A Genetically Encoded Approach for Breaking Chromatin Symmetry

Bradley J. Lukasak\textsuperscript{1,2}, Robert E. Thompson\textsuperscript{1,2}, Michelle M. Mitchener\textsuperscript{1}, Vanessa J. Feng\textsuperscript{1}, John D. Bagert\textsuperscript{1}, and Tom W. Muir\textsuperscript{1}\textsuperscript{*}

Author Affiliations

\textsuperscript{1}Department of Chemistry, Princeton University, Frick Chemistry Laboratory, Princeton, NJ 08544

\textsuperscript{2}B.J.L. and R.E.T. contributed equally.

*Corresponding Author, Email: muir@princeton.edu

Table of Contents: 38 Pages

I. Methods: S2-S20
II. Safety Statement: S21
III. Reagent Table: S21
IV. References: S21-S22
V. Supplemental Figures 1-13: S23-S36
General Materials and Methods

Common reagents and chemicals were purchased from Millipore Sigma (St. Louis, MO) unless stated otherwise and were used without further purification. Fmoc amino acids for use in solid-phase peptide synthesis (SPPS) were purchased from either Matrix Innovations (Quebec City, Canada) or Novabiochem (Darmstadt, Germany). Coupling reagent (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from Oakwood (Estill, SC). Trityl ChemMatrix peptide resin was purchased from Biotage (Charlotte, NC). Trifluoracetic acid (TFA) was purchased from Halocarbon (North Augusta, SC). Oligonucleotide primers for cloning and generation of 601 DNA were purchased from Integrated DNA Technologies (Coralville, IA) or Millipore Sigma (St. Louis, MO). Phusion Hot Start Flex DNA Polymerase and Gibson Assembly Master Mix were purchased from New England Biolabs (Ipswich, MA). One Shot BL21(DE3) chemically competent E. coli cells and subcloning efficiency DH5α competent E. coli were generated in house from cells purchased from Invitrogen (Carlsbad, CA). DNA purification kits for plasmid purification and 601 DNA purification were purchased from QIAGEN (Valencia, CA). All plasmid sequences were verified by Sanger sequencing performed by GENEWIZ (South Plainfield, NJ). Acrylamide, TEMED, APS, Criterion Empty Cassettes, and Immuno-blot PVDF membrane (0.2 µM) were purchased from Bio-Rad (Hercules, CA).

Analytical-scale reverse-phase high performance liquid chromatography (RP-HPLC) was conducted on an Agilent 1100 Series or an Agilent 1260 Infinity system equipped with a C18 Vydac column (5 µM, 4.6 x 150 mm) at a flow rate of 1 mL/min. Semi-preparative RP-HPLC was conducted on an Agilent 1260 Infinity system equipped with a Waters XBridge BEH C18 column (5 µM, 10 x 250 mm) at a flow rate of 4 mL/min. Preparative-scale RP-HPLC was conducted on a Waters prep LC system consisting of a Waters 2545 Binary Gradient Module and a Waters
2489 Ultraviolet (UV)-Visible detector equipped with a C18 Vydac column (10 μM, 22 x 250 mm). HPLC solvents were H₂O with 0.1% TFA (Solvent A) and 90% acetonitrile in water with 0.1% TFA (Solvent B). Proteins and peptides were characterized by electrospray ionization mass spectrometry (ESI-MS) on a Bruker Daltonics MicroTOF-Q II mass spectrometer.

Coomassie-stained SDS-PAGE gels were imaged on an Odyssey system (LI-COR). Western blots were imaged using HRP-labelled secondary antibodies on an ImageQuant LAS-4000 (GE Healthcare Life Sciences). Native PAGE DNA gels were stained with ethidium bromide and imaged on an ImageQuant LAS-4000 (GE Healthcare). Densitometry measurements were performed using Image Studio Lite (LI-COR) or ImageJ (National Institutes of Health). Molecular graphics and analyses were performed with UCSF Chimera v.1.13.1, developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco. Statistical analyses were conducted in Prism v.9.2.0.

**Cloning of SpyCatcher/Tag Histone Fusions**

All cloning was performed utilizing Phusion Hot Start Flex DNA polymerase using the manufacturer’s instructions and primers from IDT or Sigma-Aldrich. Gibson assembly was used to insert SpyCatcher002 or SpyTag002 into previously described histone expression plasmids.¹ Further mutations or truncations were made by quick-change mutagenesis or by inverse PCR. All plasmid sequences were codon optimized for *E. coli* expression and verified by GENEWIZ. Note, that unless otherwise stated all H3 constructs contained C96A and C110A mutations.

**pET-His6-SpyCatcher-H3:** A sequence encoding SpyCatcher (His₆-tagged) fused to histone H3 by a short linker sequence with a TEV site (underlined) was generated by a two-fragment Gibson assembly from a G-block encoding His6-SpyC and a previously reported vector encoding H3.¹
GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQQGPSPGMTEEDSATHIKFSKRDEGDRELA
GATMELRDSSGKTISTWISDGHVDFYLYPGKYTEAQPDGYEVATAITFTVNEQGQVTV
NGEATKGDAHTGSSGSGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPATG
GVKKPHRYRPGTVALREIRRKYKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEA
AEAYLVLGFEDTNLAAIHAKRVTIMPKDIQLARRIRGERA

**pET-His6-SpyCatcher-H3D81A**: A sequence encoding SpyCatcher (His$_6$-tagged) fused to histone mutant H3D81A by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His6-SpyCatcher-H3, generated in this study.

GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQQGPSPGMTEEDSATHIKFSKRDEGDRELA
GATMELRDSSGKTISTWISDGHVDFYLYPGKYTEAQPDGYEVATAITFTVNEQGQVTV
NGEATKGDAHTGSSGSGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPATG
GVKKPHRYRPGTVALREIRRKYKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEA
AEAYLVLGFEDTNLAAIHAKRVTIMPKDIQLARRIRGERA

**pET-His6-SpyCatcher-H3R83A**: A sequence encoding SpyCatcher (His$_6$-tagged) fused to histone mutant H3R83A by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His6-SpyCatcher-H3, generated in this study.

GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQQGPSPGMTEEDSATHIKFSKRDEGDRELA
GATMELRDSSGKTISTWISDGHVDFYLYPGKYTEAQPDGYEVATAITFTVNEQGQVTV
NGEATKGDAHTGSSGSGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPATG
GVKKPHRYRPGTVALREIRRKYKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEA
AEAYLVLGFEDTNLAAIHAKRVTIMPKDIQLARRIRGERA

**pET-His6-SpyCatcher-H3E97K**: A sequence encoding SpyCatcher (His$_6$-tagged) fused to histone mutant H3E97K by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His6-SpyCatcher-H3, generated in this study.
GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGEATKGDHAHTGSSGSGGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEAAKAYLVGLFEDTNLAIAHKRVTIMPKDIQLARRIRGERAPET-His6-SpyCatcher-H3.3: A sequence encoding SpyCatcher (His₆ tagged) fused to histone H3.3 (containing native cysteines) by a short linker sequence with a TEV site (underlined) was generated by a two fragment Gibson assembly from a G-block encoding for His6-SpyC and a previously reported vector encoding for H3.3.²

GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGEATKGDHAHTGSSGSGGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEAAKAYLVGLFEDTNLAIAHKRVTIMPKDIQLARRIRGERA

pET-His6-SpyCatcher-H3tr(15-135): A sequence encoding SpyCatcher (His₆-tagged) fused to histone mutant H3tr(15-135) by a short linker sequence with a TEV site (underlined) was generated by Inverse PCR from the vector pET-His6-SpyCatcher-H3, generated in this study.

GSSHHHHHHSSGLVPRGSHMVTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGEATKGDHAHTGSSGSGGGSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEAAKAYLVGLFEDTNLAIAHKRVTIMPKDIQLARRIRGERA

pET-His5-Sumo-SpyTag-H3: A sequence encoding SUMO (His₅-tagged) and SpyTag fused to histone H3 by a short linker sequence with a TEV site (underlined) was generated by a two-
fragment Gibson assembly from a G-block encoding His5-SUMO-SpyC and a previously reported vector encoding H3.¹

GSSHHHHHGSGLVPRGSASMSDSEVNQEAKeAkvKpeVPeVPethInLkVsdGSeIfFkIkKtT
PLRRlMeAFaKrqGKeMdSLRFLYDGiRIQAdQTPedLMDendDiIEAhReQigGgVpTiVm
VDAYkRYKgSgEgGgGgSSGGgGgGSeNyFQaRTKQtArkStggKapRkQlAtKAArKsAp
ATGGVkkPkhryrPgtValReIrryQkSTeLLIrKLpFqRLvReIAqDFKtDLRFqSSAvmAL
QeaaEaylQgLFDtnlAAIAHAKRVtIMpKdIqLARRIRGERA

**pET-His5-Sumo-SpyTag-H3D81A:** A sequence encoding SUMO (His5-tagged) and SpyTag fused to histone mutant H3D81A by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His5-SUMO-SpyTag-H3, generated in this study.

GSSHHHHHGSGLVPRGSASMSDSEVNQEAKeAkvKpeVPeVPethInLkVsdGSeIfFkIkKtT
PLRRlMeAFaKrqGKeMdSLRFLYDGiRIQAdQTPedLMDendDiIEAhReQigGgVpTiVm
VDAYkRYKgSgEgGgGgSSGGgGgGSeNyFQaRTKQtArkStggKapRkQlAtKAArKsAp
ATGGVkkPkhryrPgtValReIrryQkSTeLLIrKLpFqRLvReIAqDFKtDLRFqSSAvmAL
QeaaEaylQgLFDtnlAAIAHAKRVtIMpKdIqLARRIRGERA

**pET-His5-Sumo-SpyTag-H3R83A:** A sequence encoding SUMO (His5-tagged) and SpyTag fused to histone mutant H3R83A by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His5-SUMO-SpyTag-H3, generated in this study.
ATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLAFQSSAVMAL
QEEAEAYLVGLFEDTNLAIIHAKRVTIMPKDIQLARRIRGERA

**pET-His5-Sumo-SpyTag-H3E97K:** A sequence encoding SUMO (His$_5$-tagged) and SpyTag fused to histone mutant H3E97K by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His5-SUMO-SpyTag-H3, generated in this study.

GSSHHHHHGSGLVPGRSASMDSDEVNQEAKPEVKPEVKPETHINLKVDGSSEIFFKIKKTT
PLRRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNIDIEAHREQIGGCVPTIVM
VDAYKRYKGSGEGGGSSGSGGSENLYFQARTKQTARKSTGGKAPRKLATKARKSAP
ATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMAL
QEEAKAYLVGLFEDTNLAIIHAKRVTIMPKDIQLARRIRGERA

**pET-His5-Sumo-SpyTag-H3tr(15-135):** A sequence encoding SUMO (His$_5$-tagged) and SpyTag fused to histone mutant H3tr(15-135) by a short linker sequence with a TEV site (underlined) was generated by Inverse PCR from the vector pET-His5-SUMO-SpyTag-H3, generated in this study.

GSSHHHHHGSGLVPGRSASMDSDEVNQEAKPEVKPEVKPETHINLKVDGSSEIFFKIKKTT
PLRRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNIDIEAHREQIGGCVPTIVM
VDAYKRYKGSGEGGGSSGSGGSENLYFQARTKQTARKSTGGKAPRKLATKARKSAP
ATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMAL
QEEAKAYLVGLFEDTNLAIIHAKRVTIMPKDIQLARRIRGERA

**pET-His5-Sumo-SpyTag-H3A96C/A110C:** A sequence encoding SUMO (His$_5$-tagged) and SpyTag fused to histone H3 (with its native cysteine residues at positions 96 and 110) by a short linker sequence with a TEV site (underlined) was generated by quick change mutagenesis from the vector pET-His5-SUMO-SpyTag-H3, generated in this study.
GSSHHHHHGSLVPRGSASMDSDEVNQEAKPEVKPEVKPETHINLKVSDBGSEIFFKIKKTT
PLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIIEAHREQIGG
VPTIVM
VDAYKRYKGSGESGGSSGSSGGSSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAP
ATGGVKKPHRYRPRTVALREIRRYQKSTELLIRKLPHQRLVREIAQDFKTDLRFQSSAVMAL
QEAACEAYLVLGFLEDTNLCAIHAKRVTIMPKDIQLARRIRGERA

pET-His5-Sumo-SpyTag-H3K18C: A sequence encoding SUMO (His5-tagged) and SpyTag fused to histone H3 K18C by a short linker sequence with a TEV site (underlined) was generated by a two fragment Gibson assembly from a G-block encoding for His5-SUMO-SpyC and a previously reported vector encoding for H3. Used for the generation of H3K18Ub.

GSSHHHHHGSLVPRGSASMDSDEVNQEAKPEVKPEVKPETHINLKVSDBGSEIFFKIKKTT
PLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIIEAHREQIGG
VPTIVM
VDAYKRYKGSGESGGSSGSSGGSSENLYFQARTKQTARKSTGGKAPRKQLATKAARKSAP
ATGGVKKPHRYRPRTVALREIRRYQKSTELLIRKLPHQRLVREIAQDFKTDLRFQSSAVMAL
QEAACEAYLVLGFLEDTNLCAIHAKRVTIMPKDIQLARRIRGERA

Preparation of DNA for Nucleosome Reconstitution

Standard 601 DNA: A plasmid containing multiple copies of the 147 bp Widom 601 DNA sequence flanked by EcoRV sites was transformed into DH5α E. coli cells. The plasmid was isolated and digested with EcoRV and then purified by precipitation with PEG6000.3

Generation of 601 DNA by PCR: The DNA used for the assembly of dinucleosomes was prepared by PCR amplification from a plasmid encoding the Widom 601 nucleosome positioning sequence as previously reported with minor modifications.4 Briefly, double stranded blunt-end DNA was amplified using the Pfu-Sso7d polymerase prepared in-house (polymerase-expressing E. coli cells and purification protocols were a kind gift from Jeffrey Barrick, UT Austin). The DNA was purified using anion-exchange chromatography or Qiagen miniprep spin columns.
207-bp 601 DNA: DNA with 45 and 15 bp DNA overhangs, 5’ and 3’ of the minimal 601 DNA sequence, respectively, was synthesized by PCR as described above and used to make mononucleosomes for the ACF remodeling assays. 601 sequence in bold; PstI site in bold and underlined.

5’_TACGGCGACCACCGAGATCTACACCAGCGTGTCAGTCACCAGCGTGACAGGATGTATA_TATCTGACACGTGCGTGGAGACAGGGATGATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAACGCAGGGGACAGCGCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCTGCAGCACCGGGATTCTCCAGCATCAGAGACCTAGG_3’

Restriction Digestion of 601 DNA: To generate double-stranded DNA with phosphorylated overhangs for nucleosome ligations, DNA was digested with the DraIII-HF restriction enzyme in CutSmart buffer overnight at 37 °C (5 units per microgram of DNA). The digested DNA was then purified via either anion exchange chromatography or Qiagen miniprep spin columns.

187-bp 601 DNA: For generation of asymmetric H3K4me3 nucleosomes and ligation to stimulatory nucleosomes. 601 sequence in bold; DraIII site in bold and underlined.

5’_CTACTGGGTACGGCGAGACAGGATGTATATATCTGACACGTGCCCTGGAGACTAGGGAGT_AATCCCCCTTGGCGGTTAAAACGCGGGGACAGCGCGTACGTGCGTGTATTAAGCGCGTGCTAGCTGCTGCTGCA_3’

184-bp 601 DNA: For generation of asymmetric WT and tr nucleosomes and ligation to H3K4me3 nucleosomes. 601 sequence in bold; DraIII site in bold and underlined.

5’_GCATACGCAGTGAGGCCGCACACAGGATGTATATATCTGACACGTGCCTGGAGACACAGGGAGTAGGGAGTAATCCCCTTGGCGGTTAAAACGCGGGGACAGCGCGTACGTGCGTGTATTAAG_3’
CGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCTGCAGCACCGGGATTCTCCAGTATT
CGAGGCCGTTC_3’

**Solid-Phase Peptide Synthesis**

The SpyT-H3K4me3(1-14) $\alpha$-thioester peptide was manually synthesized by following the general protocols outlined below.

**Loading Trityl-OH ChemMatrix Resin:** The resin was swelled in DCM (5 min), followed by treatment with 10% thionyl chloride in DCM for 3 h. The functionalized resin was washed with DCM, DMF, and 10% DIEA in DMF. The resin was loaded by agitation in the presence of Fmoc-carbazate (5 equiv. relative to functionalized resin) and DIEA (10 equiv.) in DMF. Then the resin was washed extensively with DMF.

**Fmoc Deprotection:** N-terminal Fmoc groups were removed by agitation of the resin in 20% piperidine in DMF containing 0.1 M HOBt (1x1 min, 1x20 min at room temperature). Deprotection solution was washed out extensively with DMF.

**Amino Acid Coupling:** Standard amino acids were coupled to deprotected resin by treating with 5 equiv. of Fmoc-protected amino acid, 5 equiv. PyAOP, and 10 equiv. of DIEA in DMF (2x30 min). Following all coupling reactions, the resin was washed with DMF.

**N-terminal Acetylation:** The N-terminus of the peptide was acetylated following Fmoc deprotection by treatment with 10% acetic anhydride and 10% DIEA in DMF for 20 minutes. Following acetylation, the resin was washed thoroughly with DMF and then DCM. The fully assembled resin-bound peptides were then dried under reduced pressure.

**Full Deprotection and Formation of Peptide $\alpha$-Thioester:** The peptide was cleaved from resin and deprotected using a cocktail of 95% TFA, 2.5% triisopropylsilane, and 2.5% water for 2 h at room temperature. Cleavage products were precipitated in cold diethyl ether. After precipitation,
the crude peptide was converted from a peptide-hydrazide to a C-terminal mercaptoethylsulfonate (MES) thioester by treatment with NaNO₂ (10 eq.) in activation buffer (6 M Gdn.HCl, 200 mM phosphate, pH 3.0) for 20 min at -20 °C. Following activation, MESNa (100 eq.) was added and the reaction was incubated for 15 min at room temperature at pH 7.0. The crude peptide α-thioester was purified by semi-preparative RP-HPLC using a gradient of 10-50% Solvent B over 40 min (see Figure S9B for characterization).

Ac-VPTIVMVDAYKRYKGSGGGSGGGSENLYFQARTK(me3)QTARKSTGGK-MES

SpyTag-H3K4me3 Semi-Synthesis

Full length SpyT-H3K4me3 was generated by semi-synthesis using methods described previously. In brief, SpyT-H3K4me3(1-14) α-thioester was incubated with H3A15C(15-135) in ligation buffer (6M Gdn.HCl, 100 mM phosphate, 20 mM TCEP, 100 mM TFET, pH 7.5). Upon completion of the ligation reaction (as judged by HPLC and ESI-MS analysis), the crude ligation product was subjected to radical desulfurization using VA-044. For the desulfurization step, the ligation reaction was first purged with argon for 5 min. Next, stock solutions of TCEP (1 M) and glutathione (0.5 M) were added to the crude ligation to yield final concentrations of 200 and 100 mM, respectively. Solid VA-044 was added to obtain a final concentration of 20 mM. The desulfurization reaction was allowed to proceed overnight at 37 °C and was then purified by semi-preparative RP-HPLC using a gradient of 45-65% Solvent B (see Figure S9C for characterization).

Semi-Synthesis of Disulfide Linked Ubiquitylated H3 (SpyT-H3K18CssUb)

Synthesis of SpyT-H3ssUb (referred to in the text as H3K18Ub) was performed according to previously described protocols. Briefly, HPLC-purified SpyT-H3K18C (1 equiv.) activated as a disulfide with 2,2′-dithiobis(5-nitropyridine) and HPLC-purified Ub-SH (2 equiv.) were co-dissolved in 1.5 mL reaction buffer (6 M Gdn.HCl, 1 M HEPES, pH 6.93). The reaction was
incubated for 1 h at room temperature, and the sample was quenched with 0.5 mL 25% HPLC Solvent B. Disulfide-linked H3-Ub was purified on a semi-preparative RP-HPLC column using a 40–65% gradient of HPLC Solvent B at 65 °C (see Figure S6E for characterization).

**Expression and Purification of KDM5B (1-814)**

KDM5B was expressed and purified similarly to that previously described for an analogous KDM5A construct with minor modifications. KDM5B(1-814) bearing an N-terminal His8 tag was produced in Sf9 cells using a baculovirus expression system, using bacmid generated using the MultiBac system according to the manufacturer’s instructions (Geneva Biotech). Sf9 cells were transfected at 27°C with purified bacmid according to the manufacturer’s instructions (Baculovirus Expression Vector System, Thermo Fisher Scientific). Supernatant (containing virus) was collected after 96 h, filtered and supplemented with 2% v/v FBS to give the P1 viral stock. Amplification of virus into P2 and P3 viral stocks was carried out through iterative fold dilution of viral stock into Sf9 suspension cell cultures containing penicillin/streptomycin at a density of 1.5 x10^6 cells per mL. To generate P2 virus, P1 viral stock was diluted 10-fold in cell culture and incubated at 27 °C until cells reached 50% viability according to trypan plus staining. The media was filtered and 2% FBS added to afford P2 viral stocks. To generate P3 virus for protein production, P2 virus was diluted 100-fold and cells were incubated at 27 °C until cells reached 50% viability according to trypan plus staining. The media was filtered and 2% FBS added to afford P3 viral stock. For recombinant protein production, P3 virus was diluted 1:100 into a Sf9 suspension culture containing penicillin/streptomycin at 1.5 x10^6 cells per mL at 27 °C in the dark. After 60h, the cells were harvested by centrifugation. The cells were then lysed via sonication in lysis buffer (25 mM HEPES pH 7.5, 350 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, complete protease inhibitor cocktail). The lysate was cleared through centrifugation at 17,000g for 30 min and incubated with TALON Cobalt resin (Takara) for 1h at 4 °C. The resin was washed with lysis buffer and eluted with elution buffer (25 mM HEPES pH 7.5, 100 mM
NaCl, 0.5 mM MgCl₂, 100 mM imidazole, 10% glycerol). The eluted protein was purified by size exclusion chromatography on a Superdex 200 Increase 10/300 column (GE healthcare), equilibrated and run with 40 mM HEPES pH 7.5, 50 mM KCl, 10% Glycerol. Pure fractions, as judged by SDS-PAGE, were combined, aliquoted for single use, flash frozen, and stored at -80 °C (see Figure S12 D & E for characterization).

**Expression and Purification of Recombinant Histones**

Recombinant human histones H2A, H2B, H3, and H4 were expressed in *E. coli* and purified following standard procedures with minor alterations. BL21(DE3) cells were transformed with plasmids for expression of histones and grown at 37 °C in Luria-Bertani (LB) media supplemented with antibiotics (Kan 50 µg/mL or Amp 100 µg/mL) until an OD₆₀₀ of 0.6 was reached. Expression of histones was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM). After 3 to 4 h of expression at 37 °C, cells were pelleted by centrifugation (4000 g, 15 min, 4 °C). Cell pellets were resuspended in 40 mL of lysis buffer (20 mM Tris.HCl, pH 7.6, 200 mM NaCl, 1 mM EDTA, 5 mM BME, 1 mM PMSF, 1% Triton X-100) per liter of expression. Cells were lysed by sonication (35% amplitude, 5 cycles of 30 seconds on/30 seconds off). Lysate was cleared by centrifugation (35,000g, 30 min, 4 °C) to pellet inclusion bodies. Inclusion bodies were resuspended in lysis buffer lacking Triton X-100 and pelleted by centrifugation (35,000g, 30 min, 4 °C). Histones were extracted from inclusion bodies by resuspension in 20 mL (per liter of expression) of extraction buffer (8 M urea, 3 M thiourea, 20 mM phosphate, 5 mM BME, pH 7.5) for 2 h at room temperature on a nutator. Extract was cleared by centrifugation (35,000g, 30 min, 16 °C) and the inclusion body extract supernatant batch-bound to Sepharose SP FF resin (15 mL of resin per liter of expression) that was equilibrated with urea wash buffer (8 M urea, 20 mM phosphate, 200 mM NaCl, 5 mM BME, pH 7.5) for 1 h at 4 °C. The resin was then washed with 10 column volumes of urea wash buffer and eluted using elution buffer (6M Gdn.HCl, 20 mM Tris.HCl, 5 mM BME, pH 7.5). Protein-containing fractions were identified via
absorption at 280 nm and pooled. Histones were then purified by preparative RP-HPLC using a gradient of 20-73% Solvent B over 60 min.

**Expression and Purification of SpyCatcher Histone Fusions**

Recombinant wild-type, mutant, and truncated His6-SpyCatcher-H3 proteins were expressed and purified in BL21(DE3) cells. Cells were transformed with expression plasmids (pET) and grown in LB medium supplemented with antibiotic (Kan 50 µg/mL) and 1% glucose. The cultures were agitated at 37 °C until an OD_{600} of 0.6-0.8 was reached, at which point protein expression was induced by addition of IPTG (0.5 mM). After 3 h, cells were pelleted by centrifugation (4000g, 20 min) and stored at -80 °C prior to further processing. Cell pellets were resuspended in 20 mL of cold lysis buffer (50 mM Tris.HCl pH 7.5, 500 mM NaCl, 1% Triton X-100, 20 mM thiodiglycol, 5 mM BME, 1 mM TCEP, and 1 mM PMSF) per liter of expression. Cells were lysed by sonication (3 min 20 sec total, 35% amplitude) and the lysate cleared by centrifugation (35,000g, 30 min). The inclusion bodies were washed with 20 mL of IB wash buffer (50 mM Tris.HCl pH 7.5, 20 mM thiodiglycol, 5 mM BME, and 1 mM TCEP) and were then isolated by centrifugation (35,000g, 30 min). Histones were extracted from inclusion bodies by resuspension in 20 mL IB extraction buffer (6 M Gdn.HCl, 20 mM Na_{2}HPO_{4} pH 7.5, 20 mM thiodiglycol, 1 mM BME, and 1 mM TCEP) and incubation at room temperature for 1 h on a nutator. The extract was cleared by centrifugation (35,000g, 30 minutes), the supernatant applied to Ni^{2+}-NTA agarose beads equilibrated with IB extraction buffer, and tagged proteins bound to the resin overnight at 4 °C. The beads were washed with 50 mL of IB extraction buffer and bound proteins eluted in IMAC elution buffer (6 M Gdn.HCl, 20 mM Na_{2}HPO_{4} pH 7.5, 300 mM imidazole, 20 mM thiodiglycol, 1 mM BME, and 1 mM TCEP). Eluted His6-SpyC-H3 was purified by semi-preparative HPLC (45-65% Solvent B over 30 min, 65 °C) and protein identity confirmed by mass spec (see **Figure S1A, S5B & C, S7A-C** for characterization).
Expression and Purification of SpyTag Histone Fusions

Recombinant wild-type, mutant, and truncated His5-SUMO-SpyTag-H3 proteins were expressed and purified in BL21(DE3) cells as described for SpyCatcher histones with the following minor changes. Following IMAC elution, the His5-SUMO-SpyT-H3 fusion was dialyzed against SUMO cleavage buffer (2 M urea, 20 mM Na₂HPO₄ pH 7.5, 20 mM thiodiglycol, 5 mM BME, and 1 mM TCEP) for 16 h at 4 °C with His6-Ulp1 protease. The dialyzed sample was supplemented with solid Gdn.HCl to approximately 4 M to solubilize precipitated proteins. The sample was flowed through 5 mL of Ni²⁺-NTA agarose to remove cleaved His5-SUMO, Ulp1, and uncleaved protein. Eluted SpyT-H3 was purified by semi-preparative HPLC (45-65% HPLC B over 30 minutes, 65 °C) and protein identify confirmed by mass spec (see Figure S1B, S5A, S7D-E for characterization).

¹⁵N-labeled Protein Expression

Uniformly isotopically labeled His6-SpyC-H3.1(C96A-C110A) was expressed in BL21(DE3) cells grown in minimal M9 media supplemented with ¹⁵N-labeled ammonium chloride (1 g/L). Cells were grown to OD₆₀₀ = 0.6-0.8 at 37 °C, and protein expression was induced using 0.5 mM IPTG for 3 h at 37 °C. The cells were harvested by centrifugation, and the protein purified according to standard protocols for purification of His6-SpyC-H3 conjugates (see Figure S5C for characterization).

Refolding of H2A/H2B Dimers

Histone dimers were formed using standard methods. Histones H2A and H2B were dissolved in unfolding buffer (6 M Gdn.HCl, 20 mM Tris.HCl pH 7.5, 0.5 mM EDTA, 10 mM DTT) and their concentrations determined by absorption at 280 nm. Histones were combined in an equimolar ratio in unfolding buffer at a final protein concentration of 1 mg/mL. The resulting mixture was dialyzed three separate times against refolding buffer (10 mM Tris.HCl pH 7.5, 2 M NaCl, 0.5
mM EDTA, 1 mM DTT) at 4 °C (for 4, 4, and 18 h, respectively). Dimers were purified from aggregates by size-exclusion chromatography on a Superdex S200 10/300 increase column. Fractions were analyzed by SDS-PAGE and pure fractions were pooled, concentrated, diluted to give a final concentration of 50% glycerol, and stored at -20 °C.

Refolding of Asymmetric H3/H4 Tetramers

Spytethered H3/H4 tetramers were refolded by combining His6-SpyC-H3 (1.0 equiv., 20 µM), SpyT-H3 (1.2 equiv., 24 µM), and H4 (2.4 equiv., 48 µM) in histone unfolding buffer (10 mM Tris. HCl pH 7.5, 6 M Gdn.HCl, 10 mM DTT) and adjusting the pH of the solution to 7.0. This mixture was dialyzed against Spytagging buffer (10 mM Tris.HCl pH 7.0, 1 M Gdn.HCl, 10 mM thiodiglycol, 1 mM DTT, 10% glycerol) at 4 °C for 16 h. The mixture was then dialyzed against histone refolding buffer (10 mM Tris.HCl pH 7.5, 2 M NaCl, 1 mM DTT) at 4 °C for 2 x 4 h. Refolded tetramers were added to Ni²⁺-NTA resin (1 mL slurry/mL of refolding mixture) equilibrated with refolding buffer. Tetramers were batch-bound at 4 °C for 1 h. The beads were then washed 3x with 10 column volumes of refolding buffer and tetramers eluted with refolding buffer supplemented with 250 mM imidazole. The eluate was concentrated in a centrifugal filter (10 kDa MW cutoff) to less than 500 µL, filtered, and tetramers purified by injection onto a Superdex 200 Increase 10/300 SEC column equilibrated in freshly degassed refolding buffer containing 0.1 mM EDTA. Fractions were analyzed by SDS-PAGE, pooled, and concentrated using a centrifugal filter. Glycerol was added to 50% (v/v) and purified tetramers were stored at -20 °C. Yield as determined by A280 (represented as an overall recovery following the workflow) ranged between 10-25%.

Reconstitution of Asymmetric Mononucleosomes

Nucleosomes were made as previously described with slight modifications. Briefly, pure tethered tetramers (50 pmol) were combined with H2A/H2B dimers (100 pmol) and 601 DNA (50 pmol)
in 50 μL of cold 2 M TEK buffer (10 mM Tris.HCl pH 7.6, 2 M KCl, 0.1 mM EDTA, 1 mM DTT). Assembly mixtures were placed in Slide-A-Lyzer MINI dialysis devices and dialyzed against 200 mL nucleosome assembly start buffer (10 mM Tris.HCl pH 7.6, 1.4 M KCl, 0.1 mM EDTA, 1 mM DTT) for 1 h at 4 °C. After 1 h, 320 mL of nucleosome end buffer (10 mM Tris.HCl pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT) was added at a rate of 1 mL/min using a peristaltic pump. Then the nucleosomes were incubated at 37 °C for 30 min to promote proper DNA positioning and subsequently dialyzed against 200 mL of nucleosome end buffer (2 x 2 h). For cleavage of the SpyCatcher/Tag, TEV protease (500 nM) was added to assembled nucleosomes and incubated overnight at 4 °C. To remove TEV and the cleaved SpyCatcher/Tag, nucleosome assemblies were placed in a 50 kDa cutoff spin filtration unit and diluted 10-fold. Small proteins were removed by centrifugation (14,000g, 5 min) and this step was repeated twice. Pure nucleosomes were collected, analyzed on a 5% TBE native gel, and stored at 4 °C.

**Generation of H3K4me3/H3: H2BK120Ub/H2B Dual Asymmetric Nucleosomes**

Nucleosomes containing asymmetry with H3 and H2B were generated following previously established protocols with minor alterations (see Figure S8A). Tethered H3K4me3/WT tetramers and H2A/H2BK120Ub dimers were combined in a ratio of 1:1.2 with 1 equivalent of 601 DNA. H2BK120Ub dimers were prepared as described previously. These ratios produce roughly equivalent amounts of hexasomes and nucleosomes following the nucleosome reconstitution and SpyCatcher/Tag removal protocol described above. The mixture of hexasomes and nucleosomes were then diluted to 0.5 μM in complementation buffer (20 mM Tris.HCl, 50 mM KCl, 5 mM TCEP, pH 7.6) and H2A/H2B-CfaN-TwinStreptag dimers were added in a ratio of approximately 1:1 and incubated at room temperature for 60 minutes. Complementation was complete after 60 minutes as determined on a native 5% TBE gel. Asymmetric nucleosomes were purified by addition of 0.02% (v/v) IGEPAL CA-630 (Sigma-
Aldrich) and subsequent incubation with Strep-Tactin Superflow Plus resin (QIAGEN). Approximately 200 µL of resin pre-washed with complementation buffer was used for each 300 pmol of 601 DNA. Following overnight incubation for 1 hour at room temperature, the resin was washed with 4 volumes of nucleosome wash buffer (20 mM Tris, 0.5 M NaCl, pH 7.6 and 0.02% (v/v) IGEPAL CA-630) and 2 volumes of nucleosome splicing buffer (10 mM Tris, 250 mM KCl, 0.1 mM EDTA, 1 mM TCEP, 10 mM 2-mercaptoethanol, pH 7.6 and 0.02% (v/v) IGEPAL CA-630). Nucleosomes were eluted by thiolysis on-resin using a catalytically dead CfaC intein (bolded residues indicate mutations that prevent splicing):

VKIISRKSLGTQNVYDIGVEKDHNFLLKNGLVASAAFN

Two bead volumes of dead CfaC (20 µM) in nucleosome splicing buffer was added to the resin and incubated overnight at 4°C with rotation. Eluted H3K4me3/H3:H2BK120Ub/H2B nucleosomes were isolated from the resin using empty Micro Bio-Spin columns (Bio-Rad) and the flow through was concentrated and exchanged into 10 mM TEK buffer using 30 kDa MWCO ultrafiltration centrifugal concentrators (Sartorius). Nucleosome quality was assessed on a Native 5% TBE gel and concentrations were determined by A260 (see Figure S8B for characterization).

**Dinucleosome Ligation**

Mononucleosomes containing complementary sticky end overhangs were made as described above using the relevant histones and pre-digested 601 DNA. Mononucleosome building blocks (400 nM) were mixed in ligation buffer (100 mM Tris.HCl pH 7.5, 6 mM MgCl₂, 1 mM ATP, 10 mM DTT, 10 U/mL T4 DNA ligase) and incubated at room temperature for 30 min. The ligated dinucleosomes were then dialyzed against nucleosome end buffer for 2 h at 4°C. Dinucleosomes were precipitated by addition of 100 mM MgCl₂ to a final concentration of 10 mM. The resulting mixture was incubated at 30°C for 15 min and was subsequently centrifuged at 21,000g for 15 min at 4°C to pellet dinucleosomes. The supernatant was discarded, and the
pellet resuspended in 30 µL of 10 mM nucleosome end buffer. To remove residual magnesium, the resuspended dinucleosomes were dialyzed against nucleosome end buffer for 1 h. Pure dinucleosomes were assessed on a 5% TBE native gel and their concentrations determined via absorption at 260 nm (see Figure 5C, S12C for characterization).

**ACF Remodeling Assay**

Human ACF complex was expressed and purified as described previously.² Remodeling assays were conducted using a REA-based method, as in previous investigations.²,⁹ Each remodeling reaction used 10 nM mononucleosome substrate and 0.8 nM ACF in a total reaction volume of 60 µL. Assays were conducted in REA buffer (12 mM HEPES, pH 7.6, 4 mM Tris, 60 mM KCl, 10 mM MgCl₂, 10% glycerol, 0.02% (v/v) IGEPAL CA-630) in the presence of 2 U µL⁻¹ PSTI-HF restriction enzyme. To allow for cutting of any free DNA, all reaction components except ATP were incubated for 20 min at 30 °C. ATP was then added to a final concentration of 2 mM to initiate remodeling. Remodeling reactions were carried out for a total of 64 min at 30 °C, with 6 µL aliquots removed at 0, 2, 4, 8, 16, 32, and 64 min and immediately quenched with 9 µL of quench buffer (20 mM Tris, 70 mM EDTA, pH 8.0, 10% (v/v) glycerol, 2% SDS, 20 U/mL Proteinase K). One additional sample was prepared without ATP and was incubated for 64 min at 30 °C to serve as a negative control. Following quenching, all samples were incubated for 1 h at 37 °C to allow for protein digestion. Samples (DNA remaining) were analyzed on a 5% TBE gel stained with SYBR Gold Nucleic Acid Gel Stain and remodeling rates calculated as described previously.⁹

**Thermal Stability Assay**

Stability of nucleosomes was measured using a SYPRO Orange thermal stability assay.¹⁰ Nucleosomes were assembled using minimal 601 DNA (147 bp). SYPRO orange was supplied as a 5000x solution and used at a final concentration of 5x for the assay. Assays were conducted
by mixing 9 µL of nucleosomes (0.5 to 1.0 µM) with 1 µL of 50x SYPRO orange in a 384-well plate. Samples were analyzed in a ViiA 7 Real-Time PCR system by increasing the temperature of the samples by 1 °C at 1-minute intervals from 25 to 95 °C and measuring the dye fluorescence. Fluorescence measurements were fitted to a standard sigmoid function to calculate $T_{\text{half}}$ values. $T_{\text{half}}$ value statistics were the average ± standard deviation of three independently fit experimental replicates. Fits were conducted using Python v.2.7.10 utilizing the optimize.curve_fit function of the SciPy library (v.0.15.1).

**KDM5B Demethylation Assay**

Dinucleosomes (150 nM ‘601’) were incubated with KDM5B (320 nM) in demethylation buffer (50 mM HEPES, 50 mM KCl, 1 mM α-ketoglutarate, 2 mM L-ascorbic acid, 100 µM Mohr’s Salt), for 32 min at 30 °C. Reactions were quenched by addition of 2x SDS loading buffer supplemented with 40 mM EDTA and then boiled for 5 min. Proteins were separated by SDS-PAGE (12% Bis-Tris, 165 V, 60 min) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, Ca) using a semi-dry blotter (20 V, 30 min). The membrane was blocked with 5% dry milk in TBST (25 mM Tris, 137 mM NaCl, 3 mM KCl, 0.1% Tween-20, pH 8.5). To assess demethylation, the membrane was incubated with α-H3K4me2 (1:1000 in 3% milk in TBST) overnight at 4 °C. Excess primary antibody was then removed and the membrane washed with TBST (3 x 5 min). For visualization of the primary antibody, the membrane was incubated with Rb-HRP (1:4000 in TBST) for 1 h at room temperature. The membrane was washed with TBST and imaged using the ImageQuant LAS-4000 on the chemiluminescence setting. To evaluate gel loading, the above procedure was repeated using an α-H4 (1:1000 in 3% milk in TBST) and Ms-HRP (1:4000 in TBST) secondary antibody.
**Safety Statement:** No unexpected or unusually high safety hazards were encountered.

**Reagent Table:**

| Antibodies         | Source                    | Identifier   |
|--------------------|---------------------------|--------------|
| Anti-H3K4me2       | Abcam                     | ab32356      |
| Anti-H4            | Abcam                     | ab31830      |
| Anti-H3K4me3       | Abcam                     | ab8580       |
| Anti-H2B           | Abcam                     | ab1790       |
| HRP-Conjugated rabbit secondary | Jackson ImmunoResearch | 11-035-144  |
| HRP-Conjugated mouse secondary | Jackson ImmunoResearch | 11-035-003  |
| IRDye® 680RD Goat anti-rabbit IgG | Licor              | 926-68071    |

**References:**

(1) Luger, K.; Rechsteiner, T. J.; Flaum, A.; Waye, M. M.; Richmond, T. J. Characterization of Nucleosome Core Particles Containing Histone Proteins Made in Bacteria. *J. Mol. Biol.* **1997**, *272*, 301–311.

(2) Dann, G. P.; Liszczak, G. P.; Bagert, J. D.; Müller, M. M.; Nguyen, U. T. T.; Wojcik, F.; Brown, Z. Z.; Bos, J.; Panchenko, T.; Pihl, R.; et al. ISWI Chromatin Remodellers Sense Nucleosome Modifications to Determine Substrate Preference. *Nature* **2017**, *548*, 607–611.

(3) Dyer, P. N.; Edayathumangalam, R. S.; White, C. L.; Bao, Y.; Chakravarthy, S.; Muthurajan, U. M.; Luger, K. Reconstitution of Nucleosome Core Particles from Recombinant Histones and DNA. *Methods Enzymol.* **2003**, *375*, 23–44.

(4) Diehl, K. L.; Ge, E. J.; Weinberg, D. N.; Jani, K. S.; Allis, C. D.; Muir, T. W. PRC2 Engages a Bivalent H3K27M-H3K27me3 Dinucleosome Inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 22152–22157.

(5) Nguyen, U. T. T.; Bittova, L.; Müller, M. M.; Fierz, B.; David, Y.; Houck-Loomis, B.; Feng, V.; Dann, G. P.; Muir, T. W. Accelerated Chromatin Biochemistry Using DNA-Barcoded Nucleosome Libraries. *Nat. Methods* **2014**, *11*, 834–840.

(6) Debelouchina, G. T.; Gerecht, K.; Muir, T. W. Ubiquitin Utilizes an Acidic Surface Patch to Alter Chromatin Structure. *Nat. Chem. Biol.* **2017**, *13*, 105–110.

(7) Torres, I. O.; Kuchenbecker, K. M.; Nnadi, C. I.; Fletterick, R. J.; Kelly, M. J. S.; Fujimori, D. G. Histone Demethylase KDM5A Is Regulated by Its Reader Domain through a Positive-Feedback Mechanism. *Nat. Commun.* **2015**, *6*, 6204.
(8) Becker, P. B.; Luger, K.; Rechsteiner, T. J.; Richmond, T. J. *Chromatin Protocols*. 1999, 1–16.

(9) Bagert, J. D.; Mitchener, M. M.; Patriotis, A. L.; Dul, B. E.; Wojcik, F.; Nacev, B. A.; Feng, L.; Allis, C. D.; Muir, T. W. Oncohistone Mutations Enhance Chromatin Remodeling and Alter Cell Fates. *Nat. Chem. Biol.* 2021, 17, 403–411.

(10) Taguchi, H.; Horikoshi, N.; Arimura, Y.; Kurumizaka, H. A Method for Evaluating Nucleosome Stability with a Protein-Binding Fluorescent Dye. *Methods* 2014, 70, 119–126.
Figure S1: (A) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of His6-SpyC-H3 fusion expressed and purified from BL21 cells. (B) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of SpyT-H3 fusion expressed and purified from BL21 cells. (C) Assessment of the spy tethering reaction by SDS-PAGE using a SpyT-H3:SpyC-H3 ratio of 1.2:1.0. The reaction was monitored over time in either 2 M (lanes 1 through 4) or 1 M (lanes 5 through 8) guanidine. Proteins were visualized by Coomassie staining.
Figure S2: (A) Workflow for generation of spytethered heterotypic octamers. H3 heterodimers are combined with histones H2A, H2B, and H4 under denaturing conditions, followed by gradual dialysis into 2 M NaCl. (B) Tethered octamers were purified by size exclusion chromatography (SEC). The octamer peak is boxed in the chromatogram. (C) SDS-PAGE analysis of spytethered octamer fractions from SEC (proteins visualized by Coomassie staining).
Figure S3: (A) Workflow for generation of spytethered H3/H4 tetramers. His6-SpyC-H3 is combined with excess SpyT-H3 and 2.4 equiv. H4 under denaturing conditions, followed by dialysis into 1 M Gdn.HCl to allow for spytagging overnight. The spytagged mixture is then dialyzed into 2 M NaCl to form tetramers. Crude tetramers are purified by Ni-NTA affinity chromatography followed by SEC. (B) SEC chromatogram of tethered tetramers. WT Tetramer (pink) and WT octamer (green) SEC chromatograms are for comparison. The tethered tetramer peak is boxed, from which fractions were taken and assessed by SDS-PAGE. (C) SDS-PAGE analysis of crude spytethered tetramers, Ni-NTA eluted tetramers, and tetramer fractions from SEC (proteins visualized by Coomassie staining).
Figure S4: (A) Workflow for generation of asymmetric mononucleosomes. Tethered tetramers are combined with 2 equiv. of H2A/H2B dimers and 1 equiv. 601 DNA. The mixture is gradually dialyzed into 10 mM KCl, after which SpyTags are removed through proteolytic TEV cleavage. Cleaved SpyTag and TEV are then removed using 50 kDa spin concentrators. (B) Native PAGE analysis of spytethered nucleosomes (DNA visualized by ethidium bromide staining). Lane 1: spytethered mononucleosomes. Lane 2-6: titration of TEV protease for removal of SpyTags. (C) Native PAGE analysis of SpyTag and TEV removal (DNA visualized by ethidium bromide staining). Lane 1: Uncleaved nucleosomes; Lane 2: cleaved nucleosomes; Lane 3: nucleosome concentrate; Lane 4: filtrate. (D) Denaturing SDS-PAGE analysis of SpyTag and TEV removal. Lane 1: Uncleaveled nucleosomes; Lane 2: cleaved nucleosomes; Lane 3: nucleosome concentrate. (E-F) Raw sigmoidal fits for dimer melt curves WT and SpyCatcher generated WT nucleosomes (WT-WT). T
half values are shown ± s.d. (n=3). (G) Calculated rate constants for ACF remodeling assays for WT and SpyCatcher generated WT nucleosomes (WT/WT).
determined using densitometry values for cut and uncut DNA. (ns=not significant, n=12 (WT), n=6 (WT/WT)).
Figure S5: (A-C) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of SpyT-H3A96C,A110C (H3CC), His6-SpyC-H3.3, and His6-SpyC-H3(\textsuperscript{15}N) fusions for proof-of-concept substrates. (D) Denaturing SDS-PAGE analysis of purified asymmetric tetramers (proteins visualized by Coomassie staining). Lane 1: WT/H3C96C110; Lane 2: WT/H3.3; Lane 3: WT/H3(\textsuperscript{15}N). (E) Native PAGE analysis of asymmetric nucleosomes pre- and post-TEV cleavage (DNA visualized by ethidium bromide staining). Lanes 1 and 2: WT/WT; Lanes 3 and 4: WT/H3A96CA110C; Lanes 5 and 6: WT/H3.3; Lanes 7 and 8: WT/H3(\textsuperscript{15}N).
Figure S6: (A) Generation of SpyT-H3 ubiquitylated at position 18 using a disulfide linkage (SpyT-H3K18CssUb). Activated SpyT-H3K18C disulfide is combined with ubiquitin acylated at its C-terminus with amino-ethanethiol (UbSH) at neutral pH to form the product. (B-E) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of SpyT-H3K18C, activated SpyT-H3K18C, UbSH, and SpyT-H3ssUb for generation of asymmetric ubiquitinated nucleosomes. (F) Denaturing SDS-PAGE analysis of purified asymmetric tetramers containing WT/H3K18Ub. Note, for convenience H3K18CssUb is referred in the captions as simply H3K18Ub. (G) Native PAGE analysis of asymmetric nucleosomes pre- and post-TEV cleavage (DNA visualized by ethidium bromide staining). Lanes 1 and 2: WT/WT; Lanes 3 and 4: WT/H3K18Ub; Lane 5: WT/H3K18Ub treated with DTT to remove disulfide linked ubiquitin.
Figure S7: (A-F) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of His6-SpyC and SpyT oncohistone fusions expressed and purified from BL21 cells. (G) Denaturing SDS-PAGE analysis of purified asymmetric oncohistone tetramers (proteins visualized by Coomassie staining). (H) Ribbon diagram structure of the ATPase domain of ACF (green) and its interaction with a nucleosome (gray). Highlighted in blue is histone H3, specifically residues D81 and R83 (PDB: 6ne3). (I) Native PAGE analysis of asymmetric oncohistone nucleosomes with 45-15 bp DNA overhangs, for use in ACF remodeling assays, pre- and post-TEV cleavage (DNA visualized by ethidium bromide staining). WT-NA refers to a wild-type nucleosome generated using the normal assembly protocol (i.e. not involving spytethering). (J) Native PAGE analysis of asymmetric oncohistone nucleosomes with standard
147 bp 601 DNA, for use in thermal stability assays, pre- and post-TEV cleavage (DNA visualized by ethidium bromide staining).
Figure S8: (A) Generation of semi-synthetic SpyT-H3K4me3. Ac-SpyT-H3K4me3(1-14) MES thioester is ligated to H3A15C(15-135) followed by radical desulfurization to yield semi-synthetic histone. (B) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of Ac-SpyT-H3K4me3(1-14) MES thioester, generated by Fmoc-based SPPS. (C) Reverse-phase analytical HPLC and ESI-TOF mass spectrometry analysis of semi-synthetic SpyT-H3K4me3.
Figure S9: All ACF remodeling timecourse samples, analyzed by 5% TBE gel (DNA visualized with SYBR Gold staining). WT (tet) refers to nucleosomes prepared using traditional methods.
Figure S10: (A-F) Raw sigmoidal fits for dimer melt curves of oncohistone nucleosomes. $T_{\text{half}}$ values are shown ± s.d. (n=3).
Figure S11: (A) Domain architecture of KDM5B, displaying catalytic jumonji domains, DNA-binding ARID domain, three PHD domains, and Zn finger. Boxed region indicates construct used in studies. (B) Denaturing SDS-PAGE analysis of purified asymmetric tetramers used to generate dinucleosome substrates (proteins visualized by Coomassie staining). Lane 1: WT/WT; Lane 2: WT/tr; Lane 3: tr/tr; Lane 4: H3K4me3/WT; Lane 5: H3K4me3/tr. (C) Native PAGE analysis of mononucleosomes utilized to generate dinucleosomes (DNA visualized by ethidium bromide staining). Lanes 1 and 2: K4me3/WT; Lanes 3 and 4: K4me3/tr; Lanes 5 and 6: WT/WT; Lanes 7 and 8: WT/tr; Lanes 9 and 10: tr/tr. (D) SEC chromatogram of KDM5B (1-814). The KDM5B peak is outlined, from which fractions were taken and assessed by SDS-PAGE. (E) SDS-PAGE analysis of KDM5B SEC fractions (proteins visualized by Coomassie staining). Fractions F3 through F12 were pooled.
Figure S12: (A) KDM5B demethylation assay, 150 nM '601' site dinucleosome substrates are treated with 320 nM KDM5B and generation of H3K4me2 is assessed by western blot. (B) Immunoblot for H3K4me2 reveals KDM5B demethylase activity on dinucleosome substrates (top) and immunoblot of H4 serves as a loading control (bottom). Replicates 2 and 3 are shown above (replicate 1 is found in Figure 5C).