

Supplementary Information

Semisynthetic UbH2A Reveals Different Activities of Deubiquitinases and Inhibitory Effects of H2A K119 Ubiquitination on H3K36 Methylation in Mononucleosomes

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1. General methods

Chemical reagents were purchased from Alfa Aesar, Sigma-Aldrich, Merck, Fisher Scientific, and Acros Organics. T4 DNA ligase and restriction enzymes were obtained from New England BioLabs. Primer synthesis and gene sequencing were performed by Integrated DNA Technologies and 1st BASE in Singapore, respectively. Gene mutagenesis was achieved using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). SUMO-USP 21 was from Lifesensors. BAP1 was purchased from Boston Biochem, Cambridge, MA. PCR purification and gel extraction kits were purchased from Axygen. NSD2 and SETD2 were purchased from Sigma-Aldrich. Antibodies for ubH2A and monoubiquitin were purchased from Millipore and Enzo Life Sciences, respectively. Analytical HPLC analyses were performed using a Shimadzu HPLC system equipped with a Jupiter C18 (5 μ m, 4.6 x 250 mm) or a Vydac C8 (5 μ m, 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. Semi-preparative HPLC were performed using a semi-preparative HPLC column (Jupiter C18, 5 μ m, 10 x 250 mm) on a Shimadzu system with a flow rate of 2.5 mL/min. Preparative HPLC analyses were performed on Waters 600 systems equipped with a Vydac C4 column (10 μ m, 22 x 250 mm) with a flow rate of 10 mL/min. Detection was done with a UV-VIS-detector at 220 nm. The buffer system for all the analyses was buffer A □□H₂O (containing 0.045% TFA) and buffer B □□90% acetonitrile (ACN) in H₂O (containing 0.045% TFA). Peptide and protein masses were measured using a Thermo FINNIGAN LCQ Deca XP MAX equipped with ESI ion source. The raw spectra were deconvoluted with MagTran software. NMR studies were performed on a 400 MHz Bruker NMR equipment with CDCl₃ as the solvent.

2. Chemical synthesis of azidonorleucine and N-(N'-(4-(2,4-dimethoxyphenyl)thiazolidinyl))glyciny succinimide (9)

Azidonorleucine and compound **9** were produced according to our reported protocols.^[1]

3. Construction of H2AK119ANL (H2A 1) expression vector

Plasmid encoding the wild type *Xenopus laevis* H2A is a gift from Prof Lars Nordenskiöld in SBS, NTU, Singapore. Single mutation (K119M) was introduced into H2A using QuickChange site-directed mutagenesis with primers pQLN-H2A-K119M-F and pQLN-H2A-K119M-R. Then the mutated H2A-K119M sequence was amplified by PCR with primers pQLN-H2A-EZ-F and pQLN-H2A-EZ-R. The PCR product was purified and ligated with our previously reported pQLN plasmid,^[1] according to the manufactures' instruction (CloneEZ PCR Cloning Kit, Genescript, USA). The generated pQLN-H2A-K119M was used for expression of H2A containing ANL.

4. Construction of MetRS-NLL plasmid

The triple mutant MetRS-NLL was derived from our previous plasmid pRep4-MetRS-L13A^[1] by multiple mutations using QuickChange site-directed mutagenesis with three pairs of primers (MetRS_H301L_F and MetRS_H301L_R, MetRS-L13N-F and MetRS-L13N-R, MetRS_Y260L_F and MetRS_Y260L_R). The correct sequence was confirmed by gene sequencing.

5. Expression and Purification of H2A 1

The plasmids pRep4-MetRS-NLL and pQLN-H2A-K119M were co-transformed into Methionine auxotrophic *E. coli* strain CAG18491 (Yale CGSC). Single colony was picked up and inoculated in 100 ml M9 minimal media supplemented with 20 common amino acids with a concentration of about 40 mg/L each and two antibiotics (ampicillin 100mg/L and kanamycin 25 mg/L) at 37 °C. The next morning, the 100ml overnight cultures of *E. coli* was inoculated in 4 L of the M9 media mentioned above. When OD₆₀₀ reached 0.9, the cells were harvested by centrifugation and washed twice with M9 media without amino acids. The cells were then resuspended in 4 L of fresh M9 media supplied with 19 amino acids (40 mg/L) without methionine and ANL (1 mM) and the two antibiotics mentioned. The cells were grown at 37°C. After 30 min, isopropyl β-D-1- thiogalactopyranoside (IPTG, 1 mM) was added to the media to induce protein expression. After 4 h, the cells were harvested by centrifugation. The cell pellet was resuspended in 300 mL lysis buffer (50 mM Tris-HCl, pH 7.6). The cells were lysed through sonication. The cell debris was removed by centrifugation at 20,000 g for 30 min at 4 °C. The pellet was washed with wash buffer containing 1% Triton X-100 twice and one time without Triton X-100. Then 1 ml of DMSO was added to the pellet and the pellet was stirred for 30 min. After that 10 ml unfolding buffer (6 M Guanidinium HCl, 10 mM Tris-HCl, 10 mM DTT, pH 7.5) was added. After centrifugation, the supernatant was loaded to a 26/60 Sephacryl S-200 column and purified with gel filtration buffer (7 M de-ionized Urea, 20 mM sodium acetate, 1 M sodium chloride, 5 mM beta-mercaptoethanol, 0.5 M EDTA, pH 5.2). After FPLC purification, the protein was purified again by C4 semi-prep HPLC followed by lyophilization.

6. Expression of other recombinant histones (H2A, H2B, H3 and H4)

Recombinant histones H2A, H2B, H3 and H4 were expressed as previously described.^[2] In short, *E. coli* BL21(DE3)pLysS cells were transfected with histone expression plasmids, grown in 2 L LB medium at 37°C until OD₆₀₀=0.6 and protein expression was induced by addition of 0.5 mM IPTG for 3 h. Cells were harvested by centrifugation at 7000 g and lysed by 5 x passage through a French Press. The insoluble pellets were washed

twice with 40 mL wash buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) containing 1% triton X-100, and once with wash buffer. The pellets were extracted with extraction buffer (7 M guanidinium hydrochloride, 20 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) and purified over a Sephacryl S-200 column (1 L bed volume). After dialysis of the histone-containing fractions into water containing 1 mM DTT, the fractions were lyophilized. The histones were then further purified using preparative C-4 RP-HPLC and a gradient of 30-70% HPLC solvent B.

7. Histone octamer (HO) refolding and purification

Purified histones (H2A or ubH2A, H2B, H3 and H4) were dissolved in unfolding buffer individually (10 mM Tris-HCl pH 7.5, 7M Guanidinium HCl and 10 mM DTT). The histones were mixed in an accurate stoichiometry with 25% more H2A or ubH2A and H2B than H3 and H4. Unfolding buffer was added to adjust the final protein concentration to 1 mg/ml. The solution was dialyzed against 3 × 600 ml refolding buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA and 10 mM 2-mercaptoethanol) for 24 hours. After dialyzing, the HO solution was centrifuged twice at 18000 g for 10 min at 20°C. The solution was concentrated at 4000 rpm using vivaspin MWCO 10 kDa centrifugal concentrator (Sartorius) to about 10 mg/ml final concentration.

A Superdex 200 10/30 GL column (GE healthcare) was equilibrated in refolding buffer at 4°C and the concentrated HO solution was loaded to the column. The HO was eluted at 0.4 ml/min in refolding buffer. Collected fractions were analyzed on 15% SDS-PAGE gels. Fractions containing pure HO were pooled together and concentrated down to 3 mg/ml. Equal volume of pure glycerol were added, and mixed gently. The final concentration was measured by spectrophotometer ($OD_{276}=0.45$ equals to 1 mg/ml HO) and HO solution was stored at -20°C.

8. Widom 601-145 DNA Plasmid extraction

The Widom 601-145 DNA plasmid (pUC57) was a generous gift from Curt Alexander Davey (NTU, SBS). It contains 8 repeats Widom 601-145 DNA and was transformed to *E. coli* TOP 10 competent cells for amplification. One colony was picked from 100 µg/ml ampicillin LB agar plate and inoculated in 5 ml 2 × TY medium (all of following medium contained 100 µg/ml ampicillin). Cells were incubated at 37°C, 200 rpm. After 4 hours, 5 ml culture was transferred to 100 ml 2 × TY medium and incubated for 2 hours, and finally into 8 L 2 × TY medium for 16 hours incubation.

After 16 hours, cells were spun down at 4000 rpm, 4°C for 20 min. Cell

pellets were resuspended in 400 ml Alkaline Lysis solution I (25 mM Tris-HCl pH 8.0, 50 mM glucose and 10 mM EDTA). After fully resuspension, 800 ml Alkaline Lysis solution II (0.2 M NaOH and 1% (w/v) SDS) was added and cells were vigorously mixed for 20 min. Alkaline Lysis solution III (1400 ml) (4 M potassium acetate and 2 M acetic acid) was added and mixed gently for 20 min. The suspension was centrifuged at 10000 g for 20 min at 4°C and the supernatant was then filtered through a stack of sterile gauze pads. Isopropanol was added to the solution with the volume of 52% (v/v). After mixing, the solution was left at room temperature for 30 min. Precipitates were collected by centrifuging at 16000 g for 30 min at room temperature. Pellets were resuspended in 40 ml TE (10 mM Tris-HCl pH 8.0 and 50 mM EDTA) buffer. The solution was treated with 240 µl RNase A (10 mg/ml) and incubated at 37°C, 80 rpm overnight.

The next day, the solution was centrifuged at 10000 g, 4°C for 30 min. Phenol (20 ml) was added to the supernatant and centrifuged at 27000 g for 20 min at 20°C. The upper aqueous phase was taken out and 20 ml CIA solution (chloroform-isoamyl alcohol, 24:1) was added. The solution was centrifuged at 12000 g for 10 min at 20°C. The phenol/CIA treatment was repeated one more time to completely remove RNase A from the solution. To separate plasmid DNA from RNA fragments, 0.2 volume of 4 M NaCl and 0.4 volume of 40% PEG6000 were added to the solution to precipitate plasmids. The solution was mixed at 37°C for 5 min and incubated on ice for 30 min. Precipitated DNA was collected by centrifugation at 27000 g, 4°C for 20 min. The plasmid DNA pellet was resuspended in 15 ml TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA) and subjected twice to CIA extraction to remove PEG6000. The aqueous phase containing plasmid DNA was added with 1/10 volume of 4 M NaCl and 2.5 volume of 100% cold ethanol and incubated in -20°C for 30 min. The solution was centrifuged at 15000 g, 4°C for 10 min. The pellet was air-dried for 15 min and dissolved in TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA).

9. *EcoR* V digestion of plasmid DNA

Every single repeat of Widom 601-145 DNA has *EcoR* V restriction sites on both ends. *EcoR* V digestion generated Widom 601-145 DNA and linear empty vectors. The final *EcoR* V restriction enzyme setup was 2 mg/ml plasmid DNA, 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT and 300 unit *EcoR* V/ mg of DNA. The solution was incubated at 37°C for 24 hours. Digestion was checked on 1% agarose gel.

10. PEG fractionation of vector and Widom 601-145 DNA

The solution was added with 0.374 volume of 30% PEG6000 and 0.196

volume of 4 M NaCl and incubated on ice for 1 hour and centrifuged at 27000 g, 4°C for 20 min. The linear empty vector was in the pellet and Widom 601-145 DNA was in the supernatant. The supernatant was subjected to ethanol precipitation and dissolved in TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA). The purity of DNA was analyzed by 1% agarose gel.

11. Nuclear core particle reconstitution

The ingredients were mixed with utmost precision and exactly in this order: water, KCl, DTT, HO and DNA. HO and Widom 601-145 DNA were mixed at 1.15:1 ratio. The final reconstitution setup was 4.77 μ M HO, 4.13 μ M DNA, 2 M KCl and 10 mM DTT. The mixture was dialyzed in a MWCO 7.5 kDa Slide-A-Lyzer MINI Dialysis Device (Thermo Scientific) at room temperature against TCS-0.85, TCS-0.65, TCS-0.45 buffer for 2.5 hours each. At last, the solution was dialyzed against TCS-0 buffer for 12 hours (TCS-0 buffer: 20 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM DTT. TCS-0.85 buffer was TCS-0 with 0.85 M KCl. TCS-0.65 buffer was TCS-0 with 0.65 M KCl. TCS-0.45 buffer was TCS-0 with 0.45 M KCl). After dialyzing, the solution was spun at 18000 g, 4°C for 10 min twice and analyzed on 6% 0.25 \times TBE polyacrylamide gel (ratio of acrylamide and bisacrylamide was 37.5:1).

12. Ubiquitin(1-75)-thioester production

To increase the final yield of Ubi-thioester, we decided to use a smaller and more efficient intein (27 kDa).^[3] The human ubiquitin gene was amplified by PCR using the following primers: pTXB1-Ubi-NdeI F and Ubi75G-SapI-R. The PCR product was purified, digested by NdeI and SapI, then ligated into identically digested pTXB1 vector (New England Biolabs) to yield pTXB1-Ubi75. Then the correct insert was confirmed by sequence sequencing. The expression and purification of Ubi-thioester was performed as previous method.^[1] The final yield of ub(1-75)-MES was a little higher than that using pTYB1-UBi, which was about 8-10mg/L.

13. Synthesis of ubH2A 8 from H2A 1

8.8 mg of H2A 1 was dissolved in 300 μ L of dimethyl sulfoxide (DMSO). 3.6 μ L of Boc anhydride was added. 1.6 μ L of N-ethyl-diisopropylamine (DIEA) was added. The reaction mixture was kept at room temperature for 1 h and diethyl ether was added to precipitate out the Boc-protected form H2A 2. The ether-precipitate was next dissolved in 300 μ L DMSO followed by addition of 30 μ L of 1 M aqueous TCEP. After 3 h reduction reaction at room temperature, ether was added to the reaction mixture to precipitate the reduced product H2A 3. The precipitated H2A 3 was then dissolved in 330 μ L DMSO, and 2.6 mg of compound 9 and 6 μ L of DIEA were added. The reaction was

monitored by ESI-MS analysis. After 90 min, the crude product H2A **4** was obtained by ether precipitation. For global Boc deprotection, the crude protein **4** was treated with 200 μ L of TFA/TIS/H₂O (95/2.5/2.5) for 20 min. After ether precipitation, the crude deprotection product was purified by C4 reverse-phase HPLC to give 4.9 mg of **5** (56% overall yield starting from **1**). To remove the thiazolidine protection group of **5**, 4.9 mg of **5** was treated with 1 mL of the reaction solution (6 M Gdn.HCl, 0.2 M phosphate, 0.4 M MeONH₂, pH 4) for 5h at 37 °C. 3.5 mg of deprotected product **6** was obtained after C4 reverse-phase HPLC purification (yield about 70%). For a typical ubiquitination reaction, 3.5 mg of **6** and 4.4 mg of Ub(1-75)-MES were dissolved in 335 μ L of ligation buffer (6M Gdn.HCl, 0.2 M phosphate, 30 mM TCEP, 25 mM MPAA, pH 8.0). After 24 h, the ligation product was isolated by C4 semi-prep HPLC purification. 3.9 mg of ligation product **7** was obtained. To remove the auxiliary group, 3.9 mg of **7** was treated with 200 μ L of TFA/TIS/H₂O (95/2.5/2.5) for 20 min on ice. 3.3 mg of the final product **8** was obtained after ether precipitation.

14. Deubiquitination assay

UCH-L3 (Boston Biochem, Cambridge, MA) was diluted to a concentration of 5 μ M in reduction buffer (50 mM Tris HCl, 150 mM NaCl, 15 mM DTT, pH 7.5) and incubated at room temperature for 15 min. Reduced UCH-L3 (1 μ M) was then combined with ubH2A (10 μ M), respectively, in 25 μ L hydrolysis buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.5) and transferred to a 37 °C incubator. A small aliquot was taken and mixed with 5 μ L 6X SDS page loading dye at 30 min, 60 min, 90 min, 120 min, 150 min, respectively. Controls were performed without UCH-L3. The samples were analyzed by 15% SDS-PAGE and stained with Coomassie Blue.

SUMO-USP21 (LifeSensors) or BAP1 (Boston Biochem, Cambridge, MA) were mixed on ice with mononucleosomes containing ubH2A (about 2.1 μ M) in an enzyme- to-substrate ratio of 1:1 in 20 μ l deubiquitination buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM DTT) and reactions were incubated at 37°C. A small aliquots (i.e. containing 1 μ g nucleosomes) was removed at indicated time points and reactions were stopped by addition of SDS sample loading buffer and incubation at 95°C for 5 min for following western blot analysis.

15. Western blot

After chemical synthesis of ubH2A, protein samples including monoubiquitin, wild type H2A and the synthesized ubH2A was analyzed on 15% SDS-PAGE and transferred to a PVDF membrane. A Western blot was

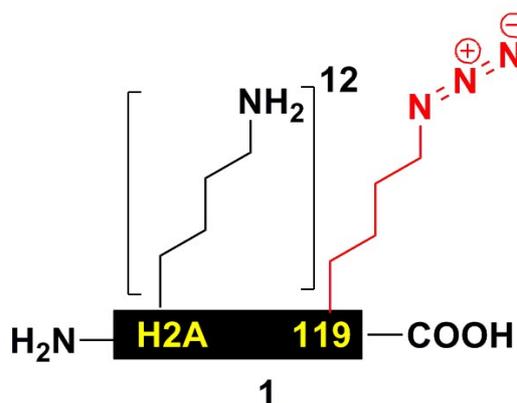
performed using a linkage specific ubH2A (K119) antibody (Millipore, 05-678) at 1:2000. The ubH2A were visualized by chemilluminescence (SuperSignal West Dura Trial Kit, Pierce, USA).

After deubiquitinase assays, each lane was loaded with about 1 μ g of mononucleosome containing ubH2A treated with USP21 or BAP on 15% SDS-PAGE. The proteins on the gel were then electrotransferred to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked by 10 mL of 5% w/v non-fat milk powder in TBS-T buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 for 2.5 h at room temperature. A 1:3300 dilution of ubiquitin mAb P4D1 (HRP conjugated) (Enzo Life Sciences) was added to the blocking mixture and incubated for 1 h at room temperature. The ubH2A and monoubiquitin were visualized by chemilluminescence (SuperSignal™ West Femto Maximum Sensitivity Substrate, Invitrogen, USA).

16. Histone lysine methyltransferase assays

The histone methyltransferase assays were performed in a 40 μ l reaction mixture containing 10 μ l NCP (2.1 μ M), 2 μ l NSD2 or SETD2, 80 μ M S-adenosylmethionine (NEB) in the histone methyltransferase buffer (25mM Tris. HCl, pH 7.4, DTT 5mM) for 1 h at 30 °C. The reaction products were separated by 15% SDS-PAGE, then transferred to PVDF membranes and subjected to western blot. The antibody used was Anti-Histone H3 (tri methyl K36) antibody - ChIP Grade (ab9050) and Anti-Histone H3 (di methyl K36) antibody - ChIP Grade (ab9049) from Abcam.

17. Raw and deconvoluted ESI-MS data of key intermediates during ubH2A synthesis



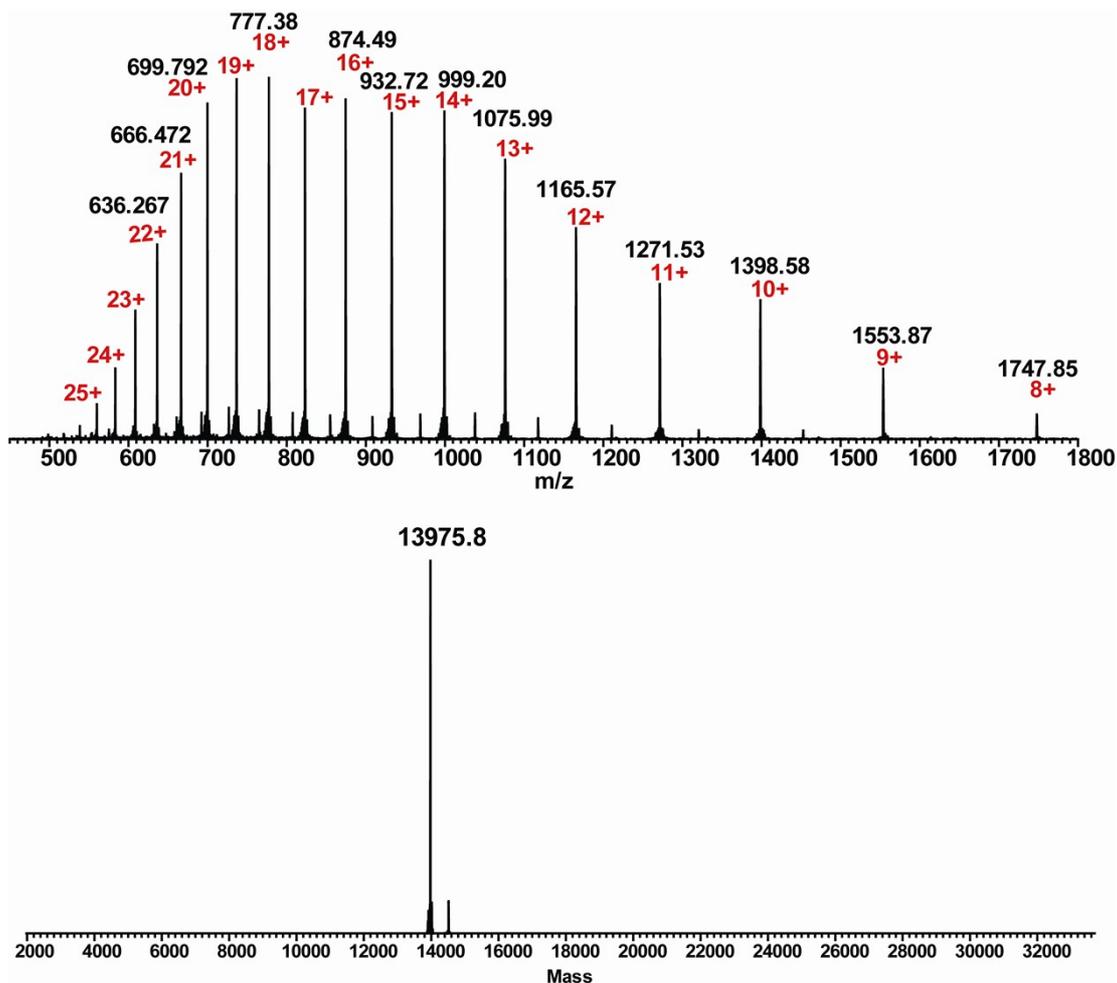
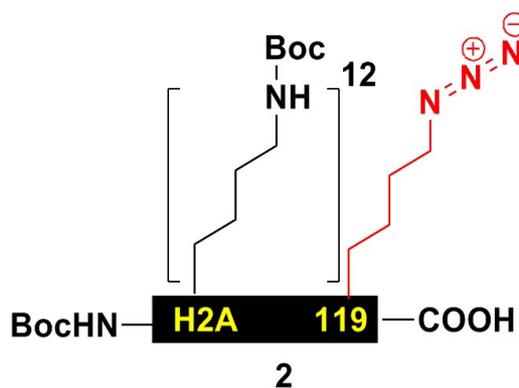


Figure S1. Raw and deconvoluted ESI-MS data of purified H2A 1 (Average molecular mass calculated 13976.02; found 13975.8).



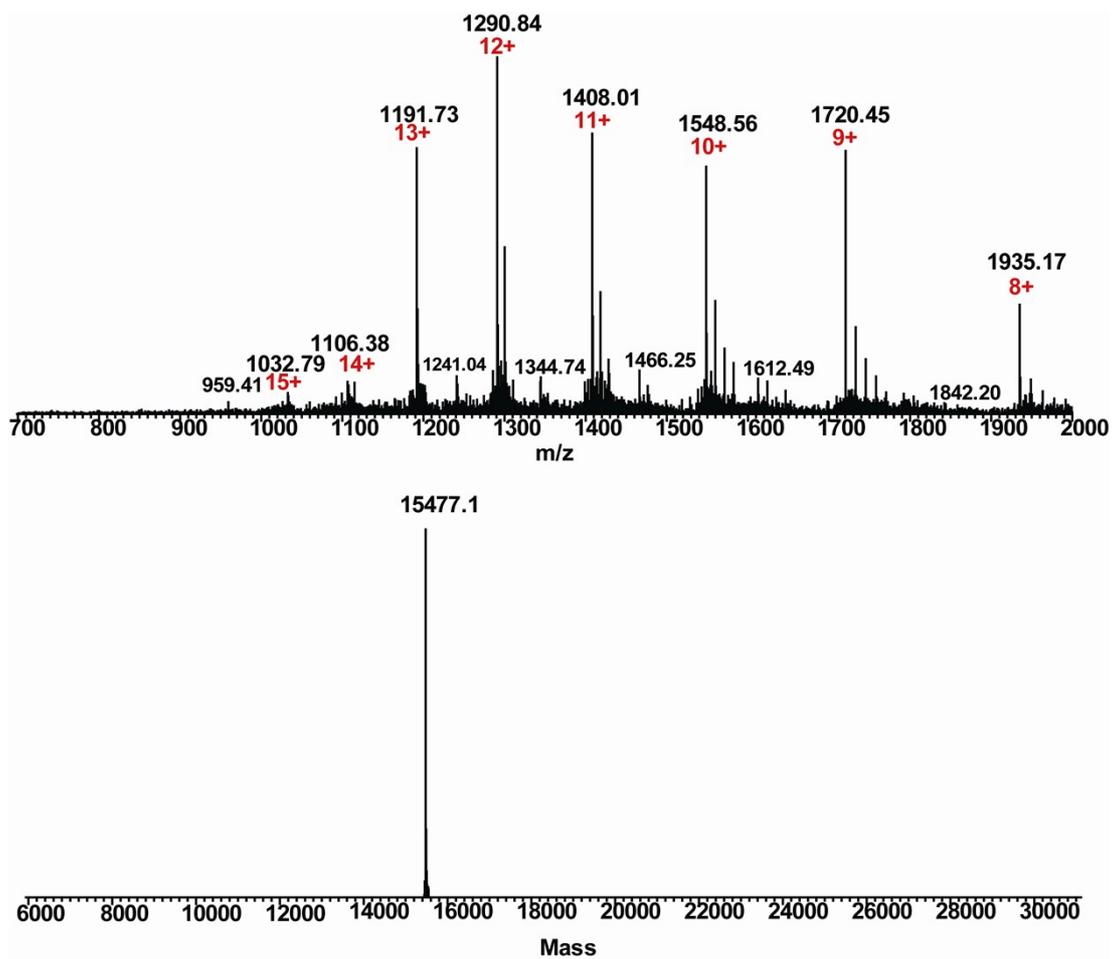


Figure S2. Raw and deconvoluted ESI-MS data of crude H2A 2 (H2A2Boc₁₅). Average molecular mass calculated 15477.76; found 15477.1).



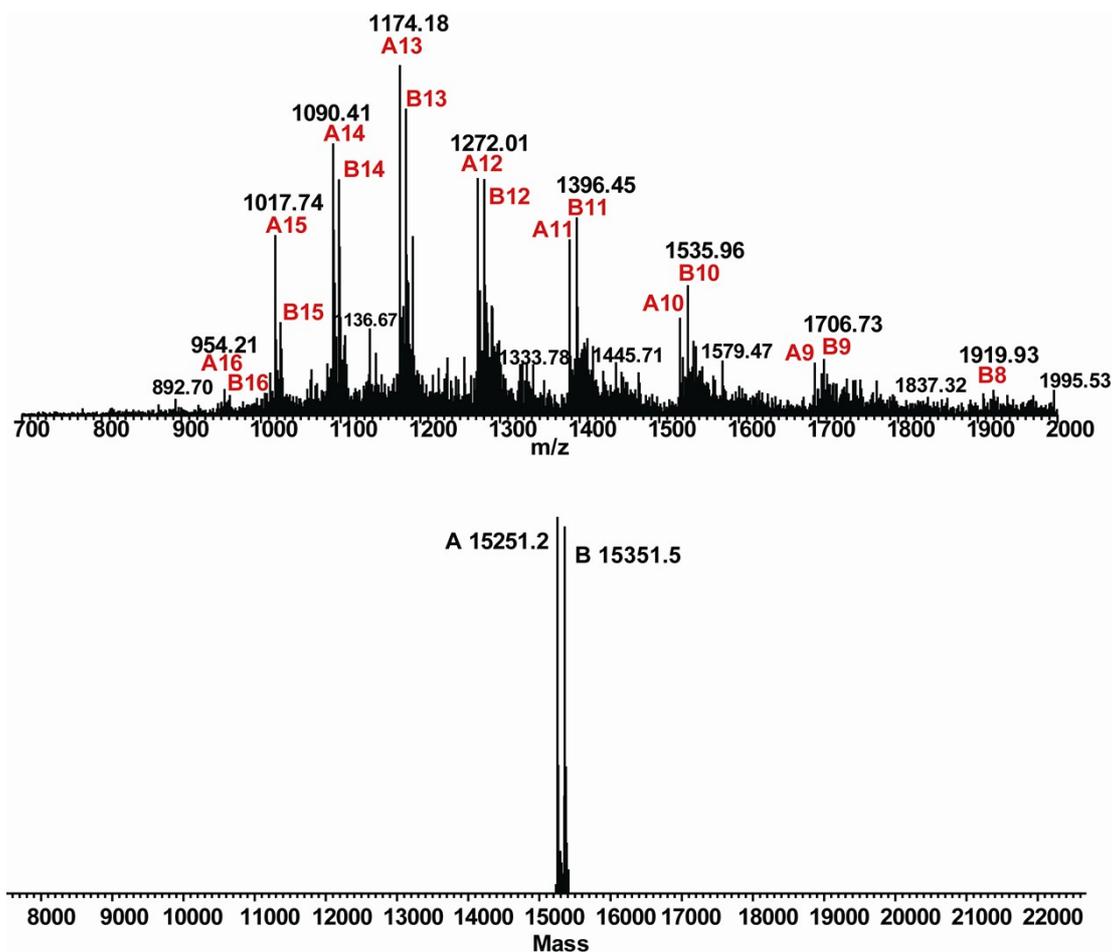
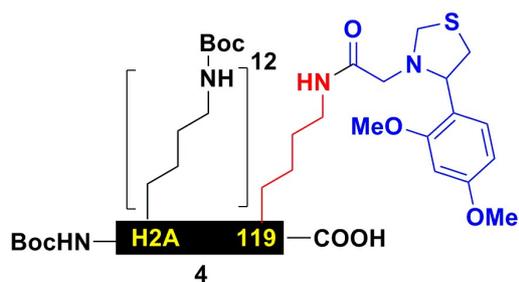


Figure S3. Raw and deconvoluted ESI-MS data of crude H2A **3** (H2A3Boc₁₃: average molecular mass calculated 15250.52, found 15251.2; H2A3Boc₁₄: average molecular mass calculated 15350.64, found 15351.5).



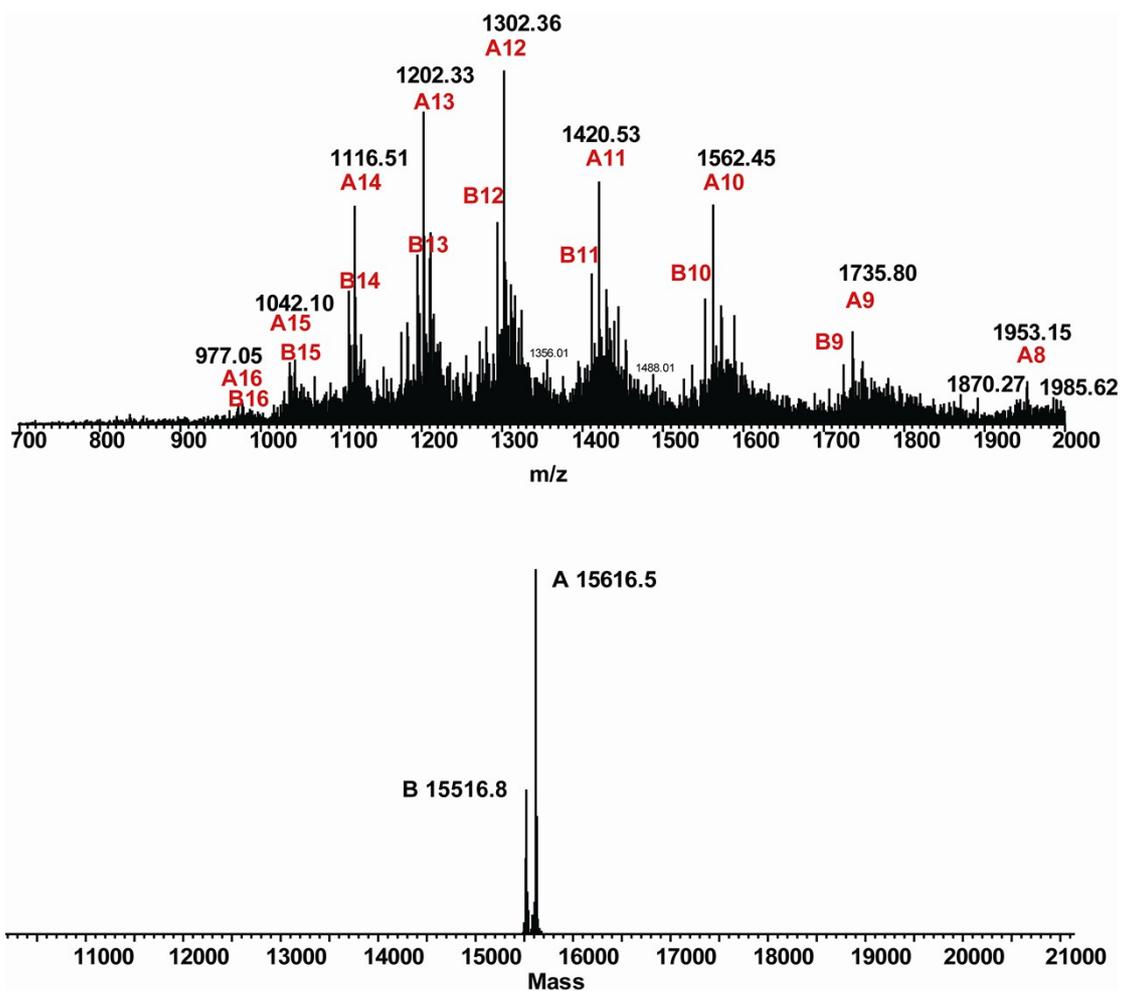
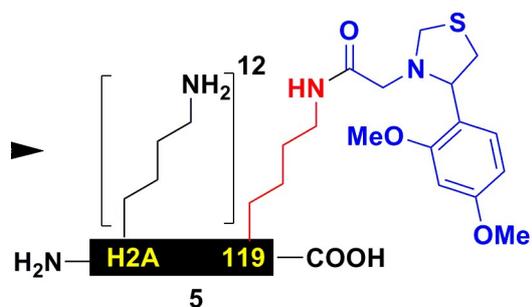


Figure S4. Raw and deconvoluted ESI-MS data of crude H2A 4 (H2A4Boc₁₃: average molecular mass calculated 15515.85, found 15516.8; H2A4Boc₁₄: average molecular mass calculated 15615.97, found 15616.5).



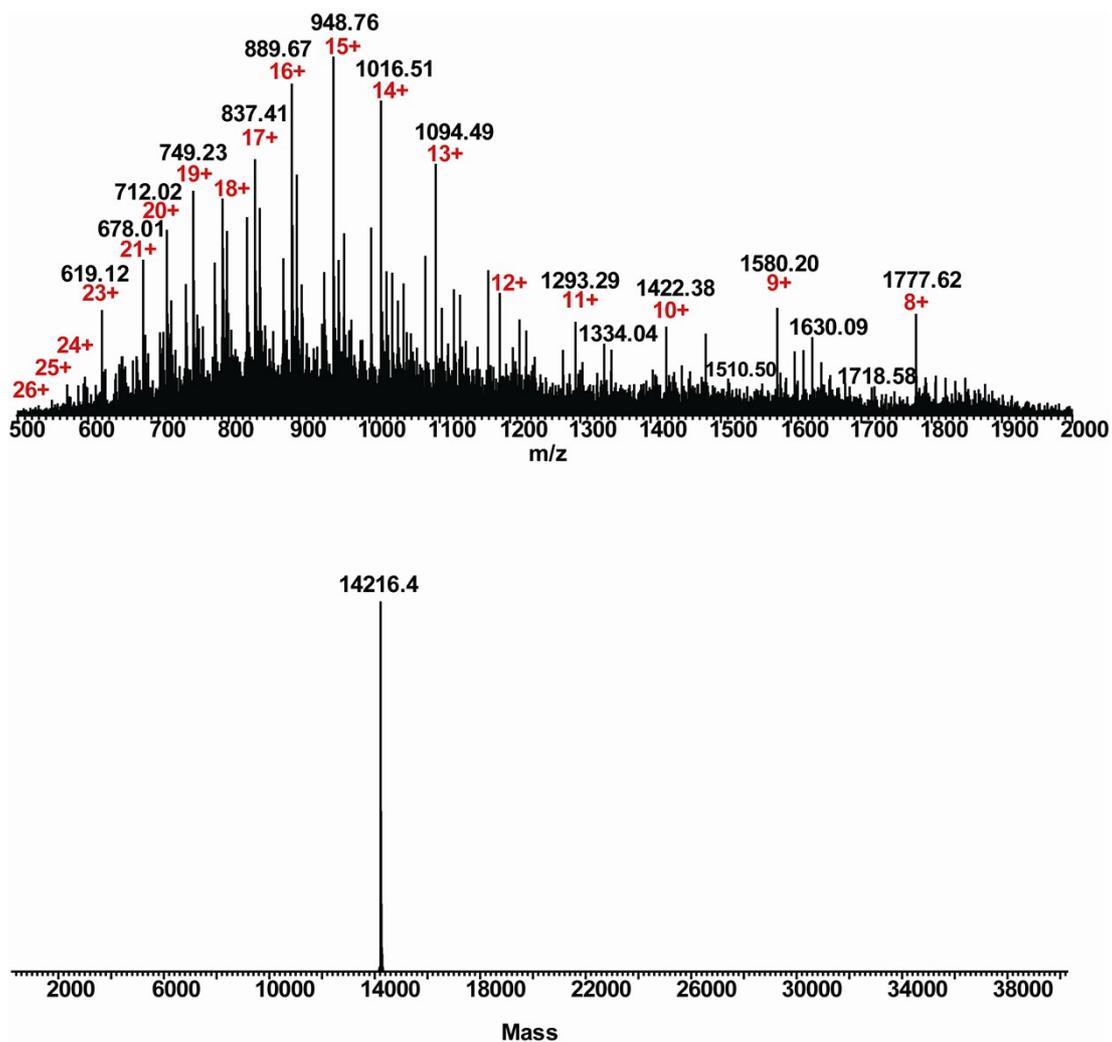
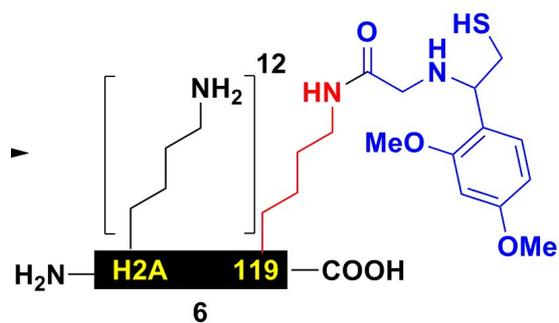


Figure S5. Raw and deconvoluted ESI-MS data of purified H2A **5** (average molecular mass calculated 14215.36; found 14216.4).



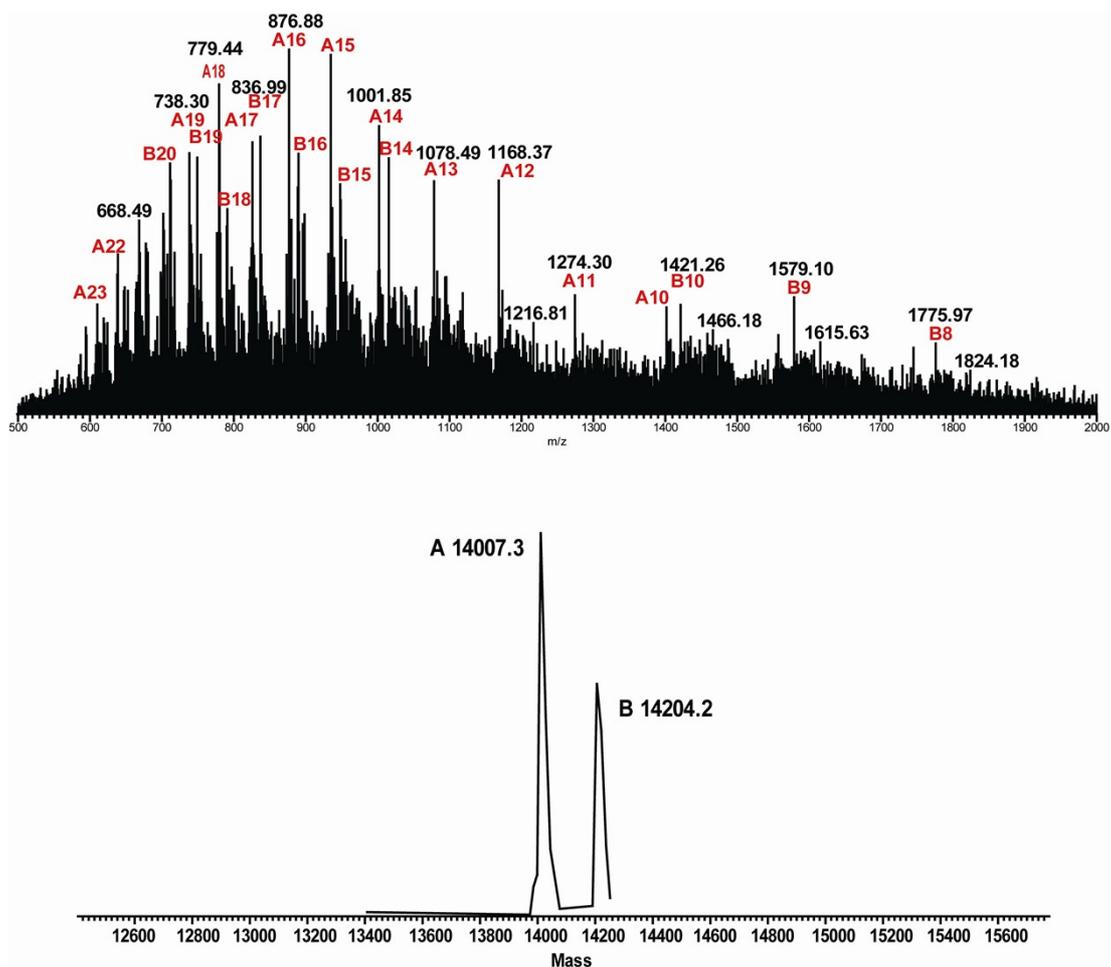
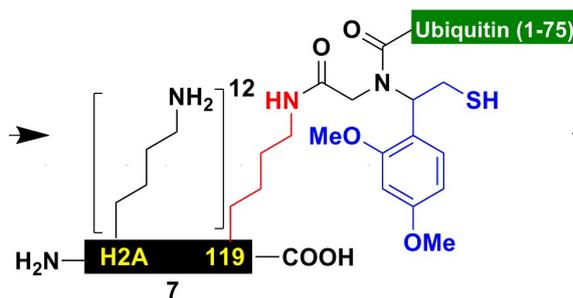


Figure S6. Raw and deconvoluted ESI-MS data of purified H2A **6** (average molecular mass calculated 14203.35; found 14204.2). Peak A is the MS adduct, which is caused by the instability of auxiliary in MS detection condition.



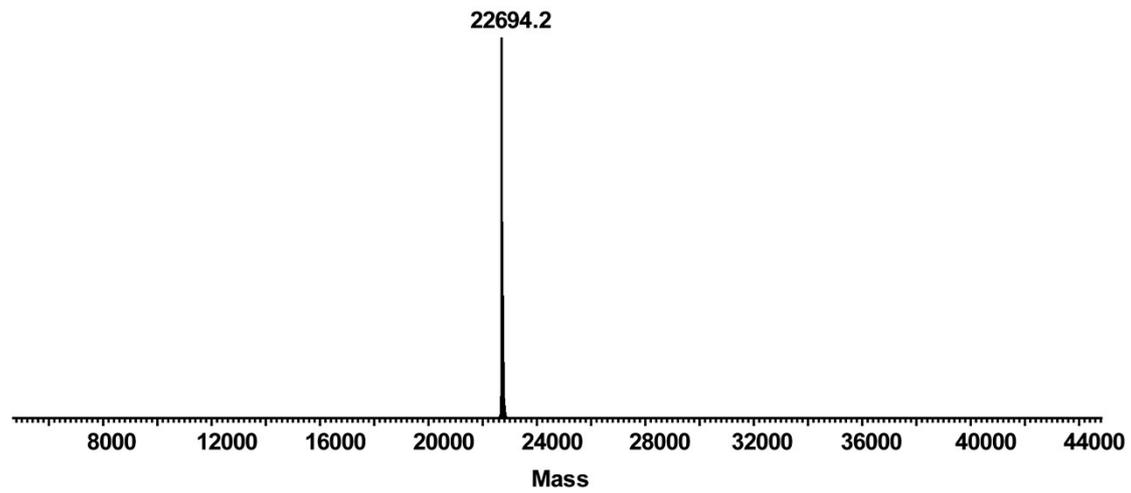
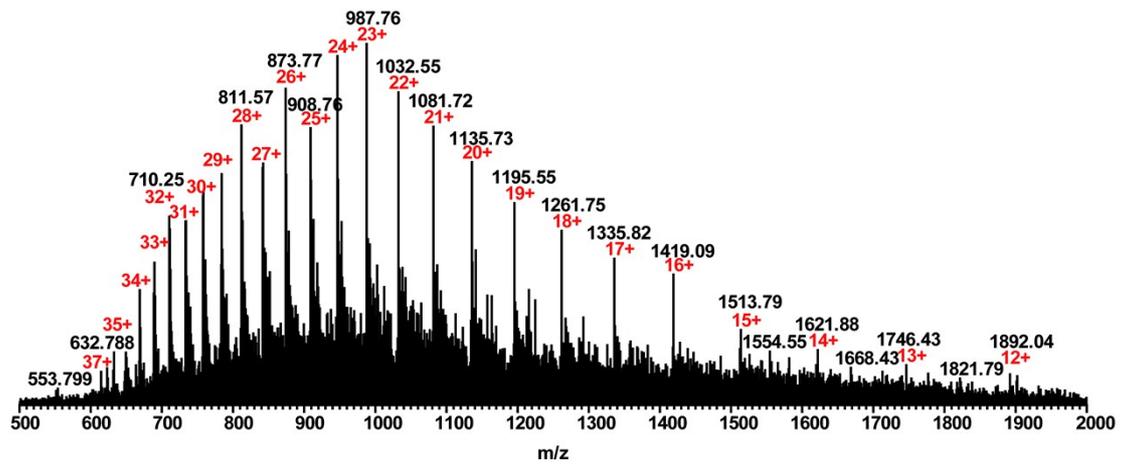
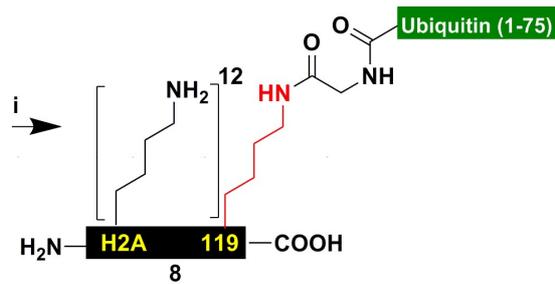


Figure S7. Raw and deconvoluted ESI-MS data of purified H2A 7 (average molecular mass calculated 22693.02; found 22694.2).



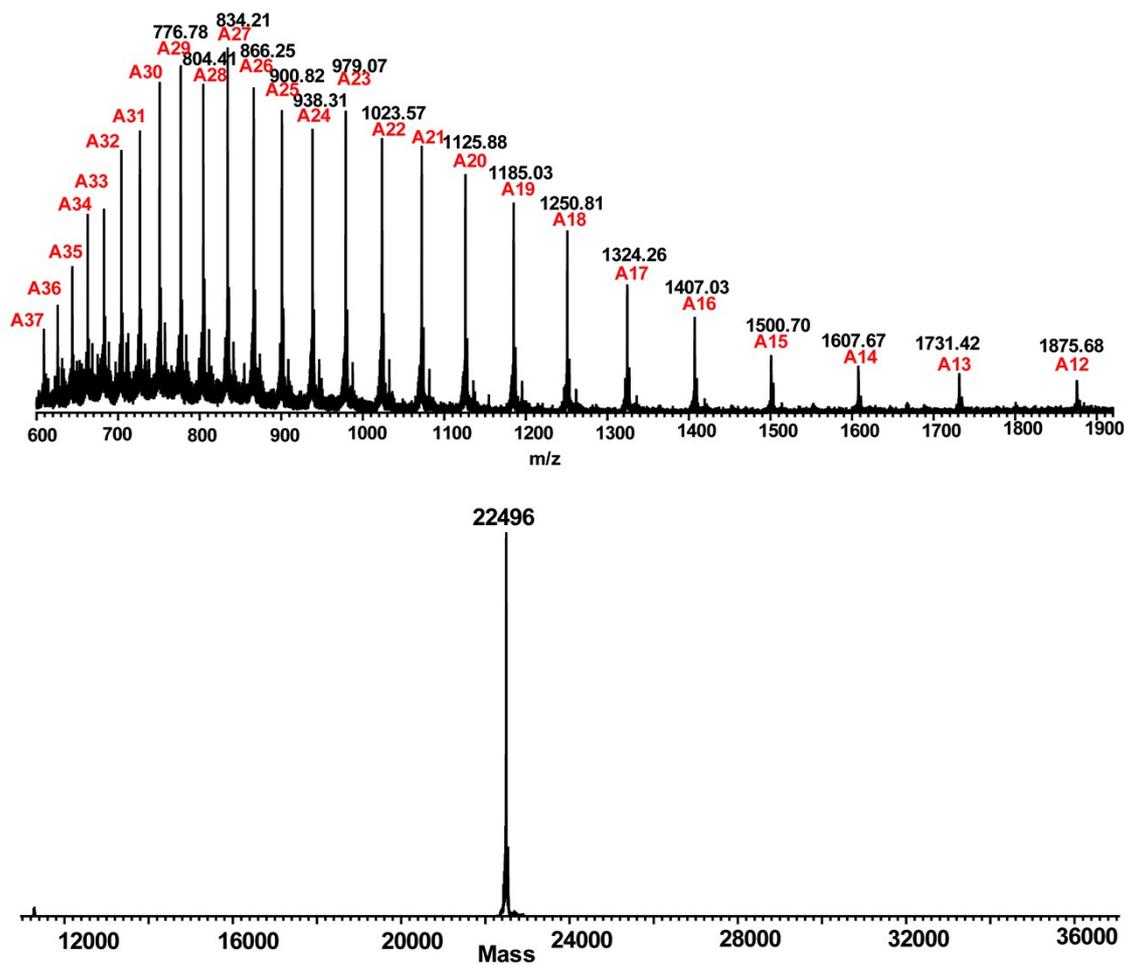


Figure S8. Raw and deconvoluted ESI-MS data of purified H2A 8 (average molecular mass calculated 22496.75; found 22496).

Primers	Sequences
pQLN-H2A-K119M-F	5'-GTGCTGCTGCCCAAGATGACCGAGAGTTCCAAG-3'
pQLN-H2A-K119M-R	5'-CTTGGA ACTCTCGGT CATCTTGGGCAGCAGCAC-3'
pQLN-H2A-EZ-F	5'- AAGAGGAGAAATTAACCATGTCAGGAAGAGGCAAAC- 3'
pQLN-H2A-EZ-R	5'- CAAGCTCAGCTAATT AAGCTT TCACTTGCTCTTGGCC GAC-3'
MetRS_H301L_F	5'-GATATTGTTTACTTCctcAGCCTGTTCTGGCCTG-3'
MetRS_H301L_R	5'-CAGGCCAGAACAGGCTgagGAAGTAAACAATATC-3'
MetRS-L13N-F	5'- CTGGTGACGTGCGCAAACCCGTACGCTAACGGC- 3'
MetRS-L13N-R:	5'- GCCGTTAGCGTACGGGTTTGCGCACGTCACCAG- 3'
MetRS_Y260L_F	5'-GGACGCACCGATTGGCCTCATGGGTTCTTTCAAG- 3'
MetRS_Y260L_R	5'-CTTGAAAGAACCCATGAGGCCAATCGGTGCGTCC- 3'
pTXB1-Ubi-NdeIF	5'-GGGAATTCATATGCAGATCTTCGTCAAGACGT-3'
Ubi75G-SapI-R	5'-GTTGCTCTTCCGCAACCTCTTAGTCTTAAGACAAG- 3'

Table S1 The primers used in this study.

Reference:

1. Yang R, Bi X, Li F, Cao Y, Liu CF (2014) Native chemical ubiquitination using a genetically incorporated azidonorleucine. Chem Commun (Camb) 50: 7971-7974.
2. Li F, Allahverdi A, Yang R, Lua GBJ, Zhang X, et al. (2011) A Direct Method for Site-Specific Protein Acetylation. Angewandte Chemie International Edition 50: 9611-9614.
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mediated site-specific peptide ubiquitylation. *Angew Chem Int Ed Engl*
46: 2814-2818.