Supporting information for:

A bi-terminal protein ligation strategy to probe chromatin structure during DNA damage

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Materials

1-hydroxybenzotriazole hydrate (HOBt), 2,2,2-trifluoroethanethiol (TFET), 2-mercaptoethanol (βME), 4-mercaptophenylacetic acid (MPAA), dimethyl sulfoxide (DMSO), diethylether, dithiothreitol (DTT), methylthioglycolate (MTG), piperidine 99%, extra pure, phenylsilane ($PhSiH_3$), tetrakis(Triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$), triisopropylsilane (TIS), tris(carboxyethyl)phosphine (TCEP), and sodium 2-mercaptoethanesulfonate (MESNa) were from Sigma Aldrich.

Coomassie brilliant Blue R-250, glycerol, bromophenol blue, trifluoroacetic acid (TFA) HPLC grade, imidazole extra pure, LB agar, LB broth were from Fisher Scientific. Alexa Fluor 568 NHS ester (Alexa568), Alexa Fluor 647 NHS ester (Alexa647) were from Thermo Fisher.

Ampicillin sodium salt, chloramphenicol, guanidine hydrochloride (GdmHCl) pure, ethylenediaminetetraacetic acid (EDTA), isopropyl-beta-D-thiogalactopyranoside (IPTG), nicotinamide adenine dinucleotide (NAD), tetramethylethylenediamine (TEMED), tris(hydroxymethyl)aminomethane (Tris) ultrapure, Triton X-100 (Sigma Aldrich), urea crystalline, L-arginine hydrochloride were from AppliChem.

Amino acids for peptide synthesis (Fmoc-Ala-OH, Boc-Cys(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(PO(PBzl)OH)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH), Fmoc-Tyr(tBu)- Wang Resin (100-200 mesh) and 2-chlorotrityl chloride resin were from Novabiochem.

1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) was from ChemPep. N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was from PTI Technologies. 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was from Wako.

3-Aminopropyltriethoxysilane 99% (APTES), dichloromethane (DCM), diisopropylethylamine (DIPEA), dimethylformamide (DMF) was from Acros Organics. Acetonitrile (ACN) HPLC gradient grade was from (Avantor), GelRed was from Biotium, GdmHCl ultrapure from MP Bio, hydrazine monohydrate was from VWR, kanamycin sulfate and triethylamine for HPLC was from Roth. 30% Acrylamide/bis (29:1), ammonium persulfate and certified molecular biology agarose were from BioRad.

2-log ladder, BsaI-HF, dNTP set, DpnI, DraIII-HF, EcorV-HF, Phusion High-fidelity DNA polymerase, Q5 DNA polymerase, RNAse A, T4 DNA ligase, T5 DNA exonuclease, Taq DNA ligase, DH5a, BL21 DE3, BL21(DE3)plysS and BL21 Rosetta 2 chemically competent cells were distributed through BioConcept from New England Biolabs.

Instruments

An HT Infors AG incubator was used for incubation of bacteria in protein expression. Cells were harvested for further processing in 1L centrifuge tubes using an Avanti J-20 XPI centrifuge from Beckman Coulter. Lysis was carried out with a Vibra-cell VCX 750 Sonics & Materials sonicator.

Size exclusion chromatography and ion exchange purification was performed on an AKTA Pure FPLC system from GE Healthcare. Size-exclusion was done using an S200 10/300GL column from GE Healthcare using isocratic elution with a flow rate of 0.5 mL/min over 1.05 column volumes and collected as 250 µL fractions in 96-well plates. Cation exchange purifications were done using HiTrap SP HP (5 mL) columns from GE Healthcare.

Reaction vessels from Peptides International were used for manual steps in solid-phase peptide synthesis and the automated parts done on a Tribute instrument from Peptides International Inc. An Agilent 1260 series instrument with an Agilent Zorbax C18 column (5 µm, 4.6 x 150 mm) was used for analytical reversed phase high-performance liquid chromatography (RP-HPLC). The mobile phases used were 0.1% TFA in water (RP-HPLC solvent A), and 90% acetonitrile, 0.1% TFA in water (RP-HPLC solvent B). Typically a gradient of 0-70% solvent B over 30 min at a flow rate of 1 mL/min was used. Preparative scale purifications were similarly conducted on Agilent 1260 preparative HPLC systems. A Zorbax C18 preparative column (7 µm, 21.2 x 250 mm) or a semi-preparative column (5 µm, 9.4 x 250 mm) was employed at flow rates of 20 mL/min or 4 mL/min, respectively.

Absorbance spectra were recorded with an Agilent 8453 UV-Vis spectrophotometer. SDS-PAGE, native PAGE and agarose gels were imaged using a ChemiDoc MP imaging system from BioRad. Fluorescence spectra were acquired with a Fluorolog®-3 spectrofluorometer (Horiba Jobin Yvon).

Single-molecule experiments were performed on a micro-mirror TIRF system[1] (MadCityLabs) using Coherent Obis Laser lines at 405 nm, 488 nm, 532 nm and 640 nm, a 100x NA 1.49 Nikon CFI Apochromat TIRF objective (Nikon) as well as an iXon Ultra EMCCD camera (Andor), operated by custom-made Labview (National Instruments) software.

Methods

Synthesis of peptide 2

2: H2A.X 135-142 (S139ph, A135C): CTQAS(ph)QEY

Automated peptide synthesis of peptide 2 was performed using a Tribute peptide synthesizer on a 0.1 mmol scale using preloaded Fmoc-Tyr(tBu)-Wang resin. Following a general protocol, amino acids were used in a 5 equivalent (eq.) excess for coupling. β-branched amino acids and arginines were double-coupled. Fmoc-deprotection was achieved with 20 % (v/v) piperidine in DMF. In the automated synthesis, amino acids were dissolved with 4.8 eq. HBTU and 10 eq. DIPEA in DMF, added to the resin and coupled for 30 min. The resin was washed with DMF between coupling and deprotection steps. Following completion of the synthesis, resins were washed with DMF, DCM and MeOH and dried. The peptide was cleaved from the resin by incubation with 10 mL/g of a mixture of TFA/TIS/H_2O (95/2.5/2.5%) for 3 h at RT and filtered through a frit. Crude peptides were precipitated by addition of cold diethyl ether, followed by centrifugation and dissolving of the pellet in 50% ACN. The crude peptide was lyophilized, followed by purification by analytical HPLC (**Figure S1d**) and analysis by ESI-MS (**Figure S1e,f**).

Synthesis of fragment 5b, H2A.X(1-20)K15ub

Synthesis of peptide 5a

5a: H2A.X(1-20)K15(εNCys)-$NHNH_2$: SGRGKTGGKARAKAK(εN-Cys)SRSSR-$NHNH_2$

For the introduction of K15 ubiquitylation, the N-terminal tail of H2A.X was synthesized as a C-terminal hydrazide, and carrying a cysteine attached to the ε-amino group of lysine 15.

Preparation of pre-loaded hydrazine resin

Hydrazide resin was prepared as described[2]. Briefly, 0.85 mmol 2-chlorotrityl chloride resin reacted with 2.55 mmol (444 µL) DIPEA and 2.6 mmol hydrazine monohydrate (82 µL) in DMF at 0° C. Unreacted resin was capped with methanol and washed with DMF. 1 g of Fmoc-Lys(Boc)-OH (5 eq., 2.125 mmol) was weighed into a vial, activated with HBTU and DIPEA (4.3 mmol, 740 µmol) and added to the resin. Coupling was allowed to proceed for 30 min, repeated with 2 equivalents of amino acid, followed by washes with DMF, DCM and methanol before drying in a desiccator. Resin loading was determined by deprotection of the Fmoc with 20% piperidine and measurement at 301 nm in triplicate samples.

Peptide synthesis of 5a

The automated synthesis of **5a** was performed as described above for peptide **2**. After automated synthesis, a cysteine was coupled to the ε-amino group of lysine 15 for ubiquitin coupling. To this end, 0.1 mmol peptidyl

resin was washed with DCM and allowed to swell for 30 min. 1 mL dry DCM and 2.4 mmol (294 µL) $PhSiH_3$ was added followed by stirring and addition of 3 mL DCM with 0.025 mmol $Pd(PPh_3)_4$ and treatment for 30 min to deprotect Alloc. The resin was washed with DCM and the treatment with $PhSiH_3$ and $Pd(PPh_3)_4$ was repeated twice. The resin was then washed with 0.5% (v/v) DIPEA in DMF, 0.5% (w/v) sodium diethyldithio carbamate in DMF, DCM/DMF (1:1) and 0.5% (w/v) HOBt in DMF. 5 eq. of the Boc-Cys(Trt)-OH amino acid were activated with 4.76 eq. of HATU from a 0.5 M stock in DMF for 2 min and 10 eq. DIPEA for 1 min followed by addition to the resin and incubation with stirring for 30 min. Coupling of Boc-Cys(Trt)-OH to the ε-amino group of lysine 15 was repeated and the resin washed with DMF,DCM and methanol before drying in a desiccator. Peptide **5a** was cleaved from the resin as described for peptide **2** (above), purified by preparative RP-HPLC and analyzed by analytical RP-HPLC (**Figure S2g**) and ESI-MS (**Figure S2h,i**).

Semi-synthesis of H2A.X(1-20)K15ub, 5b

Recombinant production of Ub-MES

Ubiquitin(1-75) was cloned in frame with a single-chain version of the split-intein Npu, containing a C-terminal mutation of the catalytic asparagine and the +1 cysteine (in the extein) to alanine:

Ub-Npu(AA): *MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR GG*-CLSYETEILTVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVD GQMLPIDEIFERELDLMRVDNLPNIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASAAFNHHHHHH

The construct was expressed in BL21(DE3)plysS cells (induction for 4h with 0.5 mM IPTG), the cells were lysed and the Ub-intein fusion was purified over a Ni:NTA affinity column. The protein was eluted with 600 mM imidazole in 20 mM Tris-HCl, pH 6.8 and 200 mM NaCl elution buffer. 100 mM MESNa and 1 mM TCEP were added and the intein cleavage was let to proceed overnight. Ub-MES was purified by preparative RP-HPLC using a gradient of 0-70% B, analyzed by analytical RP-HPLC (**Figure S2j**) and ESI-MS (**Figure S2k,l**).

One-pot ligation and desulfurization for the generation of 5b, H2A.X(1-20)K15ub

About 8.5 mg (~1 µmol) Ub-MES was dissolved in 130 µl ligation buffer (6M GdmHCl, 0.2M phosphate buffer, pH 7.0) to get to a protein concentration of 7.5 mM. The Ub-MES solution was transferred to a tube containing 1.4 mg lyophilized peptide **5a**, and 0.88 µl MTG were added, resulting in a MTG concentration of 75 mM. Then, 9 µl of 0.5 M TCEP solution (at neutral pH) was added to ensure reducing conditions. The ligation solution was incubated at RT and monitored by RP-HPLC and ESI-MS (**Figure S2m**). Upon reaching completion, the product was desulfurized in the same pot by the addition of an equal volume of 0.5 M TCEP solution, along with GSH and VA-044 to final concentrations of 40 mM and 20 mM, respectively. Desulfurization was monitored by RP-HPLC and ESI-MS. When complete, the product was purified by

semipreparative RP-HPLC using a gradient of 0-70% B in 45min. Fractions with the pure product (RP-HPLC **Figure S2n** and ESI-MS **Figure S2o-p**) were pooled, lyophilized and stored at -20 °C for later use.

Synthesis of 3, γH2A.X

Production of 1a, H2A.X(1-135)-Npu^N

1a,H2A.X(1-135)-Npu^N-6xH: SGRGKTGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGHYAERVGAGAPVYLAAVLEYLT AEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGGVTIAQGGVLPNIQAVLLPKKTSATVGPKAPSGGKK*CLSYETEILT VEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDEIFEREL DLMRVDNLPHHHHHH*

Protein **1a** was expressed in T7 express cells from a pet3a plasmid. Cell cultures were grown at 37°C in LB media supplemented with 100 µg/mL ampicillin until an OD600 of 0.6 was reached. Protein expression and purification was performed following ref.[3]. Briefly, expression was induced by addition of IPTG to a final concentration of 0.5 mM. Expression of the protein was allowed to continue until 3 h post-induction. Cells were harvested, cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 1 mM βME, 1 Roche EDTA-free protease inhibitor tablet / 50mL). Cells were lysed by freeze-thawing and sonication. The inclusion body pellet was washed twice with 7.5mL of histone lysis buffer with 1% triton-X and once without triton-X.

The inclusion body pellet was subsequently resolubilized in 10 mL resolubilization buffer (6 M GdmHCl, 50mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM imidazole) for 20min, then centrifuged for 10 min at 21000 x g to recover the supernatant, which was transferred to an equilibrated HisTrap FF column. The column was washed with wash buffer (resolubilization buffer + 50 mM imidazole) and then eluted with 15mL of elution buffer (resolubilization buffer + 500 mM imidazole). The eluted protein was purified by preparative RP-HPLC and analyzed by analytical RP-HPLC (**Figure S1a**) and ESI-MS (**Figure S1b-c**).

Expression and purification of Npu^C peptide

Npu^C(AA)-GyrA: MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASAAFNSGG-*CITGDALVALPEGESVRIADIVPGARP NSDNAIDLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLLWKLIDEIKPGDYAVIQRSA FSVDCAGFARGKPEFAPTTYTVGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHAFI TNGFVSHAHHHHHH*

The Npu^C peptide, in fusion to the GyrA intein and containing a dual alanine mutation (Npu^C-AA-GyrA-6xH) was prepared as previously described[4]. The protein was expressed in BL21(DE3)plysS cells. From a pre-culture, 4 x 1 L of LB medium with chloramphenicol and ampicillin were inoculated and grown at 37° C until an OD600 of 0.5-0.9 was reached. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM followed by incubation for another 3-4 h. Cells were harvested by centrifugation, homogenized

with Ni-NTA lysis buffer (50 mM phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0) and flash frozen. Cells were lysed by thawing and sonication followed by collection of the supernatant by centrifugation. The supernatant was purified by Ni-NTA affinity chromatography. The supernatant was bound to the Ni-NTA resin for 30-45 min. The resin was washed with lysis buffer containing 20mM imidazole and the protein eluted with the same buffer containing 300 mM imidazole. Thiolysis was initiated by addition of cysteine methyl-ester (CysOMe) and TCEP to final concentrations of 100 mM and 5 mM respectively, followed by reaction for 16 h during which a precipitate formed. The pellet containing the desired peptide was collected, redissolved in resolubilization buffer and purified by preparative RP-HPLC using a gradient of 20-50% B in 45 min. Fractions containing the purified peptide were pooled, analyzed by RP-HPLC (**Figure S1g**) and ESI-MS (**Figure S1h-i**), lyophilized and stored at -20°C for later usage.

Ligation to produce 3, γH2A.X

Around 3.2 mg (0.12µmol) of the lyophilized protein **1a**, H2A.X (1-134)-Npu^N was dissolved in 50 µL denaturing buffer (6 M GdmHCl, 200 mM phosphate buffer, pH 7.5). This was diluted with 150 µL 2x MESNa urea buffer (4 M urea, 0.2 M MESNa, pH 7.0) and transferred to 1.0 mg (1.0 µmol) of the C-terminal peptide **2**. Relative to the protein, 0.25 molar equivalents of NpuC peptide from a 2 mM stock solution in water was added. The reaction volume was increased to 300 µL with water, resulting in a final concentration of 2 M urea and 0.1 M MESNa. Then 1.34 µL MTG was added to a final concentration of 50 µM and samples were taken repeatedly to monitor the reaction progress by RP-HPLC. After completion of the ligation reaction 300 µL TCEP solution (2 M urea, 0.5 M TCEP, pH 7), 70 µL of a 0.4 M glutathione dissolved in TCEP solution and 70 µL radical initiator VA-044 (0.2 M in water) were added. The pH was adjusted to 6.5, the sample was overlaid with argon and incubated at 42° C. The desulfurization reaction was continuously monitored for progress by RP-HPLC and ESI-MS and upon completion, the reaction solution was diluted with 40% RP-HPLC solvent B to a final volume of 4.5 mL. The desulfurized product **3** was purified by preparative RP-HPLC (gradient 40-70% B in 30min) and analyzed by analytical RP-HPLC (**Figure 1b**) and ESI-MS (**Figure 1c**).

<u>Synthesis of 6, γH2A.X K15ub</u>

Production of 1b His-SUMO-H2A.X(21-142)A21C-Npu^N

<u>1b,</u> <u>SUMO-H2A.X(21-134, A21C)-NpuN:</u> *MSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAF AKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG-*
CGLQFPVGRVHRLLRKGHYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGGVTIA QGGVLPNIQAVLLPKKTSATVGPKAPSGGKK-
CLSYETEILTVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLP IDEIFERELDLMRVDNLPHHHHHH

Protein **1b** was expressed in T7 express cells from a pET3a plasmid and purified applying the same protocol as for protein **1a** (above). The final protein **1b** was purified and analyzed by analytical RP-HPLC (**Figure S2a**) and ESI-MS (**Figure S2b,c**).

SUMO protease Ulp1 expression

Ulp1 was expressed and inclusion bodies were prepared using the same protocol as for protein **1a**. Inclusion bodies were resolubilized in imidazole resolubilization buffer (6 M GdmHCl, 50 mM Tris pH 7.5, 100mM NaCl, 5mM imidazole) and the insoluble cell debris removed by centrifugation. The supernatant was loaded onto pre-equilibrated Ni-NTA resin (~1mL). The resin was allowed to bind for 45-60 min, followed by washes with 3 x 5 CV of resolubilization buffer with 25 mM imidazole, and finally by elution with 4 x 2CV of the same buffer in 300 mM imidazole. The purified Ulp1 enzyme was immediately used without further purification.

One-pot ligation and SUMO-deprotection to produce 4b, H2A.X(22-142)A21C A135C S139ph

Around 4.3 mg (0.16 µmol) of the lyophilized SUMO-H2A.X-Npu^N protein was dissolved in 60 µL resolubilization buffer (6 M GdmHCl, 200 mM phosphate, pH 7.5). This was diluted with 150 µL MESNa buffer (4 M urea, 0.2 M MESNa, pH 7.0) and transferred to 1.0 mg (1.0 µmol) of the C-terminal peptide **2**. Relative to the protein, 0.25 molar equivalents of NpuC peptide from a 2 mM stock solution in water was added. The reaction volume was increased to 300 µL with water, resulting in a final concentration of 2 M urea and 0.1 M MESNa. Then 1.34 µL MTG was added to a final concentration of 50 µM and samples were taken repeatedly to monitor the reaction progress by RP-HPLC. After the ligation reaction reached completion, 30 µL of a solution of 250 mM arginine, 50 mM cysteine and 50 mM DTT in 2 M urea and 200 mM phosphate buffer, pH7.5 was added. Then, 10µL of Ulp1 enzyme solution was added (5 µg), and the SUMO cleavage was monitored by RP-HPLC and ESI-MS (**Figure 2b**)**.** The final product, **4b**, was purified by semi-preparative HPLC (40-70%B in 30min) analyzed by analytical HPLC (**Figure S2d**) and ESI-MS (**Figure S1e-f**).

One-pot ligation and desulfurization to generate 6, γH2A.X K15ub

Approximately 0.6 mg (~0.05 µmol) of protein **4b** was weighed into a tube. The N-terminal ubiquitylated peptide **5b** was activated by *in situ* oxidation on ice. To this end, **5b** was dissolved in 100 µL degassed ligation buffer (6 M GuaHCl, 0.2 M Pi pH 3.0) and cooled to -20° C followed by addition of 3 µL 0.5 M $NaNO_2$ to a final concentration of 15 mM. Oxidation was allowed to proceed 5 min after which 50 µL MESNA ligation buffer (0.3 M MESNA, 6 M GuaHCl, 0.2 M Pi pH 7.0) was added. The pH was further adjusted to 7.0 by addition of NaOH. The peptide thioester was transferred to the lyophilized protein **4b**. 0.67 µL MTG was added to a final concentration of 50 µM. After 30 min of reaction 1.5 µL TCEP (0.5 M in ligation buffer) was added to a final

concentration of 5 mM to ensure reducing conditions. The reaction was monitored by RP-HPLC (**Figure 2d**) and ESI-MS.

Upon completion of the reaction, the desulfurization reaction was initiated by addition of TCEP buffer, VA-044 and GSH as described above. The reaction was monitored by RP-HPLC and ESI-MS and upon reaching completion, the product was purified by semipreparative RP-HPLC to yield the desired product, protein **6**. Protein **6** was further analyzed by analytical RP-HPLC (**Figure 2d**) and ESI-MS (**Figure 2e**), lyophilized and stored at -20°C for later use in octamer refolding.

Synthesis of 7, H2A.X K15ub

Production of 1c, His-SUMO-H2A.X(21-142)A21C

1c, SUMO-H2A.X(21-142, A21C): *GSSHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSS EIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG-*
CGLQFPVGRVHRLLRKGHYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGGVTIA
QGGVLPNIQAVLLPKKTSATVGPKAPSGGKKATQASQEY

Protein **1c** was expressed in Rosetta 2 cells from a pET3a plasmid. Cell cultures were grown at 37°C in LB media supplemented with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol until an OD600 of 0.6. Protein expression and purification was performed following ref. [3]. Briefly, expression was induced by addition of IPTG to a final concentration of 0.5 mM. Expression of the protein was allowed to continue until 3 h post-induction. Cells were harvested, cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 1 mM βME, 1 Roche EDTA-free protease inhibitor tablet / 50mL). Cells were lysed by freeze-thawing and sonication. The inclusion body pellet was washed twice with 7.5mL of histone lysis buffer with 1% triton-X and once without triton-X. Protease refolding and cleavage was done by dialysis of **1c** and Ulp1 against urea cleavage buffer (2M urea, 75mM Tris-HCl, 150mM NaCl, 5mM DTT, 25mM L-arginine, 5mM L-cysteine, pH 7.5) for 16h. The protein was precipitated by TCA precipitation (1:4 vol. ratio) and recovered by centrifugation. The pellet was redissolved in 10 mL 6 M GmdHCl solution, TCEP was added to a final concentration of 5 mM and incubated for 30 min. The solution was then diluted with an equivalent volume RP-HPLC solvent 30%B and purified by RP-HPLC using a gradient of 30-70%B in 45min. The final purified truncated histone was lyophilized and stored for later reactions.

Ligation to produce 7, H2A.X K15ub

Purified, SUMO-deprotected **1c** was ligated to the ubiquitylated H2A.X N-terminal peptide **5b**, followed by desulfurization as described above. The product, **7**, was purified by semipreparative RP-HPLC and was further analyzed by analytical RP-HPLC and ESI-MS (**Figure S4d-f**) followed by lyophilization for later use in octamer refolding.

Unmodified histone expression and purification

H2A: SGRGKQGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYMAAVLEYLTAEILELAGNAARDNKKT
RIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLPKKTESHHKAKGK

H2A.X: SGRGKTGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGHYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKT
RIIPRHLQLAIRNDEELNKLLGGVTIAQGGVLPNIQAVLLPKKTSATVGPKAPSGGKKATQASQEY

H2B: PEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIAGEAS
RLAHYNKRSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKYTSAK

H3 (C110A): ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREI
AQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTNLAAIHAKRVTIMPKDIQLARRIRGERA

H4: SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDAVTYTEHAKRKT
VTAMDVVYALKRQGRTLYGFGG

Human *wild-type* histones were expressed in BL21(DE3)plysS cells from genes inserted into a pET3a plasmid. Cell cultures were grown at 37° C in LB media supplemented with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol until an OD600 of 0.6. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM. Expression of the protein was allowed to continue until 3 h post-induction. Cells were harvested, cell pellets were resuspended in lysis buffer (20 mM Tris pH 7.5, 1 mM EDTA, 200 mM NaCl, 1 mM βME, 1 Roche EDTA-free protease inhibitor tablet / 50mL). Cells were lysed by freeze-thawing and sonication. The inclusion body pellet was washed twice with 7.5 mL of lysis buffer with 1% triton and once without triton.

Histones were resolubilized in resolubilization buffer (6 M GdmHCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM βME, pH 7.5), dialyzed into urea buffer (7M urea, 10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 5 mM βME, pH 7.5) followed by purification by cation exchange (using a HiTrap SP HP 5 mL column). Collected fractions were analyzed by SDS-PAGE, followed by final purification using preparative RP-HPLC. Collected fractions were characterized by analytical RP-HPLC and ESI-MS, lyophilized and stored at -20° C until use in octamer refolding.

Histone octamer refolding

In a typical octamer refolding reaction, 0.4-1.5 mg of each of the pure lyophilized human histones were dissolved in unfolding buffer (6 M GmdHCl, 10 mM Tris-HCl, 5 mM DTT, pH 7.5). The exact concentration was determined by UV spectroscopy, using the following extinction coefficients: $\varepsilon_{280nm,H2A}$ = 4470 $M^{-1}cm^{-1}$, $\varepsilon_{280nm,H2B}$ = 7450 $M^{-1}cm^{-1}$, $\varepsilon_{280nm,H3}$ = 4470 $M^{-1}cm^{-1}$, $\varepsilon_{280nm,H4}$ = 5960 $M^{-1}cm^{-1}$. Equimolar amounts of H3 and H4 were then mixed along with 1.05 equivalents of H2A and H2B at 1 mg ml^{-1} and octamers were refolded by

dialysis against refolding buffer (2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, pH 7.5). The refolded octamers were subsequently purified by gel filtration on a Superdex S200 10/300GL column. Collected fractions were analysed by SDS-PAGE, and octamer containing fractions were pooled and concentrated to ~50µM octamer concentration (**Figure S6**). Finally, glycerol was added to a final concentration of 50% (v/v), concentrations were determined by UV spectroscopy and octamer stocks were stored at -20° C.

Convergent DNA ligations for 12x601 arrays

Chromatin DNA was produced as shown in **Figure S5a** and described in ref. [5]. Briefly, recombinant pieces recP1 and recP5 were expressed in bacterial cells and purified. P2, P3 and P4 were produced by PCR reactions and purified. Each piece was digested with the restriction enzymes BsaI-HF and DraIII-HF, which generate non-palindromic unique overhangs. Digested DNA fragments were purified by PEG precipitation to remove small fragments which may re-ligate. For preparative ligations, 30-60 pmol of each DNA piece was used for large-scale ligation to generate the intermediates in combined volumes of 200-400 µL: P2 was ligated to P1 in 20% excess for 2 h in 1x T4 DNA ligase buffer with 60 U of ligase, then P3 was added in 20% excess relative to P2 and ligation allowed to proceed overnight. P4 was ligated to P5 in 20% excess for 12-16 h (**Figure S5b,** step I). The pieces were purified by PEG precipitation using a stepwise (0.5% steps) increase in PEG from 7.0% to 8.0% (**Figure S5b,** step II). Pellets containing the purified desired chromatin DNA intermediates were redissolved in 60 µL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), were pooled and stored for later ligations. 15-35 pmol of the 6x601 intermediates were mixed using 5-10% excess P4-P5, the biotinylated anchor was added as well as 1x T4 DNA ligase buffer with 60 U of ligase. The mixture was then left to ligate for 10-16 h (**Figure S5b,** step III). The formation of the product was confirmed by agarose gel electrophoresis and purified by step-wise PEG precipitation in the range 5.0-6.0% (**Figure S5b**, step IV). The pellets were redissolved in TE(10/0.1) and analyzed by gel electrophoresis to pool the purified double-labeled array DNA (**Figure 3b**).

Chromatin array reconstitution on 12-mer DNA fragments and analysis

Chromatin assembly was performed, following ref [6]. Chromatin arrays were reconstituted on a scale of ~20 pmol (calculated based on nucleosome positioning sequences). Fluorescently labeled chromatin array DNA was mixed with equimolar MMTV buffer DNA (to avoid overloading the chromatin with octamers), NaCl was added to a final concentration of 2 M, followed by the addition of equimolar equivalents of histone octamers with or without modifications. The mixture was transferred to a micro-dialysis tube and dialyzed with a linear gradient from TEK2000 (TE buffer containing 2000 mM potassium chloride) to TEK10 over 16-18 h. The dialysis tube was transferred to 200-600 mL TEK10 for another 1 h of dialysis. The chromatin assembly

mixture was further centrifuged at 21000 x g for 10 min and the supernatant was collected. The concentration of the crude assembly was finally determined by UV-VIS spectrometry. For gel analysis, 0.25-0.50 pmol of chromatin assembly sample was diluted to a volume of 10 µL with TEK10 and 25% sucrose was added to a final concentration of 5-7%. This was run in 0.7% agarose gels made with 0.25x TB and using the same as running buffer at 90 V for 90-100 min (**Figure 3b**).

For ensemble FRET analysis, MMTV DNA and free MMTV nucleosomes had to be removed. To this end the crude assembly was mixed with an equal volume of 6 mM Mg^{2+} for precipitation on ice for 10 min followed by 10 min centrifugation at 21000 x g. The supernatant was removed and the chromatin pellet was re-dissolved in a similar volume of TEK10 as present prior to addition of magnesium (**Figure 3b**).

Chromatin assembly was assessed by ScaI-digestion of the arrays, liberating individual nucleosomes. Chromatin arrays were mixed with an equal volume of 1 x CutSmart buffer and 10 U of ScaI-HF restriction enzyme, followed by digestion for 5-7 h at room temperature. ScaI digests were analyzed by agarose gel-electophoresis or native PAGE using 0.5% TBE gels. Gels were visualized first in fluorescence channels and then stained with GelRed for visualization of both fluorescing and non-fluorescent DNA and nucleosomal/chromatin bands. Only chromatin arrays that did show full nucleosomal occupancy in ScaI digest samples were used for further experiments.

Ensemble FRET measurements on chromatin

Fluorescence spectra were recorded on a Fluorolog-3 fluorescence spectrometer. The settings used on the fluorescence spectrometer were: 575 nm excitation with 4 nm slit width and detection of the fluorescence emission in the range 585-700 nm with 5 nm slit width. Chromatin samples isolated after magnesium precipitation were diluted to a final volume of typically 220-250 µL resulting in a concentration giving a spectral count of around 90000-130000 cps for the donor prior to compaction. The sample was then split in 4 x 50 µL volumes. TEK10 and Mg^{2+} from stocks of 10 mM or 50 mM was added along with TEK10 to a final volume of 55 µL 5 min prior to measuring. After standing 5 min, the sample was transferred to the fluorescence micro-cuvette for measurement of the spectra twice. This was done for all the samples in the range 0-4 mM Mg^{2+} (**Figure S7**).

Preparing of flow chambers for single-molecule TIRF

Flow chambers for TIRF experiments were prepared following refs. [6,7]. Briefly, borosilicate glass slides with 2 rows of 4 holes and borosilicate coverslips were sonicated in glass containers for 20 min in ultra-pure water, then in acetone and ethanol. They were cleaned in piranha solution (25% v/v H_2O_2 and 75% v/v H_2SO_4) in the same glass containers for 1h followed by washing with water until reaching neutral pH again. A 500 mL

Erlenmeyer flask was cleaned in the same way. The flask, coverslips and slides were all sonicated in acetone for 10 min. 3% v/v (3-aminopropyl)triethoxysilane was prepared in acetone in the flask and used to immerse the slides and coverslips, followed by a 20 min incubation. The slides and coverslips were then washed in ultra-pure water and dried with N_2. On each slide, four flow-chambers were assembled from one glass slide and one coverslip separated by double-sided 0.12 mm tape. Open ends were sealed with epoxy glue and the silanized slides stored under vacuum in the freezer until use.

Before measurements, the flow chambers were passivated by attachment of a PEG brush. To this end, the silanized flow chambers were fitted with pipette tips as inlet reservoir and outlet sources in each of the 2x4 holes. The tips were glued in place with epoxy glue. Subsequently, 350 µL of 0.1 M tetraborate buffer at pH 8.5 was used to dissolve ~1 mg of biotin-mPEG(5000kDa)-SVA, and 175 µL from this was transferred to 20 mg mPEG(5000kDa)-SVA to generate a transparent clouding-point solution after 10 s of centrifugation. This was mixed to homogeneity with a pipette and centrifuged again for 10 s before 40-45 µL aliquots were loaded into each of the four channels in the flow chamber. The PEGylation reaction was allowed to continue for the next 2½-4 h, after which the solution was washed out with degassed ultra-pure water.

smTIRF measurements

Measurements were performed as described before[5]. Briefly, imaging was carried out with a micro-mirror TIRF system[1]. For each experiment, the flow chambers were washed with 200-300 µL T50 (10 mM Tris-HCl, pH 8, 50 mM NaCl), followed by incubation with 50 µL 0.2 mg mL^{-1} neutravidin for 5 min. After a wash with 400-500 µL T50, 0.5-2 µL of chromatin assembly reaction at a concentration of 5-40 ng μL^{-1} was loaded into the chamber. Chromatin was loaded until reaching a density of 150-400 chromatin arrays in a 25 x 50 µm field of view. The channel was washed with T50 followed by exchange to imaging buffer (40 mM KCl, 50 mM Tris-HCl, 2 mM Trolox, 2 mM nitrobenzyl alcohol (NBA), 2 mM cyclooctatetraene (COT), 10% glycerol and 3.2% glucose) supplemented with GODCAT (100 x stock solution: 165 UmL^{-1} glucose oxidase, 2170 UmL^{-1} catalase). For imaging, a programmed sequence was employed to switch the field of view to a new area followed by adjusting the focus. Then the camera was triggered to acquire 1300-2000 frames with 532 nm excitation and 100 ms time-resolution followed by a final change to 640 nm excitation.

From acquired movies, the background was extracted in ImageJ using a rolling ball algorithm. Trace extraction and analysis was performed in custom-written MATLAB software. The donor and the acceptor images were non-isotropically aligned using a transformation matrix generated from 8-10 sets of peaks appearing in both the donor and the acceptor channels. Peaks were automatically detected in the initial acceptor image prior to donor excitation and the same peaks were selected in the donor channel. Peaks tightly clustered, close to the edges or above a set intensity threshold in either the donor or the acceptor

channels indicating aggregation were removed from analysis. The analysis was then limited to the peaks appearing in both the donor and the acceptor channel and these traces were extracted for further analysis.

From traces of donor- (F_D) and acceptor (F_A) fluorescence emission intensity, FRET efficiency (E_{FRET}) traces are calculated as follows:

$$E_{FRET} = \frac{F_A - \beta F_D}{F_A - \beta F_D + \gamma F_D} \text{ and } \gamma = \frac{\Delta F_{A,bleach}}{\Delta F_{D,bleach}}$$

where β denotes the bleed-through of acceptor fluorescence into the donor channel, and γ is the sensitivity ratio of the detection system for donor and acceptor fluorescence. The values of β = 0.161 and γ = 0.436 were experimentally determined for the dye pair Alexa568/647 in our experimental setup. From fluorescence time-traces, E_{FRET} histograms were constructed, using a bin size of 0.02. E_{FRET} histograms of each trace of length > 5 s were normalized to total counts. Final histograms for each independent measurement were fitted using 2 or 3 Gaussian functions $\sum_i A_i e^{-\left(\frac{x-c_i}{\sigma_i}\right)^2}$. For fit values, see **Table S2**.

Traces were selected based on the following criteria: 1) Initial total fluorescence of the donor and the γ-corrected acceptor of > 2000 counts over baseline (at 900 EM gain). 2) At least 5 s prior to bleaching of acceptor or donor. 3) Single bleaching event for donor or acceptor. 3. a) if acceptor bleaches first; leads to anticorrelated increase in donor to same total fluorescence level as prior to bleaching. 3.b) if donor bleaches first, the acceptor dye must still be fluorescent when directly probed at the end of the experiment. 4) Bleaching of the donor dye during the 120 s of acquisition to allow an unambiguous determination of background levels.

Supplementary references

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Supplementary Tables

Number	Name	Calculated mass	Yield
Proteins			
1a	H2A.X(1-134)-NpuN	26681.6	n.a.
1b	His-SUMO-H2A.X(22-134)A21C-NpuN	35797.8	n.a.
1c	His-SUMO-H2A.X(22-142)	26283.9	n.a.
Peptides			
2	H2A.X(136-142) A135C S139ph	1008.9	47%
5a	H2A.X(1-20)K15(Cys)-NHNH2	2163.5	6.7 %
Semisynthetic proteins			
3	γH2A.X	15093.3	44 %
4b'	His-SUMO-H2A.X(22-142)A21C A135C S139ph	24241.6	n.a
4b	H2A.X(22-142)A21C A135C S139ph	13129.1	57 %
5b	H2A.X(1-20)K15(Ub)-NHNH2	10621.0	80 %
6	γH2A.X K15ub	23654.2	24%
7	H2A.X K15ub	23574.3	49 %
Other			
Ub	Ubiqutin-MES	8630.9	n.a.
N	NpuC-AA	4733.4	n.a.

Supplementary Table 1: Summary of peptides and proteins employed in the study.

Sample	$[Mg^{2+}]$ (mM)	n	A_1	c_1	σ_1	A_2	c_2	σ_2
γH2A.X	0	231	2.80	0.17	0.20	2.63	0.42	0.20
γH2A.X	0	150	2.26	0.13	0.20	3.20	0.39	0.20
γH2A.X	0	128	2.74	0.14	0.20	2.53	0.40	0.20
γH2A.X	1	109	1.46	0.08	0.20	4.14	0.44	0.20
γH2A.X	1	70	1.94	0.16	0.20	3.55	0.47	0.20
γH2A.X	1	136	2.62	0.12	0.20	2.82	0.41	0.20
γH2A.X	2	157	2.47	0.14	0.20	2.82	0.45	0.20
γH2A.X	2	67	2.05	0.17	0.20	3.70	0.56	0.19
γH2A.X	2	99	1.90	0.16	0.20	3.49	0.51	0.20
γH2A.X	4	152	1.61	0.18	0.22	3.81	0.59	0.20
γH2A.X	4	145	1.75	0.23	0.22	4.09	0.60	0.17
γH2A.X	4	98	2.02	0.19	0.20	3.41	0.57	0.20
γH2A.X K15Ub	0	97	3.20	0.08	0.20	2.07	0.33	0.20
γH2A.X K15Ub	0	59	3.68	0.17	0.20	1.80	0.34	0.20
γH2A.X K15Ub	0	56	3.28	0.12	0.20	1.65	0.31	0.20
γH2A.X K15Ub	1	61	3.77	0.14	0.20	1.54	0.41	0.20
γH2A.X K15Ub	1	48	2.20	0.04	0.20	3.01	0.27	0.20
γH2A.X K15Ub	1	50	3.67	0.19	0.20	1.39	0.55	0.20
γH2A.X K15Ub	2	30	2.86	0.02	0.20	2.30	0.27	0.20
γH2A.X K15Ub	2	46	2.49	0.05	0.20	2.84	0.37	0.20
γH2A.X K15Ub	2	72	2.64	0.07	0.20	2.37	0.37	0.20
γH2A.X K15Ub	4	85	2.94	0.07	0.20	2.13	0.45	0.20
γH2A.X K15Ub	4	111	2.01	0.06	0.20	3.21	0.35	0.20
γH2A.X K15Ub	4	58	2.26	0.08	0.20	3.00	0.44	0.20
H2A.X	0	74	2.10	0.24	0.26	2.71	0.40	0.20
H2A.X	0	156	3.61	0.12	0.22	2.32	0.35	0.11
H2A.X	1	46	2.04	0.17	0.20	3.97	0.50	0.17
H2A.X	1	154	2.72	0.12	0.22	3.18	0.43	0.15
H2A.X	2	57	2.02	0.19	0.20	4.12	0.58	0.17
H2A.X	2	183	2.13	0.11	0.22	3.98	0.50	0.15
H2A.X	4	76	1.69	0.15	0.23	4.00	0.60	0.18
H2A.X	4	256	1.70	0.09	0.22	4.94	0.60	0.15

Supplementary Table 2: Parameters of Gaussian fits to E_{FRET} distributions for all chromatin fibers. Here, n denotes the number of traces analyzed for each experimental repeat. A_i, c_i and σ_i are the parameters of the Gaussian fits, as described above.

Supplementary Figures

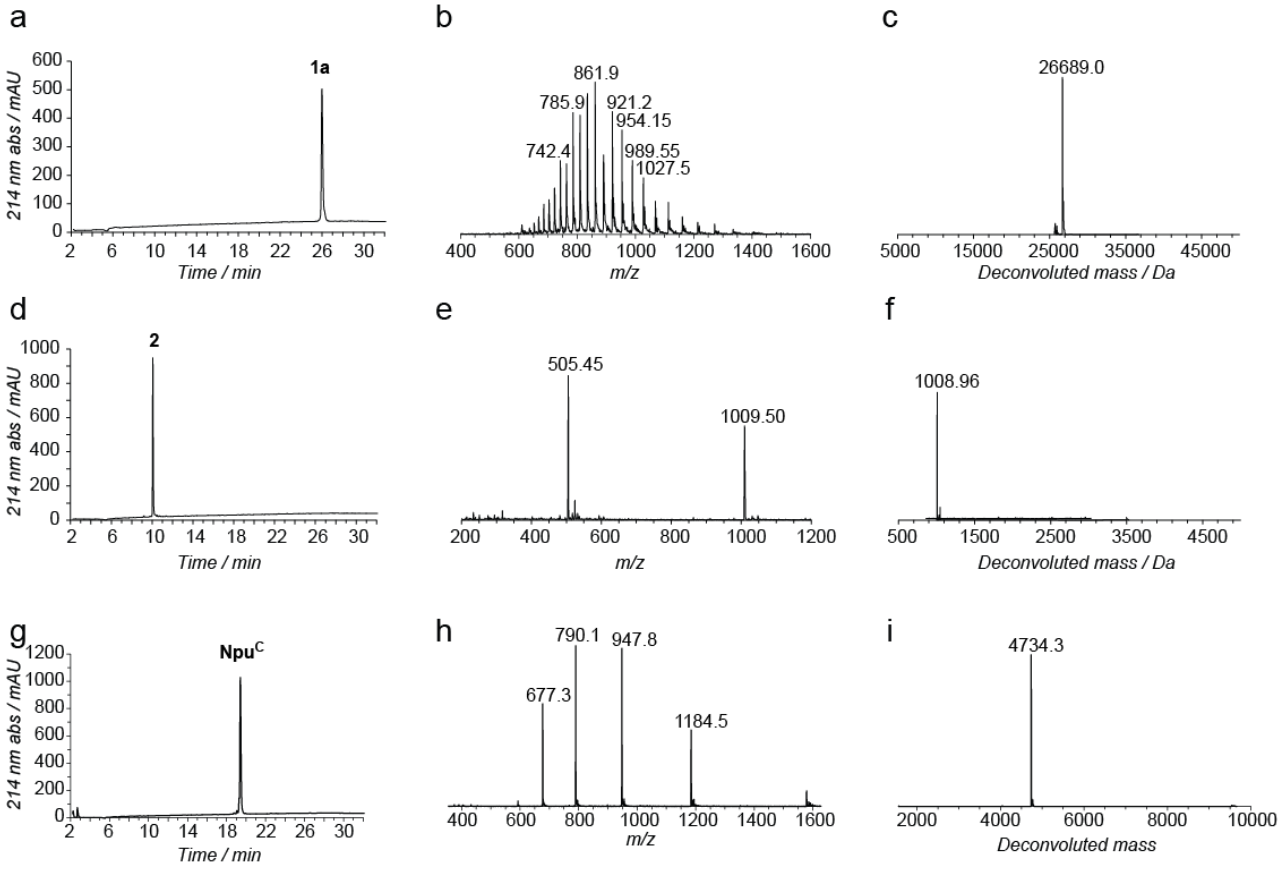
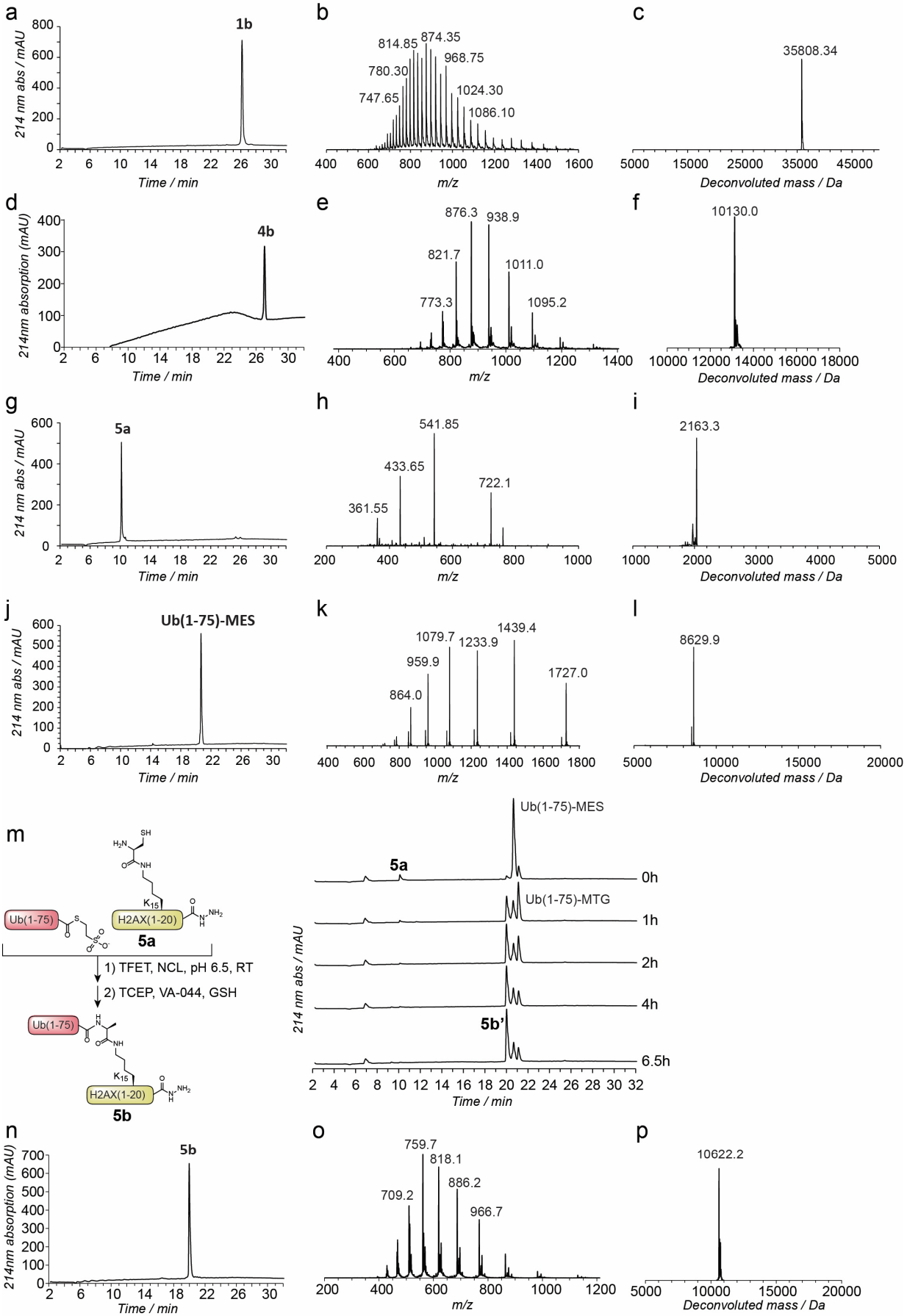


Figure S1: Starting materials for the synthesis of γH2A.X (3). (**a**) RP-HPLC chromatogram of H2A.X(1-134)-Npu^N **1a** after purification. (**b**) ESI-MS analysis and (**c**) deconvoluted spectrum of **1a**, M_{obs} = 26689.0 Da. M_{calc} = 26681 Da. (**d**) RP-HPLC chromatogram of H2A.X 135-142 (A135C, S139ph), **2**, after purification. (**e**) ESI-MS analysis and (**f**) deconvoluted spectrum of **2**, M_{obs}=1009.0 Da. M_{calc}=1008.9 Da. (**g**) RP-HPLC chromatogram of Npu^C-AA-CysOMe peptide after purification. (**h**) ESI-MS analysis of NpuC peptide, M_{obs} = 4734.3 Da. M_{calc} = 4733.4 Da. (**i**) Deconvoluted MS of (**h**).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1b
214 nm abs / mAU
Time / min
b
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874.35
968.75
780.30
1024.30
747.65
1086.10
m/z
c
35808.34
Deconvoluted mass / Da
d
4b
214nm absorption (mAU)
Time / min
e
876.3
938.9
821.7
1011.0
773.3
1095.2
m/z
f
10130.0
Deconvoluted mass / Da
g
5a
214 nm abs / mAU
Time / min
h
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433.65
722.1
361.55
m/z
i
2163.3
Deconvoluted mass / Da
j
Ub(1-75)-MES
214 nm abs / mAU
Time / min
k
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1439.4
1233.9
959.9
1727.0
864.0
m/z
l
8629.9
Deconvoluted mass / Da
m
Ub(1-75)
H2AX(1-20)
5a
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2) TCEP, VA-044, GSH
Ub(1-75)
H2AX(1-20)
5b
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5a
0h
Ub(1-75)-MTG
1h
2h
4h
5b'
6.5h
214 nm abs / mAU
Time / min
n
5b
214nm absorption (mAU)
Time / min
o
759.7
818.1
886.2
709.2
966.7
m/z
p
10622.2
Deconvoluted mass / Da

Figure S2: Synthesis of γH2A.X K15ub. (**a**) RP-HPLC chromatogram of SUMO-H2A.X(21-134, A21C)-Npu^N, **1b,** after purification. (**b**) ESI-MS analysis and (**c**) deconvoluted spectrum of **1b**, M_{obs}=35808.3 Da. M_{calc}=35798 Da. (**d**) RP-HPLC analysis of γH2A.X. (21-142, A21C, A135C), **4b,** after purification. (**e**) ESI-MS analysis and (**f**) deconvoluted spectrum of **4b** after purification. M_{obs} = 10130.0 Da. M_{calc} = 10129.1 Da. (**g**) RP-HPLC chromatogram of H2A.X 1-20 K15 ε-Cys peptide hydrazide, **5a**. (**h**) ESI-MS analysis and (**i**) deconvoluted spectrum of **5a**, M_{obs}=2163.3 Da. M_{calc}=2163.5 Da. (**j**) RP-HPLC chromatogram of purified Ub(1-75)-MES. (**k**) ESI-MS analysis and (**l**) deconvoluted spectrum of Ub(1-75)-MES, M_{obs} = 8629.9 Da. M_{calc} = 8630.9 Da. (**m**) Ligation of ubiquitin thioester (Ub(1-75)-MES) to **5a**, resulting in the ligation product H2A.X(1-20)K15ub(G76C)-$NHNH_2$, **5b'**. Left: Scheme of reaction. Right: Monitoring of reaction by RP-HPLC after 0h, 1h and 4h respectively. (**n**) RP-HPLC analysis of the desulfurized ligation product H2A.X(1-20) K15ub (76A)-$NHNH_2$, **5b**, after purification. (**o**) ESI-MS analysis and (**p**) deconvoluted spectrum of **5b**, M_{obs} = 10622.2 Da. M_{calc} = 10621.0 Da.

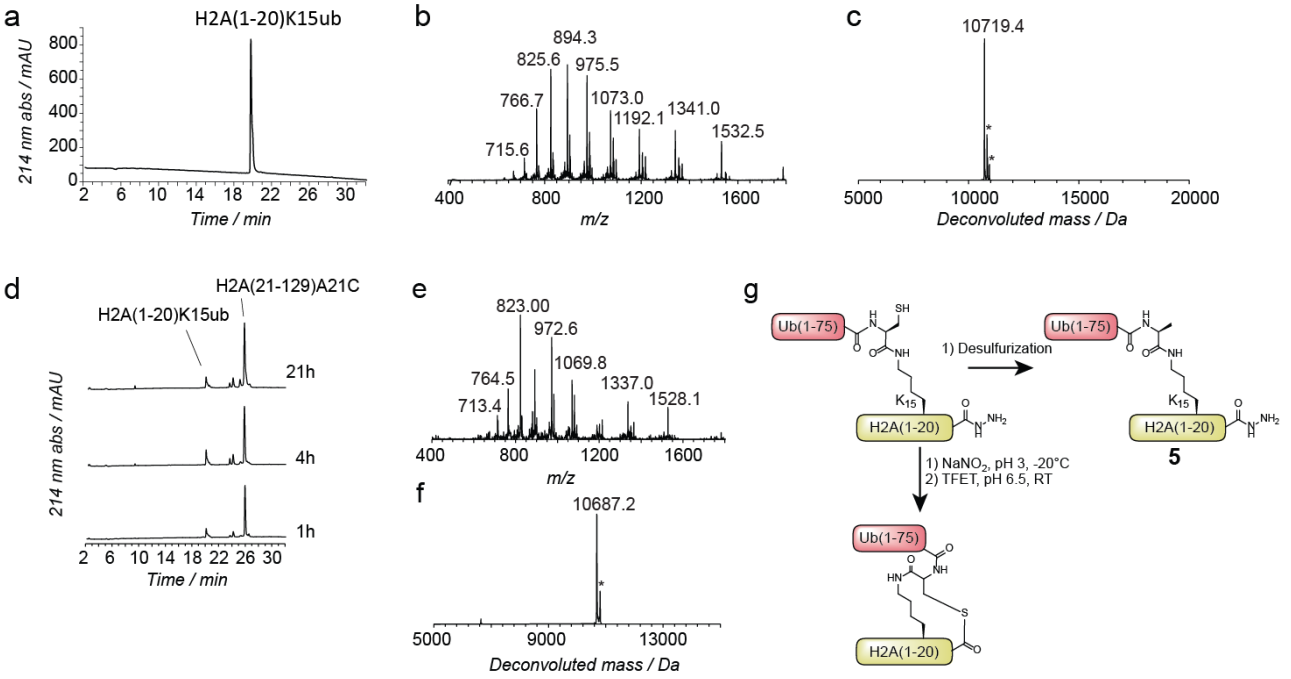

Figure S3: Intramolecular cyclization during ubiquitylation of H2A K15ub (G76C). (**a**) RP-HPLC analysis of H2A(1-20) K15ub after purification. (**b**) ESI-MS analysis and deonvoluted spectrum (**c**) of H2A(1-20) K15ub, M_{obs} = 10719.4 Da. M_{calc} = 10722.8 Da. (**d**) Ligation attempts of H2A(1-20) K15ub to H2A(21-129)A21C. The ligation is not progressing during the 21 h time, but a -32 Da side product accumulates. (**e**) ESI-MS analysis of the ligation reaction after 21 h, revealing a loss of 32 Da, corresponding to cyclization of H2A(1-20)K15ub-SR, M_{obs}=10687.2Da. M_{calc}=10690.7Da. (**f**) Deconvoluted spectrum of (**e**). (**g**) The cyclization reaction likely taking place after hydrazide to thioester conversion activation. Desulfurization prior to ligation prevents this side reaction.

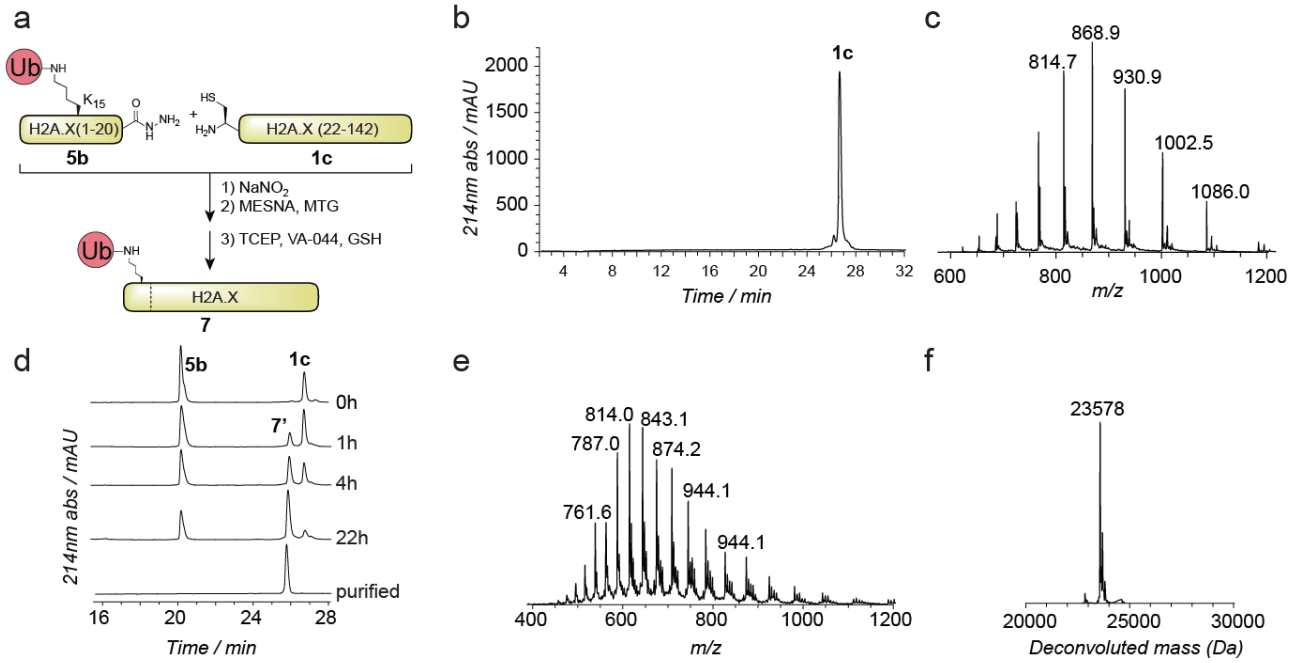

Figure S4: Formation of H2A.X K15ub. (**a**) Scheme of one-pot ligation and desulfurization of ubiquitylated N-terminal peptide to H2A.X(21-142, A21C). (**b**) RP-HPLC analysis of purified H2A.X(21-142, A21C). (**c**) ESI-MS analysis of H2A.X(21-142, A21C) (observed mass: 13018.7 Da, calculated mass: 13017.1 Da (**d**) RP-HPLC analysis of the reactions shown in (**a**). (**e**) ESI-MS analysis of purified H2A.X K15ub (observed mass: 23578.0 Da, calculated mass: 23574.3 Da). (**f**) Deconvoluted MS of (**e**).

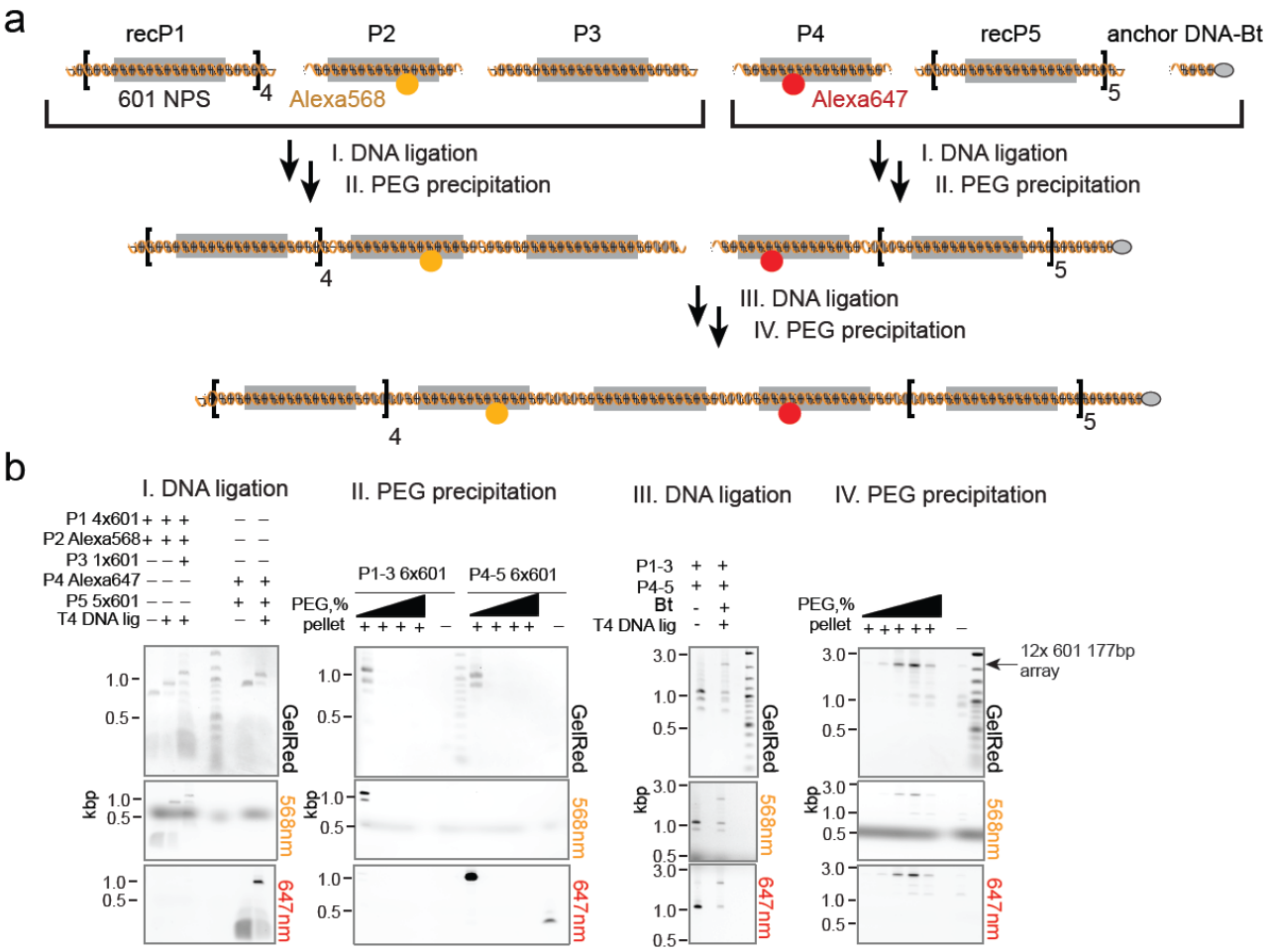

Figure S5. Preparation of dual-labeled chromatin DNA. (**a**) Convergent assembly and purification of 12x array DNA. Recombinant DNA piece recP1 (containing 4 x 601 sequences) is ligated to PCR produced piece P2 (1x 601 sequence, carrying the donor dye Alexa568) and P3 (1x601 sequence). The intermediate 6x601 is purified by PEG precipitation. A similar procedure was used to ligate PCR produced P4 (1x601, containing acceptor dye Alexa647) and recombinantly produced fragment recP5 (5x601), as well as the dsDNA anchor to produce another 6x601 intermediate. The two intermediate 6x601 pieces are ligated to produce the 12x601 array DNA with internal fluorophores and the biotin (Bt)-anchor, followed by final purification by PEG precipitation. (**b**) Chromatin DNA assembly. I. Large-scale ligations to produce intermediates analyzed by agarose gel electrophoresis. Gels were stained by GelRed and imaged using fluorescence imaging for donor (Alexa568) and acceptor (Alexa647) fluorophores. II. Enrichment of intermediates by PEG precipitation. The enriched, ligated pieces are collected from pellet fractions, whereas unligated pieces P2 and P4 are removed. III. Final ligation to produce 12x601 chromatin array DNA. IV. Final PEG purification of 12x601 chromatin DNA. Samples in Lane 3 and 4 are used for further experiments.

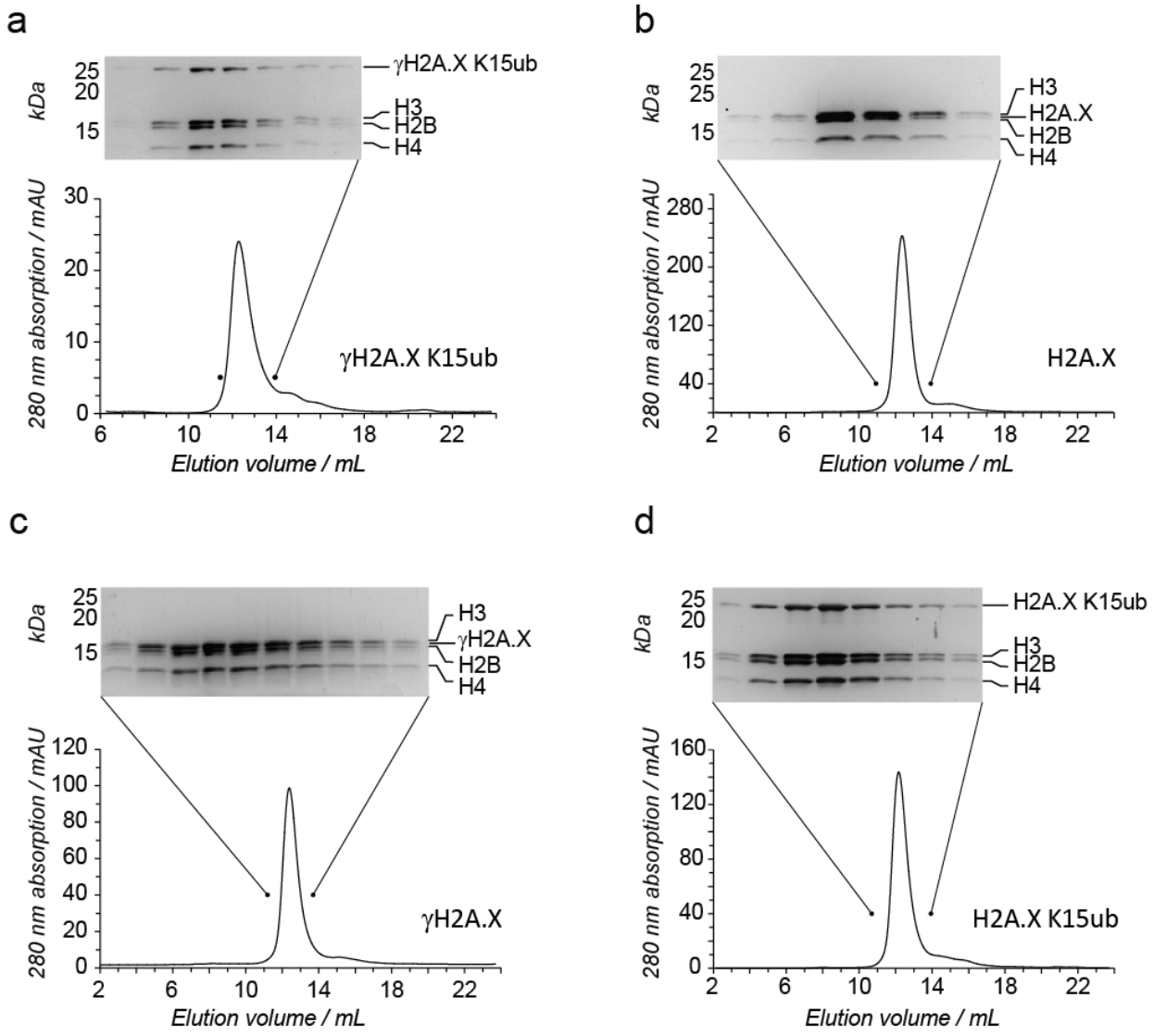

Figure S6: Analysis of assembled histone octamer variants by size exclusion chromatography and SDS-PAGE. (**a**) Gel filtration and SDS-PAGE analysis of histone octamers with γH2A.X K15ub (**6**). (**b**) Gel filtration and SDS-PAGE analysis of histone octamers with unmodified H2A.X. (**c**) Gel filtration and SDS-PAGE analysis of histone octamers with γH2A.X. (**d**) Gel filtration and SDS-PAGE analysis of histone octamers with H2A.X K15ub.

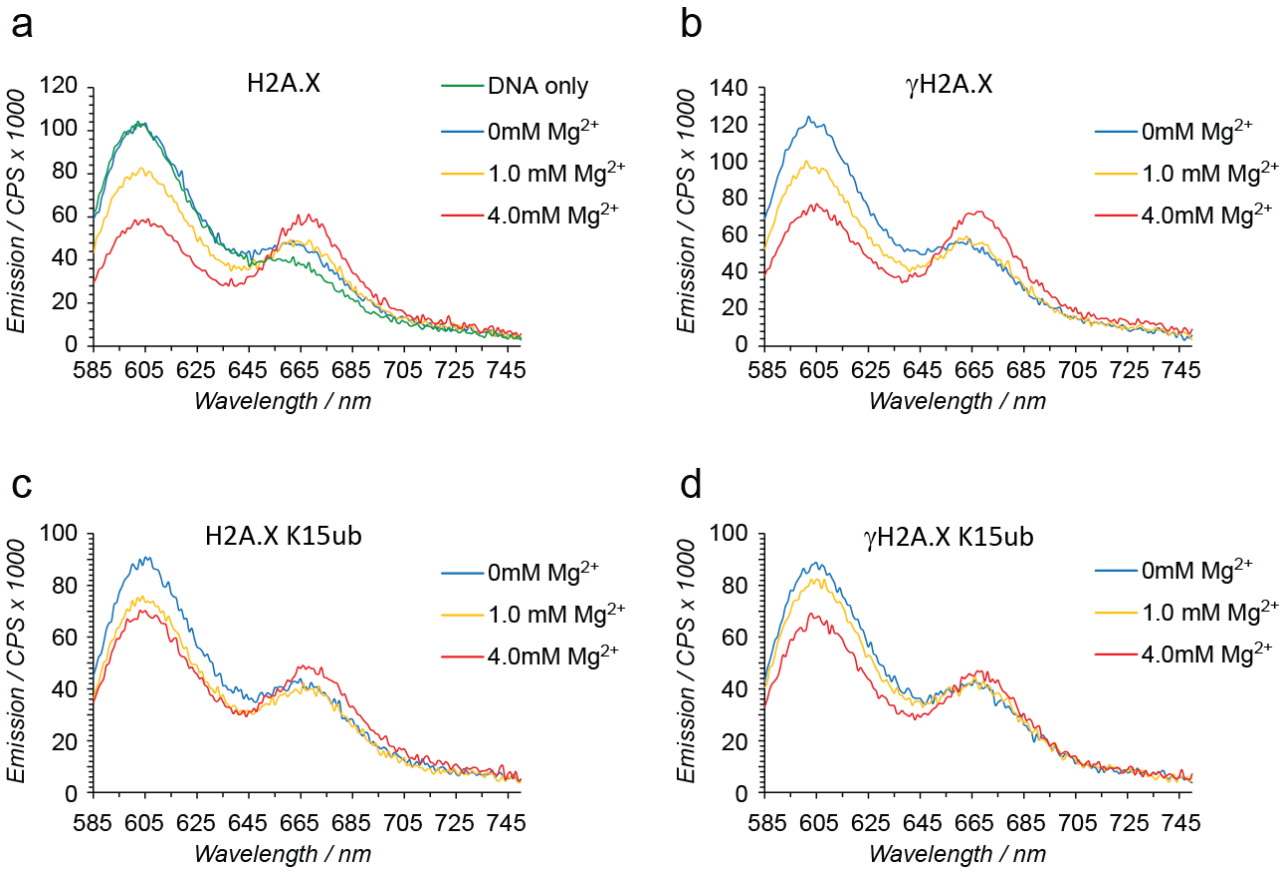

Figure S7. Ensemble FRET measurements of chromatin compaction. (**a**) Fluorescence spectra of chromatin array DNA or chromatin arrays containing unmodified H2A.X in the presence of indicated Mg^{2+} concentrations. A decrease in FRET donor emission (at 605 nm) and a concomitant increase in FRET acceptor dye emission (at 690 nm) indicates tetranucleosome stacking. (**b**) Fluorescence spectra as a function of Mg^{2+}-induced chromatin compaction assay for chromatin fibers containing γH2A.X. (**c**) Fluorescence spectra as a function of Mg^{2+}-induced chromatin compaction assay for chromatin fibers containing H2A.X K15ub. (**d**) Fluorescence spectra as a function of Mg^{2+}-induced chromatin compaction assay for chromatin fibers containing γH2A.X K15ub.