Supporting Information

SIRT2 reverses 4-oxononanoyl lysine modification on histones

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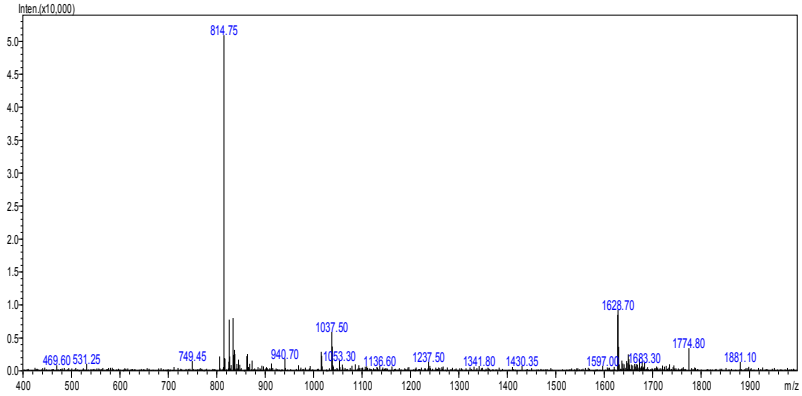
Contents of Supporting Information

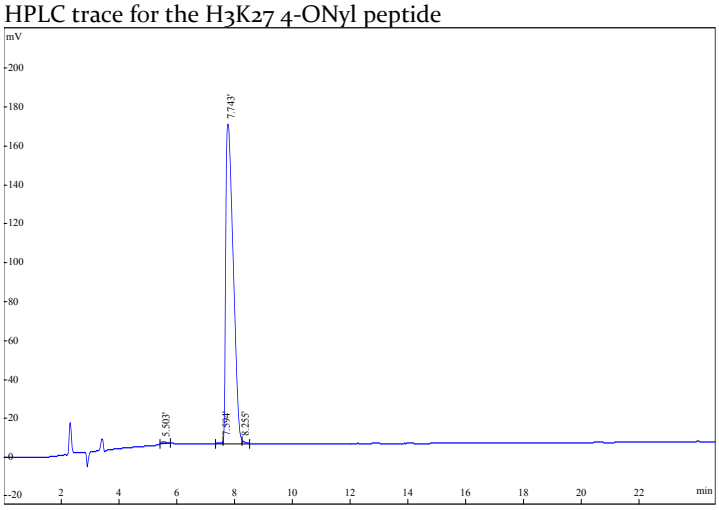
Reagents and materials. Unless otherwise noted, all the chemical reagents were purchased from Sigma-Aldrich. . Dulbecco's Modified Eagle Media (DMEM), fetal bovine serum (FBS), MEM non-essential amino acids, penicillin streptomycin and L-glutamine were purchased from Thermo Scientific. Ethylene diamine tetraacetic acid (EDTA)-free protease inhibitor was purchased from Roche Applied Science. Tetramethylrhodamine (TAMRA) Azide (catalog # T10182) were purchased from Thermo Scientific. Lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS) was purchased from Sigma. 4-Oxo-2-nonenal alkyne probe was from Cayman. LCMS was carried out on a SHIMADZU LC and Thermo LCQ FLEET MS with a Sprite TARGA C18 column (40 × 2.1 mm, 5 µm, Higgins Analytical, Inc.) monitoring at 215 and 280 nm. Solvents used in LCMS were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. Preparative HPLC purification was carried out using Targa™ Prep C18 10µm 250x20mm reverse phase column with UV detection at 215 nm and 280 nm. Analytic HPLC analysis was carried out using Kinetex XB-C18 100A, 100 mm × 4.60 mm, 2.6 μm reverse phase column with UV detection at 215 nm and 280 nm. The binding affinity study was investigated on the microscale thermophoresis instrument of Monolith NT.115 (Nanotemper Technologies GmbH, Munich, Germany). The value of K_d was calculated by Nanotemper Analysis software, v.1.2.101.

Synthesis of Peptides. Starting from Wang Resin (200 mg, 0.1mmol), the peptide synthesis was carried out using standard Fmoc/tBu chemistry O-benzotriazol-N,N,N',N'-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazol (HBTU/HOBt) protocol. The 4-ONylation of lysine residue was done by using Fmoc-Lys(Dde)-OH coupled into the peptide followed by hydrolysis of 2% hydrazine hydrate and coupling with 4-oxononanoic acid at the last. For cleavage from the resin, the resin was suspended in a solution of trifluoroacetic acid (TFA) containing phenol (5%), thioanisole (5%), ethanedithiol (2.5%), and water (5%) for 2 hrs at room temperature. The resin was filtered and the filtrate was then concentrated in vacuum. For removal of protecting groups, the above residue was treated with TFA (2 mL) for 4 hours. The crude peptides were purified by reverse phase HPLC on BECKMAN COULTER System Gold 125P solvent module & 168 Detector with a TARGA C18 column (250 × 20 mm, 10 µm, Higgins Analytical, Inc., Mountain View, CA) monitoring at 280 nm. Mobile phases used were 0.1% aqueous TFA (solvent A) and 0.1% TFA in acetonitrile (solvent B). Peptides were eluted with a flow rate of 10 mL/min with the following gradient: 0% solvent B for 5 min, then 0 % to 25% solvent B over 25 min. The identity and purity of the peptides were verified by LCMS.

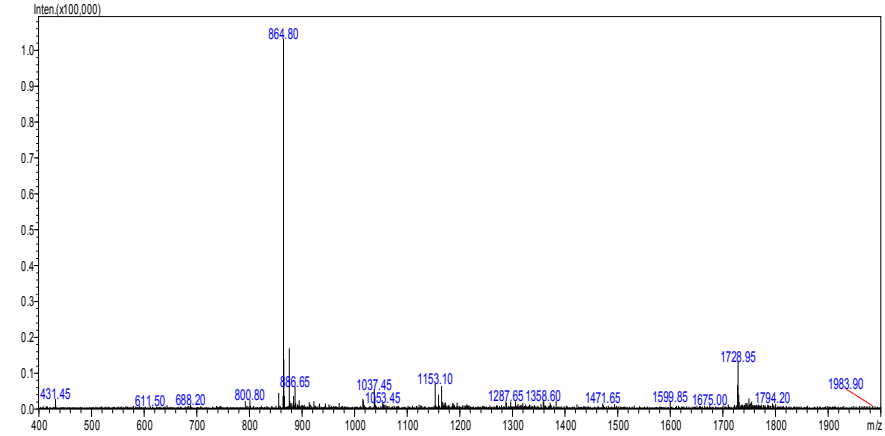
MS spectrum for the H3K27 4-ONyl peptide, TKAARK(4-ONyl)SAPATWW, LCMS (ESI) calcd. for $C_{77}H_{118}N_{20}O_{19}$ $[M+H]^+$ 1627.89, obsd. 1628.70; $[M+2H]^{2+}$ 814.45, obsd. 814.75.



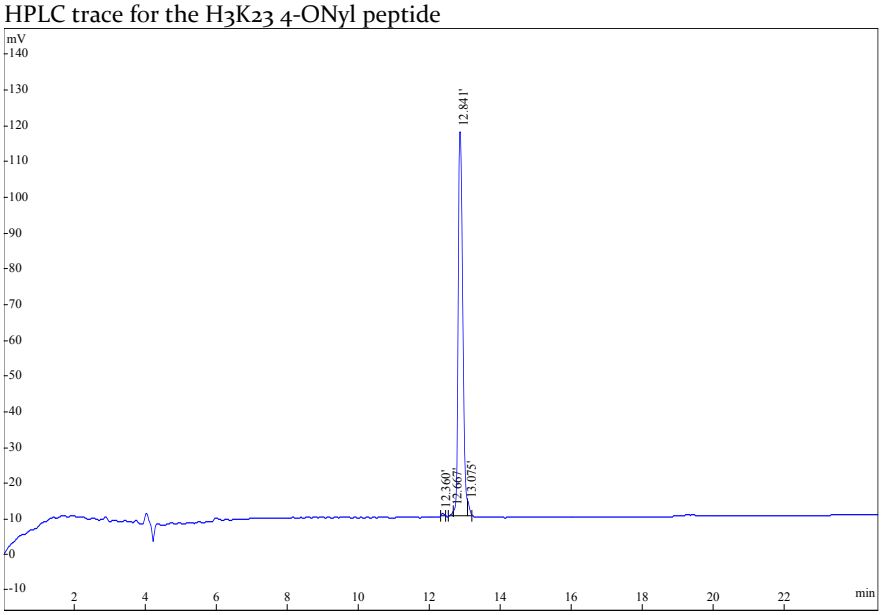
HPLC trace for the H3K27 4-ONyl peptide



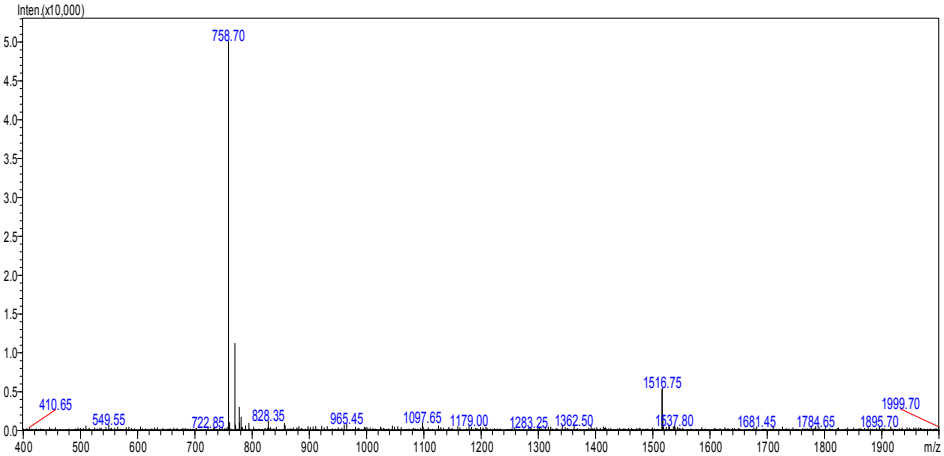
MS spectrum for the H3K23 4-ONyl peptide, KQLATK(4-ONyl)AARKSWW, LCMS (ESI) calcd. For $C_{82}H_{130}N_{22}O_{19}$ $[M+H]^+$ 1728.88, obsd. 1728.95; $[M+2H]^{2+}$ 864.50, obsd. 864.80.



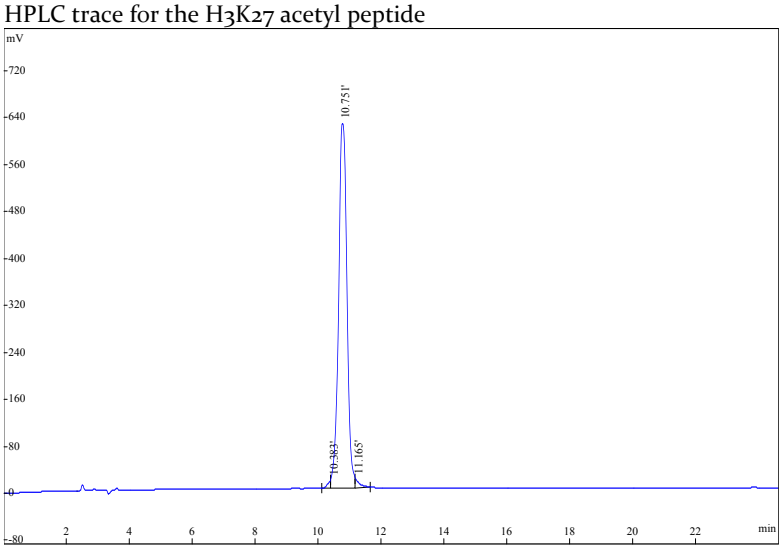
HPLC trace for the H3K23 4-ONyl peptide



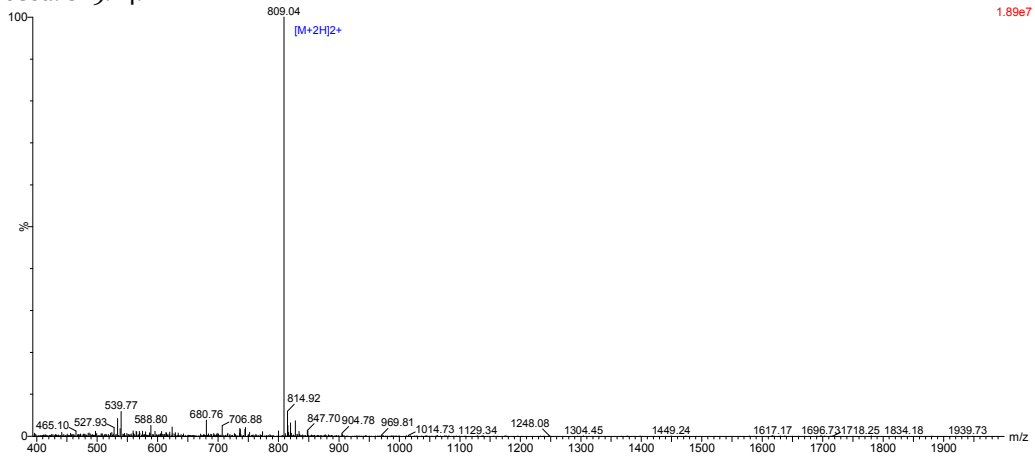
MS spectra for the H3K27 acetyl peptide, TKAARK(Ac)SAPATWW, LCMS (ESI) calcd. for $C_{70}H_{106}N_{20}O_{18}$ $[M+H]^+$ 1515.81, obsd. 1516.75; $[M+2H]^{2+}$ 758.41, obsd. 758.70.



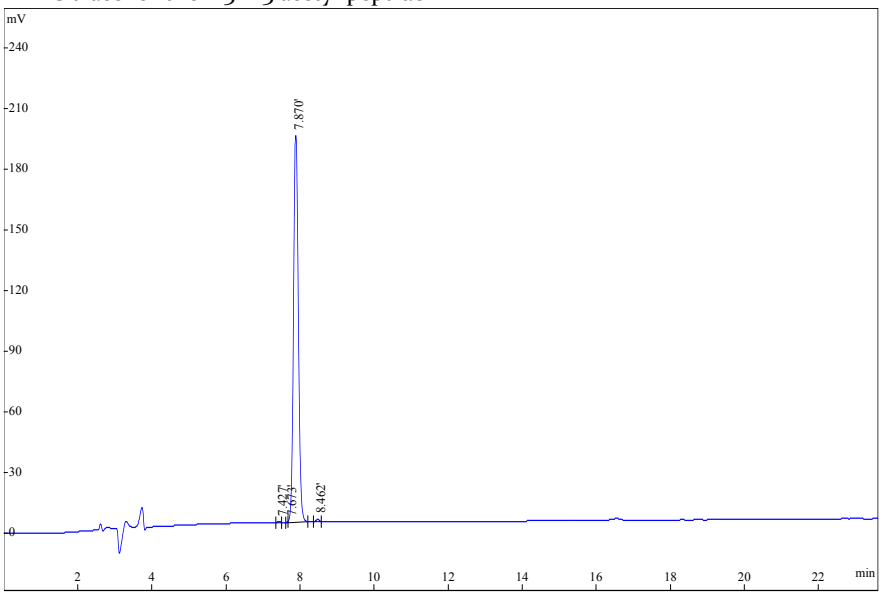
HPLC trace for the H3K27 acetyl peptide



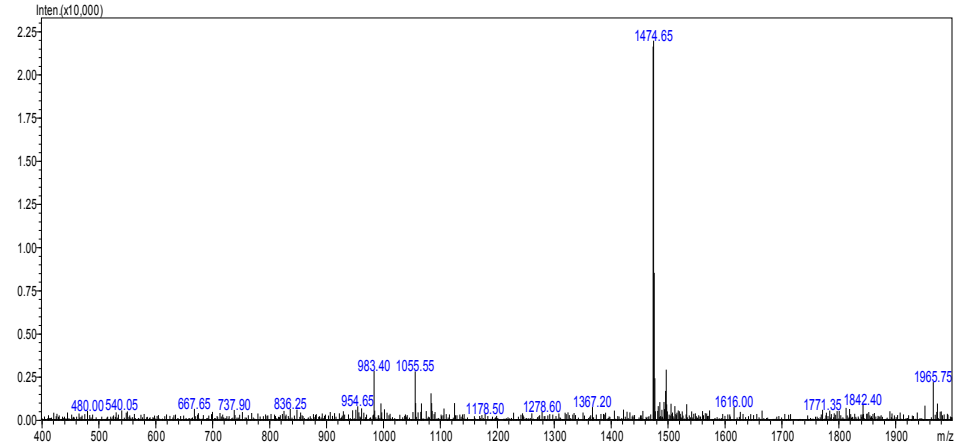
MS spectrum for the H3K23 acetyl peptide, KQLATK(Ac)AARKSWW, LCMS (ESI) calcd. for $C_{75}H_{118}N_{22}O_{18}$ $[M+2H]^{2+}$ 808.46, obsd. 809.04.



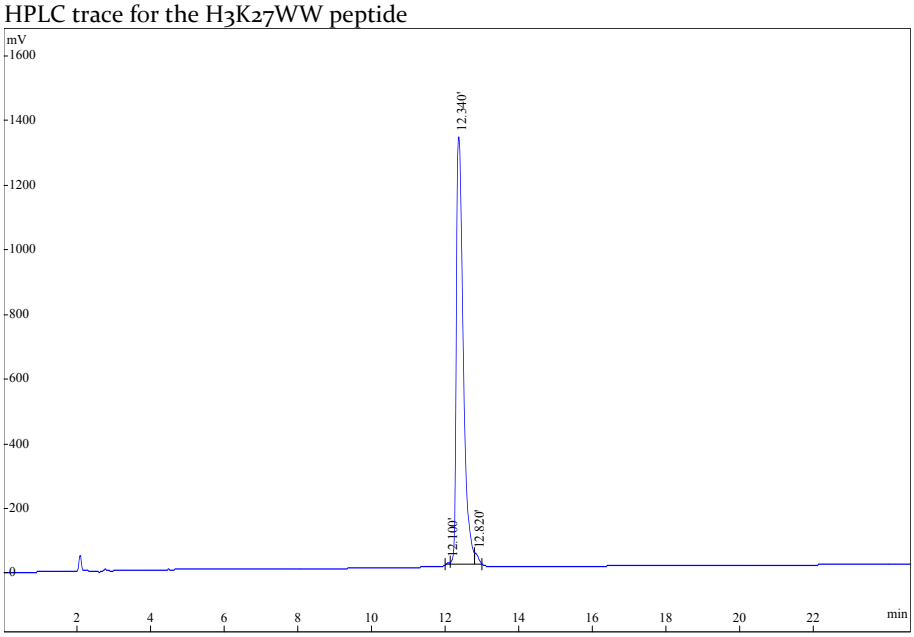
HPLC trace for the H3K23 acetyl peptide



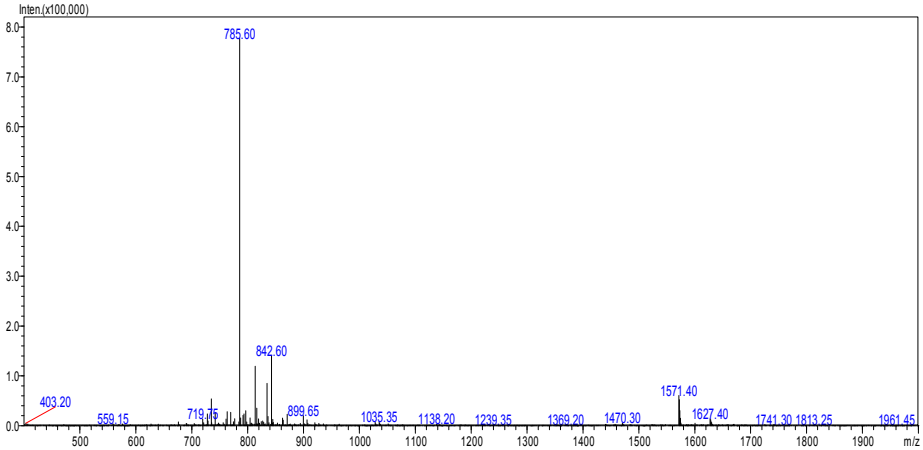
MS for the H3K27WW peptide, TKAARKSAPATWW , LCMS (ESI) calcd. for $C_{68}H_{105}N_{20}O_{17}$ $[M+H]^+$ 1474.80, obsd. 1474.65.



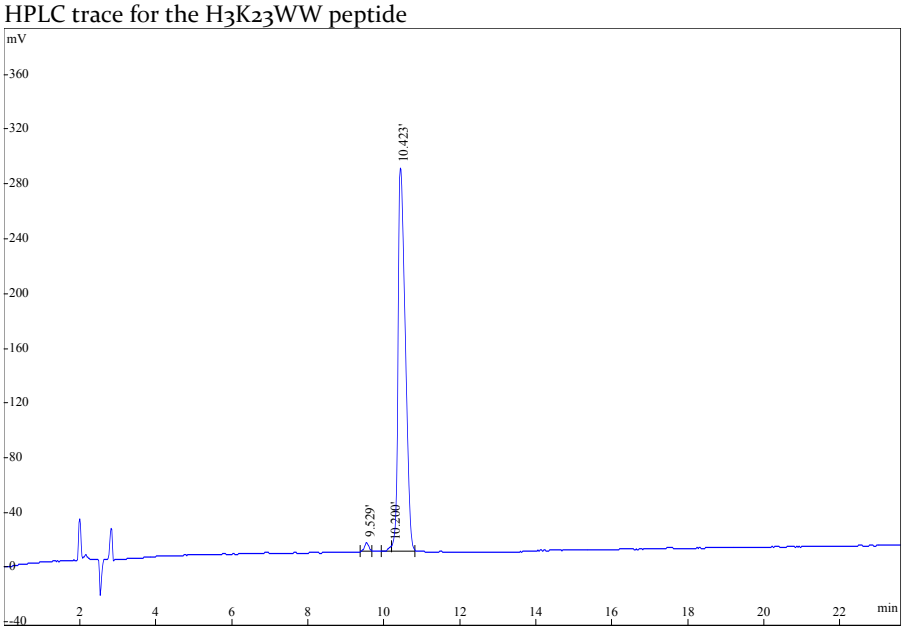
HPLC trace for the H3K27WW peptide



MS spectrum for the H3K23WW peptide, KQLATKAARKSWW, LCMS (ESI) calcd. for $C_{73}H_{116}N_{22}O_{17}$ $[M-H]^-$ 1571.88, obsd. 1571.40; $[M-2H]^{2-}$ 785.44, obsd. 785.60.



HPLC trace for the H3K23WW peptide



Expression and purification of recombinant human sirtuins. Plasmids for SIRT1 (193-747) and SIRT3 (102-399), SIRT5 (34-302), SIRT6 (1-355) expression in *E. coli* were constructed. Plasmids of SIRT2 (36-356, wide-type and mutant F119A) cloned in pET28-SUMO vector were expressed in *E.coli* Rosetta cells. To induce the expression of target proteins, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.02 mM when OD600 reached 0.4, and the culture was grown at 15 °C overnight. Cells were harvested and resuspended in lysis buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). Following sonication and centrifugation, the supernatant was loaded onto a nickel column pre-equilibrated with lysis buffer A. The target proteins were eluted with a linear gradient of Imidazole from 0 mM to 500 mM. After purification, SIRT2 was digested by ULP1 at 4 °C overnight and purified on a Highload 26/60 Superdex75 gel-filtration column (GE Healthcare Life Sciences). After concentration, the target proteins were frozen and stored at -80°C for later use. All the proteins were purified to >95% purity as assessed by SDS-PAGE analysis.

Enzymatic reactions. The enzymatic activity of human SIRT1, SIRT2, SIRT3, SIRT5 and SIRT6 to hydrolyze the H3K27 (4-ONyl) were measured by detecting the deacylated peptide using HPLC. The peptides all have two tryptophan residues attached at the C-termini to facilitate detection by ultraviolet absorption on HPLC. In short, 2 μM of purified sirtuin was incubated with 0.3 mM of acyl peptides, 1.0 mM NAD in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol (DTT) in 60 μL reactions for 1 h at 37°C. The reactions were stopped by adding 60 μL of an aqueous solution of 200 mM hydrogen chloride and 320 mM acetic acid. Samples without NAD or without sirtuin and the free lysine H3K27 peptide were treated under the same conditions and used as controls. Nicotinamide (10 mM) or TM (10 μM) molecules were used for inhibition assay. The reactions mixtures were spun at 12000 g for 10 mins and then analyzed on a Kinetex XB-C18 column (100A, 100 mm × 4.60 mm, 2.6 um, Phenomenex). Mobile phases used were ACN with 0.1% TFA (buffer A) and water with 0.1% TFA (buffer B) with a flow rate at 0.5 mL/min. The product and the substrate peaks were quantified using absorbance at 280 nm.

Determination of k_{cat} and K_m values. SIRT2 (1 μM) was incubated with different concentrations of H3K27 (4-ONyl) and H3K23 (4-ONyl) peptides bearing two tryptophans at *C*-terminus (15, 30, 60, 120, 240, 360 and 540 μM), 1 mM NAD in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT in 60 μL reactions at 37 °C for 10 min. The reactions were stopped by adding 60 μL of a solution of 200 mM hydrogen chloride and 320 mM acetic acid. The reactions mixtures were spun at 12000 g for 10 mins and then analyzed on a Kinetex XB-C18 column (100A, 100 mm× 4.60 mm, 2.6 um, Phenomenex). Mobile phases used were ACN with 0.1% TFA (buffer A) and water with 0.1% TFA (buffer B) with a flow rate at 0.5 mL/min. The product and the substrate peaks were quantified using absorbance at 280 nm and converted to initial rates, which were then plotted against the peptide concentrations and fitted using Kaleidagraph. All reactions were duplicated.

Determination of k_d values by microscale thermophoresis (MST). The microscale thermophoresis (MST) method has been described in detail elsewhere[1]. The K_d values for SIRT2 and 4-ONyl (or Acetyl) peptides were measured using the Monolith NT.115 from Nanotemper Technologies. SIRT2 was fluorescently labeled with the Monolith NT Protein Labeling Kit BLUE-NHS according to the manufacturer's protocol. Different concentrations of the modified peptide (from about 500 μM to 3.9 μM) were incubated with 100 nM of labeled SIRT2. The samples were loaded into silica capillaries (Polymicro Technologies) after incubation at

room temperature for 30 mins. Measurements were performed at 25°C in 10 mM PBS buffer, pH 7.4, with 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, by using 20% LED power and 80% MST power. Data analyses were performed using Nanotemper Analysis software, v.1.2.101.

Crystallization, X-ray data collection, and structure determination. SIRT2 and H3K23 (4-ONyl) peptide mixed at a 1:20 molar ratio and incubated on ice for 30~60 mins. Then 1 mM of carba-NAD was added into the mixture. Crystals of SIRT2 (36-356) in complexes with H3K23 (4-ONyl) peptide and carba-NAD were obtained by the hanging drop vapor-diffusion method at 291 K using commercial screens from Hampton Research. Each drop, consisting of 1 μL of 10 mg/ml protein complex solution (20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM DTT) and 1 μL of reservoir solution, was equilibrated against 400 μL of reservoir solution. The qualified crystals of SIRT2 grew within 4 weeks with a reservoir containing 12% PEG8K, 0.1 M HEPES, pH 7.5, 5% isopropanol. The mixture of 30% glycerol and the reservoir solution above was used as cryogenic liquor. The X-ray diffraction data were collected in a liquid nitrogen gas stream using the Shanghai Synchrotron Radiation Facility (SSRF) beamline 17U. Data was indexed and integrated using the program *HKL2000*[2]. The complex structure of SIRT2 with H3K23 (4-ONyl) peptide was solved by molecular replacement using the program *Phaser*[3] from the CCP4 Suit[4] with the published SIRT2 structure (PDB: 3ZGO) as the search model. Refinement and model building were performed with REFMAC5 and COOT from CCP4[4]. The X-ray diffraction data collection and structure refinement statistics are shown in Supplementary Table 1.

Cell Culture. Mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactived fetal bovine serum (FBS), 1% MEM non-essential amino acids, 1% penicillin streptomycin, and 1% L-glutamine. Cells were maintained in a humidified 37 °C incubator with 5% CO2.

RNAi experiments. Mouse SIRT2 siRNA (GE healthcare) with final concentration at 30 nM was transfected into MEF cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Corresponding concentration of Control siRNA (GE healthcare) was used as negative control. Following transfection, the cells were maintained in a humidified 37 °C incubator with 5% CO_2 for 72 h.

Metabolic labeling. MEF cells were treated with 25 μM of alkynyl-4-ONE (Cayman chemical) for 3h in a humidified 37 °C incubator with 5% CO_2. For co-incubation with SIRT2 inhibitors, MEF cells were treated with nicotinamide (50 μM) or TM (50 μM)[5] in the presence of alkynyl-4-ONE (25 μM) for 3h. For the cells transfected with SIRT2 siRNA or control siRNA, after incubation for 72h, the SIRT2 knockdown and control cells were treated with alkynyl-4-ONE (25 μM) for 3h. Following metabolic labeling, the cells were harvested, washed twice with ice-cold PBS, and pelleted at 1000 rpm for 5 min. Then the cell pellets were directly lysed or flash frozen in liquid nitrogen and stored at -80 °C.

Histone extraction. The procedure was modified from David Shechter *et al. Nature Protocols*. 2007, 6, 1447. Briefly, the harvested MEF cells pellet were resuspended with lysis buffer (10 mM Tris–HCl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF and Roche Complete EDTA-free protease inhibitors) and incubated at 4 °C with rotating for 30 minutes. The intact nuclei were pelleted by centrifuging at 10000 g for 10 mins at 4 °C. To extract histones, 0.4 N H_2SO_4 was added to resuspend the nuclei, followed by rotating at 4 °C for overnight. After centrifugation to remove the nuclei debris, histones were precipitated by adding 100% trichloroacetic acid (TCA) drop by drop (TCA final concentration: 33%). The precipitated histones were pelleted at 16,000 g for 10 min at 4 °C and washed with ice-cold acetone twice. The air-dried protein pellet was dissolved with ddH_2O and stored at -80 °C for later use.

Cu(I)-catalyzed cycloaddition/click chemistry. Briefly, in 20 μL reaction system, the 10 mM Tris pH 8.0 and 100 μM rhodamine azide for in-gel fluorescence scanning was added, followed by 1 mM tris (2-carboxyethyl) phosphine (TCEP), 100 μM 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 $CuSO_4$. The reactions were incubated for 2 hrs in the dark at room temperature.

In-gel fluorescence visualization. The click chemistry reactions were quenched by adding 4 volume of ice-cold acetone to precipitate proteins. The mixture was placed at -20 °C overnight and then was spin down at 6000 g for 5 min at 4 °C. The supernatant was discarded and the pellet was washed twice with ice-cold methanol and air-dried for 10 mins. The proteins were resuspended in 1 × loading buffer and heated at 95 °C for 10 min, and then 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).

In-solution activity assay of SIRT2 WT and F119A mutant. Histones (15 μg) extracted from Sirt2 knockdown cells treated with 25 μM of alkynyl-4-ONE were incubated with 2 μM identical concentration of BSA, SIRT2 WT, or the F119A mutant in 20 μL of reaction buffer (25 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, pH 7.5, 1 mM NAD) at 37 °C for 1 h. Then click chemistry was carried out as described above and the labeling was analyzed by in-gel fluorescence.

Western blot. The histones from SIRT2 control and knockdown cells were separated by SDS-PAGE (15%), then transferred (25 mM Tris, 192 mM Glycine, 20% methanol in deionized water) onto a PVDF membrane which was then blocked (5% BSA and 0.1% tween-20 in TBS) for 1 h at room temperature. The membrane was incubated with primary antibody from Abcam (for SIRT2, 1:1000 catalog # ab67299 and for H3, 1:2500, catalog # ab1791) diluted in TBST (0.1% Tween-20 in TBS with 5% BSA) overnight at 4°C, washed with TBST for three times, incubated with goat anti-rabbit-HRP conjugated secondary antibody (1:2000 dilution, Cell signaling) in TBST for 1h at room temperature, and then washed with TBST three times and visualized with Western blotting detection reagents (Bio-Rad).

LC-MS/MS analysis of histone 4-ONylation. Both the WT and SIRT2 KO MEF cells were treated with 1μg/mL LPS for 6 h respectively and then histones were extracted for mass analysis. For LC-ESI-MS/MS analysis, the lyophilized peptides were dissolved in 2% acetonitrile with 0.5% formic acid. The dissolved peptides were injected into an Acclaim PepMap nano Viper C18 trap column (5 μm, 100 μm × 2 cm, Thermo Dionex) at a flow rate of 20 μL/min and then separated on C18 RP nano column (5 μm, 75 μm × 50 cm, Magic C18, Bruker). The gradient for LC condition was: 5-38% acetonitrile with 0.1% formic acid from 0 to 120 min, and the flow rate was 0.3 μL/min. The MS analysis was carried out on a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA), which was interfaced with Dionex UltiMate3000 MDLC system (Thermo Dionex, Sunnyvale, CA). The Orbitrap Elite was operated in positive ion mode with spray voltage 1.6 kV and source temperature 275 ˚C. Data-dependent acquisition (DDA) mode was used by one precursor ions MS survey scan from m/z 300 to 1800 at resolution 60,000 using FT mass analyzer, followed by up to 10 MS/MS scans on 10 most intensive peaks. All data were acquired in Xcalibur 2.2 operation software (Thermo Fisher Scientific).

For data analysis, tandem mass spectra were searched against mouse subset dataset from Uniprot in Sequest HT software (v1.3). The dynamic modifications were set as following: Acetyl: +42.011 Da (Lys), Oxidation: +15.995 Da (Met), 4-ONE: +154.099 Da (Lys), Reduced 4-ONE: +156.115 Da (Lys), Methylation: +14.016 Da (Lys), Dimethylation: +28.031 Da (Lys). The static modification was set as: Carbamidomethylation: +57.021 Da (Cys).

Reference

(1) Jerabek-Willemsen, M.; Wienken, C.J.; Braun, D.; Baaske, P.; Duhr, S. *Assay Drug Dev. Technol.* **2011**, *9*, 342.
(2) W., O. Z. a. M. *Methods in Enzymology* **1997**, *276*, 307.
(3) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. *Journal of applied crystallography* **2007**, *40*, 658.
(4) Bailey, S.; Fairlamb, A. H.; Hunter, W. N. *Acta crystallographica. Section D, Biological crystallography* **1994**, *50*, 139.
(5) Jing, H.; Hu, J.; He, B.; Negron Abril, Y. L.; Stupinski, J.; Weiser, K.; Carbonaro, M.; Chiang, Y. L.; Southard, T.; Giannakakou, P.; Weiss, R. S.; Lin, H. *Cancer cell* **2016**, *29*, 607.

Table S1. Statistics for SIRT2 with H3K23 (4-ONyl) peptide and carba-NAD. Statistics for the highest-resolution shell are shown in parentheses.

	Human SIRT2-H3K23 (4-ONyl)-carba NAD
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	37.341, 117.31, 71.346
α, β, γ (°)	90, 92.65, 90
Resolution (Å)	40.00-2.00
R_{sym} or R_{merge} (%)	10.5 (54.6)
I / σ	11.97 (2.55)
Completeness (%)	99.9 (100.0)
Redundancy	3.8 (3.7)
Refinement	

Resolution (Å)	32.38-2.10
No. reflections	40356 (3137)
R_{work} / R_{free} (%)	18.30 (21.98)
No. of protein residues	590
No. of ligand/ion molecules	
H3K23 (4-ONyl)	2
Carba-NAD	2
Zn	2
No. of water	316
R.m.s deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.896

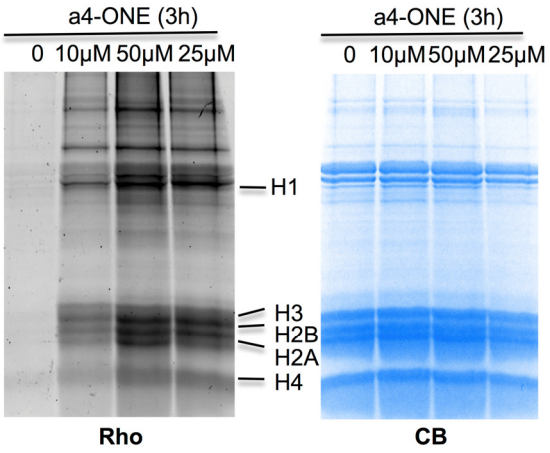


Figure S1. Dose-dependent metabolic labeling of cells with alkynyl-4-ONE (a4-ONE). MEF cells were labeled with different concentration of alkynyl-4-ONE and harvested after incubation for 3 h. The histone were extracted from the cell lysates and reacted with rhodamine-azide, resolved by SDS-PAGE, and detected by in-gel fluorescence scanning. The Coomassie blue staining was used as loading controls. Rho = rhodamine fluorescence, CB = Coomassie blue.

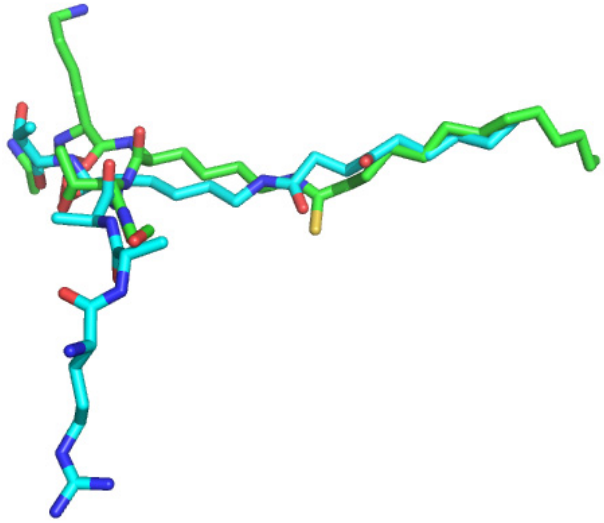
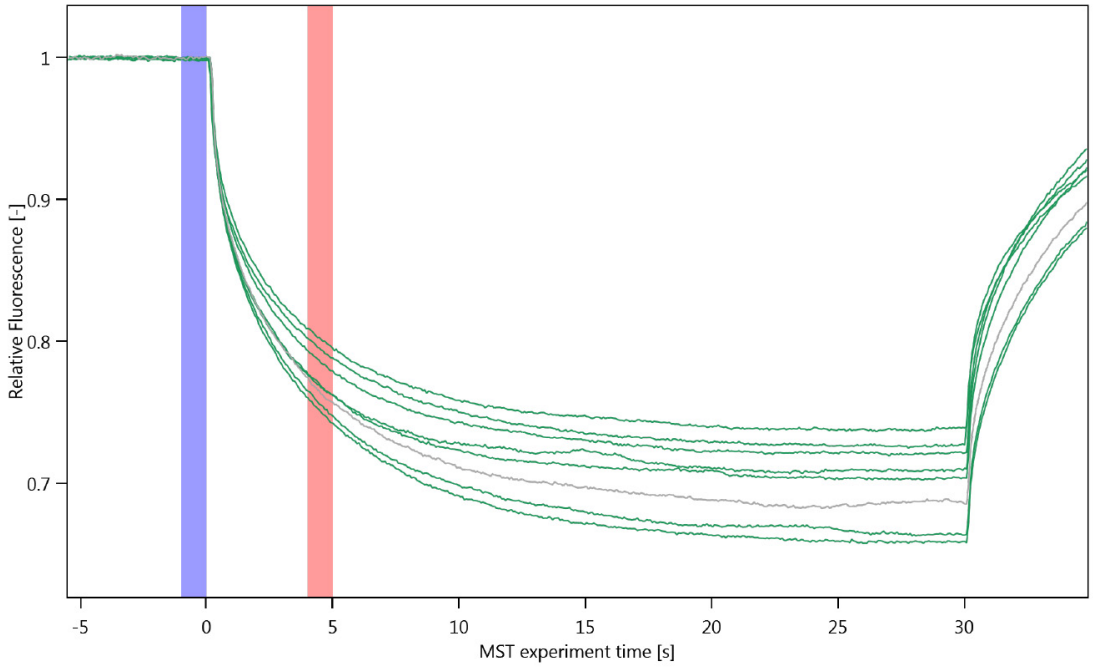
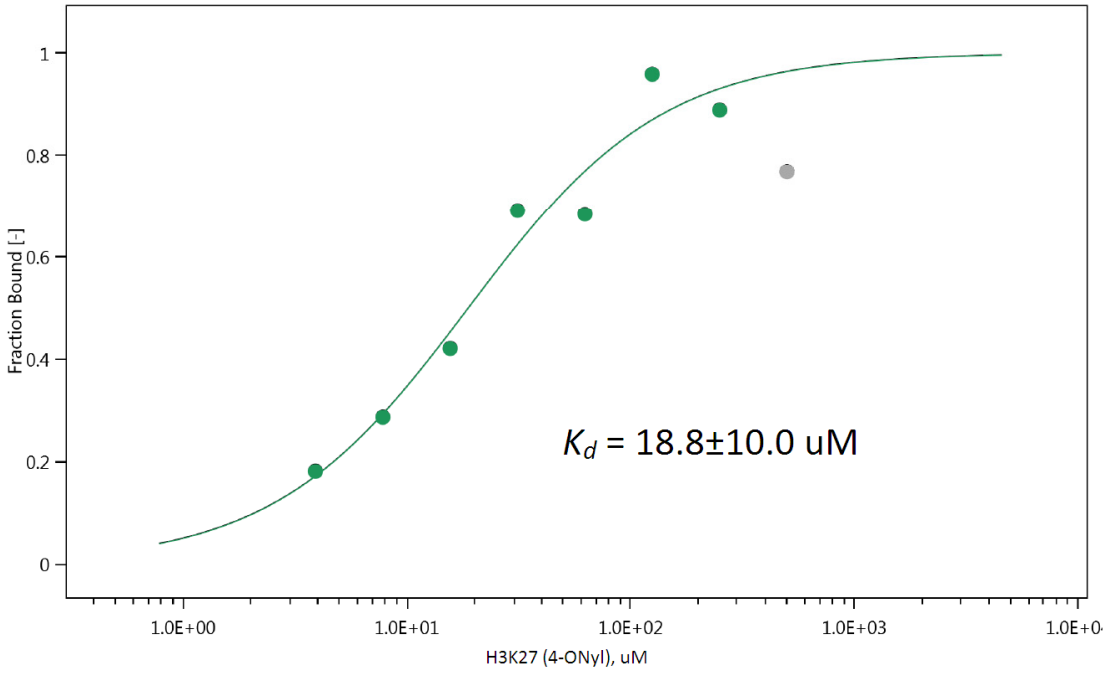


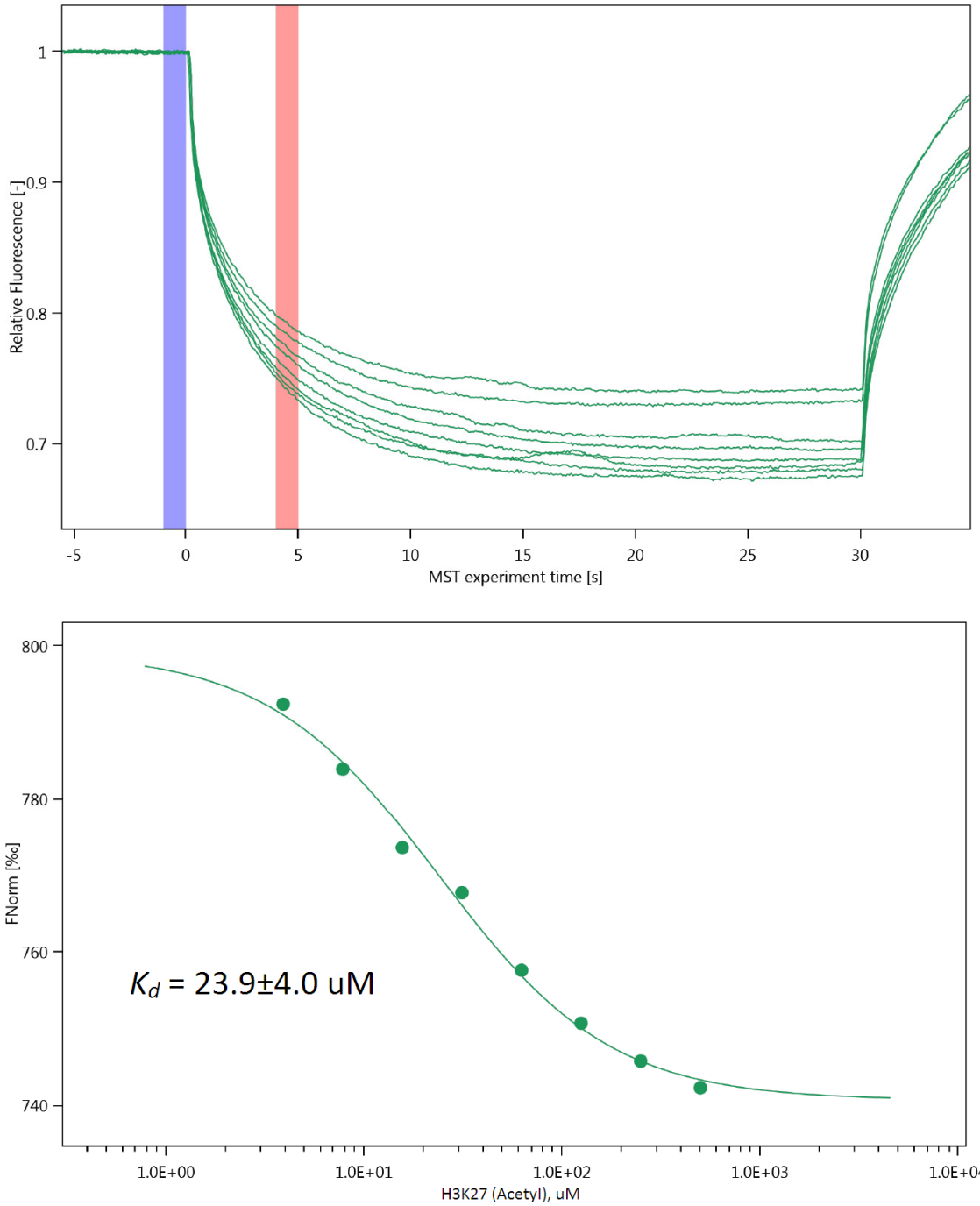
Figure S2. Alignment of the substrates binding pattern between SIRT2-H3K23 (4-ONyl)-carba NAD (cyan) and SIRT2-H3K9 (Myr) (PDB 4R8M) (green).

A

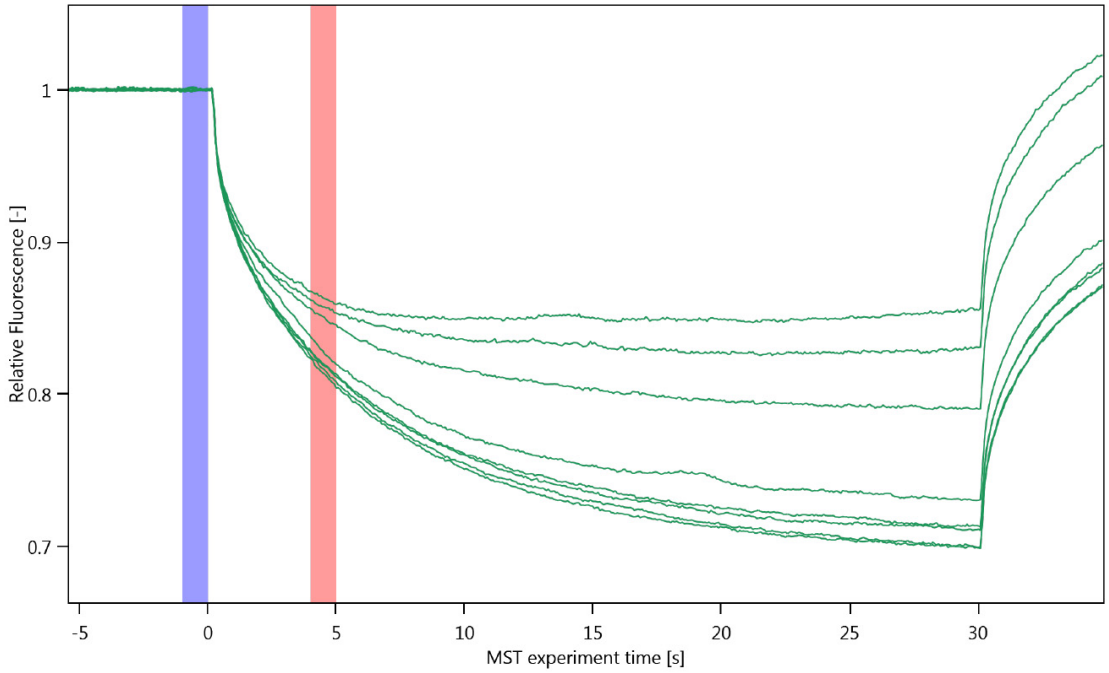

Relative Fluorescence [-]
1
0.9
0.8
0.7
-5
0
5
10
15
20
25
30
MST experiment time [s]

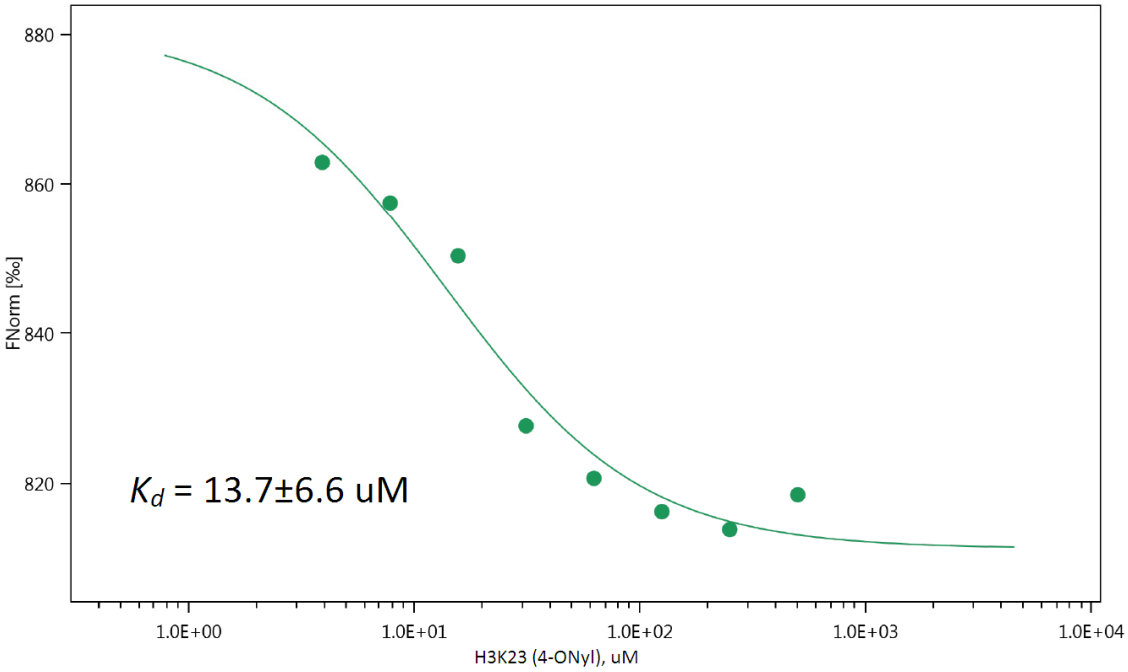

Fraction Bound [-]
1
0.8
0.6
0.4
0.2
0
K_d = 18.8±10.0 uM
1.0E+00
1.0E+01
1.0E+02
1.0E+03
H3K27 (4-ONyl), uM

B


Relative Fluorescence [-]
1
0.9
0.8
0.7
-5
0
5
10
15
20
25
30
MST experiment time [s]
FNorm [‰]
800
780
760
740
K_d = 23.9±4.0 uM
1.0E+00
1.0E+01
1.0E+02
1.0E+03
H3K27 (Acetyl), uM

C


Relative Fluorescence [-]
1
0.9
0.8
0.7
-5
0
5
10
15
20
25
30
MST experiment time [s]


FNorm [‰]
880
860
840
820
K_d = 13.7±6.6 uM
1.0E+00
1.0E+01
1.0E+02
1.0E+03
1.0E+04
H3K23 (4-ONyl), uM

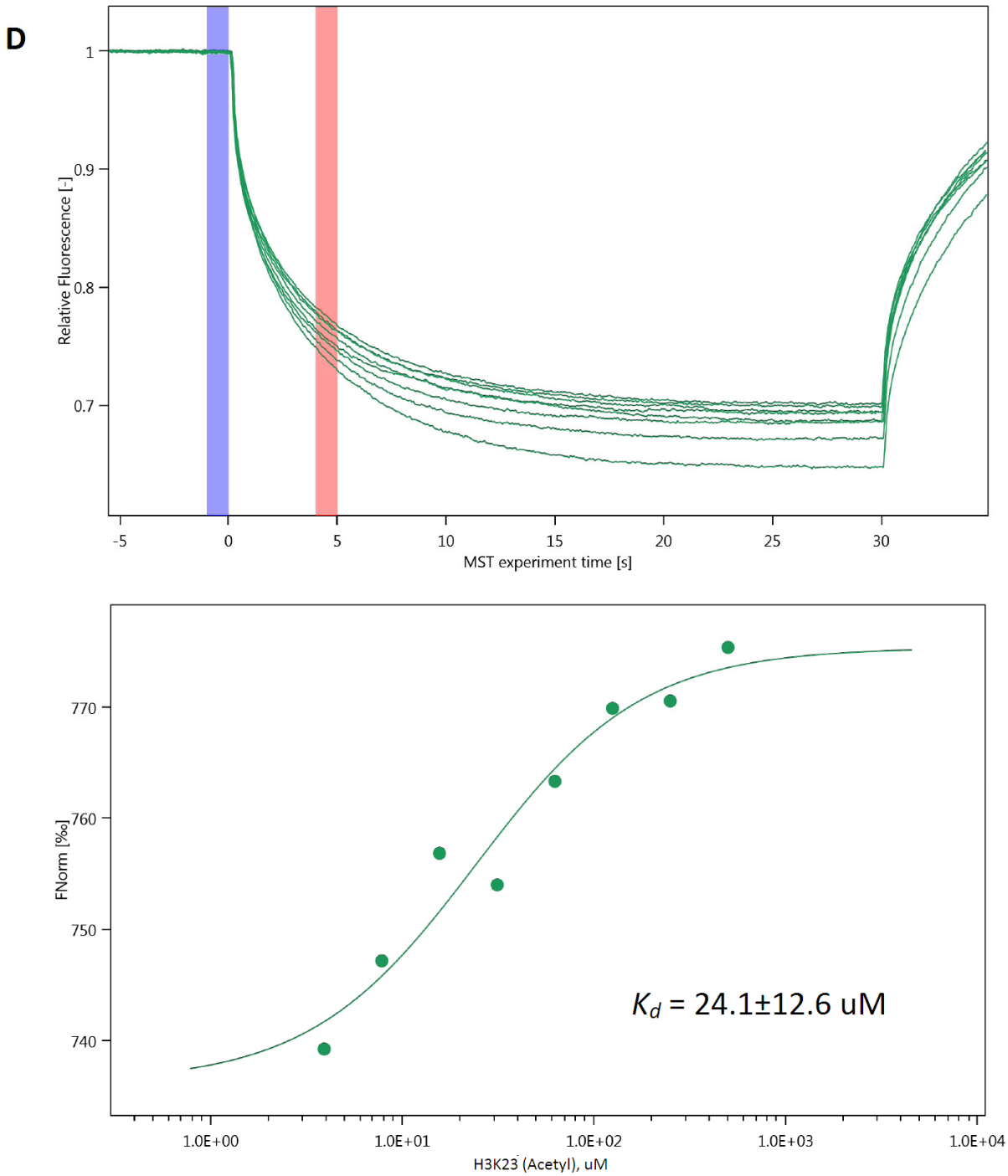


Figure S3. The K_d values of SIRT2 for 4-ONyl and acetyl peptides were measured by MST[1]. The modified peptide (500 μM to 3.9 μM) was titrated into a fixed concentration of labeled SIRT2 (100 nM). The top panels of A-D show the raw data for thermophoresis recorded at 25°C using the LED power at 20% and MST power at 80%. The bottom panels of A-D show the isotherm derived from the raw data and fitted according to the law of mass action to yield an apparent K_d[1]. (A) The K_d value of SIRT2 for H3K27 4-ONyl was 18.8 ± 10.0 μM. (B) The K_d value of SIRT2 for H3K27 acetyl was 23.9 ± 4.0 μM. (C) The K_d value of SIRT2 for H3K23 4-ONyl was 13.7 ± 6.6 μM. (D) The K_d value of SIRT2 for H3K23 acetyl was 24.1 ± 12.6 μM.

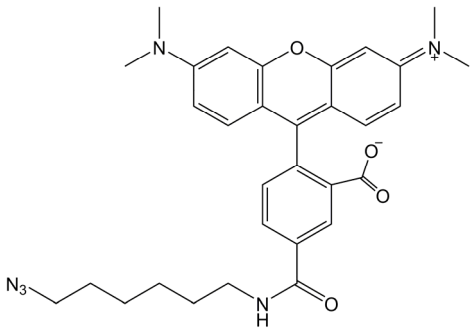


Figure S4. The structure of tetramethylrhodamine (TAMRA) azide (tetramethylrhodamine 5-carboxamido-(6-azidohexanyl)), 5-isomer.

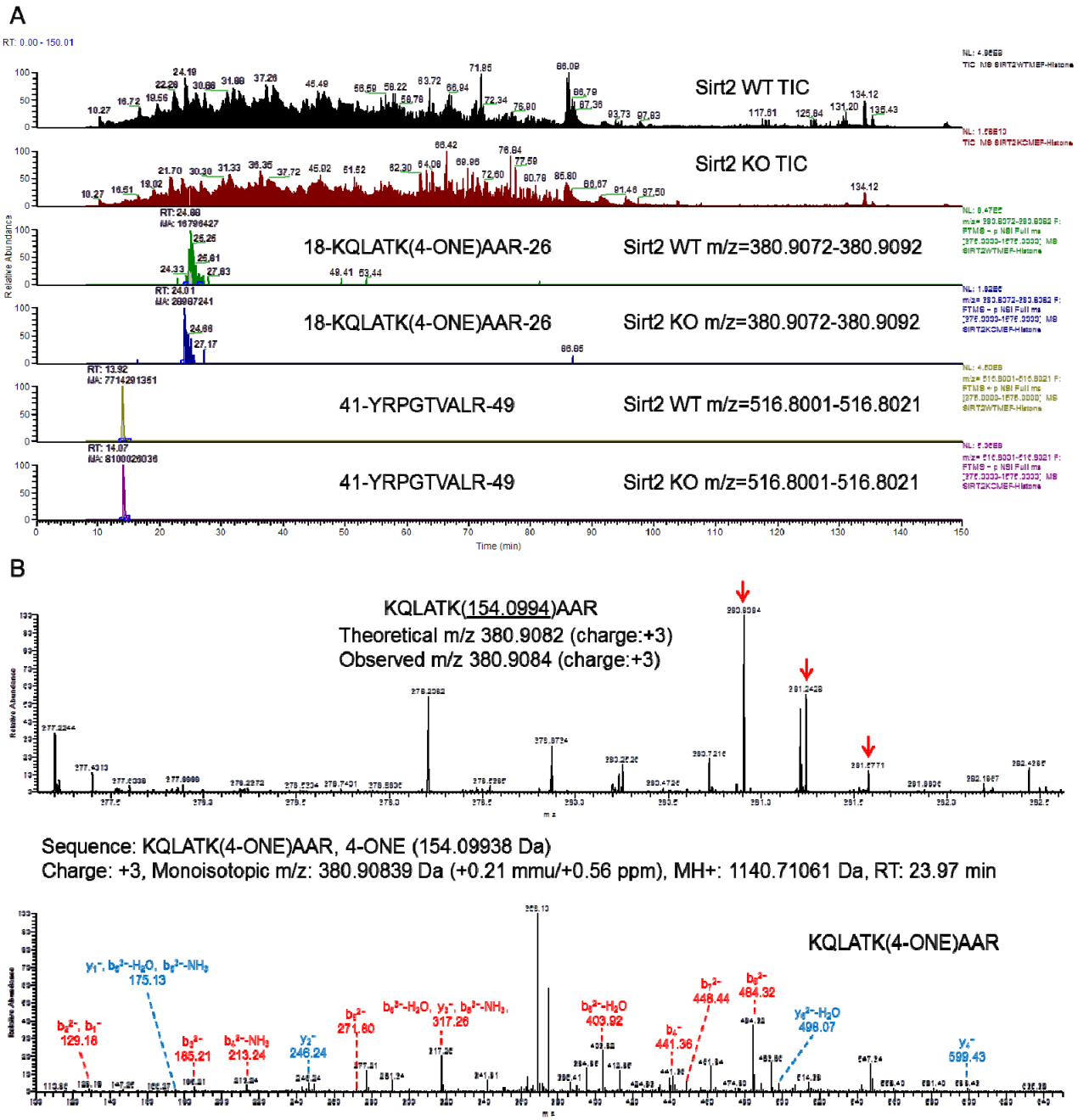


Figure S5. SIRT2 regulates endogenous 4-ONyl level on histones in mouse embryonic fibroblasts (MEFs). (A) Total ion chromatograms (TIC) and selected ion chromatograms (m/z=380.9072-380.9092: 4-ONyl H3K23, and m/z=516.8001-516.8021: unmodified H3Y41-R49) of histones from Sirt2 WT and KO MEFs. (B) The tandem MS spectrum of 4-ONyl H3K23 from histones in Sirt2 KO MEFs.