEN peptide.

References

1. Skinner D; Kelleher N Nat. Biotech 2015, 33, 717–718.
2. Bondarenko P; Second T; Zabrouskov V; Makarov A; Zhang ZJ Amer.Soc. Mass Spectrom 2009, 20, 1415–1424
3. Bush D; Zang L; Belov A; Ivanov A; Karger B Anal. Chem 2016, 88, 1138–1146. [PubMed: 26641950]
4. Cappadocia L; Lima C Chem Rev 2017 doi:10.1021/acs.chemrev.6b00737
5. Seeler J; Dejean A Oncogene 2001, 20, 7243–7249. [PubMed: 11704852]
6. Mukhopadhyay D and Riezman H Science 2007, 315, 201–205. [PubMed: 17218518]
7. Varshavsky A Annu. Rev. Biochem, 2012, 81, 167–176. [PubMed: 22663079]
8. Kattah M; Malynn B; Ma A J. Mol. Biol 2017, 429, 3471–3485. [PubMed: 29031697]
9. Bazzaro M Oncotarget, 2015, 6, 4159–4165. [PubMed: 25784654]
10. Kim W; Bennett E; Huttlin E; Guo A; Li J; Possemato A; Sowa M; Rad R; Rush J; Comb M; Harper J; Gygi S Mol. Cell 2011, 44, 325–340. [PubMed: 21906983]
11. Morris J; Garvi AJ Mol Biol 2017, 429, 3376–3387
12. Cannon J; Martinez-Fonts K; Robotham S; Matouschek A; Brodbelt J Anal. Chem 2015, 87, 1812–1820 [PubMed: 25559986]
13. Lee A; Geis-Asteggianti L; Dixon E; Kim Y; Kashyap T; Wang Y; Fushman D; Fenselau C. J. Mass Spectrom 2016, 51, 315–321. [PubMed: 27041663]
14. Lee A; Geis-Asteggianti L; Dixon E; Miller M; Wang Y; Fushman D; Fenselau C J. Mass Spectrom 2016, 51, 629–637. [PubMed: 28239975]
15. Strachan J; Roach L; Sokratous K; Tooth D; Long J; Garner T; Searle M; Oldham N; Layfield R; J. Prot. Res 2012, 11, 1969–1980.
16. Pickart C; Raasi S Meth Enzym 2005, 399, 21–36. [PubMed: 16338346]
17. Varadan R; Assfalg M; Fushman D Meth Enzym 2005, 399, 172 – 192.
18. Cannon J, Nakasone M, Fushman D and Fenselau C Anal. Chem 2012, 84, 10121–10128. [PubMed: 23101881]
19. Singh R; Zerath S; Kleifeld O; Scheffner M; Glickman M; Fushman D. Mpl. Cell Proteomics 2012, 11, 1595–1611.
20. Castaneda C.; Liu J; Chaturvedi A; Nowicka U; Cropp T; Fushman D J.Am.Chem.Soc, 2011, 133, 17855–17868. [PubMed: 21962295]
21. Fellers R; Greer J; Early B; Yu X; LeDuc R; Kelleher N; Thomas P. Proteomics, 2015, 15, 1235–1239. [PubMed: 25828799]
22. Nakasone M; Livnat-Levanon N; Glickman M; Cohen R; Fushman D Structure 2013, 21, 727–740 [PubMed: 23562397]
23. Wu M; Hanlon M; Eddins M; Tsui C; Rogers R; Jensen J; J. Matunis M; Weissman A; Wolberger C; Pickart C. EMBO J 2003, 22, 5241–5250. [PubMed: 14517261]
24. Saville M; Sparks A; Xirodimas D; Wardrop J; Stevenson L; Bourdon J; Woods Y; Lane D J. Biol. Chem 2004, 279, 42169–42181.. [PubMed: 15280377]

25. Cai W; Guner H; Gregorich ZR; Chen AJ; Ayaz-Guner S; Peng Y; Valeja SG; Liu X; Ge Y Mol. Cell. Proteomics 2016, 15, 703–714. [PubMed: 26598644]
26. Riley N; Westphall M; Coon J J. Prot. Res 2017, 16, 2653–26.
27. Li H; Sheng Y; McGee W; Cammarata M; Holden D; Loo J Anal. Chem 2017, 89, 2731–2738. [PubMed: 28192979]
28. Holden D; McGee W; Brodbelt J Anal. Chem 2016, 88, 1008–1016. [PubMed: 26633754]

Figure 1A.

foundation maps of the two proteins that constitute a branched protein. Top: Rub1-T72R.

Bottom: Ub-D77. The top structure is designated the modifier protein based on the predominance of b/c ions. The bottom structure is designated the anchor protein because both b/c and y/z ions are detected. A window or gap is indicated in green in Ub-D77 between the series of b/c ions and of y/z ions, which contains a potential site of attachment.

Figure 1B.
Final fragment ion map and structure assigned to the branched protein Rub1(T72R)—⁴⁸Ub(D77).).

Figure 2A.

Foundation map the MS/MS spectrum of product A mapped on UbCH5b-H₆. The window or gap between the series of b/c ions and y/z ions is indicated in green.

Figure 2B.

Final fragment ion map and structure of product A as the branched protein Ub(K0)—¹⁴⁴UbCH5b-H6.

Figure 3A:

Foundation map of Ubch5b mapped with the MS/MS spectrum of product B. The window or gap between the series of b/c ions and y/z ions is indicated in green.

Figure 3B.

Final fragment ion map and structure of product B as the branched protein Ub(K0)—¹⁰¹UbCH5b-H₆.

Figure 4A.
Foundation map of Ubch5b mapped with the MS/MS spectrum of product C.

Figure 4B.
Final fragment ion map and structure of product C as the branched protein Ub(K0)—⁸⁵UbCH5b-H₆

Figure 5.

Sequential fragmentation maps localize two Alloc groups remaining on the ubiquitin modifier protein and confirm modifications on the anchor octadecapeptide. The final structure is shown (bottom) for Ub(K29Alloc, K48Alloc)—⁹PTEN carrying Allocs at K29 and K48 in the ubiquitin chain.

Table 1.

Candidate proteins

Accession#	Description	Matched spectra
Rubl T72R	Rubl T72R imitation	83
Q03919	WT Rubl	18
UbD77	Ub D77 mutation	7
POCH09	Ubiquitin-60S ribosomal protein L40	3
Ub	mono-Ubiquitin	1

Table 2.

Comparison of the observed mass with masses calculated for combinations of linked candidate proteins.

Protein conjugate	Theoretical mass
	(MH+, monoisotopic, Da)
Rub1-UbD77	17345.38
Rub1T72R-Rub1	17299.49
Rub1T72R-UbD77	17286.39
Rub1-Ub	17230.35
Rub1T72R-Ub	17171.36
Ub-Ub	17102.22
Rub1T72R-Rub1T72R	17240.50
Observed Mass (MH+, monoisotopic): 17286.42 Da	