Biochemical and Biophysical Research Communications 436 (2013) 223–229

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The parallel reaction monitoring method contributes to a highly sensitive polyubiquitin chain quantification

Hikaru Tsuchiya, Keiji Tanaka *, Yasushi Saeki *

Laboratory of Protein Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

ARTICLE INFO

Article history:
Received 14 May 2013
Available online 31 May 2013

Keywords:
Ubiquitin
Proteasome
Proteolysis
Yeast
Mass spectrometry

ABSTRACT

Ubiquitylation is an essential posttranslational protein modification that is implicated in a diverse array of cellular functions. Although cells contain eight structurally distinct types of polyubiquitin chains, detailed function of several chain types including K29-linked chains has remained largely unclear. Current mass spectrometry (MS)-based quantification methods are highly inefficient for low abundant atypical chains, such as K29- and M1-linked chains, in complex mixtures that typically contain highly abundant proteins. In this study, we applied parallel reaction monitoring (PRM), a quantitative, high-resolution MS method, to quantify ubiquitin chains. The ubiquitin PRM method allows us to quantify 100 attomole amounts of all possible ubiquitin chains in cell extracts. Furthermore, we quantified ubiquitylation levels of ubiquitin-proline-β-galactosidase (Ub-P-βgal), a historically known model substrate of the ubiquitin fusion degradation (UFD) pathway. In wild-type cells, Ub-P-βgal is modified with ubiquitin chains consisting of 21% K29- and 78% K48-linked chains. In contrast, K29-linked chains are not detected in *UFD4* knockout cells, suggesting that Ufd4 assembles the K29-linked ubiquitin chain(s) on Ub-P-βgal *in vivo*. Thus, the ubiquitin PRM is a novel, useful, quantitative method for analyzing the highly complicated ubiquitin system.

1. Introduction

Protein ubiquitylation is an essential post-translational modification responsible for targeted protein degradation, cellular signaling activation, DNA damage response, and intracellular protein trafficking, and protein quality control [1–3]. Ubiquitylation is achieved by the concerted action of activating (E1), conjugating (E2) and ligating (E3) enzymes, some of which possess elongating activities that support the generation of polyubiquitin chains. Proteins can be modified through the conjugation of monoubiquitin or polyubiquitin chains of variable lengths on any of the seven Lys (K) residues (K6, K11, K27, K29, K33, K48 or K63) or the amino-terminal Met (M1) of the ubiquitin monomer. Ubiquitin chains can thus be connected by at least eight different homotypic linkages, as well as by a range of heterogeneous chains (forked or mixed) [3]. Occasionally, efficient protein polyubiquitylation requires additional conjugation factor, so-called E4 enzyme, that supports the elongation of ubiquitin chains [4,5]. The topologically distinct polyubiquitin chains result in the different biological consequences. For example, K48-linked chains function as a targeting signal for proteasomal destruction, whereas K63-linked chains are used in DNA repair and the trafficking of membrane proteins. However, the functions of atypical chains linked through M1, K6, K11, K27, K29, or K33 are only beginning to emerge, and the roles of mixed or branched chains are unknown [6]. Ubiquitin receptors, many of which display specificity or preference for ubiquitin chain types or length, play a key role in decoding the signals embedded in the structure of ubiquitin chains [7].

To understand the biological significance of different ubiquitin chain topologies, it is essential to study the dynamics of protein ubiquitylation in endogenous settings [8]. Currently, a MS-based strategy is a most powerful and sensitive method to analyze endogenous ubiquitylation events in cells [6]. The ubiquitin-absolute quantification (AQUA) method was established to quantify individual ubiquitylated protein substrates [9] or affinity-captured ubiquitin chain mixtures from cells [10]. The method involves using isotopically labeled internal standard ubiquitin peptides. Peptides in the sample are generated by digestion of polyubiquitin chains with trypsin. Both unlabeled sample peptides and isotopically labeled internal standards can be analyzed by selected reaction monitoring (SRM) on a triple quadrupole (QQQ) MS or by

* Corresponding authors. Address: Laboratory of protein metabolism, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Fax: +81 3 5316 3152.

E-mail addresses: tanaka-kj@igakuken.or.jp (K. Tanaka), saeki-ys@igakuken.or.jp (Y. Saeki).

0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.05.080

narrow window extracted ion chromatograms on a high resolution Orbitrap MS [9,11,12]. Nevertheless, in the complex mixtures that typically contain highly abundant species of ubiquitin chains (e.g., K48 chain), MS-based quantification of the low abundant atypical chains, such as K29-linked and M1-linked chains, is still limited [12].

Recently, a new targeted proteomics method using a next generation, quadrupole-equipped Orbitrap instrument (Q Exactve), called parallel reaction monitoring (PRM), has been proposed [13,14]. The Q Exactive is like a QQQ except that the third quadrupole is replaced with a high-resolution, high-mass accuracy Orbitrap mass analyzer [13]. Whereas in SRM, all transitions are monitored one at a time, PRM allows parallel detection of all transitions in a single analysis. PRM yielded quantitative data over a wider dynamic range than SRM in the complex biological matrix due to PRM's high selectivity in the mass-to-charge domain [14,15].

In this study, we applied the PRM method to quantify polyubiquitin chains in biological matrices and succeeded in the quantification of all the linkages from 50 amol to 100 fmol. Using the ubiquitin PRM, we dissected the ubiquitylation of ubiquitin-proline-β-galactosidase (Ub-P-βgal), a substrate of the ubiquitin fusion degradation (UFD) pathway [16], and unambiguously identified that K29-linked ubiquitin chains are attached to Ub-P-βgal *in vivo*. Thus, the ubiquitin PRM is a highly sensitive and reliable method to analyze the complicated ubiquitin system.

2. Materials and methods

2.1. Yeast strains

Saccharomyces cerevisiae strains used are listed in Supplementary Table 1.

2.2. Immunoprecipitation and Western blotting

Yeast cultures were grown in SC medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, 2% glucose, and 20 mg/l uracil) to an OD_{600} of 0.8 at 28 °C. Cells (200 OD_{600}) were harvested and lysed using glass beads in 400 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 10 μM MG132, 10 mM iodoacetamide, and 1 X complete protease inhibitor cocktail (Roche, EDTA free)). After addition of 600 μl of lysis buffer containing 0.2% TX-100, the extracts were centrifuged at 20,000 x *g* for 20 min. The supernatants were recovered and immunoprecipitated with 11 μg anti-βgal monoclonal antibody (Promega) preadsorbed with 1.5 mg of Dynabeads Protein G (Life Technologies) for 30 min at 4 °C. After washing three times with lysis buffer containing 0.2% TX-100, the proteins were eluted with 1 X NuPAGE LDS sample buffer for 10 min at 70 °C. The proteins were separated by SDS–PAGE on 4–12% NuPAGE Bis-Tris gels with MES buffer (Life Technologies). For mass spectrometric analysis, the gels were stained with Bio-Safe Coomassie (BioRad).

For Western blotting, the proteins were blotted onto a PVDF membrane using the NuPAGE immunoblotting system (Life Technologies). The membranes were probed with anti-βgal or anti-ubiquitin monoclonal antibody (P4D1, HRP conjugated, Santa Cruz).

2.3. Preparation of internal AQUA peptides

Synthetic isotopically labeled peptides were purchased from Sigma (Custom AQUA peptides) or Thermo Fisher Scientific (AQUA Ultimate grade) as listed in Table 1. The peptides were dissolved or diluted in 10% acetonitrile (ACN)/0.1% formic acid (FA) and mixed at a final concentration of 200 fmol/μl. The peptide mixtures were divided to small aliquots and stocked at −80 °C. We noted that the use of Protein Lobind tube (Eppendorf) significantly improved the stabilities of the ubiquitin peptides.

2.4. Trypsin digestion and sample preparations

In-gel trypsin digestion was carried out as described in a protocol by Phu et al. [12] with modifications. After the gels were extensively washed with Milli-Q water (Millipore), gels were excised, diced into 1 mm^3 pieces, and destained by 1 ml of 50 mM ammonium bicarbonate (AMBC)/30% ACN with agitation for 1 h, then the gels were further washed by 1 ml of 50 mM AMBC/50% ACN for 1 h. Finally, 100% ACN wash was performed to ensure complete gel dehydration. Trypsinization solution (20 ng/μl) was prepared on ice by dilution of modified sequencing grade trypsin (Promega) using pre-chilled 50 mM AMBC/5% ACN pH 8.0. Trypsin solution was subsequently added to gel pieces at approximately equivalent

Table 1
List of the ubiquitin peptides used in PRM analysis.

Abbreviation	Peptide sequence	Precursor m/z (charge state)	Product ions for PRM	RT min
TITLE	TITLEVESSDTIDNVLK	882.449 (+2)	y_3^+, y_5^+, y_6^+, y_8^+, y_{10}^+, y_{11}^+, y_{12}^+, y_{14}^+	53.76
	TITLEVESSDTIDNV[HeavyV]K	885.456 (+2)		
EST	ESTLHLVLR	534.314 (+2)	y_3^+, y_4^+, y_5^+, y_6^+, y_7^+, y_8^+	39.37
	ESTLHLVL[HeavyL]R	537.823 (+2)		
K6 (ox)	M[Oxid]QIFVK[di-GlyGly]TLTGK	465.927 (+3)	y_3^+, y_4^+, y_5^+, y_6^+, y_7^+	38.62
	M[Oxid]QIFVK[di-GlyGly]TL[HeavyL]TGK	468.266 (+3)		
K11	TLTGK[di-GlyGly]TITLEVESSDTIDNVK	793.415 (+3)	y_5^+, y_7^+, y_8^+, y_9^+, y_{10}^+, y_{11}^+,	57.54
	TLTGK[di-GlyGly]TITLEVESSDTIDNV[HeavyV]K	795.419 (+3)		
K27	TITLEVESSDTIDNVK[di-GlyGly]SK	698.358 (+3)	y_5^+, y_6^+, y_7^+, y_8^+, y_9^+, y_{10}^+, y_{11}^+	47.85
	TITLEVESSDTIDNV[HeavyV]K[di-GlyGly]SK	700.363 (+3)		
K29	SK[di-GlyGly]IQDK	416.730 (+2)	y_2^+, y_3^+, y_4^+, y_5^+	21.44
	SK[di-GlyGly]I[HeavyI]QDK	420.238 (+2)		
K33	IQDK[di-GlyGly]EGIPPDQQR	546.613 (+3)	y_3^+, y_4^+, y_5^+, y_6^+	22.42
	IQDK[di-GlyGly]EGIPP[HeavyP]DQQR	548.618 (+3)		
K48	LIFAGK[di-GlyGly]QLEDGR	487.6 (+3)	y_3^+, y_5^+, y_6^+, y_8^+, y_9^+	40.52
	LIFAGK[di-GlyGly]QL[HeavyL]EDGR	489.939 (+3)		
K63	TLSDYNIQK[di-GlyGly]ESTLHLVLR	561.805 (+4) 748.738 (+3)	y_5^+, y_9^+, y_{10}^+	57.64
	TLSDYNIQK[di-GlyGly]ESTLHLVL[HeavyL]R	563.559 (+4) 751.077 (+3)		
M1 (ox)	GGM[Oxid]QIFVK	448.239 (+2)	y_2^+, y_3^+, y_4^+, y_5^+, y_6^+	28.53
	GGM[Oxid]QIFV[HeavyV]K	451.246 (+2)		

The oxidation state of Met containing peptides is denoted by (ox) for sulfoxide. In the peptide sequences, isotopically labeled amino acids (Heavy: $^{13}C^{15}N$), ubiquitylation site (di-GlyGly), and the Met oxidation (Oxid) are in the blankets. For each peptide, the optimal precursor ion(s), product ions, and retention times are shown.

volume and incubated on ice for 30 min. Another small volume of trypsin solution was added to gel samples and incubated at 37 °C for overnight. Digests were quenched and extracted by addition of 50 µl of 50% ACN/0.1% trifluoroacetic acid (TFA) for 1 h by shaking. The digested peptides were recovered into fresh Protein Lobind tubes and additional extraction step was performed with 70% ACN/0.1% TFA for 30 min. The extracted peptides were combined and concentrated to 15 µl by a speed-vac. The concentrated peptides were mixed with 50 fmol of ubiquitin AQUA peptides, 2.5 µl of 1% TFA, and 2.5 µl of 0.05% H_2O_2 in a total volume of 25 µl and were incubated at 4 °C for overnight to oxidize Met [12]. Note that we used 0.1% TFA instead of FA for sample preparation to improve the peak shape of K29-linked ubiquitin peptide for reliable quantification.

2.5. Liquid chromatography and mass spectrometry

A nanoflow UHPLC instrument (Easy nLC 1000, Thermo Fisher Scientific) was coupled on-line to a Q Exactive MS (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Because K29-linked ubiquitin peptide is very hydrophilic, the samples were directly loaded onto a C18 analytical column (Reprosil-Pur 3 µm, 75 µm id x 12 cm packed tip column, Nikkyo Technos Co. Ltd.). For ionization, 1.8 kV of liquid junction voltage and 250 °C capillary temperature were used. Reversed phase chromatography was performed using the Thermo EASY-nLC 1000 with a binary buffer system consisting of 0.1% FA (solvent A) and 100% ACN/0.1% FA (solvent B) with a flow rate of 300 nl/min. For shotgun analysis, the peptides were separated using a 12 min 2-step

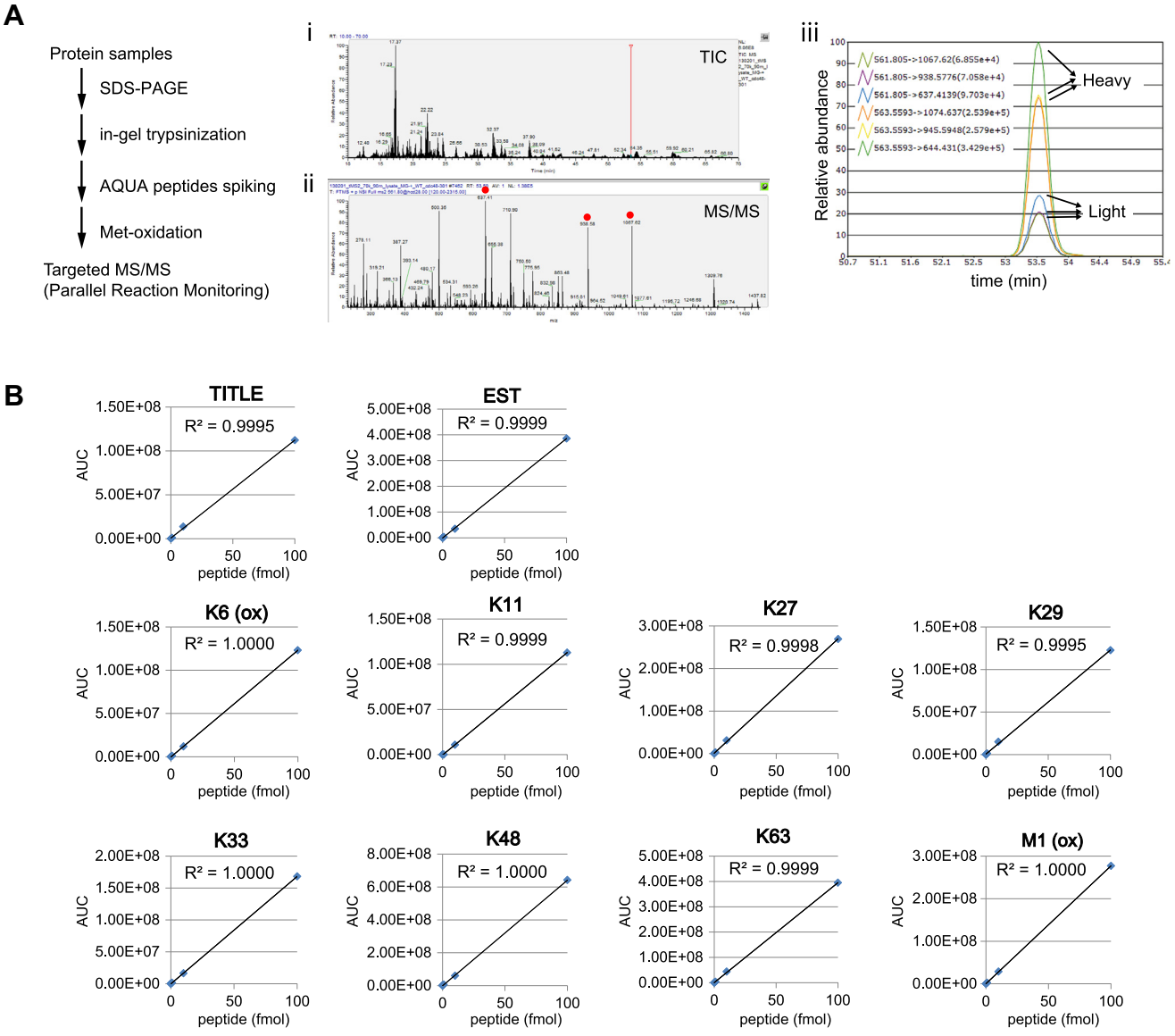

Fig. 1. Ubiquitin quantification by parallel reaction monitoring analysis. (A) Workflow for the absolute quantification of ubiquitin peptides by parallel reaction monitoring (PRM). After proteins are separated by SDS–PAGE, the gel regions of interest were excised from the gel and subjected to in-gel trypsin digestion. Ubiquitin AQUA peptides were spiked in the extracted peptides and treated with hydrogen peroxide for oxidation of Met-containing peptides. The samples were analyzed by a Quadrupole-Orbitrap MS in targeted MS/MS mode. (i) Total ion chromatogram (TIC) of PRM analysis of wild-type yeast lysate with 10 fmol of Ub AQUA peptides. (ii) MS/MS spectra of endogenous K63 peptide (+4, m/z = 561.805). The product ions used in the PRM analysis were indicated by red circles (y_5^+, y_9^+, and y_{10}^+). (iii) The chromatogram of selected product ions of both peptides. Identical elution times were observed between the light (endogenous) and heavy (internal control) counterparts. (B) Linearity range of the dilution curves established by PRM analysis from the dilution series of five internal standards (50 amol–100 fmol) prepared in 500 ng of *E. coli* digest. For all ubiquitin peptides except TITLE (See Table 1 for their abbreviations), a limit of detection of 50 amol was determined (Supplementary Fig. 1).

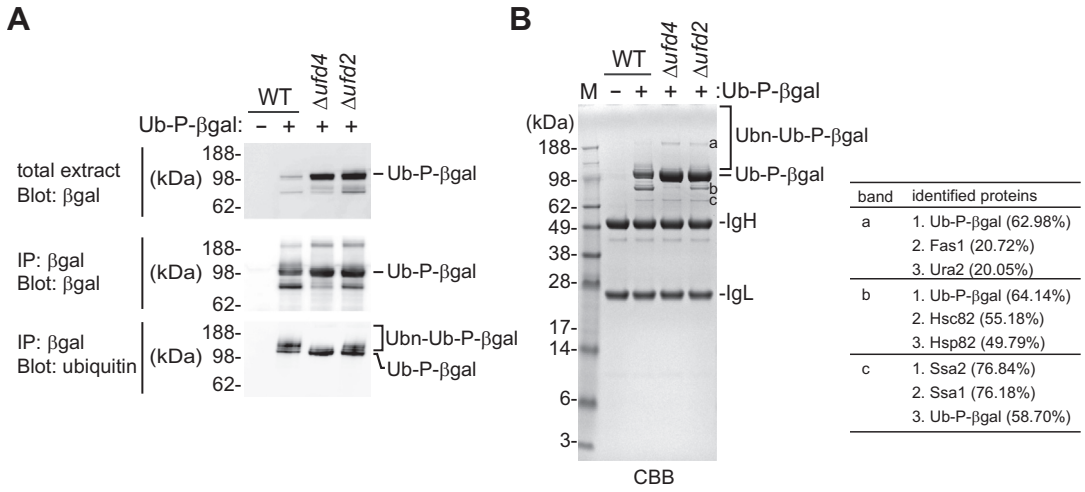

Fig. 2. Expression levels of Ub-P-βgal in mutant cells. (A) Steady state levels of Ub-P-βgal in BY4741 (WT: wild-type), E3 knockout (Δ*ufd4*), and E4 knockout (Δ*ufd2*) cells. Total cell extracts of the cells stably expressing Ub-P-βgal (WT, YHT20; Δ*ufd4*, YHT24; Δ*ufd2*, YHT26) were analyzed by Western-blotting with anti-βgal (upper). The cells were lysed and Ub-P-βgal was purified on anti-βgal beads and analyzed by Western-blotting with anti-βgal or anti-ubiquitin antibodies (middle and bottom). Polyubiquitinated (Ubn) species of Ub-P-βgal are indicated by a half square bracket. (B) SDS–PAGE analysis of the purified Ub-P-βgal. Purified proteins in (A) were subjected to SDS–PAGE followed by Coomassie Brilliant Blue-staining. The protein bands corresponding to Ub-P-βgal, the IgG heavy chain (IgH), and the IgG light chain (IgL) are indicated. Unassigned protein bands (a–c) were analyzed by LC-MS/MS. Identified proteins with high score are listed in right table and the sequence coverage of the identified proteins is indicated in parenthesis. The gel regions analyzed by ubiquitin-PRM in Fig. 3 are indicated by Ubn-Ub-P-βgal.

gradient (0–35% for 10 min and 35–100% for 2 min of solvent B). The Q Exactive was operated in the data dependent mode, using Xcalibur software, with survey scans acquired at a resolution of 70,000 at m/z 200. Up to the top 10 most abundant isotope patterns with charge 2 ~ 5 from the survey scan were selected with an isolation window of 1.2 Th and fragmented by HCD with normalized collision energies of 28. The maximum ion injection times for the survey scan and the MS/MS scans were 60 ms, respectively and the ion target values were set to 3e6 and 1e6, respectively. Selected sequenced ions were dynamically excluded for 90 sec. For parallel reaction monitoring (PRM) quantification, the peptides were separated using an 80 min 3-step gradient (0–10% in 5 min, 10–25% in 70 min, and 25–80% in 5 min of solvent B). The Q Exactive was operated in the targeted MS/MS mode, using Xcalibur software, with time-scheduled acquisition of the 11 pairs of isotopically labeled peptides/endogenous peptides in +/− 6 min retention time windows (list in Table 1) with 2 Th individual isolation windows. The target ion values was 1e6, and maximum ion fill times was 200 ms. Fragmentation was performed with normalized collision energy of 27 and MS/MS scans were acquired with a at a resolution of 70,000 at m/z 200. We used a hydrophilic MS vial (ProteoSave, AMR Inc.) to avoid nonspecific peptide adsorption. To characterize the linear range of ubiquitin quantification, we diluted the ubiquitin AQUA peptide mixtures in 0.1% TFA, 0.05% H_2O_2, and 100 ng/μl and *Escherichia coli* matrix (MassPREP, Waters) and incubated at 4 °C for overnight. Then, 5 μl of samples corresponding to 0.05, 0.1, 1, 10, and 100 fmol of ubiquitin peptides with 500 ng *E. coli* matrix were subjected to the PRM analysis.

2.6. Protein identification and quantification from MS data

For protein identification, MS spectra were analyzed using Protein Discoverer software version 1.3 (Thermo Fisher Scientific). The fragmentation spectra were searched against UniProt database with the amino acid sequence information of Ub-P-βgal using the SEQUEST search engine. The precursor and fragment mass tolerances set to 10 ppm and 20 ppm, respectively. Oxidation of Met and protein N-terminal acetylation were chosen as variable modifications for database searching. Peptide identification was filtered at 1% false discovery rate. For ubiquitin quantification, raw files were processed by PinPoint software version 1.3 (Thermo Fisher Scientific). Ion chromatograms were extracted with a mass tolerance of 5 ppm for PRM data. The area under the curve (AUC) of selected fragment ion (PRM analysis, list of selected transitions in Table 1) was calculated for each sampling point based on the co-elution profiles of differently labeled peptides. The AUCs of each individual PRM transition were then summed to obtain AUCs at the peptide level [15]. The linearity range of measurement of each peptide was defined as the range of spiked peptide amounts for which the relative difference between deduced values of peptide area ratio from regression analysis and experimentally determined ones was lower than 15%. The composite MS/MS spectrum of each targeted peptide was reconstructed from the AUCs of the three to eight transitions selected for the peptide. The identity of targeted peptides was verified by the correlation between the composite MS/MS spectrum and the MS/MS spectral library entry, assessed by the calculation of a Bonferroni corrected *p*-value of correlation [17]. The peptide that gave a *p*-value less than 0.05 was considered to be confidently confirmed by the library match.

3. Results and discussion

3.1. Ubiquitin quantification by Q-Q-Orbitrap mass spectrometry

Based on the previous MS method to use synthetic di-Gly-tagged peptides as internal standards for quantifying ubiquitin linkages [9,12], we established a more precise method to measure ubiquitin chains by using a quadrupole-equipped Orbitrap in targeted MS/MS mode (Fig. 1). Protein samples were subjected to SDS–PAGE followed by in-gel trypsinization according to the previous study [12]. To minimalize sample loss and experimental errors, the extracted peptides were directly subjected to LC-MS/MS analysis. After spiking with ubiquitin AQUA peptides, the samples were treated with 0.05% H_2O_2 in 0.1% TFA at 4 °C for 12 h to oxidize the Met-containing peptides, M1- and K6-linkages, according to a previous study [12]. Under the condition, we confirmed that >95% of the Met-peptides were converted to the oxidized form (data not shown). During instrumental setup, we found that K29-linked

peptide was not efficiently trapped to a pre-column and was eluted as a broad peak in the nanoflow UHPLC system probably due to the hydrophilicity. To overcome this problem, we used one-column setting that peptide samples were directly loaded onto the analytical column. We also noticed that 0.1% TFA instead of 0.5% FA for sample preparation dramatically improves the peak shape of K29-linked ubiquitin peptide (see Supplementary Figs. 1 and 2). To minimalize nonspecific adsorption to tubes or vials, especially occurred in K63-linked peptide, we used a hydrophilic tube and MS vial throughout the analysis.

We first analyzed wild-type yeast lysate by PRM (Fig. 1A and Table 1). The lysate (10 μg) was separated by SDS–PAGE and the gel region above 62 kDa was subjected in-gel trypsinization. The extracted peptides were spiked with 10 fmol of the ubiquitin AQUA peptides and analyzed by Q Exactive in targeted MS/MS mode. As expected, total ion chromatogram (TIC) was relatively complex even in the targeted MS/MS mode. Ion chromatograms were extracted with a mass tolerance of 5 ppm from at least 3 fragment ions (Fig. 1A, ii for K63-linked peptide). The area under the curve (AUC) of selected fragment ions was calculated for each sampling point based on the co-elution profiles of differently labeled peptides (Fig. 1A, ii for K63-linked peptide). We optimized PRM transition, the charge state of precursor ions and well resolved fragment ions, by manually or using PinPoint software [15]. The AUCs of each individual PRM transition were then summed to obtain AUCs at peptide level. Using the ubiquitin PRM, all the ubiquitin peptides except M1- and K27-linkages were unambiguously detected from yeast extract (data not shown).

To establish standard curves of ubiquitin peptides in biological samples, the ubiquitin AQUA peptide mixtures were serially diluted in *E. coli* matrix and subjected to the PRM analysis (Fig. 1B). Because two peptides, TITLE and EST (see Table 1), detected easily even in complex samples, total ubiquitin levels were assessed by these ubiquitin peptides (data not shown). We could detect

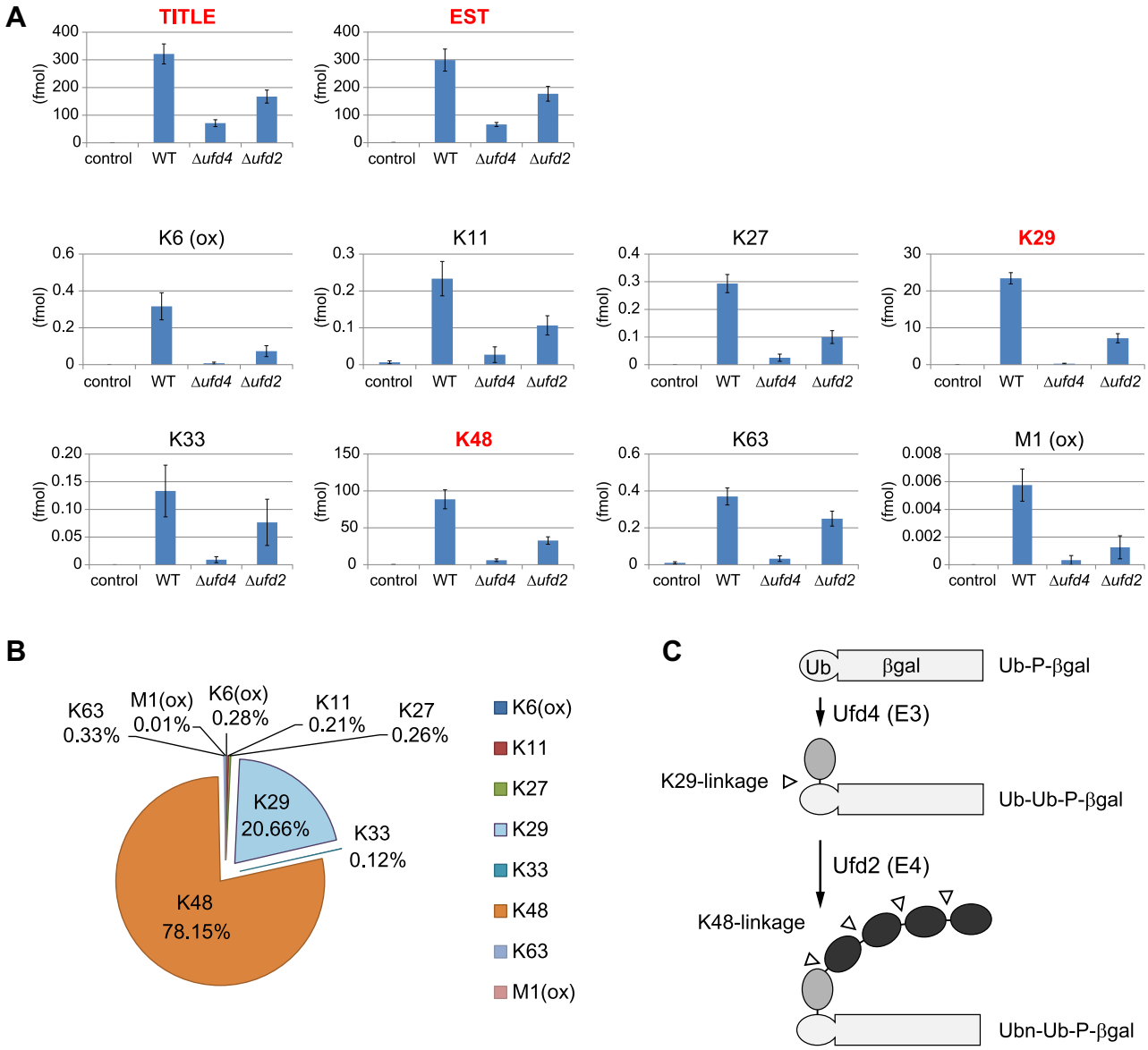

Fig. 3. Ufd4 attaches K29-linked ubiquitin chains to Ub-P-βgal *in vivo*. (A) Summary of ubiquitylation levels on Ub-P-βgal by ubiquitin-PRM. The samples from BY4741, YHT20, YHT24, and YHT26 cells were simply denoted as control, WT, Δ*ufd4*, Δ*ufd2*, respectively. Total ubiquitin were quantified by two ubiquitin peptides, TITLE and EST. The ubiquitin peptides with high abundance (>1 fmol) are indicated in red text. (mean ± s.e.m.; $n = 3$ biological replicates). (B) Pie chart of the abundance of ubiquitin linkages. (C) A possible model of the UFD pathway. Ufd4 recognizes UFD substrate and attaches K29-linked chain. Subsequently, Ufd2 elongates the ubiquitin chains via K48-lined chains.

all the ubiquitin peptides from a limit of detection between 50–100 amol in the presence of biological matrices. Standard curves were obtained with $R^2 > 0.999$ (Fig. 1B and Supplementary Fig. 1). By the ubiquitin PRM, quantitation can be performed across at least 3 orders of magnitude with all the ubiquitin peptides being quantifiable from 0.1 fmol to >100 fmol. From our knowledge, the ubiquitin PRM method is highest sensitive method to quantify ubiquitins to date.

3.2. Quantification of the ubiquitylation levels of a ubiquitin fusion degradation substrate

The UFD pathway is responsible for ubiquitylation of the fusion proteins that bear a "non-removable" N-terminal Ub moiety, which serves as a degron [16]. The UFD pathway is conserved in both yeast and mammalian cells [18]. Previous work has identified the key ubiquitylating enzymes of the UFD pathway in *S. cerevisiae*, including E2 Ubc4 and Ubc5, the HECT E3 Ufd4, and the Ub-chain elongation factor Ufd2 (called E4) [4,16,19–21]. Ufd4 is a 167 kDa HECT-domain E3 ligase that was shown to recognize the N-terminal ubiquitin degron via its N-terminal domain and ubiquitylate the UFD substrates [4,16,22]. In addition, Ufd4 targets the E2 Ubc7 [23] and the DNA alkyltransferase Mgt1 (O-6-methylguanine-DNA methyltransferase) for proteasomal degradation [24]. Interestingly, it was recently shown to interact with the N-end rule E3 Ubr1, leading to enhanced activities of both the N-end rule and the UFD pathways [25]. Because the ubiquitylation of the UFD substrate requires the presence of K29 of the ubiquitin moiety of the substrate [4,16], Ufd4 is likely to assemble K29-linked ubiquitin chains on the ubiquitin-moiety of the UFD substrate. However, recent studies have suggested that Ufd4 might assemble K48-linked polyubiquitin chains [25,26]. We previously investigated the concept that Ufd4 and Ufd2 assemble different chain types, i.e., Ufd4 and Ufd2 assemble K29- and K48-linked ubiquitin chains, respectively. Although our *in vitro* study clearly suggested that Ufd2 assembles K48-linked chains onto the Ufd4-preassembled chains, we failed to detect putative K29-linked chains by Ufd4 [20]. Because one of the utility of developed ubiquitin PRM method is to dissect such multi-step ubiquitylation by sequentially acting enzymes, we decided to analyze the ubiquitylation of the UFD substrate.

To investigate the UFD substrate, we generated the yeast cells that chromosomally expressing Ub-P-βgal. As expected, strong accumulations of Ub-P-βgal were detected in both Δ*ufd4* and Δ*ufd2* cells, but the ubiquitylation levels were different among the wild-type, Δ*ufd4* and Δ*ufd2* cells (Fig. 2A). Consistent with previous results, the polyubiquitylation of Ub-P-βgal was easily detected in the wild-type and Δ*ufd2* cells while weak ubiquitylation was observed in the Δ*ufd4* cells (Fig. 2A, bottom). The immunoprecipitated Ub-P-βgal were further analyzed by SDS–PAGE (Fig. 2B). Minor protein bands that associated with Ub-P-βgal were identified as dimerized form of the substrate itself and protein chaperones, Hsp70 and Hsp90 family (Fig. 2B, right table), indicating that high purity of the immunoprecipitated Ub-P-βgal sufficient for the ubiquitin quantification.

The gel region of polyubiquitylated Ub-P-βgal was excised and subjected to the ubiquitin PRM analysis (Fig. 3). In wild-type cells, Ub-P-βgal is modified with 310 fmol of total ubiquitin, assessed by two peptides, TITLE and EST. Of these, K29-linked peptide (∼23 fmol) and K48-linked peptides (∼89 fmol) were prominent than other linkages (Fig. 3A). Because the ubiquitin-moiety of Ub-P-βgal is involved in the calculation of total ubiquitin, the percentage of the ubiquitin linkages could be underscored (∼30% of total ubiquitin). Nevertheless, the PRM analysis clearly suggested that the polyubiquitin chains assembled on Ub-P-βgal consist of ∼21% of K29- and ∼78% of K48-linked chains, respectively (Fig. 3B). In contrast to the wild-type cells, total ubiquitylation levels were decreased to ∼69 fmol and both K29- and K48-linked chains were markedly reduced in the Δ*ufd4* cells (Fig. 3A). In the Δ*ufd2* cells, the total ubiquitin level as well as both chains was decreased by half, suggesting that compromised chain elongation results in the substrate stabilization and that other factor(s) working downstream of Ufd4 might exist in the cells. Combined with the previous result that Ufd2 assembles K48-linked chains *in vitro* [20], these results suggested that Ufd4 is a unique ubiquitin ligase that can assemble K29-linked ubiquitin chains in yeast. Interestingly, other ubiquitin linkages including K6-, K11-, K27-, K33- and K63-linkages were unambiguously identified in the ubiquitin chains, although the sum of their percentage is only 1.2% of total linkages, suggesting that such chains were mistakenly incorporated into the UFD substrate, probably by Ufd4 *in vivo*.

In conclusion, we established the ubiquitin PRM method to quantify the ubiquitylation levels with highest sensitivity even in the complex biological samples. Using the ubiquitin PRM, we revealed that Ufd4 assembles K29-linked ubiquitin chains on the UFD substrate *in vivo*.

Acknowledgments

We thank Dr. F. Ohtake and Mr. H. Yoshihara for comments on the manuscript. We are grateful to Dr. A. Varshavsky for the pUb23-P-βgal vector and Dr. I. Sadowski and Euroscarf staff for pIS373 plasmid. This work was supported by JSPS KAKENHI Grant Numbers 22687009, 24112008 (to Y.S.), and 21000012 (to K.T.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.080.

References

[1] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[2] D. Komander, M. Rape, The ubiquitin code, Annu. Rev. Biochem. 81 (2012) 203–229.
[3] C. Grabbe, K. Husnjak, I. Dikic, The spatial and temporal organization of ubiquitin networks, Nat. Rev. Mol. Cell Biol. 12 (2011) 295–307.
[4] M. Koegl, T. Hoppe, S. Schlenker, H.D. Ulrich, T.U. Mayer, S. Jentsch, A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly, Cell 96 (1999) 635–644.
[5] B. Crosas, J. Hanna, D.S. Kirkpatrick, D.P. Zhang, Y. Tone, N.A. Hathaway, C. Buecker, D.S. Leggett, M. Schmidt, R.W. King, S.P. Gygi, D. Finley, Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities, Cell 127 (2006) 1401–1413.
[6] A. Williamson, A. Werner, M. Rape, The Colossus of ubiquitylation: decrypting a cellular code, Mol. Cell 49 (2013) 591–600.
[7] K. Husnjak, I. Dikic, Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions, Annu. Rev. Biochem. 81 (2012) 291–322.
[8] F. Ikeda, N. Crosetto, I. Dikic, What determines the specificity and outcomes of ubiquitin signaling?, Cell 143 (2010) 677–681
[9] D.S. Kirkpatrick, N.A. Hathaway, J. Hanna, S. Elsasser, J. Rush, D. Finley, R.W. King, S.P. Gygi, Quantitative analysis of *in vitro* ubiquitinated cyclin B1 reveals complex chain topology, Nat. Cell Biol. 8 (2006) 700–710.
[10] E.J. Bennett, T.A. Shaler, B. Woodman, K.Y. Ryu, T.S. Zaitseva, C.H. Becker, G.P. Bates, H. Schulman, R.R. Kopito, Global changes to the ubiquitin system in Huntington's disease, Nature 448 (2007) 704–708.
[11] P. Xu, D.M. Duong, N.T. Seyfried, D. Cheng, Y. Xie, J. Robert, J. Rush, M. Hochstrasser, D. Finley, J. Peng, Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation, Cell 137 (2009) 133–145.
[12] L. Phu, A. Izrael-Tomasevic, M.L. Matsumoto, D. Bustos, J.N. Dynek, A.V. Fedorova, C.E. Bakalarski, D. Arnott, K. Deshayes, V.M. Dixit, R.F. Kelley, D. Vucic, D.S. Kirkpatrick, Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals, Mol. Cell. Proteomics 10 (2011). M110 003756.
[13] A. Michalski, E. Damoc, J.P. Hauschild, O. Lange, A. Wieghaus, A. Makarov, N. Nagaraj, J. Cox, M. Mann, S. Horning, Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer, Mol. Cell. Proteomics 10 (2011). M111 011015.

[14] A.C. Peterson, J.D. Russell, D.J. Bailey, M.S. Westphall, J.J. Coon, Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics, Mol. Cell. Proteomics 11 (2012) 1475–1488.
[15] S. Gallien, E. Duriez, K. Demeure, B. Domon, Selectivity of LC-MS/MS analysis: Implication for proteomics experiments, J. Proteomics 81 (2013) 148–158.
[16] E.S. Johnson, P.C. Ma, I.M. Ota, A. Varshavsky, A proteolytic pathway that recognizes ubiquitin as a degradation signal, J. Biol. Chem. 270 (1995) 17442–17456.
[17] R. Kiyonami, A. Schoen, A. Prakash, S. Peterman, V. Zabrouskov, P. Picotti, R. Aebersold, A. Huhmer, B. Domon, Increased selectivity, analytical precision, and throughput in targeted proteomics, Mol. Cell. Proteomics 10 (2011). M110 002931.
[18] N.P. Dantuma, K. Lindsten, R. Glas, M. Jellne, M.G. Masucci, Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells, Nat. Biotechnol. 18 (2000) 538–543.
[19] Y. Xie, A. Varshavsky, UFD4 lacking the proteasome-binding region catalyses ubiquitination but is impaired in proteolysis, Nat. Cell Biol. 4 (2002) 1003–1007.
[20] Y. Saeki, Y. Tayama, A. Toh-e, H. Yokosawa, Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage, Biochem. Biophys. Res. Commun. 320 (2004) 840–845.
[21] T. Hoppe, Multiubiquitylation by E4 enzymes:'one size' doesn't fit all, Trends Biochem. Sci. 30 (2005) 183–187.
[22] D. Ju, X. Wang, H. Xu, Y. Xie, The armadillo repeats of the Ufd4 ubiquitin ligase recognize ubiquitin-fusion proteins, FEBS Lett. 581 (2007) 265–270.
[23] T. Ravid, M. Hochstrasser, Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue, Nat. Cell Biol. 9 (2007) 422–427.
[24] C.S. Hwang, A. Shemorry, A. Varshavsky, Two proteolytic pathways regulate DNA repair by cotargeting the Mgt1 alkylguanine transferase, Proc. Natl. Acad. Sci. USA 106 (2009) 2142–2147.
[25] C.S. Hwang, A. Shemorry, D. Auerbach, A. Varshavsky, The N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases, Nat. Cell Biol. 12 (2010) 1177–1185.
[26] O. Braten, N. Shabek, Y. Kravtsova-Ivantsiv, A. Ciechanover, Generation of free ubiquitin chains is up-regulated in stress and facilitated by the HECT domain ubiquitin ligases UFD4 and HUL5, Biochem. J. 444 (2012) 611–617.