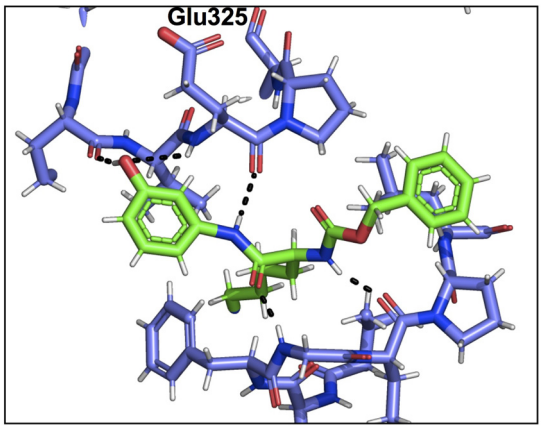
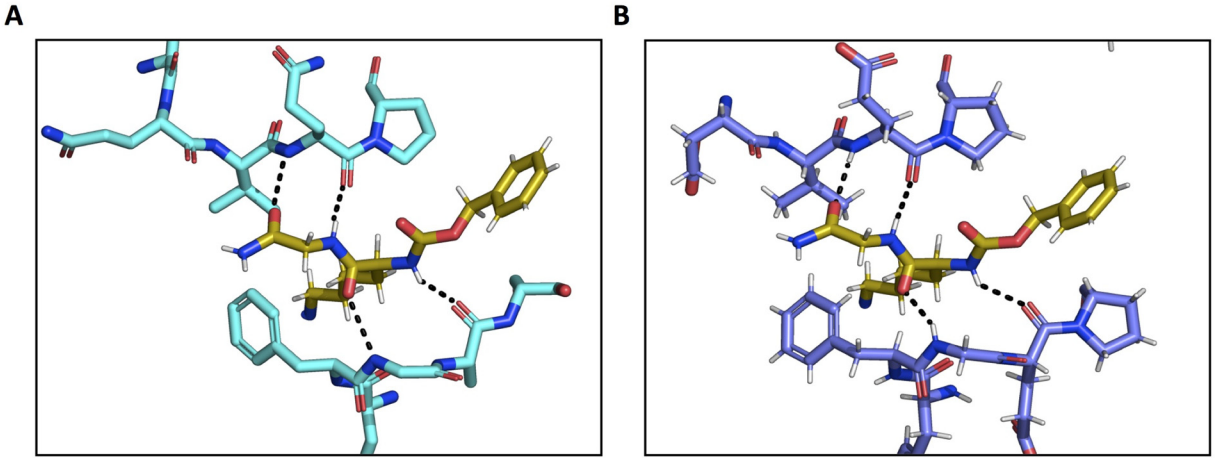


Supplemental Figure 1. Modeling of JH-T4 with the established Sirt3 - myristoyl H3K9 peptide crystal structure (5BWN).



Supplemental Figure 2. Modeling of NH-TM with the established (A) Sirt2 (4r8m)[1] and (B) Sirt3 (5BWN) - myristoyl H3K9 peptide crystal structures.

Materials and Methods

Reagents, antibodies and plasmids. TM and JH-T4 were synthesized as previously described.[2] TM and JH-T4 were dissolved in ethanol (EtOH). For studies with NH-TM, all compounds were dissolved in dimethyl sulfoxide (DMSO). For western blots, the acetyl-p53 (CST #2525) (1:1000, o/n 4˚C) and anti-rabbit conjugated to horseradish peroxidase (CST #2525) were purchased from cell signaling technologies and used at the indicated dilutions and incubation time. Anti-Flag M2 conjugated to horseradish peroxidase (A8592) (1:7500, 1 hr, rt), and the anti-acetyl-α-tubulin (6-11B-1) (MABT868) antibodies were purchased from Sigma-Aldrich. The Anti-Flag M2 affinity agarose was from Sigma. For immunofluorescence, the secondary CY3 goat-anti-mouse (A10521) was purchased from Life Technologies and used at a 1:1000 dilution for 1 hour at room temperature. For ectopic overexpression of K-Ras4a, pCMV5-Flag-K-Ras4a was cloned as previously described.[3] Alk14 was synthesized as previously described.[4]

Cell Culture and Transfection. MCF-7, MDA-MB-231, MDA-MB-468 and HME-1 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen). ASPC-1, and NCI-H23 cells were cultured in RPMI media supplemented with 10% FBS. HCT116 cells were cultured in McCoy's 5A media with 10% FBS. Mammary epithelial cell growth medium (MEGM; Lonza) supplemented according to manufacturer's instruction was used to culture MCF10A cells. All cells were maintained at 37˚C with 5% CO_2.

Expression and Purification of Sirtuins. Human SIRT1, SIRT2, SIRT3 and SIRT6 were expressed and purified as previously described.[2, 5]

***In vitro* deacylation assay** (with and without pre-incubation). *In vitro* IC_{50} values were determined using the same method previously reported.[2, 6] Briefly, for assays done with pre-incubation various concentrations of inhibitors dissolved in ethanol were added to the purified enzymes. The samples were placed at 37˚C for 15 min, after which the substrate peptides were added and placed at 37˚C for a set period of time as described in previous publications. For these assays either the H3K9-Ac or H3K9-Myr peptides were used. For the assays without preincubation, the enzyme, inhibitor and substrates were all added simultaneously, and the reactions were allowed to run for the defined period of time as previously described. [2, 6]

Western Blots. Western blots were performed as previously described. Briefly, protein samples were lysed in 4% SDS lysis buffer supplemented with protease inhibitor cocktail (Sigma) and universal cell nuclease (Thermo). After determining the protein concentration by BCA assay, the samples were normalized, denatured and resolved by 12% SDS PAGE at a constant voltage of 200 V for approximately 50 min. The proteins were then transferred to Polyvinylidene difluoride (PVDF) membrane at a constant current of 330 mA for 90 min to 120 min. After transferring, the membrane was blocked in 5% bovine serum albumin (BSA) in TBST buffer (0.1% Tween-20, 25 mM Tris-HCl pH 7.6, 150 mM NaCl) for 1 hr at room temperature. The antibodies were added according to the manufacture instructions. After primary antibody incubation, the membrane was washed three times with TBST buffer for 5 minutes each. The appropriate secondary antibody was added to the sample at a ratio of (1:3000) for 1 hr at room temperature in 5% BSA in TBST. After secondary antibody incubation, the membrane was washed 5 times with TBST buffer for 5 min each. After the final wash the membranes were developed using ECL Plus (Pierce).

Detection of Ac-α-Tubulin Levels by Immunofluorescence. The same method previously reported was used.[6] Briefly, 2×10^5 MCF-7 cells were seeded in 35mm glass bottom dishes from MatTek. After 24 hr cells were treated either with 25 μM of inhibitor or vehicle control for 6 hr. After 6 hr the cells were washed three times with 1× PBS and fixed by placing the cells in ice cold

methanol for 10 min. Cells were treated with 0.1% Triton-X in PBS for 10 min to permeabilize the membrane. The cells were then washed with 1× PBS three times, for 5 min each. After the last wash, the cells were placed in 1% BSA in TBST (25 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 30 min at room temperature. After blocking, the Ac-α-tubulin antibody was added to the samples at a 1:100 dilution in 1% BSA in TBST, and the samples were placed at 4˚C overnight. The samples were washed three times with TBST (5 min per wash), and the secondary antibody (CY3) was added to the samples (1:1000, in 1% BSA in TBST) for 1 hr at room temperature. After 1 hr, the samples were washed three times with TBST for 5 min each and then mounted with DAPI-Fluoromount G (Southern Biotech). Samples were imaged using a Zeiss 880i confocal microscope. Fiji software was used for analysis.[7]

Detection of K-Ras4a Fatty Acylation after *in vitro* treatment with SIRT2. pCMV5-Flag-K-Ras4a was transfected into HEK-293T cells. After 24 hours, the cells were treated with 50 μM Alk14 for 6 hours. The cells were collected and lysed with 1% NP40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, and 1% NP40) supplemented with protease inhibitor cocktail. To isolate K-Ras4a, anti-Flag M2 affinity agarose was added to the samples. After 2 hours incubation at 4˚C, the affinity agarose was washed three times with 1 mL of 0.2% NP40 wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.2% NP40). The affinity gel was then washed three times with 50 mM Tris pH 8.0, and aliquoted equally into 8 tubes. The affinity gel was then suspended in 25 μl of assay buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT) with 5 μM SIRT2 with 1 mM NAD and the noted inhibitor at the indicated concentration and placed at 37 °C for 1 h. After the enzymatic reaction, the affinity gel was again washed three times with 1 mL of 0.2% NP40 wash buffer. After the last wash, the affinity gel was dried and resuspended in 20 μL of 0.2% NP40 wash buffer. To each tube, Rh-N_3 (3 μL of 1 mM solution in DMF, final concentration 200 μM), followed by TBTA (1 μL of 10 mM solution in DMF, final concentration 500 μM), $CuSO_4$ (1 μL of 40 mM solution in H_2O, final concentration 2 mM), and TCEP (1 μL of 40 mM solution in H_2O, final concentration 2 mM) were added, and the click chemistry reaction was allowed to proceed for 30 minutes at room temperature in the dark. The reaction was quenched by adding 10 μL of 6 × protein loading buffer (60 mM Tris pH 6.8, 0.12 % SDS (w/v), 47% glycerol, 0.6 M DTT and 0.0006% bromophenol blue (w/v)) and heating the sample at 95 °C for 10 min. The sample was then treated with hydroxylamine (pH 8.0, final concentration 300 μM) at 95 °C for 7 min. To visualize the fatty acylation levels by in gel fluorescence, the samples were resolved by 12% SDS-PAGE. Fluorescence signal was detected using a Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences, Piscataway, NJ) with the emission/excitation settings of Green (532 nm)/580BP30 with a PMT setting of 500 V (normal sensitivity). Fiji software was used for analysis.[7]

Detection of K-Ras4a Fatty Acylation after in cell treatment with different inhibitors. HEK-293T cells were transfected with pCMV5-Flag-K-Ras4a, cultured for 24 hours, and then were treated with the indicated concentration of inhibitor for 6 hours. The media was then changed with the indicated concentration of inhibitor and 50 μM of Alk14 for an additional 6 hours. The samples were collected and lysed with 1% NP40 lysis buffer supplemented with protease inhibitor cocktail. After determining the protein concentration using a Bradford assay, 0.5-1 mg of whole cell lysate was aliquoted out, and diluted to a protein concentration of 1mg/1mL. To the samples, 10 μL of pre-washed M2 affinity agarose was added to each. After incubation for 2 hours at 4˚C, the affinity agarose was washed three times with 1 mL of 0.2% NP40 wash buffer. The same click chemistry conditions described above were used to detect fatty acylation levels.

Cell Viability Assay. In cell GI_{50} values were determined using the method previously published.[6] Briefly, 2,000-6,000 cells/ well were seeded in a 96 well dish. After 24 hr, inhibitors were added. After 72 hr, Cell Titer Blue (Promega) was added following the manufactures

protocol. GI_{50} values were determined using Prism7 software, the calculated inhibition ratios were determined comparing the viability of cells treated with different concentrations of inhibitor to the viability of cells treated with the same concentration of the vehicle control (EtOH in these studies).

Anchorage-Independent Growth Assay. In cell GI_{50} values for anchorage-independent growth were determined using the method previously published.[6] Briefly, cells were counted and 1,000 cells per well were seeded in 0.3% low melting point agar supplemented with the appropriate amount of inhibitor (Sigma Aldrich) on top of a 0.6% low melting point agar layer. Additional agar was added after 7 days of culture. Colonies were stained with nitrotetrazolium blue chloride in PBS after 14 days of culture at 37˚C and 5% CO_2. Images were taken on a BioRad imager, and the number of colonies per well was quantified using FIJI/ ImageJ software.[7]

General Synthetic Methods and Materials. Reagents were obtained from ChemImpex, Alfa Aesar and Sigma-Aldrich. All ^{1}H-NMR were performed on Bruker 500 spectrometers. All LC-MS data were obtained by Shimadzu HPLC LC20-AD and Thermo Scientific LCQ Fleet. The column used was Kinetex 5u EVO C18 100A column (30 × 2.1 mm, 5 µm). The compounds were monitored at 215 and 260 nm with positive detection mode. Solvents used were water with 0.1 % HPLC-grade acetic acid and acetonitrile with 0.1% HPLC-grade acetic acid.

Synthesis of H3K9-Ac and Myr peptides. The peptides used for the *in vitro* activity assays were synthesized using solid phase peptide synthesis as previously reported.[8]

Synthesis of JH-T4. JH-T4 was synthesized following a procedure for TM described in the literature.[2] Briefly, to a solution of N^2-((benzyloxy)carbonyl)-N^6-tetradecanethioyllysine (10 mmol) and N-methylmorpholine (10 mmol) in dimethylformamide at 0˚C, isobutyl chloroformate was added dropwise. 3-Aminophenol was added subsequently added. After 30 min at 0˚C, the reaction was warmed to room temperature and stirred overnight. The reaction was concentrated, and the product was purified using silica gel flash chromatography (hexanes: ethyl acetate = 3:2) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.53 (s, 1H), 7.68 (s, 1H), 7.39 (m, 1H), 7.32 (m, 6H), 7.12 (t, J = 8.1 Hz, 1H), 6.86 (d, J = 7.9 Hz, 1H), 6.61 (dd, J = 8.3, 2.3 Hz, 1H), 5.65 (d, J = 7.9 Hz, 1H), 5.28 – 4.89 (m, 2H), 4.35 (m, 1H), 3.61 (m, 2H), 2.57 (t, J= 7.5 Hz, 2H), 1.90 (m, 1H), 1.67 (m, 5H), 1.43 (m, 2H), 1.22 (m, 21H), 0.86 (t, J = 6.7 Hz, 3H). LCMS (ESI) calcd. for $C_{34}H_{52}N_3O_4S$ $[M+H]^+$ 598.36, observed. 598.25.

Synthesis of NH-TM. The synthesis of N^2-((benzyloxy)carbonyl)-N^6-tetradecanethioyllysine was done following the procedure in the literature[2]. To the solution of N^2-((benzyloxy)carbonyl)-N^6-tetradecanethioyllysine (.1006 g, .985 mmol), glycinamide (220 mg, 0.1985 mmol) and triethylamine (83 µL, 0.5956 mmol) in dimethylformamide (2 mL), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (419 mg, 0.2184 mmol) and hydroxybenzotriazole (365 mg, 0.2382 mmol) were added. The reaction mixture was stirred overnight at room temperature. Dimethylformamide was removed in vacuo at 70 °C. The mixture was re-dissolved in ethyl acetate, and washed by 5% sodium bicarbonate, 10% citric acid, water and brine. After washing, it was dried with sodium sulfate. The mixture was purified by column (dichloromethane: methanol) to afford the final compound (265 mg, 24% yield) as a white solid. ^{1}H NMR (500 MHz, Methanol-d4) δ 7.42 – 7.27 (m, 5H), 5.16 – 5.07 (m, 2H), 4.07 (dd, J = 8.6, 5.5 Hz, 1H), 3.82 (d, J = 16.9 Hz, 2H), 3.60 (t, J = 7.1 Hz, 2H), 2.62 – 2.57 (m, 2H), 1.85 (ddt, J = 13.7, 10.0, 6.0 Hz, 1H), 1.79 – 1.60 (m, 5H), 1.56 – 1.39 (m, 2H), 1.31 (m, J = 10.5 Hz, 20H), 0.92 (t, J = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, Methanol-d4) δ 205.08, 174.01, 172.90, 157.41, 136.67, 128.09 (2C), 127.67, 127.52 (2C), 66.44, 55.43, 45.68, 45.13, 41.77, 31.68, 30.98, 29.44, 29.41, 29.37 (2C), 29.33, 29.23, 29.09, 29.08, 28.56, 26.92, 22.93, 22.35, 13.06. LCMS (ESI) calcd. for $[M+H]^+$ 563.36, observed 563.32.

References:

[1] Y. B. Teng, H. Jing, P. Aramsangtienchai, B. He, S. Khan, J. Hu, H. Lin, Q. Hao, *Sci Rep* **2015**, *5*, 8529.

[2] H. Jing, J. Hu, B. He, Y. L. Negron Abril, J. Stupinski, K. Weiser, M. Carbonaro, Y. L. Chiang, T. Southard, P. Giannakakou, R. S. Weiss, H. Lin, *Cancer Cell* **2016**, *29*, 767-768.

[3] H. Jing, X. Zhang, S. A. Wisner, X. Chen, N. A. Spiegelman, M. E. Linder, H. Lin, *Elife* **2017**, *6*.

[4] X. Zhang, N. A. Spiegelman, O. D. Nelson, H. Jing, H. Lin, *Elife* **2017**, *6*.

[5] X. Zhang, S. Khan, H. Jiang, M. A. Antonyak, X. Chen, N. A. Spiegelman, J. H. Shrimp, R. A. Cerione, H. Lin, *Nat Chem Biol* **2016**, *12*, 614-620.

[6] N. A. Spiegelman, I. R. Price, H. Jing, M. Wang, M. Yang, J. Cao, J. Y. Hong, X. Zhang, P. Aramsangtienchai, S. Sadhukhan, H. Lin, *ChemMedChem* **2018**, *13*, 1890-1894.

[7] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat Methods* **2012**, *9*, 676-682.

[8] P. Aramsangtienchai, N. A. Spiegelman, B. He, S. P. Miller, L. Dai, Y. Zhao, H. Lin, *ACS Chem Biol* **2016**, *11*, 2685-2692.