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## Supplementary Information for

# Histone H3Q5 serotonylation stabilizes H3K4 methylation and potentiates its readout

Shuai Zhao[a,1], Kelly N. Chuh[b,1], Baichao Zhang[a,1], Barbara E. Dul[b], Robert E. Thompson[b], Lorna A. Farrelly[c,d] Xiaohui Liu[e], Ning Xu[e], Yi Xue[e], Robert G. Roeder[f], Ian Maze[c,d,2], Tom W. Muir[b,2], Haitao Li[a,g,2]

[a]MOE Key Laboratory of Protein Sciences, Beijing Advanced Innovation Center for Structural Biology, Beijing Frontier Research Center for Biological Structure, Department of Basic Medical Sciences, School of Medicine, Tsinghua University, Beijing 100084, China
[b]Department of Chemistry, Princeton University, Princeton, New Jersey 08540, USA
[c]Nash Family Department of Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York 10029, USA
[d]Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York 10029, USA
[e]National Protein Science Technology Center, School of Life Sciences, Tsinghua University, Beijing 100084, China
[f]Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10065, USA
[g]Tsinghua-Peking Center for Life Sciences, Beijing 100084, China
[1]These authors contributed equally.
[2]Correspondence: lht@tsinghua.edu.cn (H.L.), muir@princeton.edu (T.W.M.), ian.maze@mssm.edu (I.M.)

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**Supplementary Information Text**

**Materials and Methods**

***Experimental model.*** The plasmid DNAs were amplified in *Escherichia coli* DH5α (Thermo Fisher Scientific), cultured in LB medium at 37°C overnight. The recombinant proteins were overexpressed in *E. coli* BL21 (DE3) (Shenzhen KT Life technology).
HeLa cell lines were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% FBS with 100 μg $ml^{-1}$ streptomycin and 100 U $ml^{-1}$ penicillin. Cells were maintained at 37°C in a 5% $CO_2$, 95% humidified incubator.

***Materials and general laboratory methods.*** All commercially available reagents were used without further purification. Analytical and semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 series system with a Waters XBridge Peptide BEH C18 column (analytical column 300Å, 5 μm, 4.6 × 150 mm #186003524; semi-preparative column 300Å, 5 μm, 10 mm × 250 mm #186008193) employing 0.1% trifluoroacetic acid (TFA) in water (HPLC solvent A), and 90% acetonitrile and 0.1% TFA in water (HPLC solvent B) as mobile phases. Preparative scale RP-HPLC purifications were performed on a Waters prep LC equipped with a Waters 2545 Binary Gradient module, Waters 2489 UV detector, and a Vydac C18 column (10 μm, 22 × 250 mm). Electrospray ionization (ESI)–mass spectrometry (MS) analysis was performed on a MicrOTOF-Q II ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Billerica, MA).

***Peptide synthesis of serotonylated peptides.*** Serotonylated H3(1-15) and H3(1-25) peptides, varying in methylation state at position K4 were synthesized according to previously reported procedures (1). For peptides utilized in ITC, MALDI-TOF and control experiments, synthesis was conducted on Rink amide ChemMatrix resin using standard Fmoc-strategy solid-phase chemistry. For peptides used in semisynthesis of modified histones, synthesis was conducted on SEA-PS resin (Iris Biotech) using standard Fmoc-strategy solid-phase chemistry. Briefly, when necessary, Fmoc-Glu(OAll)-OH was incorporated at position 5 for on-resin serotonylation. At position 4, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Me,Boc)-OH, Fmoc-Lys(Alloc)-OH or Fmoc-Lys(me3)-OH were coupled in order to access the me0, me1, me2 or me3 methylation states, respectively. The fully assembled resin-bound peptides were allyl deprotected through treatment with Pd(0)(PPh3)4 (0.2 equivalents (equiv.)), N,N-dimethylbarbituric acid (10 equiv.) in DCM for 30 min (two treatments). The resin was then washed extensively with DCM and DMF (to remove residual Pd). To serotonylate peptides corresponding to methylation states K4me0, K4me1 and K4me3, the appropriate peptide-resins were then treated with PyAOP (1.1 equiv.) and DIEA (4.0 equiv.) in DMF, followed by direct addition of serotonin hydrochloride (2.0 equiv.) and agitation for 30 min each. To access the K4me2Q5ser peptide, the Lys(Alloc) was first converted to Lys(me2) through alloc/allyl deprotection according to the above conditions, followed by reductive alkylation with 37% formaldehyde and NaBH3CN (50 equivs. Each) in PBS/MeOH (1:1). The peptide was then serotonylated according to the conditions above. Each of the crude peptides were then cleaved from the resin and globally deprotected through treatment with 5% ethanedithiol (EDT), 2.5% iPr3SiH, 2.5% $H_2O$ in TFA for 2h at room temperature. Crude peptides were then purified by preparative C-18 RP-HPLC, and the pure peptides were characterized by analytical C-18 RP-HPLC and ESI-MS.

***Protein expression and purification.*** The cDNA encoding the histone reader proteins (the frame of each protein is shown in Table. S2) were cloned into the pRSFDuet vector (Novagen). All histone reader proteins were expressed in the E. coli BL21 (Novagen) and induced overnight by 0.2mM isopropyl β-D-thiogalactoside at 16°C in the TB medium. The collected cells were suspended in 100 mM NaCl, 20 mM Tris pH 7.5. After cell lysis and centrifugation, the supernatant was applied to HisTrap column (GE Healthcare). After washing 5 column volumes with the suspension buffer, the protein was eluted with the buffer 100 mM NaCl, 20 mM Tris pH 7.5, 500 mM imidazole. All proteins were further purified by the HiTrap SP (GE Healthcare) cation-exchange column or HiTrap Q (GE Healthcare) anion-exchange column and a HiLoad 16/60 Superdex 75 (GE Healthcare) gel filtration column using AKTA Purifier 10 systems (GE Healthcare). All proteins were stored in 100 mM NaCl, 20 mM Tris, pH 7.5 at ~ 10mg/ml in a -80°C freezer.

Human MLL1 constructs (3745-3969), as well as full-length human WDR5, RBBP5, DPY30 and ASH2L (95-628) proteins, were individually expressed in *E. coli* BL21 cells. All proteins were induced overnight with 0.2 mM isopropyl β-D-thiogalactoside at 16°C in TB medium. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM DTT). After cell lysis and centrifugation, supernatants were purified using HisTrap columns (GE Healthcare) or Glutathione Sepharose 4B beads (GE Healthcare), followed by enzyme digestion to remove tags. All proteins were further purified on HiTrap SP (GE Healthcare) cation-exchange columns

or HiTrap Q (GE Healthcare) anion-exchange columns. MLL1, WDR5 and DPY30 were further purified with an HiLoad 10/300 Superdex 75, while ASH2L and RBBP5 were further purified with a HiLoad 10/300 Superdex 200. The buffer for gel-filtration chromatography contained 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM DTT and 10% glycerol. Purified proteins were concentrated to 10-20 mg/ml and stored at -80 °C.

The KDM5B used in this study were produced in Sf9 cells using a baculovirus expression vector system. All coding sequences were subcloned into vectors (pFASTBAC and pACEBAC) compatible with bacmid generation by standard restriction enzyme cloning methods with an N-terminal HIS tag introduced for purification. 6×HIS-KDM5B constructs were transformed into DH10MultiBacTurbo *E. coli* competent cells, and bacmids were produced per manufacturer's instructions (Geneva Biotech). To generate virus for protein production, bacmids were transfected into Sf9 cells in 6-well plates. All transfection, viral amplification, and infection steps were performed in a sterile hood. Five µg of bacmid was transfected into $1 \times 10^6$ attached Sf9 cells according to the manufacturer's instructions (Bac-to-Bac Baculovirus Expression System, ThermoFisher Scientific). After transfection, cells were overlaid with 2 ml fresh medium (Sf-900III SFM, ThermoFisher Scientific) and incubated at 27 °C for 120 hrs in the dark. The supernatant was collected and cleared by centrifugation to generate the P1 virus. 2% FBS (v/v) was added. Between uses, all viral stocks were stored at 4 °C in the dark. Subsequent steps were carried out in medium containing penicillin/streptomycin. To generate the P2 virus, 2 mls of P1 virus was added to 20 ml of Sf9 cells in a sterile flask at $2 \times 10^6$ cells per ml. Cells were grown at 27 °C in suspension culture in the dark until they reached 40% viability as monitored by trypan blue staining. The culture supernatant was then collected and cleared by centrifugation, and 2% FBS (v/v) was added. To generate the P3 virus (used for protein production), 5 mls of P2 virus was added to 50 mls of Sf9 cells in a sterile flask at $2 \times 10^6$ cells per ml. Cells were grown at 27 °C in suspension culture in the dark until they reached 40% viability as monitored by trypan blue staining. The culture supernatant was then collected and cleared by centrifugation, and 2% FBS (v/v) was added. During virus amplification Sf9 cell density was kept at around $2 \times 10^6$ cells per ml, diluting if needed, until growth arrested, and viability dropped. Viruses were then directly combined with Sf9 cell cultures to generate 6×HIS-KDM5B by adding a 1:1,000 dilution of each P3 virus to Sf9 suspension cultures at $2 \times 10^6$ cells per ml. Cells were harvested by centrifugation after 72 h at 27 °C in the dark. Cells were lysed with 50 mls of lysis buffer (50 mM Tris-HCl pH 8, 1 M NaCl, 10% glycerol, 0.5 mM DTT, 0.1% TX100, 0.5 mM PMSF) per 1 L of cells. Cells with sonicated for 5 mins with an amplitude of 30% at 15 sec on 45 off cycles. The cells were then centrifuged for 30 min at 4℃ at 17,000 x g. The supernatant was then removed and bound to Ni-NTA resin (10 mls for 50 mls of lysate) for 2 hours in batch. The beads were centrifuged for 5 min at 1000 x g and 4°C and the supernatant was removed. The beads were then washed with 50 CV (500 mls) of Wash buffer (50 mM Tris-HCl pH 8, 1 M NaCl, 0.5 mM DTT, 5% glycerol). The KDM5B protein was then eluted in batch for 30 min with 10 mls of elution buffer (25 mM Tris-HCl pH 8.5, 1 M NaCl, 0.5 mM DTT, 5% glycerol, 300 mM Imidazole). The protein was then subjected to size exclusion chromatography on a Superdex 26/600 equilibrated with size exclusion buffer (25 mM HEPES pH 7.3, 1 M NaCl, 2 mM DTT, 10% glycerol). Fractions were analyzed by gel and then combined and concentrated using appropriate molecular weight cut-off centrifugal filter units (Millipore). Purity was assessed by SDS-PAGE analysis and Coomassie blue staining.

Histones H2A type 2A (AA seq. Uniprot ID Q6FI13), H2B type 1-K (AA seq. Uniprot ID O60814), H3.1 A96C_A110C (H3.1 AA seq. Uniprot P68431), and H4 (AA seq. Uniprot ID P62805) were expressed in *E. coli* and purified according to published protocols (2, 3), with minor modifications. BL21 (DE3) cells were transformed with histone expression plasmids (pET30a, Novagen), grown in LB medium at 37 °C until an OD600 of 0.6 was reached and induced by addition of IPTG (final concentration 1 mM). Following protein expression at 37 °C for 2-3 h, cells were collected by centrifugation (4000 × g, 15 min, 4 °C). The washed cell pellet was resuspended in 10 ml lysis buffer per liter cell culture (50 mM HEPES, 300 mM NaCl, 5 mM imidazole, 1 mM EDTA, 1 mM DTT, pH 7.5). Cells were lysed by sonication and centrifuged (16,000 × g, 25 min, 4 °C). The inclusion body pellet was washed twice with cold lysis buffer containing 1% Trition X-100 before being re-suspended in re-suspension buffer (6 M guanidinium chloride, 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5 at 4 °C), nutated for 2 h at 4 °C, and cleared by centrifugation (16,000 × g, 25 min, 4 °C). The isolated supernatant was carefully filtered and purified by preparative RP-HPLC using a 10-70% B over 60 mins method.

For $KDM5B_{PHD1}$ expression and purification, BL21 (DE3) cells were transformed with GST-$KDM5B_{PHD1}$-His6 expression plasmids (pET30a, Novagen), grown in LB medium at 37 °C until an OD600 of 0.5 was reached and induced by addition of IPTG (final concentration 1 mM). Following protein expression at 37 °C for 2-3 h, cells were collected by centrifugation (4000 × g, 15 min, 4 °C). The washed cell pellet was resuspended in 50 ml lysis buffer per liter cell culture (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol, 0.5 mM DTT, 0.1% Triton-X 100, 1mM PMSF). Cells were lysed by sonication (15 sec on, 45 sec off, 3 min total) and centrifuged (17,000 × g, 30 min, 4 °C). Supernatant was then bound to Ni-NTA resin for 2 h in batch at 4°C. Beads were transferred to a fritted, plastic

column where they were washed extensively with wash buffer for 50 column volumes (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.5 mM DTT, 5% glycerol). Protein was then eluted in batch for 15-20 mins with elution buffer (10 mls) (25 mM Tris-HCl pH 8.5, 1 M NaCl, 0.5 mM DTT, 5% glycerol, 300 mM Imidazole). Eluent was collected by filter beads through a clean fritted column and filtering through a 0.22 µm filter before being subjected to size exclusion chromatography on a Superdex 26/600 equilibrated with size exclusion buffer (25 mM HEPES pH 7.3, 1 M NaCl, 2 mM DTT, 10% glycerol). Fractions were analyzed by gel and then combined and concentrated using appropriate molecular weight cut-off centrifugal filter units (Millipore). Purity was assessed by SDS-PAGE analysis and Coomassie blue staining.

For the generation of N-terminally truncated histones, BL21 (DE3) cells were transformed with protein expression constructs (pET30a, Novagen) for 6×His-SUMO-H3.1(14-135) K14C_C96A_C110A (used for generation of semisynthetic histones) and 6×His-SUMO-H3.1(15-135) A15C_C96A_C110A (used as H3Δ1-14 in chromatin array formation). Following protein expression, cell pellets were lysed and inclusion bodies were re-suspended as described above (see preparation of recombinant histones). After centrifugation (16,000 × g, 25 min, 4 °C), the cleared extract was affinity purified by Ni-NTA affinity chromatography. The elution (elution buffer: 6 M guanidinium chloride, 50 mM Tris-HCl, 500 mM imidazole, pH 7.5) was dialyzed stepwise into refolding buffer (50 mM, 300 mM NaCl, 5 mM β-mercaptoethanol, pH 7.5) at 4 °C with 4 M Urea for 2 h, 1.5 M Urea for 2 h, and 1.5 M Urea for 18 h. Ulp1 protease was added during the final dialysis step to cleave of the 6×His-SUMO tag. After 6×His-SUMO cleavage, solid urea (final concentration 6 M) was added to solubilize the truncated histone, the pH adjusted to 7.5, and the mixture was purified by RP-HPLC using a 10-70% B over 60 mins method.

***Western blotting.*** For all Western blots, samples were loaded on a 12% Bis-Tris gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA) using a semi-dry transfer cell (Bio-Rad). Blots were then blocked with 5% dry milk in 1×TBST buffer before application of the indicated antibodies for analysis (see Key Resources Table). Following incubation with primary antibody, HRP-conjugated secondary antibodies were applied in blocking buffer. Following washes with 1×TBST, blots were developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher, #34577) and imaged on a GE ImageQuant LAS 4000.

***Isothermal titration calorimetry.*** For ITC measurements of $TAF3_{PHD}$, synthetic histone peptides and wild type/mutant $TAF3_{PHD}$-within a reasonable c-value range–were extensively dialyzed against ITC buffer: 0.1 M NaCl and 20 mM Tris pH 7.5. The adjusted titration concentrations were: 1 mM H3(1-15)un/H3(1-15)Q5ser peptide to 0.1 mM TAF3; 0.5 mM H3(1-15)K4me1/H3(1-15)K4me1Q5ser peptide to 0.05 mM TAF3; 0.3 mM H3(1-15)K4me2/H3(1-15)K4me2Q5ser peptide to 0.03 mM TAF3; 0.3 mM H3(1-15)K4me3/H3(1-15)K4me3Q5ser peptide to 0.03mM TAF3. Titrations were performed using a MicroCal iTC200 system (GE Healthcare) at 25°C. Each ITC titration consisted of 17 successive injections with 0.4 µl for the first, and 2.4 µl for the rest. The resulting ITC curves were processed using Origin 7.0 software (OriginLab) according to the 'One Set of Sites' fitting model.

***SPR imaging analysis.*** To measure the interaction between immobilized proteins and flowing peptides, the SPR imaging instrument (Kx5, Plexera, USA) was used to monitor the whole procedure in real-time. Briefly, a chip with well-prepared biomolecular microarray was assembled with a plastic flow cell for sample loading. The peptide samples were prepared at 20 µM concentrations in TBS buffer (20 mM Tris pH 7.5, 100 mM NaCl,) while a 10 mM glycine-HCl buffer (pH 2.0) was used as regeneration buffer. A typical binding curve was obtained by flowing phase at 2 µl/s for 300s association and then flowing running buffer for 300s dissociation, followed by 200s regeneration buffer at 3 µl/s. Binding data was collected and analyzed by a commercial SPRi analysis software (Plexera SPR Data Analysis Module, Plexera).

***MALDI-TOF analysis of enzymatic activities.*** For KDM5B assays, full length purified KDM5B (312 nM) was incubated with H3(1-25) peptide substrates (2.5 µM) in KDM5B reaction buffer (50 mM HEPES, pH 7.5, 3 µM alpha-keto glutarate, 10 µM $(NH4)_2Fe(SO_4)_2$, 500 µM L-ascorbic acid, 3 mM TCEP) for time indicated at 30°C.

For LSD1 assays, full length purified LSD1 (Sigma) was incubated in LSD1 Reaction Buffer (50 mM Tris HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.01% Tween-20, 50 nM FAD) with 2.5 uM peptide, 1.25 nM LSD1, at 30℃. For both enzymes, reactions were quenched by the addition of HPLC solvent A ($H_2O$ + 0.1% TFA) and were desalted using C18 ZipTip (Millipore) according to manufacturer's protocol before being diluted 1:1 with α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN plus 20% acetone with 0.1% TFA and spotted on a MALDI-TOF plate for analysis. Samples were analyzed using a Bruker UltrafleXtreme MALDI TOF/TOF Mass Spectrometer and data were analyzed using Bruker Compass flexAnalysis Version 3.4.

For KDM5C assays, KDM5C (200 nM, Active Motif Cat#31833) was incubated with 1 μM peptide in demethylation reaction buffer (50 mM MES pH 6.6, 7.5 mM NaCl, 50 μM $(NH4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 1 mM 2-oxoglutarate, and 2 mM ascorbate) for time indicated at 30°C, and were quenched by the addition of trifluoroacetic acid (TFA) to 0.5%. An aliquot of the quenched sample (0.5 μl) was mixed with 0.5 μl α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma-Aldrich, Italy) in 50% ACN and 0.1% trifluoroacetic acid and spotted on to a MALDI-TOF plate for analysis. The samples were analyzed by the 4800 MALDI TOF-TOF mass spectrometer (Applied Biosystems, America). The data were processed by Data Explorer software.

***LC-MS analysis of enzymatic activities.*** H3K4 methyltransferase assays were conducted by combining 1.2 μM of the $MLL_{3745-3969}$-WDR5-RbBP5-ASH2L-DPY30 complex with 10 μM histone H3 peptide and 250 μM methyl-S-adenosyl-methionine in 50 mM Tris, pH 8.5, 50 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, and 5% glycerol. Reactions were incubated at 15°C for 24 h, and stopped by the addition of TFA to 0.5% at different time points. Substrate and product peptide detection was achieved using an Ultimate 3000 UPLC chromatography system (Dionex) coupled with a Q-Exactive orbitrap mass spectrometer (Thermo Fisher, CA). A heated electrospray ionization (HESI) probe was equipped for ionization. Source parameters were set as follows: spray voltage 3000V, capillary temperature 320°C, heater temperature, 300°C, sheath gas flow rate 35Arb, auxiliary gas flow rate 10Arb. Peptides were separated using a BEH C18 column (50 × 2.1 mm, Water, Germany). Mobile phases consisted of 0.05% TFA (v/v) in LC/MS-grade water as the A phase, and acetonitrile with 0.05% formic acid (v/v) as the B phase. Linear gradients were set as follows: 0 min, 1% B; 1.5 min, 1% B; 4 min 15% B; 6 min, 98% B; 8 min, 98% B, 8.1 min 1% B; 10 min 1% B. Column chamber and sample tray were held at 45°C and 10°C, respectively. A positive-ion mode was applied for data acquisition. The mass accuracy was typically 5ppm in the mass range of m/z 200-1000. The software used for mass spectrometry data acquisition and analysis was Thermo Xcalibur 3.0.

***Pre-steady state kinetics.*** Liquid chromatography mass spectrometry was used to determine the abundance of unmodified, mono-, di- and trimethylated species in each reaction performing a similar procedure as described by Liu and colleagues (4). The percentage of total integrated area for each species was quantitatively determined using standard curves to estimate the concentration of each modified form at each time point. Calibration curves of each peptide displayed a linear relationship between spectra area and concentration (Fig. S2). The reaction curves were fitted to a kinetic model using Equations 1-3 below, as described by Patel and colleagues (5). The program MATLAB was used to globally fit Equations 1–3 to the data.

$$[A] = [A]_0 \exp(-k_1 t) \qquad \text{(Eq.1)}$$

$$[B] = \frac{[A]_0 k_1}{k_2 - k_1} [\exp(-k_1 t) - \exp(-k_2 t)] \qquad \text{(Eq.2)}$$

$$[C] = [A]_0 \{1 + \frac{1}{k_1 - k_2} [k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)]\} \qquad \text{(Eq.3)}$$

***Peptide synthesis of H3K4me3Q5(analog) peptides.*** Tryptamine and 5-Methoxytryptamine.HCl were purchased from AK Scientific (Union City, CA). 6-Hydroxytryptamine, 5-fluorotryptamine.HCl and 5,6-difluorotryptamine.HCl were purchased from Enamine (Monmouth Jct., NJ). 7-azatryptamine was purchased from 1ClickChemistry (Kendall Park, NJ). Peptides corresponding to the N-terminus of H3 [H3(1-15)] bearing serotonylated sidechains or derivatives were synthesized through Fmoc-SPPS on Rink amide ChemMatrix resin according to standard protocols, incorporating Fmoc-Glu(All)-OH at position 5 and Boc-Ala-OH at position 1. Following assembly of the primary peptide sequence, the allyl-protected glutamate was deprotected using $Pd(PPh_3)_4$ (0.2 equiv.) and *N*,*N*-dimethylbarbituric acid (10 equiv.) in $CH_2Cl_2$ (2 x 30 min). Following extensive washing with DMF, the resin-bound peptides were monoaminylated through activation with PyAOP (2 equiv.) and DIEA (2 equiv.) for 1 min, followed by addition of serotonin derivative (10 equiv.) and additional DIEA (10 equiv. for free base monoamine, 20 equiv. for monoamine hydrochloride salts) and additional agitation for 30 min. The resin was then washed successively with DMF, 1% w/v sodium diethyldithiocarbamate in DMF (to remove residual palladium), DMF and finally $CH_2Cl_2$. The resin was dried under reduced pressure and the peptides cleaved and deprotected using a cocktail of TFA/$iPr_3SiH$/$H_2O$/EDT (185:5:5:5, v/v/v/v) for 2.5 h. In the case of peptide containing 6-hydroxytryptamine, the peptide was cleaved and deprotected using Reagent K [TFA/$H_2O$/phenol/thioanisole/EDT (165:10:10:10:5, v/v/v/v/v) for 2.5 h, as the 6-hydroxyindole ring was prone to reduction by $iPr_3SiH$. The crude cleavage mixtures were concentrated under a stream of nitrogen and peptides were precapitated from ice-cold ether. Crude peptides were purified using semipreparative RP-HPLC.

***Peptide synthesis of fluorescent polarization anisotropy peptides.*** The non-fluorescent histone H3(1-15) peptide (H3 Unmod) used in fluorescent polarization binding studies was synthesized on Rink amide ChemMatrix resin using standard Fmoc-strategy solid-phase chemistry. The serotonylated H3(1-15) (H3Q5ser) was synthesized according to the procedure written above. To access the fluorescein labeled reference peptide, Fmoc-Lys(Alloc)-OH was incorporated at position 14 during synthesis. The fully synthesized, resin-bound peptides were then alloc-deprotected through treatment with Pd(0)(PPh3)4 (0.2 equivalents (equiv.)), N,N-dimethylbarbituric acid (10 equiv.) in DCM for 30 min (two treatments). The resin was then washed extensively with DCM and DMF (to remove residual Pd) before being treated with N,N'-diisopropylcarbodiimide (5.5 equiv.), 1-hydroxy-7-azabenzotriazole (5 equiv.) and 5(6)-carboxyfluorescein (5 equiv.) in DMF overnight. Synthesis of the of the N-terminally acetylated, negative control peptide (H3 NtAc) was accomplished by first deprotecting the last Fmoc-incorporated amino acid on resin and subsequently treating the peptide with acetic anhydride (20 equiv.), N,N'-diisopropylethylamine (40 equiv.) in DMF for 10 mins (2 cycles). Following extensive washing with DMF, the crude peptides were then cleaved from the resin and globally deprotected through treatment with 5% ethanedithiol (EDT), 2.5% triisopropylsilane ($iPr_3SiH$), 2.5% $H_2O$ in TFA for 3 h at room temperature. Crude peptides were then purified by preparative C-18 RP-HPLC, and the pure peptides were characterized by analytical C-18 RP-HPLC and ESI-MS.

***Generation of peptide thioesters.*** Synthetic peptide thioesters used in semisynthesis were manually synthesized on SEA-PS resin (Iris) as described above. The peptides were then converted to C-terminal thioesters according to established protocols (6). In brief, (for a 0.05 mmol scale synthesis) the crude peptide (bearing the C-terminal bis(sulfanylethyl)amino linker) was incubated in a solution of mercaptopropionic acid (MPA, 0.5 ml) and TCEP (16 equiv. relative to peptide) in 2×PBS (10 ml) pH 4.0 at 37 °C overnight. The reaction mixture was diluted with water (10 ml) and 10% aqueous TFA (5 ml), extracted with $Et_2O$ to remove MPA, lyophilized overnight and then purified by preparative RP-HPLC using a gradient of 0-30% B over 60 min.

***Preparation of modified synthetic histones.*** Full-length, modified histones were generated by semisynthesis from two pieces, as previously described (7). Briefly, H3 peptide α-thioesters (residues 1-13) were incubated with truncated histone H3 (residues 14-135, K14C) in 6 M Gdn.HCl, 0.1 M $Na_2HPO_4$, 20 mM TCEP, 0.1 M mercaptophenyl acetic acid at pH 7.5-8.0. Upon completion of the ligation reaction (monitored by HPLC and ESI-MS analysis) the ligation product was purified by preparative RP-HPLC using a gradient of 20–73% B (9:1 ACN:$H_2O$ with 0.1% TFA) over 60 min. The purified semisynthetic protein was then treated with a solution of 2-bromoethylammonium bromide (50 mM) in degassed, filtered, 6 M Gdn.HCl, 1 M HEPES, 10 mM methionine, 20 mM DTT, pH 7.8 at 27 °C to convert the non-native cysteine residue into a lysine mimic at position 14 (8). Upon full conversion to the alkylated product (monitored by HPLC and ESI-MS), the protein was purified by semi-preparative RP-HPLC using a gradient of 20-73% B over 40 min, and its identity and purity validated by HPLC and ESI-MS analyses.

***Octamer refolding.*** Histone octamers were formed using well-established protocols (9, 10). Briefly, lyophilized histones were dissolved in unfolding buffer (6 M guanidinium chloride, 20 mM Tris-HCl, 1 mM DTT, pH 7.9) and quantified by UV absorbance at 280 nm. All four core histone were combined (equimolar amounts of H3 and H4 and 1.05 equiv. H2A, H2B) and the final concentration was adjusted to 1 mg/mL protein. The mixture was then dialyzed against folding buffer (2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.9) for 4, 18, and 4 h. The crude assemblies were purified by size-exclusion chromatography using a Superdex S200 10/300 increase column (GE Healthcare Life Sciences). The fractions were analyzed by SDS-PAGE (15% Tris-HCl gel) and octamers pooled. All histone octamers were diluted with 50% glycerol and stored at -20 °C at an approximate A280 of 2.0.

***DNA preparations.*** The 601 DNA for MN assemblies was prepared by expression of a plasmid, containing multiple copies of the 147 bp Widom 601 DNA sequence flanked by EcoRV sites, in DH5α *Escherichia coli* cells. After the isolation of the plasmid and EcoRV digestion, the monomeric 601 DNA was purified from the linearized plasmid backbone by precipitation with PEG 6000. A similar expression protocol was used for the preparation of the MMTV buffer DNA, DNA containing tetrameric repeats of the 601 sequence and DNA containing dodecameric repeats of the 601 sequences (spaced by 30 bp linkers). The biotinylated 601 DNA was prepared by large-scale PCR amplification.

***12-mer array assembly.*** The reconstitution of nucleosome arrays followed previously established protocols (9, 10) with some modifications. Octamers (ratio optimized, 1.2-1.5 eq. depending on the preparation, 12-mer DNA (12 repeats of the 601 DNA sequence separated by 30 bp linkers, 0.5-2.0 µM 601 sites), and buffer DNA (155 bp of the weak nucleosome binding MMTV sequence; necessary to ensure good reproducibility of the array reconstitution on a variety of scales (11), 0.3 eq.) was assembled in 75 µl in 2 M TEK buffer (2 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM DTT). Samples are then dialyzed against 200 ml 1.4 M TEK buffer (1.4 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM DTT) at 4°C for 1 h at which time 350 ml of 10 mM TEK buffer (10 mM KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 ml DTT) was gradually added using a peristaltic pump at 1 ml/min for a final concentration of 0.5 M. Additional dialysis against 200 ml 10 mM TEK, (2 × 2 h at 4°C) was performed before 12-mer arrays were selectively precipitated using $MgCl_2$ (15 mM final, 150 mM stock, 15 mins on ice). Following resuspension of precipitated arrays, an additional dialysis step against 10 mM TEK (2 h, 4°C) was performed to remove excess $MgCl_2$. All assembled arrays were used in experiments within 2 weeks. Reconstituted arrays were analyzed on native 1% agarose/2% polyacrylamide (APAGE) gels stained with Sybr Gold nucleic acid gel stain (Life Technologies) or a 0.9% agarose gel with ethidium bromide nucleic acid stain.

***KDM5B assays on 12-mer chromatin arrays.*** Freshly generated 12-mer chromatin arrays (150 nM unless otherwise indicated) were submitted to reaction with KDM5B (300 nM unless otherwise indicated) in KDM5B reaction buffer [50 mM HEPES, pH 7.5, α-ketoglutarate (50 µM final, 5 mM stock), L-ascorbic acid (100 µM final, 25 mM stock), tris(2-carboxyethyl)phosphine (TCEP, 3 mM final, 150 mM stock, pH equilibrated) and ammonium iron(II) sulfate hexahydrate (100 µM final, 5 mM stock)] and incubated for 1 hour at 30°C. Reactions were then quenched by the addition of 4X SDS-PAGE loading buffer (200 mM Tris-HCl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and boiled for 5 min at 98°C. Arrays were then separated on a 12% Bis-Tris gel before being transferred to PVDF membrane using a semidry transfer cell for further Western blotting according to above written procedures.

***Fluorescence anisotropy peptide binding assays.*** The $K_d$ of the reference peptide (H3 1-15, K14-fluorescein) was determined by forward titration with GST-$KDM5B_{PHD1}$ by fixing the fluorescent peptide at 1 µM and titrating in increasing concentrations of $KDM5B_{PHD1}$ in KDM5B binding buffer (50 mM Tris, pH 7.5, 10 mM NaCl, 100 mM $ZnCl_2$). Data fitting for one-site binding was performed with DynaFit (BioKin) software using a (1:1) model. For substrate peptide $K_d$ determination, fluorescent reference peptide was competed away with either unmodified H3, H3Q5ser modified or unmodified N-terminally acetylated substrate peptides. $KDM5B_{PHD1}$ and fluorescent reference peptide concentrations were fixed at 2 µM and 1 µM, respectively. For all assays, polarization and anisotropy measurements were made at 20°C using a DM302 fluorimeter (Horiba) using 490 nm excitation and 520 nm emission. For competition experiments, all replicates were normalized by rescaling from 1 to 0 such that 1 represents 100% bound peptide and 0 represents unbound peptide (100% free). Data was fitted using DynaFit software in a (1:1) model. Equations relating to data fitting can be found within references (12, 13). $K_d$ was constrained at 4.1 µM (as determined by forward titration) for competitive data fitting. An example of code used to fit data using DynaFit is provided below.

***HeLa nuclear lysate isolation.*** HeLa cells at 100% confluency were isolated (5 × 10 cm dishes) by scraping. Cell pellets were washed 2× with 1× PBS by centrifugation (5 min, 500 x g, 4°C) before being resuspended in 2 ml of Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, Roche Complete Protease Inhibitor Tablet, pH 7.6 25°C, cool to 4°C before use). Using a loose pestle, sample was homogenized with 10 strokes before being centrifuged 5 mins @ 400 x g, supernatant is removed. Pellet was again centrifuged for 20 mins, 17,000 x g at 4°C and supernatant was removed. The pellet was then resuspended in 2 ml Buffer C (20 mM HEPES, 25% Glycerol, 1.5 mM $MgCl_2$, 1.5 M NaCl, 0.2 mM EDTA, 1 mM DTT, Roche Complete Protease Inhibitor Tablet, pH 7.6 25°C, cool to 4°C before use) and using a tight pestle, sample was homogenized 10 strokes. The sample was the transferred to a 5 mL tube and was rotated for 30 mins at 4°C. Sample was cleared by centrifugation 30 mins, 13,000 x g at 4°C before dialyzing using a 2000 Da cassette overnight against Buffer D (20 mM HEPES, 20% Glycerol, 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT, Roche Complete Protease Inhibitor Tablet, pH 7.6 25°C, sterile filter, cool to 4°C before use). Following dialysis, sample was recovered and cleared by centrifugation 30 mins, 17,000 x g at 4°C and protein concentration was determined by A280.

***Chromatin dipping.*** Isolated nuclear extract (1 mg/ml diluted in Buffer D above) supplemented with α-ketoglutarate (50 µM final, 5 mM stock), L-ascorbic acid (100 µM final, 25 mM stock), tris(2-carboxyethyl)phosphine (TCEP, 3 mM final, 150 mM stock, pH equilibrated) and ammonium iron(II) sulfate hexahydrate (100 µM final, 5 mM stock),

was incubated with 100 nM chromatin array substrate for 2 h at 37°C. Following incubation, pre-washed (3× in Buffer D) magnetic streptavidin-coated beads (NEB #S1420S, 25 µl/reaction) were added directly to mixture and allowed to incubate with full rotation for 30 min at 4°C, at which time beads were harvested using a magnetic rack and washed 3× with cold Buffer D. Enriched arrays were then eluted from the beads by the addition of 2× Laemmli sample buffer containing beta-mercaptoethanol and boiling for 10 mins. Magnetic beads were removed and proteins were separated by SDS-PAGE on a 12% Bis-Tris gel before being transferred to PVDF membrane using a semi-dry transfer cell according and analyzed by Western blotting according to above written procedures.

***Antibody epitope controls.*** Peptide standards were synthesized according to above written procedures, diluted in water and concentrations were determined by HPLC (A214). For antibody controls in KDM5B reaction buffer, peptide substrates were incubated in KDM5B reaction buffer at 30°C for the times indicated before being spotted on nitrocellulose and analyzed by Western blotting.

***Quantification and statistical analysis.*** Statistical analysis was performed in GraphPad Prism 8 in Fig. 4F. Significance was determined using a Student's t-test (two-tailed, unpaired) defining significance as $P < 0.05$ where **** represents significance of $P < 0.0001$ (also indicated in the figure legend). Error bars for Figure 4D and Figure 4F represent standard deviation and the replicate number is n=3 and n=4, respectively. Replicate number "n" can be found in each figure legend.

***DynaFit.*** DynaFit software (BioKin) can be found at: http://www.biokin.com/dynafit/
An example of the code used to fit the data for the forward titration of $KDM5B_{PHD1}$ with the reference peptide can be found below:

```
[task]
  task = fit
  data = equilibria
[mechanism]
  P + LL <==> P.LL : KdL dissociation
[constants]
  KdL = 1 ?
[concentrations]
  LL = 1
[responses]
  LL = 0 ?
  P.LL = 1 ?
[data]
  variable P
  file ./tutorial/
[output]
  directory ./tutorial/output/
```

An example of the code used to fit the data for competition experiments of the reference peptide can be found below:

```
[task]
  task = fit
  data = equilibria
[mechanism]
  P + LL <==> P.LL : KdL dissociation
  P + UL <==> P.UL : KdU dissociation
[constants]
  KdL = 4.1 ; fixed !
  KdU = 1 ?

[concentrations]
  LL = 1 ; fixed!
  P = 2 ; fixed!
[responses]
```

```
   LL = 0 ?
   P.LL = 10 ?
[data]
   variable UL
   file ./tutorial/data/
[output]
   directory ./tutorial/output/
```

***Key resource table.***

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Anti-H3K4me1 | Abcam | 8895 |
| Anti-H3 (C-terminal epitope) | Abcam | 1791 |
| Anti-KDM5B | Cell Signaling Technology | 3273 |
| HRP-Conjugated rabbit secondary | Jackson ImmunoResearch | 111-035-144 |
| HRP-Conjugated mouse secondary | Jackson ImmunoResearch | 111-035-003 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Synthetic histone H3(1-15) peptide ARTKQTARKSTGGKA | Synthesized by Beijing SciLight Biotechnology | N/A |
| Synthetic histone H3(1-15)Q5serotonylation peptide ARTKQ(ser)TARKSTGGKA | This paper | N/A |
| Synthetic histone H3(1-15)K4me3Q5serotonylation peptide ARTK(me3)Q(ser)TARKSTGGKA | This paper | N/A |
| Synthetic histone H3(1-25) peptide ARTKQTARKSTGGKAPRKQLATKAA | Synthesized by Beijing SciLight Biotechnology | N/A |
| Synthetic histone H3(1-25)K4me3 peptide ARTK(me3)QTARKSTGGKAPRKQLATKAA | Synthesized by Beijing SciLight Biotechnology | N/A |
| Synthetic histone H3(1-25)K4me3Q5serotonylation peptide ARTK(me3)Q(ser)TARKSTGGKAPRKQLATKAA | This paper | N/A |
| Fluorescent polarization anisotropy peptides | This paper | N/A |
| Recombinant protein: TAF3 (aa 855-921, ref# NP_114129.1) | This paper | N/A |
| Recombinant protein: BPTF (aa 2859-2921, ref# NP_004450.3) | This paper | N/A |
| Recombinant protein: CHD1 (aa 270-443, ref# NP_001261.2) | This paper | N/A |
| Recombinant protein: DIDO1 (aa 266-325, ref# NP_071388.2) | This paper | N/A |
| Recombinant protein: ING2 (aa 210-262, ref# NP_001555.1) | This paper | N/A |
| Recombinant protein: KDM5A (aa 1609-1660, ref# NP_001036068.1) | This paper | N/A |
| Recombinant protein: KDM4A (aa 895-1008, ref# NP_055478.2) | This paper | N/A |
| Recombinant protein: MLL5 (aa 117-181, ref# NP_061152.3) | This paper | N/A |
| Recombinant protein: PHF13 (aa 229-280, ref# NP_722519.2) | This paper | N/A |
| Recombinant protein: PHF2 (aa 1-70, ref# NP_005383.3) | This paper | N/A |
| Recombinant protein: RAG2 (aa 414-487, ref# NP_033046.1) | This paper | N/A |
| Recombinant protein: SET3 (aa 116-184, ref# NP_012954.3) | This paper | N/A |

| | | |
|---|---|---|
| Recombinant protein: SGF29<br>(aa 113-254, ref# NP_612423.1) | This paper | N/A |
| Recombinant protein: SPIN1<br>(aa 50-262, ref# NP_006708.2) | This paper | N/A |
| Recombinant protein: ZCWPW1<br>(aa 246-307, ref# NP_060454.3) | This paper | N/A |
| Recombinant protein: MLL1<br>(aa 3745-3969, ref# NP_005924.2) | This paper | N/A |
| Recombinant protein: WDR5<br>(aa 1-334, ref# NP_438172.1) | This paper | N/A |
| Recombinant protein: RBBP5<br>(aa 1-538, ref# NP_005048.2) | This paper | N/A |
| Recombinant protein: ASH2L<br>(aa 95-628, ref# NP_004665.2) | This paper | N/A |
| Recombinant protein: DPY30<br>(aa 1-99, ref# NP_001308138.1) | This paper | N/A |
| Recombinant protein: KDM5B<br>(aa 1-1580, ref# NP_001300971.1) | This paper | N/A |
| Recombinant protein: KDM5B-PHD1<br>(aa 302-363, ref# NP_001300971.1) | This paper | N/A |
| Recombinant protein: KDM5C | Active Motif | Cat# 31833 |
| Experimental Models: Cell Lines | | |
| Human: HeLa cells | ATCC | CCL-2 |
| Experimental Models: Organisms/Strains | | |
| *Escherichia coli*: BL21(DE3) | Shenzhen KT Life technology | Cat#KTSM108 |
| Recombinant DNA | | |
| Plasmid: His-TAF3 | This paper | N/A |
| Plasmid: His-BPTF | This paper | N/A |
| Plasmid: His-CHD1 | This paper | N/A |
| Plasmid: His-DIDO1 | This paper | N/A |
| Plasmid: His-ING2 | This paper | N/A |
| Plasmid: His-KDM5A | This paper | N/A |
| Plasmid: His-KDM4A | This paper | N/A |
| Plasmid: His-MLL5 | This paper | N/A |
| Plasmid: His-PHF13 | This paper | N/A |
| Plasmid: His-PHF2 | This paper | N/A |
| Plasmid: His-RAG2 | This paper | N/A |
| Plasmid: His-SET3 | This paper | N/A |
| Plasmid: His-SGF29 | This paper | N/A |
| Plasmid: His-SPIN1 | This paper | N/A |
| Plasmid: His-ZCWPW1 | This paper | N/A |
| Plasmid: GST-MLL1 | This paper | N/A |
| Plasmid: GST-WDR5 | This paper | N/A |
| Plasmid: GST-RBBP5 | This paper | N/A |
| Plasmid: His-ASH2L | This paper | N/A |
| Plasmid: His-DPY30 | This paper | N/A |
| Plasmid: His-KDM5B-FL | This paper | N/A |

| | | |
|---|---|---|
| Plasmid: GST-KDM5B-PHD1 | This paper | N/A |
| Software and Algorithms | | |
| Origin7.0 | OriginLab | http://www.originlab.com/ |
| PyMOL | DeLano Scientific LLC | http://www.pymol.org/ |
| Data Explorer | Applied Biosystems | http://www.data-explorer.com/software |
| Xcalibur 3.0 | Thermo Fisher | N/A |
| DynaFit | BioKin | http://www.biokin.com/dynafit/ |

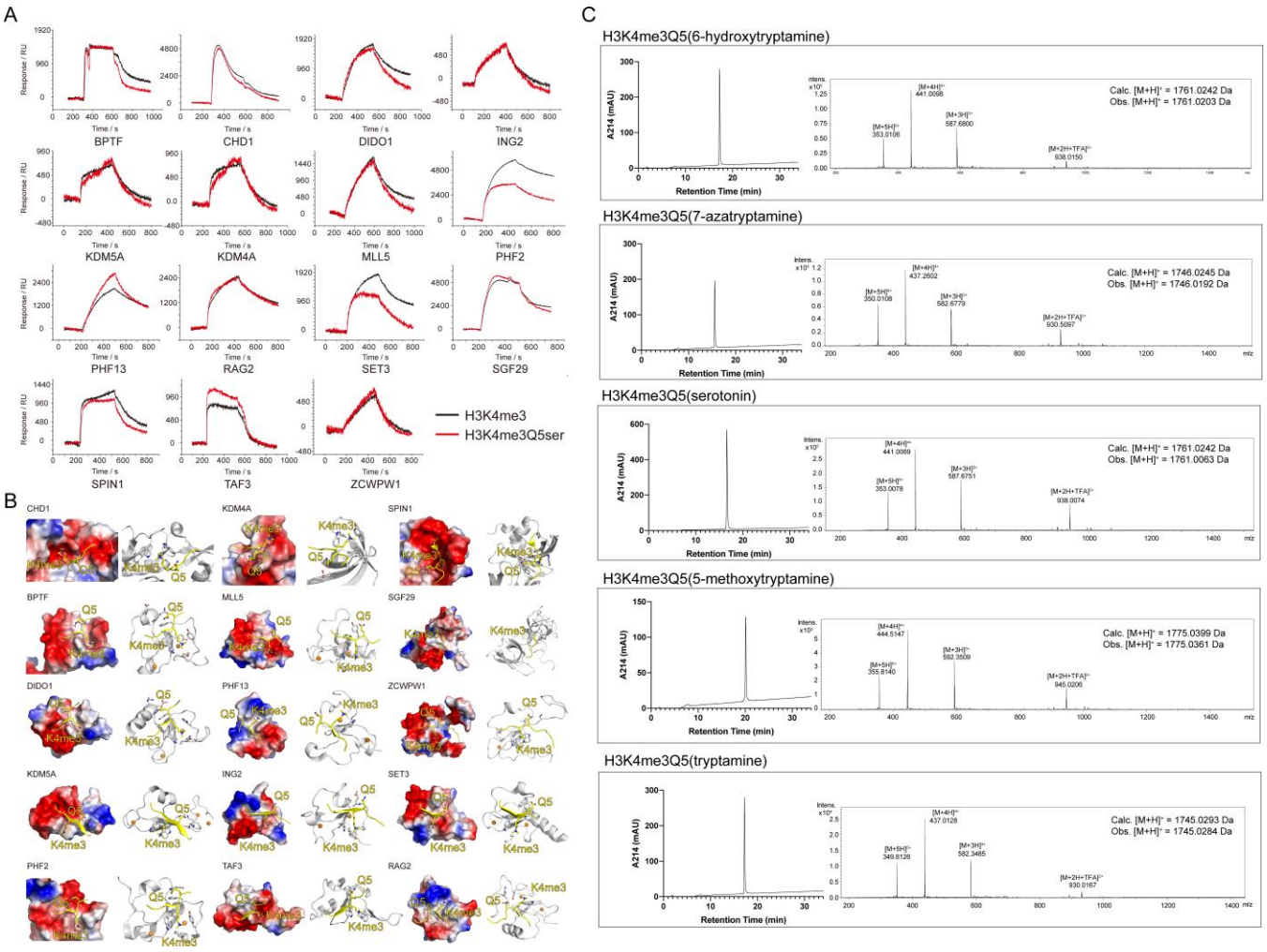


**Fig. S1. Profiling of H3K4me3 readers in response to H3K4me3Q5ser dual modifications.**
(A) SPRi detection of interactions by immobilizing H3(1-15)K4me3 and H3(1-15)K4me3Q5ser peptides on the 3D carbene chip. The binding curves to the H3K4me3 peptide are shown in black; the binding curves to the H3K4me3Q5ser peptide are shown in red. (B) Structural analysis of histone H3K4me3 readers. The solved histone H3K4me3-reader complex structures are shown in the surface mode (left) and cartoon mode (right). Histone K4me3 and Q5 residues are shown as yellow sticks. Coordinates were taken from PDB entries 2B2W for the CHD1-H3K4me3 complex (14); 2GFA for the KDM4A-H3K4me3 complex (15); 4H75 for SPIN1-H3K4me3 complex (16); 2F6J for the BPTF-H3K4me3 complex (17); 4L58 for the MLL5-H3K4me3 complex (18); 3MEA for the SGF29-H3K4me3 complex (19); 4L7X for the DIDO1-H3K4me3 complex (20); 3O7A for the PHF13-H3K4me3 complex (21); 2RR4 for the ZCWPW1-H3K4me3 complex (22); 3GL6 for the KDM5A-H3K4me3 complex (23); 2G6Q for the ING2-H3K4me3 complex (24); 5TDW for the SET3-H3K4me3 complex (25); 3KQI for the PHF2-H3K4me3 complex (26); 5WXH for the TAF3-H3K4me3 complex (27); 2V89 for the RAG2-H3K4me3 complex (28). (C) RP-HPLC and MS characterization of H3K4me3Q5(analog) peptides used in ITC studies.

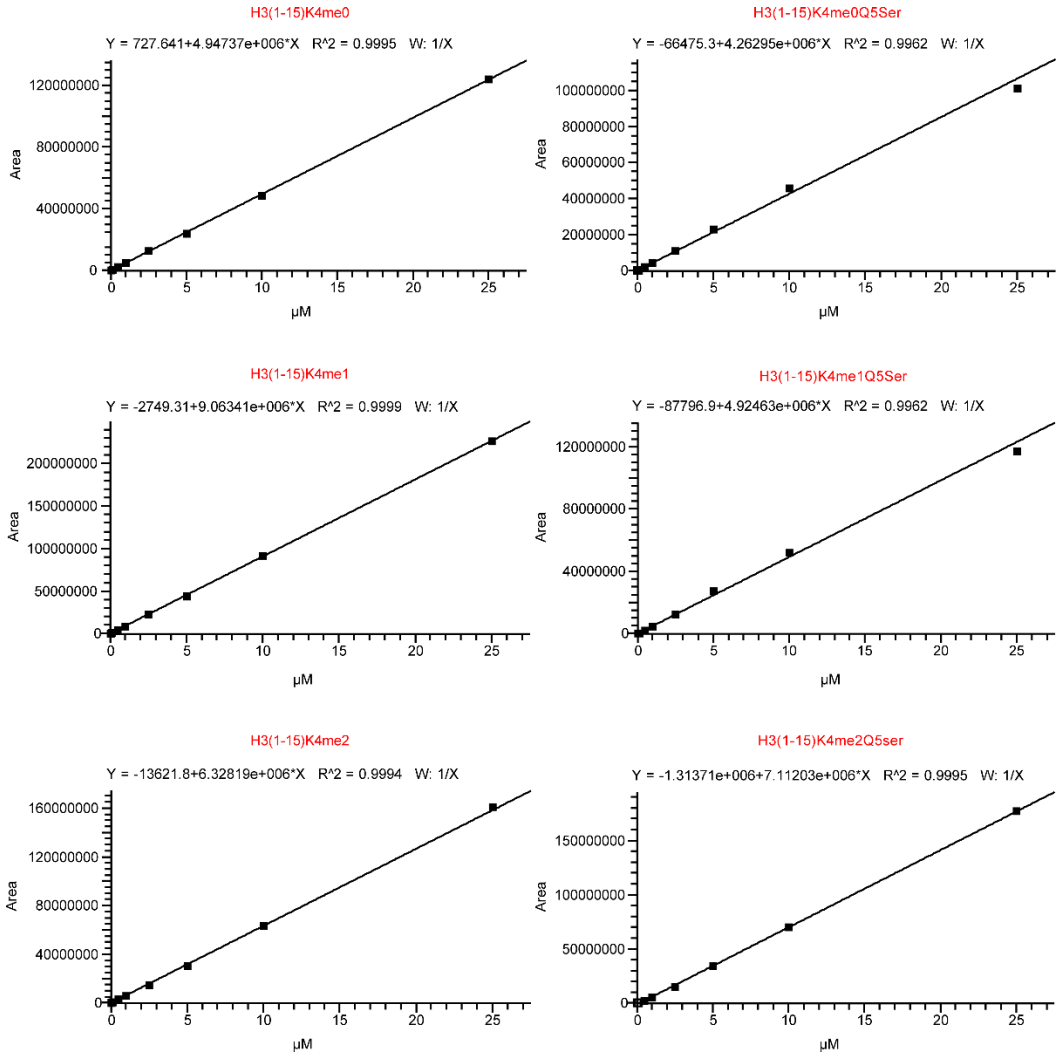


**Fig. S2. Linear detection of histone H3 peptides used for LC-MS analysis.** Unmodified *vs.* modified peptides were quantitated by LC-MS. Samples were prepared from stock solutions that were diluted at various concentrations. Results are plotted as the spectra area of the sample *vs.* the measured amount of peptide concentration. Linear regressions fitted to the data result in $R^2$ values >0.995 for all peptides.

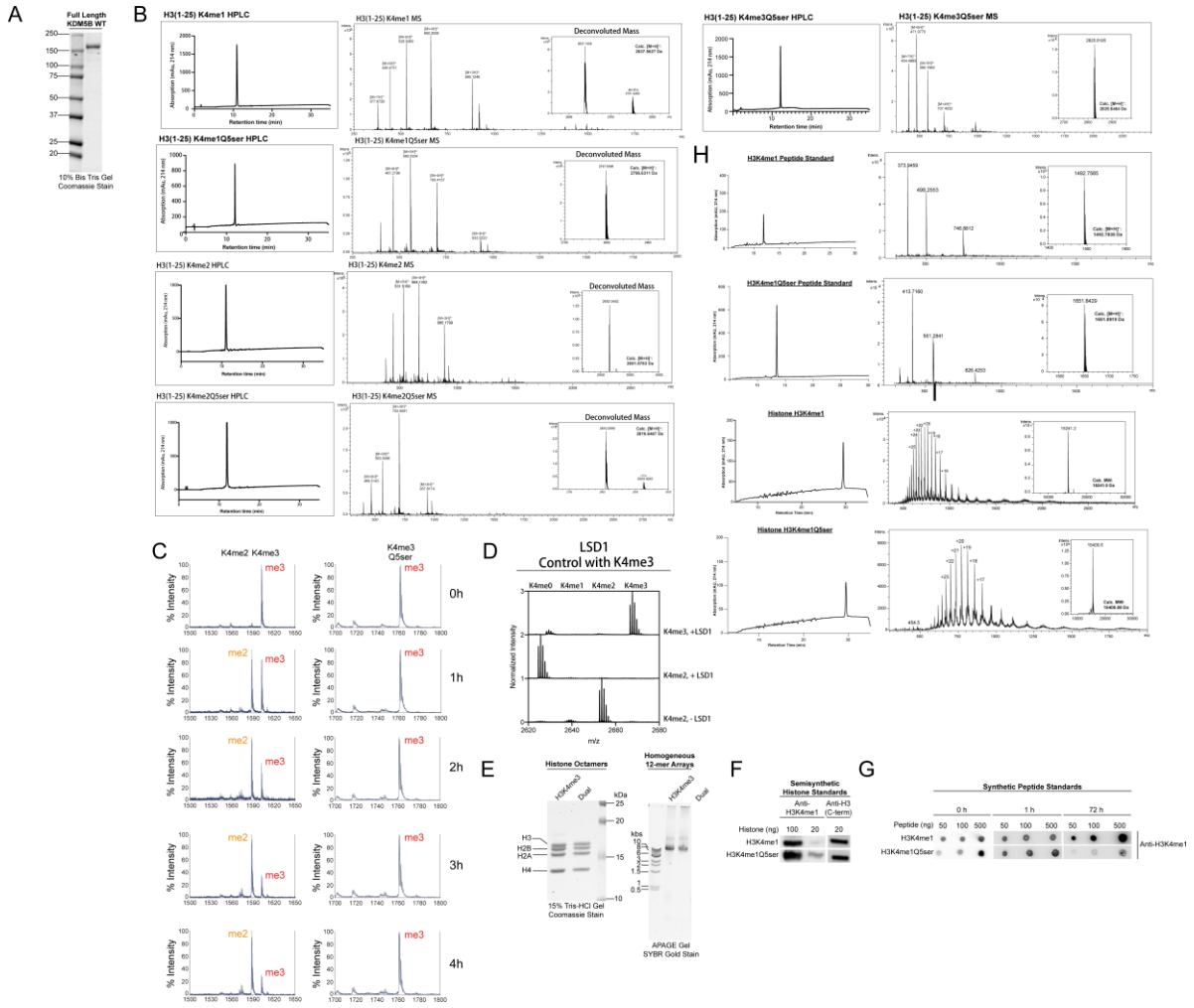


**Fig. S3. Characterization of Q5ser-modified peptide and array substrates used in Fig. 3 and antibody control experiments.** (A) SDS-PAGE gel analysis of full length KDM5B expressed in baculovirus. (B) RP-HPLC and MS characterization of synthesized peptide substrates (1-25) bearing H3K4me and Q5ser in this study. Purified peptides were eluted from a C18 RP-HPLC column using a gradient of 0-73% Solvent B (0.1% TFA in 9:1 acetonitrile/water) in Solvent A (0.1% TFA in water), detecting absorption at 214 nm. Mass spectra of purified proteins were deconvoluted (inset) and observed and calculated masses are shown. (C) MALDI-TOF mass spectrometry of KDM5C enzymatic assays using H3(1-15)K4me3 and H3(1-15)K4me3Q5ser peptides as substrates. (D) The activity of LSD1 (H3K4me2/1 demethylase) was tested against the control peptide K4me3. No activity was seen. (E) SDS-PAGE gel analysis of histone octamers formed with semisynthetic histones and subsequent AGPAGE gel analysis of formed 12-mer array substrates. (F) Histone epitope against the anti-H3K4me1 antibody. Anti-H3 (C-term epitope) was used as a loading control. (G) Peptide epitope control showing equal recognition of both H3K4me1 and H3K4me1Q5ser before and after incubation in KDM5B reaction buffer for varying times. (H) RP-HPLC and MS characterization of peptide standards (aa 1-15) H3K4me1 and H3K4me1Q5ser used for epitope controls against the anti-H3K4me1 antibody. Purified peptides were eluted from a C18 RP-HPLC column using a gradient of 0-73% Solvent B (0.1% TFA in 9:1 acetonitrile/water) in Solvent A (0.1% TFA in water), detecting absorption at 214 nm. Mass spectra of purified proteins were deconvoluted (inset) and observed and calculated masses are shown. RP-HPLC and MS characterization of semisynthetic histone proteins H3K4me1 and H3K4me1Q5ser. Purified proteins were eluted from a C18 RP-HPLC column using a gradient of 0-73% Solvent B (0.1% TFA in 9:1 acetonitrile/water) in Solvent A (0.1% TFA in water), detecting absorption at 214 nm. Mass spectra of purified proteins were deconvoluted (inset) and observed and calculated masses are shown.

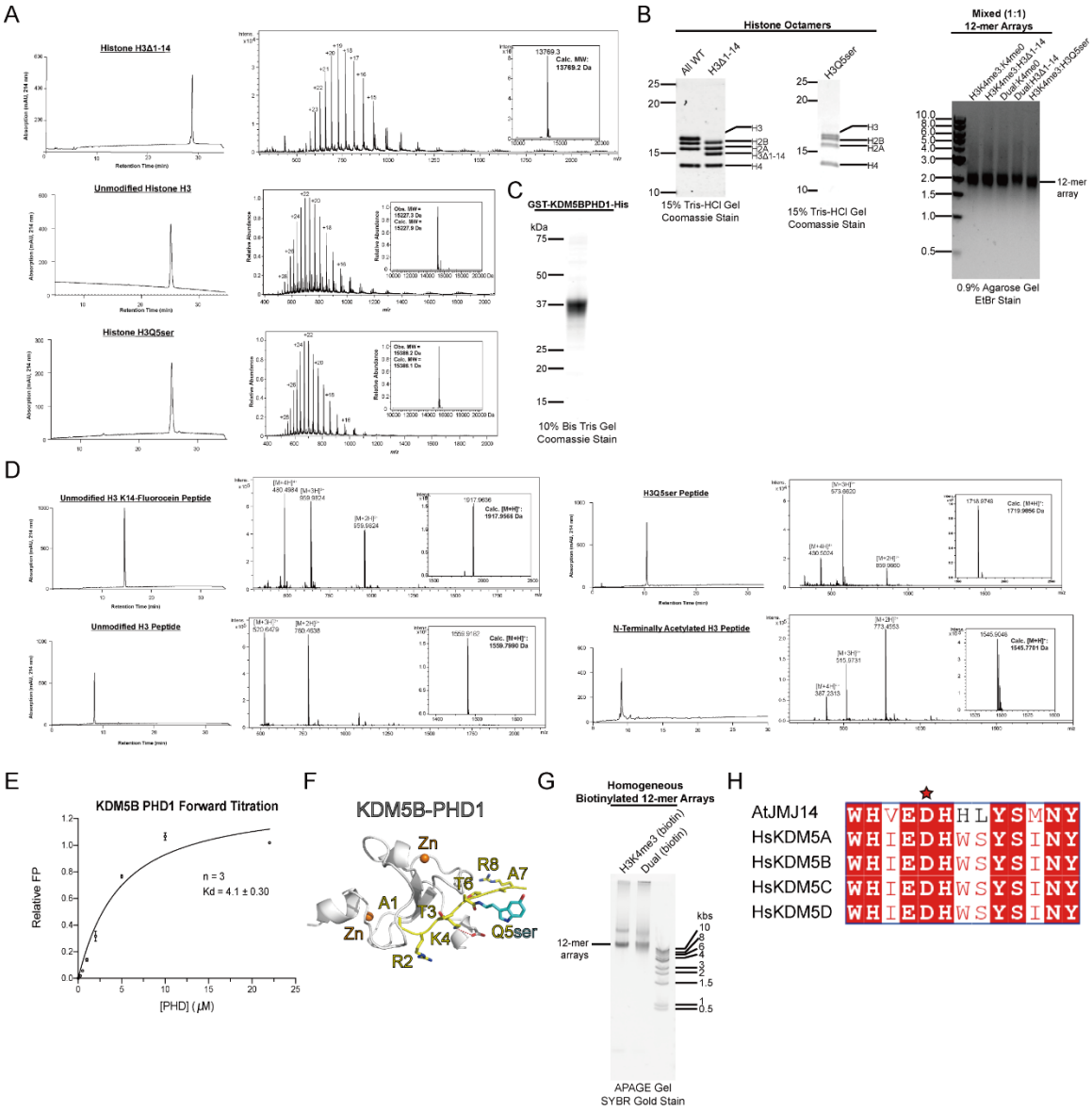


**Fig. S4. Characterization of array substrates used in Fig. 4 and peptides used in fluorescent polarization binding studies.** (A) RP-HPLC and MS characterization of histone proteins H3A15C (H3Δ1-14) and WT unmodified H3 as well as semisynthetic histone protein H3Q5ser. Purified proteins were eluted from a C18 RP-HPLC column using a gradient of 0-73% Solvent B (0.1% TFA in 9:1 acetonitrile/water) in Solvent A (0.1% TFA in water), detecting absorption at 214 nm. Mass spectra of purified proteins were deconvoluted (inset) and observed and calculated masses are shown. (B) SDS-PAGE gel analysis of histone octamers formed with histones in (A) and subsequent agarose gel analysis of formed 12-mer array substrates. (C) SDS-PAGE gel analysis of $KDM5B_{PHD1}$ expressed in *E. Coli*. (D) RP-HPLC and MS characterization of histone peptides used in fluorescent binding studies. Mass spectra of purified peptides were deconvoluted (inset) and observed and calculated masses are shown. (E) Forward titration of $KDM5B_{PHD1}$ binding with fluorescent H3(1-15) peptide. Kd of fluorescent reference peptide was calculated to be 4.1 ± 0.3 μM. Error bars represent standard deviation (n = 3). (F) The structure of $KDM5B_{PHD1}$ in complex with the histone H3 peptide (coordinates were taken from the PDB entry 2MNZ (29). The side chains of H3 peptide are shown as yellow sticks. Q5ser modification is modelled as cyan sticks. The H3Q5ser residue stretches out of the $KDM5B_{PHD1}$ binding surface. (G) AGPAGE gel analysis of biotinylated 12-mer array substrates used for chromatin dipping. (H) Sequence alignment of residues involved in H3Q5 recognition by JMJ14 and human KDM5 family proteins. The conserved aspartic acid residue is highlighted with a red asterisk.

Table S1. Thermodynamic fitting parameters for isothermal titration calorimetry.

| Protein | Peptide | *Ka* ($10^5$*$M^{-1}$) | ΔH (kcal/mol) | ΔS (cal/mol/deg) | N |
|---|---|---|---|---|---|
| TAF3 | $H3_{(1-15)}$K4un | 0.47±0.05 | -5.53±0.25 | 2.80 | 1.04±0.04 |
| | $H3_{(1-15)}$K4unQ5ser | 2.64±0.19 | -5.46±0.07 | 6.50 | 1.04±0.01 |
| | $H3_{(1-15)}$K4me1 | 5.51±0.73 | -5.52±0.10 | 7.75 | 1.07±0.01 |
| | $H3_{(1-15)}$K4me1Q5ser | 33.80±2.32 | -6.64±0.03 | 7.59 | 0.95±0.01 |
| | $H3_{(1-15)}$K4me2 | 30.70±4.61 | -7.08±0.08 | 5.94 | 1.29±0.01 |
| | $H3_{(1-15)}$K4me2Q5ser | 160.0±28.9 | -9.89±0.08 | -0.22 | 0.96±0.01 |
| | $H3_{(1-15)}$K4me3 | 58.70±15.5 | -8.48±0.16 | 2.52 | 0.94±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 515.0±156.0 | -13.55±0.10 | -10.2 | 0.95±0.01 |
| TAF3 | $H3_{(1-15)}$K4me3Q5(trypamine) | 338.0±62.9 | -11.63±0.07 | -4.57 | 1.09±0.01 |
| | $H3_{(1-15)}$K4me3Q5(5-methoxytrypamine) | 833.0±251.0 | -11.80±0.08 | -3.33 | 1.00±0.01 |
| | $H3_{(1-15)}$K4me3Q5(7-azatryptamine) | 119.0±39.2 | -11.7±0.04 | -6.91 | 1.11±0.01 |
| | $H3_{(1-15)}$K4me3Q5(6-hydroxytryptamine) | 539.0±162.0 | -12.4±0.11 | -6.10 | 1.00±0.01 |
| CHD1 | $H3_{(1-15)}$K4me3 | 0.82±0.10 | -4.68±0.18 | 6.78 | 1.03±0.03 |
| | $H3_{(1-15)}$K4me3Q5ser | 1.85±0.20 | -6.22±0.14 | 3.24 | 1.00±0.02 |
| KDM4A | $H3_{(1-15)}$K4me3 | 5.54±0.37 | -12.64±0.11 | -16.10 | 1.01±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 2.77±0.26 | -12.25±0.21 | -16.20 | 1.01±0.01 |
| ING2 | $H3_{(1-15)}$K4me3 | 1.76±0.15 | -2.84±0.03 | 14.50 | 0.98±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 3.04±0.16 | -3.03±0.02 | 14.90 | 0.96±0.01 |
| BPTF | $H3_{(1-15)}$K4me3 | 5.54±0.07 | -9.48±0.01 | -5.52 | 0.96±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 3.68±0.37 | -7.74±0.10 | -0.49 | 1.07±0.01 |
| SGF29 | $H3_{(1-15)}$K4me3 | 1.04±0.03 | -6.44±0.04 | 1.37 | 1.04±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 2.04±0.12 | -9.24±0.11 | -6.67 | 0.97±0.01 |
| KDM5A | $H3_{(1-15)}$K4me3 | 5.93±0.62 | -10.56±0.19 | -9.02 | 0.86±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 3.44±0.14 | -8.37±0.07 | -2.75 | 1.05±0.01 |
| SPIN1 | $H3_{(1-15)}$K4me3 | 19.60±2.41 | -9.51±0.10 | -3.08 | 0.95±0.01 |
| | $H3_{(1-15)}$K4me3Q5ser | 29.3±4.58 | -10.48±0.10 | -5.55 | 1.03±0.01 |
| ZCWPW1 | $H3_{(1-15)}$K4me3 | 0.10±0.01 | -7.43±0.03 | -6.56 | 1 (fixed) |
| | $H3_{(1-15)}$K4me3Q5ser | 0.49±0.01 | -6.68±0.05 | -0.97 | 1.08±0.06 |

**Table S2.** The protein information of histone H3K4me3 readers.

| No. | Protein | Reader module | Construct |
|---|---|---|---|
| 1 | BPTF | PHD | ref# NP_004450.3 2859-2921 |
| 2 | CHD1 | Chromo | NP_001261.2 270-443 |
| 3 | DIDO1 | PHD | ref# NP_071388.2 266-325 |
| 4 | ING2 | PHD | ref# NP_001555.1 210-262 |
| 5 | KDM5A | PHD | ref# NP_001036068.1 1609-1660 |
| 6 | KDM4A | Tudor | ref# NP_055478.2 895-1008 |
| 7 | MLL5 | PHD | ref# NP_061152.3 117-181 |
| 8 | PHF13 | PHD | ref# NP_722519.2 229-280 |
| 9 | PHF2 | PHD | ref# NP_005383.3 1-70 |
| 10 | RAG2 | PHD | ref# NP_033046.1 414-487 |
| 11 | SET3 | PHD | ref# NP_012954.3 116-184 |
| 12 | SGF29 | Tudor | ref# NP_612423.1 113-254 |
| 13 | SPIN1 | SPIN | ref# NP_006708.2 50-262 |
| 14 | TAF3 | PHD | ref# NP_114129.1 885-921 |
| 15 | ZCWPW1 | CW-Zf | ref# NP_060454.3 246-307 |

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