SUPPLEMENTARY FIGURES AND TEXT FOR:

Stability of nucleosomes containing homogenously ubiquitylated H2A and H2B prepared using semisynthesis

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General Methods

Amino acid derivatives, coupling reagents and resins were purchased from Novabiochem (Låufelfingen, Switzerland). All commonly used chemical reagents and solvents were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI) or Fischer Scientific (Pittsburgh, PA). Chemically competent DH5α and BL21(DE3)pLysS cells, as well as Ni:NTA resin were purchased from Novagen (Madison, WI). T4 DNA ligase and restriction enzymes were obtained from New England BioLabs (Ipswitch, MA). Primer synthesis and gene sequencing were performed by Integrated DNA Technologies (Coralville, IA) and Genewiz (South Plainfeld, NJ), respectively. Gene mutagenesis was achieved using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). PCR purification and gel extraction kits, as well as RNaseA were purchased from Qiagen (Valencia, CA). Criterion 15% Tris-HCl and 5% TBE gels were purchased from BioRad (Hercules, CA). Centrifugal filtration units were from Sartorius (Goettingen, Germany) and Slide-A-Lyzer dialysis cassettes and MINI dialysis units were from Pierce (Rockford, IL). Size exclusion chromatography was performed on an AKTA FPLC system from GE Healthcare (Waukesha, WI) equipped with a P-920 pump and UPC-900 monitor. Analytical reversed-phase HPLC (RP-HPLC) was performed on an Agilent 1200 series instrument (Agilent, Santa Clara, CA) with a Vydac C18 column (5 micron, 4 x 150 mm),

employing 0.1% TFA in water (HPLC solvent A), and 90% acetonitrile, 0.1% TFA in water (HPLC solvent B), as the mobile phases. Typical analytical gradients were 30-70% solvent B over 30 min at a flow rate of 1 mL/min. Preparative scale purifications were conducted on a Waters DeltaPrep 4000 system equipped with a Waters 486 tunable detector (Waters, Milford, MA). A Vydac C18 preparative column (15-20 micron, 20 x 250 mm) or a semi-preparative column (12 micron, 10 mm x 250 mm) was employed at a flow rate of 15 mL/min or 4 mL/min, respectively. ESI-MS analysis was conducted on a MicrOTOF-Q II ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). UV spectrometry was performed on an Agilent 8453 UV-Vis spectrophotometer (Agilent, Santa Clara, CA). All protein starting materials and ligation products were analyzed by C18 analytical RP-HPLC and ESI-MS.

uH2A Synthesis

Synthesis of Peptide 1:

For peptide **1**, the sequence corresponding to residues 115–129 of *xenopus laevis* H2A was synthesized on preloaded Wang resin using automatic solid-phase peptide synthesis with a Fmoc Nα protection strategy and using HBTU for amino acid activation. Standard tert-butyl side chain protection was used throughout with the following exception: the α-amino group of K119 was protected with the 4-methyltrityl (Mtt) group. (R)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid was purchased from Chem-Impex Intl. Inc. (Wood Dale, IL) and Boc-protected using Boc anhydride in the presence of sodium bicarbonate in water/dioxane at 0° C for 1h and at RT for 16 h. The protected building block was coupled to the deprotected N-terminus of the peptide, using HBTU/DIEA in DMF. Subsequently, the Mtt group on K119 was deprotected by successive incubations of the peptidyl-resin with 1% TFA in DCM containing 1% triisopropylsilane (TIS)

for 10 min intervals, until no yellow color evolved. Boc- *S*-trityl-cysteine was coupled to the ε-NH_2 of K119. Following cleavage from the resin with TFA/TIS/H2O (95:2.5:2.5) for 3 h, peptide **1** was purified by preparative RP-HPLC using a 15–40% B gradient over 60 min, yielding 11 mg purified peptide from 0.1 mmol resin. Peptide **1** was characterized by ESI-MS [$(M + H)^+$ observed = 1878.0 Da; expected =1878.0 Da] (**Figure S1b,c**).

Ubiquitin(1-75)-α-thioester (protein 2) production:

Protein **2** was produced as described in ref. [1]. For the analytical data of **2**, see **Figure S1d,e**.

H2A(1-113) cloning:

Using the *Xenopus* H2A expression plasmid[2] as a template, the sequence encompassing residues 1 to 113 was amplified using the following primers: forward: 5'- ATTTTGTTTAACTTTAAGA AGGAGATATACATATGTCAGGAAGAGGCAAACAAGG-3', reverse: 5'-GGTCCTGCCC AACATCCAGTCCTGCATCACGGGAGATGCACTAGTTGCCCTACCC-3'. The PCR product was gel purified and used as megaprimers in a second overlap extension PCR reaction with a pTXB1 plasmid (New England Biolabs) as template, following ref. [3], to insert H2A in-frame with the GyrA intein. In a second step, a His6-tag followed by a stop codon was inserted between the GyrA intein and the chitin binding domain in the pTXB1 plasmid by site-directed mutagenesis employing a QuikChange Site-Directed Mutagenesis kit, using the following primers: forward: 5'- GGGTCGTCAGCCACGCTCACCATCACCATCACCACTAAACTGGC CTCACCGGTCTGAAC-3' and reverse: 5'- GTTCAGACCGGTGAGGCCAGTTTAGTGGT GATGGTGATGGTGAGCGTGGCTGACGAACCC-3'. The correct sequence was confirmed by gene sequencing.

H2A(1-113)-α-thioester (protein 5) expression/purification:

The H2A(1-113)-GyrA-His6 construct described above was transfected into *E. coli* BL21(DE3)pLysS cells. 6 L LB broth were inoculated from an overnight pre-culture and induced with 0.5 mM IPTG during log-phase (OD600 = 0.6) for 3h at 37 °C. Subsequently, the cells were harvested by centrifugation and resuspended in wash buffer (20 mM Tris, pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5 mM TCEP) containing Roche EDTA free protease inhibitor cocktail. After one freeze thaw cycle, the cells were lysed by sonication followed by 5 passes through a French Press homogenizer. The insoluble pellets (inclusion bodies) were washed twice with 40 mL wash buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM TCEP) containing 1% triton X-100, and once with wash buffer. The inclusion bodies were solubilized in solubilization buffer (20 mM Tris, pH 7.6, 1 M NaCl, 6 M GdmCl, 5 mM imidazole, 0.5 mM TCEP) and applied to a Ni:NTA column equilibrated in the same buffer for IMAC purification. The protein was bound to the column under agitation overnight, washed with solubilization buffer containing 50 mM imidazole and the protein finally eluted with elution buffer (20 mM Tris, pH 7.6, 500 mM NaCl, 6 M GmdHCl, 500 mM imidazole, 0.5 mM TCEP). The concentration of H2A(1-113)-GyrA intein fusion was estimated by analytical HPLC and adjusted to approximately 1 mg/mL by dilution with refolding buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5 mM TCEP) containing 4 M GdmCl. The protein was then refolded by stepwise dialysis from 5 M to 0.5 M GdmCl in 4 steps of 2h each. In the last dialysis step, 5 mM mercaptoethanesulfonate (MES) was added. After refolding, the intein cleavage was initiated by addition of 100 mM MES and 5 mM TCEP. The intein thiolysis reaction was followed by analytical RP-HPLC and ESI-MS and allowed to proceed for 4 days for an approximately 35% conversion of starting material.

Subsequently, the solution was dialyzed into column buffer (20mM Tris, pH 7.2, 200 mM NaCl, 6 M GmdHCl, 1 mM MES) and applied to a second Ni:NTA column to remove the intein and uncleaved protein. The flow-through was collected and dialyzed into water containing 1 mM acetic acid. After dialysis the protein was lyophilized, redissolved in 6 M GdmCl solution and applied to semi-preparative RP-HPLC purification over a gradient of 30-70% B in 45 min. The pure fractions were combined and lyophilized to yield a total amount of 22 mg pure H2A1-113 MES α-thioester (**5**) from 6 L culture. For the analytical data of **5**, see **Figure S1f,g**.

Ligation 1 & thiazolidine deprotection:

Peptide **1**, (2.1 mg, 1.12 μmol) and ubiquitin α-thioester **2**, (5 mg, 0.58 μmol) were combined in 200 μL of ligation buffer (6 M GdmCl, 300 mM phosphate buffer, pH 7.8, 50 mM TCEP, 50 mM MPAA, degassed). The pH was adjusted to 7.8 using 5 N NaOH and the reaction was allowed to proceed for 4 h at room temperature under Ar, forming ligation product **3** (**Figure S2a**). To this solution was added 250 μL of 50% HPLC buffer B and 71.4 μL of 3.5 M methoxylamine and the pH was adjusted to 5. The reaction was allowed to proceed for 16 h at room temperature under argon at which point deprotected protein, **4**, was purified by sem-preparative RP-HPLC using a 25-45% B gradient, yielding 3.6 mg of product (for HPLC and MS analysis of **4**, see **Figure S2b,c**).

Ligation 2:

In a typical reaction, protein **4**, (2 mg, 0.2 μmol) and H2A(1-113)-α-thioester **5** (5 mg, 0.4 μmol) were combined in 160 μL ligation buffer (6 M GdmCl, 300 mM phosphate buffer, pH 7.8, 50 mM TCEP, 50 mM MPAA, degassed). The pH was adjusted to 7.8 using 5 N NaOH and the reaction was allowed to proceed at room temperature under Ar to generate **6**. The reaction progress was monitored by analytical RP-HPLC and ESI-MS. After 3 d, another 1.5 mg protein **5** (0.12 μmol) was added to account for α-thioester hydrolysis. After 4.5 d, the reaction mixture was diluted and acidified with 160 μL of 30% HPLC buffer B and purified by semi-preparative HPLC, using a 30 – 60% B gradient and yielding 1.8 mg purified product (for HPLC and MS analysis of **6**, see **Figure S2d,e**).

Desulfurization:

In a typical reaction, 1.6 mg of protein **6** were dissolved in desulfurization buffer (6M GdmCl, 100 mM phosphate buffer, pH 6.5, 250 mM TCEP, 40 mM glutathione, 16 mM VA-061) and incubated under an Ar atmosphere for 12 h at 37^{o}C and then another 16 h at RT. The reaction progress was monitored by RP-HPLC and ESI-MS. After complete desulfurization, the solution was acidified by addition of 50% HPLC buffer B and purified by RP-HPLC over a 0-70%B gradient, yielding 0.8 mg of pure final uH2A **7** (for HPLC and MS analysis, see **Figure 1f,g**).

Other Experimental Procedures

Production of wild-type histones and uH2B:

Unmodified, recombinant *Xenopus* histones H2A, H2A(N110C), H2B, H2B(T115C), H3(C110A)[4] and H4 were expressed as described in ref. [5] with some modifications. In brief, *E. coli* BL21(DE3)pLysS cells were transfected with histone expression plasmids, grown in 6 L LB medium at 37°C until OD_{600} 0.6 and protein expression was induced by addition of 0.5 mM IPTG for 3 h. Cells were harvested by centrifugation at 7000 x g and lysed by 5 x passage through a French Press. The insoluble pellets were washed twice with 40 mL wash buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) containing 1% triton X-100, and once with wash buffer. The pellets were soaked in 0.5 mL DMSO and extracted with extraction buffer (7M GdmCl, 10 mM Tris pH 8, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF) under agitation for 1 h. The suspension was clarified by centrifugation and then dialyzed against unfolding buffer A (7M urea, 10 mM Tris pH 8, 1 mM EDTA, 100 mM NaCl, 5 mM DTT, 0.2 mM PMSF). The solution was then applied to a HiTrap SP FF 5mL column, equilibrated in unfolding buffer A. The histones were eluted using a gradient over 15 column volumes from unfolding buffer A to unfolding buffer B (7M urea, 10 mM Tris pH 8, 1 mM EDTA, 1 M NaCl, 1 mM DTT, 0.2 mM PMSF). The histones were then further purified using preparative C-18 RP-HPLC and a gradient of 30-70% HPLC solvent B. The recombinant histones were analyzed by analytical HPLC and ESI-MS (H2A: $[M+H]^+$ calculated = 13,951 Da, $[M+H]^+$ observed = 13,952±4 Da, H2A(N110C): $[M+H]^+$ calculated = 13,940 Da, $[M+H]^+$ observed = 13,941±4 Da, H2B: $[M+H]^+$ calculated = 13,818 Da, $[M+H]^+$ observed = 13,820±4 Da, H2B(T115C): $[M+H]^+$ calculated = 13820.0, $[M+H]^+$ observed = 13819.8 H3(C110A):

$[M+H]^+$ calculated = 15,240 Da, $[M+H]^+$ observed = 15,241±4 Da), H4: $[M+H]^+$ calculated = 11,237 Da, $[M+H]^+$ observed = 11,238±4 Da. uH2B was synthesized from a synthetic peptide and expressed proteins and purified as described in ref. [4] (see **Figure S3** for analytical data).

Histone labeling:

For labeling with Alexa 488-C_5-maleimide, histones H2A(N110C) or H2B(T115C) were dissolved in labeling buffer (300 mM phosphate buffer, pH 7, 6M GmdHCl, degassed) to a concentration of about 100 μM. To this solution, 2 equivalents (200 μM) of TCEP were added and the solution was incubated for 30 min at RT. Subsequently, 3 equivalents of Alexa 488-C_5-maleimide were dissolved in DMSO were added stepwise. The progress of the reaction was monitored by RP-HPLC and ESI-MS. Upon completion of the labeling reaction, the histones were purified by semi-preparative HPLC using a gradient of 30-70% HPLC buffer B. For the analytical data for $H2A_{A488}$ and $H2B_{A488}$, see **Figure S6**.

Histone refolding and purification

For dimer or tetramer refolding, 0.1mg-2.0mg of lyophilized recombinant histones (unmodified or A488 labeled) and semi-synthetic ubiquitylated histones (as appropriate) were dissolved in a volume of unfolding buffer (6M GdmCl, 20mM Tris, 5mM DTT, pH =7.5 at 4°C) to a concentration of 2.0 mg/mL. The exact concentrations were determined by measuring absorption at 280 nm or 493 nm (for A488-labeled histones) using UV spectrometry, using the extinction coefficients for the histones calculated using the protparam online-tool (http://web.expasy.org/protparam) or using the extinction coefficient 72000 $M^{-1}cm^{-1}$ for the dye. Based on these concentration measurements, the histones (H2A/H2B for dimers, H3/H4 for tetramers) were mixed in equimolar ratios to a final protein concentration of 1.0 mg/mL. The histone solution was transferred to a Slide-A-Lyzer dialysis cassette (7000 MWCO) and dialyzed against 3 x 500mL (2 changes) of refolding buffer (2M NaCl, 10mM Tris, 1mM EDTA, 1mM DTT, pH = 7.5 at 4°C), for at least 4 h per buffer change. The dialyzed solution was centrifuged at 16.3 x g for 10 min. to remove aggregates and unfolded protein. The supernatant was concentrated to 200-400µL using 500 Vivaspin 5000 MWCO centrifugal concentrators.

Dimers or tetramers were purified by gel filtration using a Superdex 200 10/300GL 24 mL column in refolding buffer at a flow rate of 0.5mL/min. The stoichiometry of histones and the purity of collected fractions was assessed by SDS-PAGE. The desired fractions were pooled and concentrated using Vivaspin 500, 5000 MWCO centrifugal concentrators to a volume of 100-200µL. The refolded and purified subcomponents were stored on ice for usage in titrations or gel assays within 3 weeks of refolding. For purification data for the refolding of unlabeled histone dimers, see **Figure S5**, for the refolding of labeled dimers see **Figure S7**.

DNA production

Based on the 177 bp 601 DNA repeat[6], we constructed a 207 bp DNA fragment containing an internal 601 sequence[7]. At the 5'-end of the 601 sequence, we placed a PstI site followed by a BglII and an EcoRV site and 23 bp of linker DNA, and at the 3'-end we included 26 bp of linker DNA, followed by an EcoRV and a BamHI site. The construct was generated using the following primers: sense, 5'- AGTCGCTGCAGAGATCTGATATCAAGGCATCATGCTGTT CAA-3' and antisense 5'- GACTGGATCCGATATCAGATCCATGGACCCTATACGCGG-3'. Following ref. [8], 8 tandem repeats of the 207-601 DNA sequence were produced by sequential cloning steps. An 8-mer repeat of the 153 bp 601 sequence (153-601) was produced as described previously[5].

Purification of the DNA was performed as outlined in refs. [8,9], with some modifications. 6 L of terrific broth (1.2% (w/v) Bacto Tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4) were inoculated with *E. coli*, transfected with the respective plasmids. The cells were grown to OD_{600} = 0.8, subsequently chloramphenicol was added to a concentration of 170 mg/L and the incubation was continued for another 16 h. The cells were harvested by centrifugation, washed with water and subsequently resuspended in 20 mL lysis buffer (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) containing 10 mg lysozyme. After 30 min incubation on ice, alkaline lysis solution (0.2 N NaOH, 1% SDS) was added, and the mixture was gently agitated. After lysis, 30 mL of high salt solution (3 M potassium acetate, 1.8 M formic acid) was added and the solution was incubated on ice for 30 min. The genomic DNA was removed by centrifugation at 12000 x g for 30 min (all following centrifugations were similarly performed). The plasmid DNA (with contaminating RNA) was precipitated with 2x volumes of cold ethanol and redissolved in 12 mL of acetate-MOPS buffer

(0.1 M sodium acetate, 0.05 M MOPS, pH 8). After a further ethanol precipitation the pellet was redissolved in 4 mL of water. To this solution, an equal volume of LiCl solution (5 M LiCI, 0.05 M MOPS, pH 8) was added and the mixture was kept on ice for 15 min. After centrifugation, removing most contaminating RNA, the supernatant was heated to 60^{o}C for 10 min and the sample centrifuged again. The 601-207 or 153 plasmid was subsequently precipitated from the supernatant using ice cold ethanol and redissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). To this solution, 10 μg/mL RNAseA was added and the solution was incubated for 30 min. at 37^{o}C. Subsequently, 3 rounds of phenol-chloroform extractions were performed, followed by an isopropanol precipitation and ethanol wash. After redissolving the plasmid in TE buffer, it was further precipitated with 10% (w/v) PEG6000 and 500 mM NaCl. To release the 601-DNA fragments, the pellet was redissolved in TE buffer and to this solution 40U EcoRV (NEB) per nanomole of cleavage site present in the plasmid was added, followed by an incubation at 37^{o}C for 16 h. The plasmid backbone was subsequently precipitated by adding 7.5% PEG600 in TE buffer containing 500 mM NaCl and incubating on ice followed by centrifugation. The 207-601 or 153-601 DNA remaining in the supernatant was then ethanol precipitated and further cleaned by 3 consecutive rounds of phenol-chloroform extractions, followed by an isopropanol precipitation and ethanol wash. Finally, the 207-601 or 153-601 DNA was dissolved in TE buffer, the concentration was determined by UV and the aliquots were stored at -20^{o}C until use.

Nap1 cloning, expression and labeling

The mammalian Nap1 gene (*mus musculus*, NP_056596.1) was obtained in pET-15b as a gift from Robert Roeder (Rockefeller University). A TEV protease cleavage site was introduced between the His6-tag and the N-terminus of mNap1: sense: 5'- CGCATCACCATCACCATCA

CGAGAATTTGTATTTTCAGTCCATGGCCGACATTGACAAC -3', antisense: 5'- GTTGTC AATGTCGGCCATGGACTGAAAATACAAATTCTCGTGATGGTGATGGTGATGCG-3' using a QuikChange reaction. In addition, 4 cysteine residues (C88, C132, C255, C258) were mutated to serine using multisite QuikChange with the following primers: C88: 5'-GAATGCTC TCAAGAATCTTCAGGTTAAAAGTGCACAGATAGAAG-3', C132: 5'-AATCTATGAACCT ACAGAAGAAGAAAGCGAGTGGAAACC-3', C255, C258: 5'-CCAGAGATTATGGGCAGT ACAGGGAGCCAGATAGATTGGA-3', The sequence of the construct was confirmed by DNA sequencing.

For mNap1 expression, the plasmid was transformed into *E. coli,* BL21(DE3) cells. 3 L LB medium were inoculated with an overnight culture and grown to OD 0.6. The culture was cooled to 18^{o}C and induced with 0.4 mM IPTG for 16h. The cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris, pH 7.6 at 4^{o}C, 0.5 mM EDTA, 10% glycerol, 100 mM NaCl, 10 mM imidazole, 0.1 % NP-40, 1 mM beta-mercaptoethanol, Roche EDTA free protease inhibitors). The cells were subsequently lysed using a French press, the solution clarified and applied to a Ni:NTA column (2.5 mL). The protein was bound for 3 h, then the column was washed with lysis buffer containing 50 mM imidazole, finally the protein was eluted with 150 mM imidazole. The elutions were combined and dialyzed into buffer B (20 mM Tris, pH 7.6, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.1 mM PMSF). TEV protease was added in a 1:100 molar ratio and the solution was incubated at 4 ^{o}C for 16h. Subsequently, the solution was applied to another 2.5 mL of Ni:NTA resin and the flow-through collected. The flow-through was bound to a HiTrap Q HP column (5 mL) and eluted with buffer B in a gradient from 100 – 500 mM NaCl. The fractions containing mNap1 were combined, the

buffer exchanged to buffer C (20 mM Tris, pH 7.6, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol, 0.1 mM TCEP) using PD-10 desalting columns, aliquoted and stored at -80^{o}C until use.

yNap1(C200A, D201C, C249A, C272A, C414A) was expressed with an N-terminal His_6 tag followed by a thrombin and a TEV site. yNap1 was then purified by Ni affinity purification over Ni:NTA resin (elution at 300 nM NaCl) followed by ion exchange over a MonoQ column. Pure fractions were collected, glycerol was added to 20% and the fractions were flash frozen in liquid N2 for storage.

Both, yNap1 and mNap1 were labeled as follows: Nap1 in buffer D (buffer C containing 500 mM NaCl) was allowed to warm to RT, at a concentration of about 1 mg/mL. 10 molar equivalents of Alexa 546-C_5-maleimide were dissolved in DMSO and added in small portions to the Nap1 solution while stirring. Subsequently, the labeling mixture was agitated for 1 h at 4^{o}C. The progress of the reaction was followed by analytical RP-HPLC and ESI-MS. If necessary, more TCEP and Alexa 546-C_5-maleimide were added. Upon complete labeling, the protein was applied to a gel filtration column (S200 10/300), the dimer peak was collected and stored in buffer D at 4^{o}C for no longer than 14 days. For analytical data of mNap1 and yNap1, see **Figures S4** and **S9**.

Tetrasome reconstitution:

For a typical tetrasome reconstitution reaction, 2 μM of refolded tetramers and 2 μM of either 153-601 or 207-601 DNA were added to 100 μL of refolding buffer (10 mM Tris, pH 7.6, 1 mM EDTA, 2M NaCl). The mixture was transferred into a Slide-A-Lyzer MINI dialysis unit and dialyzed at 4°C against reconstitution buffer containing 1.4 M KCl, 1.2 M KCl, 1 M KCl, 0.8 M KCl, 0.5 M KCl and 300 mM KCl for 90 min each, followed by a final dialysis step against

reconstitution buffer containing 300 mM KCl. The quality of the assembly was assessed by separation on a Criterion 5% TBE gel run in 0.5x TBE buffer, followed by staining with ethidium bromide, as well as in a test nucleosome reconstitution with Nap1 and dimers (**Figure S8**).

Gel based assays:

Reassembly reactions were performed as follows: 4 pmol tetrasomes were combined with 8 pmol of the respective fluorescently labeled dimers (H2A-H2BA488, uH2A-H2BA488, H2AA488-H2B, H2AA488-uH2B) and varying amounts of competitor dimers bound to 40 pmol mNap1 in 20 µL of reassembly buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 2 mM DTT). The tubes were quickly mixed and spun down, then incubated for 4h at RT. For analysis, 10 µL of 25% sucrose were added to each sample and the mixture was separated on a Criterion 5% TBE gel run in 0.5x TBE buffer, followed by fluorescence scanning, staining with ethidium bromide and finally Coomassie Brilliant Blue. Competition experiments for the unmodified histone dimers, H2AA488-H2B and H2A-H2BA488 resulted in similar results – those were averaged and appear in **Figure 2** combined as "H2A-H2B".

Fluorescence measurements:

Fluorescence measurement buffer (300mM NaCl, 50mM Tris, 1mM DTT, 0.1mg/mL BSA, pH = 7.5 at RT, filtered, degassed) was prepared on the day of measurement by adding DTT and RP-HPLC purified BSA (centrifuged, filtered) from concentrated stock solutions to a previously degassed solution of the salts, followed by readjusting the pH and filtering. All cuvettes (polymethacrylate fluorescence cuvettes) were incubated with 3 mL of the measurement buffer

for 30-60 min prior to adding any histones to lower protein binding. The fluorescence measurements were carried out with a Fluorolog®-3 Model FL3-11 (Horiba Jobin Yvon). The measurement chamber was held at a constant temperature of 22.5°C by an Advanced Series AC 200 thermostat (Thermo Scientific) connected to an Arctic series refrigerated bath circulator A 25 water bath (Thermo Scientific).

Measurement of H2A-H2B dimer affinity for Nap1:

All fluorescence spectra were determined with an excitation wavelength of 545 nm and an excitation slit width of 4.0-4.5 nm as well as an emission slit width of 5.0 nm in the emission range from 560-660 nm, with an integration time of 0.1 s. Nap1 labeled with Alexa 546 C_5 maleimide was diluted to a concentration of 0.1 – 0.5 μM in measurement buffer. The exact concentration was determined by UV spectrophotometry at 554 nm and the stock was used to prepare the 0.25 nM measurement solution of Nap1. Before starting the titration, the sample was allowed to stand for 1.5-2 h. Main stocks of the H2A-H2B dimers with and without ubiquitin were diluted with refolding buffer (containing 2M NaCl) to a similar concentration in the range of 20-30 μM. The exact histone dimer concentrations of these stocks were determined by measuring absorption at 280 nm or 493 nm (for A488-labeled histones) using UV spectrometry, using the extinction coefficients for the histones calculated using the protparam online-tool (http://web.expasy.org/protparam) based on the amino acid sequence or using the extinction coefficient 72000 $M^{-1}cm^{-1}$ for the dye. From these stocks 10x and 100x dilution stocks were made by dilution with measurement buffer.

All titrations were performed using 4 cuvettes in a 4-way cuvette holder. Unmodified H2A-H2B, uH2A-H2B and H2A-uH2B were titrated into cuvette 1, 2 and 3 respectively.

Measurement buffer was titrated into cuvette 4 as reference. For each point in the titration, three measurements were taken with an excitation wavelength of 545nm and an excitation slit width of 4.0-4.5 nm as well as an emission wavelength/slit width of 571nm/5.0nm with an integration time of 2.0s. After each addition the solutions were mixed by pipetting and allowed to stand for 5 min. At the end of the titration, the reversibility of the binding was tested by addition of NaCl to a concentration >1M to each of the cuvettes and confirming that the sample cuvettes reached a level of fluorescence similar to the reference.

The ratio between the samples and the reference for each of the points were calculated and the ratio for each of the titration points normalized against the initial and the final single-point measurements using the formula:

$$\text{Norm. F. C.} = \frac{\mathrm{R_{obs}} - \mathrm{R_i}}{\mathrm{R_f} - \mathrm{R_i}}$$

Where Norm. F.C. is the normalized fluorescence change, R_{obs} is the observed ratio and R_i and R_f are the initial and final ratios, respectively.

The normalized fluorescence change as a function of the concentration of dimers was plotted and analyzed with the quadratic form of the binding equation:

$$y = \frac{K_D + c_R + c_L}{2c_R} - \frac{((K_d + c_R + c_L)^2 - 4c_R c_L)^{1/2}}{2c_R}$$

Where K_D is the dissociation constant, c_R is the concentration of Nap1 and c_L is the concentration of added dimer.

Measurement of histone dimer affinity in the formation of the mononucleosome:

All spectra were measured with excitation wavelength/slit width 485nm/2.5nm and emission slit width 5.0 in the emission wavelength range 500nm-660nm. Protein stocks were prepared as described above. All titrations were performed using 4 cuvettes containing 0.5 nM of H2A-$H2B_{A488}$, uH2A-$H2B_{A488}$, $H2A_{A488}$-H2B and $H2A_{A488}$-uH2B, respectively. An initial donor-only spectrum was recorded. Subsequently, $yNap1_{A546}$ was added to each of the cuvettes to a concentration of 8 nM, and the initial donor-acceptor spectrum was measured. The samples were allowed to stand for 30-60min before the titrations were initiated. During the titrations, tetrasomes were added to each of the four cuvettes, the solutions were carefully mixed by pipetting and allowed to equilibrate for 5 min. 3 measurements were taken for each measurement point using an excitation wavelength/slit width 485 nm/2.5 nm and using emission wavelengths of both 518 nm and 571 nm with a slit width of 5.0 nm and an integration time of 2.0 s. From the titration data and the initial donor-only spectrum, the FRET efficiency was calculated at all points of the titration using

$$E_{FRET} = 1 - (F_{DA}/F_D)$$

with F_D denoting the donor-only emission and F_{DA} denoting the emission of the donor in the presence of the acceptor. The FRET efficiencies were normalized against the initial and end-point measurements using the equation described above. The data were then plotted as a function of the concentration of tetrasomes and fitted to a numerical model as described below.

Data analysis

Based on the coupled equilibria for Nap1 dependent nucleosome assembly described in **Figure 3a** a numerical model was implemented using Matlab (The MathWorks, Natick, MA). Following[10,11], the equations of the equilibria were given as:

1. Nap1:tetramer association: $NT = (Nap * Tet)^{n1} / K_1^{n1}$

2. Tetramer:DNA association: $TD = (Tet * DNA)^{n3} / K_3^{n3}$

3. Nap1:dimer association: $ND = (Nap * Dim) / K_2$

4. Nucleosome formation: $NCP = (Dim * TD) / K_4$

where *Nap* indicates the concentration of free *Nap1*, *Tet* the concentration of free $(H3\text{-}H4)_2$ tetramers, *DNA* the concentration of free nucleosomal DNA and *Dim* the concentration of free H2A-H2B dimers. Concerning the complexes, *NT* denotes the concentration of Nap1:tetramer complex, *TD* the concentration of DNA:$(H3\text{-}H4)_2$ tetramer complex, *ND* the concentration of Nap1:H2A-H2B dimer complex and *NCP* the concentration of nucleosomes. For equations 1. and 2., Hill coefficients (n_1 and n_2) were used for modeling according to ref. [10]. For the equations 3. and 4., whose parameters were measured in this work, Hill coefficients were not used to fit the experimental data and thus not used in the analysis.

And for the concentrations:

$Nap_{total} = Nap + NT + ND$

$Tet_{total} = Tet + NT + TD + NCP$

$Dim_{total} = Dim + ND + NCP$

$DNA_{total} = DNA + TD + NCP$

Here, the suffix *total* denotes total concentrations of the respective species. For analysis, we used the following parameters from ref. [10]: K_1 = 1.0 x 10^{-8}, n_1 = 1.4; K_3 = 0.9 x 10^{-9}, n_3 = 1.1. The system of equations was solved by using the *fsolve* function of the Matlab Optimization Toolbox. For fitting, the *lsqnonlin* function was employed using the Levenberg Marquardt algorithm. Confidence intervals were calculated using the *nlparci* function.

SUPPLEMENTARY TABLE

Table S1: K_D values of nucleosome formation

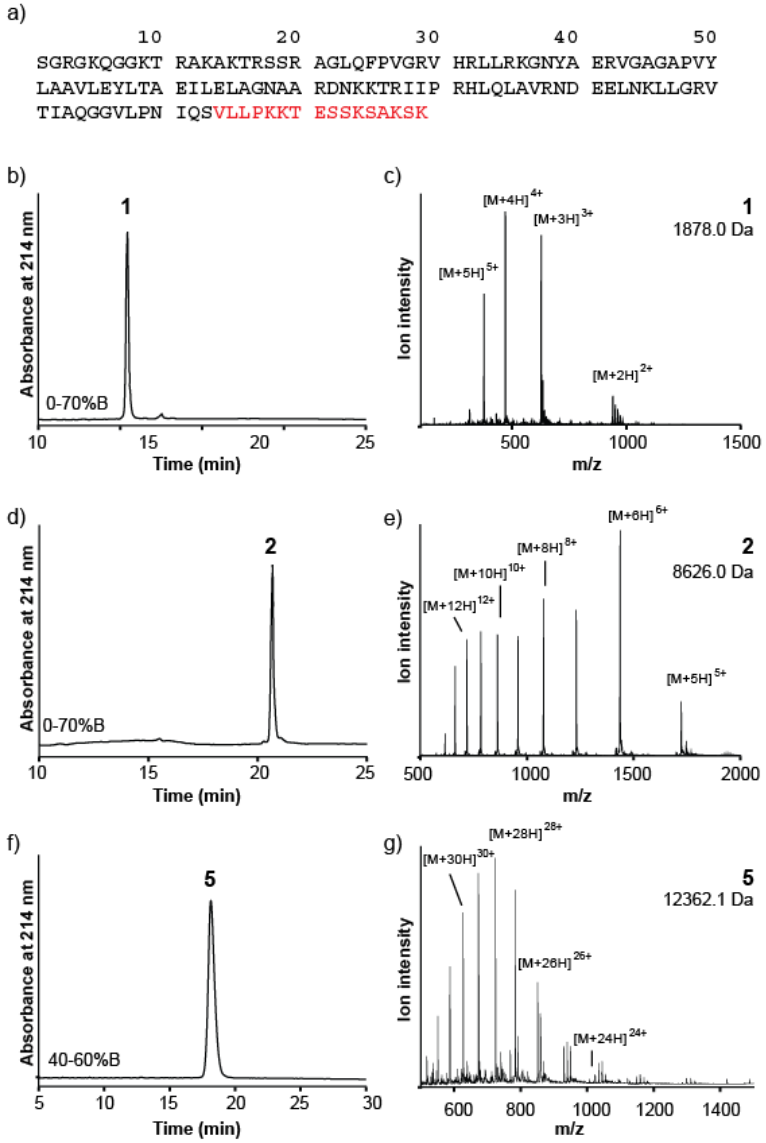
binding partners		*step*	K_D (nM)[3]
mNap1$_{A546}$ [1]	H2A-H2B	K_2	0.81 (± 0.11)
	uH2A-H2B	K_2	0.52 (± 0.07)
	H2A-uH2B	K_2	0.38 (± 0.05)
yNap1$_{A546}$ [2]	H2A-H2B	K_2	20.4 (± 4.53)
	uH2A-H2B	K_2	12.2 (± 3.81)
	H2A-uH2B	K_2	42.0 (± 4.80)
Tetrasome	H2A-H2B$_{A488}$	K_4	0.77 (± 0.15)
	uH2A-H2B$_{A488}$	K_4	1.47 (± 0.22)
	H2A$_{A488}$-H2B	K_4	0.66 (± 0.10)
	H2A$_{A488}$-uH2B	K_4	1.21 (± 0.19)

[1] Performed with mNap1(C88S, C132S, C255S, C258S), labeled at C388 with A546

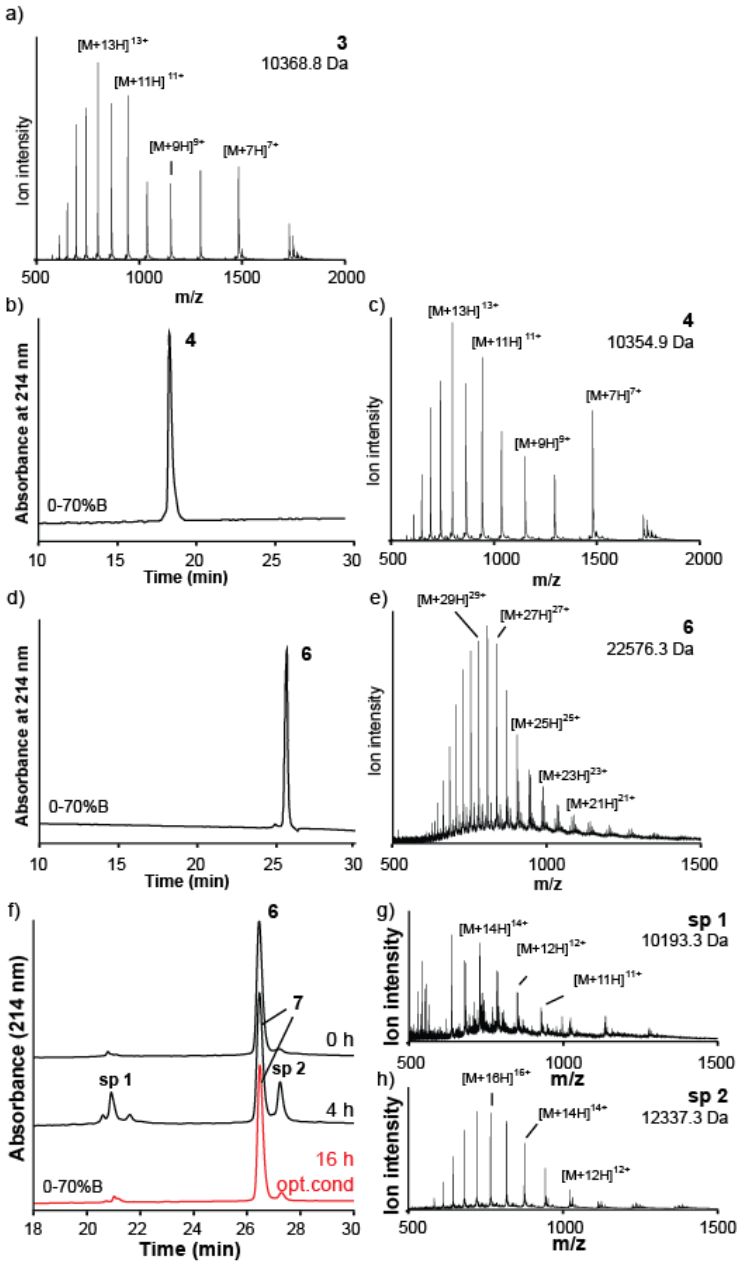
[2] Performed with yNap1(C200A, D201C, C249A, C272A, C414A), labeled at C201 with A546 and containing an N-terminal His$_6$-Tag for purification.

[3] Reported are confidence intervals obtained from the fit to the combined datasets of n = 2-3 independent experiments.

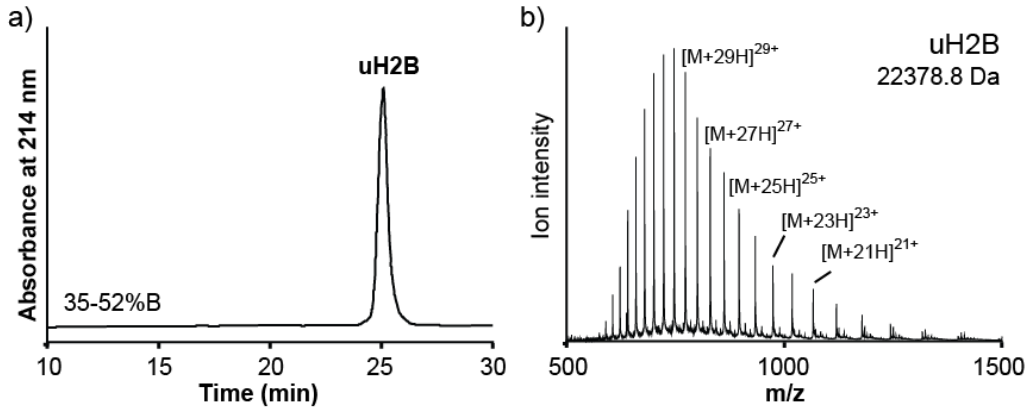
SUPPLEMENTARY FIGURES



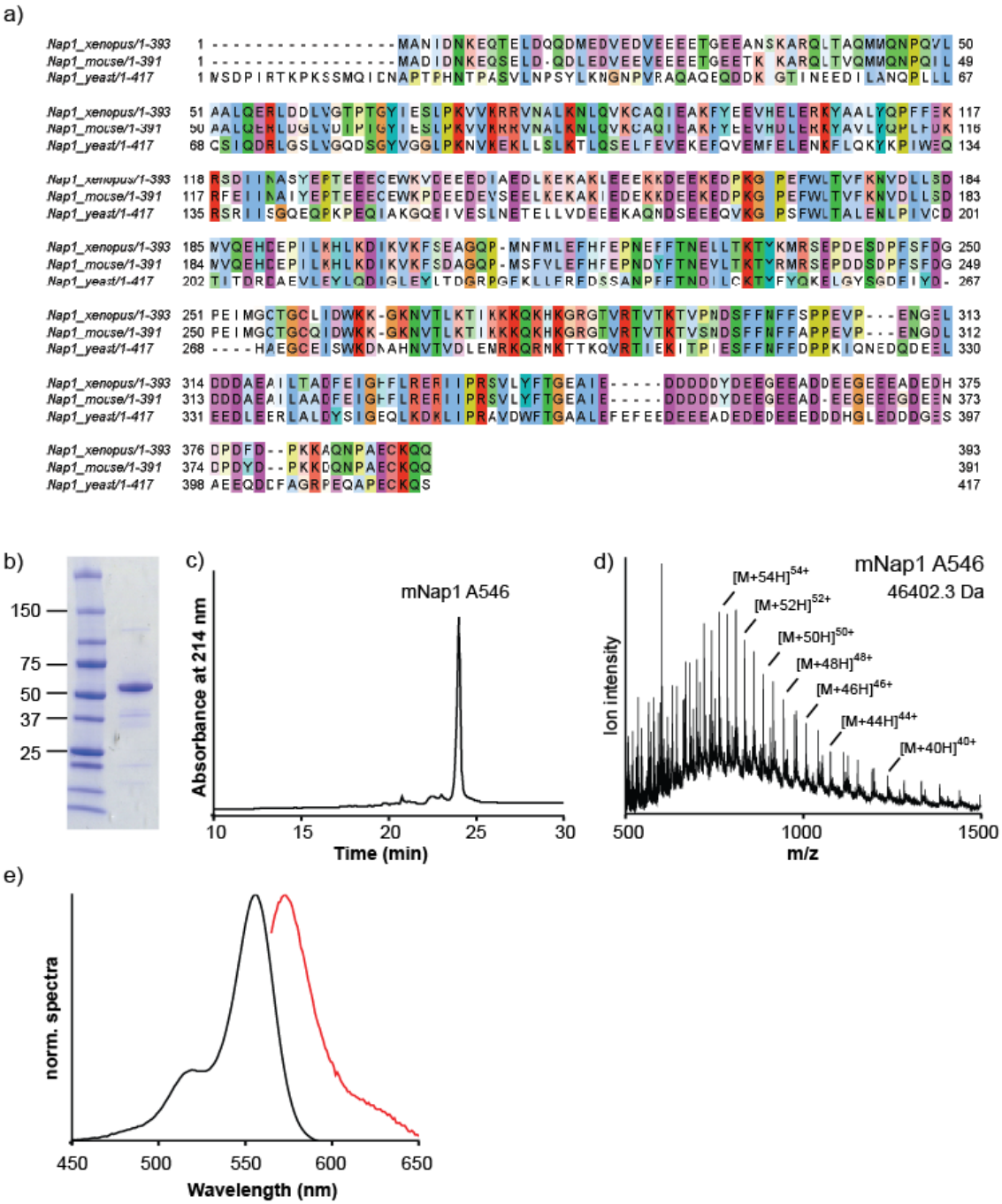
Supplementary Figure 1: Analysis of starting materials. **a)** Amino acid sequence of *xenopus laevis* H2A. The C-terminal peptide used for uH2A synthesis is indicated in red. **b)** Analytical RP-HPLC analysis of synthetic peptide **1**. **c)** ESI-MS analysis of peptide **1** [$(M+H)^+$ observed = 1878.0 Da, $(M+H)^+$ expected = 1878.0 Da]. **d)** Analytical RP-HPLC analysis of ubiquitin(1-75)-α-thioester **2**. **e)** ESI-MS analysis of **2** [$(M+H)^+$ observed = 8626.0 Da, $(M+H)^+$ expected = 8626.8 Da]. **f)** Analytical RP-HPLC analysis of H2A(1-113) -α-thioester **5**. **g)** ESI-MS analysis of **5** [$(M+H)^+$ observed = 12362.1 Da, $(M+H)^+$ expected = 12361.3 Da]



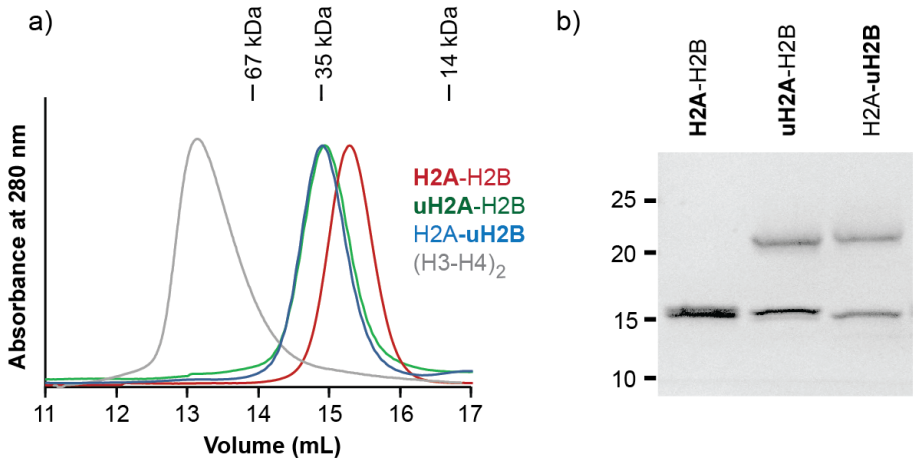
Supplementary Figure 2: Analysis of intermediates. **a)** ESI-MS analysis of branched protein **3** [$(M+H)^+$ observed = 10368.8 Da, $(M+H)^+$ expected = 10369.0 Da]. **b)** Analytical RP-HPLC analysis of deprotected branched protein **4**. **c)** ESI-MS analysis of **4** [$(M+H)^+$ observed = 10354.9 Da, $(M+H)^+$ expected = 10357.0 Da]. **d)** Analytical RP-HPLC analysis of ligation product **6**. **e)** ESI-MS analysis of ligation product **6** [$(M+H)^+$ observed = 22576.3 Da, $(M+H)^+$ expected = 22576.1 Da]. **f)** RP-HPLC analysis of the desulfurization of 6 to yield the final product 7, under standard and optimized conditions. **g-h)** ESI-MS analysis of side products of the desulfurization reaction.



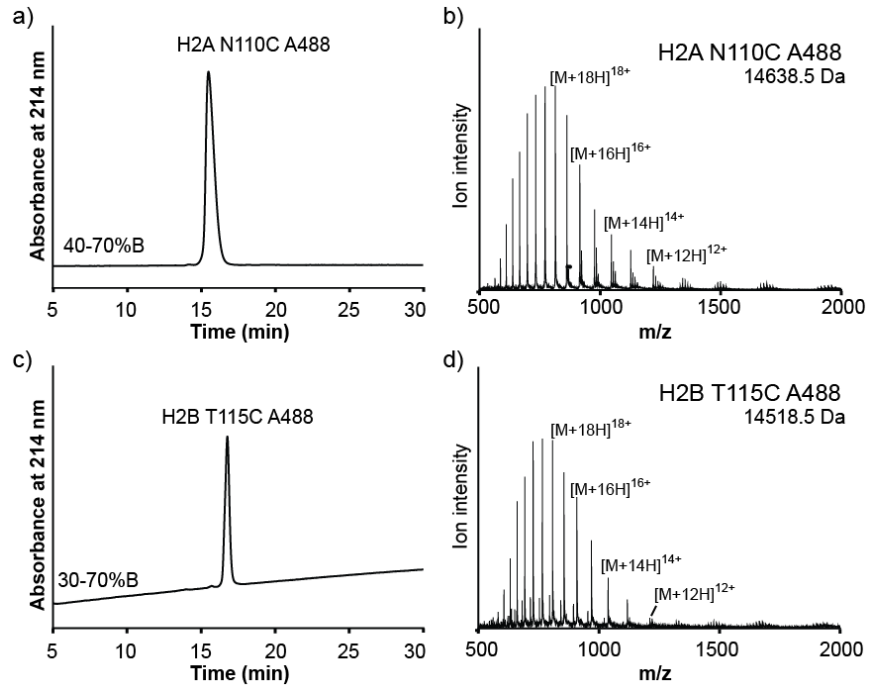
Supplementary Figure 3: uH2B synthesis: a) RP-HPLC analysis of uH2B, b) ESI-MS analysis of uH2B [$(M+H)^+$ observed = 22378.8 Da, $(M+H)^+$ expected = 22378.8 Da].



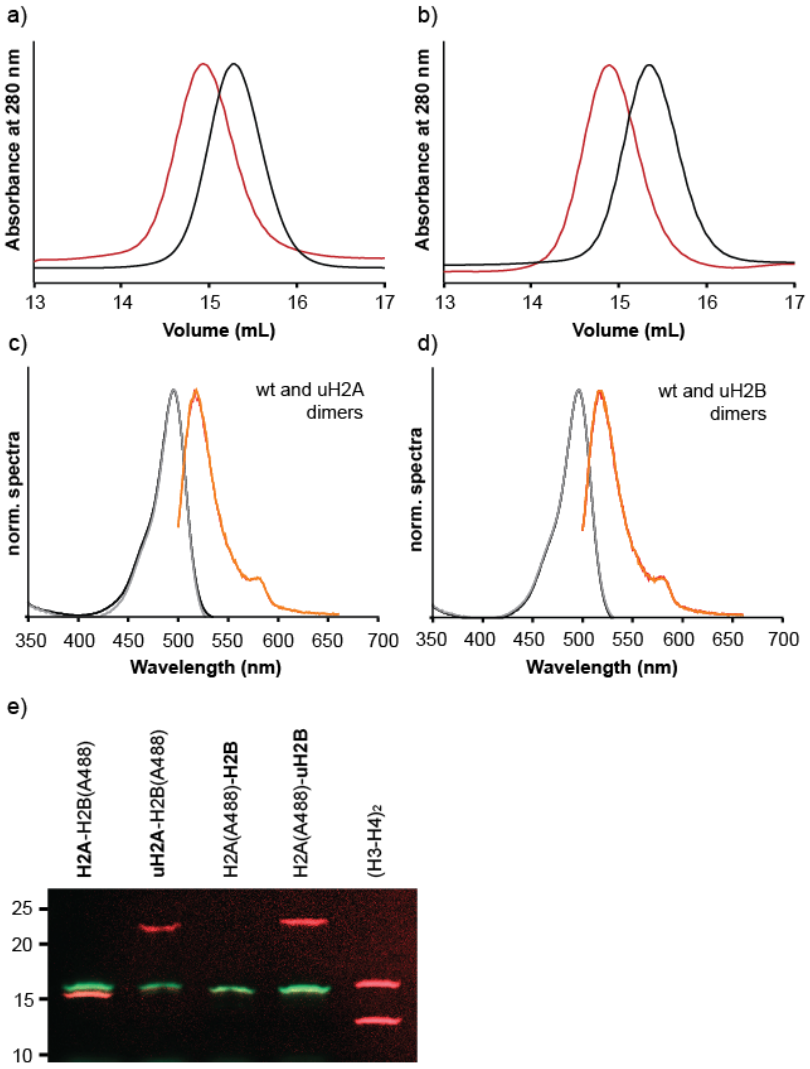
Supplementary Figure 4: Expression and labeling of mNap1: **a)** Sequence alignment of xenopus, mouse and yeast Nap1. **b)** SDS-PAGE analysis of purified mNap1 prior to labeling with A546. **c)** RP-HPLC analysis of $mNap1_{A546}$ after labeling reaction. **d)** ESI-MS analysis of mNap1(A546) [$(M+H)^+$ observed = 46402.0 Da, $(M+H)^+$ expected = 46403 Da ($+Na^+$)]. **e)** UV absorbance (black) and fluorescence emission (red) spectra of $mNap1_{A546}$.



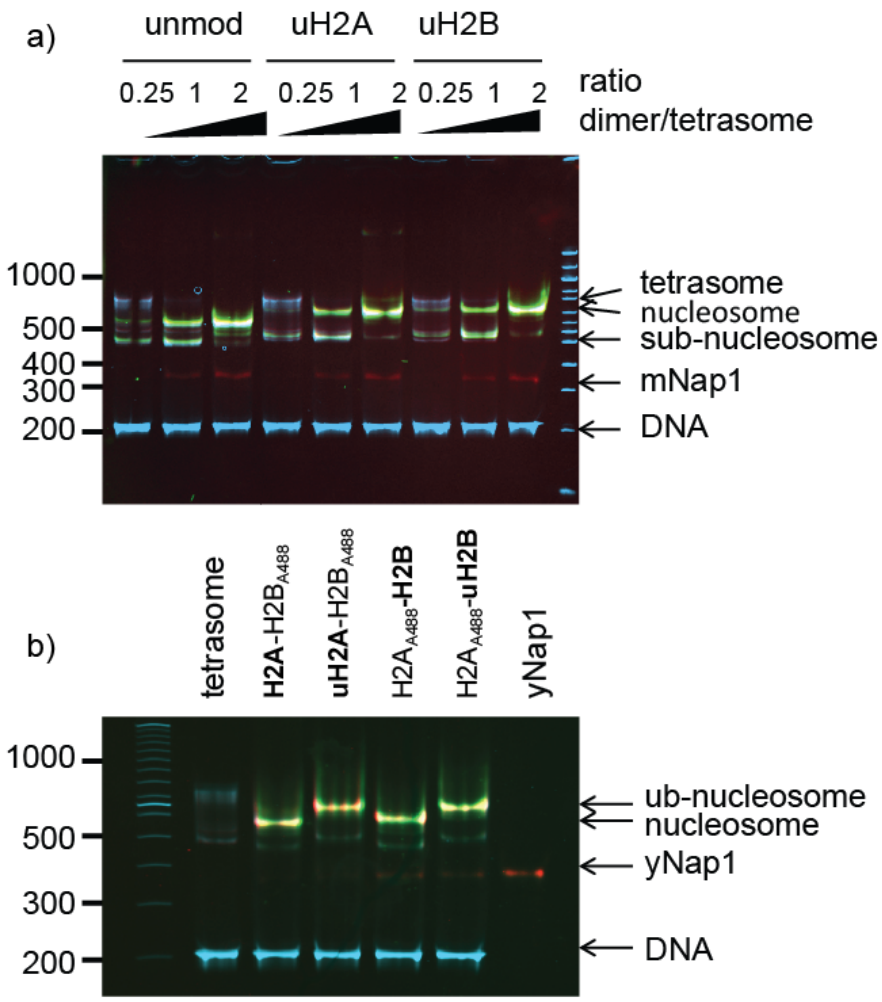
Supplementary Figure 5: Formation of unmodified, uH2A and uH2B containing dimers and $(H3\text{-}H4)_2$ tetramers. **a)** FPLC elution profiles of indicated refolded dimers from an S200 gel filtration column. **b)** Analysis of dimers by SDS PAGE, stained with Coomassie blue.



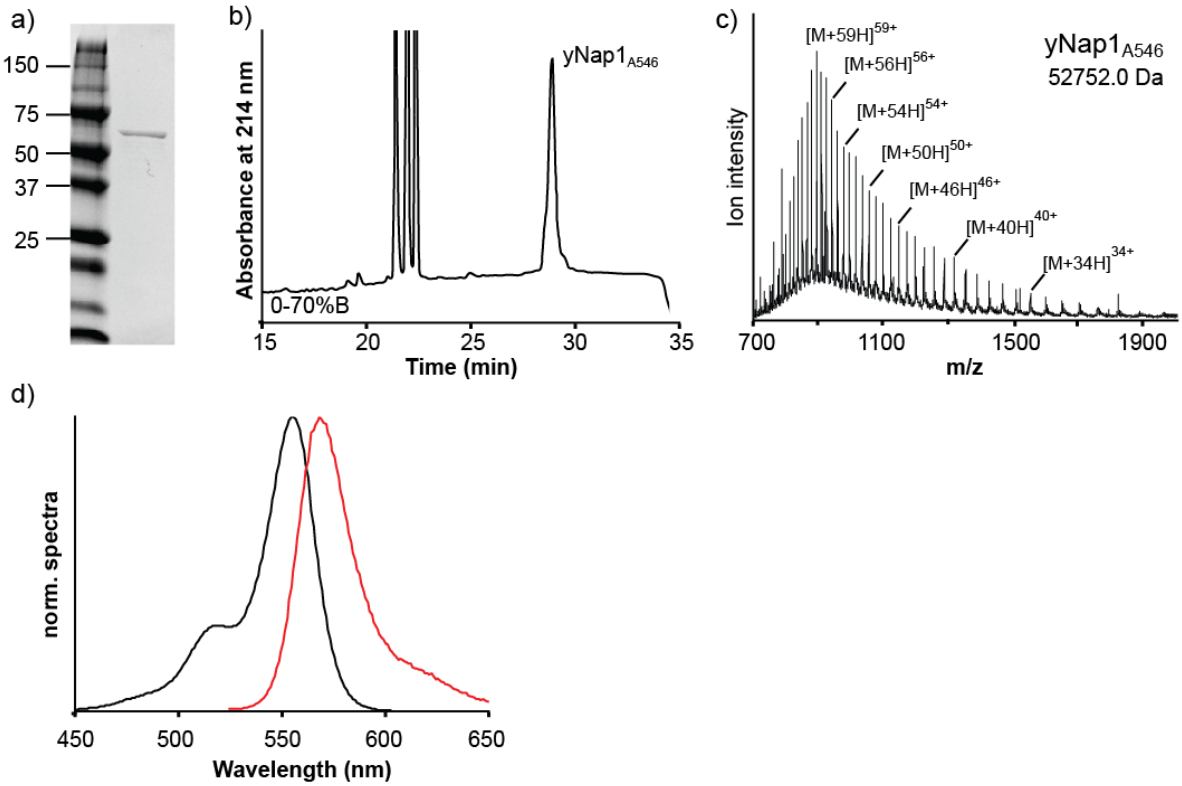
Supplementary Figure 6: Formation of labeled histones. **a)** RP-HPLC analysis of A488 labeled H2A N110C. **b)** ESI-MS analysis of H2A N110C A488 [$(M+H)^+$ observed = 14638.5 Da, $(M+H)^+$ expected = 14637.9 Da]. **c)** RP-HPLC analysis of A488 labeled H2B T115C. **d)** ESI-MS analysis of H2B T115C A488 [$(M+H)^+$ observed = 14518.5 Da, $(M+H)^+$ expected = 14517.7 Da].



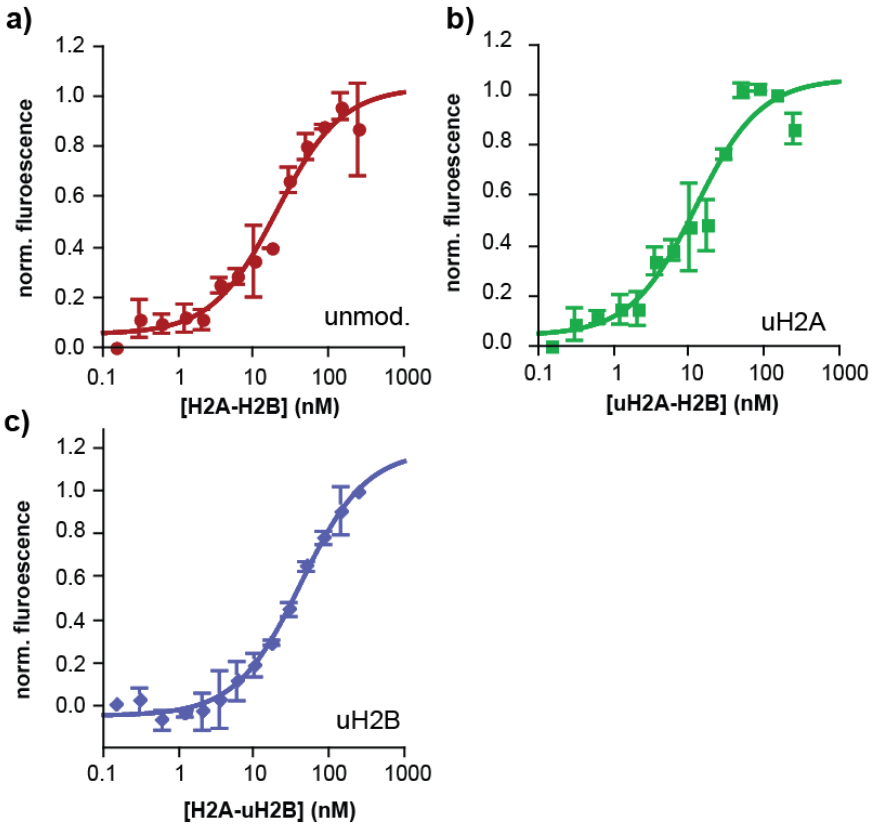
Supplementary Figure 7: H2A-H2B dimer formation. **a)** Gel filtration (S200) elution profiles for H2A-H2B(A488), black, and uH2A-H2B(A488), red. **b)** Gel filtration (S200) elution profiles for H2A(A488)-H2B, black, and H2A(A488)-uH2B, red. **c)** UV absorbance (black, grey) and fluorescence emission spectra (red, orange) for H2A-H2B(A488) and uH2A-H2B(A488), respectively. **d)** UV absorbance (black, grey) and fluorescence emission spectra (red, orange) for H2A(A488)-H2B and H2A(A488)-uH2B, respectively. **e)** SDS PAGE analysis of dimers and tetramer species used in the experiments. Histones are separated by SDS-PAGE and visualized by fluorescence scanning (green) and Coomassie stain (red). Note that in lane 3 H2A(A488) and H2B co-migrate, due to the effect of the A488 chromophore on H2A gel migration.



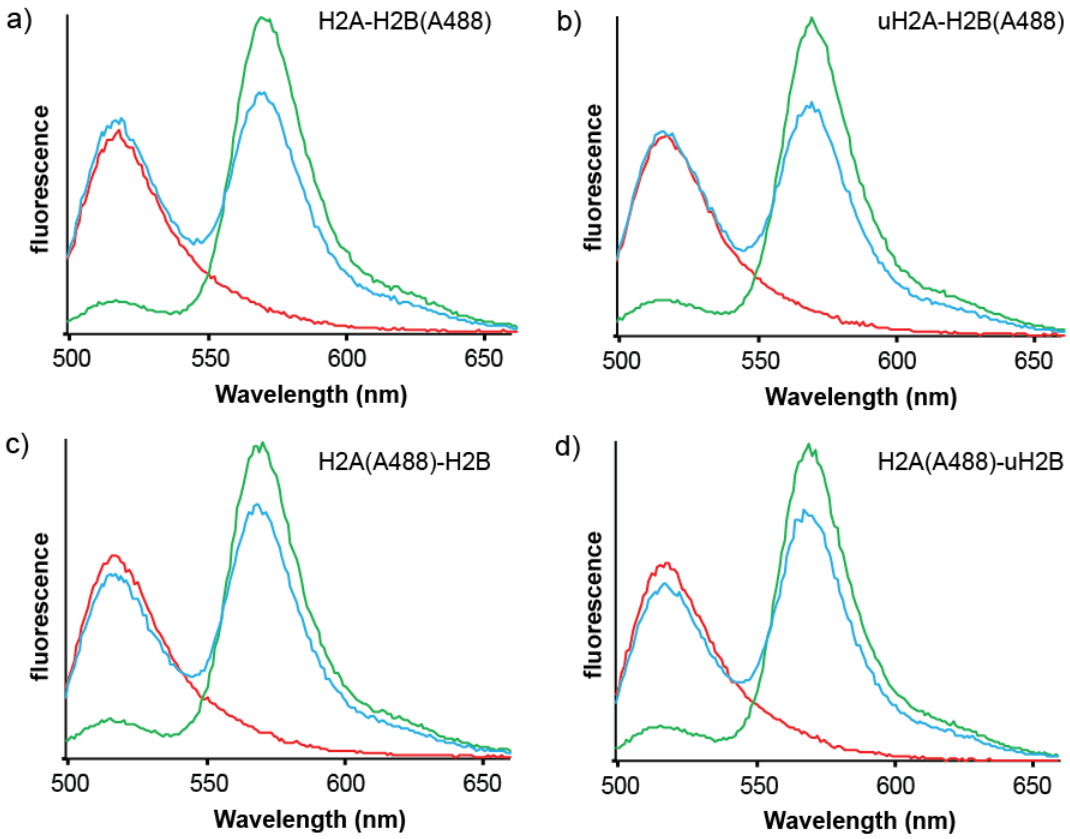
Supplementary Figure 8: Nucleosome formation with ubiquitylated and fluorescent dimers. (Green: fluorescence emission, red: Coomassie Brilliant Blue protein stain, Blue: Ethidium bromide DNA staining). **a)** Titration of preformed $(H3\text{-}H4)_2$:DNA tetrasomes (4 pmol) with fluorescent dimers as indicated ("unmod" corresponds to H2B T115C A488 containing dimers, "uH2A" to uH2A containing dimers and "uH2B" to uH2B containing dimers) in the presence of mNap1 (8 pmol). The species are identified according to refs. [12,13]. "Sub-nucleosome" denotes a complex lacking a full complement of histones. **b)** Nucleosomes formed by combining pre-formed tetrasomes (4 pmol) with 2 equivalents of histone dimer:yNap1 complexes containing the indicated histone dimers. "Ub-nucleosome" denotes nucleosomes containing 2 ubiquitylated histone species. All reactions were performed in 20 µL reassembly buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 2 mM DTT).



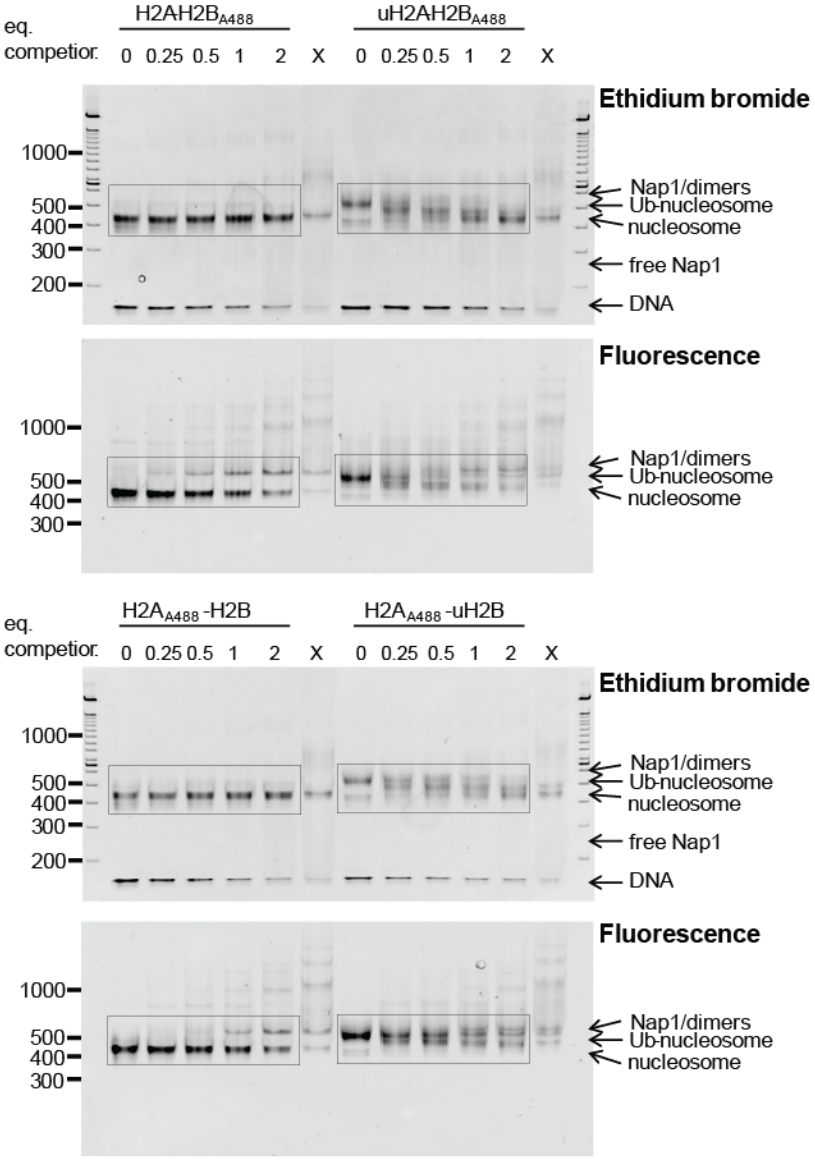
Supplementary Figure 9: Purification and labeling of yNap1. **a)** SDS-PAGE analysis of yNap1, Coomassie stained. **b**) HPLC analysis of yNap1 after A546 labeling (0-70% B). The triple peak at 22 min is unreacted chromophore, which is removed by gel filtration after labeling. **c)** ESI-MS analysis of $yNap1_{A546}$ peak at 29 min, showing full labeling: [$(M+H)^+$ observed = 52752.0 Da, $(M+H)^+$ expected = 52751.0 Da]. **d)** UV absorption (black) and fluorescence emission (red) spectra of $yNap1_{A546}$.



Supplementary Figure 10: Binding curves of **a)** unmodified, **b)** uH2A containing and **c)** uH2B containing unlabeled dimers to yNap1. The solid lines are fits of a reversible one-site binding equation with a floating start- and end-point to the experimental data. For fitting results, see **Table 1**. The error bars denote the standard error of the mean of two independent experiments.



Supplementary Figure 11: Fluorescence spectra of chaperone assisted mono-nucleosome formation. **a)** H2A-H2B$_{A488}$ alone (red), in complex with yNap1$_{A546}$ (green) and after addition of 100 nM of tetrasomes (blue). **b)** uH2A-H2B$_{A488}$ alone (red), in complex with yNap1$_{A546}$ (green) and after addition of 100 nM of tetrasomes (blue). **c)** H2A$_{A488}$-H2B alone (red), in complex with yNap1$_{A546}$ (green) and after addition of 100 nM of tetrasomes (blue). **d)** H2A$_{A488}$-uH2B alone (red), in complex with yNap1$_{A546}$ (green) and after addition of 100 nM of tetrasomes (blue). The spectra show high FRET upon complex formation, and quantitative recovery after titration of tetrasomes.



Supplementary Figure 12: Full gels from Figure 2. The boxes denotes the region shown in Figure 2. Note that lanes 6 and 12 contained a large excess of competitor dimers resulting in the formation of nonspecific aggregates, as observed in the disintegration of the nucleosomal band in the ethidium bromide stains. Thus, these lanes (marked by X) were excluded from the analysis.

Supplementary references

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