Supporting Information

Chemical Reporter Facilitates the Detection and Identification of

Lysine HMGylation on Histones

Xiucong Bao, Ying Xiong, Xin Li and Xiang David Li*

Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, China.

Email: xiangli@hku.hk

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General Methods and Materials

Unless otherwise noted, all the chemical reagents were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Media (DMEM), pre-Stained protein, pre-case polyacrylamide gels (4-12% Bis-Tris gels) and high-capacity streptavidin beads were purchased from Thermo Scientific. EDTA-free protease inhibitor was purchased from Roche. Anti-CPS1 (sc-376190) and anti-MDH2 (sc-293474) antibodies were purchased from Santa Cruz. Anti-Sirt5 (#8782) and anti-actin (#4970S) antibodies were purchased from Cell Signaling technology. Anti-Histone H2B (ab1790), anti-Histone H3 (ab1791) and anti-KAT2A (ab18381) antibodies were purchased from Abcam. Anti-AF9 (A300-595A) was purchased from Bethyl Laboratories. Goat anti-rabbit-HRP conjugated secondary antibody and goat anti-mouse-HRP conjugated secondary antibodies were purchased from Thermo Scientific. All contrast/brightness adjustments on the images were performed in ImageJ and applied to the whole gels and blots.

All Fmoc-protected amino acids, resin for solid-phase peptide synthesis, and coupling reagents were purchased from GL Biochem. In-solution reactions were monitored by TLC silica gel 60 F254 from Merck. Flash column chromatography was performed with silica gel purchased from Grace. ¹H and ¹³C NMR spectra were recorded on a Bruker UltraShield 300, 400 or 400 MHz spectrometer and were calibrated using residual undeuterated solvents as internal references. Chemical shifts were reported in values (ppm), and coupling constant J were reported in Hz. Peptides were analyzed by LC-MS with an Agilent 1260 Infinity HPLC system connected to a Thermo Finnigan LCQ DecaXP MS detector. Peptides were purified on a preparative HPLC system with Waters 2535 Quaternary Gradient Module, Waters 515 HPLC pump, Waters SFO System Fluidics Organizer, and Waters 2767 Sample Manager.

Cell Culture

HeLa, HeLa S3 and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were maintained in a humidified 37 °C incubator with 5% CO₂.

Metabolic Labeling and Preparation of Cell Lysates

Cells were treated with different chemical reporters (500 μ M of HMGAM-yne for 3 h, 100 μ M of MalAM-yne for 1 h or 20 mM of 4-pentynoate for 6 h). DMSO (for MalAM-yne and HMGAM-yne) and PBS (for 4-pentynoate) were sued as negative controls. For competition assay, cells were preincubated with 20 mM of HMG for 6 h. Following metabolic labeling, cells were washed with ice-cold PBS trice and then lysed with ice-cold modified RIPA lysis buffer (1% NP 40, 150 mM NaCl, 50 mM HEPES, 2 mM MgCl₂, 10% Glycero,1 pH 7.4, EDTA-free Roche protease inhibitor cocktail, 1 mM phenylmethylsulfonylfluor- ide (PMSF), 0.2U Benzonase). Cell lysates were collected after centrifuging at 16,100 *g* for 15 min at 4 °C to remove cell debris. Protein concentration was determined by the BCA assay (Thermo Scientific). Cell lysates were diluted with IP buffer (150 mM NaCl, 50 mM HEPES, 2 mM MgCl₂, 10% Glycerol, pH 7.4, EDTA-free Roche protease inhibitor cocktail) to achieve final protein concentration of 1.5 mg/mL for click chemistry reactions.

Cu(I)-Catalyzed Cycloaddition/Click Chemistry

Briefly, to the prepared samples, 100 μ M azide-rhodamine for in-gel fluorescence scanning or cleavable azide-biotin for streptavidin enrichment was added, followed by 1 mM tris(2carboxyethyl)phosphine (TCEP), 100 μ M tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA) and finally the reactions were initiated by the addition of 1 mM CuSO₄. The reactions were incubated for 1.5 h at room temperature in dark.

In-gel Fluorescence Visualization

The click chemistry reactions were quenched by adding 1 volume of $2 \times$ sample loading buffer. The proteins were heated at 85 °C for 8 min and resolved by SDS-PAGE. The labeled proteins were visualized by scanning the gel on a Typhoon 9410 variable mode imager (excitation 532 nm, emission 580 nm). Streptavidin affinity enrichment of biotinylated proteins

Streptavidin Affinity Enrichment of Biotinylated Proteins

HMGAM-yne-labelled cell lysate were reacted with azide-biotin (100 μ M, 5 mM stock in DMSO), TCEP (1 mM, 50 mM stock in ddH₂O freshly prepared), TBTA (100 μ M, 10 mM stock in DMSO) and CuSO₄ 5H₂O (1 mM, 50 mM stock in ddH₂O freshly prepared) for 1.5 h at room temperature. Proteins were precipitated by acetone (4 volume) at -20 °C for overnight. Precipitated proteins were centrifuged at 3500 g for 5 min at 4 °C and washed with ice-cold methanol twice. To capture the biotinylated proteins by streptavidin beads, the air-dried protein pellet was dissolved in 2 mL PBS (with 20 mM EDTA, 4% SDS and 10% Glycerol) and subsequently heated at 75 °C for 10 min, diluted by PBS to reduce SDS concentration down to 0.5%. Pre-washed streptavidin beads were then incubated with this protein solution at room temperature for 1.5 h on end-over-end rotator. After sequentially washed six times with PBS (with 0.2% SDS), 6M Urea (in PBS with 0.1% SDS), 250 mM NH₄HCO₃ (with 0.05% SDS), the enriched proteins were eluted by incubating with elution buffer (25 mM Na₂S₂O₄, 250 mM NH₄HCO₃ and 0.05% SDS) for 1 h. The eluted proteins were then dried down with SpeedVac.

Enzymatic Reactions.

The enzymatic activities of human Sirtuins were measured by detecting the removal of HMG group from H3K9hmg peptide. 5 μ M of each Sirtuin protein was incubated with 500 μ M of peptide and 1 mM of nicotinamide adenine dinucleotide (NAD) in a reaction buffer containing 20 mM Tris-HCl buffer (pH 7.5) and 1 mM DTT at 37 °C for 2 h. The reactions were stopped by adding 1/3 reaction volume of 20% TFA and frozen in liquid N₂ immediately. Samples were then analyzed by LC-MS with a Vydac 218TP C18 column (4.6 mm × 250 mm, 5 μ m, Grace Davison). Mobile phases used were 0.05% TFA in water (buffer A) and 0.05% TFA in ACN (buffer B). The flow rate for LC was 0.6 mL/min. The peptide mixtures were eluted by buffer A for 10 min, then 0-30% buffer B over 10 min. MS started to record at 10 min for each injection.

Isothermal Titration Calorimetry Measurements.

Experiments were performed at 25 °C on a MicroCal iTC200 titration calorimeter (MicroCal). The reaction cell containing 200 μ L of 150 μ M proteins was titrated with 17 injections, first 0.5 μ L, and all subsequent injections of 2 μ L of 2.5 mM peptides. The

binding isotherm was fit with Origin 7.0 software package (OriginLab) that uses a single set of independent sites to determine the thermodynamic binding constants and stoichiometry.

RNAi Experiments

30 nM of Sirt5 siRNA (Thermo Scientific) was transfected into HeLa cells with Lipofectamine 2000 Transfection Reagent (Thermo Scientific), according to the manufacturer's instructions. Corresponding concentration of control siRNA was used as negative control. Following transfection, cells were then maintained in a humidified 37 $^{\circ}$ C incubator with 5% CO₂ for another 48 h.

Immunofluorescence

HeLa cells grown on cover slips were metabolically labeled with DMSO or HMGAM-yne, fixed with 3.7% PFA, permeabilized with 0.1% Triton X-100 and then reacted with 20 μ M azide-rhodamine, 1 mM TCEP, 100 μ M TBTA and 1 mM CuSO₄ for 1 h. Cells were incubated with DAPI for 5min, washed trice with PBST (0.1% tween 20 in PBS) and then subjected to a Zeiss LSM 510 laser scanning confocal microscope.

Cellular Fractionation

Mitochondrial and nuclear extraction were performed as previously described ^[1].

Histone Extraction

Acid-extraction method was used to isolate histones from HeLa S3 cells^[2].

Sample Preparation for Mass Spectrometry

In-solution tryptic digestion of histone samples was carried out based on previous described protocols ^[3]. Histone extracts were in-solution digested either without chemical propionylation, or chemically propionylated before or after in-solution trypsin digestion. Histone proteins or peptides were treated with propionic anhydride twice to make sure fully labeling. The final resulting peptides were enriched and desalted with the StageTips. The

eluted peptides from the StageTips were dried down by SpeedVac and then resuspended in 0.5% acetic acid for analysis by LC-MS/MS.

Mass Spectrometry

Mass spectrometry was performed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). First, peptide samples in 0.1% formic acid were pressure loaded onto a self-packed PicoTip column (New Objective) (360- μ m o.d., 75- μ m i.d., 15- μ m tip), packed with 7–10 cm of reverse-phase C18 material (ODS-A C18 5- μ m beads from YMC), rinsed for 5 min with 0.1% formic acid and subsequently eluted with a linear gradient from 2 to 35% B in 150 min (A = 0.1% formic acid, B = 0.1% formic acid in ACN, flow rate ~ 200 nL/min) into the mass spectrometer. The instrument was operated in a data dependent mode cycling through a full scan (300–2,000 m/z, single μ scan) followed by 10 CID MS/MS scans on the 10 most abundant ions from the immediate preceding full scan. The cations were isolated with a 2-Da mass window and set on a dynamic exclusion list for 60 seconds after they were first selected for MS/MS. The raw data were processed and analyzed using MaxQuant setting lysine HMGylation as a variable modification to identify H-K sites in histones. A human histone fasta file was used as protein sequence searching database. Default parameters were adapted for the protein identification and quantification.

Synthesis of HMGAM-yne.



Scheme S1. Synthetic route of HMGAM-yne. TBS = *tert*-butyldimethylsilyl, THF = tetrahydrofuran, r.t. = room temperature, DMSO = dimethyl sulfoxide, Et = ethyl, DCM = dichloromethane, Ts = tosyl, Me = methyl, Py = pyridine, DIEA = N,N-diisopropylethylamine, DMF = N,N-dimethylformamide.

Preparation of 2,2,3,3,9,9,10,10-octamethyl-4,8-dioxa-3,9-disilaundecan-6-ol (1). To the solution of glycerol (5 g, 54 mmol) dissolved in 100 mL anhydrous tetrahydrofuran, imidazole (8.81 g, 129 mmol) and TBSCl (17.8 g, 118 mmol) were added at 0 °C. The mixture was warmed up and stirred at room temperature for overnight. The reaction was quenched with 50 mL water and extracted with 50 mL ethyl acetate for 3 times. The organic

layers were combined and dried over anhydrous Na₂SO₄. After filtration and concentration, the crude product was purified by flash column chromatography (hexane:ethyl acetate = 100:1 ~ 10:1) to afford compound **1** (16.02 g 93%) as a colorless oil. ¹H-NMR (CDCl₃, 400 MHz) δ 3.66–3.63 (m, 5H), 2.45 (d, *J* = 6.0 Hz, 1H), 0.89 (s, 18H), 0.07 (s, 12H). ¹³C-NMR (CDCl₃, 100 MHz) δ 71.98, 63.57, 26.00, 18.40, -5.30. The spectroscopic data match with the previous report ^[4].

Preparation of 2,2,3,3,9,9,10,10-octamethyl-4,8-dioxa-3,9-disilaundecan-6-one (2). To the solution of oxalyl chloride (4.68 g, 36.6 mmol) dissolved in 30 mL anhydrous dichloromethane at -78 °C, dimethyl sulfoxide solution (7.3 M, 10 mL) in anhydrous dichloromethane was slowly added over 15 min. The mixture was kept stirring at -78 °C for 20 min and compound 1 (7.82 g, 24.4 mmol dissolved in 10 mL anhydrous dichloromethane was slowly added over 10 min. The mixture was kept stirring for another 1.5 hours at -78 °C. Dry triethylamine (12.32 g, 122 mmol) was added and the mixture was kept stirring for 15 min at -78 °C before it was warmed to room temperature for another 1 hour. The reaction was guenched with 30 mL water and extracted with 30 mL ethyl acetate for 3 times. The combined organic layers were washed with 30 mL brine and dried over anhydrous Na_2SO_4 . After filtration and concentration, the crude product was purified by flash column chromatography (hexane:ethyl acetate = $100:1 \sim 10:1$) to afford compound 2 (7.62 g 98%) as colorless oil. ¹H-NMR (CDCl₃, 400 MHz) δ 4.42 (s, 4H), 0.92 (s, 18H), 0.09 (s, 12H). ¹³C-NMR (CDCl₃, 100 MHz) δ 208.72, 68.00, 25.86, 18.42, -5.45. The spectroscopic data match with the previous report^[5].

Preparation of 2,2,3,3,9,9,10,10-octamethyl-6-(prop-2-yn-1-yl)-4,8-dioxa-3,9-disilaundecan-6-ol (3). To a 50 mL flame-dried three-neck flask, mercury chloride (59 mg, 0.22 mmol), Mg (115 mg, 4.8 mmol) and one particle of iodine were added under the protection of inert gas. 10 mL dry Et₂O and 10% of propargyl bromide (46.6 μ L, 0.43 mmol, diluted with 1 mL dry Et₂O) was added at room temperature. The mixture was gently heated with hot gun till the decay of brown color in solution. The mixture was cooled to 0 °C with ice bath and another portion of propargyl bromide (420 μ L 3.9 mmol in 3 mL dry Et₂O) was added over 40 minutes. Then the mixture was kept stirring at 0 °C for another 2 hours to afford light green supernatant with pulverized magnesium precipitates. The supernatant was used *in situ* directly.

To a solution of compound **2** (800 mg, 2.31 mmol) dissolved in 5 mL dry THF, the supernatant of the above Grignard reagent was slowly added at 0 °C under Ar protection. The mixture was stirred at 0 °C for 1 hour and gradually warmed up to room temperature for another 1 hour. The reaction was quenched with 20 mL saturated NH₄Cl solution and the aqueous layer was extracted with 30 mL ethyl acetate for 3 times. The organic layers were pooled and washed with 30 mL brine, dried over anhydrous Na₂SO₄. After filtration and concentration, the crude product was purified by flash column chromatography to afford compound **3** (892 mg, 80%) as colorless oil. ¹H-NMR (CDCl₃, 400 MHz) δ 3.61–3.52 (m, 4H), 2.69 (s, 1H), 2.41 (d, *J* = 2.7 Hz, 2H), 2.00 (t, *J* = 2.6 Hz, 1H), 0.89 (s, 18H), 0.06 (s, 12H). ¹³C-NMR (CDCl₃, 100 MHz) δ 80.47, 73.67, 70.55, 64.79, 25.97, 24.40, 18.35, -5.39. HRMS (EI) calculated *m*/*z* for [M]⁺: 301.1655, found: 301.1659.

Preparation of 2-(prop-2-yn-1-yl)propane-1,2,3-triol (4). To a solution of compound **3** (4.44 g, 12.4 mmol) in 10 mL MeOH, *p*-toluenesulfonic acid (129 mg, 0.68 mmol) was added and followed by 1 mL H₂O. The resulting mixture was kept stirring at room temperature overnight. The solvent was removed *in vacuo* and the resulting residue was subjected to flash column chromatography (hexane:ethyl acetate = 1:1 ~ pure ethyl acetate) to afford compound **4** (1.51 g, 94%) as sticky oil. ¹H-NMR (MeOD, 400 MHz) δ 3.60–3.54 (m, 4H), 2.42 (d, *J* = 2.7 Hz, 2H), 2.29–2.27 (t, *J* = 2.7 Hz, 1H). ¹³C-NMR (MeOD, 100 MHz) δ 81.09, 75.02, 71.68, 65.49, 24.99. HRMS (ESI) calculated *m/z* for [M+Na]⁺: 153.0528 found: 153.0525.

Preparationof2-hydroxy-2-(prop-2-yn-1-yl)propane-1,3-diylbis(4-methylbenzenesulfonate)(5). To the solution of compound 4 (1.5 g, 11.6 mmol) in 50 mLanhydrous pyridine, TsCl (6.62 g, 34.89 mmol)was added slowly at 0 °C. The resultingmixture was kept stirring at room temperature overnight and quenched with 30 mL water.The resulting aqueous mixture was extracted with 30 mL ethyl acetate for 3 times. Theorganic layers were combined and washed with 1 M HCl for 3 times and dried overanhydrous Na₂SO₄. After filtration and concentration, the resulting residue was subjectedto flash chromatography (hexane:EA = 6:1) to afford compound 5 (4 g, 79%) as white solid.¹H-NMR (CDCl₃, 400 MHz) δ 7.77 (d, *J* = 8.3 Hz, 4H), 7.36 (d, *J* = 8.0 Hz, 4H), 4.01 (m,4H), 2.61 (s, 1H), 2.46–2.44 (m, 8H), 1.97 (t, *J* = 2.7 Hz, 1H).the solution of the solution of the

Preparation of 3-hydroxy-3-(prop-2-yn-1-yl)pentanedinitrile (6). To the solution of compound **5** (2 g, 4.57 mmol) dissolved in 5 mL anhydrous DMSO, NaCN (671 mg, 13.7 mmol) was added. The mixture was kept stirring at room temperature overnight. The reaction was quenched by 20 mL water and the aqueous layer was extracted with 20 mL ethyl acetate for 3 times. The combined organic layers were washed with 20 mL water for twice and dried over anhydrous Na₂SO₄. After filtration and concentration, the resulting residue was subjected to flash column (hexane:EA = 6:1) to afford compound **6** (480 mg, 71%) as yellow liquid. ¹H-NMR (CDCl₃, 300 MHz) δ 2.99 (s, 1H), 2.87 (s, 4H), 2.75 (d, *J* = 2.6 Hz, 2H), 2.29 (t, *J* = 2.6 Hz, 1H). ¹³C-NMR (CDCl₃, 100 MHz) δ 116.05, 77.47, 74.23, 70.73, 29.90, 28.53. HRMS (EI) calculated *m*/*z* for [M]⁺: 130.0531 found: 130.0522.

Preparation of 3-hydroxy-3-(prop-2-yn-1-yl)pentanedioic acid (7). To the solution of compound **6** (271 mg, 1.83 mmol) dissolved in 5 mL MeOH, 5 mL 35% (v/v) NaOH aqueous solution was added slowly at 0 °C. The resulting solution was kept stirring at 60 °C overnight. The mixture was diluted with 20 mL ethyl acetate and the organic layer was discarded after partition. The aqueous layer was carefully acidified to pH = 1 at 0 °C with concentrated HCI. The aqueous solution was extracted with 30 mL ethyl acetate for 3 times and organic layers were pooled and dried over anhydrous Na₂SO₄. After filtration and concentration, the resulting residue was subjected to flash column chromatography (MeOH: dichloromethane = 10:1 + 1% acetic acid) to afford compound **7** (100 mg 30%) as yellow oil. ¹H-NMR (MeOH, 300 MHz) δ 2.84–2.73 (m, 4H), 2.69 (d, *J* = 2.7 Hz, 2H), 2.37 (t, *J* = 2.7 Hz, 1H). ¹³C-NMR (MeOH, 100 MHz) δ 174.53, 80.93, 72.55, 71.79, 43.04, 30.91. HRMS (EI) calculated *m/z* for [M]⁺: 209.0426 found: 209.0418.

Preparation of HMGAM-yne. To the solution of compound **7** (110 mg, 0.59 mmol) dissolved in 1 mL anhydrous DMF, bromomethyl acetate (127 μ L, 1.3 mmol) was added slowly at 0 °C and followed by DIEA (260 μ L, 1.5 mmol), the resulting mixture was kept stirring at room temperature overnight. The reaction was diluted with 20 mL ethyl acetate and washed with 10 mL water for 3 times. The organic layers were pooled and washed with 10 mL brine and dried over anhydrous Na₂SO₄. After filtration and concentration, the resulting residue was subjected to flash column (hexane:EA = 3:1~1:1) to afford **HMGAM-yne** (155 mg 80%) as colorless oil. ¹H-NMR (CDCl₃, 400 MHz) δ 5.77 (s, 4H), 3.78 (s, 1H), 2.96–2.86 (m, 4H), 2.67(d, *J*= 2.64, 2H), 2.13–2.11 (m, 7H). ¹³C-NMR (CDCl₃, 100 MHz) δ 170.02, 169.69, 79.31, 72.36, 71.13, 41.96, 30.56, 20.80. HRMS (ESI) calculated *m*/*z* for [M+Na]⁺: 353.0849 found: 353.0844.

Synthesis of Fmoc-Lys(all)-OH.



Scheme S2. Synthetic route of Fmoc-Lys(all)-OH. DMAP = 4-dimethylaminopyridine, r.t. = room temperature, NHS = N-hydroxysuccinimide, DCC = N,N'- dicyclohexylcarbodiimide, THF = tetrahydrofuran, Fmoc = 9-fluorenylmethyloxycarbonyl, DIEA = N,N-diisopropylethylamine.

Preparation of 5-(allyloxy)-3-hydroxy-3-methyl-5-oxopentanoic acid (8). To a 25 mL round bottom flask, 3-hydroxy-3-methylglutaric acid (230 mg, 1.42 mmol) was dissolved in 6 mL acetic anhydride. The resulting solution was kept stirring at room temperature for 5 hours. The solvent was removed *in vacuo* to afford the corresponding anhydride as white solid, which was directly subjected to the following step without further purification.

To the solution of the above-obtained solid in 20 mL anhydrous toluene, allyl alcohol (482 μ L, 7.1 mmol) and DMAP (17.3 mg, 0.14 mmol) were added at room temperature. The resulting mixture was kept stirring at room temperature overnight. The mixture was diluted with 20 mL ethyl acetate and washed with 10 mL water for 3 times. The organic layer was pooled and dried over anhydrous Na₂SO₄. After filtration and concentration, the resulting residue was subjected to flash column chromatography (EA:hexane=1:1) to afford compound **8** (242 mg, 84%) as colorless liquid. ¹H-NMR (CDCl₃, 400 MHz) δ 5.93–5.88 (m, 1H), 5.36–5.26 (m, 2H), 4.64–4.63(d, *J* = 5.8 Hz) 2.76–2.64 (m, 4H), 1.41 (s, 3H). ¹³C-NMR (DMSO-d₆, 100 MHz) δ 174.85, 172.29, 133.65, 118.36, 70.68, 66.08, 46.14, 45.84, 27.72. HRMS (ESI) calculated *m/z* for [M+Na]⁺: 225.0739 found: 225.0733.

Preparation of Fmoc-Lys(all)-OH. To the solution of compound **8** (448 mg, 1.2 mmol) dissolved in 10 mL anhydrous THF, NHS (157 mg, 1.37 mmol) and DCC (282 mg, 1.37 mmol) were slowly added at 0 °C. The resulting mixture was gradually warmed to room temperature for overnight.

The resulting white suspension was filtrated through celite into a 25 mL round bottom flask. Fmoc-lysine HCl (383 mg, 0.95 mmol) and DIEA (480 µL, 2.74 mmol) were added. The resulting mixture was stirred at room temperature for overnight. The reaction was quenched by adjusting pH to 7 with 1 M HCl at 0 °C. Solvent was removed in vacuo and 5 mL H₂O was added to the mixture and pH was adjusted to 2 with 1 M HCl at 0 °C. The mixture was extracted with 30 mL dichloromethane for 3 times and the organic layer was pooled and dried over anhydrous Na₂SO₄. After filtration and concentration, the crude product was subjected to column chromatography (EA: hexane = 1:3 + 1% acetic acid) to afford **Fmoc**-Lys(all)-OH (287 mg, 55%) as colorless solid. ¹H-NMR (MeOD, 500 MHz) δ 7.79 (d, J = 7.5 Hz, 2H), 7.67 (t, J = 7.9 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.5, 2H), 5.92 (m, 1H), 5.30 (dd, J = 17.2, 1.5 Hz, 1H), 5.19 (dd, J = 10.5, 1.3 Hz, 1H), 4.56 (d, J = 5.7Hz, 2H), 4.38-4.30 (m, 2H), 4.22 (t, J = 7.0 Hz, 1H), 4.15-4.08 (m, 1H), 3.21-3.13 (m, 2H), 2.51 (s, 2H), 2.53–2.41 (m, 2H), 1.85 (mz, 1H), 1.74–1.64 (m, 1H), 1.55 (m, 2H), 1.43 (m, 2H), 1.32 (s, 3H). ¹³C-NMR (MeOD, 125 MHz) δ 176.51, 173.73, 172.44, 158.81, 145.52, 145.35, 142.74, 133.78, 128.94, 128.33, 128.31, 126.45, 126.42, 121.06, 118.61, 71.44, 68.08, 66.32, 55.65, 48.57, 47.26, 46.79, 40.13, 32.64, 30.02, 27.76, 24.46. HRMS (ESI) calculated *m*/*z* for [M+Na]⁺: 553.2550 found: 553.2549.

H3K9hmg peptide synthesis and purification.



Scheme S3. Synthetic route of H3K9hmg peptide. SPPS = solid-phase peptide synthesis, Pg = acid labile protecting group, de-allylation cocktail: 2.5% *N*-methyl morpholine, 5% acetic acid in dry dichloromethane (v/v), cleavage cocktail: 2.5% triisopropylsilane, 1.5% H₂O and, 1% thioanisole in trifluoroacetic acid (TFA) (v/v).

Peptide was synthesized on Rink-Amide MBHA resin followed standard Fmoc-based solid-phase peptide synthesis protocol. After the coupling of all amino acids, the last Fmoc group was removed, followed by the on-beads O-allylester deprotection. The resin was rinsed with 5 mL anhydrous dichloromethane for 3 times. The resin was charged in a flame-dried 10 mL round bottom flask with argon protection. To another 25 mL round bottom flask, 3.7 mL anhydrous dichloromethane was de-gassed with argon for 20 min and 100 μ L *N*-methyl morpholine and 200 μ L acetic acid were added to prepare the de-allylation cocktail. Pd(PPh₃)₄ (87 mg 0.075 mmol) was dissolved in the de-allylation cocktail and the resulting solution was subjected to the round bottom flask containing resin under argon protection. The reaction was kept stirring for 2 hours. The solution was discarded by

filtration and the beads were washed with 5% DIEA solution (v/v in dry DMF) and followed by washing with 5 mL dichloromethane for 5 times. The deprotection procedure should be repeated for another time to ensure a full removal of the allyl ester. The resulting resin was readily subjected to cleavage with cleavage cocktail containing 95% trifluoroacetic acid, 2.5% triisopropylsilane, 1.5% H₂O and, 1% thioanisole for 2 h. Peptides were purified by preparative HPLC with an XBridge Prep OBDTM C18 column (30 mm × 250 mm, 10 μ m, Waters). Mobile phase used were water with 0.1% TFA (buffer A) and 90% ACN in water with 0.1% TFA (buffer B). The purity (> 95%) and identity of peptides were confirmed by LC-MS.



Figure S1. Metabolic labeling of MDH2 and CPS1 by HMGAM-yne. (a) Immunoblotting analyses showing the enrichment of two known protein substrates of HMGylation, CPS1 and MDH2, by HMGAM-yne. (b) Immunoblotting analyses showing the metabolic labelling of MDH2 and CPS1 with HMGAM-yne in the absence or presence of HMG as a competitor. After metabolic labeling, the labeled proteins were then conjugated were conjugated to biotin and isolated by high-capacity streptavidin beads, followed by immunoblotting analysis. (c) Quantitative analysis of immunoblotting results (a-b). Error bars indicated \pm s.e. three independent biological replicates. The p values are based on the Student's t test. *p<0.05, **p <0.01, ***p <0.001.



Figure S2. Sirt5 regulates the dynamics of lysine malonylation, glutarylation and HMGylation. (a) The hydrolysis of H3K9mal/glu/hmg by Sirt5 was analyzed by liquid chromatography–mass spectrometry (LC-MS). Pink traces show ion intensity for the masses of unmodified peptide; and blue traces show ion intensity for the masses of malonylted/glutarylated/HMGylated peptide. (b) Isothermal titration calorimetry (ITC) measurement for the binding affinity of Sirt5 towards the H3K9mal/glu/hmg peptides. (c) A summary of the dissociation constants (K_d), enthalpy changes (ΔH), and andentropy changes (ΔS) of Sirt5 binding to H3K9mal/glu/hmg peptides.



Figure S3. Sirt5 regulates the dynamics of lysine HMGylation in living cell. In-gel fluorescence analyses showing that Sirt5 knockdown caused the accumulations of lysine HMGylation at some protein bands indicated with red arrow. Coomassie-blue (CB) staining showing the equal loading. Immunoblotting analysis showing Sirt5 knockdown efficiency. γ -actin was used as a loading control.



Figure S4. Metabolic labeling of mitochondrial (Mit) and nuclear (Nuc) proteins by HMGAM-yne. Nuclear extracted proteins with molecular weight around 15 kD was marked by red arrows. Immunoblotting analysis shows the purity of the mitochondrial extraction.



Figure S5. Metabolic labeling of H2B and H3 by HMGAM-yne. (a) Immunoblotting analyses showing the enrichment histone H2B and H3 by HMGAM-yne. (b) Immunoblotting analyses showing the metabolic labelling of H2B and H3 with HMGAM-yne in the absence or presence of HMG as a competitor. After metabolic labeling, the labeled proteins were conjugated to biotin and isolated by high-capacity streptavidin beads, followed by immunoblotting analysis. (c) Quantitative analysis of immunoblotting results (**a-b**). Error bars indicated \pm s.e. three independent biological replicates. The p values are based on the Student's t test. *p<0.05, **p <0.01, ***p <0.001.



Figure S6. Metabolic labeling of AF9 and KAT2A by HMGAM-yne. (a) Immunoblotting analyses showing the enrichment of two nuclear proteins, AF9 and KAT2A, by HMGAM-yne. (b) Immunoblotting analyses showing the metabolic labelling of AF9 and KAT2A with HMGAM-yne in the absence or presence of HMG as a competitor. After metabolic labeling, the labeled proteins were conjugated to biotin and isolated by high-capacity streptavidin beads, followed by immunoblotting analysis. (c) Quantitative analysis of immunoblotting results (a-b). Error bars indicated \pm s.e. from three independent biological replicates. The p values are based on the Student's t test. *p<0.05, **p <0.01, ***p <0.001.

No.	Protein	Position	Sequence	Incorporation rate %
1	Histone H4	59	⁵⁶ prVLKhmgVFLENVIR ⁶⁷	1.95
2	Histone H3	27	$^{27} {}_{\rm pr} K_{\rm hmg} SAPATGGV K^{36}$	4.8
3	Histone H3	18	¹⁸ KhmgQLATKprAAR ²⁶	<0.1
4	Histone H3	9	⁹ KhmgSTGGKhmgAPR ¹⁷	3
4	Histone H3	14	⁹ KhmgSTGGKhmgAPR ¹⁷	3
5	Histone H2A	119	¹¹⁹ prKhmgTESHHK ¹²⁵	5

 Table S1. The estimated incorporation rates of HMGylated lysine in histones.





LC–MS analysis of H3K9hmg peptide: Calculated m/z for $[M + H]^+$: 1375.77, found: 1375.68; calculated m/z for $[M + 2H]^{2+}$: 688.39, found: 688.54; calculated m/z for $[M + 3H]^{3+}$: 459.26, found: 459.42.



LC–MS analysis of H3K9mal (1-15) peptide: Calculated m/z for $[M + H]^+$: 1645.9, found: 1645.74; calculated m/z for $[M + 2H]^{2+}$: 823.45, found: 823.71; calculated m/z for $[M + 2H - CO_2]^{2+}$: 801.71, found: 801.79.



LC–MS analysis of H3K9glu peptide: Calculated m/z for $[M + H]^+$: 1345.76, found: 1345.69; calculated m/z for $[M + 2H]^{2+}$: 673.38, found: 673.62; calculated m/z for $[M + 3H]^{3+}$: 449.25, found: 449.46.





The CID MS/MS spectrum of a doubly charged tryptic-digest peptide, $_{pr}^{56}$ GVLK_{hmg}VFLENVIR⁶⁷, from histone H4.



The CID MS/MS spectrum of a doubly charged tryptic-digest peptide, $_{pr}^{27}K_{hmg}SAPATGGVK^{36}$, from histone H3.



The CID MS/MS spectrum of a doubly charged tryptic-digest peptide, ${}^{18}K_{hmg}QLATK_{pr}AAR^{26}$, from histone H3.



The CID MS/MS spectrum of a doubly charged tryptic-digest peptide, $_{pr}^{119}K_{hmg}TESHHK^{125}$, from histone H2A.

Note S3. ¹H- and ¹³C-NMR spectra for all the compounds synthesized in this study





























Supplementary References

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