Supplemental Information

Site- and Regiospecific Installation of Methylarginine Analogs into Recombinant Histones and Insights into Effector Protein Binding

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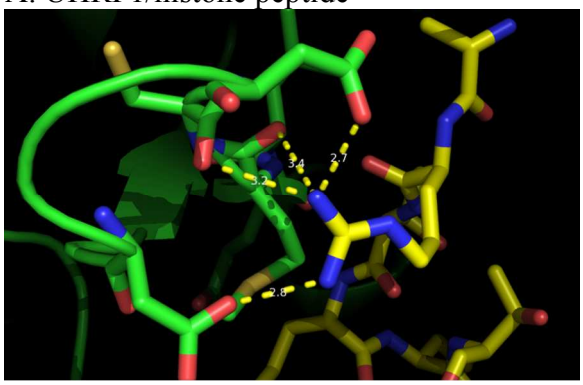
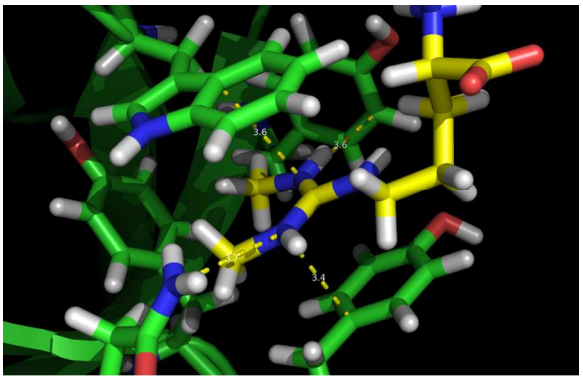
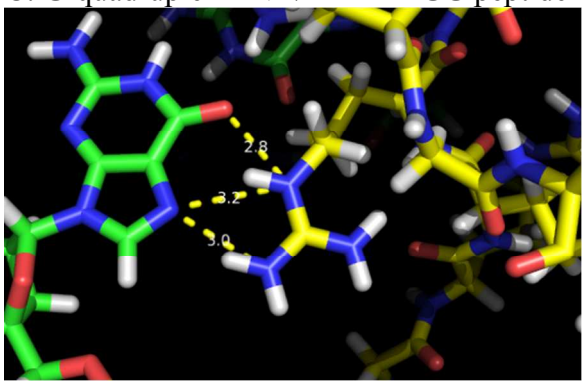
A. UHRF1/histone peptide



B. SMN/ADMA

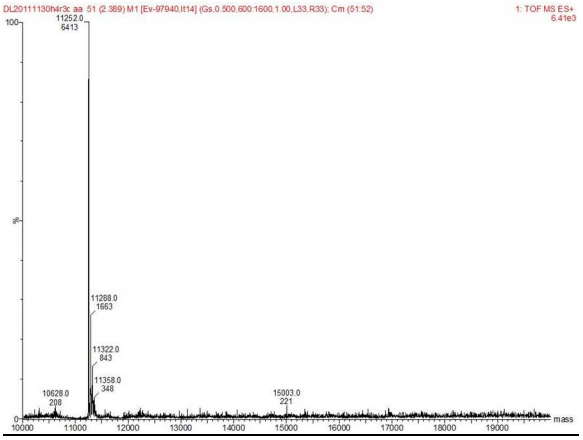


C. G-quadruplex RNA/FMRP RGG peptide

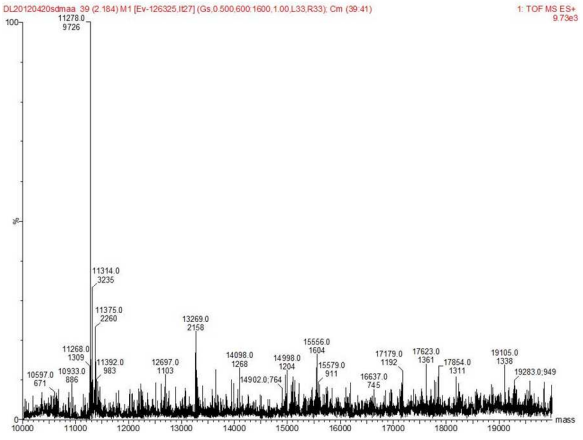


Supplemental Figure S1: Structures of representative methylarginine-dependent interactions. (A) Co-crystal structure of UHRF1 PHD finger with histone H3K4Me3 1-9 peptide (3SOW) indicates extensive binding networks localized to terminal guanidino nitrogen atoms.[1] Arginine methylation abrogates interaction. (B) NMR solution structure of SMN Tudor domain with asymmetrical dimethylarginine (4A4E) indicates hydrogen bond, pi-cation, and pi-pi stacking interactions between the protein aromatic cage binding pocket and the terminal guanidino nitrogen atoms.[2] (C) NMR solution structure of G-quadruplex RNA with FMRP RGG peptide (2LA5)—known to harbor arginine methylation that modifies FMRP association with mRNA[3]—indicates hydrogen bond interactions between a guanine base and the guanidino group of arginine, including the internal nitrogen atom.[4] In such cases, arginine analogs are expected to have a limited ability to functionally mimic native arginine. Green = interacting partner, Yellow = argininyl ligand. Dashed yellow line = distances in angstroms.

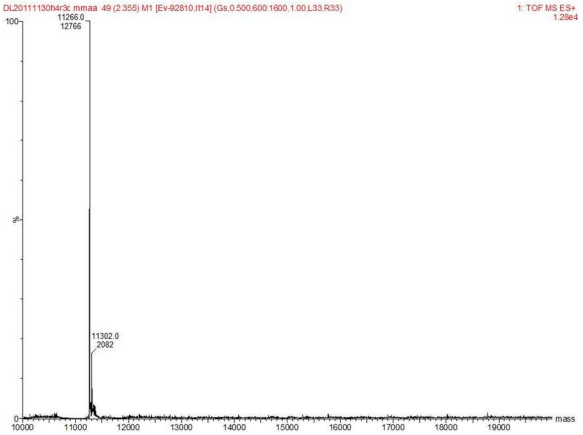
H4R3C-AA



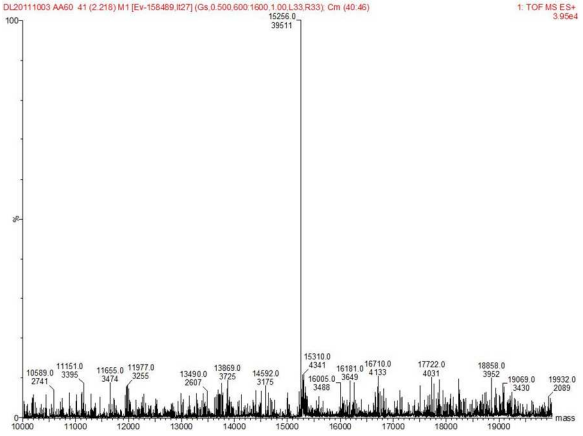
H4R3C-SDMAA



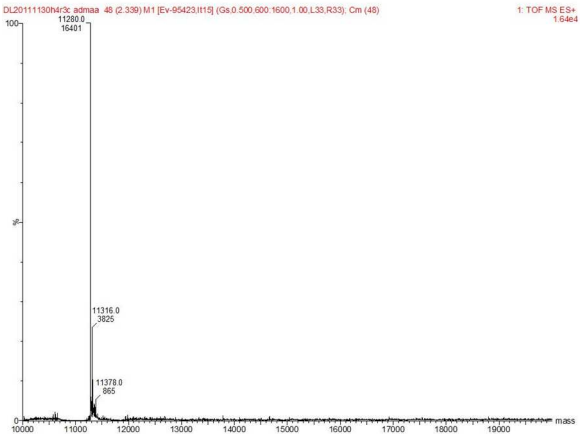
H4R3C-MMAA



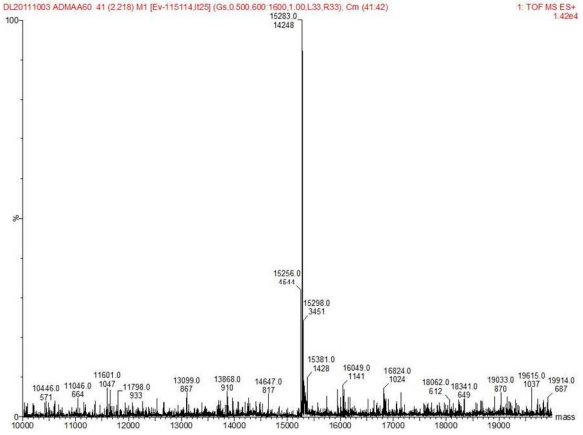
H3R2C-AA



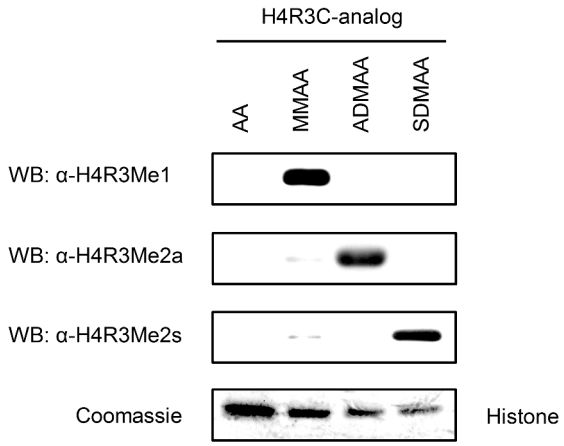
H4R3C-ADMAA



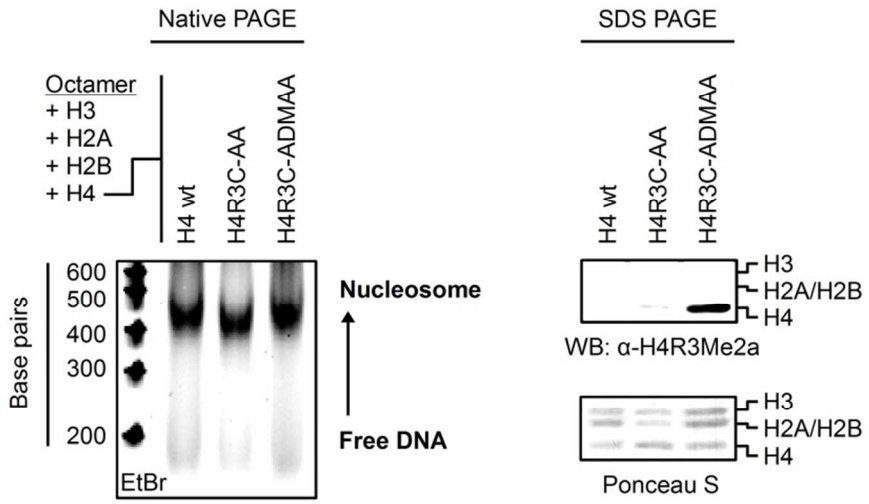
H3R2C-ADMAA



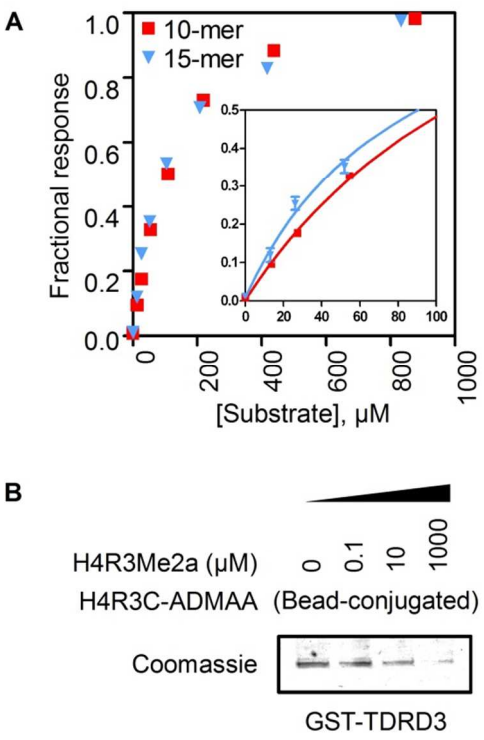
Supplemental Figure S2: De-convoluted mass spectra of analog-modified histones. Samples were taken from quenched reaction mixtures and analyzed by LC-MS. The MassLynx (Waters Corp.) software was used for ion envelope de-convolution (± 1 Da resolution, 10-20 kDa range)



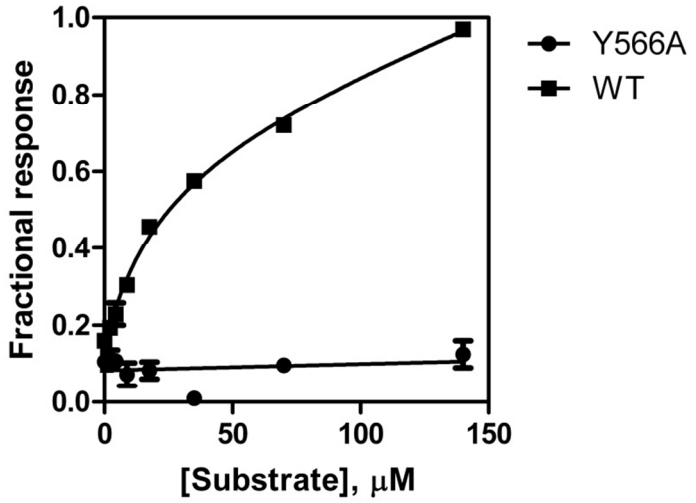
Supplemental Figure S3: Analog-modified histone H4 displays minimal antigen cross-reactivity with respective antibodies. The H4R3C-MMAA variant was weakly recognized by antibodies specific for H4R3Me2a and H4R3Me2s. The protein loading control is shown in the bottom panel.



Supplemental Figure S4: Analog-modified histones assemble into nucleosomes. Reconstitution with modified histone H4 was assessed by electrophoretic gel shift analysis (left panel). Nucleosome constituent proteins were resolved by denaturing PAGE and modification status was identified by western blot (right panel). Lower row in western blot displays protein loading control.



Supplemental Figure S5: TDRD3 Tudor domain binding assays. (A) Representative fluorescence polarization anisotropy saturation biding curves of H4R3Me2a peptides, comparing 10-mer to 15-mer substrates. Inset shows divergence of respective nonlinear regression curves. (B) Competition of GST-TDRD3 binding to peptide-conjugated beads.



Supplemental Figure S6: Fluorescence polarization saturation binding assay of TDRD3 Tudor domain Y566A aromatic cage mutant shows abrogation of binding to 10-mer H4R3Me2a peptide compared to wildtype Tudor domain. An analogous mutation in TDRD3 (Y566F) resulted in loss of binding affinity for methylated RNAPII *C*-terminal domain.[5]

General Synthesis: Chemicals were purchased from Sigma-Aldrich unless otherwise stated. RP-HPLC was conducted using a Varian ProStar system with a Phenomenex Luna C18(2) column (100 Å, 250 x 21.2 mm, 10 µm, # 00G-4253-P0). Solvent A was 0.1 % TFA in water and solvent B was 0.1 % TFA in acetonitrile. NMR spectra were collected on a 400 MHz Varian spectrometer.

Prop-2-enamidine (**1**)

To a stirred mixture containing 690 mg of ammonium chloride (12.9 mmol) in 10 mL of anhydrous toluene, at 0 ºC and under argon atmosphere, was added 6.0 mL of 2.0 M trimethylaluminum in toluene solution (12.2 mmol). The mixture was warmed to ambient temperature and stirred for 2 hr. Addition of 0.5 mL of acrylonitrile (7.6 mmol) was followed by heating to 80 ºC, and the mixture was stirred for 17 hr. The reaction was quenched at 0 ºC by addition of diethyl ether and water. The mixture was passed through filter paper, and the filtrate was concentrated at reduced pressure. Purification by RP-HPLC (0 % B / 20 min) and lyophilization of pooled fractions gave 208 mg of **1** as a white solid (3.3 mmol, 39 % yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 9.84 (s, 2H), 9.58 (s, 2H), 6.79 (d, J = 17.7 Hz, 1H), 6.62 (dd, J = 17.7, 11.0 Hz, 1H), 6.17 (d, J = 11.1 Hz, 1H). ^{13}C NMR (100 MHz, DMF-d_7) δ 164.72, 131.68, 127.16. HRMS Calcd for $C_3H_7N_2$ $[M+H]^+$: 71.0604; Found: 71.0603.

N-methylprop-2-enamidine (**2**)

To a stirred solution containing 129 mg of **8** (1.1 mmol) in 5 mL of acetonitrile, at ambient temperature, was added 200 µL of anhydrous triethylamine (1.4 mmol). The solution was stirred for 5 minute, and subsequently concentrated under reduced pressure. Purification by RP-HPLC (1 % B / 8 min) and lyophilization of pooled fractions gave 41 mg of **2** as a white solid (0.5 mmol, 45 % yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 10.37 (s, 1H), 9.59 (s, 1H), 9.08 (s, 1H), 6.69 – 6.53 (m, 2H), 6.12 (dd, J = 8.7, 1.5 Hz, 1H), 3.11 (d, J = 4.8 Hz, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 163.32, 130.20, 127.29, 29.71. HRMS Calcd for $C_4H_9N_2$ $[M+H]^+$: 85.0760; Found: 85.0762.

N,N-dimethylprop-2-enamidine (**3**)

To a stirred solution containing 180 mg of **9** (1.2 mmol) in 2 mL of anhydrous acetonitrile, at ambient temperature, was added 170 µL of anhydrous triethylamine (1.2 mmol). The solution was stirred for 1 minute, and subsequently concentrated under reduced pressure. Purification by RP-HPLC (0 % B / 5 min, 0-15 % B / 5 min, 15-100 % / 5 min) and lyophilization of pooled fractions gave 105 mg of **3** as an oily white solid (1.1 mmol, 82 % yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 9.60 (s, 1H), 9.28 (s, 1H), 6.84 (dd, J = 17.2, 11.3 Hz, 1H), 6.46 (d, J = 17.2 Hz, 1H), 6.09 (d, J = 11.3 Hz, 1H), 3.35 (s, 3H), 3.34 (s, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 163.30, 131.24, 126.67, 41.27, 40.32. HRMS Calcd for $C_5H_{11}N_2$ $[M+H]^+$: 99.0917; Found: 99.0913.

N,N′-dimethylprop-2-enamidine (**4**)

To a solution containing 15 mg of **12** (0.084 mmol) in 500 µL of DMSO, at ambient temperature, was added 10 uL of triethylamine (0.072 mmol). The solution was mixed by

shaking for 1 min, and subsequently concentrated by lyophilization. The concentrate was resuspended and purified by RP-HPLC (1-5 % B / 5 min, 5-25 % / 14 min) to give 7.8 mg of **4** as a clear colorless oil (0.080 mmol, 95% yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 9.83 (s, 1H), 9.32 (s, 1H), 6.84 (dd, J = 17.5, 11.3 Hz, 1H), 6.38 (d, J = 17.2 Hz, 1H), 6.14 (d, J = 11.1 Hz, 1H), 3.15 (s, 3H), 3.05 (d, J = 3.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.26, 130.39, 124.37, 30.16, 28.67. HRMS Calcd for $C_5H_{11}N_2$ $[M+H]^+$: 99.0917; Found: 99.0918.

Ethyl 3-hydroxypropionimidate hydrochloride (**5**)

To a stirred solution of 10 v/v % EtOH / 2.0 M HCl in diethyl ether, at ambient temperature and under argon atmosphere, was added 3 mL of 3-hydroxypropionate (43.9 mmol). Gaseous hydrochloric acid was bubbled into the solution for 3 hours. Concentration under reduced pressure gave 6.484 g of pure **5** as a hygroscopic white solid (42.4 mmol, 97 % yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 5.62 (broad s, 1H), 4.67 (q, *J* = 7.0 Hz, 2H), 3.89 (t, *J* = 6.0 Hz, 2H), 2.99 (t, *J* = 6.0 Hz, 2H), 1.43 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 179.71, 70.68, 58.47, 37.81, 14.15. HRMS Calcd for $C_5H_{12}NO_2$ $[M+H]^+$: 118.0863; Found: 118.0861.

3-hydroxy-*N*-methylpropionamidine (**6**)

To a stirred solution containing 1.1 g of compound **5** (7.2 mmol) in 4 mL anhydrous methanol, at ambient temperature and under argon atmosphere, was added 0.90 mL of 33 wt % ethanolic methylamine (7.2 mmol). The solution was stirred for 19 hr, and subsequently concentrated under reduced pressure. The resultant white solid was used without further purification.

3-hydroxy-*N,N*-dimethylpropionamidine (**7**)

To a stirred solution containing 1.0 g of compound **5** (6.5 mmol) in 4 mL of anhydrous methanol, at ambient temperature and under argon atmosphere, was added 3.6 mL of 2.0 M methanolic dimethylamine (7.2 mmol). The solution was stirred for 16 hr, and subsequently concentrated under reduced pressure. The concentrate was resuspended in 0.1 N HCl and lyophilized to give 731 mg of **7** as a glassy white solid (6.3 mmol, 97 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.40-9.00 (m, 1.5H), 9.00-8.40 (m, 1.5H), 5.32 (s, 1H), 3.72 (t, *J* = 5.9 Hz, 2H), 3.23 (s, 3H), 3.10 (s, 3H), 2.78 (t, *J* = 6.1 Hz, 2H).^{13}C NMR (100 MHz, DMSO-d_6) δ 165.88, 57.99, 40.38, 33.96, 33.90. HRMS Calcd for $C_5H_{13}N_2O$ $[M+H]^+$: 117.1022; Found: 117.1022.

3-chloro-*N*-methylpropionamidine (**8**)

To a stirred solution containing crude reaction mixture of **6** in 10 mL of anhydrous DMF, at ambient temperature and under argon atmosphere, was added 2.1 g of triphenylphosphine (7.9 mmol) and 760 μL of carbon tetrachloride (7.9 mmol). The solution was stirred for 36 hr, and subsequently concentrated under reduced pressure. The concentrate was diluted in 0.1 % aqueous formic acid, and the yellowish white precipitate was removed through filter paper. The aqueous extract was purified by RP-HPLC (0-10 % B / 8 min). Combined fractions were lyophilized to give 49 mg of **8** as a white solid (0.41 mmol, 6 % yield over 2 steps). ^{1}H NMR (400 MHz, DMF-d_7) δ 10.82 (s, 1H), 9.91 (s, 1H), 9.16 (s, 1H), 4.18 (t, *J* = 6.6 Hz, 2H), 3.20 (t, *J* = 6.6 Hz, 2H),

3.02 (d, *J* = 4.9 Hz, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 166.42, 42.07, 36.38, 29.62. HRMS Calcd for $C_4H_{10}ClN_2$ $[M+H]^+$: 121.0527; Found: 121.0519.

3-chloro-*N,N*-dimethylpropionamidine (**9**)

To a stirred solution containing 731 mg of compound **7** (6.3 mmol) in 10 mL of anhydrous DMF, at ambient temperature and under argon atmosphere, was added 1.39 g of triphenylphosphine (5.3 mmol) and 510 µL of carbon tetrachloride (5.3 mmol). The solution was stirred for 22 hr, and subsequently concentrated under reduced pressure. The concentrate was diluted in water, washed twice with dichloromethane, and washed once with diethyl ether. The aqueous extract was purified by RP-HPLC (0-3 % B / 15 min). Combined fractions were lyophilized to give 179 mg of **9** as clear oil (1.2 mmol, 19 % yield). ^{1}H NMR (400 MHz, DMF-d_7) δ 9.81 (s, 1H), 9.14 (s, 1H), 4.09 (t, *J* = 6.8 Hz, 2H), 3.43 (s, 3H), 3.35 (t, *J* = 6.7 Hz, 2H), 3.30 (s, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 166.06, 41.57, 41.41, 40.28, 34.67. HRMS Calcd for $C_5H_{12}ClN_2$ $[M+H]^+$: 135.0684; Found: 135.0678.

3-hydroxy-*N*-methylpropanamide *tert*-butyldimethylsilyl ether **(10)**

To a stirred solution containing 1 g of 3-hydroxyl-*N*-methylpropanamide (9.7 mmol; Chembridge, San Diego, CA, USA) and 1.9 g of imidazole (28 mmol; Acros Organics, Geel, Belgium) in 2 mL of anhydrous DMF, at ambient temperature and under argon atmosphere, was added 1.9 g of *tert*-butyldimethylsilyl chloride (12.7 mmol). The solution was stirred for 24 hr, then diluted in DCM. The solution was washed with 10 w/v % aqueous citrate (pH 4.5), and the extracted organic phase was dried over sodium sulfate. Purification by silica chromatography (100 % EtOAc) and concentration of combined fractions gave 1.61 g of **10** as colorless clear oil (7.41 mmol, 76 % yield). ^{1}H NMR (400 MHz, CDCl3) δ 3.86 (t, J = 5.7 Hz, 2H), 2.79 (d, J = 4.9 Hz, 3H), 2.39 (t, J = 5.7 Hz, 2H), 0.89 (s, 9H), 0.07 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.42, 59.61, 36.50, 25.95, 25.74, 18.07, -5.54. Calcd for $C_5H_{11}N_2$ $[M+H]^+$: 218.1571; Found: 218.1566.

3-hydroxy-*N,N′*-dimethylpropionamidine *tert*-butyldimethylsilyl ether **(11)**

To a stirred solution containing 590 mg of triethyloxonium tetrafluoroborate (3.1 mmol) in 3 mL of anhydrous DCM, at ambient temperature and under argon atmosphere, was added 450 mg of **10** (2.1 mmol). The reaction was stirred for 1 hr, then 2.1 mL of 2.0 M methylamine solution in THF (4.1 mmol) was added and stirred for an addition 1 hr. The resultant white precipitate was removed with Whatman filter paper and washed with EtOAc. The concentrated filtrate was purified by silica chromatography (5 % MeOH/EtOAc) to give 317 mg of **11** as a white solid (1.38 mmol, 67 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.25 (s, 1.5H), 8.62 (s, 1.5H), 3.85 (t, J = 6.1 Hz, 2H), 3.00 (d, J = 4.1 Hz, 3H), 2.78 (d, J = 4.8 Hz, 3H), 2.73 (t, J = 6.1 Hz, 2H), 0.85 (s, 9H), 0.04 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.73, 59.35, 32.62, 30.12, 28.41, 25.65, 17.84, -5.66. HRMS Calcd for $C_{11}H_{27}N_2OSi$ $[M+H]^+$: 231.1887; Found: 231.1886.

3-bromo-*N,N′*-dimethylpropionamidine (**12**)

To a stirred solution containing 120 mg of triphenylphosphine dibromide (0.28 mmol) in 1.5 mL DCM, at ambient temperature and under argon atmosphere, was added 50 mg of **11** (0.22 mmol) in 0.5 mL DCM. The solution was stirred for 20 hr, and subsequently quenched with 0.1 % TFA/water, and the aqueous phase was purified by RP-HPLC (1-5 % B / 5 min, 5-25 % / 14 min). Combined fractions were concentrated by lyophilization to give 15 mg of **12** as a clear colorless oil (0.084 mmol, 39 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.89 (s, 1H), 3.75 (t, J = 6.9 Hz, 2H), 3.15 (t, J = 6.9 Hz, 2H), 3.01 (d, J = 4.8 Hz, 3H), 2.82 (d, J = 4.9 Hz, 3H). ^{13}C NMR (100 MHz, DMF-d_7) δ 166.27, 33.55, 30.96, 29.57, 28.18. HRMS Calcd for $C_5H_{12}BrN_2$ $[M+H]^+$: 179.0178; Found: 179.0180.

Protein alkylation with analog precursors: Arginine-to-cysteine mutant proteins were suspended in 500 mM HEPES, 3 M Guanidinium hydrochloride, 1 mM TCEP, pH 7.8 buffer at a concentration of approximately 2 mg/mL. These solutions were mixed for 1 hr at room temperature. To this, 100 equivalents of analog precursor (one of compounds **1-4**) in DMSO were added. The reaction solutions were mixed for approximately 1 hr, or until complete conjugation of protein as determined by LC-MS (Supplemental Figure S2). Compound **1** was especially reactive, and required frequent monitoring to avoid over-alkylation. The reactions were quenched with 100 equivalents of 1.4 M aqueous BME. The analog-modified proteins were purified by centrifugal filtration through a 10,000 MWCO membrane (Amicon Ultra device, Millipore) against several charges of 1 mM aqueous BME. The resultant protein solutions were lyophilized for long-term storage at -80 ºC to avoid oxidation. Mass spectrometric analysis of modified proteins stored under these conditions showed minimal oxidation after more than 6 months of storage and several freeze/thaw cycles. Amidines are susceptible to hydrolysis from prolonged exposure to aqueous base (conversion to corresponding carboxylic acids upon hydrolysis is detectable by loss of 13 *m/z* units per methyl group removed).

Intact tandem mass spectrometric analysis using ETD: Recombinant H4R3C-ADMAA was dissolved in 50% MeOH/1% formic acid to a concentration of 1 pmol/μL and loaded into a Proxeon ES380 nanospray offline emitter. The sample was nanosprayed into an LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo). Intact protein was measured in both the linear ion trap and the Orbitrap, where the Orbitrap resolution was set to 60000. The 15+, 16+ and 17+ charge states were individually isolated and subjected to collisional activation (CID and HCD) and electron transfer dissociation (ETD). The represented spectrum was obtained from 20 summed high resolution (15000) ETD product ion scans (10 ms activation time) of the 17+ charge state. MS/MS data was analyzed by hand using the mutant gene sequence of H4R3C plus the mass of the appended analog and by the MS-product feature of Protein Prospector (UCSF).

Antibody recognition of methylarginine analogs was performed using the following primary antibodies:
Rabbit polyclonal to H4R3Me2a- (39705, Active Motif)
Rabbit polyclonal to H4R3Me2s- (ab5823, Abcam)
Rabbit polyclonal to H4R3Me1- (ab17339, Abcam)
Rabbit polyclonal to glutathione-*S*-transferase - (G7781, Sigma-Aldrich)

TDRD3 protein:

Construct #1: GST-TDRD3 in pGEX 6p-1 vector (Human, BC060876, residues 495-650; gift from Bedford group, University of Texas M.D. Anderson Cancer Center, see SI reference 6).
Expression: Vector was transformed into *E. coli* BL21 cells. A saturated overnight pre-culture was used to inoculate 6 L of LB containing ampicillin. The culture was grown to OD_{600} ~ 1.0 at 37 ºC, then it was induced with IPTG to a final concentration of 0.4 mM. Following incubation for 3 hr at 37 ºC, the cells were pelleted by centrifugation. The cells were lysed in 1x PBS (supplemented with 1 mM DTT and Roche Complete protease inhibitor cocktail) using sonication on ice. The lysate was cleared, and the resultant supernatant was mixed with washed GSH-sepharose beads (GE Healthcare) in 50 mM TrisHCl, 1 mM DTT, pH 7.4 (binding buffer). Elution of protein off beads was initiated with addition of 10 mM GSH in the aforementioned binding buffer. Fractions containing GST-TDRD3 were pooled, buffer exchanged into binding buffer, and concentrated by spin filtration. The collected protein was partitioned into aliquots, frozen in liquid nitrogen, and stored at -80 ºC.

Construct #2: His_6-MBP-TDRD3 (Human, BC060876, residues 495-650) in pMCSG9 vector (from DNASU). Ligation independent cloning primers: 5'-ATT GGA TTG GAA GTA CAG CTT CT-3' and 5'-ATT GGA AGT GGA TAA CGG ATC CG-3'.
Expression: Vector was transformed into *E. coli* Rosetta pLysS cells. A saturated overnight pre-culture was used to inoculate 6 L of Terrific broth (Sigma) containing ampicillin and chloramphenicol. The culture was grown to OD_{600} ~ 0.6 at 37 ºC, then it was induced with IPTG to a final concentration of 0.4 mM. Following incubation for 6 hr at 37 ºC, cells were pelleted by centrifugation. The cells were lysed in 50 mM HEPES, 50 mM NaCl, 0.01 % tween-20, 1 mM BME, 2 mM imidazole, pH 7.5 (binding buffer) + complete protease inhibitor tablet (Roche) using sonication on ice. The lysate was cleared, and the resultant supernatant was mixed with washed Ni-NTA beads (Qiagen) in binding buffer. Elution of protein off beads was initiated with addition of 150 mM imidazole in the aforementioned binding buffer. Fractions containing His_6-MBP-TDRD3 were pooled, buffer exchanged into binding buffer, and concentrated by spin filtration. The collected protein was partitioned into aliquots, frozen with liquid nitrogen, and stored at -80 ºC.

Construct #3: His_6-MBP-TDRD3 Y566A mutant construct was created by site-directed mutagenesis of construct #2. Mutagenesis primers: 5'-GAT GAA TGT TTT GCA CTT GCT TGG GAA GAC AAC AAG TTT T-3' and 5'-AAA ACT TGT TGT CTT CCC AAG CAA GTG CAA AAC ATT CAT C-3'.
Expression: Same as construct #2

Histone proteins:

Wildtype histones: *Xenopus* H2A, H2B, H3 (contains C110A that shows no change in function[7]), and H4 in pET3a expression vectors were gifts from Narlikar group, UCSF (see SI reference 8)
Mutant histones: H4R3C and H3R2C mutant constructs were created by site-directed mutagenesis. The primary amino acid sequences of these proteins contain only one cysteine residue that directs the site of analog conjugation.
H4R3C primers: 5'-ATA CAT ATG TCT GGT TGT GGT AAA GCT-3' and 5'-TTT ACC ACC TTT ACC ACA ACC AGA CAT ATG TAT -3'

H3R2C primers: 5'-GGA GAT ATA CCA TGG CCT GTA CCA AGC AGA C-3' AND 5'-GTC TGC TTG GTA CAG GCC ATG GTA TAT CTC C-3'.
Expression: See preparation described in SI reference 9.

PRMT proteins were purchased from the following sources:

PRMT1 (Sigma, Human, residues 2-end, *N*-terminal GST-tag): recombinant, expressed in *E. coli*, Cat. # SRP0140.

PRMT5 (Sigma, Human, residues 2-end, *N*-terminal DDDDK-tag): recombinant, expressed in FreeStyle 293-F cells, Cat. # SRP0145.

PRMT6 (BPS Bioscience, Human, 2-end, *N*-terminal His-tag): recombinant, expressed in *E. coli*, Cat. # 51049.

Nucleosome reconstitution: Nucleosomes were reconstituted following the protocol described in SI reference 10. The 601 positioning DNA sequence used in the assembly was synthesized by Genelink. Reconstituted nucleosomes were purified by sedimentation through a 5 mL 10-30% glycerol gradient.

Synthesized Peptides: Peptides were synthesized with Fmoc-protected amino acids (Chem-Impex and EMD Millipore,) using a Liberty 1 Microwave Peptide Synthesizer (CEM). The following peptides were purified by RP-HPLC and analyzed using a PE-Biosystems (Life Technologies Corporation) Voyager Elite STR MALDI-TOF to confirm product identity by mass:

P1: SGRGKGGKGLGKGGAGCG; Found *m/z* = 1503.7
P2: SGR(Me2a)GKGGKGLGKGGAGCG; Found *m/z* = 1531.7

The synthesized peptides **P1** and **P2** were subsequently biotinylated by the following procedure: To a stirred flask containing 1.8 g of synthesized peptide (~0.001 mmol) in 0.5 mL of 1xPBS (pH 7.5), was added 0.5 mg (0.001 mmol) of *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide in 50 μL of DMSO. The reaction mixture was allowed to react for 2 hr at room temperature. Then, the peptide was purified by RP-HPLC.

P1(biotin): SGRGKGGKGLGKGGAGC(biotin)G; Found *m/z* = 1956.7
P2(biotin): SGR(Me2a)GKGGKGLGKGGAGC(biotin)G; Found *m/z* = 1985.0

P3: SGCGKGGKGLGKGGAGK(biotin)G; Found *m/z* = 1701.4

The synthesized peptide **P3** was subsequently alkylated by analog precursors following a modified version of the aforementioned protein alkylation protocol. Briefly, 2 mg (~0.001 mmol) of **P3** was reacted with a 10-fold molar excess of either compound **1** or **3**. Then, the peptide was purified by RP-HPLC.

P3(AA): SGC(AA)GKGGKGLGKGGAGK(biotin)G; Found *m/z* = 1773.4

P3(ADMAA): SGC(ADMAA)GKGGKGLGKGGA GK(biotin)G; Found *m/z* = 1801.7

Purchased custom peptides:
SGRGK GGKGLGKGGAK(TAMRA); United Peptide
SGR(Me2a)GKGGKGLGKGGAK(TAMRA); United Peptide
SGR(Me2a)GKGGKGLK(5-FAM); Genscript
SGRGKGGKGLK; Genscript
SGR(Me2a)GKGGKGLK; Genscript
SGCGKGGKGLK; Genscript
SGR(Me2a)GKGGKGLK(TAMRA); Genscript
SGCGKGGKGLK(TAMRA); United Peptide

Fluorescence polarization saturation binding assay: Protein and substrate solutions were made in 50 mM HEPES, 50 mM NaCl, 2 mM DTT, 0.01 v/v % Tween-20, pH 7.5 buffer. After addition of all components to a 384-well plate (Corning NBS, low volume, black), samples were shielded from light and incubated at room temperature for 20 min. Using a Molecular Devices SpectraMax M5e plate reader, the fluorescence polarization was determined. For rhodamine-tagged probes, ex/em wavelengths were set to 544/590 nm; and, $[probe]_{final}$ = 10 nM. For fluorescein-tagged probes, ex/em wavelengths were set to 485/538 nm; and, $[probe]_{final}$ = 1 nM. Between sample reads, 100 ms of settling time was allowed. The resultant data was analyzed with GraphPad Prism 5 software. Fluorescence polarization change was plotted versus final [TDRD3], and the data was fit to a one-site total binding model for the determination of K_d:

$$y = \frac{y_{max}[TDRD3]_{final}}{K_d + [TDRD3]_{final}} Slope_{nonspecific}[TDRD3]_{final} + y_{control}$$

Fluorescence polarization competition assay: To a solution of $[TDRD3]_{final}$ = 350 μM and $[probe]_{final}$ = 1 μM, was added a series of competitor peptide solutions in assay buffer (probe = 15-mer H4R3Me2a peptide, *C*-terminal rhodamine). The samples were shielded from light and incubated at room temperature for 20 min. Plate reader setting were identical to those used in the saturation binding assays.

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