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Characterization of Polyubiquitin Chain Structure by Middle-down Mass Spectrometry

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SUMMARY

Ubiquitin (Ub) is a 76 amino acid polypeptide that modifies a wide range of proteins in the types of monomer or polymers, and functional consequence of ubiquitination is modulated by the length and topologies of polyUb chains. Whereas polyUb chains are usually analyzed by fully trypsin digestion and mass spectrometry (MS), we present here a middle-down strategy to characterize the structure of polyUb chains by high-resolution mass spectrometry (MS). Under optimized condition, native folded polyUb is partially trypsinized exclusively at the R74 residue, generating a large Ub fragment (1–74 residues termed UbR74) and its ubiquitinated form with a diglycine tag (UbR74-GG). The molar ratio between UbR74 and UbR74-GG reflects the length of homogeneous polyUb chains (i.e. 1:1 for dimer, 1:2 for trimer, 1:3 for tetramer, and so on). Moreover, lysine residues in ubiquitin used for chain linkages are detectable by MS/MS and MS/MS/MS of large GG-tagged Ub fragments. The strategy was validated using a number of ubiquitin polymers, including K48-linked human di-Ub, K63-linked human tetra-Ub, as well as His-tagged polyUb chains purified from yeast under native condition. The potential of this strategy to analyze polyUb chains with mixed linkages (e.g. forked chains) is also discussed. Together, this middle-down MS strategy provides a novel complementary method for studying the length and linkages of complex polyUb chain structures.

INTRODUCTION

Ubiquitin, a highly conserved small protein of 76 residues, posttranslationally modify numerous substrates by a cascade of enzymatic reactions through Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3). Protein ubiquitination regulates almost all cellular processes, such as proteasome-mediated degradation^{1, 2}, protein sorting³, inflammation⁴ and DNA-repair⁵. During the enzymatic reactions, the carboxyl group of Gly76 of ubiquitin forms an isopeptide bond with, most typically, the ε-amino group of a lysine residue within substrates. In addition to monoubiquitination, polyUb chains are often assembled by successive attachment of ubiquitin to any of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) of the previously conjugated ubiquitin⁶, providing an opportunity for enhancing structural diversity by the formation of various topologies with various length. Moreover, the N-terminal amine is another possible docking site to form linear polyUb chain⁷. Conversely, the ubiquitin moiety on the substrates can be removed by the action of deubiquitinating enzymes (DUBs)^{8–10}. Compared to approximately 500 human kinase members¹¹, 16 E1s, 53 E2s, 527 E3s, and 184 DUBs have been predicted in the human proteome¹², indicating that the scope of protein ubiquitination is as large as that of phosphorylation events in cells.

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The signaling of protein ubiquitination is thought to rely on the length and linkage of ubiquitin chains^{13, 14}. Conventional K48-linked polyUb chains target substrates to 26S proteasome-mediated degradation¹⁵, and a Ub tetramer is the minimal length for efficient degradation¹⁶. K29-linked chains are also involved in protein degradation in the ubiquitin-fusion degradation (UFD) pathway^{17, 18}. Interestingly, polyUb chains attached to a UFD substrate are heterogeneous polymers that are initiated at K29 but elongated through K48 residue^{18, 19}. In contrast, K63 linkage acts in a number of non-proteolytic processes³, although its proteolytic role in the ubiquitin-proteasome system has not been excluded^{20, 21}. Similarly, monoUb functions as a signal in protein sorting and chromatin remodeling³. It is possible that the formation of distinct polyUb chains provide structural diversities that contribute to the specificity of ubiquitin pathways. Thus, the development of analytic tools for ubiquitinated proteins and polyUb chain structures is highly important to study molecular mechanisms underlying ubiquitin signaling events.

Proteomic analyses of endogenous ubiquitinated species have become possible owing to recent development of mass spectrometry-based sequencing technologies with subfemtomolar sensitivity^{22–24}. In cells, there is often a dearth of native ubiquitinated proteins that have to be pre-enriched by affinity approaches before MS analyses^{13, 25, 26}. The isolated Ub-conjugates are usually analyzed by the bottom-up approach, in which the proteins are digested by trypsin into small peptides and then subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for identification. Moreover, ubiquitination sites in modified proteins are identifiable by mass spectrometry according to the mass shift caused by the modification, as trypsin cleaves original ubiquitin molecule into a dipeptide (GG) remnant with monoisotopic mass of 114.043 Da^{6, 27, 28}. The analysis of GG-modified peptides may be facilitated by chemical derivatization of their multiple N-termini²⁹, and alternative digestion with Glu C was also used for mapping ubiquitination sites³⁰.

This strategy also enables direct determination of lysine residues involved in polyUb assembly. Global analysis of Ub-conjugates in yeast has revealed startling complexity of polyUb chains: all seven lysine residues can be used for the formation of Ub-Ub linkages⁶. Interestingly, forked polyUb chains have also been detected in cells, because one Ub tryptic fragment has both K29 and K33 modified by the GG tag, termed as K29+K33-linked chains⁶. More recently, other forked polyUb chains (K6+K11, K11+K27, and K27+K29) have also been reported in vivo and/or in vitro^{31, 32}. The forked chains were proposed to be poor substrates of proteasome-associated deubiquitinating enzymes, and thus to delay protein degradation³².

Mass spectrometry has been further used for quantifying the abundance of different lysine linkages of polyUb chains on substrates. Using synthetic isotope-labeled GG-peptides as internal standards and the selected reaction monitoring (SRM) strategy, the polyUb linkages on numerous substrates such as cyclin B1²¹, the EGF receptor³³, and yeast total Ub-conjugates³⁴, were analyzed in quantitative details. Recently, top-down mass spectrometry emerges as a technology to analyze intact proteins without proteolysis³⁵, and has been successfully used to map comprehensive posttranslational modifications on histones³⁶. Alternatively, a “middle-down” technology analyzes large protein fragments after limited proteolysis, taking the advantages of both bottom-up and top-down methods^{37, 38}. Here we report a middle-down approach in which polyUb chains are only cleaved once at R74, producing an almost intact Ub of 74 residues with or without GG tags. Both the length and topologies of polyUb chains can be simply measured by this novel strategy without the requirement of isotope-labeled internal standards. The advantages and limitations of the middle-down strategy are also discussed.

EXPERIMENTAL SECTION

Purification of ubiquitin polymers under native condition

Native human Ub derivatives (monomer, dimer and tetramer) were purchased (Boston Biochem Inc, Cambridge, MA). His-tagged Ub polymers were isolated from yeast by nickel affinity chromatography³⁹ and glycerol gradient centrifugation. Yeast strain SUB592 was grown in YPD medium at 30°C to 0.2 A600, and then added with CuCl₂ to 0.1 mM to induce Ub expression under CUP1 promoter. The cells were harvested at 0.7 A600 and lysed using glass beads in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 0.005% SDS, 0.1% glycerol, 5 mM imidazole, 2 mM β-mecaptoethanol, and 10 mM iodoacetamide). The cell lysates were clarified by centrifugation at 200,000 g for 30 min and loaded on a nickel column. The column was sequentially washed with 200 bed volume (V) of washing buffer (the same as the lysis buffer) and 10 V of 5 mM imidazole in elution buffer (50 mM NH₄HCO₃, 2 mM β-mecaptoethanol, and 10 mM iodoacetamide) to remove salt and SDS. Ub-conjugates were step-eluted with 8 V of elution buffer containing titrated imidazole (40 and 500 mM). The eluents were analyzed by SDS gel electrophoresis and silver-staining. Most of free Ub monomer was eluted by 40 mM imidazole.

Free His-tagged Ub polymers eluted by 500 mM imidazole were further fractionated on a glycerol-density gradient centrifugation, in which 50-μl nickel column eluent was loaded onto a 2-ml 10–40% (v/v) linear glycerol gradient made of the elution buffer. Centrifugation was carried out at 200,000 g for 8 hr at 4°C in a Beckman TLS 55 rotor. Fractions (100 μl each) were collected from the top of the tube, and analyzed by SDS-PAGE and silver staining.

Ubiquitin digestion under native condition

Digestion reactions (~10 μl of total volume) usually contained 5 μg/ml ubiquitin and 5 μg/ml of trypsin (Promega, Madison, WI) in a buffer of 50 mM ammonium bicarbonate (pH 7.8) at 37°C or at room temperature. During the optimization step, bovine serum albumin (BSA) was added in as an extra substrate to adjust enzyme-to-substrate ratio. After incubation, the reactions were terminated by the addition of formic acid to 1%, and then subjected to LC-MS analysis.

Reverse phase LC-MS

The full-length or partially digested ubiquitin samples were loaded on a reverse phase column (100 μm i.d. × 12 cm) packed with fused silica C₈ resin (magic 200 Å of 5 μm, Michrom Bioresources, Auburn, CA), washed and eluted during a 20–40% gradient (buffer A: 0.4% acetic acid, 0.005% heptafluorobutyric acid, 5% acetonitrile; buffer B: 0.4% acetic acid, 0.005% heptafluorobutyric acid, 95% acetonitrile; flow rate: ~0.3 μl/min) as previously described⁴⁰. Eluted ubiquitin molecules were ionized and detected by an LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA). The LTQ was used to acquire spectra of MS (30,000 AGC target, 50 ms maximum ion time), MS/MS (8 *m/z* isolation width, 50% collision energy, 10,000 AGC target, 300 ms maximum ion time), and MS/MS/MS (3 *m/z* isolation width, 30% collision energy, 10,000 AGC target, 300 ms maximum ion time). The Orbitrap was used to collect MS scans (1,000,000 AGC target, 1,000 ms maximum ion time) and MS/MS scans (8 *m/z* isolation width, 50% collision energy, 1,000,000 AGC target, 2,000 ms maximum ion time). No MS/MS/MS scans were acquired by the Orbitrap due to weak signals.

Assignment of ion peaks in the tandem MS spectra

The ion peaks in the spectra were manually assigned according to the charge state and derived mass. The collision-induced dissociation (CID) in LTQ primarily generates *b* and *y* ions. As the MS/MS spectra collected by LTQ and Orbitrap were highly similar, the high-resolution

data from Orbitrap allowed direct determination of charge states, and thus simplified the process of peak assignment.

Quantitative analysis of polyUb length

The length of polyubiquitin was calculated based on the abundance ratio of UbR74 and UbR74-GG (Fig. 1). As each Ub derivative is detected in multiple charge states in MS scans, we compared the ion currents from 7+ charges to 12+ charges. Both ion currents in MS scans were quantified using Xcalibur software (Thermo Finnigan, San Jose, CA).

The UbR74-GG/UbR74 ratio for non-forked polyUb chains with different length was computed according to their relative abundance. Assuming that in a mixture the levels of monomer, dimer and trimer were m%, d% and t%, respectively, the final UbR74-GG/UbR74 ratio is equal to $(d\% * 1/2 + t\% * 2/3) / (m\% + d\% * 1/2 + t\% * 1/3)$. For purified yeast His-myc-Ub sample, the amount of monomer, dimer and trimer was estimated to be ~70%, ~25% and ~5%, respectively, according to the stained gel. Therefore, the UbR74-GG/UbR74 ratio was computed to be $(25\% * 1/2 + 5\% * 2/3) / (70\% + 25\% * 1/2 + 5\% * 1/3)$, which is equal to 0.19.

RESULTS AND DISCUSSION

A middle-down strategy to analyzing polyubiquitin structures

As all seven lysine residues and the N-terminal amine in ubiquitin are used for polyUb chain assembly, the chain structure may be much more diverse than previously anticipated, with the complexity rising exponentially with the length of the chain. For example, Ub dimer would display eight forms (Fig. 1B), whereas Ub trimer would exhibit two classes of configurations: branched chains with 64 forms (i.e. 8×8 possibilities) (Fig. 1C and 1D) and forked chains with 28 forms (i.e. $8 \times 7/2$ possibilities) (Fig. 1E). Traditional bottom-up mass spectrometric analysis requires complete trypsin digestion of polyUb chains, and some structure information is lost during the digestion. For instance, in a tri-Ub sample with both K29 and K48 linkages, it is difficult to know if two Ub molecules are conjugated to different Ub molecules (Fig. 1D), or to the same Ub molecule as in a forked chain (Fig. 1E). To address this problem, we partially trypsinized ubiquitin polymers under native condition, in which trypsin cleaves ubiquitin at only one site (R74) in the C-terminal tail, as other tryptic sites in the sequence are generally inaccessible owing to compact ubiquitin folding⁴¹. Thus, it would be simple to monitor how many GG-tags co-modify a single Ub molecule by mass shift. In the case of a K48-linked Ub dimer (Fig. 1B), it is excised into equal amount of two large peptides, UbR74 (amino acid M1 to R74) and UbR74-1GG-K48 (UbR74 with one GG tag at the K48 site). A Ub trimer with mixed K29 and K48 linkages (Fig. 1D) is digested into three large Ub fragments of equal abundance (UbR74, UbR74-1GG-K29 and UbR74-1GG-K48). In contrast, a forked trimer with K29 and K48 linkages (Fig. 1E) produces two large fragments (UbR74:UbR74-2GG-K29K48) with 2:1 ratio.

To validate the specificity of trypsin digestion of native ubiquitin, we used the LC-MS approach to analyze human ubiquitin before and after partial digestion. As expected, intact ubiquitin was readily ionized in a range of charge states (6+ to 12+, Fig. 2A) and the charge states were derived from the mass of ubiquitin and from high-resolution isotopic distribution (Fig. 2B). Because digestion efficiency is dependent on the concentration of trypsin, enzyme-to-substrate ratio and incubation time, we fixed the enzyme concentration at 5 µg/ml and then altered the trypsin-to-substrate ratios in the reaction. After overnight incubation, the ubiquitin sample was completely cleaved on R74 site at 1:1 and 1:3 trypsin-to-substrate ratios but not at 1:30 ratio (Fig. 2C). A time-course experiment indicated that, with 1:3 trypsin-to-substrate ratio, ~50% human ubiquitin was excised in 20 minutes, and the digestion was essentially complete in one hour. More importantly, extended incubation to as long as 9 hours generated no significant

amount of any other large ubiquitin fragments (Fig. 2D), supporting the high digestion specificity of native ubiquitin under this condition.

Measurement of polyubiquitin length by the middle-down strategy

This middle-down strategy is also useful to measure the length of polyUb chains through the ratio between UbR74 and UbR74-GG fragments. Whereas UbR74 is generated from monoUb or end-cap ubiquitin (i.e. the distal end ubiquitin moiety in polyUb polymers), UbR74-GG is produced from the other ubiquitin domains in the polymers (Fig. 1). In the case of homogenous polyUb chains, the molar ratio of UbR74 and UbR74-1GG is anticipated to be 1:1 for dimer, 1:2 for trimer, 1:3 for tetramer, and so on.

To confirm the predicted results on polymer length, we first analyzed a sample of K48-linked human ubiquitin dimer under the optimized condition above. The UbR74 and UbR74-1GG were eluted with identical retention time during reverse phase liquid chromatography (Fig. 3A), and were detected with almost equal signal intensities in MS survey scans (Fig. 3B). The results indicated that a small GG tag on UbR74 had essentially no effect on its elution or ionization in the LC-MS, and therefore their signals could be directly quantified and compared in MS scans. Moreover, the analysis was also performed using a K63-linked human ubiquitin tetramer as a substrate, and the UbR74-1GG/UbR74 ratio was approximately equal to 3.0 (Fig. 3C). As the UbR74 and UbR74-1GG fragments can be quantified by any of detected charge states, we examined the data from 7+ to 12+ charge states and found the UbR74-1GG/UbR74 ratios were 0.99 ± 0.02 and 2.94 ± 0.07 for the dimer and tetramer, respectively (Fig. 3D), highly consistent with the expected results.

In bottom-up proteomics, the length of polyUb chains could be calculated upon absolute measurement of GG-tagged peptides and total ubiquitin²¹. The quantification relies on a series of stable-isotope labeled ubiquitin peptides as internal standards, and the accuracy is influenced by the purity and quantification of all internal standards. In contrast, the middle-down strategy dramatically simplifies quantification steps without the requirement of stable-isotope labeled peptides, and thus improves the precision of quantification. For the tested dimer and tetramer, relative standard errors of the mean are approximately 2%, and the measured UbR74-1GG/UbR74 ratios differ only 1–2% compared to the anticipated results.

Identification of modified Lys residues in polyUb chains by tandem mass spectrometry

To identify precise residue(s) modified by the ubiquitin GG tag, we optimized fragmentation condition for ubiquitin and found a set of product ions that were unique to different linkages. Several precursor ions (10+, 9+, 8+ and 7+) of UbR74-1GG-K63 were subjected to collision-induced dissociation (CID), and the MS/MS spectrum of 9+ charged ion displayed the most informative pattern (data not shown). The collision energy was also adjusted and fixed at 50% (data not shown). Under the dissociation condition, the MS/MS spectra of human UbR74, UbR74-1GG-K48 and UbR74-1GG-K63 were acquired by low- and high-resolution mass spectrometers (i.e. LTQ and Orbitrap). The patterns of MS/MS spectra from the same precursor ions were similar (data not shown), and the high-resolution data allowed the determination of charge state of product ions, which facilitated the process of assigning product ions to individual peaks (Fig. 4). Comparison of these assigned spectra revealed main breakage sites during the fragmentation (Fig. 4) and derived a series of six products ions (y_{12}^{2+} , y_{16}^{3+} , y_{22}^{3+} , y_{38}^{4+} , y_{42}^{4+} , and y_{56}^{6+}) that were capable of distinguishing polyUb linkages in MS/MS spectra (Table 1). As expected, the two strongest product ions (y_{38}^{4+} and y_{56}^{6+}) were generated from proline-mediated fragmentation in ubiquitin. In addition, we examined the MS/MS/MS spectra of a strong MS/MS ion (i.e. y_{38}^{4+} ion) and found that they were also useful to define ubiquitin-modified residues (Fig. 5).

To differentiate individual ubiquitination sites, the GG-tagged R74 ubiquitin must be dissociated between adjacent lysine residues during fragmentation. In this analysis, the fragmentation sites of CID are able to assign all but two neighboring linkages (K27 and K29) (Fig. 4, Table 1). In addition to the method of CID that has preference for certain residual positions (e.g. proline)⁴², the development of electron capture dissociation (ECD)⁴³ and electron transfer dissociation (ETD)⁴⁴ may provide a better approach to break multiple-charged precursor Ub ions and to produce a more informative pattern with a richer ladder of productions.

Analysis of His-tagged polyUb chains isolated from yeast

To apply the middle-down strategy to real biological samples, we analyzed His-myc-tagged polyUb chains that were purified by native nickel affinity chromatography and glycerol gradient centrifugation from yeast (*S. cerevisiae*). On a silver-stained SDS gel, the sample exhibited three major protein bands corresponding to Ub monomer (~70%), dimer (~25%) and trimer (~5%) (Fig. 6A). During LC-MS analysis of the sample, the Ub monomer was shown as the predominant peak (data not shown). However, examination of the peak revealed the co-elution of at least three main forms of yeast ubiquitin: unmodified (11+, 1012.2 *m/z*), N-terminal acetylated (11+, 1016.1 *m/z*), and N-terminal Met cleaved (11+, 1003.1 *m/z*) (Fig. 6B). It is well known that the initiator Met from nascent polypeptides is commonly acetylated by N-terminal acetyltransferases⁴⁵ or removed by methionine aminopeptidase *in vivo*⁴⁶. After digestion under native condition, the tagged ubiquitin molecules were cleaved into UbR74 with and without a single GG tag (Fig. 6B). The deconvoluted mass spectra before and after limited proteolysis were also shown (Fig. 6C). The mass of yeast UbR74 indicated that the full-length His-myc-Ub was cleaved only at two sites: Arg74 and another lysine residue in the myc tag (Fig. 6D). The cleavage in the tag eliminated the problem of heterogeneity caused by N-terminal modifications. No double-GG tagged UbR74 was found in the experiment, suggesting that the level of forked Ub trimer in the sample is under the limitation of detection sensitivity. The UbR74-1GG/UbR74 ratio was measured to be approximately 0.20, consistent with the predicted value of 0.19 from the measurement on the SDS gel (see **Experimental Section**).

The polyUb linkages were further characterized by tandem MS analysis of UbR74-1GG (Fig. 6D, Table 1). In the spectra, the presence of 1142.0 *m/z* ion but not 1113.5 *m/z* ion indicated that the y_{38}^{4+} ion was produced from K48 or K63-linked polyUb. Consistently, the y_{42}^{4+} ion displayed a value of 1248.9 *m/z* instead of 1220.4 *m/z*. Furthermore, a set of K48-linkage-specific ions were detected, including y_{12}^{2+} (733.4 *m/z*), y_{16}^{3+} (662.1 *m/z*), and y_{22}^{3+} (872.0 *m/z*), whereas only one K63-linkage-specific ion (y_{22}^{3+} , 910.0 *m/z*) was present at a much weaker level. It should be mentioned that one of characteristic production (y_{56}^{6+}) cannot be used for analyzing yeast ubiquitin, because three Ub residues in yeast (Ser19, Asp24 and Ser28) is different from that in human (Pro19, Glu24 and Ala28), and especially the change of 19th proline residue results in the loss of a main breaking site for generating this product ion. Together, the data strongly suggested the yeast sample of free Ub polymers were mainly linked through K48 site and some through K63 residue.

CONCLUSIONS

Combining partially tryptic digestion and LC-MS, we developed a strategy to analyze the length and linkages of polyUb chains. We optimized the digestion condition to achieve exclusive cleavage at R74 residue in both human and yeast ubiquitin, and demonstrated the feasibility of this approach using multiple commercially available substrates and a real biological sample of tagged yeast ubiquitin. This middle-down strategy provides a novel method for comprehensive analyses of polyUb chains from various biological sources.

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Abbreviations

Ub	ubiquitin
MS	mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry

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Figure 1. A strategy to analyze polyUb chains using middle-down mass spectrometry

Folded ubiquitin is digested only at a single R74 site by trypsin, leading to the generation of an almost full-length ubiquitin (UbR74) and linkage-specific GG-tagged UbR74, such as UbR74-1GG-K48 (one GG tag through K48 residue). Listed are the structures of several ubiquitin forms before and after partial trypsin digestion, including monomer (A), dimer (B), homogeneous and heterogeneous trimers (C and D, respectively), and forked trimer (E).

Figure 2. Optimization of partial digestion condition for native ubiquitin

(A) Human ubiquitin monomer was detected as multiple charge states during LC-MS. The most abundant isotopic ions for all charge states (m/z) are indicated. (B) The distribution of isotopic ions with ten positive charges measured by high resolution Orbitrap mass spectrometry. (C) The effect of trypsin/substrate ratio on the digestion efficiency of ubiquitin. Human ubiquitin was incubated with trypsin at 5 $\mu\text{g}/\text{ml}$ overnight at 37 $^{\circ}\text{C}$. Bovine serum albumin was added in to adjust trypsin/substrate ratio as indicated. The digested products were analyzed by LC-MS to detect the UbR74 fragment. (D) The kinetics of ubiquitin digestion. Human ubiquitin was digested with trypsin at 5 $\mu\text{g}/\text{ml}$ and enzyme-to-substrate ratio of 1:3. An aliquot was taken at 20 min, 1 hr, 3 hr and 9 hr for mass spectrometric analyses.

Figure 3. Determination of the length of polyUb polymers by the middle-down strategy
 (A) K48-linked Ub dimers were digested into equal molar amount of UbR74 and UbR74-1GG-K48. The co-elution of UbR74 fragment (mass range: 845.7–846.7 m/z) and its GG-tagged form (mass range: 857.1–858.1 m/z) during C_8 reverse phase chromatography. (B) The measured ratio of the two Ub fragments by LC-MS. (C) K63-linked Ub tetramers were trypsinized into UbR74 and UbR74-1GG-K63 with expected 1:3 ratio. (D) The quantification of two Ub fragments based on different charge state, ranging from 7+ to 13+. The expected ratios are shown in dashed lines. When summarizing the ratio for Ub tetramer, the data point of charge state +13 was removed as an outlier due to weak signal.

Figure 4. Determination of ubiquitination sites in the large Ub fragments by MS/MS spectra

The CID spectra of human UbR74 and two GG-modified forms were acquired by high-resolution Orbitrap. The sequence of the Ub fragments and their major dissociation sites are shown, with all potential ubiquitination sites underlined. Major product ions in the MS/MS spectra are assigned. y_{56}^{6+} , for example, indicates the y_{56} ion with six positive charges. Main characteristic product ions of the Ub fragments are highlighted in grey.

Figure 5. Analysis of ubiquitin-modified sites in the large Ub fragments by MS/MS/MS spectra
 The CID spectra for the y_{38}^{4+} ions of different Ub derivatives were acquired by the LTQ mass spectrometer. Major product ions in the spectra are assigned, and those specific to the Ub linkages are highlighted.

Figure 6. Middle-down MS analysis of tagged Ub polymers in yeast

(A) Recombinant yeast ubiquitin was fused with His and myc tags at the N-terminus (His-myc-yUb). Native free polyUb chains were purified from yeast by Nickel affinity chromatography and glycerol gradient centrifugation, which was analyzed on a silver-stained gel. (B) MS analysis of the His-myc-yUb sample before and after partially tryptic digestion. (C) The deconvoluted mass spectra (1+ charge) of five major Ub ions. (D) The MS/MS spectra of yUbR74-1GG acquired using LTQ in profile mode. Some regions of the spectra were zoomed for the display: 650–850 m/z , 10 fold; 850–1015 m/z , 5 fold; 1015–1145 m/z and 1155–1400 m/z , 2 fold. Linkage-specific product ions were highlighted.

Table 1
Identification of Ub linkages in human or yeast polyUb by tandem mass spectrometry*

	precursor (9+) ^{\$}	MS2						MS3 of y ₃₈ ⁴⁺ (1142.0 m/z)		
		y ₁₂ ²⁺	y ₁₆ ³⁺	y ₂₂ ³⁺	y ₃₈ ⁴⁺	y ₄₂ ⁴⁺	y ₅₆ ^{6+[#]}	y ₁₆ ²⁺	y ₂₂ ³⁺	y ₂₂ ³⁺
UbR74	939.9	733.4	662.1	872.0	1113.5	1220.4	1070.5			
UbR74-IGG-K6	952.5	733.4	662.1	872.0	1113.5	1220.4	1070.5			
UbR74-IGG-K11	952.5	733.4	662.1	872.0	1113.5	1220.4	1070.5			
UbR74-IGG-K27	952.5	733.4	662.1	872.0	1113.5	1220.4	1089.6			
UbR74-IGG-K29	952.5	733.4	662.1	872.0	1113.5	1220.4	1089.6			
UbR74-IGG-K33	952.5	733.4	662.1	872.0	1113.5	1248.9	1089.6			
UbR74-IGG-K48	952.5	733.4	662.1	872.0	1142.0	1248.9	1089.6	992.7	872.0	
UbR74-IGG-K63	952.5	790.4	700.1	910.0	1142.0	1248.9	1089.6	1049.7	910.0	

* The values listed are average m/z.
\$ The m/z values of +9 charged yeast tagged Ub fragments are slightly different: yUbR74, 1034.3; UbR74-IGG, 1047.0.
Yeast ubiquitin does not produce intensive y56 ion due to Pro-to-Ser amino acid change.