Electronic Supplementary Material (ESI) for Chemical Science.


# Supplementary Information

## Examining histone modification crosstalk using immobilized libraries established from ligation-ready nucleosomes

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## Experimental procedures

### 1. Materials and general methods

Standard Fmoc-amino acid derivatives and HBTU (2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate) were purchased from GLS (Shanghai, China). Amino acid derivatives were obtained from Novabiochem (Darmstadt, Germany), Bachem (Schwerte, Germany) and Iris Biotech (Marktredwitz, Germany). Tentagel R RAM and Tentagel S Trt-Ala Fmoc resin were purchased from Rapp Polymere (Tübingen, Germany). Other chemicals and organic solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck Novabiochem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), (Bachem, Bubendorf, Switzerland), Iris Biotech (Marktredwitz, Germany), Thermo Fisher Scientific (Waltham, USA), J. T. Baker (Deventer, Netherlands), VWR (Leuven, Belgium), Biosolve (Valkenswaard, Netherlands) and Th. Geyer (Renningen, Germany).

***High-performance liquid chromatography:*** Analytical RP-HPLC was performed on a Shimadzu LC10 system with a Nucleosil C18 column (100 Å, 5 µm, 4.6 × 250 mm, Macherey-Nagel, Düren, Germany), with eluents A (0.1% TFA in water) and B (80% ACN, 0.1 % TFA in water). Samples were eluted with a gradient of 5-95% B over 20 min at a flow rate of 1.5 mL/min. Absorption was detected at 218 nm.
Preparative purifications were carried out on a Varian ProStar 210 HPLC device equipped with a preparative Reprosil C18 column (100 Å, 5 µm, 20 × 250 mm, Dr. Maisch, Germany) at a flow rate of 13 mL/min with eluents A and B. For histone H3 depsipeptides a gradient of 5-40% B was used. All other peptides were eluted with a gradient of 5-95% B over 50 min. Absorption was detected at 218 nm. Collected fractions were analyzed by LC-MS and lyophilized.

***Mass spectrometry:*** LC-MS measurements of peptides were performed on a LC-MS2020 System (Shimadzu, Kyoto, Japan) equipped with a Kinetex C18 column (2.6 µm, 2.1 × 100 mm, Phenomenex, Aschaffenburg, Germany). 0.1% formic acid (FA) in water (A) and 80% ACN, 0.1% FA in water (B) were used as eluents. A gradient of 5-95% B over 12.75 min was applied at a flow rate of 0.2 mL/min. Absorption was detected at 218 nm and the ESI-MS was operated in positive mode.

### 2. Peptide Synthesis

***Synthesis strategy:*** The synthesis of the depsipeptide library was performed by automated SPPS after formation of the ester bond between threonine and glycine. During synthesis, pseudoproline or Dmp dipeptide building blocks at positions 5-6 (QT), 12-13 (GG) and 21-22 (AT) were installed (Supporting Scheme 1). Couplings of pseudoprolines and modified amino acids ($K(Me)_2$, K(Ac) and S(ph)) were performed manually. A list of the IntN-ΔH2A and H3 depsipeptides can be found in table S1. Peptide synthesis requires chemicals and organic solvents classified as hazardous. Please refer to the risk and safety instructions for safe handling of these chemicals.

***Automated peptide synthesis:*** Peptides were synthesized by solid-phase peptide synthesis (SPPS) applying standard Fmoc-based synthesis strategy in 25 µmol or 50 µmol scale on a ResPep (INTAVIS, Cologne, Germany) or a Syro I (Multisyntech, Witten, Germany) peptide synthesizer. TentaGel R RAM resin or TentaGel S Trt-Ala Fmoc resin were used as solid support and following standard amino acid building blocks were used: Fmoc-Ala-OH, Fmoc-Ile- OH, Fmoc-Pro-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc- Trp(Boc)-OH,

Fmoc-Thr(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)- OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Ser(tBu)-OH.
Automated coupling reactions of amino acid building blocks (5 eq) were coupled twice with 2-(1H-Benzotriazol-1-yl)-1,1,3,3 tetramethyluroniumhexafluorophosphate (HBTU) (4.5 eq) and NMM (*N*-methylmorpholine) (300 mM in DMF) as base for 30 min. Fmoc deprotection was accomplished by treating the resin twice with 20% or 40% piperidine in DMF for 8 min each, followed by washing of the resin with DMF (6 × 200 µL).

***Depsipeptide formation:*** The formation of an ester bond between threonine and glycine was achieved *via* the Steglich reaction (Fig. S12).[1] TentaGel R RAM resin was deprotected with 20% piperidine in DMF (3 × 1 mL) for 5 min and washed with DMF (3 × 2 min) and DCM (3 × 2 min). Glycolic acid (10 eq) was coupled with HBTU (9 eq) in NMM (400 mM in DMF) for 15 min twice. In a round bottom flask provided with a $CaCl_2$ drying tube, Fmoc-Thr(tBu)-OH (20 eq) was dissolved in anhydrous DCM and cooled down to 0 °C, before N,N'-diisopropylcarbodiimide (DIC) (10 eq) was added. After 20 min under stirring at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in DMF and half of the solution was added to the resin with catalytic amounts of 4-dimetyhylaminopyridine (DMAP). After 30 min the reaction was repeated and the resin was washed with DMF (3× 2 min) and DCM (3 × 2 min).

***Manual peptide synthesis:*** Commercially available building blocks 6-(Fmoc-amino)hexanoic acid (Fmoc-Ahx-OH) (Iris Biotech), Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Lys$(Me)_2$-OH·HCl, Fmoc-Lys(Ac)-OH, Fmoc-Gly-(Dmb)Gly-OH, Fmoc-Ala-Thr(psiMe,Mepro)-OH, Fmoc-Gln(Trt)-Thr(psiMe,Mepro)-OH (all Merck Novabiochem), Fmoc-L-Arg$(Me)_2$(Pbf)-OH (Bachem, Bubendorf, Switzerland) and 5(6)-Carboxytetramethylrhodamine (TAMRA) (Carbosynth (Berkshier, UK) were coupled manually. Manual coupling of the amino acids (2.5 eq) was performed with PyOxim (Novabiochem) (2.5 eq) in NMM (400 mM in DMF) for 1 h or overnight under shaking.

***Peptide cleavage and purification:*** Deprotection of amino acid side chains and cleavage from the resin was achieved by treating the resin with a solution (7 mL) of TFA/phenol/TIPS/water (85:5:5:5) for 3 h under shaking at room temperature. Incubation was repeated with the same solution (1.5 mL) for 30 min twice, followed by washing the resin with DCM (3 mL). The combined solutions were concentrated under reduced pressure and the peptide precipitated in cold (-80 °C) diethyl ether (40 mL). After centrifugation (10 min, 4000 × g, -4 °C) the pellet was washed with cold diethyl ether (20 mL) and centrifuged (10 min, 4000 × g, -4 °C). The pellet was dissolved in water/ACN and lyophilized. Crude peptides were purified by preparative RP-HPLC. Purified peptides were analyzed by LC-MS (Figs. S13 to S16)

## 3. Biochemical methods

***Bacterial protein expression and purification:*** *E. coli* BL21(DE3) cells (Agilent, Waldbronn, Germany) were transformed with pET23b(+) plasmids encoding for $His_6$-tagged Sortase A and Sortase A mutant F40 [2] and cultured in LB media supplemented with ampicillin (100 µg/mL) at 37°C under shaking (160 rpm). At an $OD_{600}$ of 0.5-0.6 expression was induced with Isopropyl-β-D-thiogalactopyranosid (IPTG) (1 mM) and expression was performed at 20 °C overnight under shaking (160 rpm). Cells were harvested by centrifugation (5000 × g, 15 min, 4 °C), resuspended in sortase lysis buffer (20 mM Tris-HCl, 300 mM

NaCl, 20 mM imidazole, pH 7.5) and lysed on an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada). The lysate was centrifuged (20,000 × g, 30 min, 4 °C) and recombinant proteins were purified on Ni-NTA Superflow resin (Quiagen, Venlo, Netherlands). After incubation of the lysate with the resin for 1 h, beads were washed with Ni-NTA sortase washing buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5) and eluted with Ni-NTA sortase eluting buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM imidazole, pH 7.5). Eluted sample was dialyzed against storage buffer (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 7.5), flash-frozen in liquid nitrogen and stored at -80°C.

*E. coli* Rosetta pLysS cells (Merck Millipore Novagen, Darmstadt, Germany) were transformed with pET16b-$His_6$-eCFP-hHP1ß or pET11b-$His_6$-hHP1ß and cultured in 2xYT media supplemented with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL). At an $OD_{600}$ of 0.3-0.5 expression was induced with IPTG (0.3 mM) and expression was performed at 18 °C overnight under shaking (160 rpm). Cells were harvested by centrifugation (5000 × g, 15 min, 4 °C), resuspended in HP1 lysis buffer (50 mM $NaP_i$, 300 mM NaCl, 10 mM imidazole, pH 7.9, cOmplete EDTA-free protease inhibitor cocktail (Sigma Aldrich)) and lysed on an EmulsiFlex-C5 homogenizer. The lysate was centrifuged (20,000 × g, 45 min, 4 °C) and the recombinant protein was purified on Ni-NTA Superflow resin. After incubation of the lysate with the resin for 2 h, beads were washed with Ni-NTA HP1 washing buffer (50 mM $NaP_i$, 1 M NaCl, 30 mM imidazole, pH 7.9) and slowly eluted with Ni-NTA HP1 elution buffer (50 mM $NaP_i$, 300 mM NaCl, 300 mM imidazole, pH 7.9). Fractions were analyzed by SDS-PAGE and combined fractions were dialyzed (Spectra/Por Membrane, 6-8 kDa cutoff, Spectrum Laboratories) against HP1 storage buffer (20 mM $N(EtOH)_3$, 300 mM NaCl, 0.1 mM EDTA, pH 7.5). Protein concentrations were determined using a NanoDrop ND-1000 device (VWR Peqlab, Radnor, USA). Protein solutions were aliquoted and stored at -20 °C.

***Polyacryl gel electrophoresis (SDS-PAGE):*** Protein sample buffer (5 × SDS sample buffer: 250 mM Tris, 10% SDS, 30% glycerol, 0.5 M DTT, 0.02% bromophenol blue) was added to protein samples and heated for 5 min at 97 °C. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used as marker. 12% SDS-PAGE gels were used for all protein samples except samples containing histone proteins, in which case 4–20% Mini-PROTEAN TGX precast protein gels were used. Separation was performed at 120 V for around 70 min. Fluorescence was detected by UV radiation at 312 nm using a LAS-3000 imager (Fujifilm, Minato, Japan). Protein staining was performed with Coomassie Brilliant Blue staining solution (80 mg Coomassie Brilliant Blue G250 (Sigma Aldrich), 3 mL 37% HCl in 1 L water).

***Western blotting:*** Protein samples, separated by SDS-PAGE, were transferred onto PVDF membranes (Amersham Hybond LFP 0.2 µm, GE Healthcare, Freiburg, Germany) by western blotting using a wet tank system (Mini Trans-Blot Cell, Bio-Rad Laboratories, Hercules, USA). After protein transfer membranes were blocked with blocking buffer (5% low fat powdered milk in PBS) for 1 h at room temperature and washed with PBST (0.1% Tween 20 (v/v) in PBS). Anti-histone H3 primary antibody (1:1000, ab1791, Abcam, Cambridge, UK) was diluted in primary incubation buffer (5% low fat powdered milk in PBS) and the membrane was incubated for 2 h at room temperature or at 4 °C overnight. After this, the membrane was washed with PBST (3 × 5 min) and incubated with the secondary antibody. Goat anti-rabbit HRP (1:5000, sc-2004, Santa Cruz Biotechnology, Dallas, USA) was diluted in HRP secondary incubation buffer (5% low fat powdered milk in PBST) and donkey anti-rabbit IRDye 680RD (1:5000, P/N 926-68073, LI-COR Biosciences, Bad Homburg, Germany) in IRDye-secondary incubation buffer (5% low fat powdered milk in PBST with 0.01% SDS). The membrane was incubated with secondary antibody solution for 1 h at room temperature or at 4 °C overnight. After washing with PBST (3 × 5 min) and PBS (2 × 3 min) membranes treated with the HRP conjugated

secondary antibody were developed with Pierce ECL Western Blotting Substrate Kit (Thermo Fisher Scientific). Chemiluminescence was detected with a LAS-3000 imaging system and IRDye conjugated antibodies were detected using an Odyssey Imaging system (LI-COR Biosciences).

**4. Nucleosome reconstitution**

***Preparation of DNA:*** The DNA template used for nucleosome library preparation (147+3RS) had several modifications introduced by PCR from the original 147 base pair Widom 601 positioning sequence.[3] First, it was extended at the 5'- end with the recognition sites of HindIII, EcoRI and EcoRV using the 147+3RS Fwd primer 5'-([biotin]CAAGCTTGAATTCGATATCCTGGAGAATCCCGGTGCCGAGGC-3'). Second, a biotin tag linked via a tetra ethylene glycol linker was added to the very 5' end also with the 147+3RS Fwd primer. Third, Cy5 was added to the 3'- end of the sequence using the 147 Rev primer (147 Rev primer: 5'-[Cy5]ACAGGATGTATATATCTGACACGTGCCTG-3'. 147 DNA for MST experiments was amplified by PCR using the 147 Fwd primer (5'-CTGGAGAATCCCGGTGCCGAGGC-3') together with the 147 Rev primer (5'-[Cyanine5]-ACAGGATGTATATATCTGACACGTGCCTG-3'). 187 DNA for MST experiments was amplified by PCR from a pBluescriptII KS(+) 1x601 plasmid template[3] using 187 Fwd (5'-CCACCGCGGTGGCGGCCGCCCTG-3') and 187 Rev (5'-[Cyanine5]-AGTCGCTGTTCAATACATGCACAGGAT-3') primers, containing the 601 sequence at its center. DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Aldrich P2069) followed by overnight isopropanol precipitation at -20 °C. After centrifugation, pellets were washed with 70% ethanol, air-dried and resuspended in water. DNA was further purified using a PCR purification kit (Zymo Research, D4031). The quality of DNA was assessed by determining the ratio of absorbance at 260 nm vs. 230 nm (2.0-2.2 considered as good quality) and via agarose gel electrophoresis.

**Expression and purification of recombinant histones:** *Xenopus laevis* core histones [4] were expressed in *E. coli* BL21 (DE3) RIL cells in YT medium. Protein expression was induced at 37 °C at an $OD_{600}$ of 0.4-0.6 with 0.5 mM IPTG for 4 h and shaking (160 rpm). Inclusion bodies were prepared as described [4] and solubilized in unfolding buffer (7 M deionized urea, 20 mM Tris-HCl, 10 mM DTT, pH 7.5). The solubilized material was dialyzed against urea chromatography buffer (10 mM Tris-HCl, 7M deionized urea, 1 mM EDTA, 100 mM NaCl, 2 mM DTT, 0.2 mM PMSF, pH 7.5) and loaded onto a Q Sepharose column in front of a SP Sepharose column (GE Healthcare, Freiburg, Germany). After washing with five column volumes of urea chromatography buffer, the Q Sepharose column with bound DNA and contaminating proteins was removed. Histone proteins were eluted from the SP Sepharose column using a linear gradient from 0.1 to 1 M NaCl in urea chromatography buffer. Purified histones were dialyzed extensively against water, lyophilized and stored at -80 °C.

***Reconstitution of histone octamers:*** Lyophilized purified core histones H2A/IntC-ΔH2A, H2B, H4 or modified H3/ΔH3 were dissolved in unfolding buffer (10 mM Tris-HCl, 7 M guanidinium hydrochloride, 10 mM DTT, pH 7.5) and mixed to equimolar ratios. The histone mixture was extensively dialyzed at 4°C against RB high buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, 1 mM DTT, pH 7.5) with at least three changes of dialysis buffer. Histone octamers were concentrated to 10-20 mg/mL using Amicon Ultra centrifugal filter units (Millipore, Billerica, USA) with a 30 kDa cutoff and purified on a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare, Freiburg, Germany). Peak fractions were pooled and concentrated to at least 2 mg/mL. Histone octamers were stored in 50 % (v/v) glycerol at 20 °C.

***Nucleosome reconstitution:*** The reconstitution of nucleosomes was largely done as described.[5] Histone octamers and DNA were mixed in RB high buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5) at a molar ratio of 1.2-1.3. Using a peristaltic pump, a gradient to RB low buffer (10 mM Tris-HCl, 25 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5) was applied during dialysis at 4 °C over 36 h. After reconstitution IntC-ΔH2A ligation-ready nucleosomes were dialyzed against AB25 buffer (50 mM Tris-HCl, 25 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol, pH 7.0) (Fig. S17).

**5. Synthesis of nucleosomal libraries**

***SML of H3 in solution:*** Histone H3 depsipeptides were ligated to immobilized ΔH3 ligation-ready nucleosomes by sortase-mediated ligation (SML). H3 depsipeptide (6 μM) and sortase (wildtype Sortase A or Sortase A mutant F40, 3μM) were incubated in sortase buffer at a total volume of 50 μL with H3-ligation-ready nucleosomes (10 nM). Reactions were incubated at 37 °C under shaking (300 rpm) and samples were taken at different time points. Reactions were stopped by adding protein sample buffer (5x) and heated for 5 min at 97 °C. Samples were analyzed by SDS-PAGE and western blotting. H3 depsipeptides (450 μM) were further ligated to biotinylated carrier peptide (170 μM) with wt Sortase A (3 μM) for 4-5 h in sortase buffer. The reaction mixture was lyophilized, analyzed by LC-MS and immobilized in streptavidin coated High Capacity 96-well plates as described below.

***Immobilization in on 96-well plates:*** Black streptavidin coated High Capacity 96-well plates (Thermo Fisher Scientific) were washed (3 × 200 μL) with nucleosome buffer (10 mM Tris, 25 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5) and 50 μL of 'ligation-ready' ready nucleosomes (0.9 μM) in nucleosomes buffer were added and incubated overnight at room temperature. The solution was removed and stored at 4 °C for reusing. Plates were washed (3 × 200 μL) with nucleosomes wash buffer (20 mM $N(EtOH)_3$, 300 mM NaCl, 0.1 mM EDTA, 5% glycerol, pH 7.5), followed by washing (10 × 200 μL) with nucleosomes buffer and stored at 4 °C in nucleosomes buffer.

***SML on solid support:*** Histone H3 depsipeptides were ligated to immobilized ΔH3 ligation-ready nucleosomes by sortase-mediated ligation (SML). Depsipeptides without phosphorylated serine 28 were ligated by adding the histone H3 depsipeptide (6 μM) and Sortase A (3 μM) in sortase buffer (50 μL) and incubation for 2 h at 37 °C under shaking. Depsipeptides containing phosphorylated serine 28 were ligated by adding the histone H3 depsipeptide (12 μM) and Sortase A (3 μM) in sortase buffer (50 μL) and incubation for 2 h at 37 °C under shaking. After adding additional histone H3 depsipeptide (12 μM) the plates were incubated for another 2 h at 37 °C under shaking. The reaction solution was removed and wells were washed (3 × 200 μL) with nucleosome buffer and library plates were stored at 4 °C in nucleosome buffer. Three additional washing steps were performed prior to the assays to ensure removal of excess peptides (see below).
Conversion ratios were analyzed by denaturing ligated nucleosomes with SDS-PAGE protein sample buffer (50 μL) at 95 °C for 5 min. The solution was removed from the well, concentrated by heating and analyzed by western blotting. Replicates with n of 3-6 were performed and conversion ratios were calculated with the ImageStudioLite imaging program (LI-COR) for each SML per well and eventually, a final total conversion was calculated with the mean and standard deviation for each modified histone H3 nucleosome and the entire histone H3 library.

***Protein trans-splicing reaction:*** Protein *trans*-splicing (PTS) based on the M86 split intein mutant from *Ssp* DnaB [6] was performed in solution for ligation-ready nucleosomes reconstituted with IntC-ΔH2A. H2A-IntN peptide (1.35 µM) was added to a solution of IntC-ΔH2A/ΔH3 ligation-ready nucleosomes (0.9 µM) in buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 300 mM NaCl, 10% glycerol, pH 7.0) and the ligation was carried out overnight at 25 °C while shaking. After the reaction, the solutions were stored at 4 °C and used for immobilization on 96-well plates. Analysis of PTS reactions was accomplished by SDS-PAGE and LCMS analysis. Conversion ratios were calculated by measuring the fluorescence intensity of TAMRA and Cy5 after immobilization and release of ligated nucleosomes from streptavidin-coated 96-well plates (see below). Replicates with n of 3-6 were performed.

***Analysis of PTS by LC-MS:*** PTS reaction solutions were prepared for LC-MS analysis by solid-phase extraction. The nucleosome solution (137.5 µL) was brought to a final concentration of 1 M NaCl with 1% TFA and heated for 5 min at 60 °C. A stationary solid phase (Empore Octadecyl C18 Extraction Disk 47 mm) (Supelco, Sigma Aldrich) was fixed in a pipette tip and activated by centrifugation (1300 × g, 2 min) of methanol (100 µL), LC-MS solvent B (100 µL) and LC-MS solvent A (100 µL) through the disk. The nucleosome samples were loaded onto the solid phase by centrifugation (500 × g, 5 min), followed by washing with LC-MS solvent A (2 × 20 µL). Elution was performed with LC-MS solvent B in 50% formic acid by centrifugation (500 × g, 2 min) and the eluate was directly injected into a LC-MS2020 System (Shimadzu, Kyoto, Japan) equipped with a ReproSil-Pur C8 column (100 Å, 5 µm, 4 × 250 mm, Dr. Maisch). 0.1% formic acid (FA) in water (A) and 80% ACN, 0.1% FA in water (B) were used as eluents and an adapted protocol [7] with a flow rate of 0.8 mL/min. A method with different gradients (6.3-25% B over 25 min, 25-55% B over 70 min, 55-100% B over 20 min) was used. The ESI-MS was operated in positive mode.

***Calculation of the conversion ratio of PTS reactions:*** After protein *trans*-splicing, nucleosomes were immobilized on 96-well plates and washed with pulldown washing buffer (20 mM $N(EtOH)_3$, 150 mM NaCl, 0.1 mM EDTA, 15% glycerol, pH 7.5). The release of immobilized nucleosomes was performed by adding 1 µL of EcoRI (10 u/µL) (Thermo Scientific) in 49 µL EcoRI buffer (50 mM Tris, 20 mM $MgCl_2$, 50 mM NaCl, 0.1 mg/mL BSA, PH 7.5) to each well and incubation for 1 h at 37 °C, shaking at 180 rpm. After releasing from solid support, fluorescence intensities of TAMRA (excitation: 550 nm, emission: 575 nm) and Cy5 (excitation: 649 nm, emission: 670 nm) in each well were directly measured on a TECAN Infinite 200 PRO plate reader. Replicates with n of 3-6 were performed. Calibration curves of the fluorophores Cy5-147 and TAMRA were used for calculating concentrations of semisynthetic TAMRA-H2A and nucleosomes in each well. The concentration of newly synthesized histones was related to the concentration of nucleosomes in a ratio of 2:1 (TAMRA : Cy5) and a conversion yield for PTS ligation was obtained for each well. Mean and standard deviations were calculated for each PTS reaction.

**6. Assays with nucleosomal libraries**

***eCFP-HP1 binding assay:*** Pulldown experiments based on fluorescence intensity readouts were carried out with the prepared H3 and H2A+H3 nucleosome libraries. All following steps were performed at room temperature under shaking (600 rpm) and without light exposure. Wells were washed (3 × 200 µL) with pulldown buffer (20 mM $N(EtOH)_3$, 300 mM NaCl, 0.1 mM EDTA, 1 mg/mL BSA, 15% glycerol, pH 7.5) for 30 s, followed by blocking once with 100 µL pulldown blocking buffer (20 mM $N(EtOH)_3$,

300 mM NaCl, 0.1 mM EDTA, 1 mg/mL BSA, 0.1 mg/mL ssDNA, 15% glycerol, pH 7.5) for 10 min. Incubation was performed with 50 µL protein solution (75 µM eCFP-HP1 in pulldown buffer) for 30 min. After that, wells were washed (4 × 200 µL) with pulldown washing buffer (20 mM $N(EtOH)_3$, 150 mM NaCl, 0.1 mM EDTA, 15% glycerol, pH 7.5) for 30 s. Fluorescence intensity of eCFP was measured after adding 50 µL pulldown washing buffer to each well on a TECAN Infinite 200 PRO plate reader (excitation at 434 nm, emission at 477 nm, 6 x 6 reads per well).
Removal of bound eCFP-HP1 allowed reusing of nucleosome libraries. To this end, plates were washed (10 × 200 µL) with plate washing buffer (20 mM $N(EtOH)_3$, 500 mM NaCl, 0.1 mM EDTA, 15% glycerol, pH 7.5) for 5 min at room temperature while shaking (600 rpm) and stored at 4 °C in nucleosome buffer. Removal of eCFP-HP1 and retention of immobilized NPCs were confirmed by recording the eCFP and Cy5 fluorescent signals.
Pulldown screens of H3 nucleosomal libraries were performed in triplicate with a newly synthesized library for each replicate. Pulldown screens with H3+H2A nucleosomal libraries were performed in triplicate with a total of two synthesized libraries, one of which was reused after eCFP-HP1 removal. Pulldowns with immobilized H3 peptides ligated to biotinylated carrier peptide were performed in the same way.

***eCFP-HP1ß recruitment analysis:*** Fluorescence intensities of the eCFP signals were adjusted to the amounts of immobilized nucleosomes by dividing the eCFP signal intensities with the Cy5 signal intensities. The mean of the eCFP/Cy5 signal of pulldowns with unmodified NPCs was set to 1 and the remaining eCFP/Cy5 signals were adjusted accordingly representing fold-changes relative to eCFP-HP1β enrichment on unmodified nucleosomes. Mean and standard deviations were calculated for each modified nucleosome. Analysis of pulldowns with immobilized H3 peptides ligated to biotinylated carrier peptide was performed in the same way using the TMARA signal of the carrier peptide for normalization.

**7. Assays with reconstituted nucleosomes**

**SML of H3 in solution:** 250 µM of H3K9me2 or H3K9me2S28ph H3 (1 – 33) depsipeptide was mixed with 85 µM ΔH3 and 20 µM Sortase A in SML reaction buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl, 1 mM DTT, 500 mM Arginine, pH 7.5) and incubated at 37 °C for 2 h. The buffer of the reaction mix was exchanged to SAU 200 buffer (7 M urea, 200 mM NaCl, 20 mM NaAc, 2 mM DTT, 1 mM EDTA, pH 5.2) using HiTrap Desalting Columns, 5 ml (GE Healthcare, Freiburg, Germany). Next, the sample was applied to a SP HP, 1 ml ion exchange chromatography column (GE Healthcare, Freiburg, Germany). Samples were eluted with a linear gradient to 100% SAU 1000 buffer (7 M urea, 1 M NaCl, 20 mM NaAc, 2 mM DTT, 1 mM EDTA, pH 5.2), in 50 column volumes (CV), at a flow rate of 1mL/min. The purity of fractions was assessed by SDS-PAGE. Peak fractions were pooled, dialyzed three times against 2 mM DTT in water, lyophilized and stored at -80°C.

**Quantitative binding measurements:** Isothermal calorimetry **(**ITC) measurements were performed on a MicroCal PEAQ-ITC calorimeter (Microcal, Malvern, UK) at 25 °C in binding buffer (10 mM triethanolamine, 150 mM NaCl, 0.1 mM EDTA, pH 7.5). Reaction heats were recorded by titrating 500 µM H3K9me2 or H3K9me2S28ph peptide (aa 1 - 32), spaced at 60 s intervals, into 30 µM of recombinant HP1 under constant stirring at 1,000 rpm. Data were analyzed using Sigmaplot.
For microscale thermophoresis (MST), titration series of recombinant HP1 were incubated with 20 nM

Cy5-tagged nucleosomes in binding buffer at room temperature for 15 minutes before measuring on a Monolith NT.115 instrument (NanoTemper, 30% LED power, 40% MST power). All binding measurements were performed minimally with two independent preparations of protein and nucleosomes and in multiple independent replicates ($n \geq 5$). $K_D$ values of individual measurements were determined fitting (using least squares) a binding curve derived from the law of mass action equation to the data points:

$$[AL]=½*(([A_0]+[L_0]+ K_D)-(([A_0]+[L_0]+ K_D)^2 -4*[A_0]*[L_0])^{1/2})$$

$[A_0]$, concentration of fluorescent molecule; $[L_0]$, concentration of ligand/ binding partner; [AL], concentration of the complex of A and L.
$K_d$ values from individual measurements were averaged to report the final dissociation constants in Figure 3E. To define the uncertainty parameters of the averaged $K_d$, we performed error propagation using the following formula:

$$\sigma_{ave} = \frac{\sqrt{(\sigma_1)^2 + (\sigma_2)^2 + \cdots (\sigma_n)^2}}{n}$$

where $[\sigma_{ave}]$, standard deviation of the averaged $K_d$; $[\sigma_1, \ldots \sigma_n]$, standard deviation of $K_d$ values from individual measurements; [n], number of experimental replicates.
The values of individual data points measured were normalized to the raw fluorescence signals of unbound and fully bound state using the formula:

$$y = ((y_0 – min)*-1)/(max – min)$$

where [max], bound value; [min], unbound value of the fitted graph. For generating the final binding graphs (Figure 3), datasets were first normalized and then averaged. Errors reported correspond to stdev. Graphs were plotted using GraphPad Prism and applying a single site binding model. Samples were statistically compared using a Student's t-test (unpaired, two-tailed).

## 8. NMR measurements

***NMR sample preparation:*** Nucleosome samples were dialyzed in NMR buffer (25 mM NaPi, 25 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 6.8) and concentrated to 200-220 µM using Amicon ultrafiltration devices with 100 kDa cutoff. Samples were placed in 5 mm NMR tubes and $D_2O$ was kept in high precision glass insert manufactured by Wildman.

***Relaxation measurements:*** The $^{15}N$ longitudinal ($R_1$), transverse ($R_2$) relaxation and steady-state heteronuclear {$^1H$}-$^{15}N$ NOE (nuclear Overhauser effect) experiments for H3 wt and H3S28E-containing nucleosomes were recorded on a 950 MHz Bruker NEO spectrometer equipped with a TCI cryogenic probe at 301 K (Fig. S18). We utilized the 2D [$^1H$,$^{15}N$] pulse programs from the Bruker library (Topspin version 4.0.4).[8, 9] The $^{15}N$-$R_1$ experiments were recorded with 12 different delays from 80 to 1200 ms for both samples. $^{15}N$-$R_2$ were recorded with 11 various delays from 4.24 to 110.24 ms. The delays were used in random order with a recycle delay (d1) of 3.5 s. For steady-state NOE we used a recycle delay of 10 s and 4 s of $^1H$ saturation of 180° pulses spaced 22 ms with the $^1H$ carrier moved on the

amide proton region at 8.2 ppm. The $^{15}$N-$R_1$ and $^{15}$N-$R_2$ values and their errors were determined by the non-linear least-squares method by fitting the measured peak heights to the two-parameter function $Y(t)=Y_0*\exp(-t/R_{1,2})$ [10], where Y(t) is the intensity after a delay of time t and $Y_0$ is the intensity at the time t=0. The errors of relaxation rates were obtained from appropriate elements of the variance-covariance matrix. The heteronuclear NOE values were determined from the ratio of signal intensities from an experiment with $^1$H saturation divided by the signal intensities from the reference experiment without saturation. The errors were determined from signal-to-noise values coming from two experiments according to the formula: $dNOE=|NOE|\cdot(stn_{sat}^{-2}+ stn_{nosat}^{-2})^{0.5}$ [11-13]. Assessment of the fast local ps range backbone motions was carried out by direct comparison of the NOE values and their errors as derived for H3 wt and H3 S28E nucleosomes. The ns time scale motions were assessed from respective relaxation rates.[14, 15] Rotational correlation times for each residue were calculated by the formula: $\tau_c= \frac{1}{4\pi\nu N}\sqrt{\frac{6R_2}{R_1}-7}$ [15-17] The resulting total correlation time errors were determined from the propagation of the errors of the individual relaxation rates.

# Supplementary data

## 9. Supporting Figures

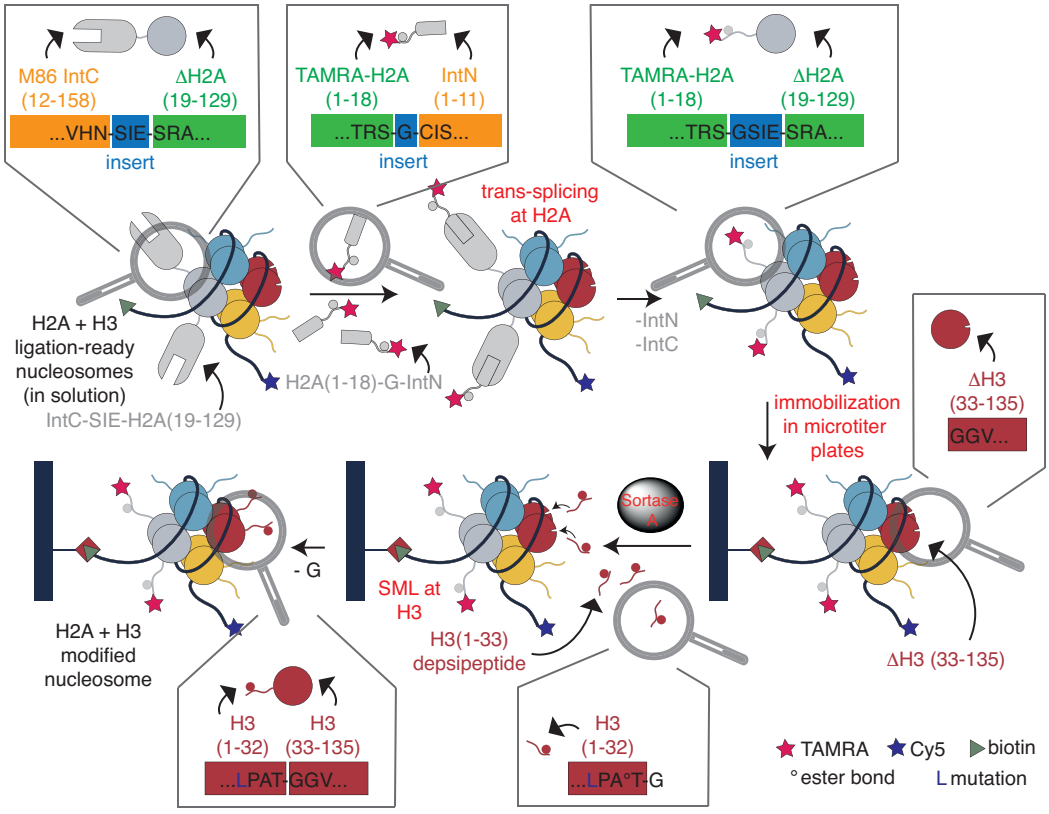



**Fig. S1: Strategy for synthesizing a H2A+H3 library using-ligation-ready nucleosomes.** The N-terminal tail of histone H2A is installed by protein trans-splicing (PTS) in solution. H2A+H3-ligation-ready nucleosomes contain N-terminal truncated H2A extended by the IntC fragment of the M86 mutant of the Ssp DnaB intein. An insert of three amino acids was incorporated in order to facilitate efficient PTS. The synthetic TAMRA-H2A(1-18)-IntN fragment contains a further single amino acid insert. Upon mixing of IntN and IntC a splice competent complex forms resulting in excision of the IntN and IntC fragments under concomitant joining of the H2A fragments. The nucleosomal splice product of H2A contains an insert of four residues. Spiced nucleosomes are immobilized by the biotin tag on streptavidin allowing removal of excess TAMRA-H2A-IntN peptides and subsequent installation of the H3 tails on solid support. Immobilized nucleosomes contain N-terminally truncated histone H3 (33-135). Synthetic H3 tails are installed by on-resin Sortase-mediated ligation (SML) using H3(1-32) depsipeptide substrates. Ligation of the H3 tail results in the introduction of an A28L mutation in order to facilitate the SML reaction. The amino acid sequences at the ligation sites are shown in single letter code.

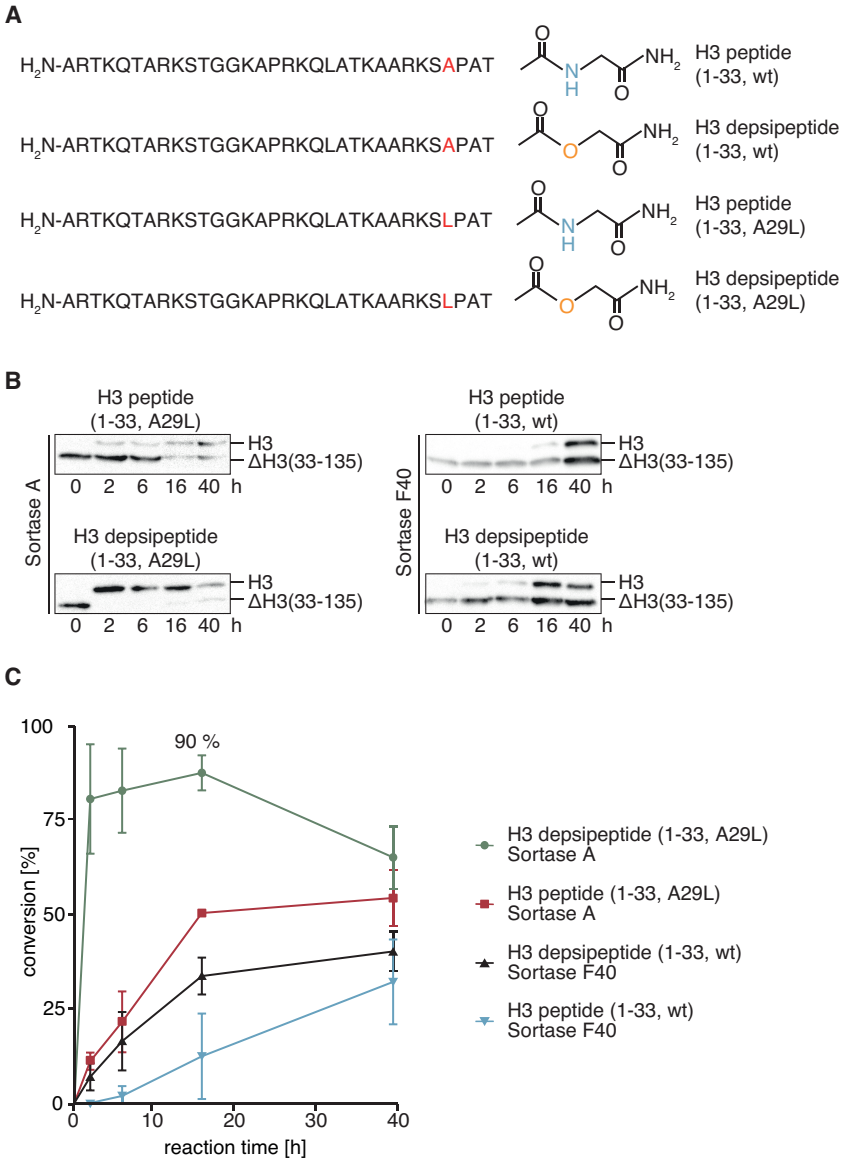



**Fig. S2: Test of SML with wt and evolved F40 mutant Sortase A.** (**A**) Amino acid sequences of peptides and depsipeptides analyzed in nucleosomal SML assays in solution. H3 peptides with A29L (red) mutation are substrates of wt Sortase A, while peptides with the native sequence can be ligated by the F40 Sortase A mutant. (**B**) Representative western blot analysis of SML reactions. Ligated full-length H3 possesses different electrophoretic mobility than truncated ΔH3. (C) Time course of ligation reactions with all four combinations of peptides, depsipeptides, wt and F40 Sortase A. The ratio of ligation product and starting material was defined as conversion and calculated from the intensities of the western blot signals of full-length H3 and ΔH3. The conversion was plotted against the reaction time showing that ligation reactions peeked at 16h. The subsequent decrease in conversion is explained by Sortase A catalyzed hydrolysis of the ligation products that occurs after consumption of the stating materials.

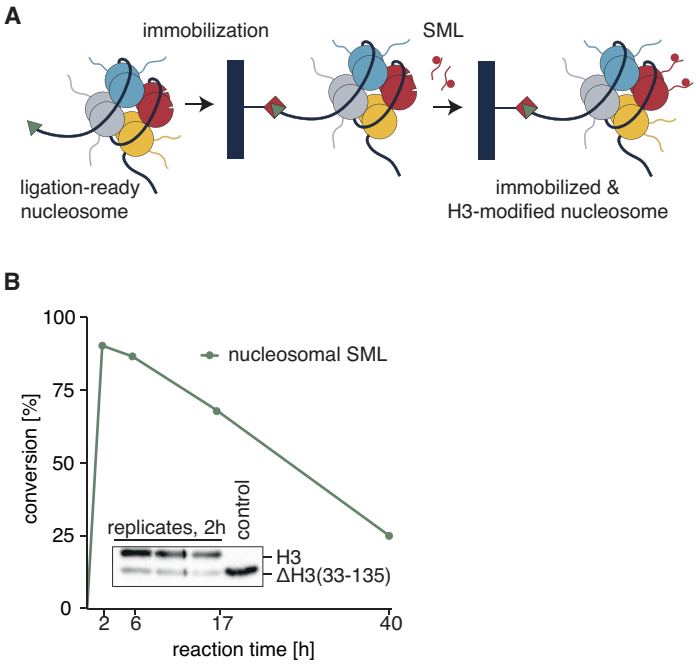



**Fig. S3: SML on 'ligation-ready' nucleosomes.** (**A**) Scheme of SML with immobilized H3 ligation-ready nucleosomes. Immobilization is achieved via a biotin handle on the nucleosomal DNA. The subsequent SML reaction is performed on-surface allowing removal of excess peptide, leaving group, and Sortase A by discarding the supernatant after the ligation reaction. (**B**) Time course of nucleosomal, on-resin SML. The reaction peeks after 2 h under optimized conditions. Western blot analysis of three replicates at the ligation peek at 2 h is shown.

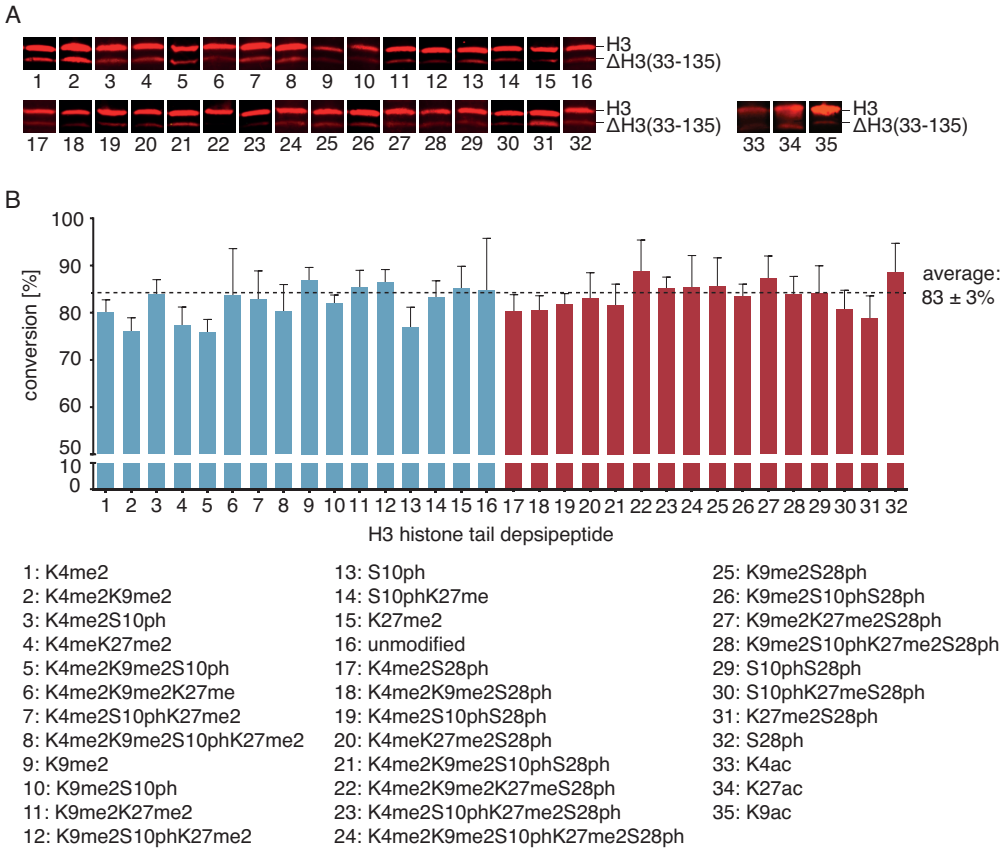



**Fig. S4: Library of H3 modified nucleosomes.** (**A**) Representative western blot analysis of the on-resin SML reactions with depsipeptides of the H3 library. The upper bands correspond to the ligation products (full-length H3). Western blots were developed with fluorescently labeled secondary antibodies. (**B**) Analysis of ligation yields from three replicates. Ligation reactions with H3 depsipeptides containing no S28ph modification are shown in blue. Ligation reactions with H3 depsipeptides containing S28ph are shown in red. The average ligation yield was 83 ± 3 %.

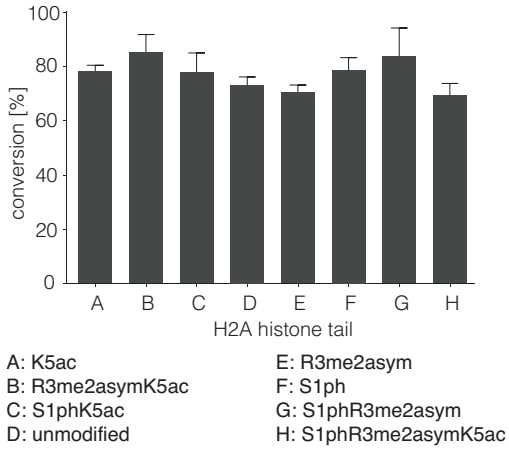



**Fig. S5: Library of H2A+H3 modified nucleosomes.** Analysis of nucleosomal PTS reactions in solution. The conversion rate was calculated from the ratios of the TAMRA signal of the ligated H2A tail and the Cy5 signal of the nucleosomes taking the 2:1 ratio of H2A tail to nucleosomes into account.

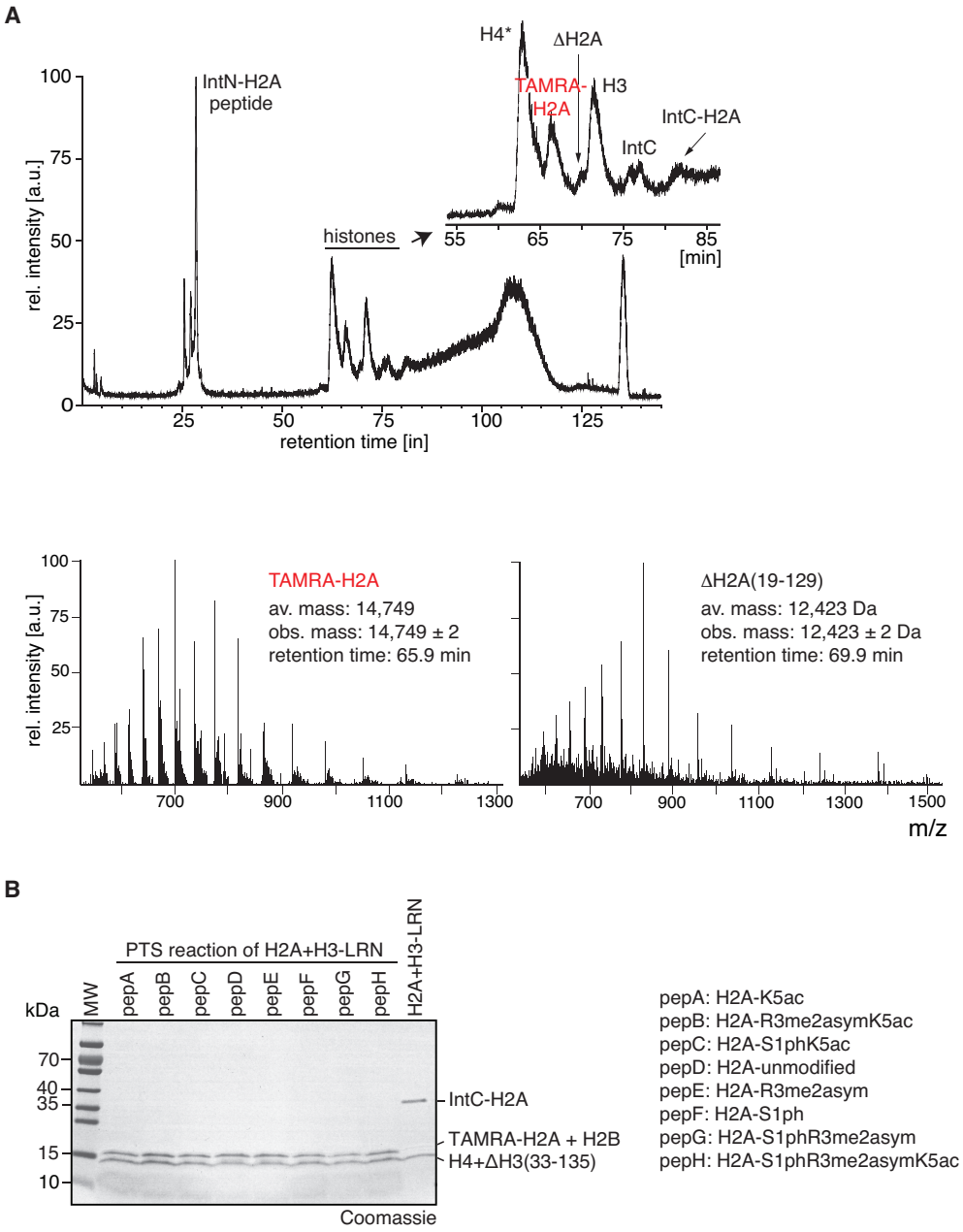



**Fig. S6: Analysis of nucleosomal PTS reactions.** (**A**) LC-MS analysis of PTS reactions. Top: The chromatogram shows the total ion count (TIC) plotted against the retention time. The reaction was performed with H2A-ligation-ready nucleosomes containing full-length H3 and pepD representing the unmodified TAMRA-H2A(1-18)-IntC substrate. The method was optimized for separating the ligation product (TAMRA-H2A) from truncated ΔH2A resulting from undesired hydrolysis of IntC. The region were histones elute are magnified, indicating only little hydrolytic cleavage of IntC. The MS spectra of TAMRA-H2A and ΔH2A are shown at the bottom. (**B**) Analysis of PTS reactions by SDS-PAGE. The PTS reactions of TMARA-H2A-IntC substrates pepA - pepH and H2A+H3-ligation-ready nucleosomes were separated on a denaturing 4% - 20% SDS-PAGE gradient gel and stained with Coomassie.

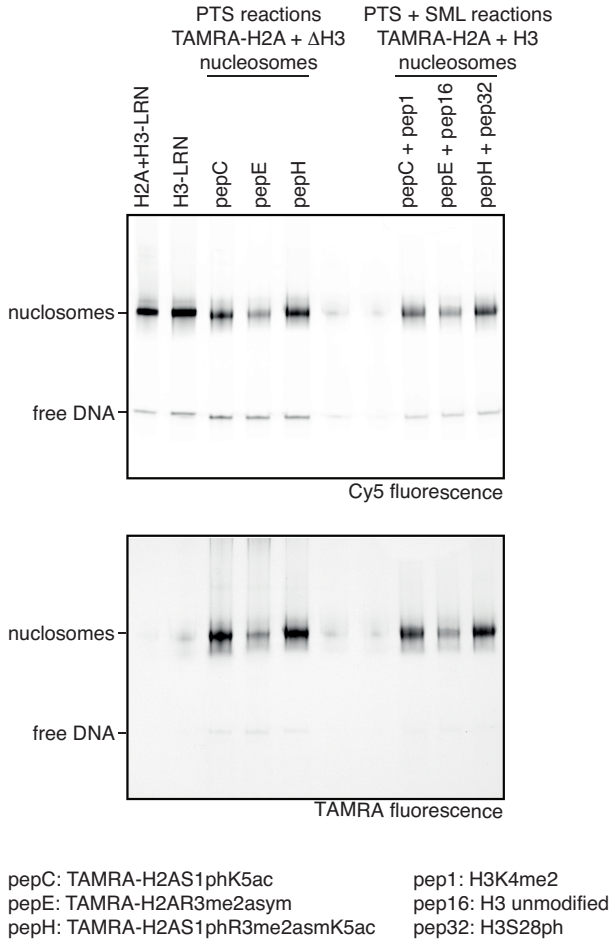



**Fig. S7: Analysis of nucleosomal integrity after PTS and SML reactions by native PAGE.** H2A+H3-ligation-ready nucleosomes, H3-ligation-ready nucleosomes, PTS reactions of H2A+H3-ligation-ready nucleosomes and selected subsequent SML reactions were analyzed by native 4% - 20% gradient PAGE. Nucleosomes were immobilized on magnetic streptavidin resin and released after ligation reactions and washing by cleavage of the DNA linker with EcoRI. Released material was separated on gradient PAGE. The gel was scanned for the Cy5 signal of the DNA label that remains with the nucleosomes after EcoRI release (top) and the TAMRA signal of TAMRA-H2A (bottom). All analyzed nucleosomes migrated as one distinct band on native PAGE indicating no nucleosomal decomposition upon ligation and immobilization. LRN: ligation-ready nucleosomes.

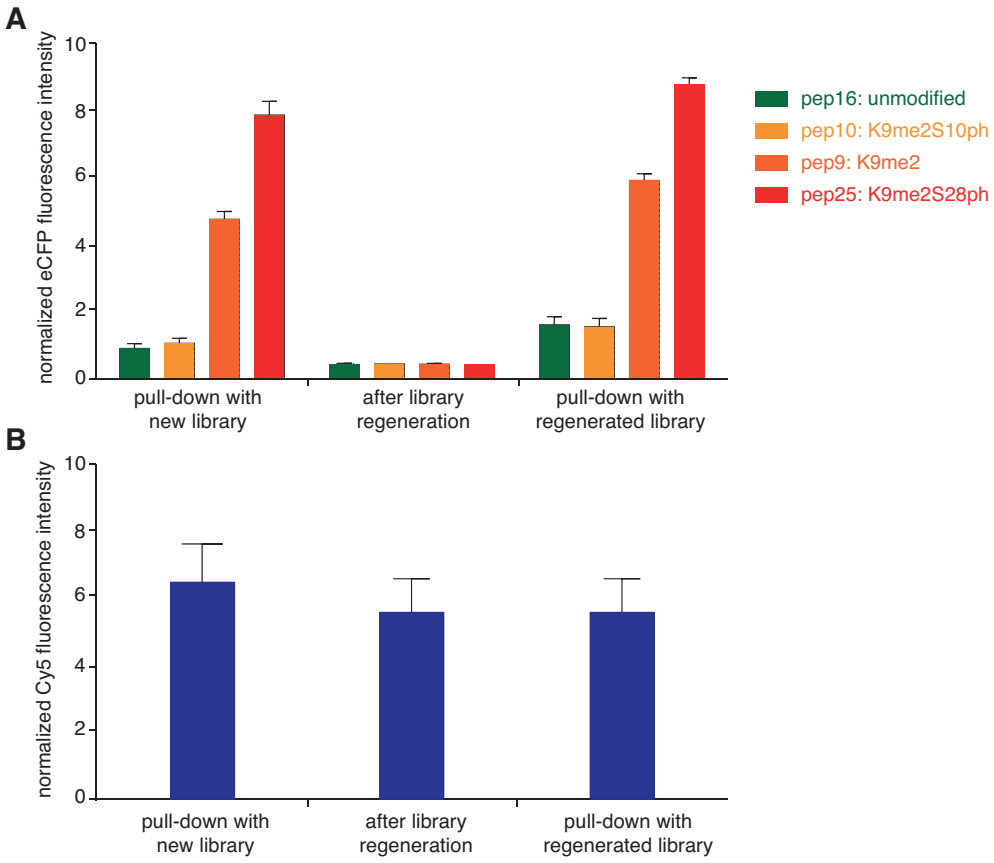



**Fig. S8: Analysis of library regeneration.** (**A**) Nucleosomal libraries can be regenerated after HP1 pull-down reactions by a stringent washing protocol removing bound binding modules. Pull-down reactions with representative nucleosomes showing distinct HP1 recruitment abilities were performed with newly synthesized nucleosomes. Binding of eCFP-HP1 was detected by fluorescence readouts showing the expected interaction pattern. After applying the wash protocol indicated in ESI chapter 6 the eCFP signal was reduced to background indicating quantitative removal of bound HP1. The regenerated nucleosomes were probed with eCFP-HP1 for a second time showing the same interaction pattern. (**B**) Signal of the Cy5 label of nucleosomes after pull-down and washing procedures as described in (A). The Cy5 fluorescence indicates only limited nucleosome wash-out by the regeneration procedure.

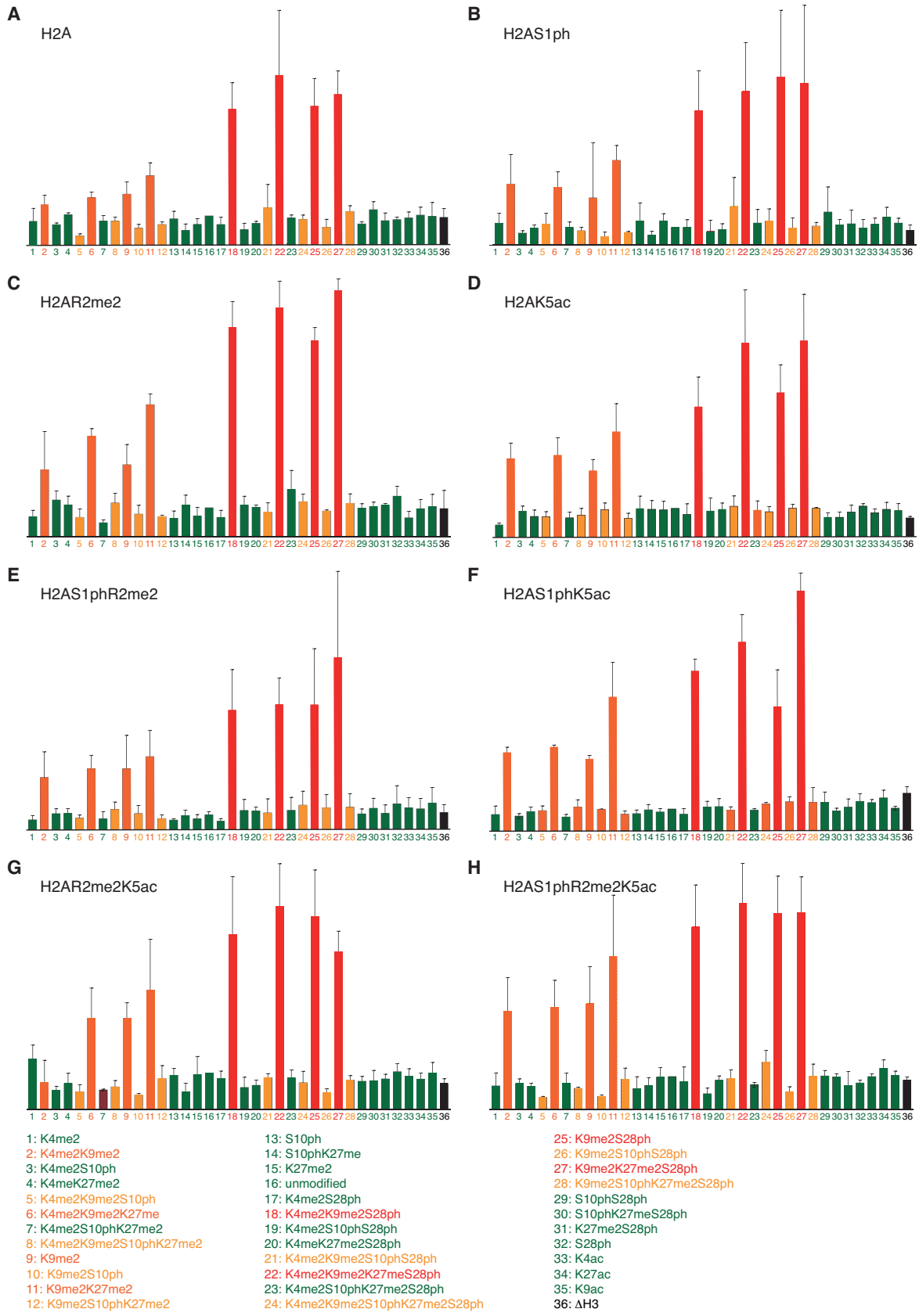



**Fig. S9: Results of screening H2A+H3 library with eCFP-HP1.** The H2A+H3 nucleosomal library was screened for binding retention of eCFP-HP1. (**A-H**) Diagrams correspond to results obtained different modification patterns on the H2A-tail. Columns in each graph correspond to the H3 tail modifications specified at the bottom. Pulldowns were performed in triplicate with sets of H2A+H3 libraries, one of which was reused after eCFL-HP1β removal. Mean normalized values as arbitrary units are blotted. Error bars correspond to standard deviation.

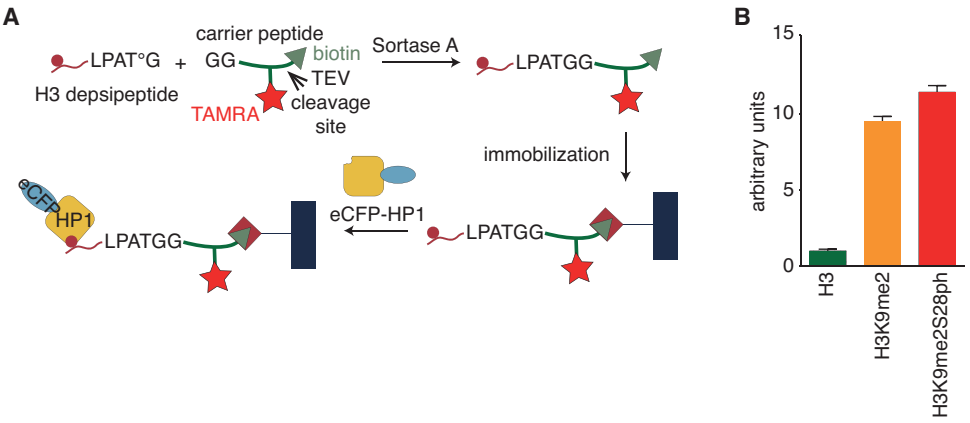



**Fig. S10: Analysis of $His_6$-eCFP-HP1β binding to H3-tail peptides.** (**A**) Scheme of assembly and immobilization of H3 peptides for eCFP-HP1 pulldown. Three H3 depsipeptides (H3 unmodified, H3K9me2, H3K9mesS28ph) were ligated to a carrier peptide by sortase A. The carrier peptide was equipped with a biotin handle for immobilization, a TMARA fluorophore for quantification of immobilized material, a cleavage site for TEV protease allowing release from the solid support and an N-terminal Gly serving as acceptor in the SML reaction. Ligation products were immobilized on streptavidin-coated High Capacity 96-well plates for eCFP-HP1 binding assays. (**B**) Results of eCFP-HP1 binding assays from three independent repeats. Error bars correspond to standard deviation.

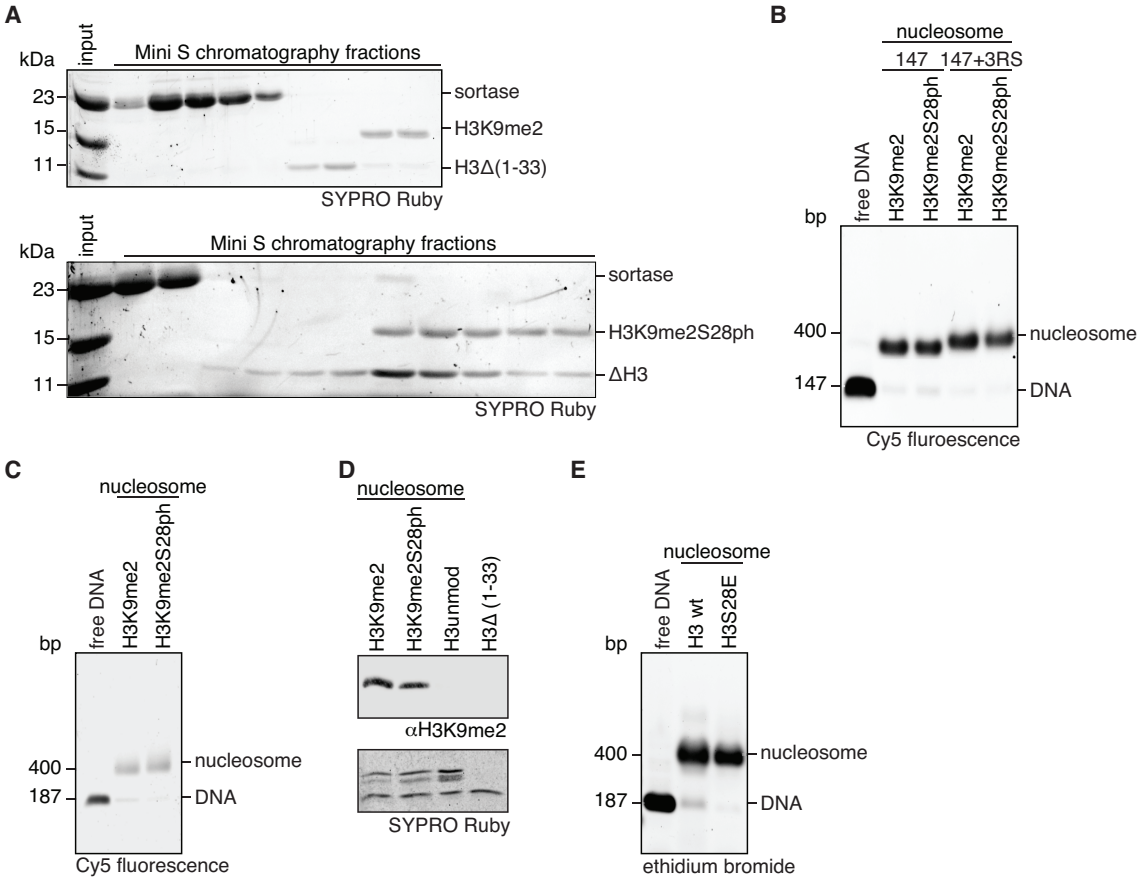



**Fig. S11: Assembly of 'designer' nucleosomes for quantitative measurements.** (**A**) SDS-PAGE analysis of fractions of chromatography purification of sortase-mediated ligation products for H3K9me2 (top) and H3K9me2S28ph (bottom). (**B, C**) Agarose gel electrophoresis of 'designer' 147, 147+3RS and 187 nucleosomes reconstituted using H3K9me2 or H3K9me2S28ph. (**D**) Western blot analysis of recombinant nucleosomes containing H3, H3K9me2 or H3K9me2S28ph histones using anti-H3K9me2 antibodies to show equal incorporation levels. SYPRO Ruby staining of the membrane served as loading control. (**E**) Agarose gel electrophoresis of nucleosome samples for NMR reconstituted on 187 bp 601 DNA and containing $^{15}$N labeled H3 wt or the H3S28E mutant protein.

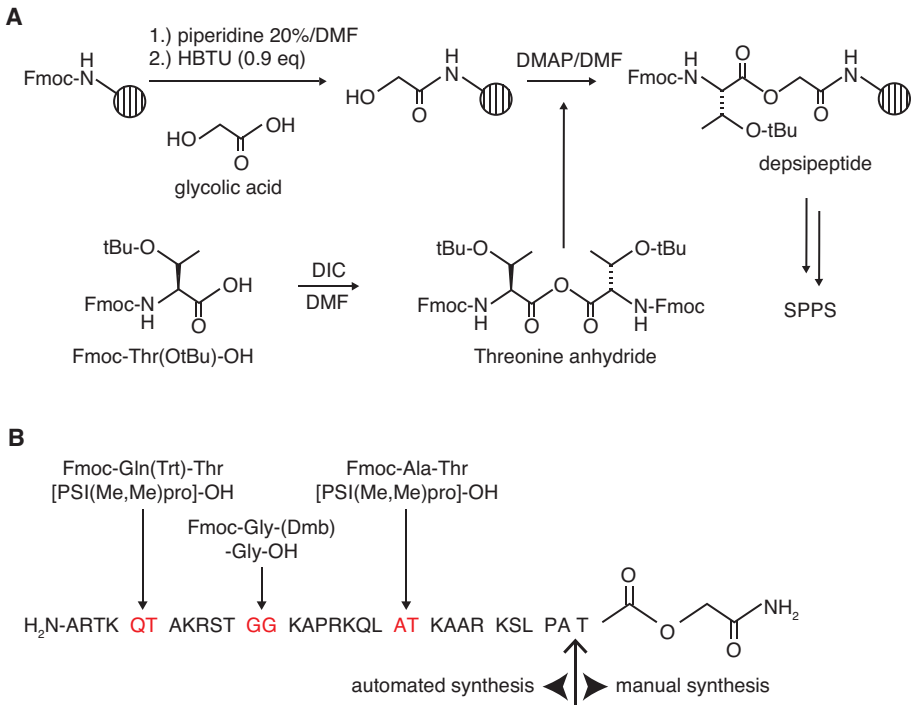
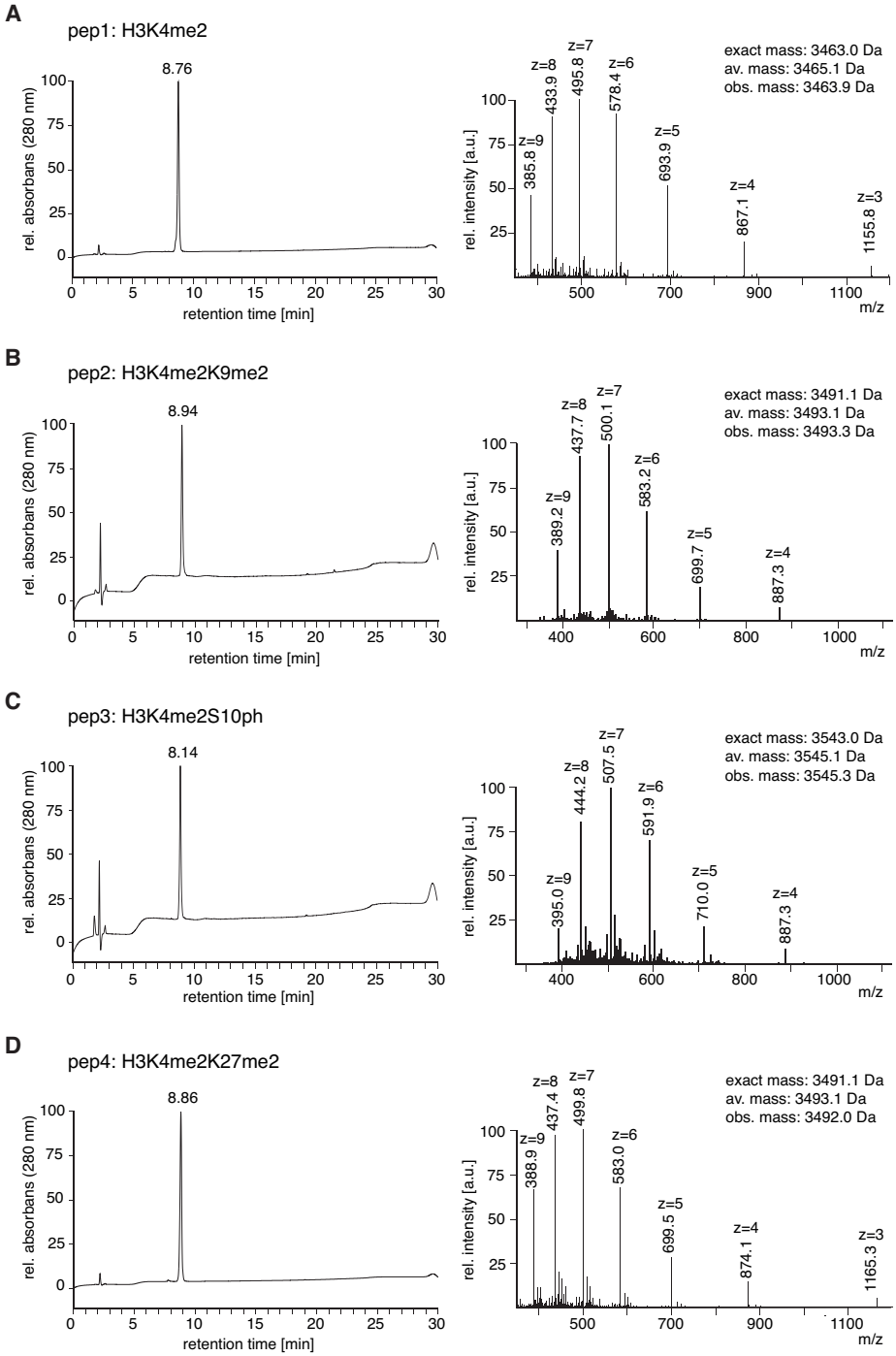



**Fig. S12:** Formation of depsipeptide bond and H3 depsipeptides. (**A**) Depsipeptide bonds between glycolic acid and Threonine were formed by coupling glycolic acid to the solid support followed by coupling of Fmoc-Thr(otBu)-OH as symmetric anhydride under 4-dimethylaminopyridine (DMAP) catalysis. (**B**) H3 depsipeptide synthesis was facilitated by installation of pseudoproline and Dmb dipeptide building blocks during synthesis at the indicated positions.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rel. intensity [a.u.]
m/z
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z=8
z=7
z=6
z=5
z=4
z=3
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433.9
495.8
578.4
693.9
867.1
1155.8
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437.7
500.1
583.2
699.7
887.3
C
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591.9
710.0
887.3
D
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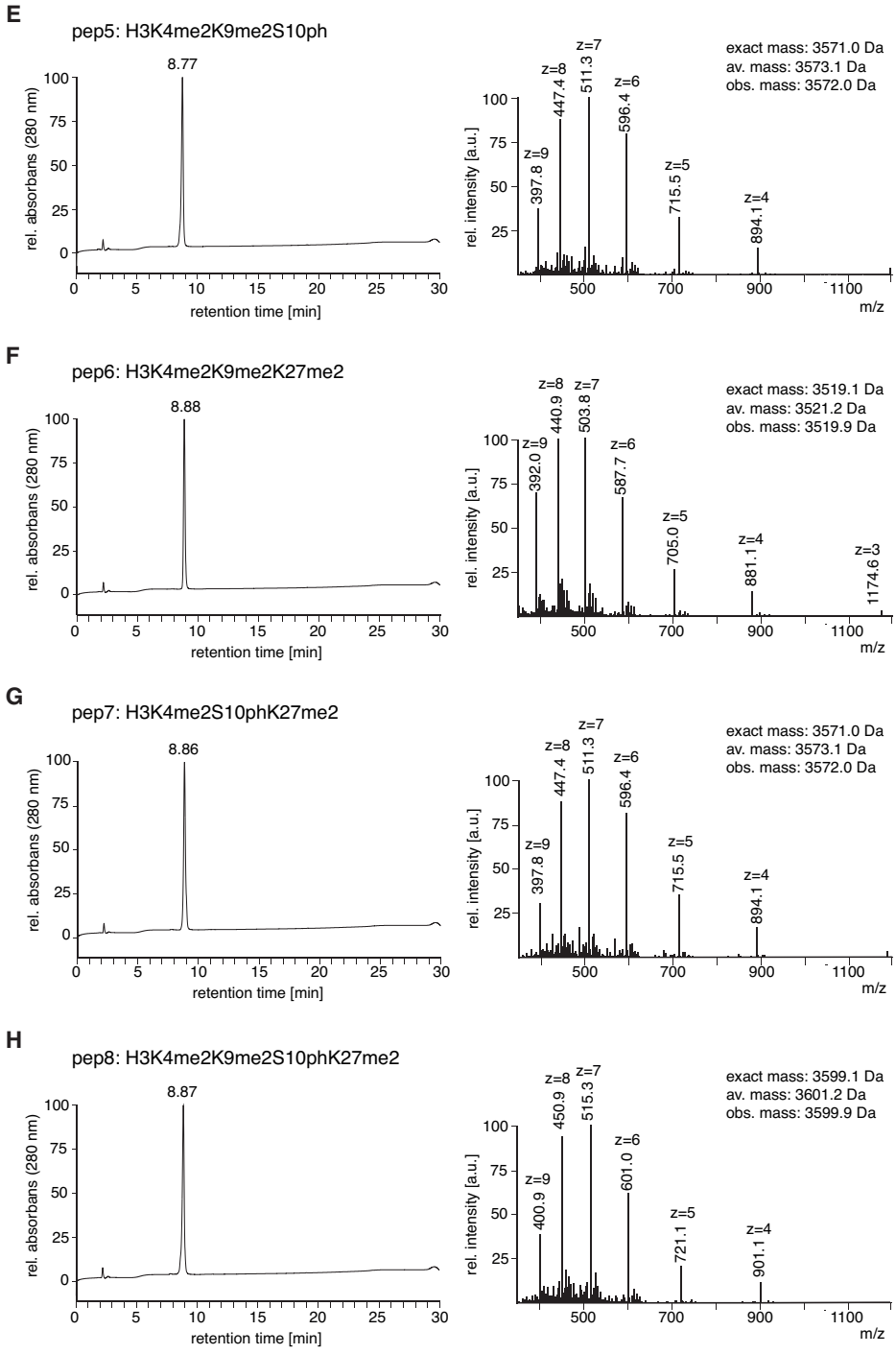
437.4
499.8
583.0
699.5
874.1
1165.3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z=5
z=4
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447.4
511.3
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894.1
rel. intensity [a.u.]
m/z
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440.9
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587.7
705.0
881.1
1174.6
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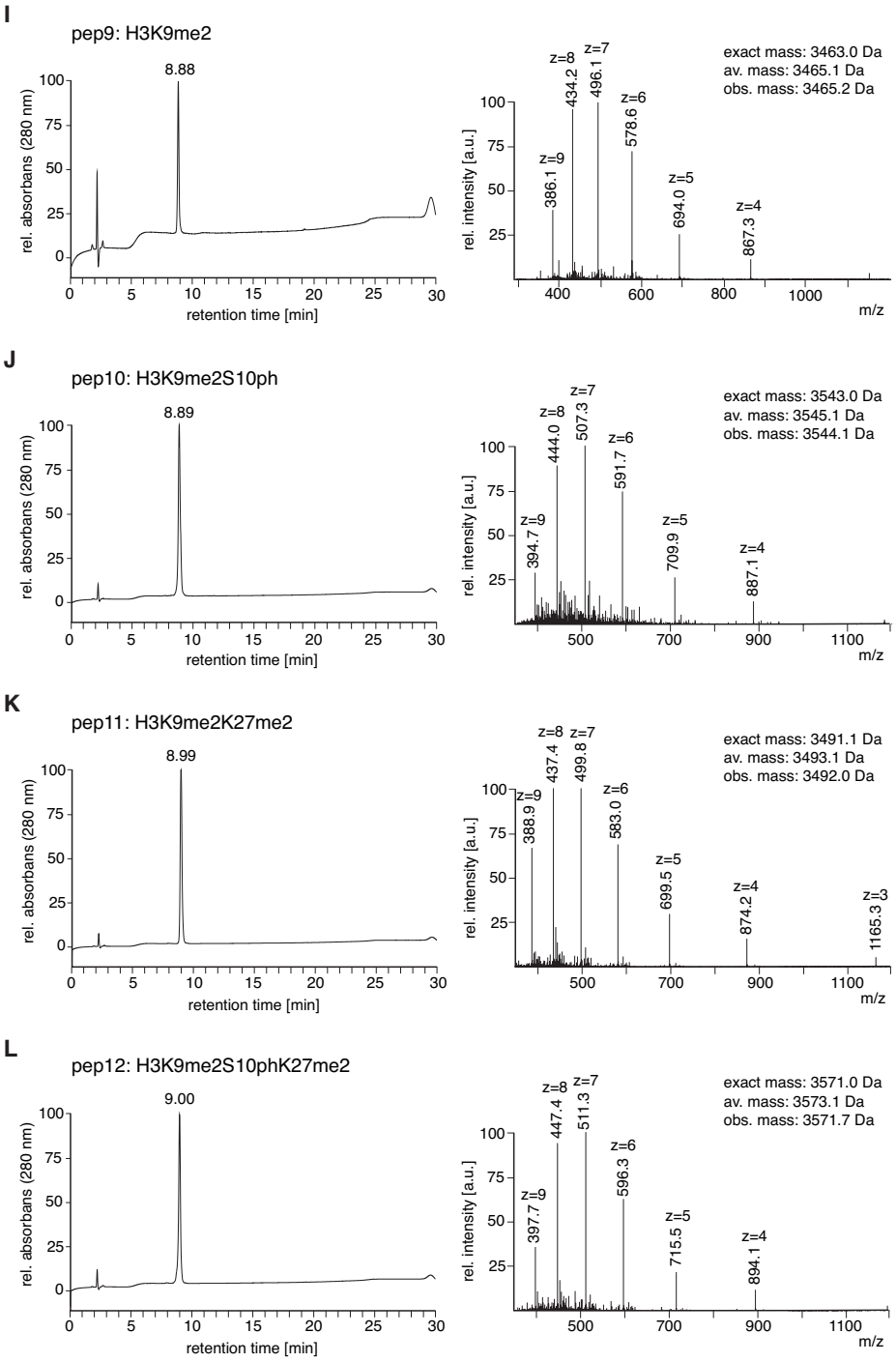
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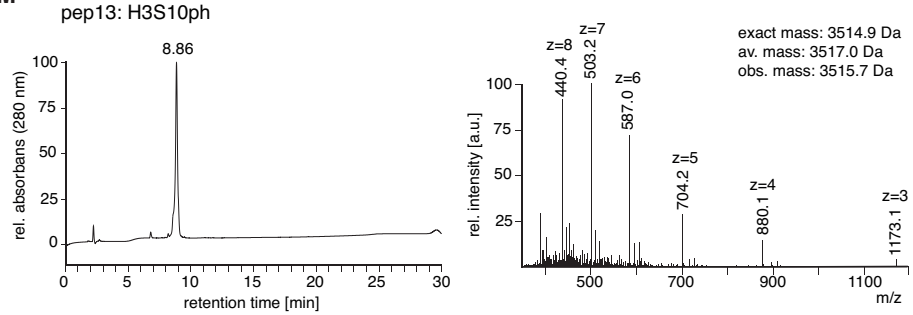
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m/z

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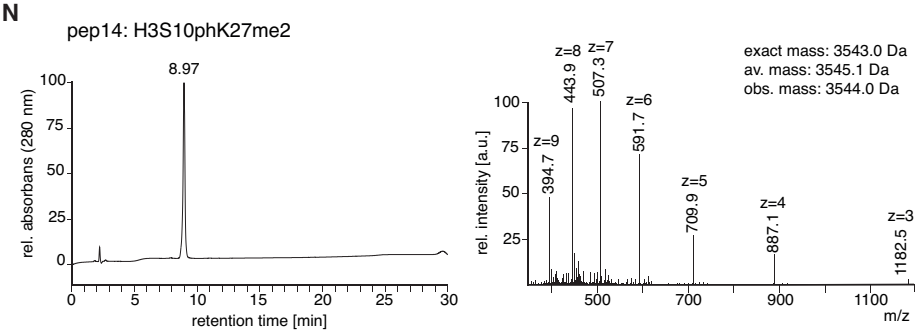
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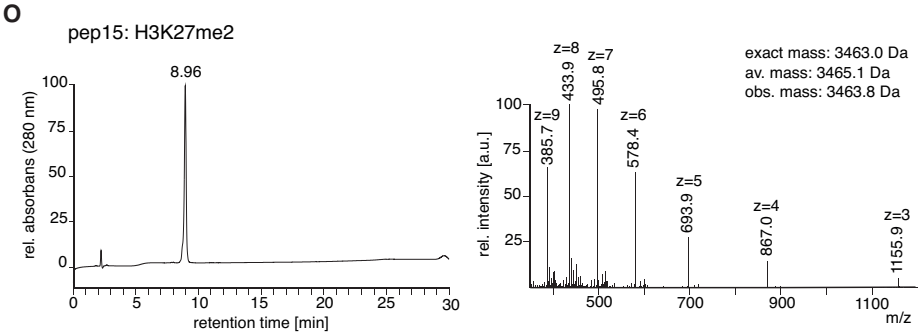
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m/z

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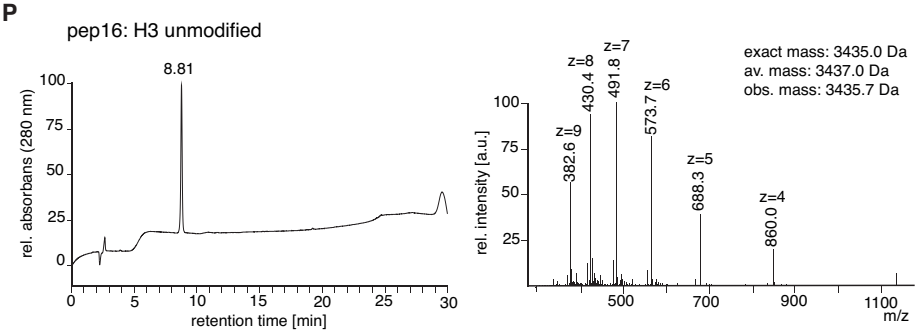
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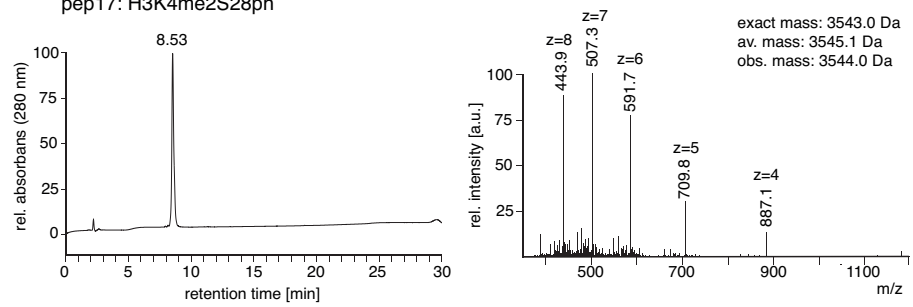
m/z

**Q**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z=5
709.8
z=4
887.1
rel. intensity [a.u.]
m/z

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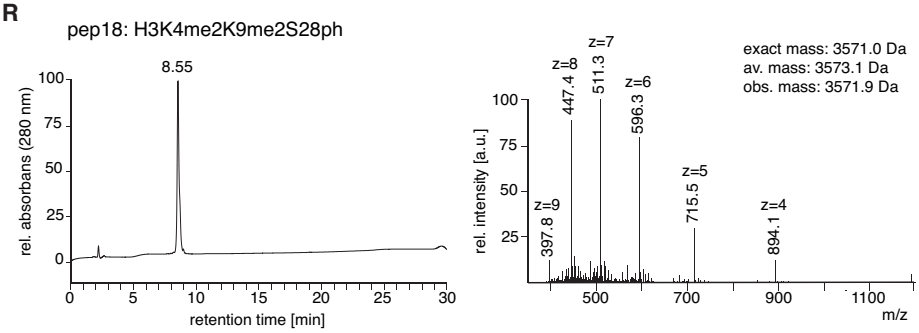
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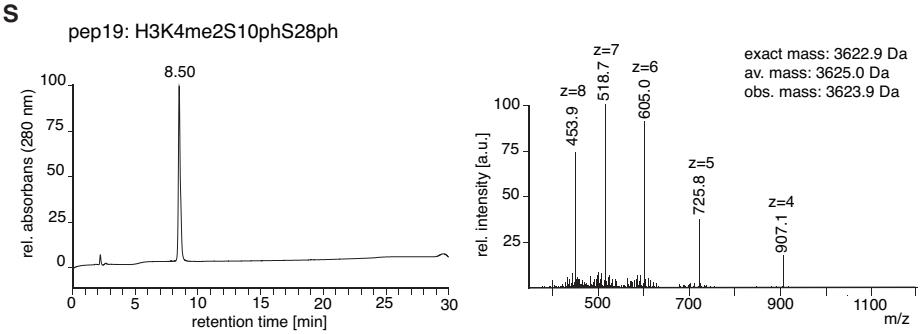
rel. intensity [a.u.]
m/z

**S**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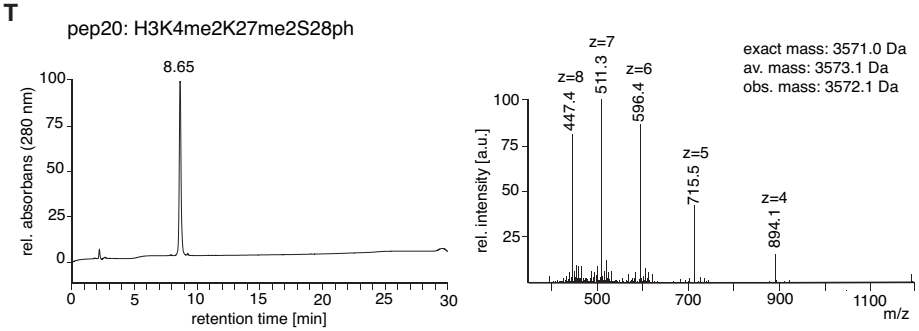
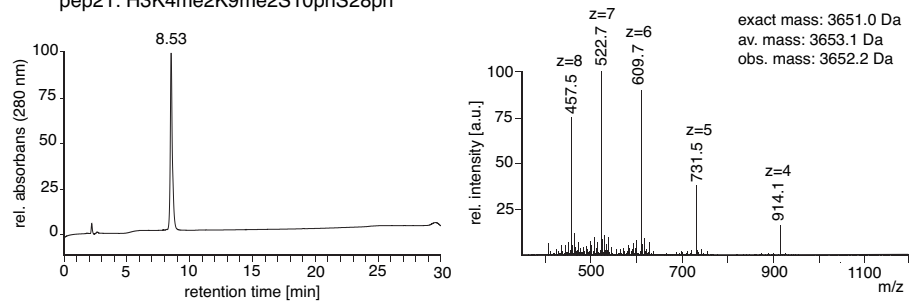
m/z

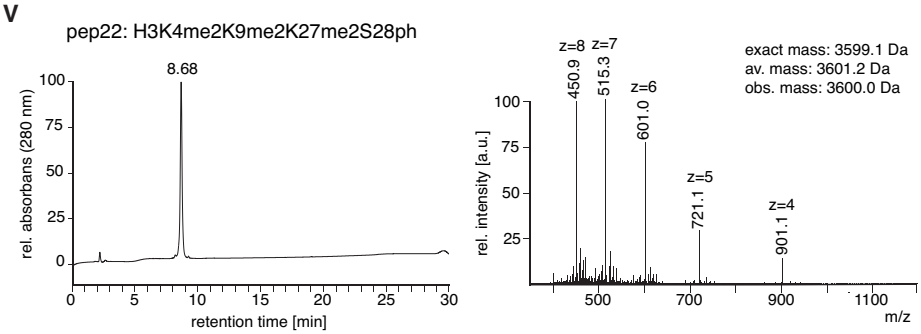
**T**


pep20: H3K4me2K27me2S28ph
8.65
rel. absorbans (280 nm)
retention time [min]
exact mass: 3571.0 Da
av. mass: 3573.1 Da
obs. mass: 3572.1 Da
z=8
447.4
z=7
511.3
z=6
596.4
z=5
715.5
z=4
894.1
rel. intensity [a.u.]
m/z

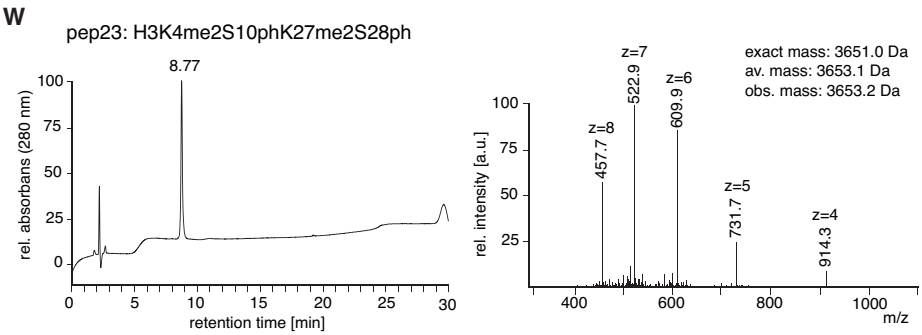
**U**


pep21: H3K4me2K9me2S10phS28ph
8.53
rel. absorbans (280 nm)
retention time [min]
exact mass: 3651.0 Da
av. mass: 3653.1 Da
obs. mass: 3652.2 Da
z=8
457.5
z=7
522.7
z=6
609.7
z=5
731.5
z=4
914.1
rel. intensity [a.u.]
m/z

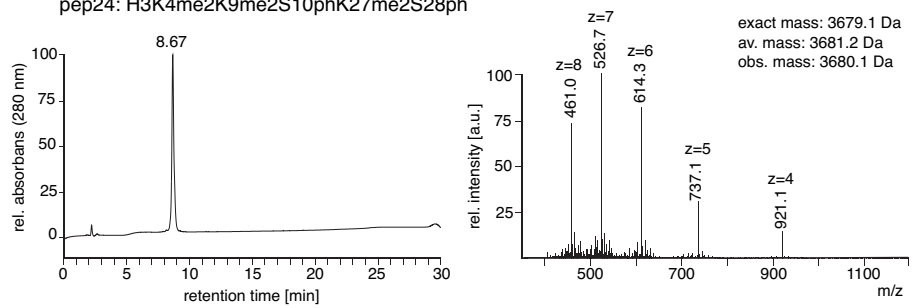
**V**


pep22: H3K4me2K9me2K27me2S28ph
8.68
rel. absorbans (280 nm)
retention time [min]
exact mass: 3599.1 Da
av. mass: 3601.2 Da
obs. mass: 3600.0 Da
z=8
450.9
z=7
515.3
z=6
601.0
z=5
721.1
z=4
901.1
rel. intensity [a.u.]
m/z

**W**

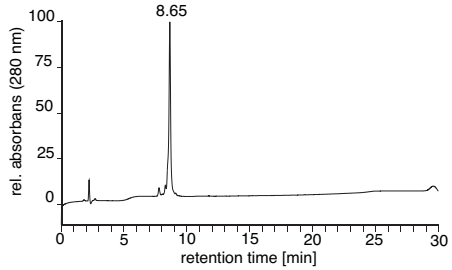

pep23: H3K4me2S10phK27me2S28ph
8.77
rel. absorbans (280 nm)
retention time [min]
exact mass: 3651.0 Da
av. mass: 3653.1 Da
obs. mass: 3653.2 Da
z=8
457.7
z=7
522.9
z=6
609.9
z=5
731.7
z=4
914.3
rel. intensity [a.u.]
m/z

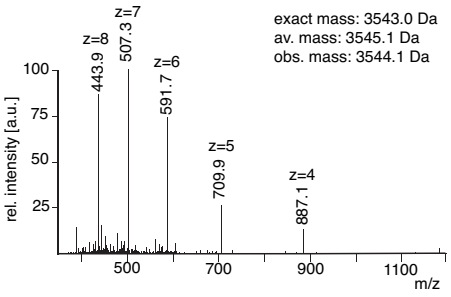
**X**


pep24: H3K4me2K9me2S10phK27me2S28ph
8.67
rel. absorbans (280 nm)
retention time [min]
exact mass: 3679.1 Da
av. mass: 3681.2 Da
obs. mass: 3680.1 Da
z=8
461.0
z=7
526.7
z=6
614.3
z=5
737.1
z=4
921.1
rel. intensity [a.u.]
m/z

**Y**

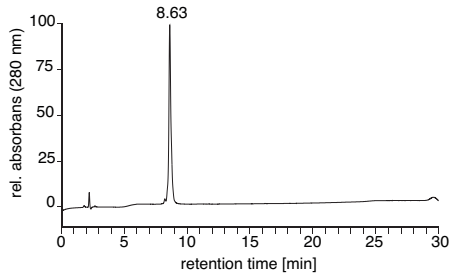
pep25: H3K9me2S28ph

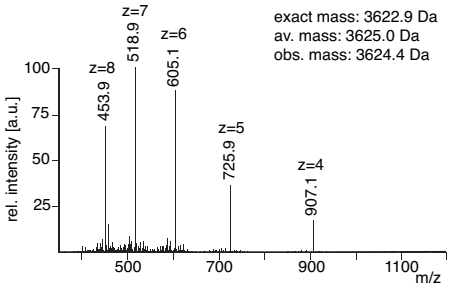



**Z**

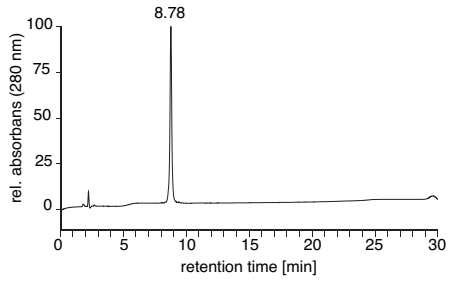
pep26: H3K9me2S10phS28ph

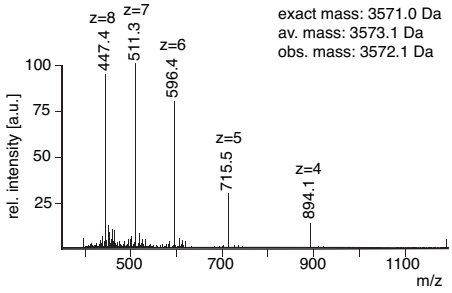



**A1**

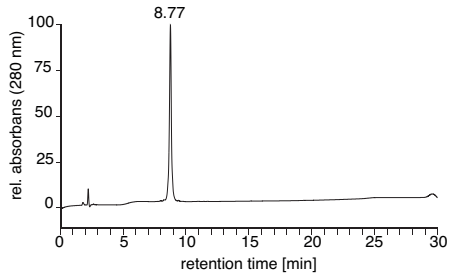
pep27: H3K9me2K27me2S28ph

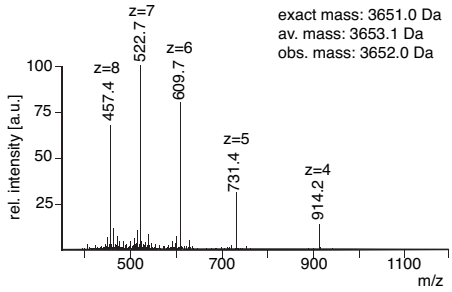



**A2**

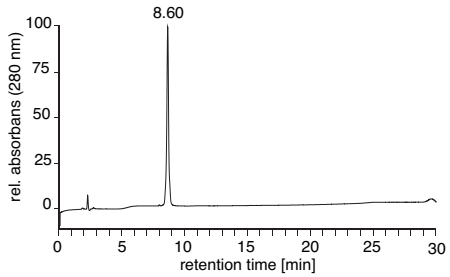
pep28: H3K9meS10ph2K27me2S28ph

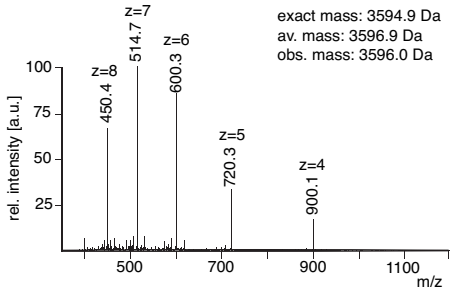


**A3**

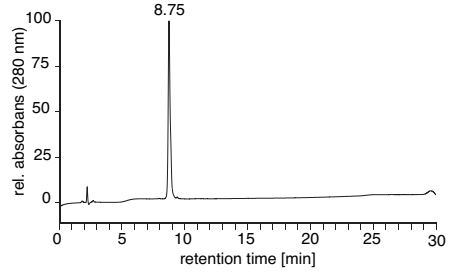
pep29: H3S10phS28ph

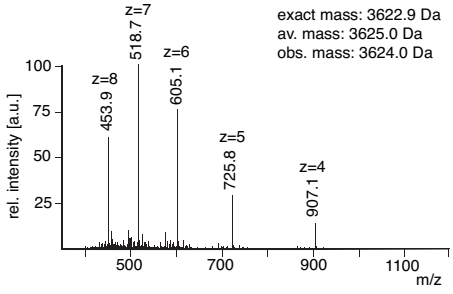



**A4**

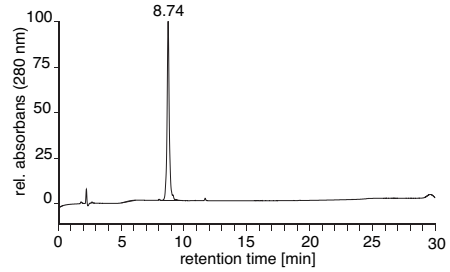
pep30: H3S10phK27me2S28ph

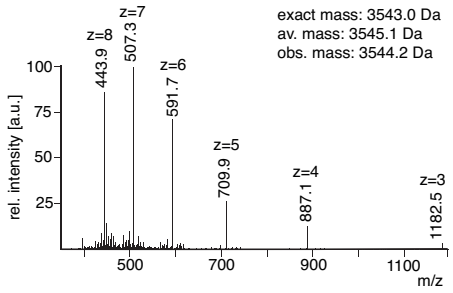



**A5**

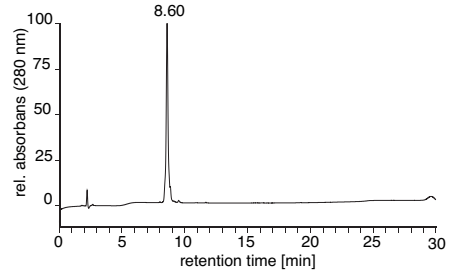
pep31: H3K27me2S28ph

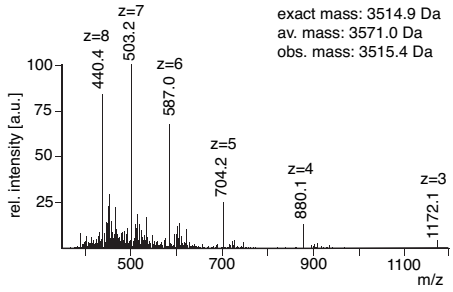



**A6**

pep32: H3S28ph



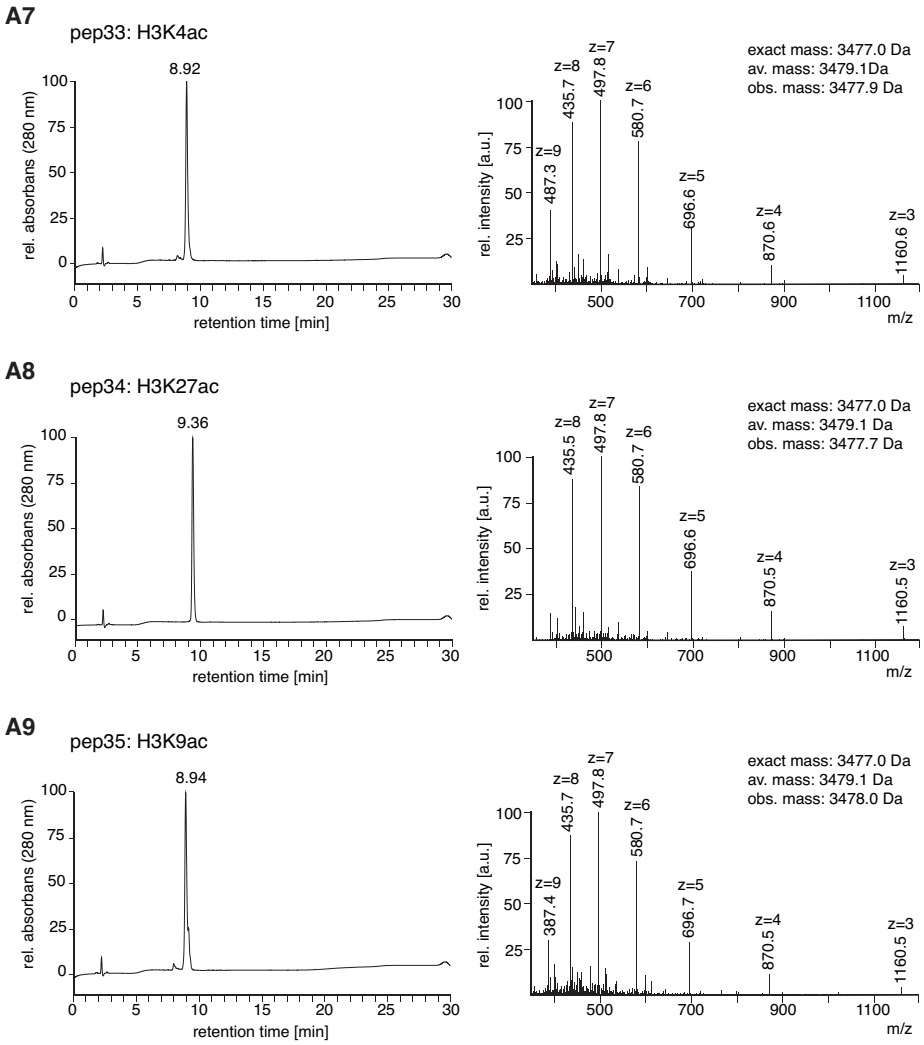
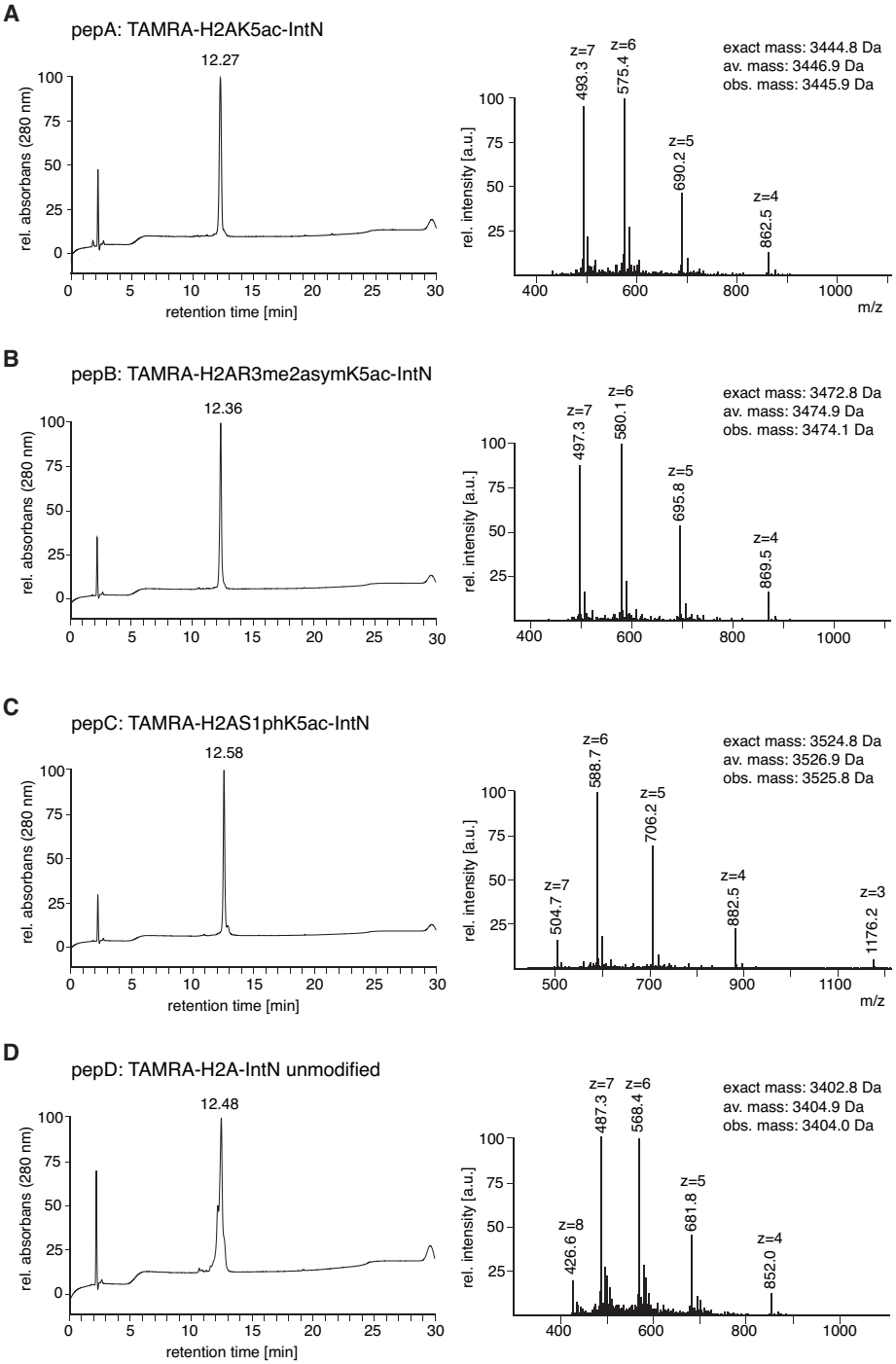



**Fig. S13: LC-MS analysis of H3 depsipeptides used in this study.**


A
pepA: TAMRA-H2AK5ac-IntN
12.27
rel. absorbans (280 nm)
retention time [min]
z=7
493.3
z=6
575.4
z=5
690.2
z=4
862.5
exact mass: 3444.8 Da
av. mass: 3446.9 Da
obs. mass: 3445.9 Da
rel. intensity [a.u.]
m/z
B
pepB: TAMRA-H2AR3me2asymK5ac-IntN
12.36
rel. absorbans (280 nm)
retention time [min]
z=7
497.3
z=6
580.1
z=5
695.8
z=4
869.5
exact mass: 3472.8 Da
av. mass: 3474.9 Da
obs. mass: 3474.1 Da
rel. intensity [a.u.]
C
pepC: TAMRA-H2AS1phK5ac-IntN
12.58
rel. absorbans (280 nm)
retention time [min]
z=7
504.7
z=6
588.7
z=5
706.2
z=4
882.5
z=3
1176.2
exact mass: 3524.8 Da
av. mass: 3526.9 Da
obs. mass: 3525.8 Da
rel. intensity [a.u.]
m/z
D
pepD: TAMRA-H2A-IntN unmodified
12.48
rel. absorbans (280 nm)
retention time [min]
z=8
426.6
z=7
487.3
z=6
568.4
z=5
681.8
z=4
852.0
exact mass: 3402.8 Da
av. mass: 3404.9 Da
obs. mass: 3404.0 Da
rel. intensity [a.u.]

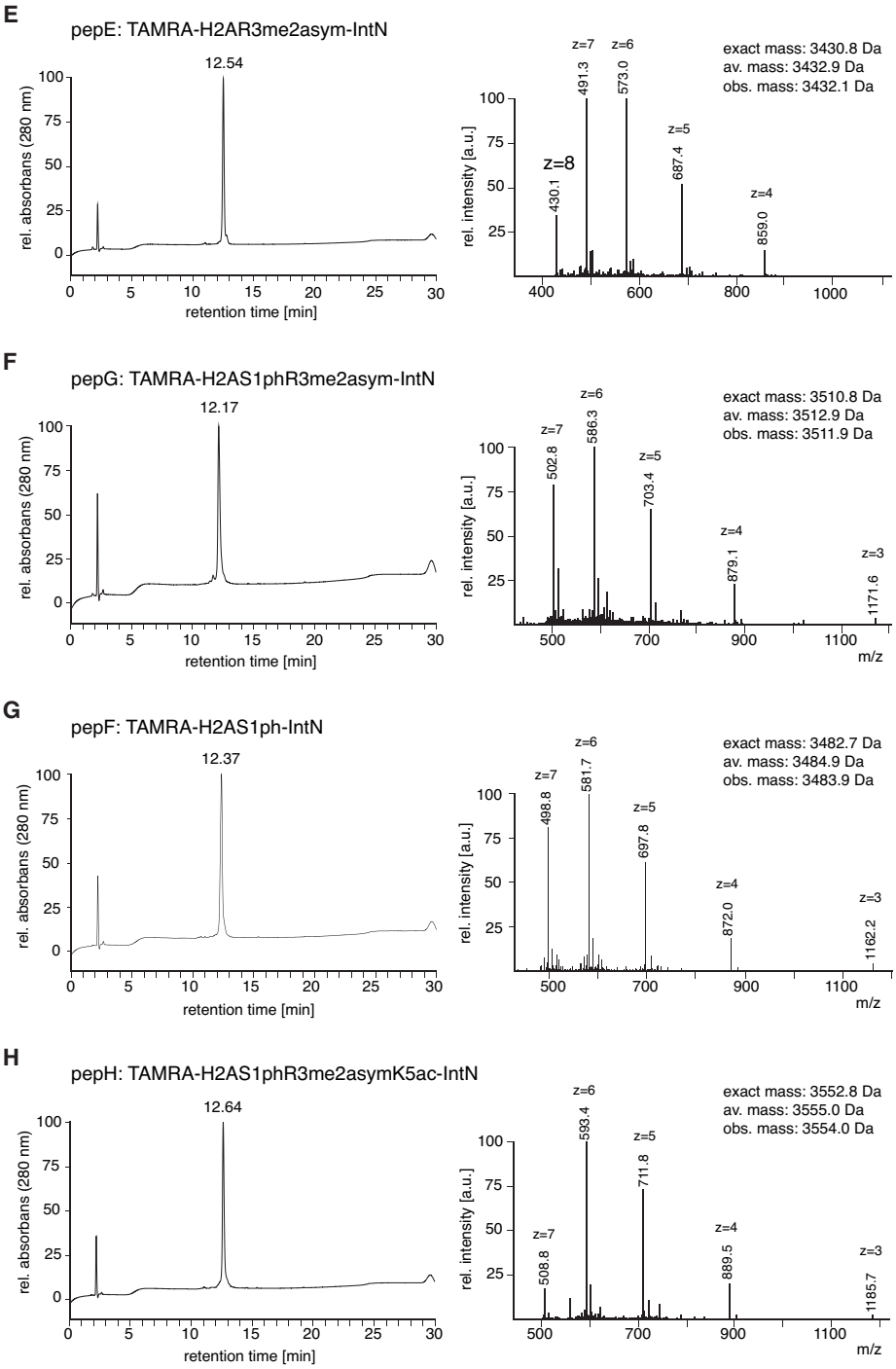



**Fig. S14: LC-MS analysis of H2A peptides used in this study.**

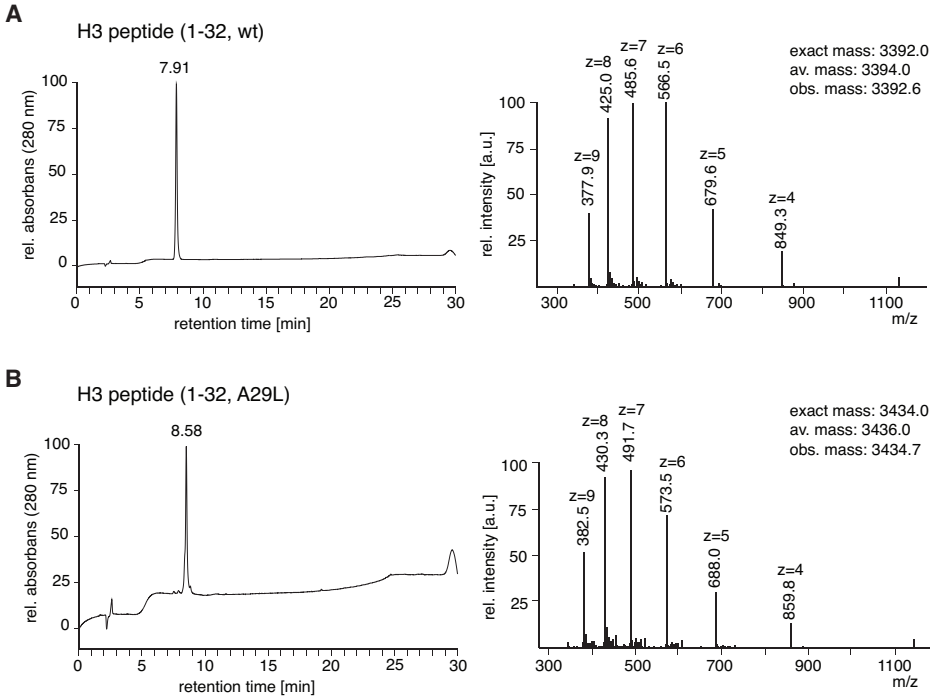



**Fig. S15: LC-MS analysis of H3 peptides used in this study.** The peptides were used for establishing nucleosomal ligations in solution (see Fig. S1). The H3 depsipeptide (1-32, A29L) is identical to pep19 (Fig. S9 P).

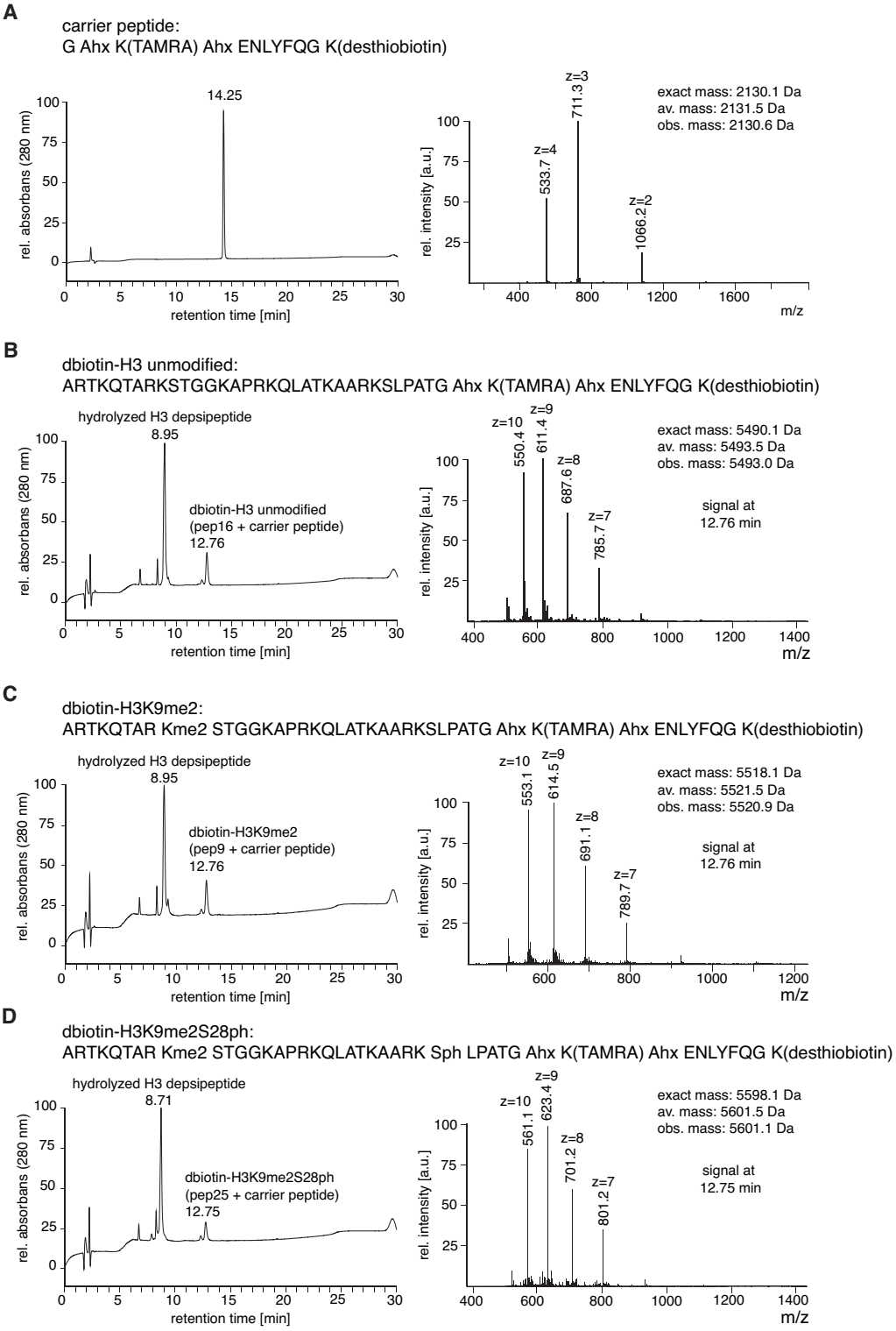



**Fig. S16: LC-MS analysis of carrier peptide and peptide ligation reactions.** The peptides and ligation products were used for investigating HP1-binding on peptide level (see Fig. S6).

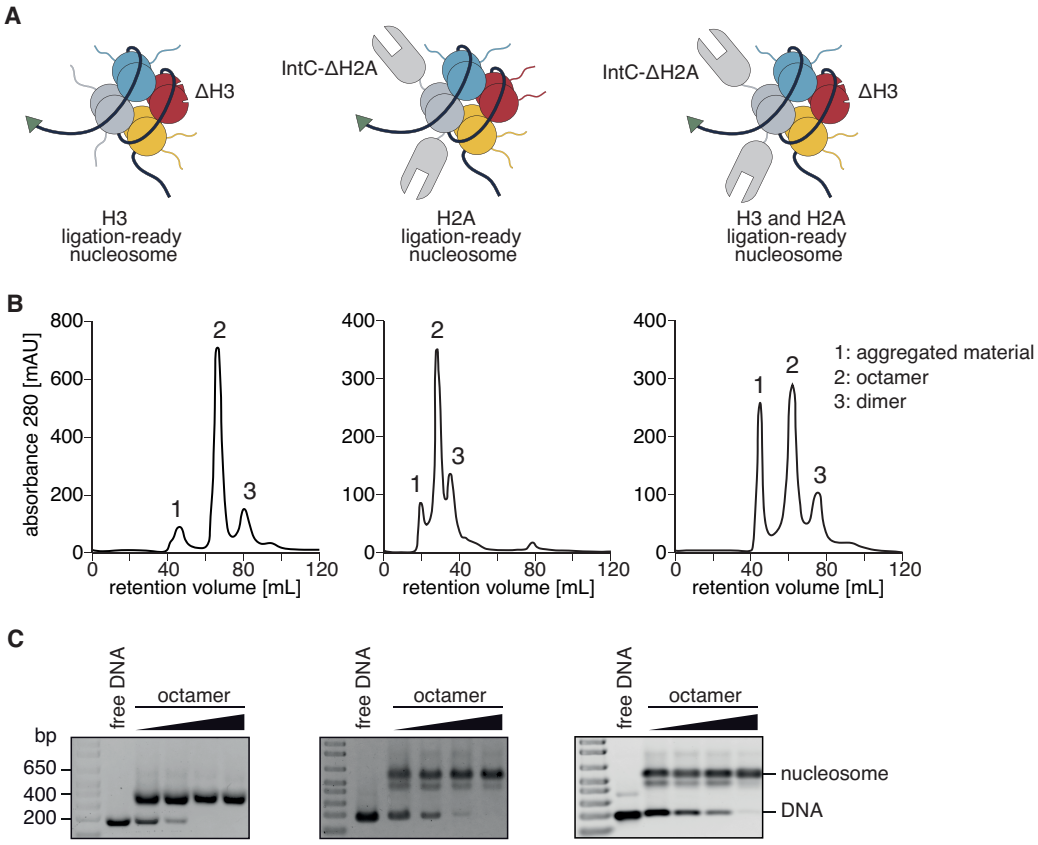



**Fig. S17: Reconstitution of H3-, H2A, and H3+H2A-ligation-ready nucleosomes.** (**A**) Scheme of ligation-ready nucleosomes. (**B**) Purification of histone octamers by FPLC. (**C**) Reconstitution reactions of nucleosomes at different ratio of DNA : histone octamer. DNA: 147 bp 601 Widom sequences.

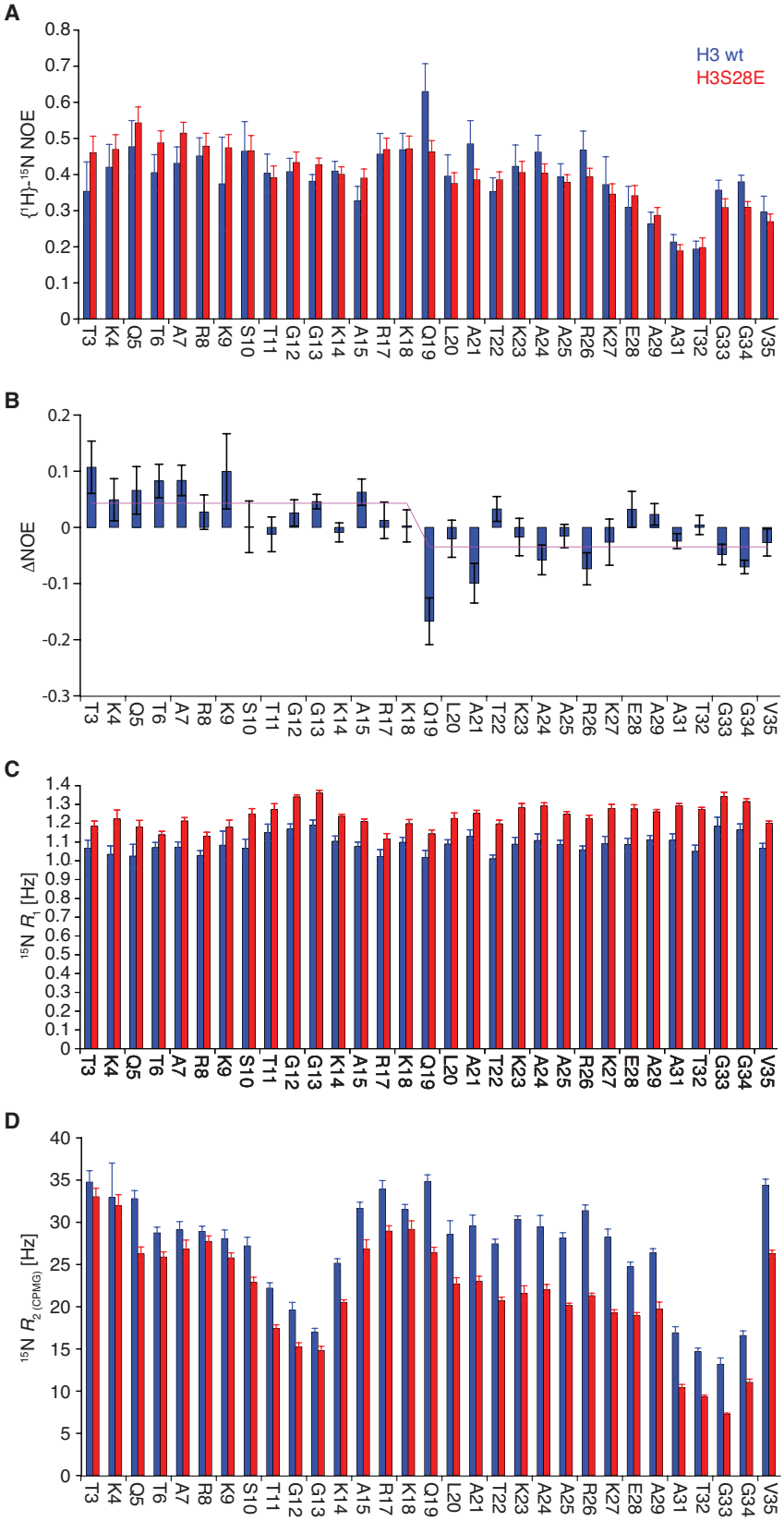



**Fig. S18: NMR analysis of nucleosomal H3S28E in comparison to H3 wt.** (**A**). NOE (nuclear Overhauser effect) values for H3 (blue) and H3S28E-containing (red) nucleosomes as collected at 950 MHz ($^1$H frequency) at 301K. (**B**) Difference between the steady-state heteronuclear {$^1$H}-$^{15}$N NOE values from H3 and H3 S28E-containing nucleosomes. (**C, D**) $^{15}$N longitudinal ($R_1$) and transverse ($R_2$) relaxation rates.

## 10.Supporting Tables

| Peptide ID | H3 histone tail depsipeptide |
|---|---|
| pep 1 | ART Kme2 QTARKSTGGKAPRKQLATKAARKSLPATG |
| pep 2 | ART Kme2 QTAR Kme2 STGGKAPRKQLATKAARKSLPATG |
| pep 3 | ART Kme2 QTARK Sph TGGKAPRKQLATKAARKSLPATG |
| pep 4 | ART Kme2 QTARKSTGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 5 | ART Kme2 QTAR Kme2 Sph TGGKAPRKQLATKAARKSLPATG |
| pep 6 | ART Kme2 QTAR Kme2 STGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 7 | ART Kme2 QTARK Sph TGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 8 | ART Kme2 QTAR Kme2 Sph TGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 9 | ARTKQTAR Kme2 STGGKAPRKQLATKAARKSLPATG |
| pep 10 | ARTKQTAR Kme2 Sph TGGKAPRKQLATKAARKSLPATG |
| pep 11 | ARTKQTAR Kme2 STGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 12 | ARTKQTAR Kme2 Sph TGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 13 | ARTKQTARK Sph TGGKAPRKQLATKAARKSLPATG |
| pep 14 | ARTKQTARK Sph TGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 15 | ARTKQTARKSTGGKAPRKQLATKAAR Kme2 SLPATG |
| pep 16 | ARTKQTARKSTGGKAPRKQLATKAARKSLPATG |
| pep 17 | ART Kme2 QTARKSTGGKAPRKQLATKAARK Sph LPATG |
| pep 18 | ART Kme2 QTAR Kme2 STGGKAPRKQLATKAARK Sph LPATG |
| pep 19 | ART Kme2 QTARK Sph TGGKAPRKQLATKAARK Sph LPATG |
| pep 20 | ART Kme2 QTARKSTGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 21 | ART Kme2 QTAR Kme2 Sph TGGKAPRKQLATKAARK Sph LPATG |
| pep 22 | ART Kme2 QTAR Kme2 STGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 23 | ART Kme2 QTARK Sph TGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 24 | ART Kme2 QTAR Kme2 Sph TGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 25 | ARTKQTAR Kme2 STGGKAPRKQLATKAARK Sph LPATG |
| pep 26 | ARTKQTAR Kme2 Sph TGGKAPRKQLATKAARK Sph LPATG |
| pep 27 | ARTKQTAR Kme2 STGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 28 | ARTKQTAR Kme2 Sph TGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 29 | ARTKQTARK Sph TGGKAPRKQLATKAARK Sph LPATG |
| pep 30 | ARTKQTARK Sph TGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 31 | ARTKQTARKSTGGKAPRKQLATKAAR Kme2 Sph LPATG |
| pep 32 | ARTKQTARKSTGGKAPRKQLATKAARK Sph LPATG |
| pep 33 | ART Kac QTARKSTGGKAPRKQLATKAARKSLPATG |
| pep 34 | ARTKQTARKSTGGKAPRKQLATKAAR Kac SLPATG |
| pep 35 | ARTKQTAR Kac STGGKAPRKQLATKAARKSLPATG |

| Peptide ID | H2A tail IntN peptide |
|---|---|
| pep A | TAMRA-SGRG Kac QGGKTRAKAKTRSG-CISGDSLISLA |
| pep B | TAMRA-SG Rme2asym G Kac QGGKTRAKAKTRSG-CISGDSLISLA |
| pep C | TAMRA- Sph GRG Kac QGGKTRAKAKTRSG-CISGDSLISLA |
| pep D | TAMRA-SGRGKQGGKTRAKAKTRSG-CISGDSLISLA |
| pep E | TAMRA-SG Rme2asym GKQGGKTRAKAKTRSG-CISGDSLISLA |
| pep F | TAMRA- Sph GRGKQGGKTRAKAKTRSG-CISGDSLISLA |
| pep G | TAMRA- Sph G Rme2asym GKQGGKTRAKAKTRSG-CISGDSLISLA |
| pep H | TAMRA- S Sph G Rme2asym G Kac QGGKTRAKAKTRSG-CISGDSLISLA |

| Peptide ID | further peptides used in this study |
|---|---|
| carrier peptide | G Ahx K(TAMRA) Ahx ENLYFQG K(desthiobiotin) |
| dbiotin-H3 unmodified | ARTKQTARKSTGGKAPRKQLATKAARKSLPATG Ahx K(TAMRA) Ahx ENLYFQG K(desthiobiotin) |
| dbiotin-H3K9me2 | ARTKQTAR Kme2 STGGKAPRKQLATKAARKSLPATG Ahx K(TAMRA) Ahx ENLYFQG K(desthiobiotin) |

| | |
|---|---|
| dbiotin-H3K9me2S28ph | ARTKQTAR Kme2 STGGKAPRKQLATKAARK Sph LPATG Ahx K(TAMRA) Ahx ENLYFQG K(desthiobiotin) |
| H3 peptide (1-32, wt) | ARTKQTARKSTGGKAPRKQLATKAARKSAPATG |
| H3 peptide (1-32, A29L) | ARTKQTARKSTGGKAPRKQLATKAARKSLPATG |

**Table S1: Sequences of all peptides and ligation products used in this study.**

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