SI Appendix

Identification of a Functional Hotspot on Ubiquitin Required for Stimulation of Methyltransferase Activity on Chromatin

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Supplementary Note about Ub ligation directly to the mononucleosome

Methyltransferase assays and nucleosome ligations were quantified by taking the total amount of the H2B-Ub, in a nucleosomal context, into account. Accordingly, native PAGE gel bands (either non, mono, or di-Ub species) stained with ethidium bromide or an alternative DNA staining agent such as syber gold were quantified using ImageJ software. Gel lanes were normalized to the total amount of mononucleosomes (at the time of 60 min) to determine the absolute amount of each ubiquitylated species. Note, we assumed that the different Ub mutants had a negligible affect on nucleosome visualization. This resulted in the calculation of the abundance of each type of nucleosome (A_x, where x = non, mono, di):

$A_{non} = non/total_{60}$, $A_{mono} = mono/total_{60}$, $A_{di} = di/total_{60}$

Where non, mono, di = quantification of the respective gel band;

$total_{60}$ = non + mono + di at the 60 min. time point

The total amount of the ubiquitylation in the nucleosome was calculated by taking into account that the mono species contained one copy of H2BK120C and one copy of H2B-Ub per MN:

Total H2B-Ub = $A_{di} + (0.5 \times A_{mono})$

hDot1L methyltransferase results were adjusted by the total amount of ubiquitylated species, setting the wild-type H2B-Ub to 1, and termed Ub efficiency:

Ub efficiency = Scintillation Counts per minute/Total H2B-Ub

Materials

Amino acid derivatives, coupling reagents and resins were purchased from Novabiochem (Laufelfingen, Switzerland). The thiol activating reagents 2,2'-dithiobis(5-nitropyridine) (DTNP), 5, 5'-Dithiobis(2-nitrobenzoic acid) (DTNB), and cystamine dihydrochloride were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). [^{3}H]-S-adenosyl methionine, Amplify solution and Sephacryl S-200 resin were obtained from GE Healthcare (Waukesha, WI). S-adenosyl methionine was obtained from New England Biolabs (Ipswich, MA). ^{15}N labeled ammonium chloride was purchased from Cambridge Isotopes. All other commonly used chemical reagents and solvents were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI) or Fischer Scientific (Pittsburgh, PA). Chemically competent DH5alpha, BL21(DE3), and BL21(DE3)pLysS cells were purchased from Novagen (Madison, WI). The pTXB1 vector, restriction enzymes, T4 DNA ligase, chitin resin, and NiNTA resin were obtained from New England BioLabs (Ipswitch, MA). Primer synthesis and gene sequencing were performed by Integrated DNA Technologies (Coralville, IA) and Genewiz (South Plainfeld, NJ), respectively. Criterion 15% Tris-HCl and 5% TBE gels were purchased from BioRad (Hercules, CA). Centricons were from Sartorius (Goettingen, Germany) and dialysis cassettes were from Pierce (Rockford, IL). PCR purification and gel extraction kits were purchased from Qiagen (Valencia, CA).

Equipment

Size-exclusion and ion-exchange chromatography were performed on an AKTA FPLC system from GE Healthcare equipped with a P-920 pump and UPC-900 monitor.

Analytical reversed-phase HPLC (RP-HPLC) was performed on Hewlett-Packard 1100 and 1260 series instruments with a Vydac C18 column (5 micron, 4 x 150 mm), employing 0.1% TFA in water (A), and 90% CH3CN, 0.1% TFA in water (B), as the mobile phases. Typical analytical gradients were 0-73% B over 30 min at a flow rate of 1 mL/min. Preparative HPLC was carried out on a Waters prep LC system comprised of a Waters 2545 Binary Gradient Module and a Waters 2489 UV detector. A Vydac C18 process column (15-20 micron, 50 x 250 mm) or a semi-preparative column (12 micron, 10 mm x 250 mm) was employed at a flow rate of 30 mL/min, or, 4 mL/min, respectively. ESI-MS analysis was conducted on a Sciex API-100 single quadrupole spectrometer or Bruker Daltonics MicrOTOF-Q II mass spectrometer. All protein starting materials and ligation products were analyzed by C18 analytical RP- HPLC and ESI-MS. Scintillation counting was performed on a LKB Wallac 1209 RackBeta Primo Liquid scintillation counter. All fluorescent measurements were done on a Fluorolog-3 fluorescence spectrometer (HORIBA Jobin Yvon, Edison NJ).

Cloning, expression and isolation of ubiquitin mutants

Ubiquitin mutants were prepared using either an ubiquitin GyrA-intein Chitin Binding Domain fusion (Ub-GyrA-CBD) or an ubiquitin NPU intein 6XHis-tag fusion (Ub-NPU-6XHis) protein with the following human ubiquitin sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDY
NIQKESTLHLVLRLRGG

Mutagenesis of pUb-GyrA

All ubiquitin library mutants were made by site-directed mutagenesis using a pUb plasmid template previously engineered with ubiquitin(1-75) in a pTXB1 vector and mutagenic primers as follows[1]:

pUb1. Forward primer (Ub T9A/G10A/K11A/T12A) 5'-CAT ATG CAG ATC TTC GTG AAG ACT CTG GCT GCT GCG GCC ATC ACT CTC GAA GTG GAG CCG-3' and reverse primer (Ub T9A/G10A/K11A/T12A) 5'- CTC GGC TCC ACT TCG AGA GTG ATG GCC GCA GCA GC C AGA GTC TTC ACG AAG ATC TGC ATA TG-3'.

pUb2. Forward primer (Ub Q2A) 5'- CTTTAAGAAGGAGATATACAT ATG GCG ATC TTC GTG AAG ACT CTG AC -3' and reverse primer (Ub Q2A) 5' -GT CAG AGT CTT CAC GAA GAT CGC CAT ATG TAT ATC TCC TTC TTA AAG-3' and forward primer (UbL15A/E16A) 5'- CT CTG ACT GGT AAG ACC ATC ACT GCC GCA GTG GAG CCG AGT GAC ACC ATT GAG -3' and reverse primer (UbL15A/E16A) 5' - CTC AAT GGT GTC ACT CGG CTC CAC TGC GGC AGT GAT GGT CTT ACC AGT CAG AG-3' and forward primer (Ub K29A) 5'- C ACC ATT GAG AAT GTC AAG GCA GCG ATC CAA GAC AAG GAA GGC ATC CC-3' and reverse primer (Ub K29A) 5'- GGG ATG CCT TCC TTG TCT TGG ATC GCT GCC TTG ACA TTC TCA ATG GTG -3'

pUb3. Forward primer (Ub K6A) 5'- GATATACAT ATG CAG ATC TTC GTG GCG ACT CTG ACT GGT AAG ACC ATC AC-3' and reverse primer (Ub K6A) 5'- GTG ATG GTC TTA CCA GTC AGA GTC GCC ACG AAG ATC TGC ATA TGT ATA TC-

3’ and forward primer (Ub T66A/H68A) 5’- GAC TAC AAC ATC CAG AAA GAG TCC GCC CTG GCC CTG GTA CTC CGT CTC AGA GGT TG-3’ and reverse primer (Ub T66A/H68A) 5’-CAA CCT CTG AGA CGG AGT ACC AGG GCC AGG GCG GAC TCT TTC TGG ATG TTG TAG TC-3’.

pUb4. Forward primer (Ub E18A/P19A/S20A/D21A) 5’- GGT AAG ACC ATC ACT CTC GAA GTG GCG GCG GCT GCC ACC ATT GAG AAT GTC AAG GCA AAG-3’ and reverse primer (Ub E18A/P19A/S20A/D21A) 5’-CTT TGC CTT GAC ATT CTC AAT GGT GGC AGC CGC CGC CAC TTC GAG AGT GAT GGT CTT ACC-3’.

pUb5. Forward primer (Ub T22A/E24A/N25A) 5’-CT CTC GAA GTG GAG CCG AGT GAC GCC ATT GCG GCT GTC AAG GCA AAG ATC CAA GAC AAG -3’ and reverse primer (Ub T22A/E24A/N25A) 5’-CTT GTC TTG GAT CTT TGC CTT GAC AGC CGC AAT GGC GTC ACT CGG CTC CAC TTC GAG AGT G-3’.

pUb6. Forward primer (Ub Q31A/D32A/K33A/E34A) 5’- C ATT GAG AAT GTC AAG GCA AAG ATC GCA GCC GCG GCA GGC ATC CCT CCT GAC CAG CAG AG -3’ and reverse primer (Ub Q31A/D32A/K33A/E34A) 5’- CCT CTG CTG GTC AGG AGG GAT GCC TGC CGC GGC TGC GAT CTT TGC CTT GAC ATT CTC AAT G-3’.

pUb7. Forward primer (Ub P37A/P38A/D39A) 5’- GCA AAG ATC CAA GAC AAG GAA GGC ATC GCT GCT GCC CAG CAG AGG TTG ATC TTT GCT GGG-3’ and

reverse primer (Ub P37A/P38A/D39A) 5'- CCC AGC AAA GAT CAA CCT CTG CTG GGC AGC AGC GAT GCC TTC CTT GTC TTG GAT CTT TGC-3'.

pUb8. Forward primer (Ub G47A/K48A/Q49A) 5'- GAC CAG CAG AGG TTG ATC TTT GCT GCG GCA GCG CTG GAA GAT GGA CGC ACC CTG TCT G-3' and reverse primer (Ub G47A/K48A/Q49A) 5'-CAG ACA GGG TGC GTC CAT CTT CCA GCG CTG CCG CAG CAA AGA TCA ACC TCT GCT GGT C-3'.

pUb9. Forward primer (Ub E51A/D52A/G53A/R54A) 5'- G TTG ATC TTT GCT GGG AAA CAG CTG GCA GCT GCA GCC ACC CTG TCT GAC TAC AAC ATC C-3' and reverse primer (Ub E51A/D52A/G53A/R54A) 5'- CTG GAT GTT GTA GTC AGA CAG GGT GGC TGC AGC TGC CAG CTG TTT CCC AGC AAA GAT CAA C-3'.

pUb10. Forward primer (Ub D58A/Y59A/N60A) 5'- G CTG GAA GAT GGA CGC ACC CTG TCT GCC GCC GCC ATC CAG AAA GAG TCC ACC CTG CAC C-3' and reverse primer (Ub D58A/Y59A/N60A) 5'- GGT GCA GGG TGG ACT CTT TCT GGA TGG CGG CGG CAG ACA GGG TGC GTC CAT CTT CCA GC-3'.

pUb11. Forward primer (Ub Q62A/K63A/E64A) 5'- CGC ACC CTG TCT GAC TAC AAC ATC GCG GCA GCG TCC ACC CTG CAC CTG GTA CTC CGT C-3' and reverse primer (Ub Q62A/K63A/E64A) 5'- GAC GGA GTA CCA GGT GCA GGG TGG ACG CTG CCG CGA TGT TGT AGT CAG ACA GGG TGC G-3'.

pUb12. Forward primer (UbL71/R72/L73/R74A) 5'- G AAA GAG TCC ACC CTG CAC CTG GTA GCC GCT GCC GCA GGT TGC ATC ACG GGA GAT GCA CTA G-3' and reverse primer (UbL71/R72/L73/R74A) 5' CTA GTG CAT CTC CCG TGA TGC AAC CTG CGG CAG CGG CTA CCA GGT GCA GGG TGG ACT CTT TC-3'.

Preparation of ubiquitin aminoethanethiol analogs by thiolysis of GyrA intein fusions

Ubiquitin aminoethanethiol analogs were prepared as described.[2] Typical yields were 4-6 mg per liter for each protein. The purest fractions were pooled and analyzed by analytical HPLC, and mass spectrometry **(Figure S14)**

Mutagenesis of ubiquitin in NPU intein 6XHis tagged vector

Selected ubiquitin mutants were generated by site-directed mutagenesis using a previously described pUb-NPU-6XHis plasmid template [3] and mutagenic primers as follows:

pUb13. Forward primer (UbL8A) 5'-GCA GAT CTT CGT GAA GAC TGC GAC TGG TAA GAC CAT CAC T-3' and reverse primer (Ub L8A) 5'- AGT GAT GGT CTT ACC AGT CGC AGT CTT CAC GAA GAT CTG C -3' and forward primer (UbI44A) 5'-CTG ACC AGC AGA GGT TGG CCT TTG CTG GGA AAC AGC-3' and reverse primer (UbI44A) 5'- GCT GTT TCC CAG CAA AGG CCA ACC TCT GCT GGT CAG-3'.

pULL. Forward primer (UbL71/73A) 5'-C CTG CAC CTG GTA GCC CGT GCC AGA GGT GGT TGT TTA AGC TAT GAA ACG GAA ATA TTG AC-3' and reverse primer

(UbL71/73A) 5'-GT CAA TAT TTC CGT TTC ATA GCT TAA ACA ACC ACC TCT GGC ACG GGC TAC CAG GTG CAG G-3'.

pURR. Forward primer (UbR72/74A) 5'- GAG TCC ACC CTG CAC CTG GTA CTC GCT CTC GCA GGT GGT TGT TTA AGC TAT GAA ACG G -3' and reverse primer (R72/74A) 5' C CGT TTC ATA GCT TAA ACA ACC ACC TGC GAG AGC GAG TAC CAG GTG CAG GGT GGA CTC -3'.

pUbL71A. Forward primer 5'- G AAA GAG TCC ACC CTG CAC CTG GTA GCC CGT CTG AGA GGT TGC ATC ACG GGA GAT GCA C -3' and reverse primer 5' G TGC ATC TCC CGT GAT GCA ACC TCT CAG ACG GGC TAC CAG GTG CAG GGT GGA CTC TTT C -3'

pUbL73A. Forward primer 5'- G AAA GAG TCC ACC CTG CAC CTG GTA CTG CGT GCC AGA GGT TGC ATC ACG GGA GAT GCA C -3' and reverse primer 5' G TGC ATC TCC CGT GAT GCA ACC TCT GGC ACG CAG TAC CAG GTG CAG GGT GGA CTC TTT C -3

pHA-Ub/uLL. Forward primer (HA_tag-Ub/uLL) 5'- GAA GGA GAT ATA CAT ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT CAG ATC TTC GTG AAG-3' and reverse primer (HA_tag-Ub/uLL) 5'- CTT CAC GAA GAT CTG AGC GTA ATC TGG AAC ATC GTA TGG GTA CAT ATG TAT ATC TCC TTC – 3'.

Preparation of ubiquitin aminoethanethiol analogs by thiolysis of NPU intein His fusions

E. coli BL21(DE3) cells were transformed with plasmids containing the Ubiquitin-NPU-6XHis fusion or ubiquitin mutant-NPU-His tag fusions and grown in 6L of Luria-Bertani (LB) media (100 ug/L ampicillin) at 37 °C until an OD600 of 0.6. Overexpression of the desired proteins was induced by the addition of 0.5 mM IPTG and the cells were grown for an additional 5 h at 30 °C. The cells were then harvested by centrifugation at 10k x *g* for 15 min and the cell-pellet was resuspended in buffer B (50 mM Sodium Phosphate, 200 mM NaCl, 1 mM EDTA, pH 6.0). The cells were lysed by passage through a French Press and the soluble fraction separated from insoluble cellular debris by centrifugation at 18.5-20k x *g* for 20 min. After filtration through a 0.45 μm filter, supernatants were bound to a 5 mL Ni-NTA column, pre-equilibrated with ten column volumes of buffer B, for 30 min at 4 °C. The resin was washed with 10 column volumes of buffer B, followed by 10 column volumes of column buffer B containing 20 mM imidazole, and eluted with 10 column volumes of buffer B containing 250 mM imidazole. Following dialysis into buffer B (containing no imidazole), Ub and Ub mutants were cleaved from the respective NPU-6XHis fusions by incubation with 10 column volumes of buffer B containing 50 mM of cystamine dihydrochloride, and 50 mM of Tris(2-carboxyethyl) phosphine, pH 7.2 for 10 h. The eluted proteins, bearing the desired C-terminal aminoethanethiol appendage, were subsequently purified by C18 process RP-HPLC employing a gradient of 25-55% B, over 60 min, followed by a second purification by C18 semi-preparative HPLC with a gradient of 25-55% B over 60 min to remove residual cystamine. Typical

yields were 10-30 mgs per liter for each protein. The purest fractions were pooled and analyzed by analytical HPLC, and mass spectrometry **(Figure S14).**

Circular Dichroism (CD) Analysis of Ub and uLL

CD measurements were performed on an Applied Photophysics Chirascan spectropolarimeter at 25°C using a 1mm path length cuvette. Protein concentrations were 30 μM in 10 mM potassium phosphate, 100 mM potassium fluoride buffer pH 7.5. Spectra were recorded from 260 to 190 nm using 1 nm steps and an averaging time of one second.

Preparation of ubiquitin α-thioesters

All constructs were expressed, purified and dialyzed into Buffer B similar to Ub aminoethanethiol constructs and then thiolyzed with mercaptoethane sulfonate (MES) as published.[3] Briefly, ubiquitin and Ub mutants were cleaved from the respective NPU intein fusions, after a dialysis step into buffer B, by incubation with 10 column volumes of buffer B containing 80 mM MES and 10 mM of Tris(2-carboxyethyl) phosphine, pH 7.2 for 18 h. The eluted proteins, bearing the desired C-terminal MES α-thioester, were subsequently purified by C18 process RP-HPLC employing a gradient of 25-55% B, over 60 min, and the purest fractions were pooled and analyzed by analytical HPLC, and mass spectrometry. Typical yields were 10-30 mgs per liter per protein.

Preparation of ^{15}N labeled uLL

E. coli BL21(DE3) cells were transformed with a plasmid encoding the ubiquitin mutant-NPU-His tag fusion and grown in 1L of M9 minimal media containing ^{15}N labeled ammonium chloride (100ug/L ampicillin) at 37 °C until an OD600 of 0.6. Overexpression of the desired protein was induced by the addition of 0.5 mM IPTG and the cells were grown for an additional 4 h at 37 °C. The uLL-NPU-His fusion was prepared exactly as the aminoethanethiol constructs but then thiolyzed with dithiothreitol (DTT). Briefly, uLL was cleaved from the respective NPU intein fusion, resulting in a free carboxyl group, after a dialysis step into buffer B, by incubation with 10 column volumes of buffer B containing 80 mM DTT, pH 8.5 for 10 h. The eluted protein was subsequently purified by C18 process RP-HPLC employing a gradient of 25-55% B, over 60 min, and the purest fractions were pooled and analyzed by analytical HPLC, and mass spectrometry **(Figure S14)**. Typical yields were 5 -10mgs per liter.

NMR Analysis of uLL

NMR spectra of uLL in 20 mM Tris, pH 7.5, 10 mM KCl, and 1 mM EDTA at 1 mM protein were recorded at 30°C on a Varian Inova 800-MHz spectrometer. All recorded data were processed using NMRPipe and analyzed using Sparky (http://www.cgl.ucsf.edu/home/sparky/).

Mutagenesis of histone H3

H3C110S was prepared using the previously described *xenopus laevis* H3.1 construct in a pET3a plasmid as a template [1] using the mutagenic primers: Forward primer (H3C110S) 5'-GCT CTC TTT GAG GAC ACC AAC CTG AGC GCC ATC CAC GCC

AAG AGG GTC ACC ATC ATG C-3' and reverse (H3C110S) 5' G CTA GAT GGT GAC CCT CTT GGC GTG GAT GGC GCT CAG GTT GGT GTC CTC AAA GAG TGC-3'.

Expression of recombinant histones

All histones, including mutant histones (e.g. H2BK120C, H2AN110C), were prepared as previously reported from the indicated expression vectors.[1,2,4] The purest fractions from HPLC purification were pooled and analyzed by analytical HPLC and mass spectrometry (**Figures S15**).

Fluorescent labeling of H2A(N110C)

H2AN110C was labeled with fluorescein as previously reported.[4] The purest HPLC fractions were pooled and analyzed by analytical HPLC, and mass spectrometry (**Figure S15**).

Synthesis of ubiquitylated H2B-Ub constructs by asymmetric disulfide formation

H2B-Ub and H2B-Ub mutants were prepared according to published protocols.[2] The purest HPLC fractions from HPLC purification were pooled and analyzed by analytical HPLC, and mass spectrometry (**Figures S16**).

Preparation of H2B-Ub conjugates by expressed protein ligation

H2B-Ub and H2B-uLL were prepared using an established sequential expressed protein ligation procedure.[5] Note, the ubiquitin in these semisynthetic constructs harbored a

G76A mutation. The purest fractions from HPLC purification were pooled and analyzed by analytical HPLC, and mass spectrometry (**Figures S16**).

Histone octamer formation

Histone octamers were formed as previously described.[4] Briefly, lyophilized histones were resuspended in unfolding buffer (20 mM Tris, pH 7.5, 7 M guanidinuim hydrochloride), combined in equimolar amounts and dialyzed into refolding buffer (10 mM Tris, pH 7.5, 2 M NaCl, 1 mM ETDA) for 3 x 2 h. The refolded histone octamers were concentrated and purified by size-exclusion chromatography (Superdex 200 10/300). Fractions were analyzed by SDS-page and the fractions containing equivalent amount of all histones were pooled, concentrated, and stored at -20 °C after the addition of 50% glycerol (v/v) (**Figure S17)**.

DNA preparation

A 153bp segment containing the 601 DNA sequence was prepared as previously described[6] with slight alterations. A plasmid containing 30 copies of the 147 base pair 601 DNA flanked by EcoRV sites (153 bp in total length) was assembled following the general protocol.[6] The plasmid was prepared via alkaline lysis, digested with EcoRV, and purified from the vector with 10% polyethylene glycol-3500 precipitation on ice followed by centrifugation at 26,00 g for 30 min. The 153bp construct was further purified by isopropanol and ethanol precipitation, centrifuged, resuspended in TE buffer (10 mM Tris pH 7.5, and 1 mM EDTA), and quantified by UV spectroscopy and stored in aliquots at -20 ºC.

For array formation, a plasmid containing 12 copies of a 177 base pair repeat of the 601 nucleosome positioning sequence (12-177-601) flanked by EcoRV sites was purified from a 6 L culture of DH5alpha cells as previously described [6]. The 12-177-601 sequence was obtained by preparative digestion of the plasmid followed by selective precipitation of the fragment with 6% polyethylene glycol-6000 on ice followed by centrifugation at 26,000 g for 30 min. After phenol extraction and ethanol precipitation, the DNA was redissolved in TE buffer (10 mM Tris pH 7.5, 1 mM EDTA), quantified by UV spectroscopy and stored in aliquots at -20 °C.

Mononucleosome/nucleosome array reconstitution

Mononucleosomes and nucleosomal arrays were prepared by a gradual dialysis of DNA and histone octamers (ratios were empirically optimized) into a low salt buffer, or by serial dilution.[4] Partial micrococcal nuclease digestion and restriction enzyme digests were utilized to assess array quality (**Figure S13**). Chromatinized plasmids containing various H2B-Ub constructs were prepared as previously reported.[7,8]

Fluorescence–based chromatin compaction assay

Fluorescence measurements were performed in measurement buffer (10 mM Tris, pH 7.8, 10 mM KCl) containing appropriate amounts of $MgCl_2$ as previously described [4]. For measurements in the absence of Mg^{2+}, the buffer contained 0.1 mM EDTA. All buffers were degassed before use by sonication. Nucleosomal array concentrations were 50 nM (per nucleosome) for all measurements. Samples containing different Mg^{2+}

concentrations were prepared fresh by mixing an equivalent volume of array stock (100 nM per nucleosome) with measurement buffer containing twice the final amount of $MgCl_2$ and equilibrating for 15 min at RT before measurement. Measurements were performed on a Fluorolog-3 instrument (HORIBA Jobin Yvon) equipped with automated dual polarizers and using a Sub-Micro Fluorometer cell (Starna Cells) with 10-mm path length. The excitation wavelength was 480 nm with a bandwidth of 5 nm, and emission was recorded at 520 nM with 5 nm bandwidth. Six to eight measurements were taken per sample with an integration time of 5 sec for V/V, V/H, H/H and H/V polarizer settings (excitation/emission, V: vertical polarization, H: horizontal polarization). The final anisotropy was calculated by the formula $SSA = (I_{VV} - G \times I_{VH})/(I_{VV} + 2 \times G \times I_{VH})$, with $G = I_{HV}/I_{HH}$. Vertically polarized emission intensity was monitored as a function of $[Mg^{2+}]$ for all samples to ensure no loss of signal (**Figure S13d**). Data presented is the average of 3 independent experiments ± s.e.m.

Synthesis of Int^C-H2B-Ub and H2B-uLL for in vivo delivery

The Int^C-H2B-Ub branched protein consists of human histone H2B residues 117-125, ubiquitinated at lysine 120, and fused to the C-terminus of Npu^C (referred herein as Int^c). The protein contains two point mutations: A117C in the histone H2B backbone and G76A in ubiquitin. uLL additionally contains the two UbL71/73A mutations.

The linear sequence corresponding to the Int^C-H2B(117-125) fusion was synthesized as a C-terminal carboxylic acid on a Wang ChemMatrix resin using the Liberty Microwave Synthesizer. H2B residues Cys^{117} and Lys^{120} were incorporated as Fmoc-Cys(Acm)-OH

(Novabiochem) and Fmoc-Lys(Alloc)-OH, respectively. Following chain assembly, the Alloc group was deprotected, and Fmoc-Cys(Trt) was coupled to the ε-amine of histone H2B Lys^{120}. Following Fmoc deprotection, the peptide was cleaved from the resin and purified by RP-HPLC with 20-35 % B to yield the desired Acm-protected peptide.

The final product Int^{C}-H2B-Ub and Int^{C}-H2B-uLL was formed by expressed protein ligation (EPL) of HA-Ub-MES and the branched Int^{C}-H2B(117-125) peptide followed by radical desulfurization and Acm deprotection. EPL was initiated by dissolving the reactants (~1 mM each) in ligation buffer (6 M guanidine, 300 mM phosphate buffer, 15 mM TCEP, 50 mM MPAA, pH 7.8) previously degassed with Ar. The reaction was allowed to proceed overnight at room temperature and the ligated product isolated by RP-HPLC purification using a 25-60% solvent B gradient. Radical desulfurization was performed by dissolving the ligation product (1.5 mM) in degassed desulfurization buffer (6 M guanidine, 100 mM phosphate, 40 mM reduced glutathione; pH 6.5) in the presence of 20 mM radical initiator VA-061 (Wako Pure Chemical Industries; Osaka, Japan) followed by overnight incubation. The desulfurized product was purified by RP-HPLC using 30-50% solvent B linear gradient and dissolved to 1 mg/mL in 25% acetic acid/H_2O. Acm deprotection was then performed by adding 2.5 eq. mercury(II) acetate for 2.5 hr. with agitation. The deprotection was quenched by addition of Acm quenching solution (6 M guanidine, 100 mM DTT, and 0.1 % TFA) and incubation at r.t. for 1 h. The final products Int^{C}-H2B-Ub and Int^{C}-H2B-uLL was purified by RP-HPLC using a 30-50% B gradient **(Figure S16)**.

In nucleo H2B-Ub semisynthesis and subsequent H3K79 methylation assays

293T cells (10^7) were transfected with a plasmid encoding the fusion construct, H2B(1-116)-Int^N, using lipofectamine (Invitrogen) according to manufacture's protocol. After 24 hours, cells were harvested on ice in cold PBS and flash frozen. The cell pellet was resuspended in hypotonic lysis buffer (10 mM Tris base, 15 mM sodium chloride, and 1.5 mM magnesium chloride and protease inhibitors; pH 7.6) to osmotically rupture the plasma membrane while leaving nuclei intact. The lysate was centrifuged at 400g for 5 minutes to separate nuclei from the cytoplasmic fraction, and the pellet (nuclear fraction) was resuspended in hypotonic lysis buffer for homogenization with 10 strokes of a loose pestle Dounce homogenizer to help lyse cells and rid nuclei of contaminating membranous organelles. Nuclei were centrifuged and equilibrated in nucleus delivery buffer (20 mM Hepes, 1.5 mM magnesium chloride, 150 mM potassium chloride, 1 mM DTT, 1 mg/mL BSA, 1 mM ATP, protease inhibitors, pH 7.6) via centrifugation/resuspension. Nuclei (0.5 mL in nucleus delivery buffer) were then treated with the relevant split intein fusion construct (Int^C-H2B(117-125)-Ub or Int^C-H2B(117-125)-uLL) at 1 μM and in the presence of SAM and Acetyl-CoA (10 μM each). Reactions were allowed to proceed for 30 minutes at 37 °C before being washed with nucleus delivery buffer and then quenched by the addition of iodoacetamide to a final concentration of 80 mM. (Note, iodoacetamide reacts with key cysteines in the intein to block splicing). Nuclei were again washed and resuspended in 100 μL of sucrose-based buffer (10 mM Tris base, 0.25 M sucrose, 3 mM calcium chloride and protease inhibitors, pH 7.5). After a short sonication (5 seconds, 35% amplitude on a rod sonicator), 1 μL of MNase (NEB) was added and allowed to react for 20 minutes at 37 °C. The reaction was

quenched by the addition of 5 mM EGTA, after which it was diluted with an equal volume of lysis buffer (10 mM Tris-HCl, 100 mM sodium chloride, 1 mM EDTA, 0.5 mM EGTA, 0.1 % sodium deoxycholate, 0.5 % N-lauroylsarcosine and protease inhibitors, pH 8) and triton was added to a final concentration of 1 %. Reactions were cleared by a 10 minutes 10,000 g centrifugation after and resolved by SDS-PAGE followed western blotting with the indicated antibodies.

Mutagenesis of hDot1L(1-416)

hDot1L(1-416)C44S/C74S/C178S (referred as hDot1L in the main text)

MGEKLELRLKSPVGAEPAVYPWPLPVYDKHHDAAHEIIETIRWVSEEIPDLKLAMENYV
LIDYDTKSFESMQRLSDKYNRAIDSIHQLWKGTTQPMKLNTRPSTGLLRHILQQVYNHS
VTDPEKLNNYEPFSPEVYGETSFDLVAQMIDEIKMTDDDLFVDLGSGVGQVVLQVAAAT
NSKHHYGVEKADIPAKYAETMDREFRKWMKWYGKKHAEYTLERGDFLSEEWRERIANTS
VIFVNNFAFGPEVDHQLKERFANMKEGGRIVSSKPFAPLNFRINSRNLSDIGTIMRVVE
LSPLKGSVSWTGKPVSYYLHTIDRTILENYFSSLKNPKLREEQEAARRRQQRESKSNAA
TPTKGPEGKVAGPADAPMDSGAEEEKAGAATVKKPSPSKARKKKLNKKGRKMAGRKRGR
PKK

A 6XHis-Sumo tagged cysteine-less hDot1L(1-416) construct was prepared and cloned into a pET30 vector prepared via sequence and ligation-independent cloning using a 6XHis-SUMO PCR product and a PCR product prepared from the previously reported hDot1L(1-416) construct[1] where all cysteine residues (C44S/C74S/C178S) were mutated to serine. The mutagenic primers used prepare a cysteine-less hDot1L(1-416):

Forward primer (C44S) 5'- ACC ATC CGA TGG GTC AGT GAA GAA ATC CCG G -3' and reverse, 5'- CCG GGA TTT CTT CAC TGA CCC ATC GGA TGG T -3'. Forward primer (C74S) 5'- AGC ATG CAG AGG CTC AGC GAC AAG TAC AAC C -3' and reverse, 5'- GGT TGT ACT TGT CGC TGA GCC TCT GCA TGC T -3'. Forward primer

(C178S) 5'- TGC TGC TGC CAC CAA CAG CAA ACA TCA CTA TGG -3' and reverse, 5'-CCA TAG TGA TGT TTG CTG TTG GTG GCA GCA GCA-3'.

Primers to create hDot1L(1-416) PCR product:

Sumo-hDot1 5'-GCT CAC AGA GAA CAG ATT GGT GGT ATG GGG GAG AAG CTG GAG CTG -3'

hDot1L–pET30 vector 5'-CCG CAA GCG CGG GCG CCC CAA GAA GTA GGA ATT CGA GCT CCG TCG ACA AGC TTG CGG C-3'

hDot1L(1-416) preparation

E. coli BL21(DE3) cells were transformed with plasmids containing the 6XHis-SUMO-hDot1L(1-416) fusion and grown in 6L of Luria-Bertani (LB) media (50 ug/L kanamycin) at 37 °C until an OD600 of 0.6. Overexpression of the desired protein was induced by the addition of 0.5 mM IPTG and the cells were grown for an additional 18 h at 18 °C. The cells were then harvested by centrifugation at 10k x *g* for 15 min and the cell-pellet was resuspended in buffer C (50 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.5 at 4 °C). The cells were lysed by passage through a French Press and the soluble fraction separated from insoluble cellular debris by centrifugation at 18.5-20k x *g* for 20 min. After filtration through a 0.45 μm filter, supernatants were bound to a 5 mL Ni-NTA column, pre-equilibrated with ten column volumes of buffer C, for 12 h at 4 °C. The resin was washed with 10 column volumes of buffer C, followed by 10 column volumes each of column buffer C containing 25mM, 50mM, and 100mM imidazole. His-SUMO-hDot1L(1-416) was eluted with 10 column volumes of buffer B containing 250mM

imidazole by collecting 1 column volume fractions which were analyzed by SDS-PAGE and the purest fractions were pooled. The His-SUMO tag was cleaved from hDot1L(1-416) at 4 °C by the addition of SUMO protease. Cleavage was monitored by SDS-PAGE, and upon 100% cleavage hDot1L(1-416) was purified by cation exchange chromatography using a High Trap SP FF 5ml column (gradient is 100mM NaCl to 1M NaCl over 10 column volumes). Fractions were analyzed by SDS-PAGE, and the purest fractions were pooled, concentrated and purified further using gel filtration. Fractions were analyzed by analytical SEC FPLC, pooled and stored at -80 °C in 50% glycerol (**Figure S12**).

hDot1L methyltransferase assays

Methyltransferase assays were performed using an optimized protocol similar to that previously described [10]. 1.5 pmol of nucleosomes and 0.2 pmol of hDot1L catalytic domain or 0.3 pmol full-length hDot1L were equilibrated in 20 μL of assay buffer (20 mM Tris 7.9, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF). 250 nCi of [^{3}H]-S-adenosyl methionine was added and the reaction was incubated at 30 °C for 15 minutes. 15 μL of the reaction mixture was run on a Criterion 5% TBE gel in 0.5x TBE buffer, followed by staining with ethiduim bromide, or Sybr Gold. The gel was incubated in Amplify solution for 15 min. and then dried and visualized by fluorography. The remaining 5 μL of the reaction mixture was spotted on Whatman p81 filter paper and washed three times with $NaHCO_3$ solution (pH 9) and air dried. Econo F Liquid Scintillation Cocktail (GE healthcare) was then added, and the samples were counted with a LKB Wallac 1209 RackBeta Primo Liquid scintillation counter. Data presented is the average of 3 to 6

independent experiments ± s.e.m.

Preparation of full-length hDot1L

Full-length hDot1L was purified from a baculovirus expression system as previously described[1,5] and stored at -80 °C in 50% glycerol (**Figure S12**).

ySet1C preparation

Recombinant ySet1C was prepared as previously described [9] and stored at -80 °C in 50% glycerol (**Figure S12**).

ySet1C methyltransferase assays

Methyltransferase assays were performed using a slightly altered protocol from previous reports [9]. Nucleosomes (3 pmol) and ySet1C (containing 50 ng of Bre2) were equilibrated in 40 μL of HEG buffer (25 mM HEPES pH 7.6, 0.1 mM EDTA, 10% Glycerol). 0.7 μCi of [^{3}H]-S-adenosyl methionine was added and the reaction was incubated at 30 °C for 2 h. 40 μL of the reaction was run on a Criterion 5% TBE gel in 0.5x TBE buffer, followed by staining with Sybr Gold stain. The gel was incubated in Amplify solution for 15 min. and then dried and visualized by fluorography.

References

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2. Chatterjee, C., McGinty, R.K., Fierz, B. & Muir, T.W. Disulfide-directed histone ubiquitylation reveals plasticity in hDot1L activation. *Nat Chem Biol* **6**, 267-9 (2010).
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9. Kim, J. et al. The n-SET domain of Set1 regulates H2B ubiquitylation-dependent H3K4 methylation. *Mol Cell* **49**, 1121-33 (2013).

Supplementary Figures

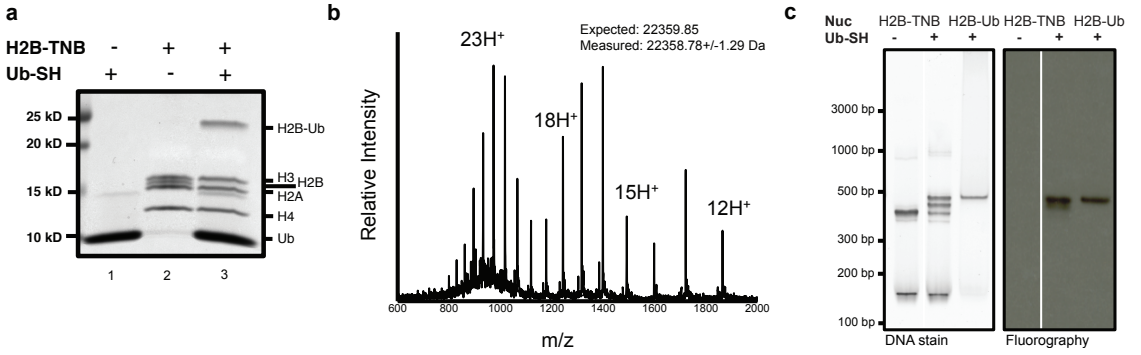


Figure S1. SDS-PAGE analysis and mass spectrometry of H2B-Ub after nucleosomal asymmetric disulfide ligation. (**a**) SDS-PAGE gel of H2B-TNB nucleosomes before and after addition of Ub-SH visualized by coomassie staining. The appearance of a band that runs at the molecular weight of H2B-Ub is present when Ub-SH is added to H2B-TNB containing nucleosomes (lane 3). (**b**) ESI-MS of purified H2B-Ub after ligation to nucleosome. (**c**) Non-cropped ethidium stained native-PAGE gel and fluorograph from Figure 1c. Note, ethidium stained DNA band in H2B-TNB lanes (1 and 2) running at ~150bp is free nucleosomal DNA.

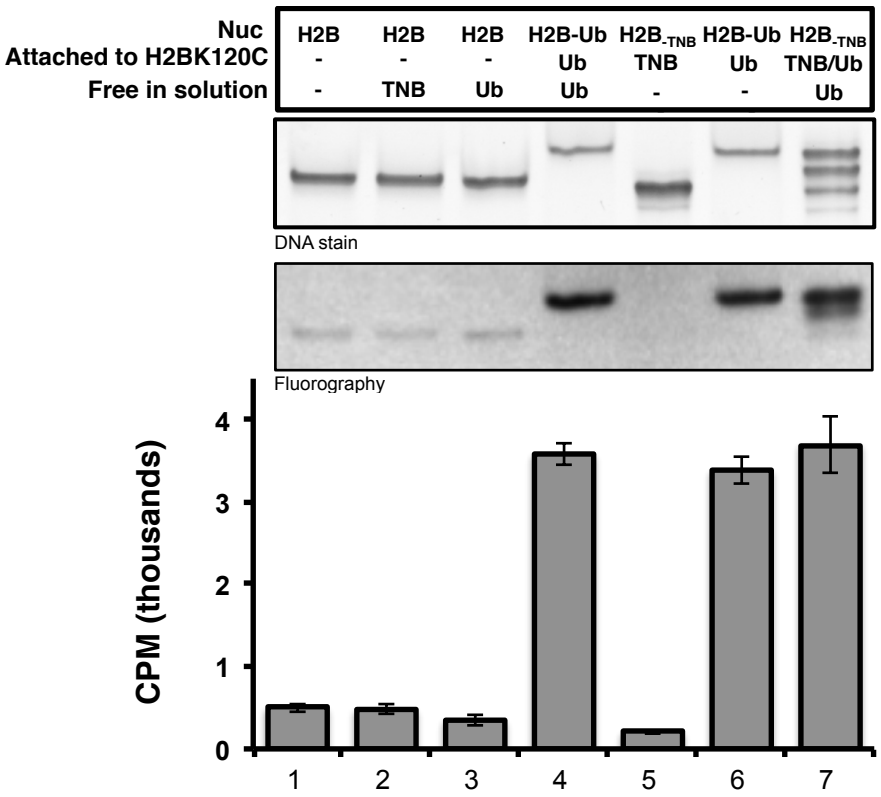


Figure S2. hDot1L methyltransferase assays on nucleosomes with H2B-Ub and Ub. ^{3}H-SAM methyltransferase assays were performed on unmodified (H2B), H2B-TNB, and H2B-Ub nucleosomes in the presence or absence of TNB or Ub. Note the row 'attached to H2BK120C' refers to all species attached to H2BK120C regardless of whether the ligation was performed at the nucleosome or histone level. Ub ligated to H2B-TNB nucleosomes (top panel, lane 7). Nucleosomes were visualized by native-PAGE followed by Sybr Gold staining (top panel), and ^{3}H-methyl incorporation was probed by fluorography (middle panel). Quantification of methylation was performed by p81 filter binding assays followed by liquid scintillation counting (bottom panel). ^{3}H-methylation was only observed in samples where Ub was ligated to H2B-TNB or in H2B-Ub nucleosomes (middle and bottom panels, lanes 4, 6, 7) and no additional stimulation was observed when Ub was present in the reaction but not ligated to the nucleosome (middle and bottom panels lane 4 versus lane 6). Error bars, s.e.m (n = 3).

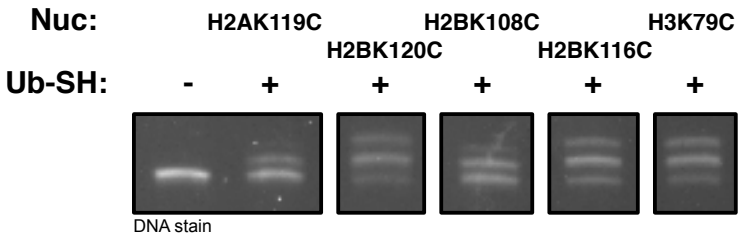


Figure S3. Direct Ub-SH ligations to other single cysteine octamer mutants. Ub-SH was ligated to activated nucleosomes prepared with single cysteine histone mutants. Nucleosomes, and ligation efficiency, were visualized by native-PAGE followed by ethidium bromide staining. The extent of reaction was dependent on the ligation site, however all nucleosomes show Ub ligation.

Ub	**MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG**
Ub1	MQIFVKTL**AAAA**ITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub2	M**A**IFVKTLTGKTIT**AA**VEPSDTIENVKA**A**IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub3	MQIFV**A**TLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKES**A**L**A**LVLRLRGG
Ub4	MQIFVKTLTGKTITLEV**AAAA**TIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub5	MQIFVKTLTGKTITLEVEPSD**A**I**AA**VKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub6	MQIFVKTLTGKTITLEVEPSDTIENVKAKI**AAAA**GIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub7	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGI**AAA**QQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub8	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFA**AAA**LEDGRTLSDYNIQKESTLHLVLRLRGG
Ub9	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQL**AAAA**TLSDYNIQKESTLHLVLRLRGG
Ub10	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLS**AAA**IQKESTLHLVLRLRGG
Ub11	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNI**AAA**STLHLVLRLRGG
Ub12	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLV**AAAA**GG
Ub13	MQIFVKT**A**TGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRL**A**FAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
uRR	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVL**A**L**A**GG
uLL	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLV**A**R**A**RGG

Figure S4. Individual ubiquitin mutant sequences. Ubiquitin mutant amino acid sequences (1-75) are shown for Ub1 through Ub13, uRR, and uLL. In Ub, all residues mutated across all the mutants are colored red and nonmutated residues are bolded. For the ubiquitin mutants the amino acid residues mutated are shown in red.

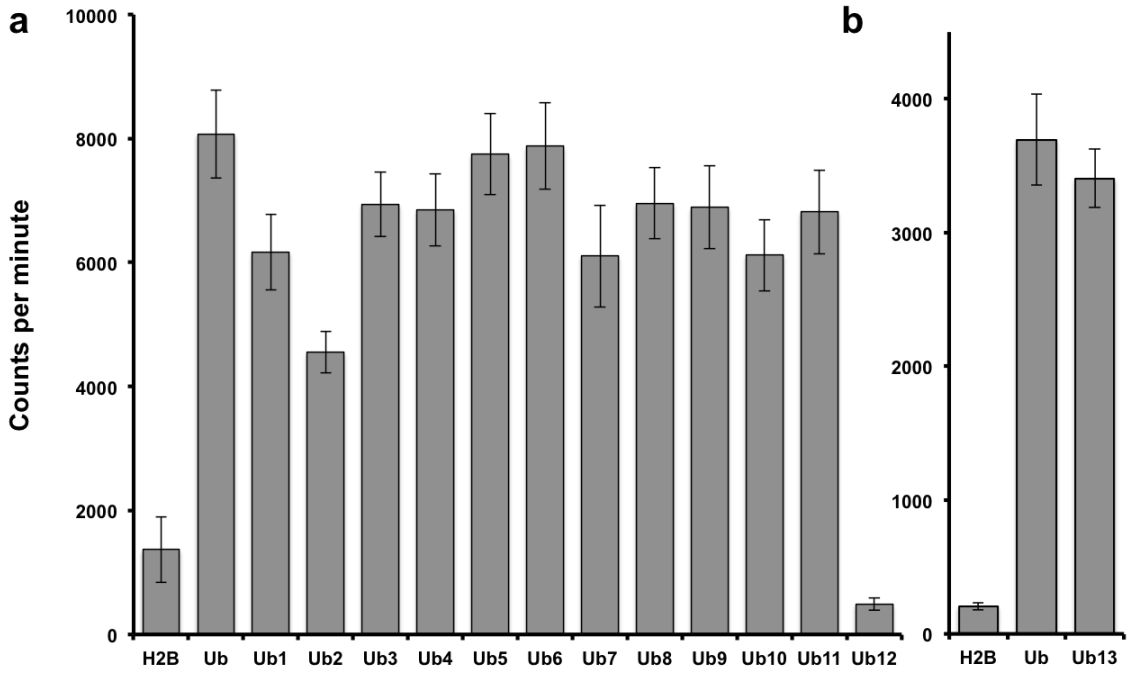


Figure S5. Raw scintillation counts used to calculate Ub efficiency in Figure 3. (**a**)^{3}H-methyl incorporation was monitored by scintillation counting of each Ub1-12 mutant as described in Figure 3, error = s.e.m. (n = 6). (**b**) ^{3}H-methyl incorporation was monitored by scintillation counting of Ub13 as described in Fig 3, error = s.e.m. (n = 3).

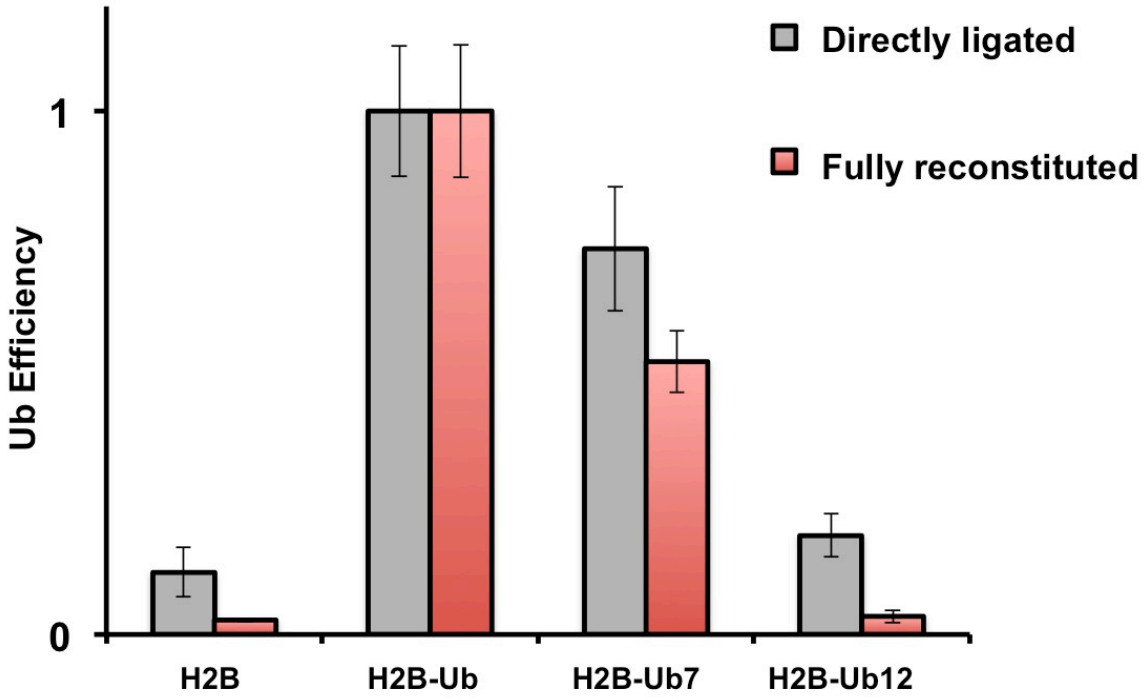


Figure S6. Comparison of hDot1L activity on H2B-Ub and H2B-Ub mutant nucleosomes prepared by H2B-Ub reconstitution at the octamer level versus direct ligation of Ub and Ub mutants to a pre-assembled nucleosome. hDot1L activity towards H2B-Ub and H2B-Ub mutants reconstituted into nucleosomes at the octamer level (referred to as fully reconstituted) are compared to H2B-Ub and H2B-Ub mutants prepared by direct Ub-SH ligation (referred to as directly ligated). ^{3}H-SAM methyltransferase assays were performed and Ub efficiency was calculated to normalize for extent of ubiquitylation (wild-type ubiquitin is set to 1, see supplementary note). Both fully reconstituted and directly ligated nucleosomes show similar results. Ub7 and Ub12 mutations are displayed Figure S4. Error = s.e.m. (n = 3-6).

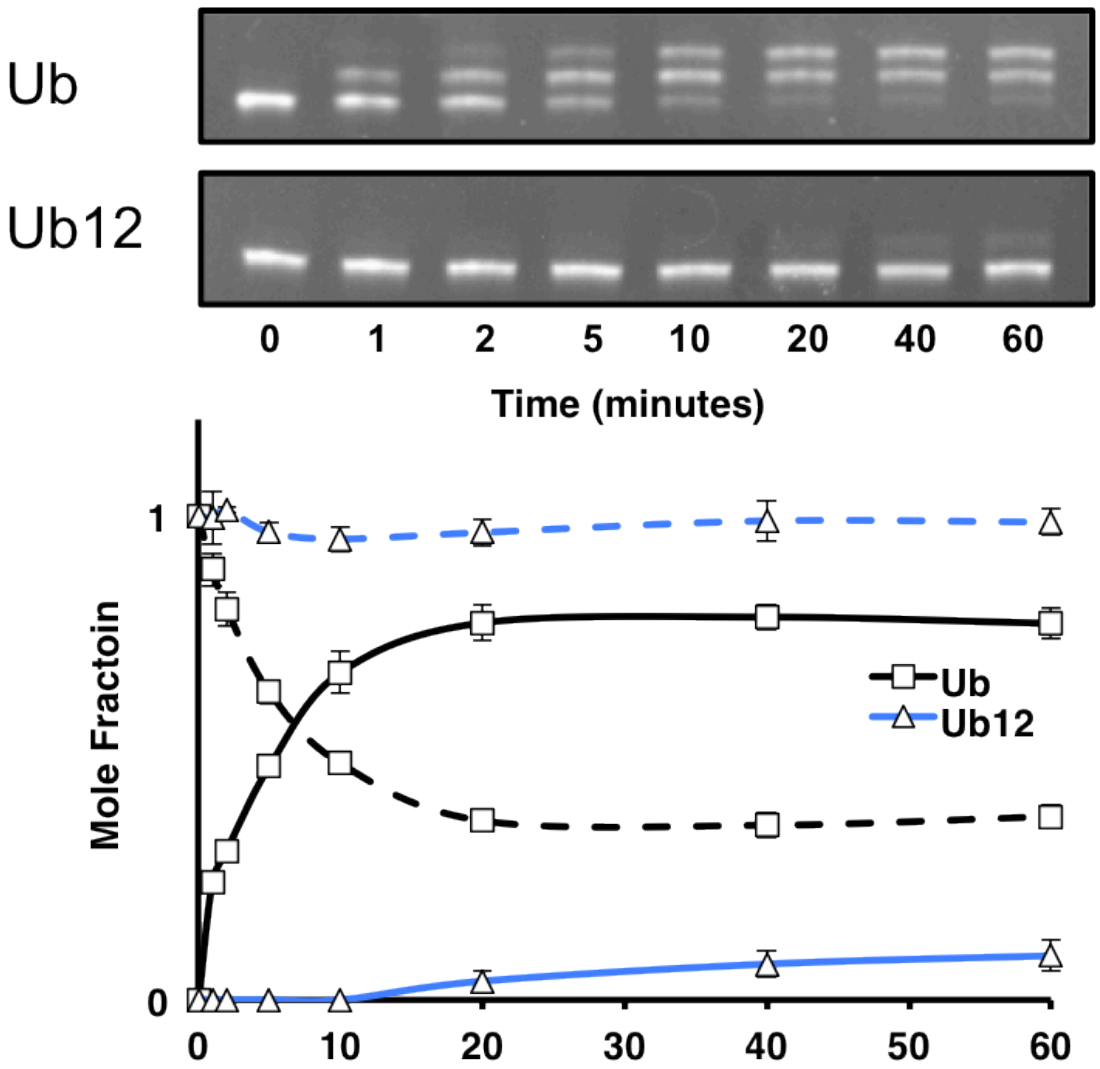


Figure S7. Ligation kinetics of Ub and Ub12 to the H2B-TNB nucleosome. Time courses of ligation assays were performed with the H2B-TNB nucleosomes and either Ub or Ub12. The extent of ligation from the native gel (top) was quantified (bottom) using the imageJ software. Dashed lines represent loss of H2B-TNB and solid lines represent gain of H2B-Ub or H2B-Ub12. Over the course of 60 min., Ub12 showed only 5% ligation to the H2B-TNB nucleosome whereas Ub showed 60%. Error = s.e.m. (n = 3).

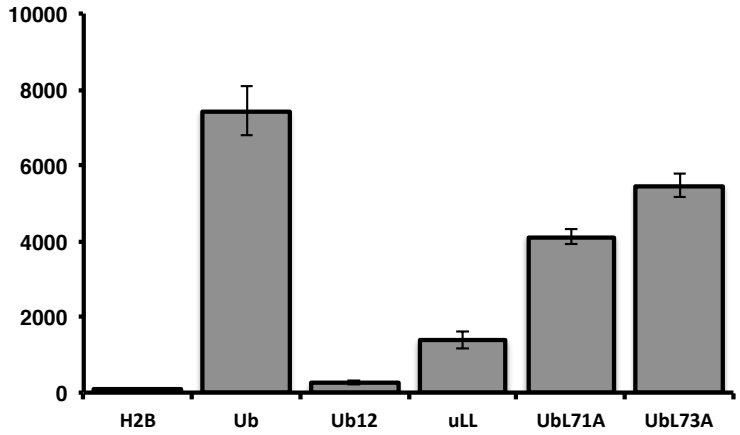


Figure S8. hDot1L assays on nucleosomes containing individual UbL71A and UbL73A point mutants. ^{3}H-SAM methyltransferase assays were performed on H2B, H2B-Ub, H2B-Ub12, H2B-uLL, H2B-Ub-L71A, and H2B-UbL73A nucleosomes. Quantification of methylation was performed by filter binding assays followed by liquid scintillation counting. Error bars, s.e.m (n = 3).

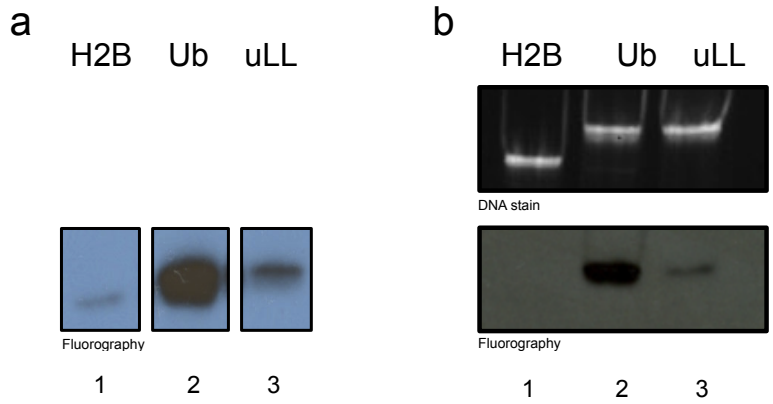


Figure S9. Full-length hDot1L assays on chromatinized plasmids and mononucleosomes. **(a)** H2B-Ub and H2B-uLL containing octamers, generated through expressed protein ligation, were reconstituted into chromatinized plasmids and ^{3}H-SAM methyltransferase assays with full-length Flag-tagged hDot1L were performed and analyzed via fluorography. **(b)** ^{3}H-SAM methyltransferase assays were performed on mononucleosomes (visualized by Sybr Gold, top panel) using full-length hDot1L. ^{3}H-methyl incorporation was visualized by fluorography. H2B-uLL was unable to stimulate hDot1L compared to H2B-Ub (bottom panel, lane 2 versus lane 3).

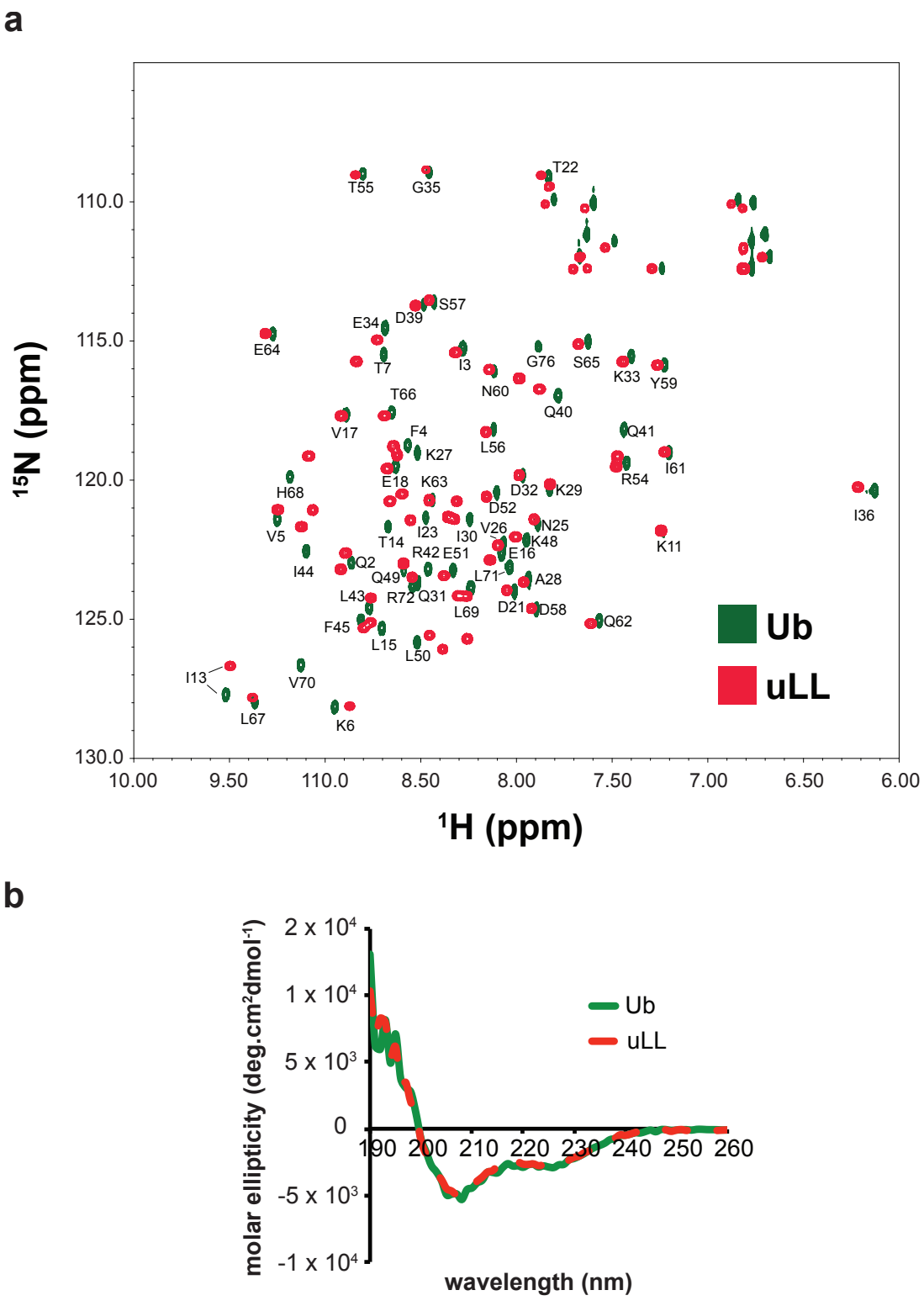


Figure S10. Structural characterization of the uLL mutant. (a) ^{1}H-^{15}N HSQC

spectrum of uniformly ^{15}N labeled uLL was compared to an HSQC spectrum of uniformly ^{15}N labeled Ub. All peaks were annotated from the BMRB databank (accession code 6457). (b) Far-uv circular dichroism spectra of Ub and uLL.

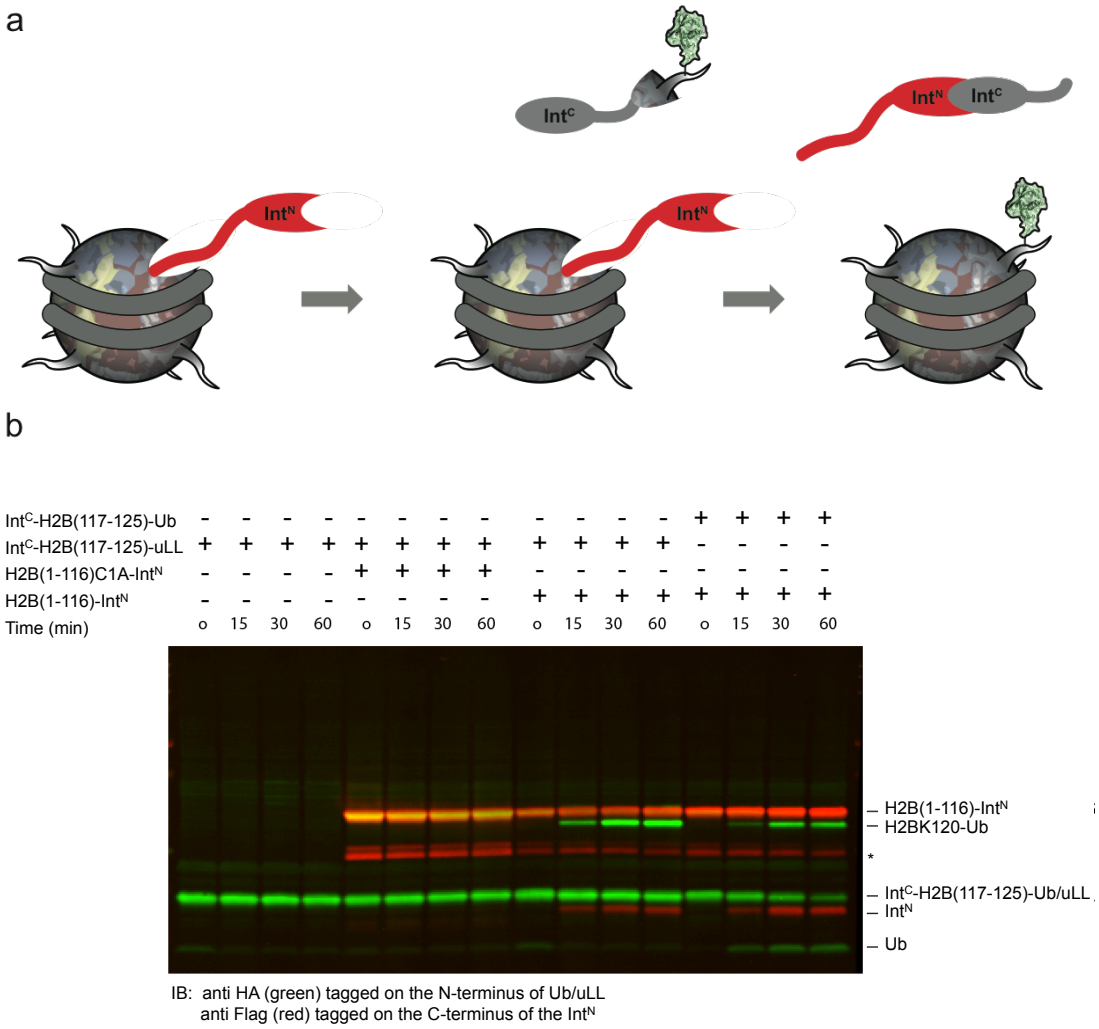


Figure S11. Time course of Int^C-H2B-Ub and Int^C-H2B-uLL ligation *in nucleo*. (**a**) Schematic of *in nucleo* semisynthesis approach used to prepare ubiquitylated chromatin. H2B(1-116)-Int^N was transfected into 293 cells and incorporated into the endogenous chromatin (depicted as one copy of H2B(1-116)-Int^N incorporated into a nucleosome, left panel). Int^c-H2B(117-125)-Ub was added to isolated nuclei containing H2B(1-116)-Int^N (middle panel). Upon association of the Int^N and Int^C fragments, intein *trans*-splicing yielded H2BK120-Ub within the endogenous chromatin (right panel). Int^N is shown in red

and Ub is shown in green to correlate with the IB signals in panel b. (**b**) Int^{C}-H2B(117-125)-Ub and Int^{C}-H2B(117-125)-uLL were added to nuclei derived from 293 cells transfected with H2B(1-116)-Int^{N} and monitored over the course of 60 min (lanes 13-16, and lanes 9-12 respectively). Non-transfected (lanes 1-4) and cells transfected with a catalytically dead intein construct, H2B(1-116)-C1A-Int^{N} (lanes 5-8) were used as controls. The appearance of a HA band (the N-terminal Ub/uLL tag) at the molecular weight of H2B-Ub is indicative of the ligation of H2B(1-116)-Int^{N} and Int^{C}-H2B(117-125)-Ub or Int^{C}-H2B(117-125)-uLL. Note, a nonspecific transfection dependent band is observed at a molecular weight lower than that of H2B(1-116)-Int^{N} but higher than the thiolysed Int^{N} (lanes 5-16, asterisk).

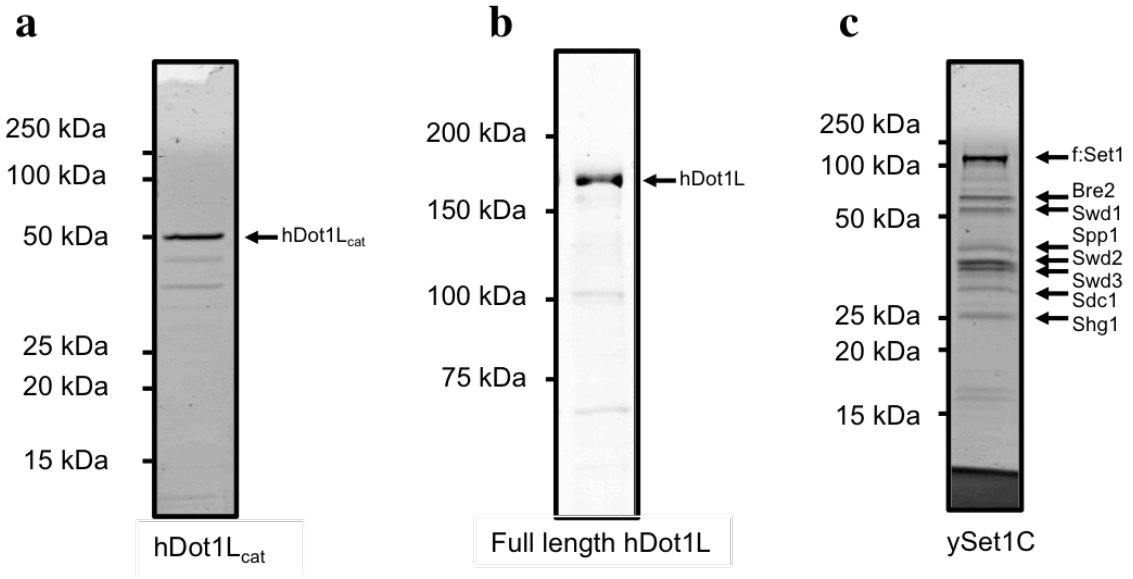


Figure S12. Analysis of hDot1L and ySet1 complex. SDS-PAGE was performed to assess the quality of hDot1L and ySet1C. **(a)** Coomassie stained gel showing hDot1L catalytic domain at a molecular weight around 50kDa (expected MW is 47kDa). **(b)** Coomassie stained gel showing full length hDot1L. **(c)** Coomassie stained gel of ySet1C. All 8 subunits can be observed.

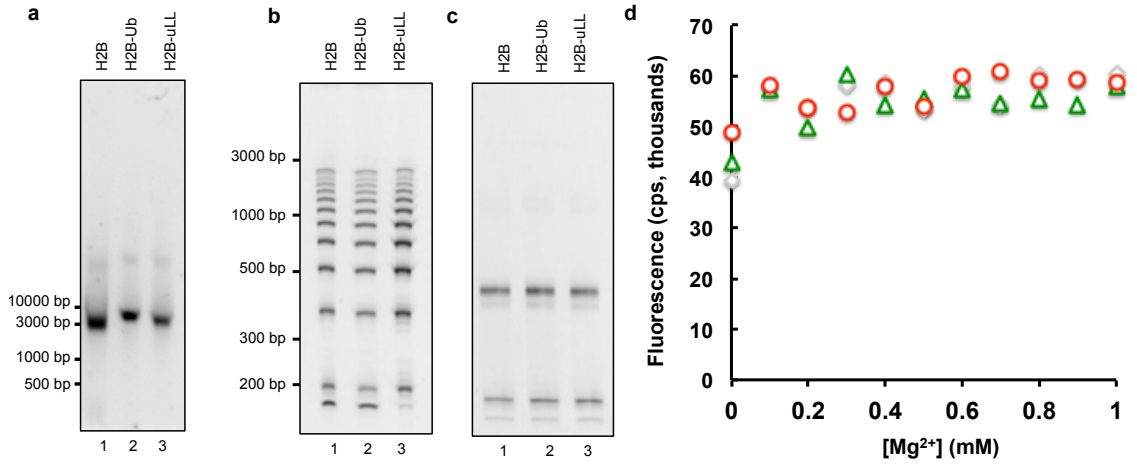
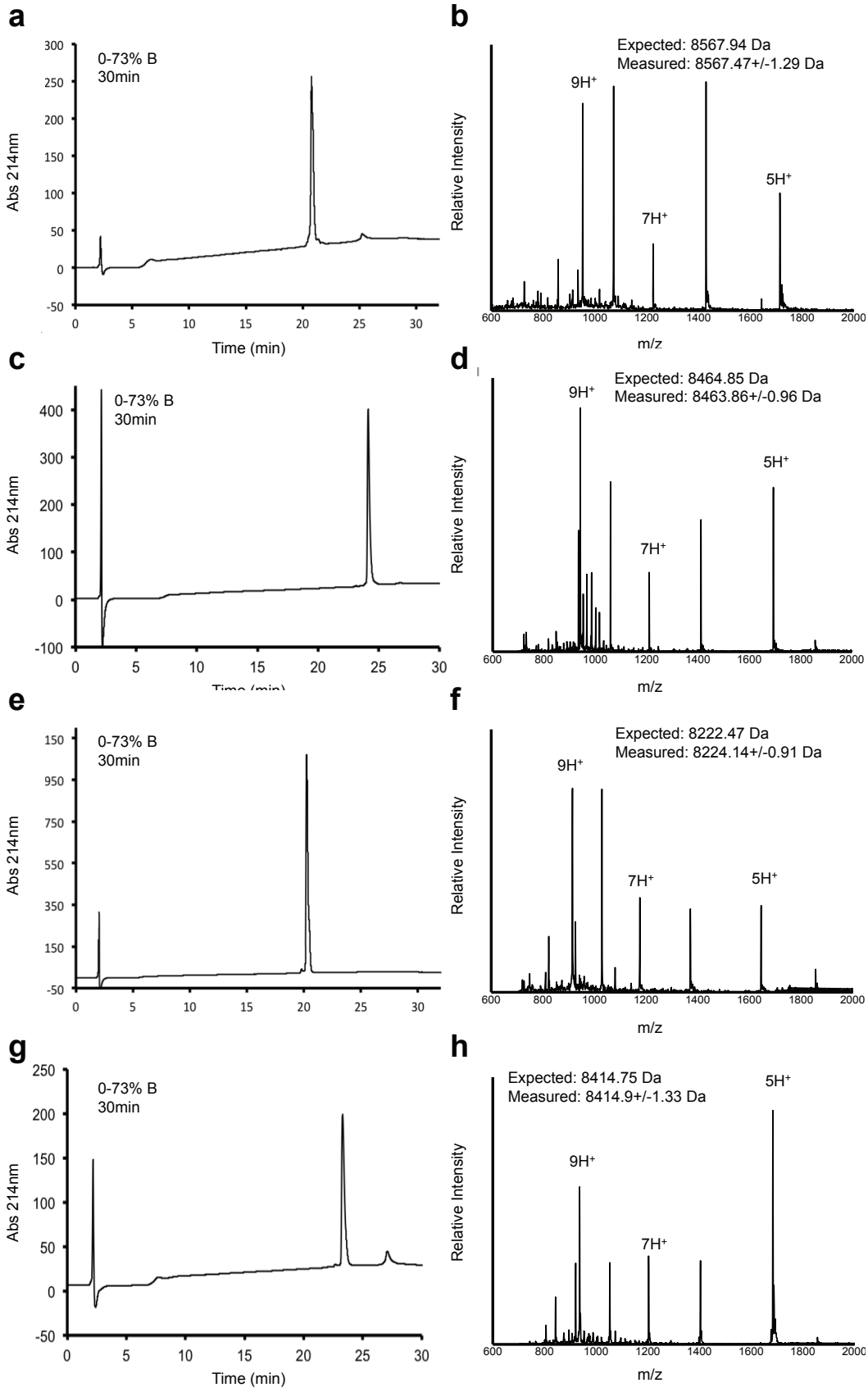


Figure S13. Reconstitution of unmodified, H2B-Ub, and H2B-uLL fluorescently labeled nucleosomal 12-mer arrays. (a) Sybr gold stained native 1% agarose, 1% polyacrylamide gel electrophoresis (APAGE) analysis of reconstituted 12-mer nucleosomal arrays containing unmodified H2B (lane 1), H2B-Ub (lane 2), and H2B-uLL (lane 3). **(b)** Sybr Gold stained native PAGE gel showing micrococcal nuclease (MNase) digests of reconstituted arrays containing unmodified H2B (lane 1), H2B-Ub (lane 2), and H2B-uLL (lane 3). **(c)** Sybr Gold stained native PAGE gel showing Sca1 restriction enzyme digests of reconstituted arrays containing unmodified H2B (lane 1), $H2B_{ss}Ub$ (lane 2), and H2B-uLL (lane 3). **(d)** Fluorescence emission of unmodified (grey diamonds), H2B-Ub (green triangles), and H2B-uLL arrays (red circles) as a function of added Mg^{2+} to the sample. Graph shows vertically polarized fluorescence emission of each sample excited with vertically polarized light. Addition of Mg^{2+} does not affect fluorescence emission in any of these substrates.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Time (min)
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Relative Intensity
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c
0-73% B
30min
Abs 214nm
d
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Measured: 8463.86+/-0.96 Da
9H+
7H+
5H+
Relative Intensity
m/z
e
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30min
Abs 214nm
f
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Measured: 8224.14+/-0.91 Da
9H+
7H+
5H+
Relative Intensity
m/z
g
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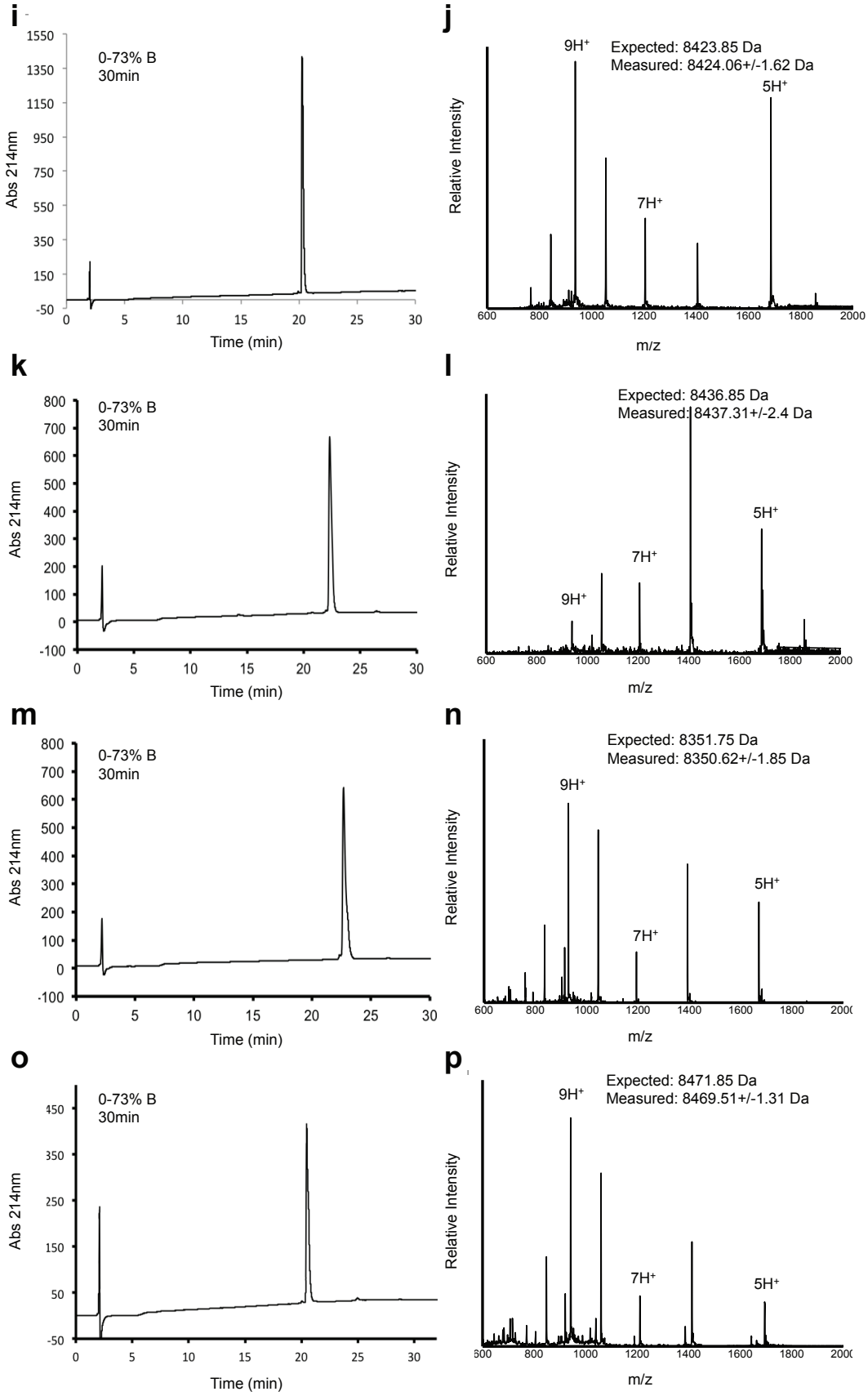
m/z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7H+
9H+
Relative Intensity
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m
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Time (min)
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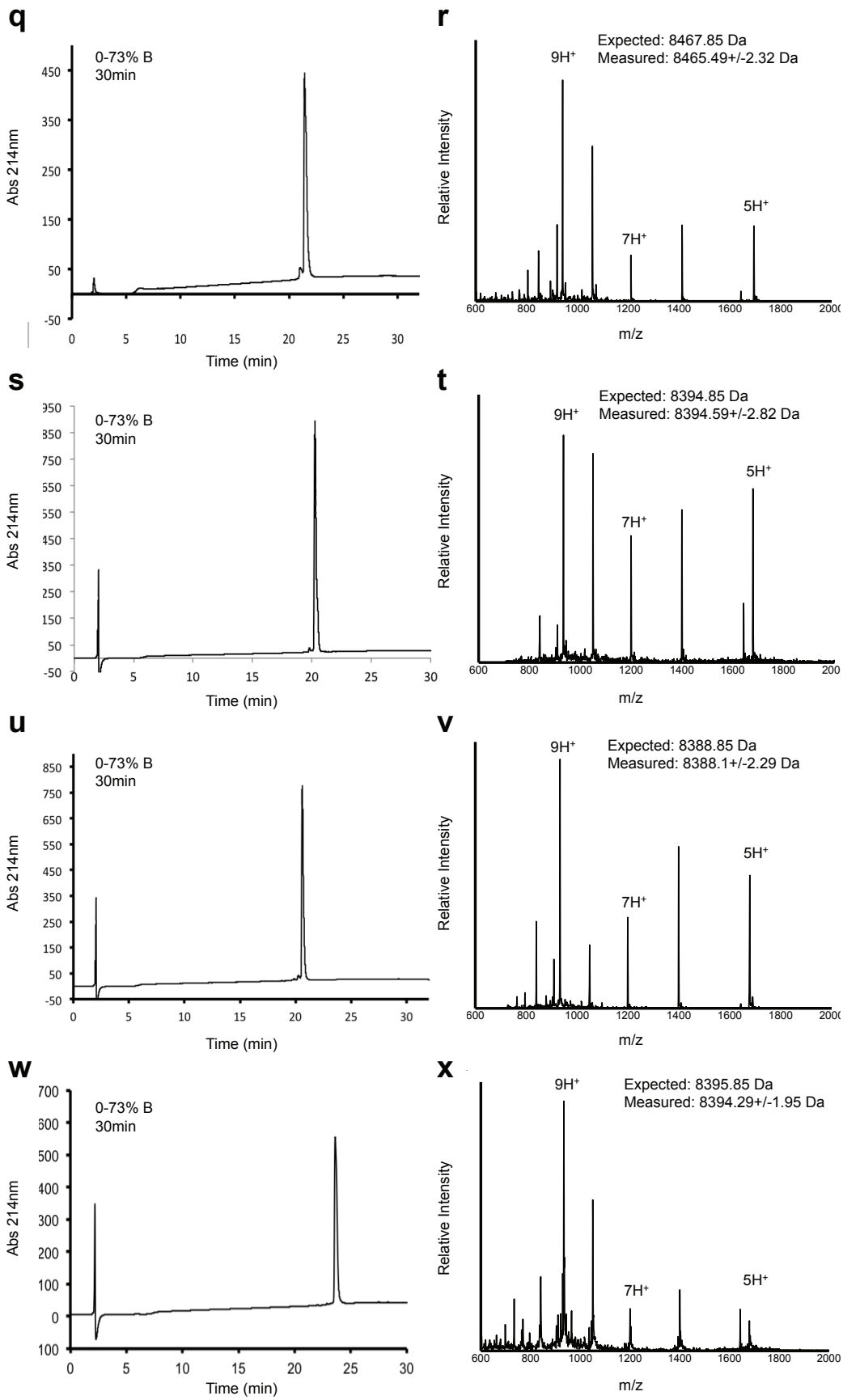
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Relative Intensity
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7H+
5H+
Relative Intensity
m/z
u
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Abs 214nm
Time (min)
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7H+
5H+
Relative Intensity
m/z
w
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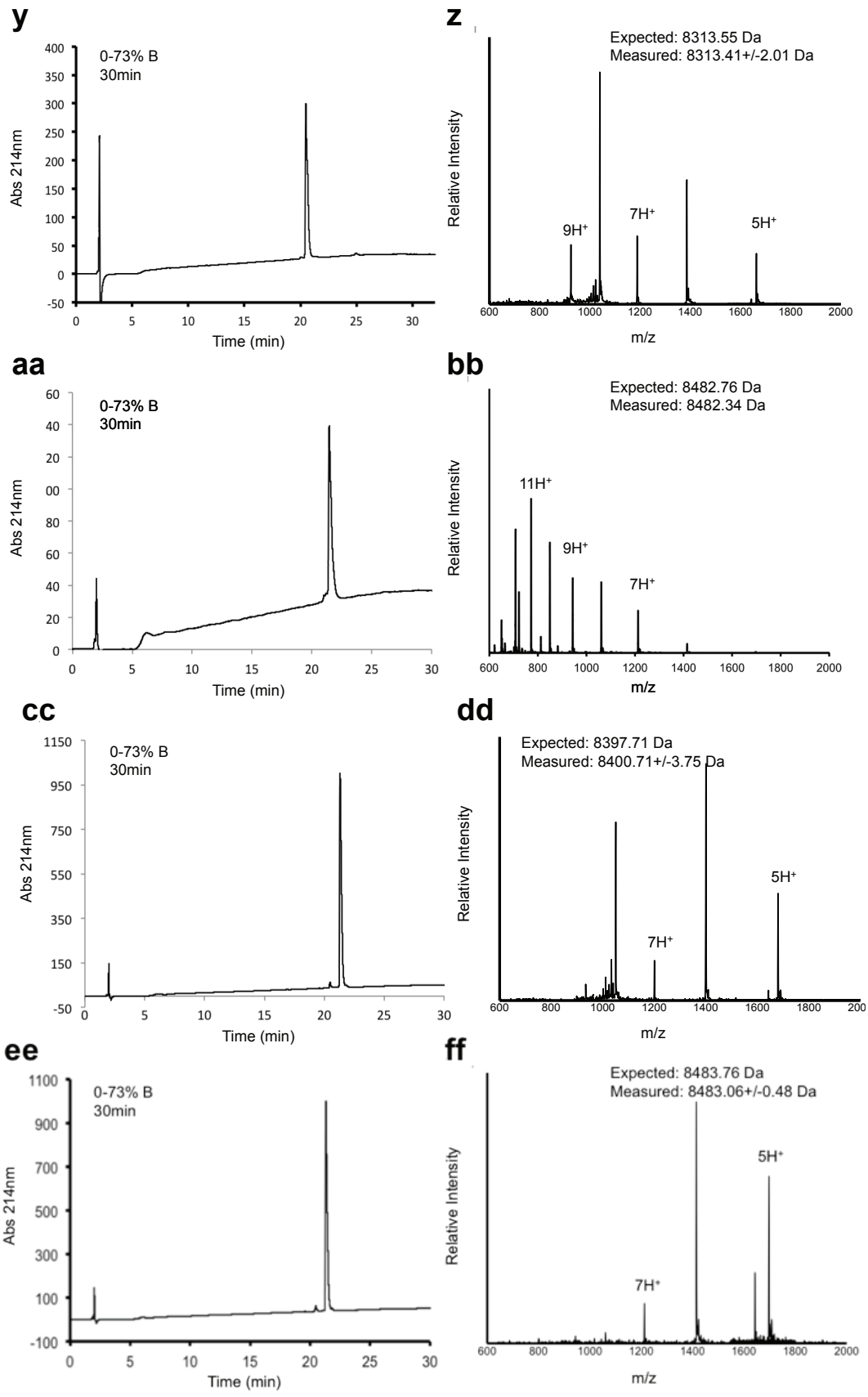
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0-73% B
30min
Abs 214nm
Time (min)
z
Expected: 8313.55 Da
Measured: 8313.41+/-2.01 Da
Relative Intensity
9H+
7H+
5H+
m/z
aa
0-73% B
30min
Abs 214nm
Time (min)
bb
Expected: 8482.76 Da
Measured: 8482.34 Da
Relative Intensity
11H+
9H+
7H+
m/z
cc
0-73% B
30min
Abs 214nm
Time (min)
dd
Expected: 8397.71 Da
Measured: 8400.71+/-3.75 Da
Relative Intensity
5H+
7H+
m/z
ee
0-73% B
30min
Abs 214nm
Time (min)
ff
Expected: 8483.76 Da
Measured: 8483.06+/-0.48 Da
Relative Intensity
5H+
7H+
m/z

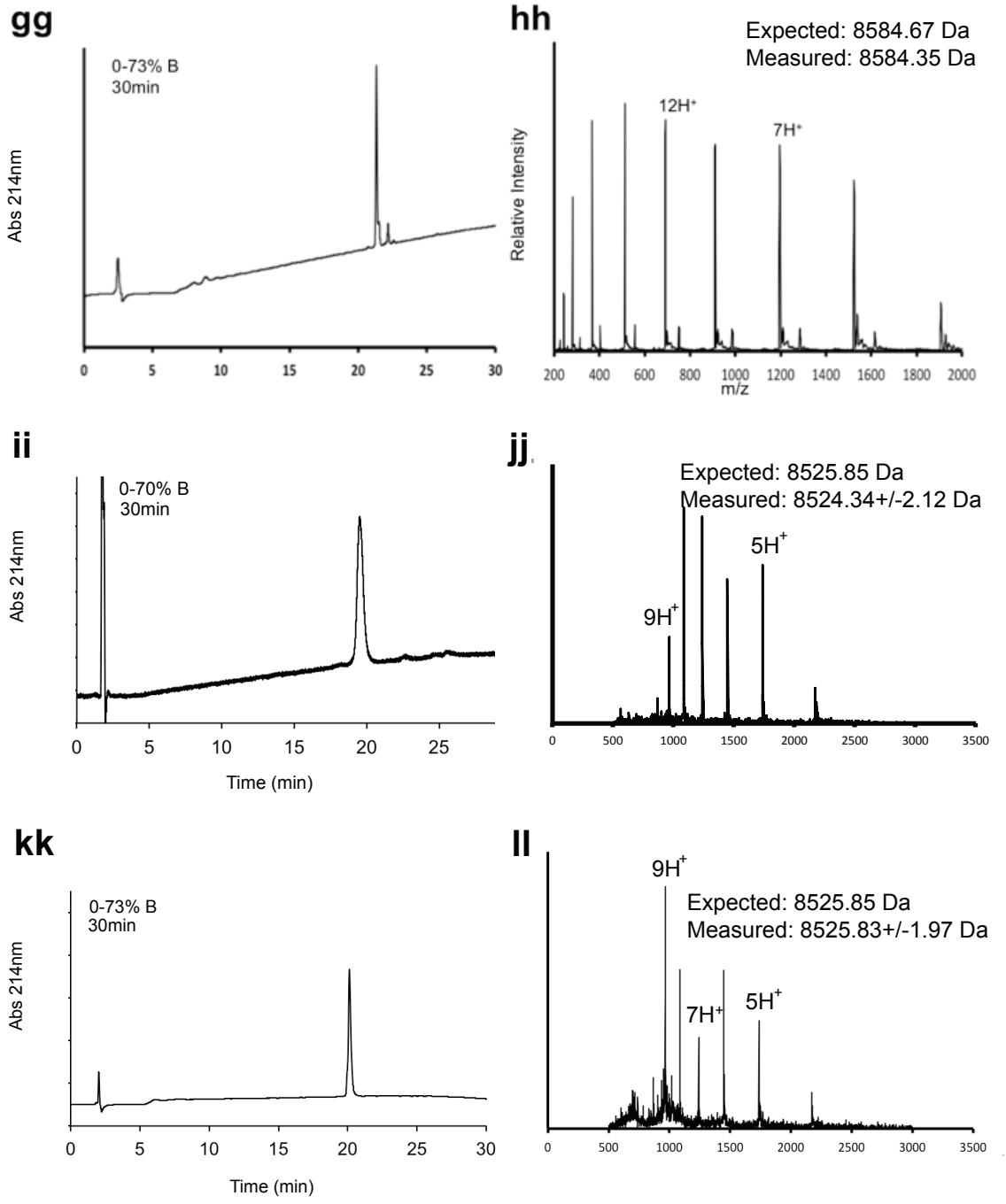


Figure S14. Characterization of Ub and Ub mutants. **(a)** C18 analytical RP-HPLC chromatogram of purified Ub-SH. **(b)** ESI-MS of purified Ub-SH**. (c)** C18 analytical RP-HPLC chromatogram of purified Ub1 **(d)** ESI-MS of purified Ub1**. (e)** C18 analytical RP-HPLC chromatogram of purified Ub2 **(f)** ESI-MS of purified Ub2**. (g)** C18 analytical

RP-HPLC chromatogram of purified Ub3 **(h)** ESI-MS of purified Ub3**. (i)** C18 analytical RP-HPLC chromatogram of purified Ub4 **(j)** ESI-MS of purified Ub4**. (k)** C18 analytical RP-HPLC chromatogram of purified Ub5 **(l)** ESI-MS of purified Ub5**. (m)** C18 analytical RP-HPLC chromatogram of purified Ub6 **(n)** ESI-MS of purified Ub6**. (o)** C18 analytical RP-HPLC chromatogram of purified Ub7. **(p)** ESI-MS of purified Ub7**. (q)** C18 analytical RP-HPLC chromatogram of purified Ub8. **(r)** ESI-MS of purified Ub8**. (s)** C18 analytical RP-HPLC chromatogram of purified Ub9. **(t)** ESI-MS of purified Ub9**. (u)** C18 analytical RP-HPLC chromatogram of purified Ub10. **(v)** ESI-MS of purified Ub10**. (w)** C18 analytical RP-HPLC chromatogram of purified Ub11. **(x)** ESI-MS of purified Ub11**. (y)** C18 analytical RP-HPLC chromatogram of purified Ub12. **(z)** ESI-MS of purified Ub12**. (aa)** C18 analytical RP-HPLC chromatogram of purified Ub13. **(bb)** ESI-MS of purified Ub13**. (cc)** C18 analytical RP-HPLC chromatogram of purified uRR. **(dd)** ESI-MS of purified uRR**. (ee)** C18 analytical RP-HPLC chromatogram of purified uLL. **(ff)** ESI-MS of purified uLL**. (gg)** C18 analytical RP-HPLC chromatogram of purified ^{15}N labeled uLL. **(hh)** ESI-MS of purified ^{15}N labeled uLL. **(ii)** C18 analytical RP-HPLC chromatogram of purified UbL71A. **(jj)** ESI-MS of purified UbL71A. **(kk)** C18 analytical RP-HPLC chromatogram of purified UbL73A. **(ll)** ESI-MS of purified ^{15}N labeled UbL73A.

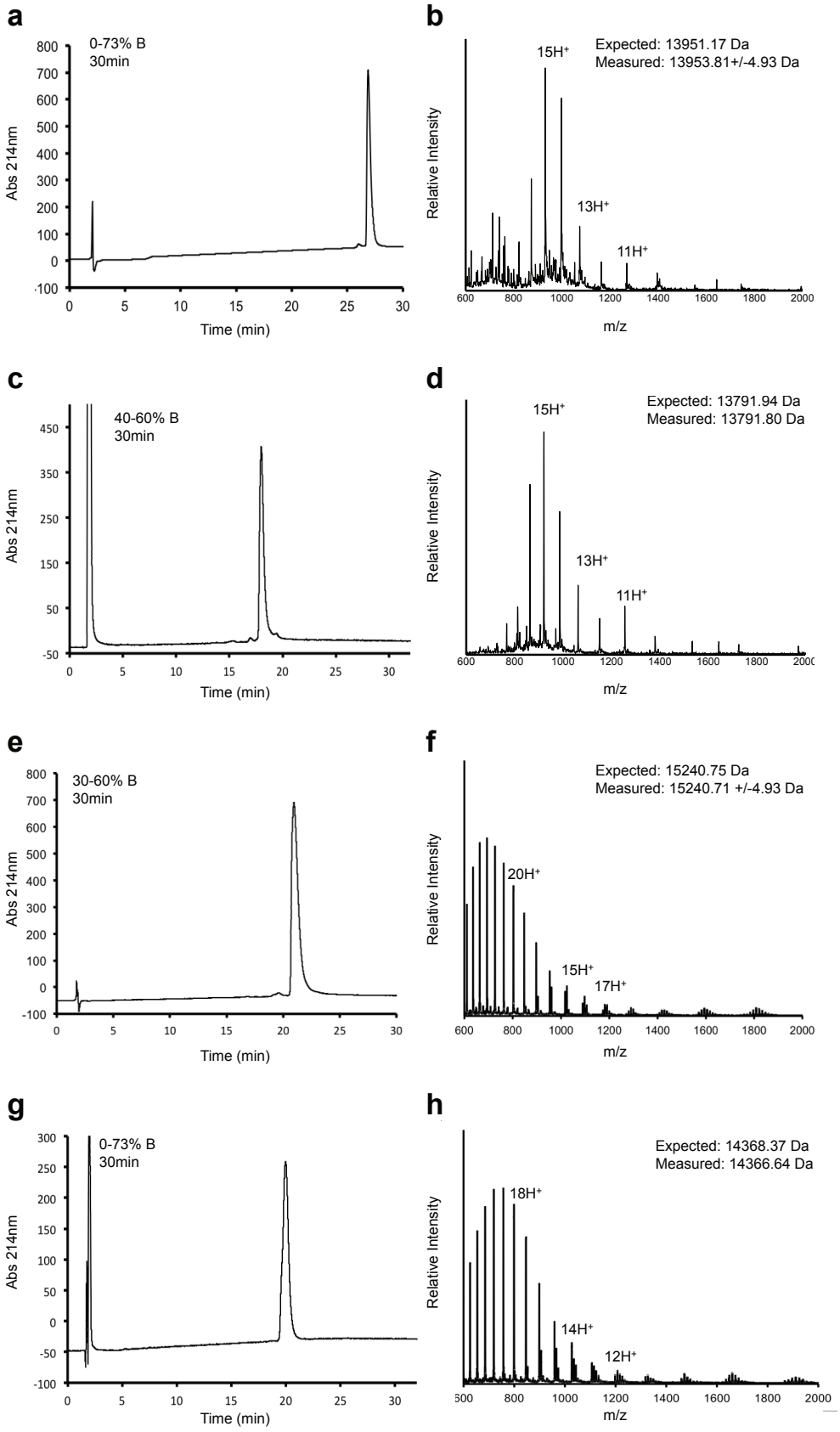
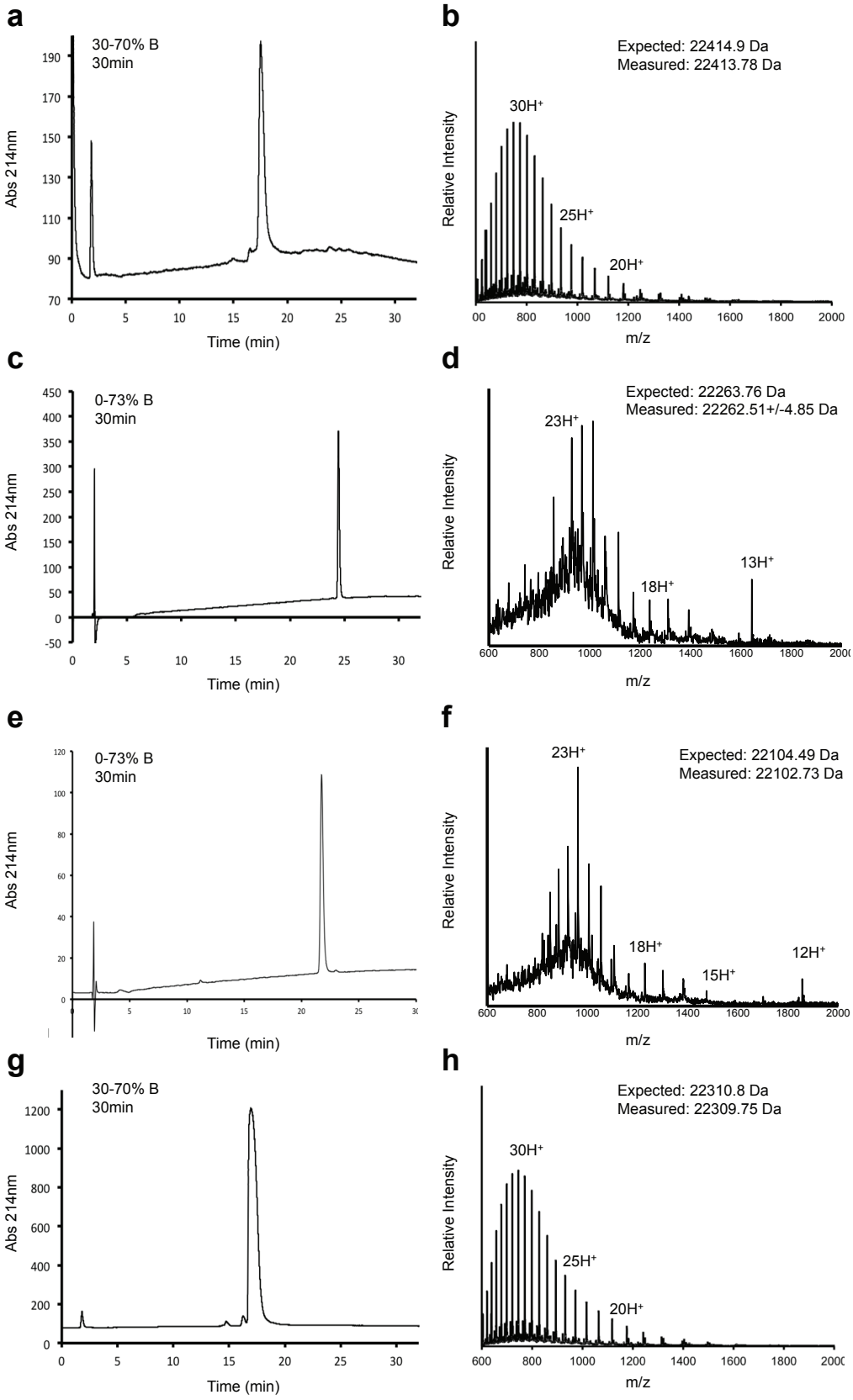

a
0-73% B
30min
Abs 214nm
Time (min)
b
15H+
13H+
11H+
Expected: 13951.17 Da
Measured: 13953.81+/-4.93 Da
Relative Intensity
m/z
c
40-60% B
30min
Abs 214nm
Time (min)
d
15H+
13H+
11H+
Expected: 13791.94 Da
Measured: 13791.80 Da
Relative Intensity
m/z
e
30-60% B
30min
Abs 214nm
Time (min)
f
20H+
15H+
17H+
Expected: 15240.75 Da
Measured: 15240.71 +/-4.93 Da
Relative Intensity
m/z
g
0-73% B
30min
Abs 214nm
Time (min)
h
18H+
14H+
12H+
Expected: 14368.37 Da
Measured: 14366.64 Da
Relative Intensity
m/z

Figure S15. Characterization of H2A, H2BK120C, H3C110S, and fluorescently labeled H2A (fH2A). (a) C18 analytical RP-HPLC chromatogram of purified H2A. **(b)** ESI-MS of purified H2A**. (c)** C18 analytical RP-HPLC chromatogram of purified H2BK120C. **(d)** ESI-MS of purified H2BK120C**. (e)** C18 analytical RP-HPLC chromatogram of purified H3C110S. **(f)** ESI-MS of purified H3C110S**. (g)** C18 analytical RP-HPLC chromatogram of purified fH2A. **(h)** ESI-MS of purified fH2A**.**


a
30-70% B
30min
Abs 214nm
Time (min)
b
Expected: 22414.9 Da
Measured: 22413.78 Da
30H+
25H+
20H+
Relative Intensity
m/z
c
0-73% B
30min
Abs 214nm
Time (min)
d
Expected: 22263.76 Da
Measured: 22262.51+/-4.85 Da
23H+
18H+
13H+
Relative Intensity
m/z
e
0-73% B
30min
Abs 214nm
Time (min)
f
23H+
Expected: 22104.49 Da
Measured: 22102.73 Da
18H+
15H+
12H+
Relative Intensity
m/z
g
30-70% B
30min
Abs 214nm
Time (min)
h
Expected: 22310.8 Da
Measured: 22309.75 Da
30H+
25H+
20H+
Relative Intensity
m/z

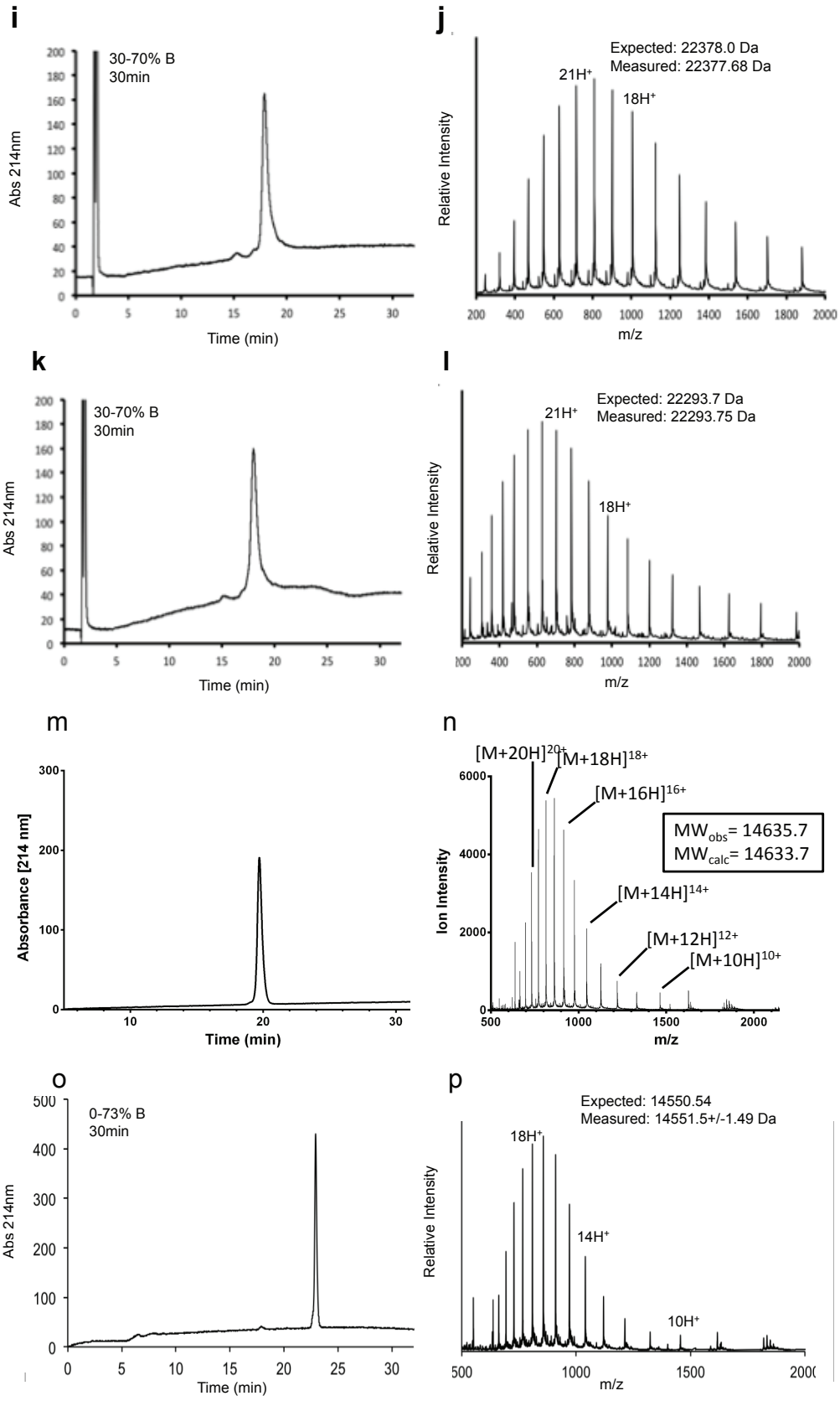

i
30-70% B
30min
Abs 214nm
Time (min)
j
Expected: 22378.0 Da
Measured: 22377.68 Da
21H+
18H+
Relative Intensity
m/z
k
30-70% B
30min
Abs 214nm
Time (min)
l
Expected: 22293.7 Da
Measured: 22293.75 Da
21H+
18H+
Relative Intensity
m/z
m
Absorbance [214 nm]
Time (min)
n
[M+20H]20+
[M+18H]18+
[M+16H]16+
MWobs= 14635.7
MWcalc= 14633.7
[M+14H]14+
[M+12H]12+
[M+10H]10+
Ion Intensity
m/z
o
0-73% B
30min
Abs 214nm
Time (min)
p
Expected: 14550.54
Measured: 14551.5+/-1.49 Da
18H+
14H+
10H+
Relative Intensity
m/z

Figure S16. Characterization of H2B-Ub, H2B-Ub mutants synthesized via assymetric disulfide formation (ss) or expressed protein ligation (epl). **(a)** C18 analytical RP-HPLC chromatogram of purified H2B-Ub(ss). **(b)** ESI-MS of purified H2B-Ub(ss)**. (c)** C18 analytical RP-HPLC chromatogram of purified H2B-Ub7(ss). **(d)** ESI-MS of purified H2B-Ub7(ss)**. (e)** C18 analytical RP-HPLC chromatogram of purified H2B-Ub12(ss). **(f)** ESI-MS of purified H2B-Ub12(ss)**. (g)** C18 analytical RP-HPLC chromatogram of purified H2B-uLL(ss). **(h)** ESI-MS of purified H2B-uLL(ss)**. (i)** C18 analytical RP-HPLC chromatogram of purified H2B-Ub(epl). **(j)** ESI-MS of purified H2B-Ub(epl)**. (k)** C18 analytical RP-HPLC chromatogram of purified H2B-uLL(epl) **(l)** ESI-MS of purified H2B-uLL(epl)**. (m)** C18 analytical RP-HPLC chromatogram of purified Int^{C}-H2B-Ub(epl) **(n)** ESI-MS of purified Int^{C}-H2B-Ub(epl). **(o)** C18 analytical RP-HPLC chromatogram of purified Int^{C}-H2B-uLL(epl). **(p)** ESI-MS of purified Int^{C}-H2B-uLL(epl).

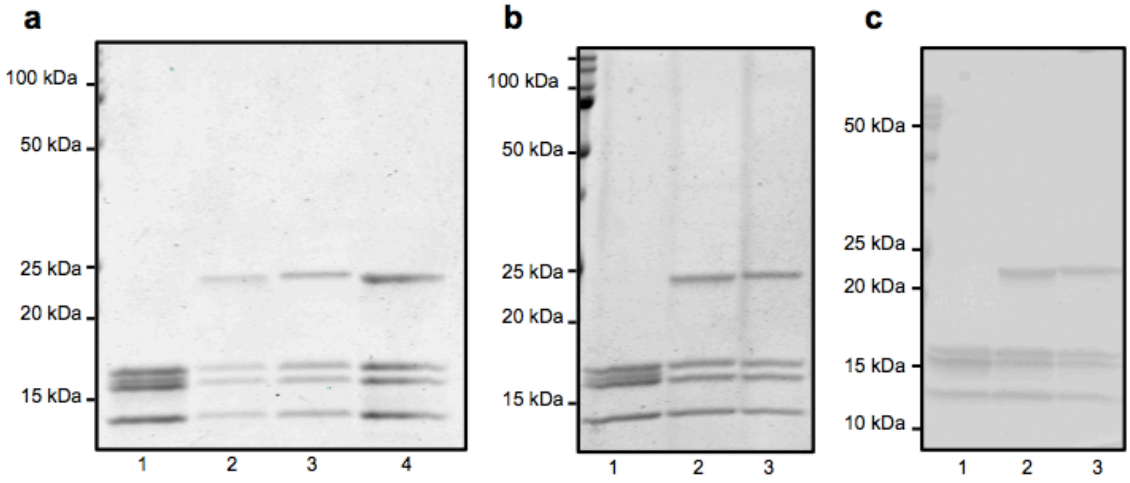


Figure S17. Histone octamers after FPLC purification. **(a)** Coomassie stained SDS-PAGE gel showing unmodified H2B (lane 1), H2B-Ub (lane 2), H2B-Ub7 (lane 3) and H2B-Ub12 (lane 4) octamers after FPLC purification. **(b)** Coomassie stained SDS-PAGE gel showing histone octamers of unmodified H2B (lane 1), H2B-Ub (lane 2), and H2B-uLL (lane 3) after FPLC purification. **(c)** Coomassie stained SDS-PAGE gel showing histone octamers of fH2A, unmodified H2B, (lane 1), fH2A, H2B-Ub (lane 2), and fH2A, H2B-uLL (lane 3) after FPLC purification.