

Supplementary information for:

Controlling the supramolecular assembly of nucleosomes asymmetrically modified on H4

Nora Guidotti^a, Carolin Lechner^{a,b} and Beat Fierz^{a,c}

a. Laboratory of Biophysical Chemistry of Macromolecules, Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland.

b. Present address: Bachem AG, Bubendorf, Switzerland

c. E-mail: beat.fierz@epfl.ch

Materials:

All solvents and reagents were purchased from commercial sources and used without further purification. Fmoc-L-Lys(Boc,Me)-OH was from Iris Biotech (Marktredwitz, Germany). All other amino acid derivatives, 2-chlorotriptyl chloride resin and 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate (HATU) were purchased from Novabiochem, Merck (Darmstadt, Germany). N,N-Dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), piperidine and phenol were from Acros Organics (Geel, Belgium). 2-(1H-benzotriazol-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate (HBTU) was from Protein Technologies Inc. (Tucson, USA). Hydrazine monohydrate was purchased from Alfa Aesar (Heysham, UK), acetonitrile (ACN) from Avantor Performance Materials (USA). Diethylether, acetic anhydride, phenylsilane, tetrakis(triphenylphosphine)palladium(0), hydroxybenzotriazole (HOBt), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Dithiothreitol (DTT), silver acetate, trifluoroacetic acid (TFA), dichloromethane (DCM), triisopropylsilane (TIS), ethanedithiol (EDT), thioanisole, sodium nitrite, L-glutathione reduced (GSH), sodium diethyldithiocarbamate trihydrate and methyl thioglycolate (MTG) were from Sigma Aldrich (Taufkirchen, Germany). 2,2'-Azobis[2-(2- imidazolin-2-yl)propane]dihydrochloride (VA-044) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other commonly used chemical reagents and buffer components were from Applichem (Darmstadt, Germany) and Fisher Scientific (Reinach, Switzerland). DNA ladders and DNA loading dyes were from New England Biolabs (Ipswich, MA, USA). Chemicals and solutions for preparation of agarose and SDS polyacrylamide gels (agarose, acrylamide, Precision Plus Protein™ All Blue Prestained Protein Standard) were purchased from BioRad (Hercules, CA, USA). Slide-A-Lyzer™ dialysis cassettes and Slide-A-Lyzer™ MINI dialysis devices were from Thermo Scientific (Rockford, IL, USA). Recombinant Set8 was purchased from Active Motif (La Hulpe, Belgium). Amersham Amplify Fluorographic Reagent was from GE Healthcare, S-[Methyl-3H]-adenosyl-L-methionine (3H-SAM) and Ultima Gold F liquid scintillation cocktail from Perkin Elmer (Waltham, MA, USA). P81 Ion Exchange Cellulose Chromatography Paper was from Reaction Biology (Malvern, PA, USA).

Instrumentation:

Reaction vessels for manual peptide synthesis as well as the automated Tribute peptide synthesiser were from Protein Technologies Inc. Bacterial cells for recombinant protein expression were grown in an HT infors AG incubator. *E. coli* cells were lysed by sonication using a Vibra-cell VCX 750 Sonics & Materials sonicator.

Sedimentations were accomplished in an Avanti J20 XPI centrifuges and rotors (JA-12 and JA-8.1000) from Beckman Coulter. The chambers for horizontal DNA electrophoresis and the Mini-Protean II system for SDS-PAGE were from BioRad. Gels were imaged using a ChemiDoc MP imaging system from BioRad. Size exclusion chromatography was performed on an ÄKTA Pure FPLC system with an S200 10/300GL column from GE Healthcare. Analytical RP-HPLC analysis was performed on an Agilent 1260 series instrument using an analytical Agilent Zorbax C18 column (column dimensions: 150 mm x 4.6 mm, 5 µm particle size) at a flow rate of 1 mL/min. All RP-HPLC analyses were done with 0.1 % (v/v) TFA in H₂O (RP-HPLC solvent A) and 90 % ACN and 0.1 % (v/v) TFA in H₂O (RP-HPLC solvent B) as mobile phases. Typically, a gradient from 0-70 % solvent A to solvent B over 30 min was used for analytical RP-HPLC analyses unless otherwise stated. Purification of proteins on a semi-preparative scale were performed on an Agilent 1260 series instrument using a semi-preparative Agilent Zorbax C18 column (column dimensions: 250 mm x 9.4 mm, 5 µm particle size) at a flow rate of 4 mL/min. Preparative RP-HPLC purifications were done on an Agilent 1260 preparative HPLC system with a preparative Agilent Zorbax C18 column (column dimensions: 250 mm x 21.2 mm, 7 µm particle size) at a flow rate of 20 mL/min. Lyophilisation was carried out with a Telstar LyoQuest freeze dryer. Electrospray ionisation mass spectrometric (ESI-MS) analysis was conducted on a Shimadzu MS2020 single quadrupole instrument connected to a Nexera UHPLC system. Mass spectra were acquired by electrospray ionisation in positive ion mode in the mass range of 200-2000 m/z. UV-Vis absorption measurements were carried out using an Agilent 8453 UV-Vis spectrophotometer. Scintillation counting was performed on a Packard Tri-Carb Liquid Scintillation Counter.

1. Peptide synthesis:

For the production of symmetrically and asymmetrically methylated H4K20 (sH4K20me1 and asH4K20me1, respectively), several peptides were synthesised. The sequences are summarised in **Table S1**. The synthetic procedures and analytical data is presented in the following.

Name	Synthetic peptide sequence	Purpose
1	Ac-SGRGK(^{iso} Inc[Acm])-GGKGLGKGG-NHNH ₂	Synthesis of asH4K20me1
1'	Ac-SGRGKGKGLGKGG-NHNH ₂	Native H4 seq., synthesis of sH4K20me1
2	H-CKRHRKVLRDNIQGITKPAIRRL-NHNH ₂	Synthesis of asH4K20me1
2a	H-CKRHRK(me)VLRDNIQGITKPAIRRL-NHNH ₂	Synthesis of asH4K20me1, sH4K20me1

Table S1: Peptides used for chemical synthesis of differently modified H4 molecules.

Preparation of Fmoc-aa-hydrazine-Cl-trityl-resin

Preparation of Fmoc-Leu-NHNH-Cl-Trt-resin and Fmoc-Gly-NHNH-Cl-Trt-resin was performed as reported previously.¹ In general, 0.5 g 2-Cl-Trt-resin (0.82 mmol, 1 eq., substitution: 1.63 mmol/g) were swollen in 3 mL DMF for 15 min at room temperature. Subsequently, the resin was cooled to 0° C and 1 mL of a solution containing DIEA (2.45 mmol, 427 µl, 3 eq.) and hydrazine monohydrate (1.64 mmol, 80 µl, 2 eq.) in DMF was added dropwise. The reaction mixture was stirred 1 h at room temperature. Then 100 µl methanol (MeOH) were added, the resin was stirred 10 min at room temperature, transferred to a reaction vessel for manual peptide synthesis and washed with DMF.

Due to the low stability of the hydrazine resin, the first amino acid Fmoc-Leu-OH or Fmoc-Gly-OH was coupled manually by standard Fmoc chemistry.² The loading of the 2-Cl-Trt-resin with hydrazine was assumed to be 50% (0.41 mmol, 1 eq.). Fmoc-Leu-OH or Fmoc-Gly-OH (2.05 mmol, 5 eq.) was first activated by addition of 3.9 mL of a 0.5 M HBTU solution (1.95 mmol, 4.76 eq.) in DMF followed by 2 min incubation at room temperature. Then, 714 μ L DIEA (4.1 mmol, 10 eq.) were added and the reaction mixture was incubated another 1 min at room temperature. The activated amino acid was added to the dry resin, incubated 30 min at room temperature and washed with DMF. To ensure high-yield, the coupling procedure was repeated. Finally, the resin was washed with DMF, DCM and MeOH and dried under vacuum. Resin substitution was determined by treating a defined amount of resin with 20% piperidine in DMF for 30 min at RT, followed by spectrophotometrical quantification of released dibenzofulven-related species.

Automated Solid Phase Peptide Synthesis (SPPS)

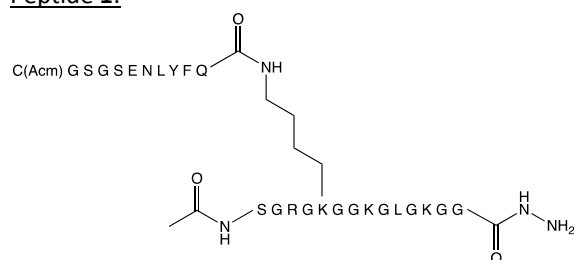
General protocol: the peptides were synthesised by the Tribute peptide synthesiser (PTI) on the previously prepared Fmoc-aa-hydrazine-Cl-Trt-resin to yield peptides with C-terminal hydrazide. The syntheses were performed on 0.1 mmol scale using Fmoc chemistry. The base-resistant groups employed to protect amino acid side chains are listed below: Arg(Pbf), Lys(Boc), Thr(tBu), Gln(Trt), Ans(Trt), Asp(OtBu), His(Trt), Cys(Trt), Ser(tBu), Tyr(tBu), Glu(tBu), Cys(Acm), Lys(Alloc). To maximise synthesis yield, amino acids were double coupled and pseudoproline dipeptide building blocks were used where necessary.

Briefly, the N-terminal Fmoc-group was deprotected by incubating the resin with 20% (v/v) piperidine in DMF. Activation of amino acid (0.5 mmol, 5 eq.) was achieved by addition of HBTU (0.48 mmol, 4.8 eq.) and DIEA (1 mmol, 10 eq.). The coupling step was performed by adding the activated amino acid to the resin, followed by 30 min incubation at room temperature. When the full-length peptide was assembled, the peptidyl-resin was washed with DMF, DCM and MeOH and dried under vacuum.

The peptide was cleaved from the resin using either 95% TFA, 2.5% TIS, 2.5% H₂O or 87.5% TFA, 5% phenol, 5% thioanisole, 2.5% ethanedithiol, 5% H₂O. The crude peptide was precipitated by addition of ice-cold diethyl ether, recovered by centrifugation, dissolved in 50% (v/v) acetonitrile in H₂O, flash-frozen and lyophilised.

Synthesis notes and analytical data for peptides

Peptide 1:



A special protocol was adopted for the synthesis of the branched peptide **1**: the first 14 residues were synthesised as reported above, on the pre-loaded Fmoc-Gly-hydrazine Cl-Trt resin and introducing the

commercially available Fmoc-Lys(Alloc)-OH at position 5. Acetylation of the N-terminus was achieved by 3x30 min incubation of the peptidyl-resin with 15 mL of a solution containing 10% (v/v) acetic anhydride, 10% (v/v) DIEA in DMF. After extensive washing with DMF and DCM, Alloc deprotection of lysine 5 was initiated. The resin was swollen for 30 min in DCM. 1 mL of dry DCM and PhSiH₃ (24 mmol, 24 eq.), followed by Pd(PPh₃)₄ (0.025 mmol, 0.25 eq.) in 3 mL dry DCM were added to the resin and incubated for 30 min at room temperature. The resin was washed with DCM and the deprotection reaction with PhSiH₃ and Pd(PPh₃)₄ was repeated two more times. The resin was thoroughly washed with DCM followed by washing with 0.5% (v/v) DIEA in DMF; 0.5% (w/v) sodium-diethyldithiocarbamate in DMF; 50% (v/v) DCM in DMF; 0.5% (w/v) HOBt in DMF and intensively washed with DMF. After Alloc deprotection, the glutamine residue was coupled manually to the ε-amino group of Lys5. Fmoc-Gln(Trt)-OH (0.5 mmol, 5 eq.) was activated by addition of 0.95 mL of a 0.5 M HATU solution (0.48 mmol, 4.8 eq.) in DMF followed by 2 min incubation at room temperature. Then, 172 μL DIEA (1 mmol, 10 eq.) were added and the reaction mixture was incubated another 1 min at room temperature. The activated amino acid was added to the peptidyl-resin, incubated 30 min at room temperature and washed with DMF. To ensure high-yield, the coupling procedure was repeated. Finally, the resin was washed with DMF. The rest of the synthesis was carried out by automated Fmoc-SPPS on the Fmoc-Gln(Trt) coupled to Lys5 side chain. Subsequent cleavage from the resin was performed as indicated above. The crude peptide was purified by preparative RP-HPLC on a linear gradient from 10 to 40% solvent B over 30 min. Pure fractions were pooled and lyophilised. The purified peptide **1** (yield 21.0%) was analysed by analytical RP-HPLC and ESI-MS (**Figure S1**).

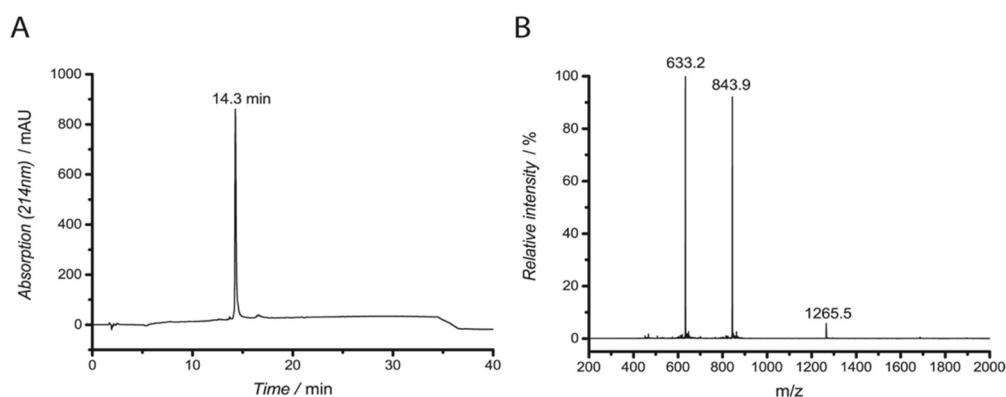


Figure S1: RP-HPLC chromatogram (A) and ESI-MS (B) of purified peptide **1**. Calculated mass: 2528.8 Da, found: 633.2 [M+4H]⁴⁺, 843.9 [M+3H]³⁺, 1265.5 [M+2H]²⁺.

Peptide **1'**:



Peptide **1'** (containing the native H4 sequence (1-14) without the *iso/nc*-tag) was synthesised on the preloaded Fmoc-Gly-hydrazine Cl-Trt resin. Acetylation of the N-terminus was achieved by 3x30 min incubation of the peptidyl-resin with 15 mL of a solution containing 10% (v/v) acetic anhydride, 10% (v/v) DIEA in DMF. Purification of the crude peptide was performed on the preparative RP-HPLC employing a linear gradient from 0 to 30% solvent B over 45 min. Fractions were analysed, pooled accordingly and lyophilised (yield: 21.5%). Purified peptide **1'** was analysed and characterised by analytical RP-HPLC and ESI-MS (**Figure S2**).

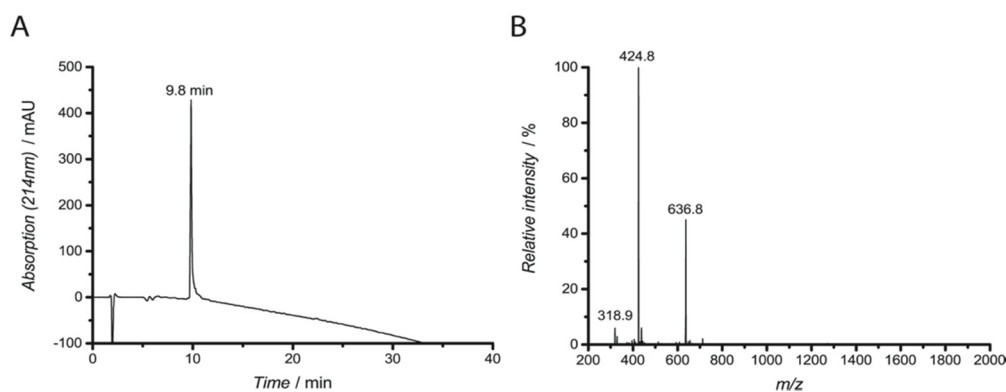


Figure S2: RP-HPLC chromatogram (A) and ESI-MS (B) of purified peptide **1'**. Calculated mass: 1271.4 Da, found: 318.9 [M+4H]⁴⁺, 424.8 [M+3H]³⁺, 636.8 [M+2H]²⁺.

Peptide 2:



Peptide **2** (the unmethylated H4 middle-fragment (15-37)) was synthesised on the preloaded Fmoc-Leu-hydrazine Cl-Trt resin. Purification of the crude peptide was performed on the preparative RP-HPLC employing a linear gradient from 10 to 40% solvent B over 45min. Fractions were analysed, pooled accordingly and lyophilised (yield: 59.5%). Purified peptide **2** was analysed and characterised by analytical RP-HPLC and ESI-MS (**Figure S3**).

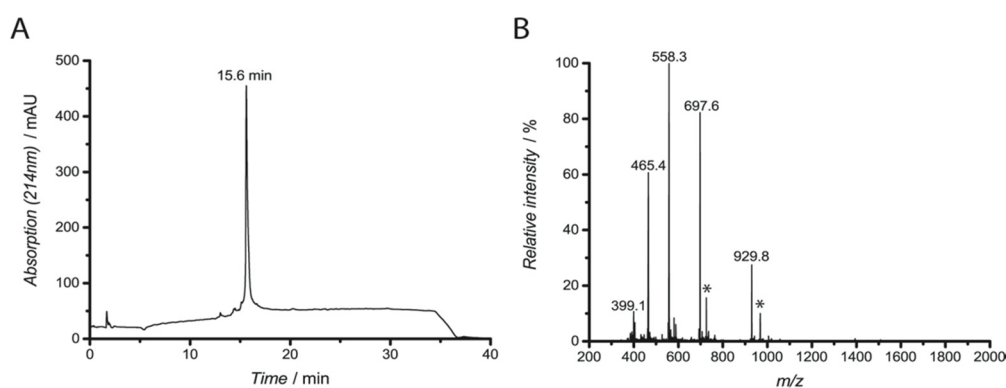


Figure S3: RP-HPLC chromatogram (A) and ESI-MS (B) of purified peptide **2**. Calculated mass: 2786.4 Da, found: 399.1 [M+7H]⁷⁺, 465.4 [M+6H]⁶⁺, 558.3 [M+5H]⁵⁺, 697.6 [M+4H]⁴⁺, 929.8 [M+3H]³⁺. Asterisks correspond to TFA adducts.

$$\text{H}-\text{CKRHRK}(\text{me})\text{VLRDNIQGITKPAIRRL}-\text{C}(=\text{O})\text{NH}_2$$

A

1400
1200
1000
800
600
400
200
0

Absorption (214nm) / mAU

15.5 min

0 10 20 30 40

Time / min

B

100
80
60
40
20
0

Relative intensity / %

561.1 701.1

467.7

401.0

934.5

200 400 600 800 1000 1200 1400 1600 1800 2000

m/z

2. Expression and purification of recombinant histone proteins

Human wild-type core histones H2A, H2B and H3(C110A) were expressed and purified as previously described.³ N-terminal truncated H4 protein (H4(38-102)A38C), **4**, was recombinantly expressed in *E. coli* BL21(DE3) cells as N-terminal fusion to a His6-SUMO tag.⁴ Bacterial cultures were grown in 12x1L LB medium (with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol) at 37° C to an OD₆₀₀ of 0.6-0.8. Protein expression was induced with 0.25 mM IPTG and cells were incubated at 37° C for 2 h. Cells were harvested by centrifugation (4000 x g, 4° C, 15 min) and cell pellets resuspended in lysis buffer (200 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.5). Cells were lysed by sonication and centrifuged (15000 x g, 4° C, 15 min). The pelleted insoluble inclusion bodies were washed twice with lysis buffer containing 1% Triton X-100 and once with lysis buffer without detergent. Inclusion bodies were solubilised in resolubilisation buffer (6 M GdmHCl, 100 mM NaCl, 50 mM Tris-HCl, 5 mM imidazole, 250 mM L-ArgHCl, 2 mM 2-mercaptoethanol, pH 7.5), centrifuged (15000 x g, 4° C, 15 min) to remove insoluble material and applied to Ni-NTA resin previously washed with 10 mM imidazole and equilibrated in resolubilisation buffer. The protein was bound to the resin under gentle agitation overnight at 4° C. The flow-through was collected and the resin was washed with 1 column volume (CV) resolubilisation buffer and 1 CV urea wash buffer (6 M Urea, 150 mM NaCl, 25 mM Tris-HCl, 5 mM imidazole, 2 mM 2-mercaptoethanol, pH 7.5). The protein was eluted with 5 x 0.5 CV urea elution buffer (6 M Urea, 150 mM

NaCl, 25 mM Tris-HCl, 300 mM imidazole, 2 mM 2-mercaptoethanol, pH 7.5). Washing and elution fractions were analysed by SDS-PAGE (12% polyacrylamide gel) and protein containing fractions were pooled.

The SUMO protease His₆-Ulp1 needed for cleavage of the His₆-SUMO-H4(38-102)A38C was expressed in *E. coli* BL21(DE3) cells. Bacterial cultures were grown in LB medium (with 50 µg/ml kanamycin) at 37° C to an OD₆₀₀ of 0.6-0.8. Protein expression was induced with 0.25 mM IPTG and cells were incubated at 37°C for 3.5 h. Harvesting and lysis of cells, isolation of inclusion bodies and purification was performed as described for His₆-SUMO-H4(38-102)A38C.

Dialysis of both proteins against dialysis buffer (1 M Urea, 150 mM NaCl, 75 mM Tris-HCl, 25 mM L-ArgHCl, 5 mM L-Cys, 2 mM 2-mercaptoethanol, pH 7.5) allowed the refolding and cleavage of the His₆-SUMO tag from the His₆-SUMO-H4(38-102)A38C fusion protein. The protease and the protein of interest were mixed to a ratio 2:1 and dialysed over night at 4°C. The cleaved H4(38-102)A38C as well as His₆-Ulp1 precipitated during the dialysis, as confirmed by analytical RP-HPLC and ESI-MS. The precipitate was recovered by centrifugation (47000 x g, 4° C, 15 min) and solubilised in resolubilisation buffer. The resolubilised proteins were applied to Ni-NTA resin previously washed with 10 mM imidazole and equilibrated in resolubilisation buffer. His₆-Ulp1 was bound to the resin under gentle agitation for 20 min at 4° C. The flow-through was collected and the resin was washed with 6 x 0.1 CV resolubilisation buffer (supplemented with 15mM imidazole). The flow-through and washing fractions were analysed by RP-HPLC, H4(38-102)A38C containing fractions were pooled and 10mM TCEP was added to avoid H4(38-102)A38C - 2-mercaptoethanol adducts. Crude H4(38-102)A38C was purified by semipreparative RP-HPLC using a linear gradient from 30-70% solvent B in 45min and collected fractions were analysed by analytical RP-HPLC and ESI-MS. Pure fractions were pooled, flash-frozen, lyophilised and stored at -20° C (**Figure S5**). Typical yields were 0.8 mg per litre of bacterial culture.

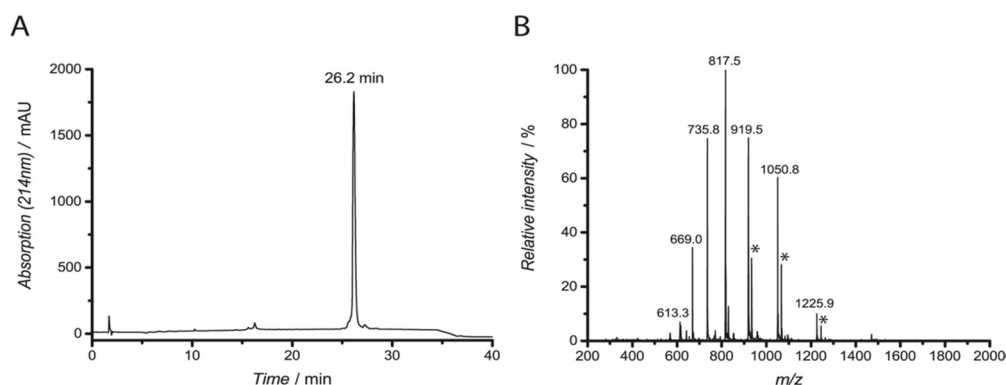


Figure S5: RP-HPLC chromatogram (A) and ESI-MS (B) of purified **4**. Calculated mass: 7348.5 Da, found: 613.3 [M+12H]¹²⁺, 669.0 [M+11H]¹¹⁺, 735.8 [M+10H]¹⁰⁺, 817.5 [M+9H]⁹⁺, 915.5 [M+8H]⁸⁺, 1050.8 [M+7H]⁷⁺, 1225.9 [M+6H]⁶⁺. Asterisks correspond to TFA adducts.

3. Synthesis of modified histone H4 proteins

Several H4 proteins were synthesised in two ligation steps each from two synthetic (**1**, **1'**, **2**, **2a**) and one recombinant fragment (H4(38-102)A38C), **4**, as summarised in **Table S2**. The detailed synthetic protocols and analytical data are given in the following.

Synthesis of H4 protein fragments and proteins		
Name	Sequence	Reaction product
	H4 fragments	
3	Ac-H4(1-37)K5(^{iso} Inc[Acm])-NHNH ₂	Ligation/desulfurisation of 1 + 2
3a	Ac-H4(1-37)K5(^{iso} Inc[Acm])K20me-NHNH ₂	Ligation/desulfurisation of 1 + 2a
3a'	Ac-H4(1-37)K20me-NHNH ₂	Ligation/desulfurisation of 1' + 2a
	H4 proteins	
5	Ac-H4K5(^{iso} Inc[Acm])-OH	Ligation/desulfurisation of 3 and H4(38-102)A38C, 4
5a	Ac-H4K5(^{iso} Inc[Acm])K20me-OH	Ligation/desulfurisation of 3a and H4(38-102)A38C, 4
5a'	Ac-H4K20me-OH	Ligation/desulfurisation of 3a' and H4(38-102)A38C, 4

Table S2: H4 fragments and H4 proteins used for the synthesis of differently modified H4 molecules.

One-pot ligation and desulfurisation

General protocol for one-pot ligation and desulfurisation: The ligation of N-terminal H4 peptides (1-14) (**1**, **1'**) and the middle fragments (15-37) (**2**, **2a**) (**Table S1**), yielding fragments **3**, **3a** and **3a'**, and the subsequent ligation of fragments **3**, **3a** and **3a'** and truncated H4 protein H4(38-102)A38C (**4**), yielding H4 proteins **5**, **5a** and **5a'** (**Table S2**), was achieved following Fang et al.,⁵ but with some modifications. Typically, peptide hydrazides (1 μmol, 1 eq.) were dissolved in ligation buffer (6 M GdmHCl, 0.2 M NaH₂PO₄, pH 3) to a final concentration of 10 mM. The peptide solution was cooled to -20° C in an ice/salt bath, followed by addition of NaNO₂ (1 μmol, 1 eq., aqueous 0.5 M stock solution) and incubation at -20° C for 20 min. MTG was added to a final concentration of 75 mM (75 μmol, 75 eq.) in order to form the thioester *in situ*, the pH was adjusted to 6.8 with NaOH and the reaction mixture was stirred at room temperature for 10 min. Subsequently, the solution containing the peptide-MTG thioester was transferred to a tube containing the N-terminal cysteine-containing peptide or truncated H4 protein (0.67 μmol, 0.67 eq.). TCEP was added to a final concentration of 25 mM (25 μmol, 25 eq.), the pH was adjusted to 6.8 and the ligation reaction was allowed to proceed for 3-16 h at 25° C. The progress of the reaction was monitored by RP-HPLC and ESI-MS analysis.

When the ligation was complete, desulfurisation of the cysteine at the ligation site was performed in the same reaction tube without prior purification of the ligation product. TCEP desulfurisation buffer (0.5 M TCEP, 6 M GdmHCl, 0.2 M phosphate, pH 7) was added to a final TCEP concentration of 0.25 M. The desulfurisation reaction was initiated by addition of VA-044 and GSH to a final concentration of 30 mM and 40 mM, respectively.⁶ The reaction mixture was incubated at 42° C for 6-16 h and the progress of the reaction was monitored by RP-HPLC and ESI-MS analysis. In the case of proteins **5** and **5a**, a tenfold dilution of the reaction mixture in combination with a decreased incubation temperature (30°C) prevented precipitation. When the desulfurisation was complete, 2 volumes of ligation buffer (pH 3) were added and the peptide was purified by semipreparative RP-HPLC.

Synthesis notes and analytical data for modified histone proteins:

Fragment 3:

The ligation of peptide **1** to peptide **2**, followed by desulfurisation of Cys15 was monitored by analytical RP-HPLC and MS. H4 fragment **3** was purified by semi-preparative RP-HPLC employing a linear gradient from 0 to 40% solvent B over 40 min and characterised by analytical RP-HPLC and ESI-MS (yield 45.7%) (**Figure S6**).

Fragment 3a:

The ligation of peptide **1** to peptide **2a**, followed by desulfurisation of Cys15 was monitored by analytical RP-HPLC and MS. H4 fragment **3a** was purified by semi-preparative RP-HPLC employing a linear gradient from 0 to 40% solvent B over 40 min and characterised by analytical RP-HPLC and ESI-MS (yield 60.5%) (**Figure S7**).

Protein 5:

The ligation of fragment **3** to truncated H4 H4(38-102)A38C (**4**), followed by desulfurisation of Cys38 was monitored by analytical RP-HPLC and MS. Protein **5** was purified by semi-preparative RP-HPLC employing a linear gradient from 30 to 70% solvent B over 30 min and characterised by analytical RP-HPLC and ESI-MS (yield 34.7%) (**Figure S8**).

Protein 5a:

The ligation of fragment **3a** to truncated H4 H4(38-102)A38C (**4**), followed by desulfurisation of Cys38 was monitored by analytical RP-HPLC and MS. Protein **5a** was purified by semi-preparative RP-HPLC employing a linear gradient from 40 to 70% solvent B over 40 min and characterised by analytical RP-HPLC and ESI-MS (yield 40.5%) (**Figure S9**).

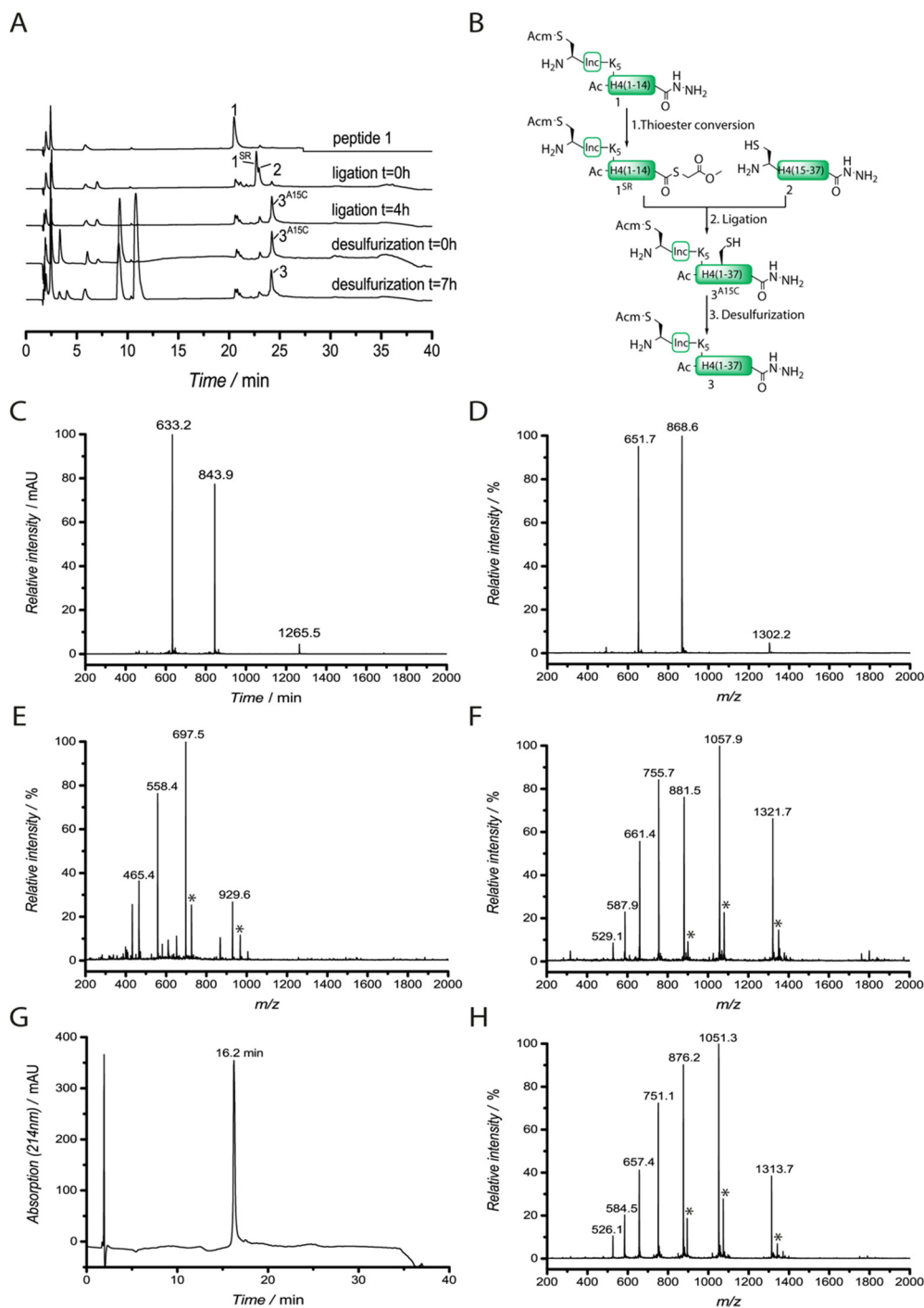


Figure S6: Synthesis of fragment **3** by one-pot ligation and desulfurisation. **A)** Analytical RP-HPLC chromatograms of the reaction steps. **B)** Scheme of the reaction steps. **C)** ESI-MS of peak **1**; Calculated mass for peptide-hydrazide **1**: 2528.8 Da, found: 633.2 [M+4H]⁴⁺, 843.9 [M+3H]³⁺, 1265.5 [M+2H]²⁺. **D)** ESI-MS of peak **1^{SR}**; Calculated mass for peptide-MTG thioester **1^{SR}**: 2602.9 Da, found: 651.7 [M+4H]⁴⁺, 868.6 [M+3H]³⁺, 1302.2 [M+2H]²⁺. **E)** ESI-MS of peak **2**; Calculated mass for peptide-hydrazide **2**: 2786.4 Da, found: 465.4 [M+6H]⁶⁺, 558.4 [M+5H]⁵⁺, 697.5 [M+4H]⁴⁺, 929.6 [M+3H]³⁺. **F)** ESI-MS of peak **3^{A15C}**; Calculated mass for ligation product (H4 fragment) **3^{A15C}**: 5283.2 Da, found: 529.1 [M+10H]¹⁰⁺, 587.9 [M+9H]⁹⁺, 661.4 [M+8H]⁸⁺, 755.7 [M+7H]⁷⁺, 881.5 [M+6H]⁶⁺, 1057.9 [M+5H]⁵⁺, 1321.7 [M+4H]⁴⁺. **G)** RP-HPLC chromatogram of purified desulfurised product **3**. **H)** ESI-MS of peak **3**; Calculated mass for purified desulfurised H4 fragment **3**: 5251.1 Da, found: 526.1 [M+10H]¹⁰⁺, 584.5 [M+9H]⁹⁺, 657.4 [M+8H]⁸⁺, 751.1 [M+7H]⁷⁺, 876.2 [M+6H]⁶⁺, 1051.3 [M+5H]⁵⁺, 1313.7 [M+4H]⁴⁺. Asterisks correspond to TFA adducts.

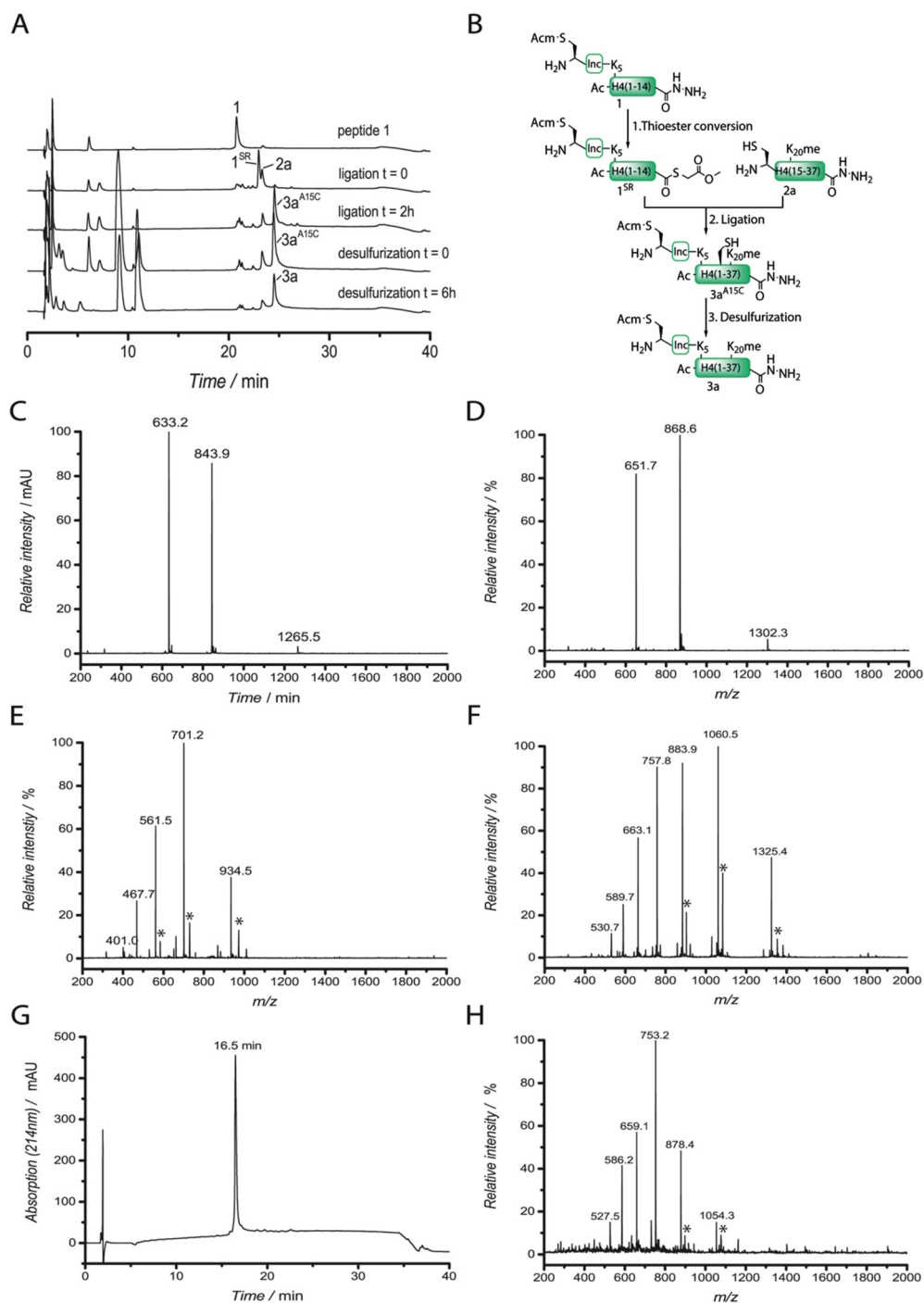


Figure S7: Synthesis of H3 fragment **3a** by one-pot ligation and desulfurisation. **A**) Analytical RP-HPLC chromatograms of the reaction steps. **B**) Scheme of the reaction steps. **C**) ESI-MS of peak **1**; Calculated mass for peptide-hydrazide **1**: 2528.8 Da, found: 633.2 [M+4H]⁴⁺, 843.9 [M+3H]³⁺, 1265.5 [M+2H]²⁺. **D**) ESI-MS of peak **1^{SR}**; Calculated mass for peptide-MTG thioester **1^{SR}**: 2602.9 Da, found: 651.7 [M+4H]⁴⁺, 868.6 [M+3H]³⁺, 1302.3 [M+2H]²⁺. **E**) ESI-MS of peak **2a**; Calculated mass for peptide-hydrazide **2a**: 2800.4 Da, found: 401.0 [M+7H]⁷⁺, 467.7 [M+6H]⁶⁺, 561.5 [M+5H]⁵⁺, 701.2 [M+4H]⁴⁺, 934.5 [M+3H]³⁺. **F**) ESI-MS of peak **3a^{A15C}**; Calculated mass for ligation product **3a^{A15C}**: 5297.1 Da, found: 530.7 [M+10H]¹⁰⁺, 589.7 [M+9H]⁹⁺, 663.1 [M+8H]⁸⁺, 757.8 [M+7H]⁷⁺, 883.9 [M+6H]⁶⁺, 1060.5 [M+5H]⁵⁺, 1325.4 [M+4H]⁴⁺. **G**) RP-HPLC chromatogram of purified desulfurised H4 fragment **3a**. **H**) ESI-MS of peak **3a**; Calculated mass for purified desulfurised H4 fragment **3a**: 5265.1 Da, found: 527.5 [M+10H]¹⁰⁺, 586.2 [M+9H]⁹⁺, 659.1 [M+8H]⁸⁺, 753.2 [M+7H]⁷⁺, 878.4 [M+6H]⁶⁺, 1054.3 [M+5H]⁵⁺. Asterisks correspond to TFA adducts.

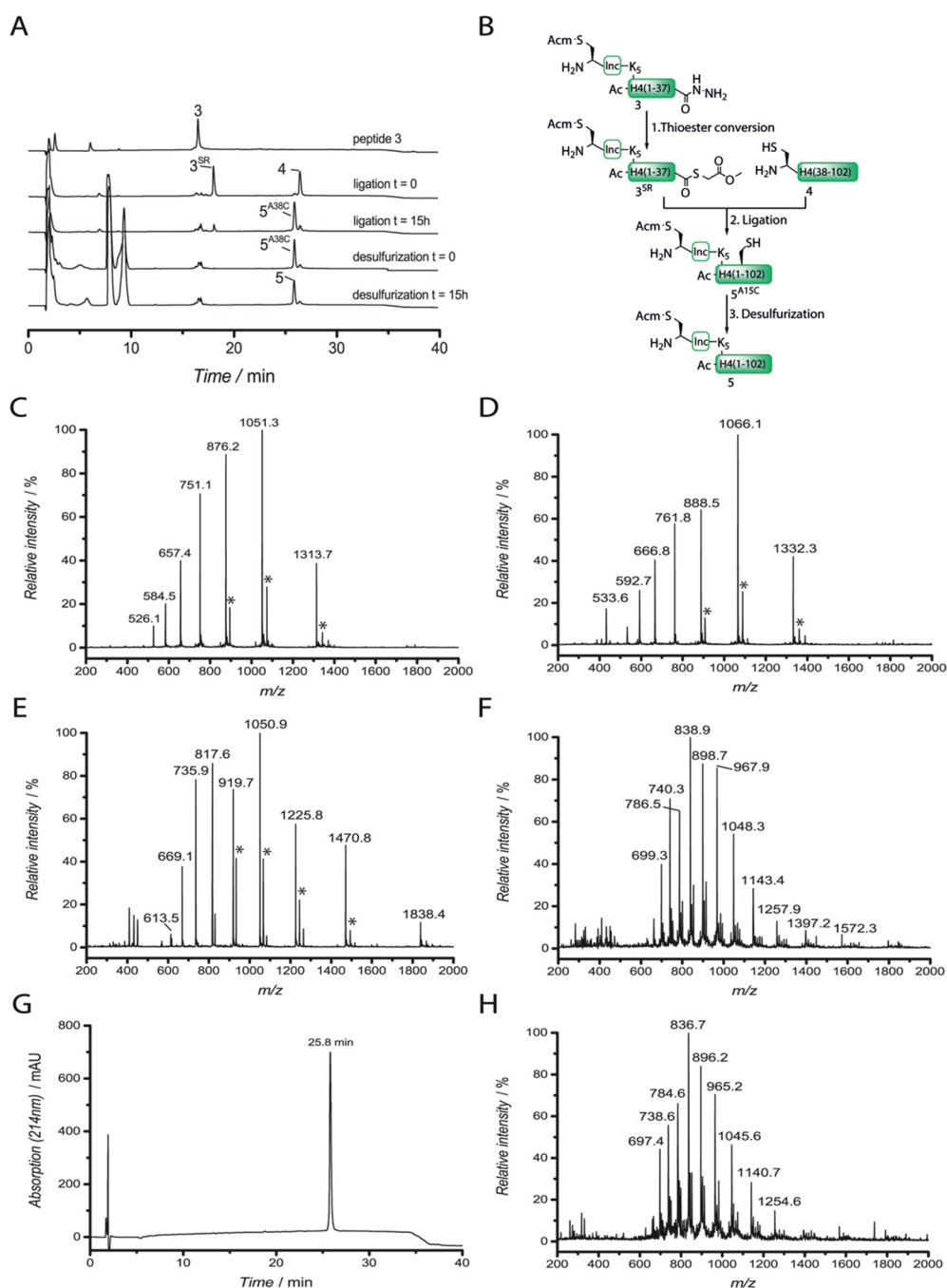


Figure S8: Synthesis of H4 protein **5** by one-pot ligation and desulfurisation. **A**) Analytical RP-HPLC chromatograms of the reaction steps. **B**) Scheme of the reaction steps. **C**) ESI-MS of peak **3**; Calculated mass for H4 fragment-hydrazide **3**: 5251.1 Da, found: 526.1 [M+10H]¹⁰⁺, 584.5 [M+9H]⁹⁺, 657.4 [M+8H]⁸⁺, 751.1 [M+7H]⁷⁺, 876.2 [M+6H]⁶⁺, 1051.3 [M+5H]⁵⁺, 1313.7 [M+4H]⁴⁺. **D**) ESI-MS of peak **3^{SR}**; Calculated mass for H4 fragment-MTG thioester **3^{SR}**: 5325.2 Da, found: 533.6 [M+10H]¹⁰⁺, 592.7 [M+9H]⁹⁺, 666.8 [M+8H]⁸⁺, 761.8 [M+7H]⁷⁺, 888.5 [M+6H]⁶⁺, 1066.1 [M+5H]⁵⁺, 1332.3 [M+4H]⁴⁺. **E**) ESI-MS of peak **4**; Calculated mass for H4(38-102)A38C (**4**): 7348.5 Da, found: 613.5 [M+12H]¹²⁺, 669.1 [M+11H]¹¹⁺, 735.9 [M+10H]¹⁰⁺, 817.6 [M+9H]⁹⁺, 919.7 [M+8H]⁸⁺, 1050.9 [M+7H]⁷⁺, 1225.8 [M+6H]⁶⁺, 1470.8 [M+5H]⁵⁺, 1838.4 [M+4H]⁴⁺. **F**) ESI-MS of peak **5^{A15C}**; Calculated mass for ligation product **5^{A15C}**: 12567.5 Da, found: 662.4 [M+19H]¹⁹⁺, 699.3 [M+18H]¹⁸⁺, 740.3 [M+17H]¹⁷⁺, 786.5 [M+16H]¹⁶⁺, 838.9 [M+15H]¹⁵⁺, 898.7 [M+14H]¹⁴⁺, 967.9 [M+13H]¹³⁺, 1048.3 [M+12H]¹²⁺, 1143.4 [M+11H]¹¹⁺, 1257.9 [M+10H]¹⁰⁺, 1397.2 [M+9H]⁹⁺, 1572.3 [M+8H]⁸⁺. **G**) RP-HPLC chromatogram of purified desulfurised H4 protein **5**. **H**) ESI-MS of peak **5**; Calculated mass for purified desulfurised H4 protein **5**: 12535.4 Da, found: 697.4 [M+18H]¹⁸⁺, 738.6 [M+17H]¹⁷⁺, 784.6 [M+16H]¹⁶⁺, 836.7 [M+15H]¹⁵⁺, 896.2 [M+14H]¹⁴⁺, 965.2 [M+13H]¹³⁺, 1045.6 [M+12H]¹²⁺, 1140.7 [M+11H]¹¹⁺, 1254.6 [M+10H]¹⁰⁺. Asterisks correspond to TFA adducts.

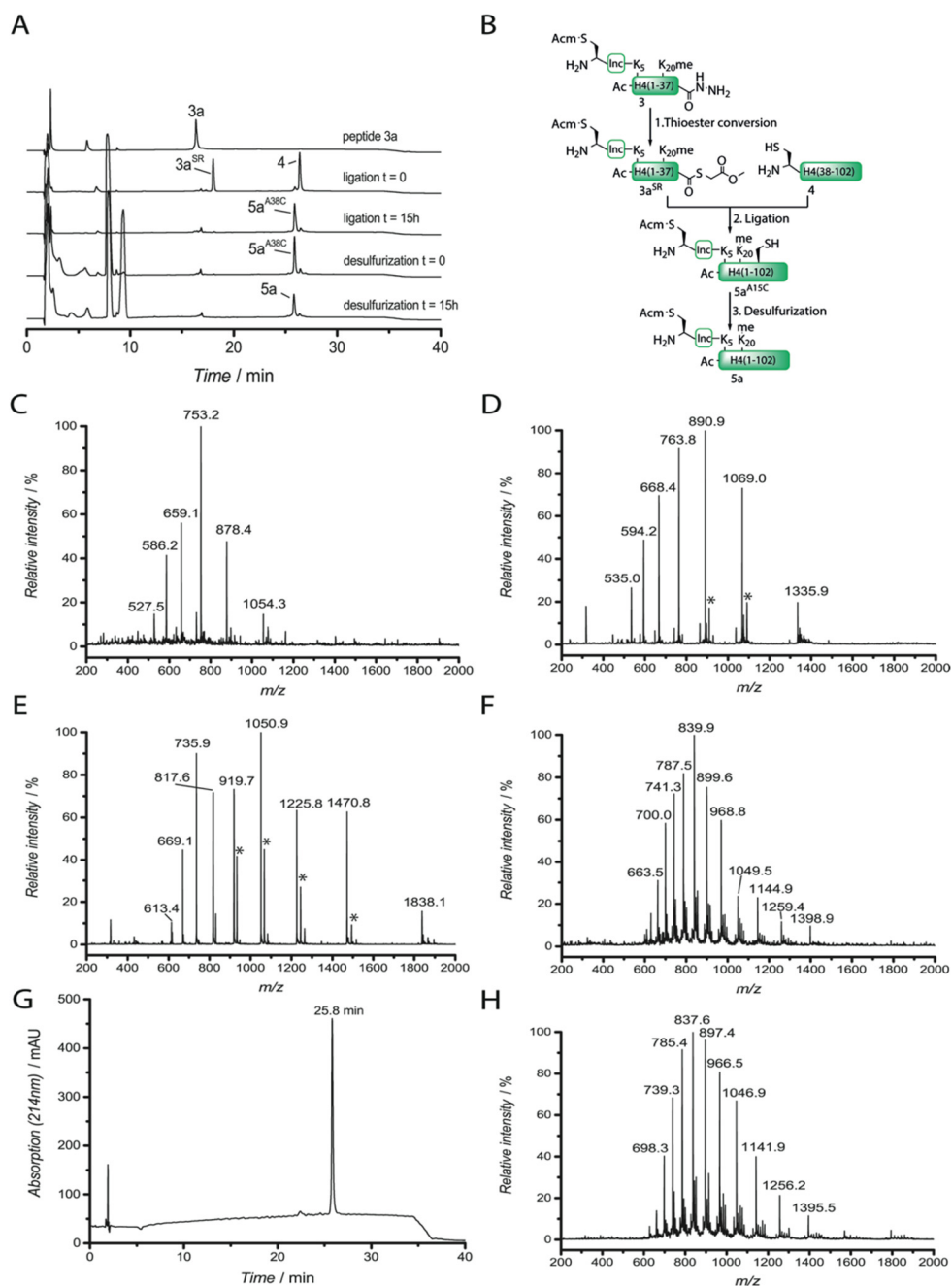


Figure S9: Synthesis of H4 protein **5a** by one-pot ligation and desulfurisation. **A)** Analytical RP-HPLC chromatograms of the reaction steps. **B)** Scheme of the reaction steps. **C)** ESI-MS of peak **3a**; Calculated mass for H4 fragment-hydrazide **3a**: 5265.1 Da, found: 527.5 [M+10H]¹⁰⁺, 586.2 [M+9H]⁹⁺, 659.1 [M+8H]⁸⁺, 753.2 [M+7H]⁷⁺, 878.4 [M+6H]⁶⁺, 1054.3 [M+5H]⁵⁺. **D)** ESI-MS of peak **3a^{SR}**; Calculated mass for H4 fragment-MTG thioester **3a^{SR}**: 5339.2 Da, found: 535.0 [M+10H]¹⁰⁺, 594.2 [M+9H]⁹⁺, 668.4 [M+8H]⁸⁺, 763.8 [M+7H]⁷⁺, 890.9 [M+6H]⁶⁺, 1069.0 [M+5H]⁵⁺, 1335.9 [M+4H]⁴⁺. **E)** ESI-MS of peak **4**; Calculated mass for truncated H4(38-102)A38C (**4**): 7348.5 Da, found: 613.4 [M+12H]¹²⁺, 669.1 [M+11H]¹¹⁺, 735.9 [M+10H]¹⁰⁺, 817.6 [M+9H]⁹⁺, 919.7 [M+8H]⁸⁺, 1050.9 [M+7H]⁷⁺, 1225.8 [M+6H]⁶⁺, 1470.8 [M+5H]⁵⁺, 1838.1 [M+4H]⁴⁺. **F)** ESI-MS of peak **5a^{A15C}**; Calculated mass for ligation product **5a^{A15C}**: 12581.6 Da, found: 663.5 [M+19H]¹⁹⁺, 700.0 [M+18H]¹⁸⁺, 741.3 [M+17H]¹⁷⁺, 787.5 [M+16H]¹⁶⁺, 839.9 [M+15H]¹⁵⁺, 899.6 [M+14H]¹⁴⁺, 968.8 [M+13H]¹³⁺, 1049.5 [M+12H]¹²⁺, 1144.9 [M+11H]¹¹⁺, 1259.4 [M+10H]¹⁰⁺, 1398.9 [M+9H]⁹⁺. **G)** RP-HPLC chromatogram of purified desulfurised H4 protein **5a**. **H)** ESI-MS of peak **5a**; Calculated mass for purified desulfurised H4 protein **5a**: 12549.5 Da, found: 698.3 [M+18H]¹⁸⁺, 739.3 [M+17H]¹⁷⁺, 785.4 [M+16H]¹⁶⁺, 837.6 [M+15H]¹⁵⁺, 897.4 [M+14H]¹⁴⁺, 966.5 [M+13H]¹³⁺, 1046.9 [M+12H]¹²⁺, 1141.9 [M+11H]¹¹⁺, 1256.2 [M+10H]¹⁰⁺, 1395.5 [M+9H]⁹⁺. Asterisks correspond to TFA adducts.

Protein 5':

Peptide **1'** was ligated to peptide **2a** and the resulting H4 fragment **3a'** was purified by semi-preparative RP-HPLC employing a linear gradient from 10 to 40% solvent B over 40 min and characterised by analytical RP-HPLC and ESI-MS (yield 37.3%) (**Figure S10**).

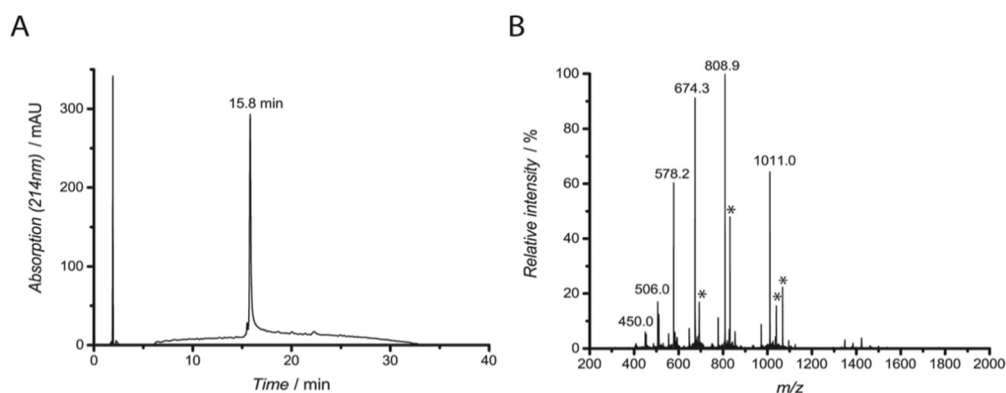


Figure S10: RP-HPLC chromatogram (A) and ESI-MS (B) of purified H4 fragment **3a'**. Calculated mass: 4039.8 Da, found: 450.0 [M+9H]⁹⁺, 506.0 [M+8H]⁸⁺, 578.2 [M+7H]⁷⁺, 674.3 [M+6H]⁶⁺, 808.9 [M+5H]⁵⁺, 1011.0 [M+4H]⁴⁺. Asterisks correspond to TFA adducts.

Subsequently, H4 fragment **3a'** was ligated to H4(38-102)A38C (**4**) and, after desulfurisation of Cys15 and Cys38, H4 protein **5a'** was purified by semi-preparative RP-HPLC employing a linear gradient from 40 to 70% solvent B over 40min and characterised by analytical RP-HPLC and ESI-MS (yield 35.2%) (**Figure S11**).

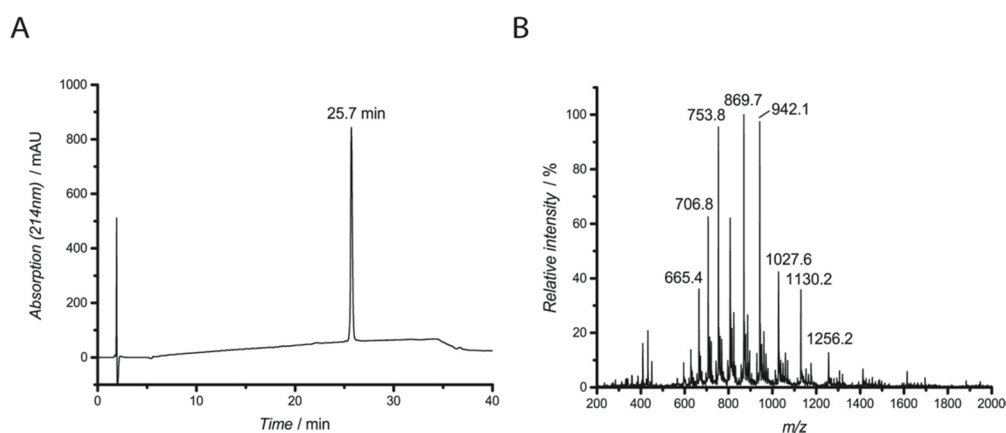


Figure S11: RP-HPLC chromatogram (A) and ESI-MS (B) of purified H4 protein **5a'**. Calculated mass: 11292.1 Da, found: 665.4 [M+17H]¹⁷⁺, 706.8 [M+16H]¹⁶⁺, 753.8 [M+15H]¹⁵⁺, 869.8 [M+13H]¹³⁺, 942.1 [M+12H]¹²⁺, 1027.6 [M+11H]¹¹⁺, 1130.2 [M+10H]¹⁰⁺, 1256.2 [M+9H]⁹⁺.

Deprotection of Cys(Acm)

Silver acetate (AgOAc) was employed to remove the Acetamidomethyl (Acm) protecting group of the Inc-tag N-terminal cysteine. Protein **5a** was dissolved in 50% (v/v) acetic acid in H₂O to a final concentration of 0.5mM and AgOAc was added to a final concentration of 15mM from a stock solution in 50% (v/v) acetic acid in H₂O. The reaction mixture was incubated at 37° C for 6h. Progression of the reaction was monitored by RP-HPLC and ESI-MS. When the reaction was complete, aqueous solution of DTT was added to a final concentration of 50mM in

order to precipitate silver. The reaction was thoroughly vortexed, centrifuged (15000xg, 4°C, 10min) and the supernatant was mixed with 3 volumes of ligation buffer (pH 3). After centrifugation (15000xg, 4°C, 10min) the protein was purified by semipreparative RP-HPLC using a linear gradient from 30-70% solvent B in 45min and pure protein **6** characterised by analytical RP-HPLC and ESI-MS (yield 62.8%) (**Figure S12** and **Table S3**).

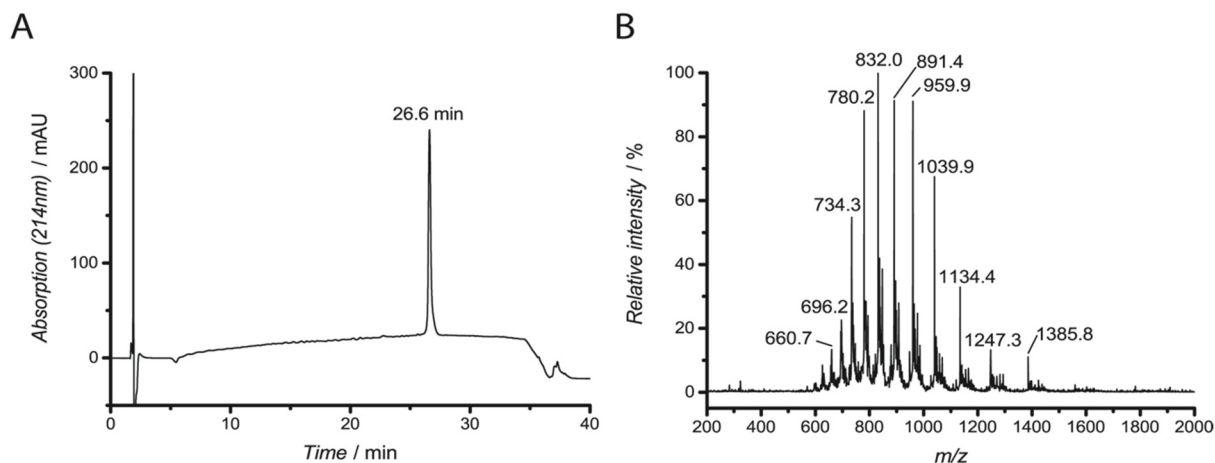


Figure S12: RP-HPLC chromatogram (A) and ESI-MS (B) of purified peptide **6**. Calculated mass: 12464.3 Da, found: 660.7 [M+19H]¹⁹⁺, 696.2 [M+18H]¹⁸⁺, 734.3 [M+17H]¹⁷⁺, 780.2 [M+16H]¹⁶⁺, 832.0 [M+15H]¹⁵⁺, 891.4 [M+14H]¹⁴⁺, 959.9 [M+13H]¹³⁺, 1039.9 [M+12H]¹²⁺, 1134.4 [M+11H]¹¹⁺, 1247.3 [M+10H]¹⁰⁺, 1385.8 [M+9H]⁹⁺.

Deprotection of Cys(Acm) and *in situ* activation of Cys with DTNB

As described above, protein **5** was first treated with AgOAc in 50% (v/v) AcOH for 6 h at 37° C to remove the Acm protecting group. Then, an equal volume of a solution of 60 mM DTNB in 6 M GmdHCl, 0.2 M phosphate, pH 7 was added to the reaction mixture. The solution was thoroughly vortexed, incubated 5 min at room temperature and centrifuged (5 min, 4° C, 15000 x g). Acetonitrile was added to the supernatant to a final concentration of 30% (v/v) and the solution was immediately subjected to semipreparative RP-HPLC purification using a linear gradient from 30-70% solvent B in 45 min. The pure protein **7** was characterised by analytical RP-HPLC and ESI-MS (yield 51.5%) (**Figure S13** and **Table S3**).

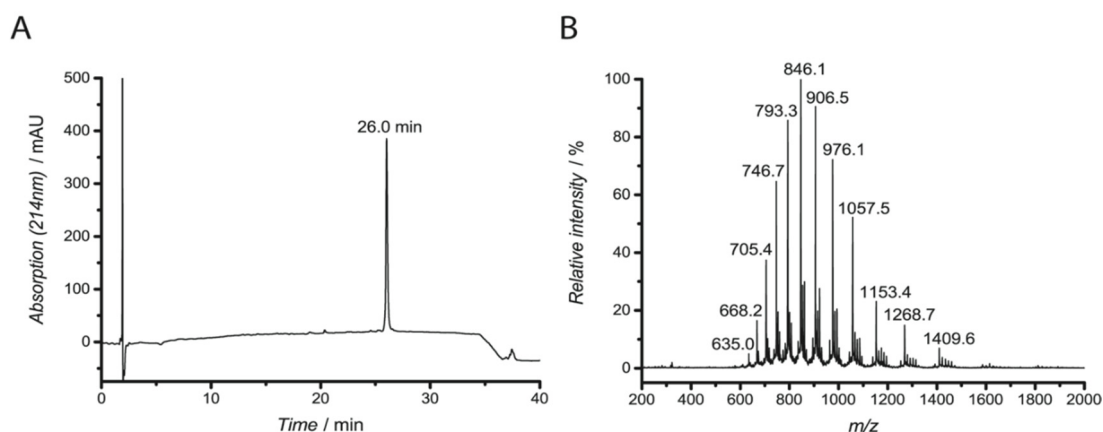


Figure S13: RP-HPLC chromatogram (A) and ESI-MS (B) of purified protein **7**. Calculated mass: 12676.5 Da, found: 635.0 [M+20H]²⁰⁺, 668.2 [M+19H]¹⁹⁺, 705.4 [M+18H]¹⁸⁺, 746.7 [M+17H]¹⁷⁺, 793.3 [M+16H]¹⁶⁺, 846.1 [M+15H]¹⁵⁺, 906.5 [M+14H]¹⁴⁺, 976.1 [M+13H]¹³⁺, 1057.5 [M+12H]¹²⁺, 1153.4 [M+11H]¹¹⁺, 1268.7 [M+10H]¹⁰⁺, 1409.6 [M+9H]⁹⁺.

4. Generation of heterodisulfide dimer proteins

Generation of heterodisulfide dimers ^{xInc} asH4K20me1		
Name	Sequence	Reaction product
6	Ac-H4(1-37)K5(^{iso} Inc)-NHNH ₂	5a Cys(Acm) deprotection
7	Ac-H4(1-37)K5(^{iso} Inc[TNB])K20me-NHNH ₂	5 Cys(Acm) deprotection and TNB activation
8	^{xInc} asH4K20me1	Heterodisulfide bond formation between 6 and 7

Table S3: Differently modified H4 molecules for the formation of the heterodisulfide ^{xInc}asH4K20me1 8.

Proteins (6, 7) were dissolved in reaction buffer (6 M GdmHCl, 0.2 M phosphate, pH 6, degassed and flushed with argon) to a final concentration of 2.5 mM for the Cys(TNB)-protein 7 and 2.3mM for the Cys-protein 6. Equal volumes of the two proteins were mixed together, incubated for 30s at room temperature and quenched by addition of 6M GdmHCl pH 2 and disulfide-linked asymmetric H4 dimers ^{xInc}asH4K20me1 (8) were immediately purified by semipreparative RP-HPLC using a linear gradient from 35-65% solvent B in 45 min. Heterodisulfide formation was confirmed by analytical RP-HPLC and ESI-MS (**Figure S14** and **Table S3**). Purified proteins were flash frozen, lyophilised and stored at -20°C (yield 45.2%).

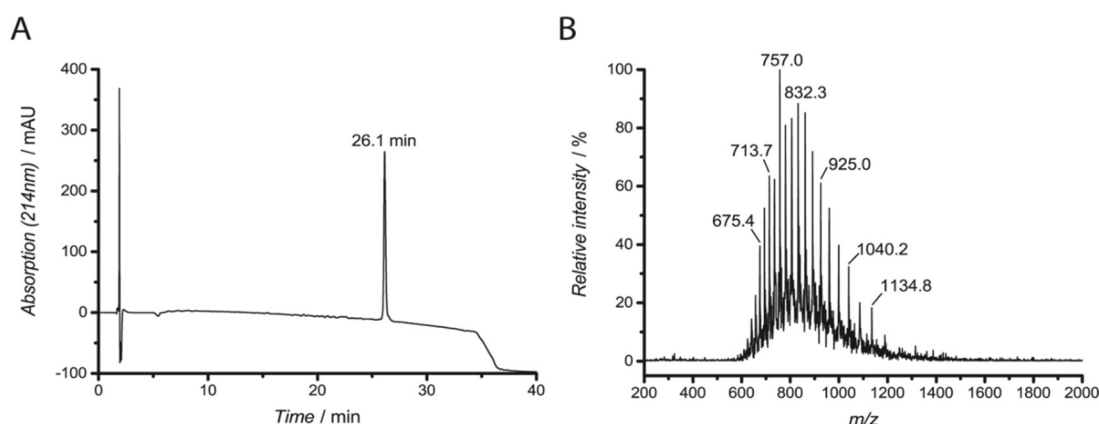


Figure S14: RP-HPLC chromatogram (A) and ESI-MS (B) of purified asymmetric H4 dimers ^{xInc}asH4K20me1 8. Calculated mass: 24940.5 Da, found: 24945.0 Da.

5. Refolding and purification of histone octamers

Lyophilised recombinant or semisynthetic modified histone proteins were dissolve in unfolding buffer (6 M GdmHCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5). The concentration of each histone was determined by UV-absorbance measurements, and calculated using the extinction coefficients and molar weights listed below:

Histone	Extinction coefficient (280nm)	Molecular weight
hH2A	4470 M ⁻¹ cm ⁻¹	13964 g/mol
hH2B	7450 M ⁻¹ cm ⁻¹	13759 g/mol
hH3_C110A	4470 M ⁻¹ cm ⁻¹	15209 g/mol
^{xInc} asH4K20me1	15025 M ⁻¹ cm ⁻¹	24940.5 g/mol

Table S4: Extinction coefficients and molecular weights of recombinant core histones and synthetic heterodisulfide H4 dimers.

1eq. of **8** was mixed with 2 eq. hH3_C110A, 2.2 eq. hH2A and 2.2eq. hH2B to a final protein concentration 0.5 mg/mL in unfolding buffer. The solution was transferred in a Slide-A-Lyzer™ dialysis cassette (7k MWCO) and dialysed at 4° C overnight against refolding buffer (2 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5). The refolded octamers were removed from the dialysis cassette and concentrated with a centrifugal concentrator Vivaspin500 (10K MWCO) to a final concentration of 40 µM. Subsequently, they were purified by size exclusion chromatography on a Superdex S200 10/300GL column and collected fractions were analysed by SDS-PAGE (15% polyacrylamide gel). Octamer containing fractions were pooled and concentrated to a final concentration of 30-40 µM. After addition of glycerol to a final concentration of 50% (v/v), octamers were analysed again by SDS-PAGE (15% polyacrylamide gel) under reducing (in presence of DTT) and non-reducing conditions (in the absence of DTT) and stored at -20° C.

6. Test of TEV isopeptide bond cleavage

0.2 mg peptide **1** (79 nmol) were dissolved in 100 µL TEV solution (1 mg/ml TEV protease in 200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH8, 2 mM DTT) and incubated at 30° C under gentle agitation. This resulted in a molar ratio of 1:22 TEV/peptide. The cleavage reaction of peptide **1** into the non-branched peptide **1'** and the free *iso*Inc-tag was monitored by analytical RP-HPLC and MS (**Figure S15**)

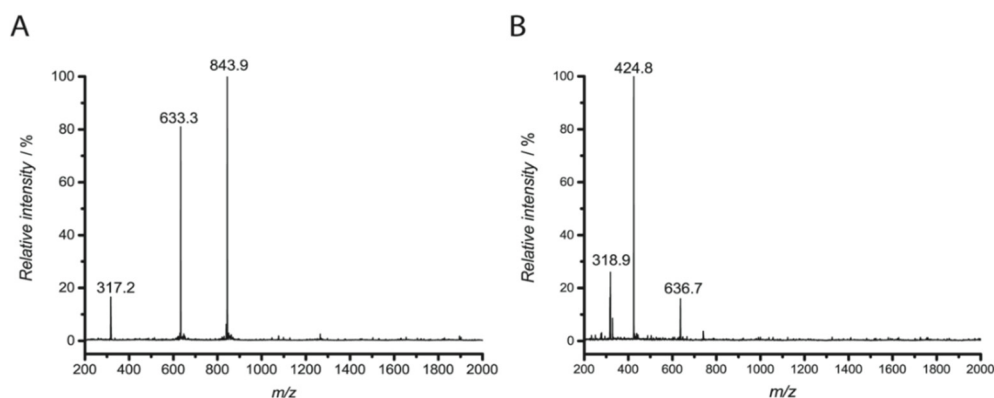


Figure S15: **A)** ESI-MS of peptide **1**. Calculated mass: 2528.8 Da, found: 317.2 [M+5H]⁵⁺, 633.3 [M+4H]⁴⁺, 843.9 [M+3H]³⁺. **B)** ESI-MS of cleaved peptide **1'**. Calculated mass: 1271.4 Da, found: 318.9 [M+4H]⁴⁺, 424.8 [M+3H]³⁺, 636.7 [M+2H]²⁺.

7. Reconstitution of nucleosomes and TEV cleavage

Nucleosomes were reconstituted as described before.⁷ Typically, the 153 base pair 601 nucleosome positioning DNA sequence⁸ (75 pmol, 1 eq.) was mixed with the refolded octamers (1-1.2 eq.) at high salt concentration and reconstitution was achieved by gradient dialysis into low salt conditions (10 mM KCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). Dialysis was performed in Slide-A-Lyzer™ MINI dialysis devices using a peristaltic pump at a flow rate of 1ml/min over 18h at 4°C. Subsequently, DTT to a final concentration of 1mM was added and the *x*_{Inc}asH4K20me1 **8** in the formed nucleosomes were reduced by incubation at 4° C for 30 min. Multiple additions of TEV protease (4 x 1 µg per 100 pmol nucleosomes) over a total time of 24 h at room temperature allowed to remove the *iso*Inc-tag. Final nucleosomes concentrations were determined by UV quantification and the reconstitution was checked by native gel electrophoresis. Removal of the Inc-tag by TEV protease was confirmed by SDS-PAGE (17% polyacrylamide gel) (**Figure S16**).

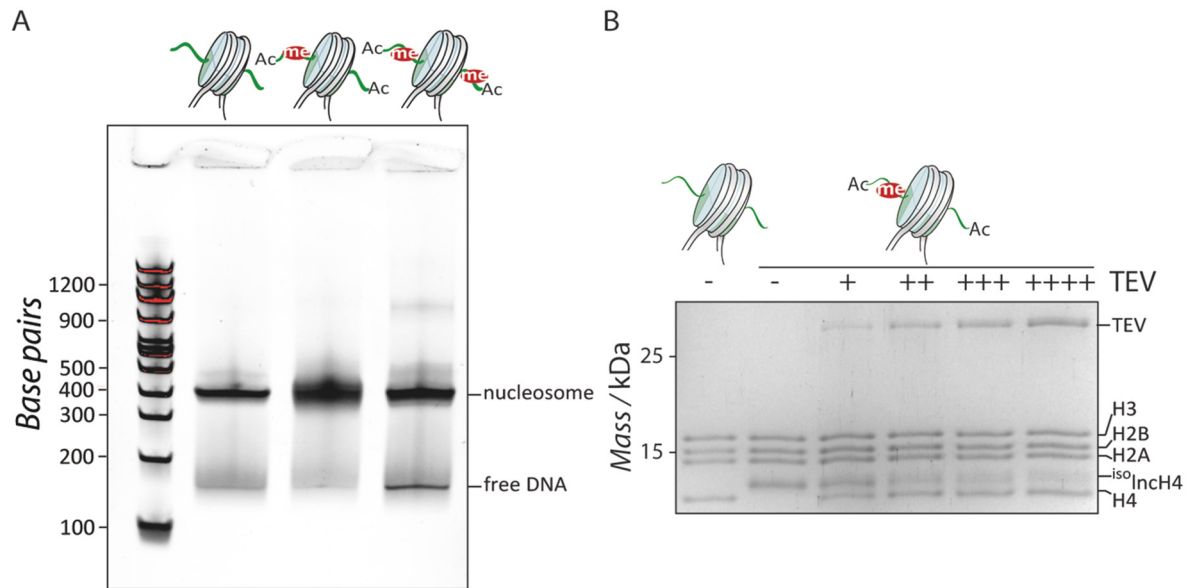


Figure S16: Uncropped full-length native 5% TBE gel analysis of reconstituted nucleosomes (A) and SDS-PAGE analysis of removal of *iso*Inc-tag by TEV protease treatment (B).

8. Methyltransferase assays with Set8

Endpoint experiments: 1 pmol of recombinant Set8 was incubated at RT for 2 h with 15 pmol of nucleosomes and 60 pmol ^3H -SAM (1 μCi) in 50 mM Tris-HCl pH 8.5, 10 mM NaCl, 2 mM MgCl_2 , 1 mM EDTA, 5 mM DTT (30 μL total volume). Salt concentration was adjusted to 30 mM. Subsequently, histones were separated by SDS-PAGE (13% polyacrylamide gel, 20 μL) and stained with Coomassie Brilliant Blue G250. After destaining, the gel was incubated for 30 min in Amersham Amplify Fluorographic Reagent and dried for 2 h at 80° C. Incorporation of ^3H in histone substrates was detected by overnight exposure to an X-ray film at -80° C (**Figure S17A**). The activity of each reaction was quantified by scintillation counting (**Figure S17B**). 5 μL reaction mixture were applied to P81 Ion Exchange Paper. The filters were dried at RT for 40 min, washed 3 x with 50 mM NaHCO_3 pH 9 and dried on a gel dryer for 2 h at 80° C. Scintillation counting was performed with 5 mL Ultima Gold F scintillation cocktail on a Packard Tri-Carb Liquid Scintillation Counter.

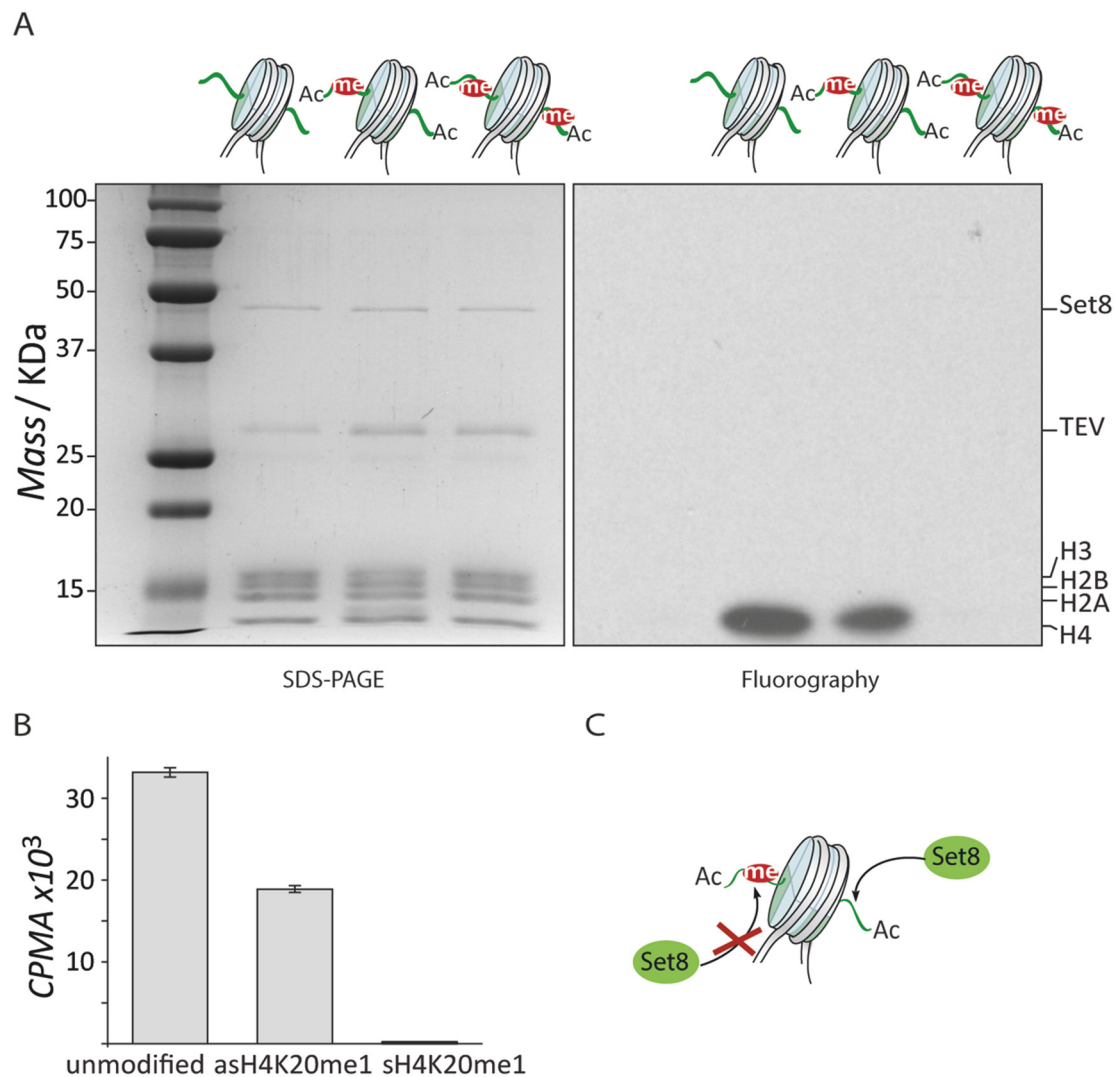


Figure S17: A) Uncropped full-length SDS-PAGE gel (left) and X-ray film (right). **B)** Scintillation counting. **C)** Model of Set8 nucleosome methylation.

Kinetic studies: 0.25 pmol of recombinant Set8 were incubated at RT for 2 h with 15 pmol of nucleosomes and 60 pmol ³H-SAM (1 μ Ci) in 50 mM Tris-HCl pH 8.5, 10 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 5 mM DTT (30 μ L total volume). Salt concentration was adjusted to 30 mM. 5 μ L sample was quenched with 5 μ L 0.2% TFA solution at the following time points: 2 min, 5 min, 10min, 30 min, 1 h and 2 h and applied to P81 Ion Exchange Paper. Filter washing and scintillation counting were performed as described above.

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