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Materials and Methods

Cell Culture. HEK293 cells were cultured in low-glucose DMEM supplemented with 10% Fetal Bovine Serum (FBS, Atlas Biologicals, Fort Collins, CO) and incubated at 37°C under 5% CO_2.

Synthetic Peptide Preparation. *Coupling Procedure*: Peptide H_2**N-E-I-P-A-E-K(alloc)-V-F-L-A-Q-**OH was synthesized by hand as follows. 0.2 mmol CTC resin (CEM, #R005) was swelled in 3 mL CH_2Cl_2 in a Bio-Rad Poly-Prep chromatography tube for 20 minutes. The first amino acid was loaded by adding 0.8 mmol (5 eq) amino acid in 2 mL of 1:1 CH_2Cl_2:DMF and 1.6 mmol (8 eq) DIEA and rotating overnight. Any unreacted resin was capped by adding 17:2:1 CH_2Cl_2:MeOH:DIEA and rotating for 1h, after which resin was washed with DMF (x5), CH_2Cl_2 (x5), and DMF (x5).

With the exception of Fmoc-Lys(alloc)-OH, subsequent amino acids were added as follows. Resin was Fmoc deprotected by adding 2 mL of 20% piperidine in DMF and rotating for 5 minutes (x2). Resin was then washed with DMF (x5), CH_2Cl_2 (x5), and DMF (x5). Amino acids were coupled by adding 0.8 mmol (4 eq) protected amino acid, 0.8 mmol (4 eq) Oxyma, and 0.8 mmol (4 eq) DIC in 3 mL DMF to the resin and rotating for 1h. Resin was washed with DMF (x5), CH_2Cl_2 (x5), and DMF (x5). Next, resin was capped by adding 2 mL of 9:1 pyridine:Ac_2O, then washed again with DMF (x5), CH_2Cl_2 (x5), and DMF (x5). Fmoc-Lys(alloc)-OH was added by deprotecting as above, then adding 0.3 mmol (1.5 eq) amino acid, 0.3 mmol (1.5 eq) HATU, 0.4 mmol (2 eq) HOAt, and 0.6 mmol (3 eq) DIEA in 3 mL DMF to the resin and rotating overnight. Resin was then washed, capped, and washed as above.

Lactoylation: Completed peptide H_2**N-E-I-P-A-E-K(alloc)-V-F-L-A-Q-**OH was lactoylated as follows. The alloc protecting group was selectively removed by adding 3 mg (0.1 eq) tetrakis(triphenylphosphine)palladium(0) and 61 µL (20 eq) phenylsilane in 3 mL DMF, then rotating 20 min and washing with CH_2Cl_2 (5x). This was repeated 2x. Resin was split and transferred into 2 new Bio-Rad Poly-Prep chromatography tubes. Resin was deprotected as above, then treated with 0.15 mmol (1.5 eq) of either L-lactic acid or D-lactic acid, 0.15 mmol (1.5 eq) HATU, 0.2 mmol (2 eq) HOAt, and 0.3 mmol (3 eq) DIEA in 3 mL DMF and rotated overnight. Resin was then washed, capped, and washed as above.

Cleavage from resin: Resin was Fmoc deprotected as above, then washed with DMF (x5) and CH_2Cl_2 (x10). Next, the peptide was cleaved from resin by addition of 3 mL cleavage cocktail, 90:5:5 TFA:H_2O:TIS, which was made fresh daily. This was rotated for 2-3 hours, then expelled into a vial, concentrated under nitrogen flow, and precipitated in 1.5 mL cold ether. The resulting residue was redissolved in 4 mL 0.1% TFA in H_2O and HPLC purified.

HPLC purification: Preparatory HPLC was performed with a SunFire Prep C18 OBD 10x150 mm reverse-phase column as the stationary phase. H_2O and MeCN buffered with 0.1% TFA were used as the mobile phase. The HPLC conditions were as follows: 10%

MeCN for 5 min, followed by 10 to 80% MeCN over 30 min, followed by 100% MeCN for 9 min, and finally 10% MeCN for 8 min. The desired HPLC fractions were combined and lyophilized to give the corresponding peptide as a TFA salt.

Characterization: The molecular weight of the peptides was determined by High Resolution Mass Spectrometry (HR-MS), which was performed at the Yale Chemical and Biophysical Instrumentation Center (CBIC). Purity of the peptides was determined to be above 95% with analytical HPLC, which was also performed at the CBIC.

Recombinant Sirtuin Assay. Adapted from Huang, et al.[1] and Jin, et al.[2]: 2 μM recombinant sirtuins (SIRT1-7, Cayman Chemicals, Ann Arbor, MI) were incubated with 0.3 mM acyl peptide, 1 mM NAD+, and 1 mM dithiothreitol (DTT) for a total of 20 μL in PBS for 24 hours at 37°C. Reactions were stopped by adding 20 μL of 200 mM HCl and 320 mM acetic acid in methanol. The reactions were then dried under N_2, resuspended in 100 μL mobile phase A, and analyzed via LC-MS/MS. A Shimadzu LC system equipped with a 50 x 2.1 mm, 5 μM Atlantis dC18 column (Waters, Dublin, Ireland) was used to chromatograph 20 μL of sample. Mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. The following LC gradient was used with a flow rate of 0.5 mL/min: 0.5 min, 2.5% B; 5.0 min, 95% B; 7.0 min, 95% B; 7.5 min, 2.5% B. The column was equilibrated between each run with 2.5% B for 2 min. MRM was conducted in positive mode with a 90 sec scan window using an AB SCIEX 4500 QTRAP with the following transitions:

Species	Q1 (m/z)	Q3 (m/z)	CE (V)
EIPAEKVFLAQ	1244.8	767.3	67
EIPAEKacVFLAQ	1286.8	1069.5	55
EIPAEKlaVFLAQ	1316.8	1170.6	53

The presence of deacylated, unmodified peptide was then quantified as percent of total peptide in the sample. Because of the differences in ionization efficiency between the unmodified peptide and lactoylated peptide, a correction parameter for ionization efficiency was calculated. The correction parameter was calculated according to Tanabe, et al.[3]; equaling the ratio of an authenticated unmodified peptide area to an authenticated lactoylated peptide area (1.685). Lactoylated peptide peak areas were then corrected and deacylation was calculated as above.

Determination of K_m and k_{cat} Values: Adapted from Jin, et al.[2]: 1 μM recombinant SIRT2 (Cayman Chemicals, Ann Arbor, MI) was incubated with varying concentrations of acyl peptides (30, 60, 120, 240, and 480 μM), 1 mM NAD+, and 1 mM DTT for a total of 20 μL in PBS for 3 hours at 37°C. Reactions were stopped by adding 20 μL of 320 mM acetic acid and 200 mM HCl in methanol. Reactions were dried under N_2 and resuspended with 100 μL mobile phase A and analyzed via LC-MS/MS as described above.

Molecular Docking. AutoDockTools version 1.5.6 (Scripps) was used for the docking simulations. MM2 energy-minimized molecules (Acetyl, L-lactoyl, and D-lactoyl lysine

peptides: AA-K*-T) were prepared in Chem3D version 16.0 (PerkinElmer). Lamarckian genetic algorithm was selected for ligand conformational searching, and rigid docking was performed on SIRT2 (PDB: 5G4C) with non-NAD ligands removed. Each molecule was docked 20 times to each protein, and the output of each docking simulation was the result of 2,500,000 evaluations. The lowest energy outputs for each interaction are displayed.

Generation of SIRT2$^{-/-}$ HEK293 Knockout Cell Lines. The biology software Benchling (San Francisco,CA) was used to design guide RNA (gRNA) oligonucleotides for SIRT2 directed at exon 4. gRNA forward sequence: 5′-CACCGCGTTCGCTCTGCATGTACC-3'. gRNA reverse sequence: 5′- aaacGGTACATGCAGAGCGAACGC-3'. gRNA inserts for SIRT2 were inserted into pSp-Cas9(bb)-2A-GFP plasmid as described by Cong et al.[4]. The PX458 plasmid containing gRNA inserts for SIRT2 was transformed into DH5α cells (New England Biolabs, Ipswich, MA). Transformed DH5α cells were then grown overnight on LB agar plates containing 50 μg/mL at 37°C. Single colonies were then grown overnight in LB supplemented with 100 μg/mL ampicillin and incubated at 37°C in a shaker at 250 RPM. A Zymo Plasmid Miniprep Kit (Zymo Research, Irvine, CA) was used to purify the plasmid DNA according to the manufacturer's protocol. Plasmid DNA concentration was determined using a Spectrophotometer NanoDrop ND-1000 (ThermoFisher Scientific, Waltham, MA). The purified plasmids were sequenced with the hU6 primer to confirm successful integration of the gRNA inserts into the PX458 plasmid.

Wild-type and GLO2$^{-/-}$ cells were seeded 200,000 cells/well in a six-well plate one day prior to transfection. Each well was incubated with a mixture of 5 μL of Lipofectamine 3000, 1.25 μL of P3000, 1.2 μg of plasmid DNA with gRNA inserts in 2 mL of Opti-MEM for 24 hours at 37°C. After 24 hours, Opti-MEM was replaced with low-glucose DMEM and cells to let cells recover for 24 hours. The cells were then scraped into warm PBS and collected for cell sorting. A BD FACSAria III in the University of Arizona Flow Cytometry Core Facility was used to perform fluorescence-assisted single cell sorting into two 96-well plates containing 100 μL low-glucose DMEM. Clones were maintained until a sufficient number of cells were obtained to assess successful knockout clones.

Chromatin Isolation. HEK293 cell pellets (WT, GLO2$^{-/-}$, SIRT2$^{-/-}$, SIRT2/GLO2$^{-/-}$) treated with 50 μM MGO, 50 μM alkynyl-MGO, or vehicle for 6 hours were resuspended by pipetting in a hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% IGEPAL, 5 mM NaB, pH 7.9) containing protease and phosphatase inhibitor cocktails (1:500 v/v, Sigma Aldrich, St. Louis, MO) and incubated on ice for 30 minutes. The nuclear fraction was then isolated via centrifugation at 1,000 x *g* for 10 minutes at 4°C. The supernatant, containing the non-nuclear fraction, was then put in a separate tube and stored in -20°C. The nuclear pellets were then resuspended in a high salt buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 25% glycerol, 5 mM NaB, pH 7.9) containing protease and phosphatase inhibitors (1:500 v/v) and rotated end-over-end overnight at 4°C. Chromatin was then isolated via centrifugation at 1,000 x *g* for 20 minutes at 4°C and resuspended in ddH_2O and sonicated into solution.

SDS-PAGE and Immunoblotting. HEK293 cells (WT, GLO2$^{-/-}$, SIRT2$^{-/-}$, SIRT2/GLO2$^{-/-}$) treated with 50 μM MGO or vehicle for 6 hours were lysed in a buffer containing 150 mM

NaCl, 50 mM HEPES, and 1% IGEPAL, pH 7.4. The lysates were denatured in SDS loading buffer and heated at 95°C for 5 minutes. Proteins were then resolved via SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and blocked with Intercept PBS Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for 30 minutes. Membranes were incubated with primary antibodies overnight at 4°C: HAGH (GLO2, 1:1000, Invitrogen, PA5-80682), SIRT2 (1:500, Cell Signaling Technologies, 12650T), , Actin (1:10,000, Sigma, A1978). Membranes were washed with TBST (TBS + 0.1% Tween-20) three times, 5 minutes each. Infrared secondaries (1:5000, Li-Cor Biosciences) were added in 1:1 blocking buffer and TBST and incubated for 45 minutes. After three additional TBST washes, the blots were imaged with a c600 Azure Imaging System (Azure Biosystems, Dublin, CA).

Reactivity-Based Protein Profiling. WT, GLO2$^{-/-}$, SIRT2$^{-/-}$, and SIRT2$^{-/-}$/GLO2$^{-/-}$ cells were treated with either 50 µM alkynyl-MGO or vehicle for 6 hours[5]. Chromatin was isolated with the method above. To generate whole cell lysates, cell pellets resuspended and sonicated in PBS containing an EDTA-free protease inhibitor pellet (Pierce, San Jose, CA) and insoluble debris were removed via centrifugation at 14,000 x *g* for 10 minutes at 4°C. 25 µg of chromatin or soluble protein was than diluted into a total of 27µL PBS. Click chemistry was performed by adding 200 µM TBTA (final concentration, TCI America, Portland, OR), 1mM $CuSO_4$ (final concentration, Acros Organics, Fair Lawn, NJ), 1mM TCEP (final concentration, Thermo Scientific, San Jose, CA), and 100 µM N_3-Biotin (Click Chemistry Tools, Scottsdale, AZ) in 3 µL to end with a final volume of 30 µL. 10 µL 4x loading buffer was added after 90 minutes of incubation at room temperature in the dark. Proteins were then separated (2.5 µg chromatin, 25 µg whole cell lysate) on a 15% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and blocked with Intercept PBS Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for 30 minutes. The blots were then probed with an IRDye 800CW Streptavidin antibody (1:5000, Li-Cor Biosciences), added in 1:1 blocking buffer and TBST (TBS + 0.1% Tween-20)) and incubated for 45 minutes. Membranes were washed with TBST three times, 5 minutes each, and visualized with a c600 Azure Imaging System (Azure Biosystems, Dublin, CA). After imaging, Ponceau S stain was used to ensure equal loading.

Quantitation of LactoylLys PTMs using SILAC QuARKMod. GLO2$^{-/-}$ or SIRT2$^{-/-}$/GLO2$^{-/-}$ cells were cultured in DMEM Flex medium for Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) (ThermoFisher Scientific) supplemented with 1 g/L sterile filtered glucose, 2 mM glutamine, 1 mM pyruvate, and 10% dialyzed FBS (Gibco). GLO2$^{-/-}$ HEK293 cells were cultured in 'heavy' media containing 0.1 g/L $^{13}C_6{}^{15}N_2$ Lysine and 0.1 g/L $^{13}C_6{}^{15}N_4$ Arginine for a minimum of 5 passages before treatment and SIRT2/GLO2$^{-/-}$ HEK293 cells were cultured in 'light' media containing natural abundance isotope amino acids for a minimum of 5 passages before treatment. Each cell line (N = 4) was treated with high glucose (25 mM glucose) in their respective media (light or heavy) for 6 hours. After treatment, cells were pelleted and lysed via sonication in lysis buffer (150 mM NaCl, 50 mM HEPES, and 1% IGEPAL, pH 7.4) containing protease and phosphatase inhibitor cocktails (1:500 v/v, Sigma Aldrich, St. Louis, MO). Insoluble debris was removed via centrifugation at 14,000 x *g* for 10 minutes at 4°C. 1.3 mg of soluble protein from each cell line was mixed and precipitated in 700 µL ice-cold acetone for > 1 hour at -20°C.

Precipitated protein was pelleted via centrifugation at 14,000 x *g* for 10 minutes at 4°C, acetone was removed, and pellets were allowed air dry. Protein pellets were resuspended in 300 µL of 50 mM NH_4HCO_3 (pH 8.0) with 1 mM CaCl (final concentration). Proteins were digested with sequencing grade trypsin (1:100 w/w, Promega) overnight at 37°C. Trypsin was denatured via boiling at 95 °C for 10 min. Once samples had cooled to room temperature, samples were spun down at 14,000 x *g* for 10 minutes at 4°C and supernatant with digested protein was transferred to a new tube. Aminopeptidase (15 µg in 10 µL) was added to 50 µg of each sample (from supernatant) and incubated overnight at 37 °C. Aminopeptidase was denatured via heating at 95 °C for 10 min and samples were cooled to room temperature. A 1:1 ratio of Heptafluorobutyric acid (HFBA) and water (15 µL) was added to each sample. Debris was removed via centrifugation at 14,000 × *g* for 10 min and lactoylLys was further analyzed via LC-MS/MS as previously described.[6] Briefly, a Shimadzu LC system equipped with a 150 x 2.1mm, 3.5 µm particle Eclipse XDB-C8 column (Agilent, Santa Clara, CA) was used to chromatograph 12 µL of supernatant. Mobile phase A: 10 mM HFBA in H_2O; mobile phase B: 10 mM HFBA in acetonitrile. With a flow rate of 0.425 mL/min the following gradient was used: 0.5 min, 5% B; 8 min, 50% B; 8.5 min, 80% B; 9.5 min, 5% B. The column was equilibrated for 5 minutes at 5% B between samples. Scheduled MRM was conducted in positive mode using an AB SCIEX 4500 QTRAP. The MRM detection window was 90 seconds with a target scan time of 0.1.16 seconds. The following parameters were used for detection:

Species	Q1 (m/z)	Q3 (m/z)	Time	CE (V)
“Light” LactoylLys	219	84	3.6	41
“Heavy” LactoylLys	227	90	3.6	41

LactoylLys was quantified as light ($SIRT2^{-/-}$/$GLO2^{-/-}$) peak area over heavy ($GLO2^{-/-}$) peak area to create a L:H ratio.

Statistical Analysis. Data were quantified using either a Michaelis-Menten non-regression model, t-test, or one-way ANOVA (when applicable for each) using a Tukey post-hoc test. Differences were considered to be significant when $P < 0.05$ with the N indicated in each figure legend. All analyses were carried out using Prism 9 for Macintosh, GraphPad Software.

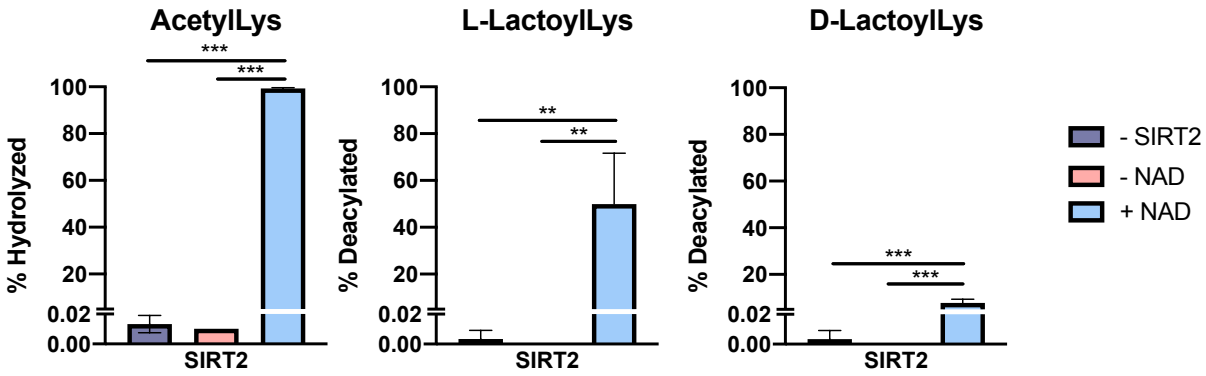

Figure S1. EIPAE-K*-VFLAQ peptides were incubated with NAD+, DTT, and recombinant SIRT2. Without NAD+, SIRT2 possesses no enzymatic activity. N = 3 **p < 0.01, ***p < 0.001. Statistical significance determines via One-Way ANOVA.

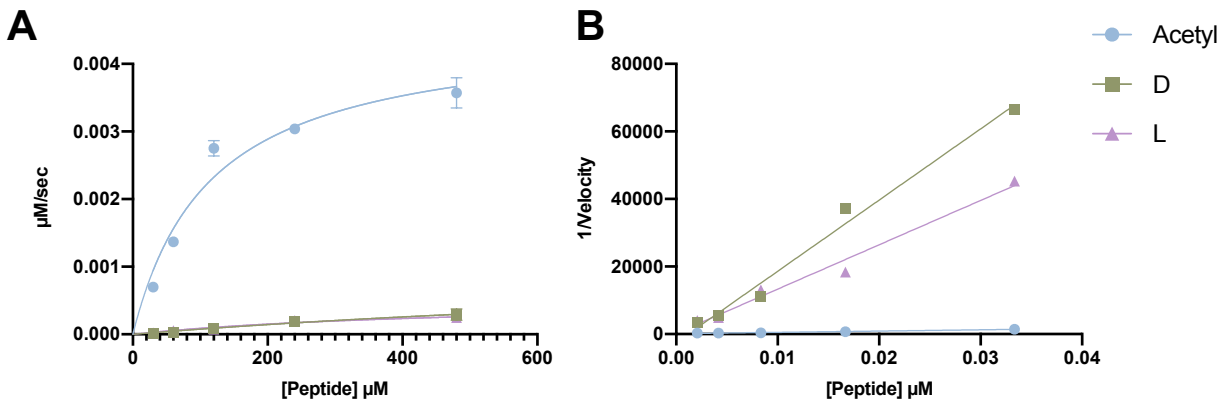

Figure S2. Enzyme Kinetics for recombinant SIRT2. (A) Michaelis-Menten curve and (B) Lineweaver-Burk plot for recombinant SIRT2 on EIPAE-K*-VFLAQ peptides. K_m ± S.E.; k_{cat} ± S.E; N = 3. Acetyl, EIPAEK$_{ac}$VFLAQ; L, EIPAEK$_{L\text{-}la}$VFLAQ; D, EIPAEK$_{D\text{-}la}$VFLAQ.

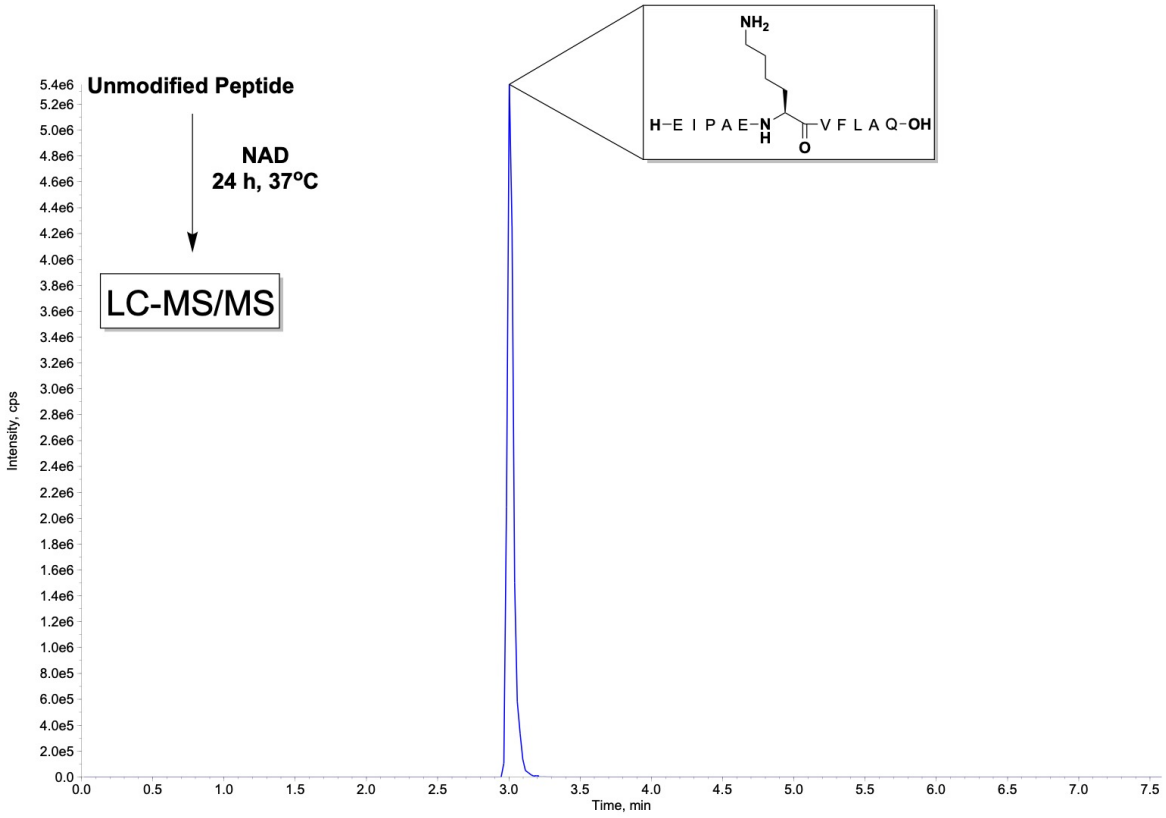

Figure S3. LC-MS/MS chromatograph of EIPAEKVFLAQ PKM2 peptide incubated with NAD+ for 24 hours at 37°C.

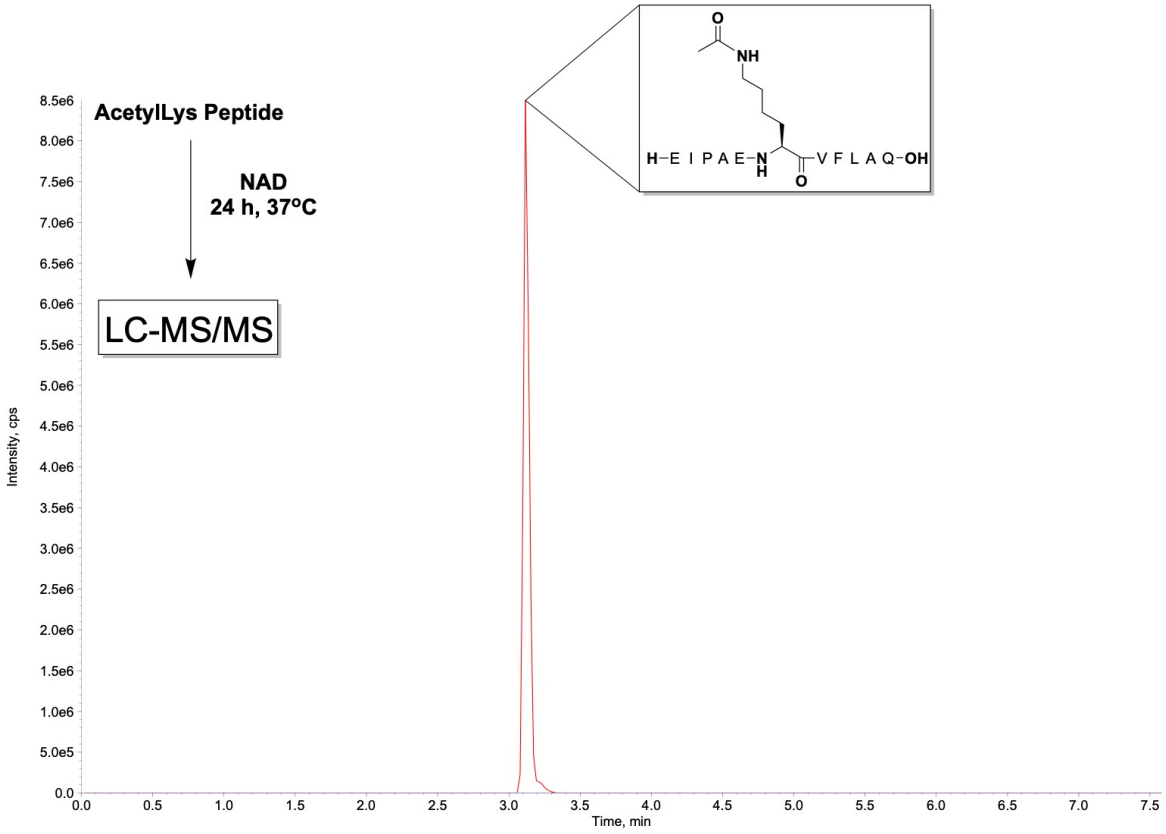

Figure S4. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with NAD+ for 24 hours at 37°C.

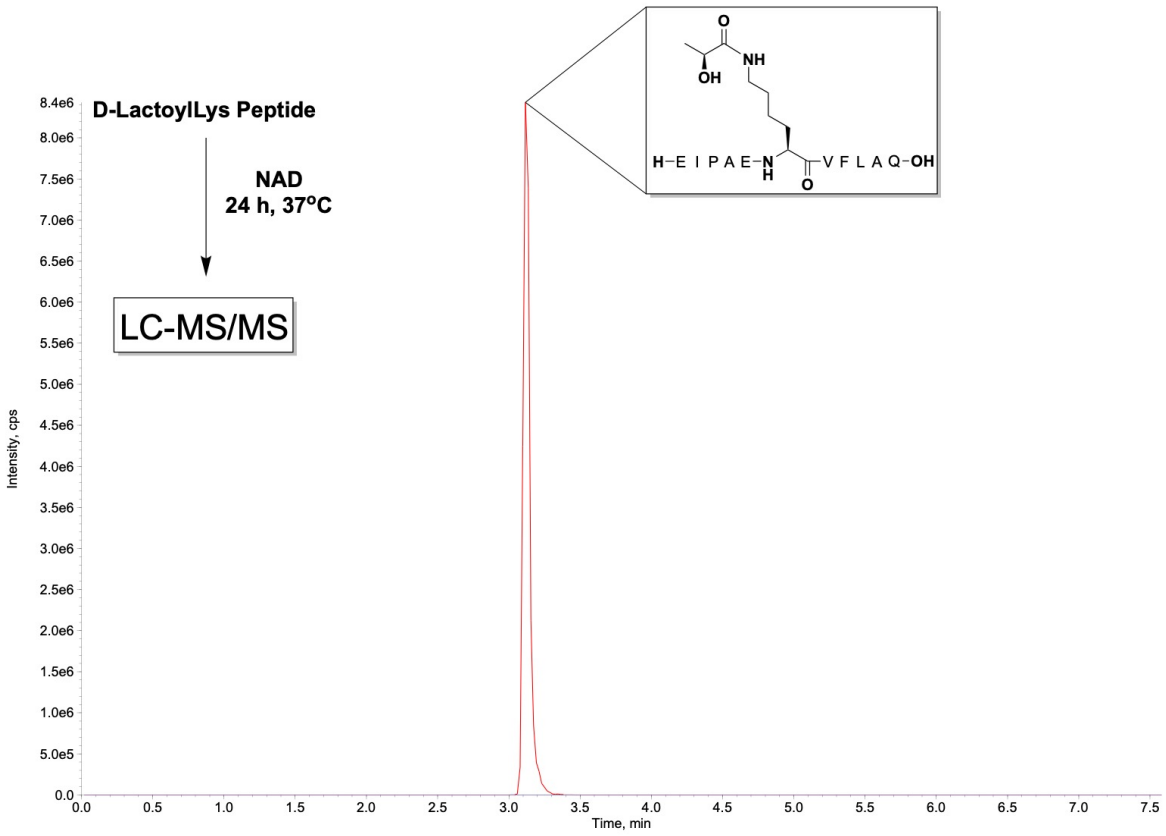

Figure S5. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with NAD+ for 24 hours at 37°C.

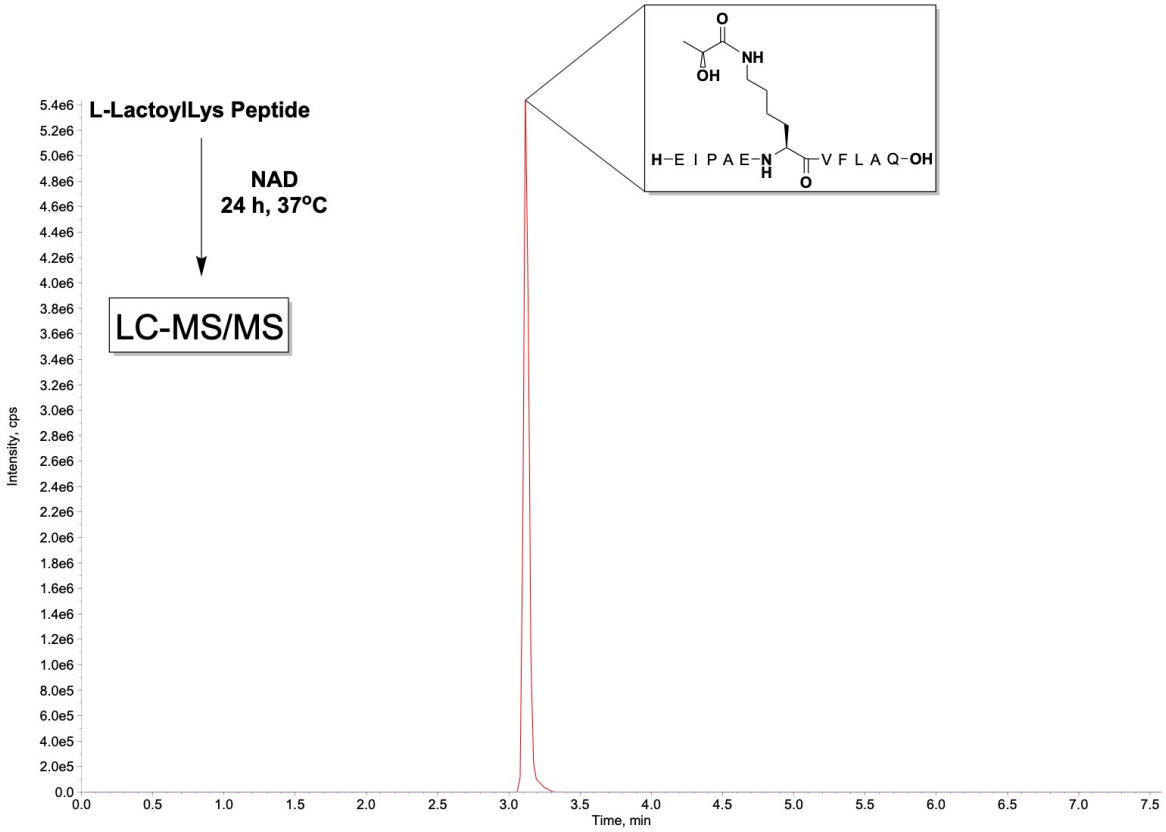

Figure S6. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with NAD+ for 24 hours at 37°C.

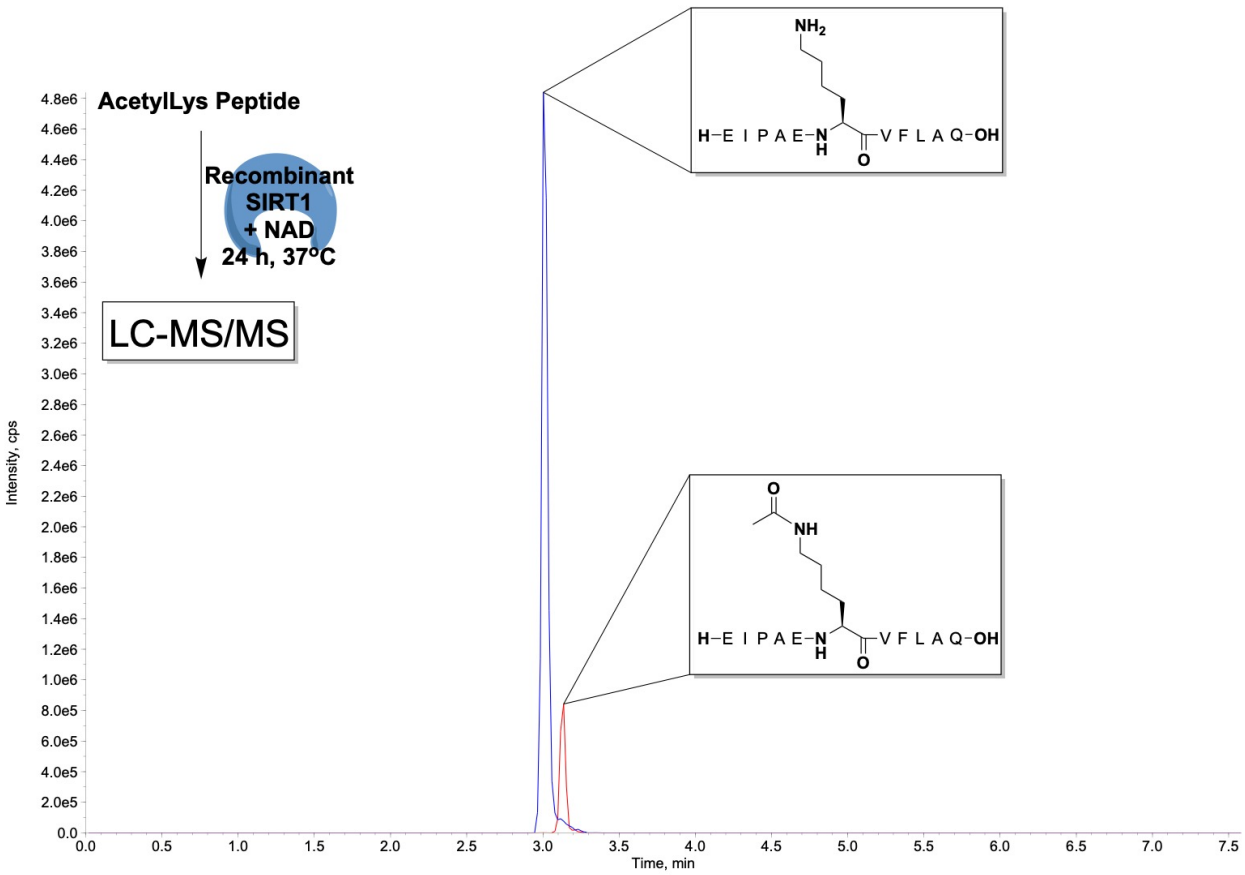

Figure S7. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT1 and NAD+ for 24 hours at 37°C.

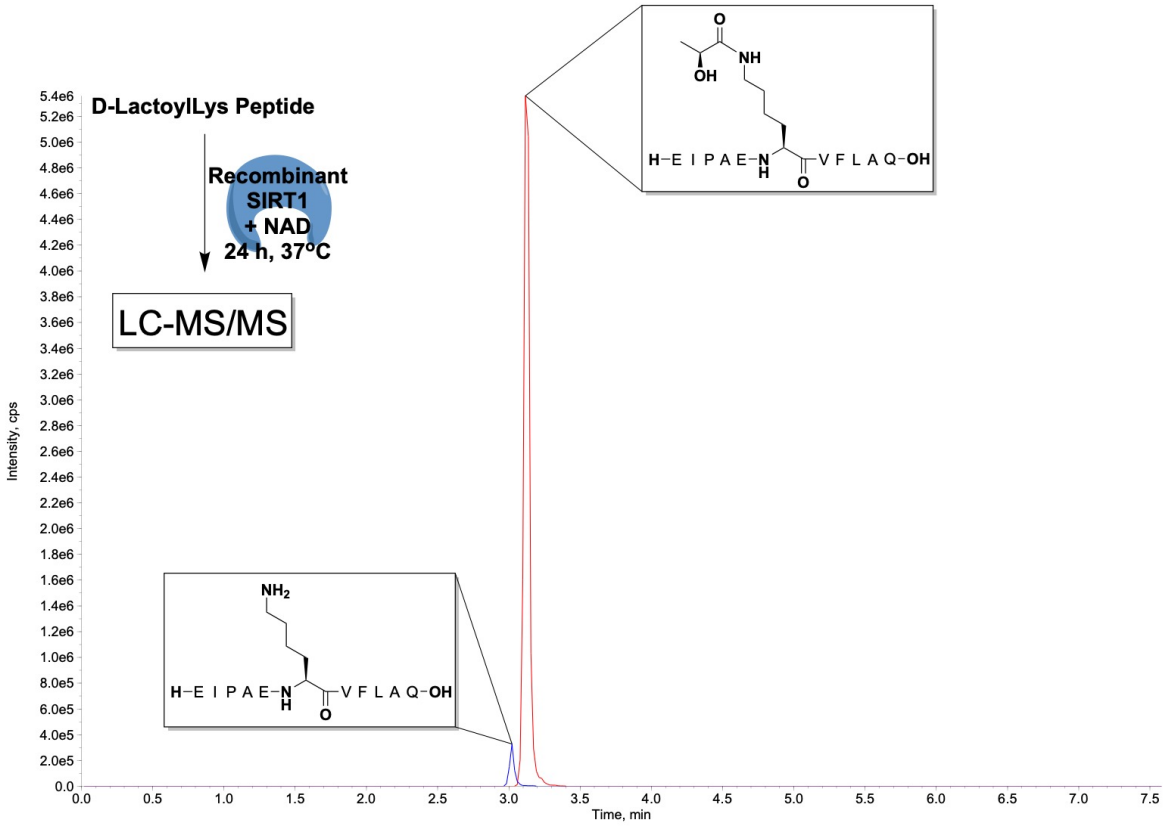

Figure S8. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT1 and NAD+ for 24 hours at 37°C.

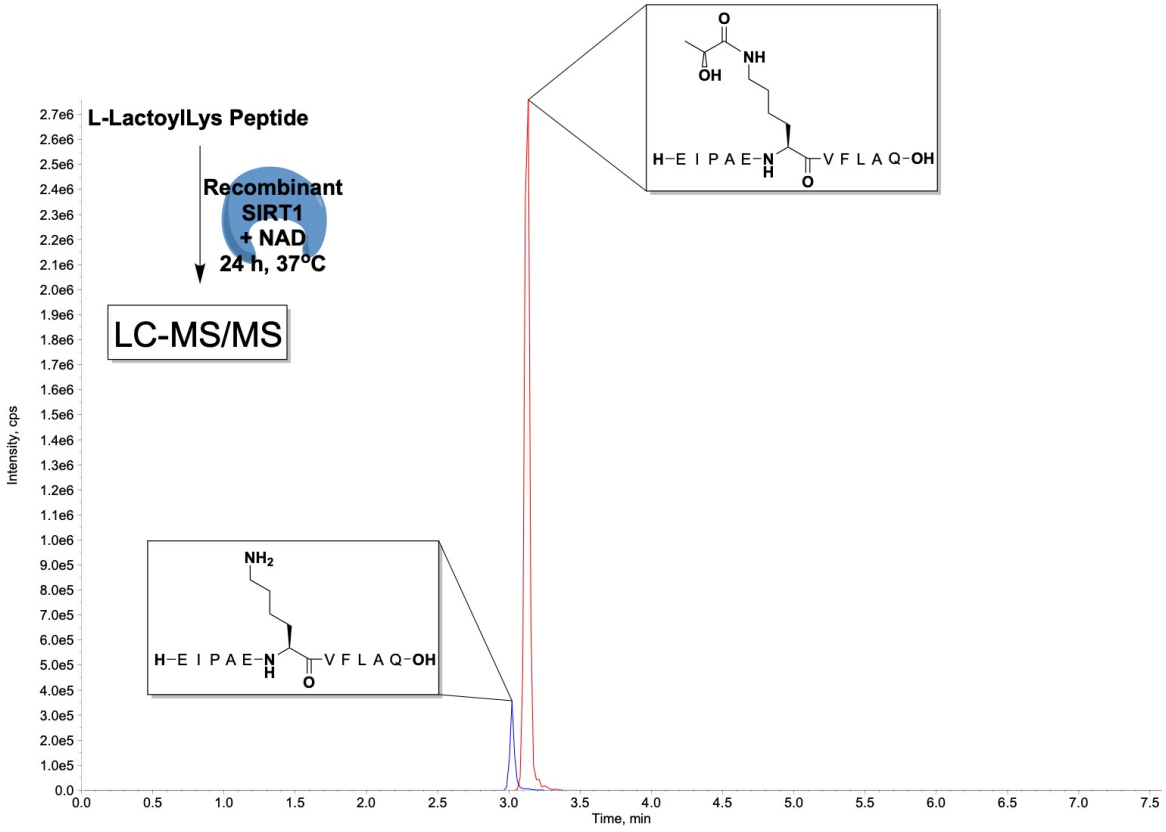

Figure S9. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT1 and NAD+ for 24 hours at 37°C.

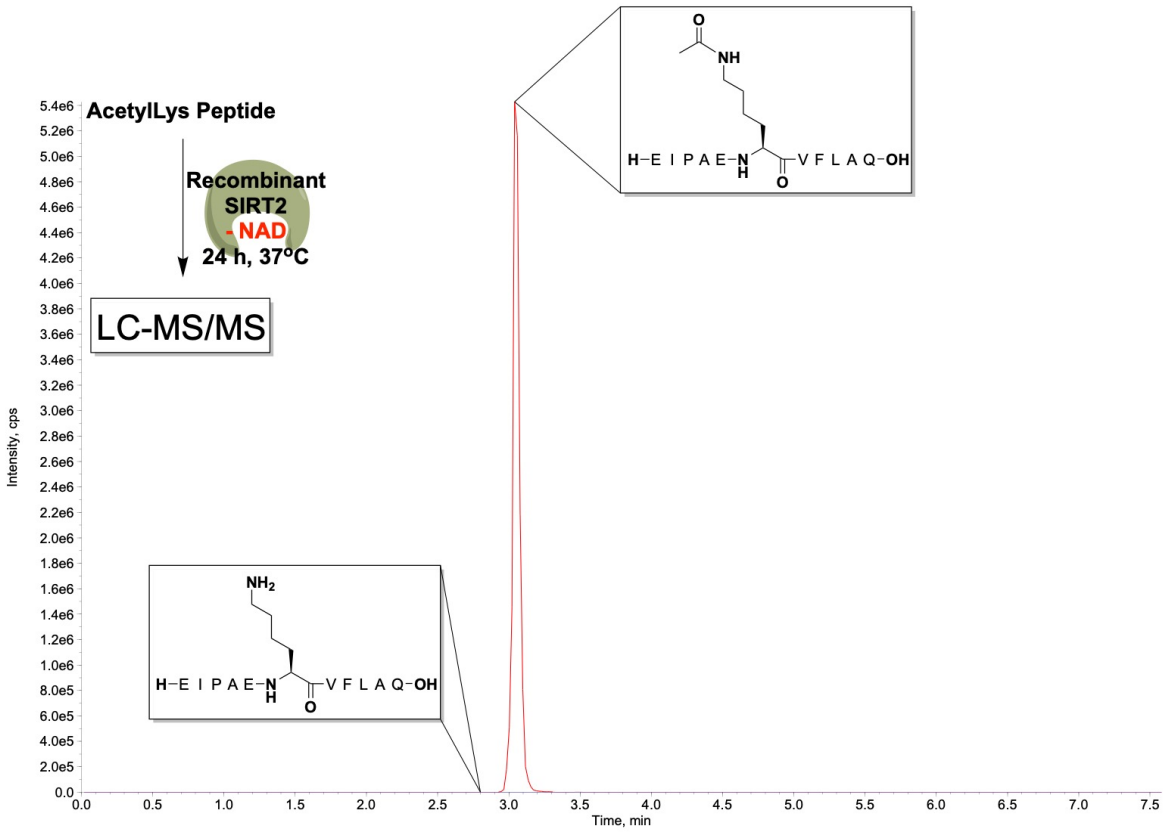

Figure S10. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT2 without NAD+ for 24 hours at 37°C.

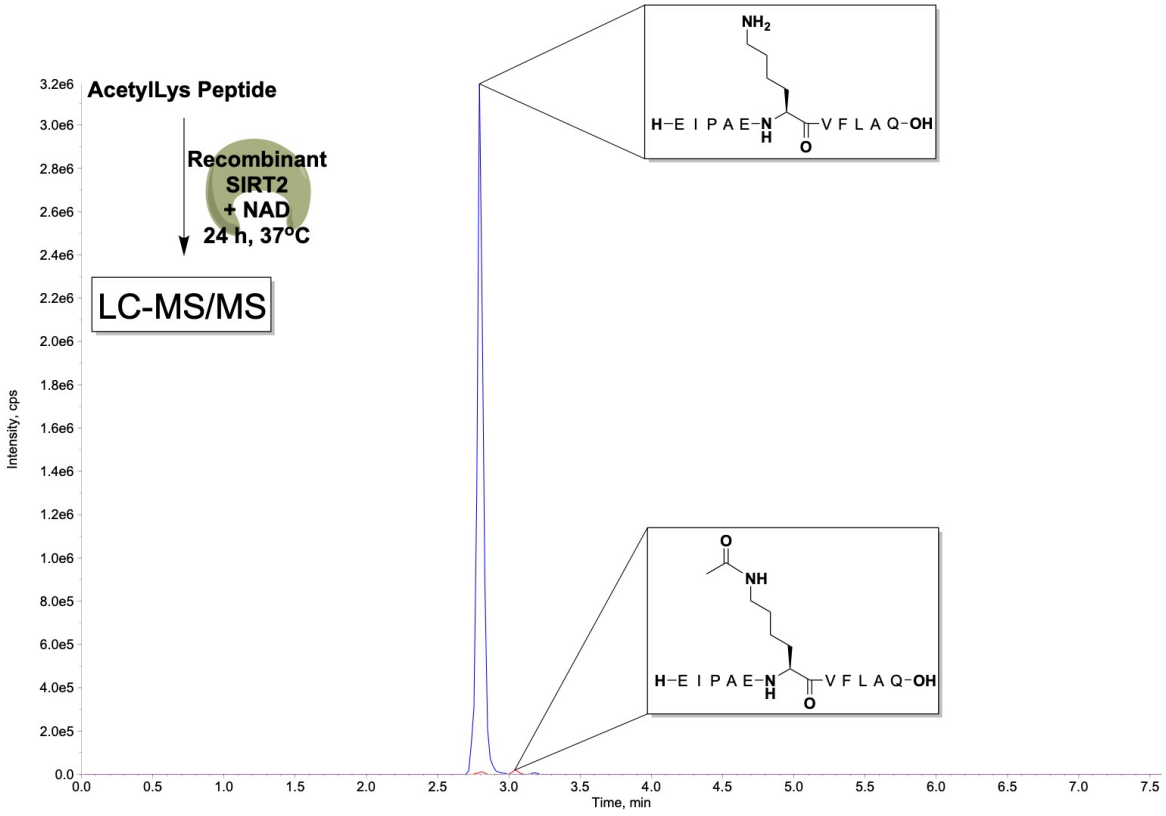

Figure S11. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT2 and NAD+ for 24 hours at 37°C.

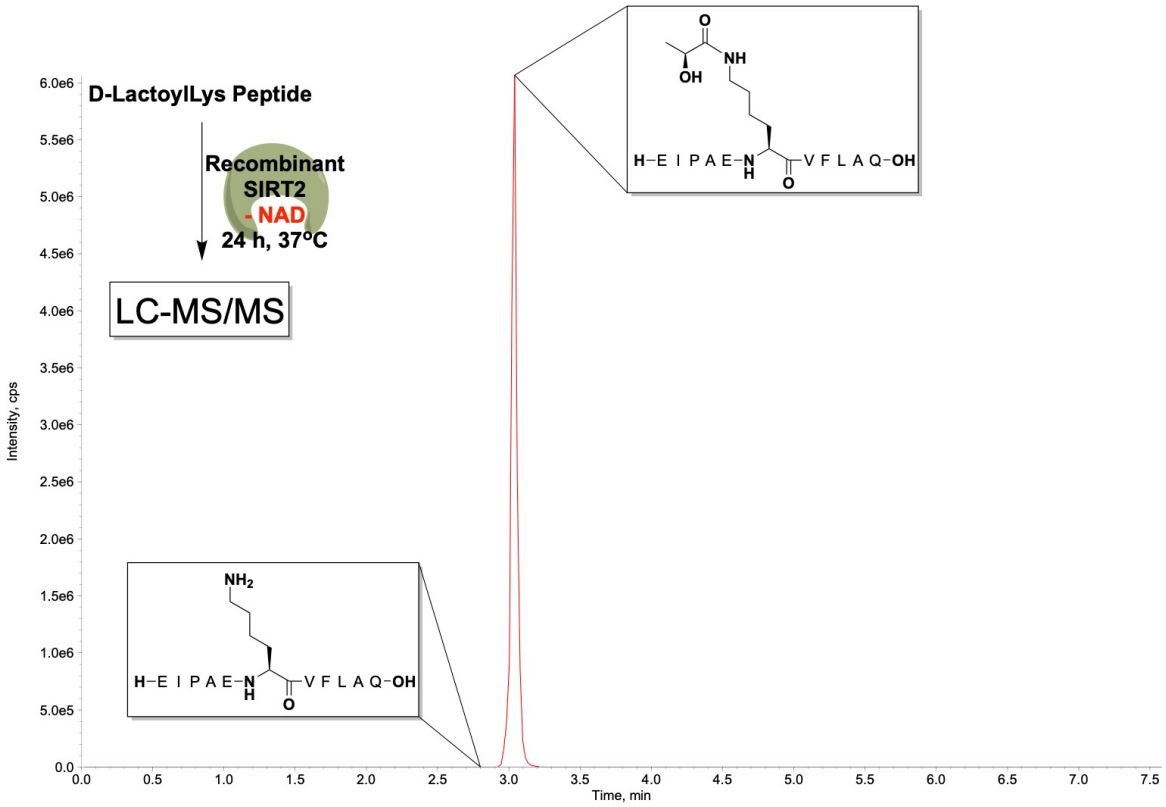

Figure S12. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT2 without NAD+ for 24 hours at 37°C.

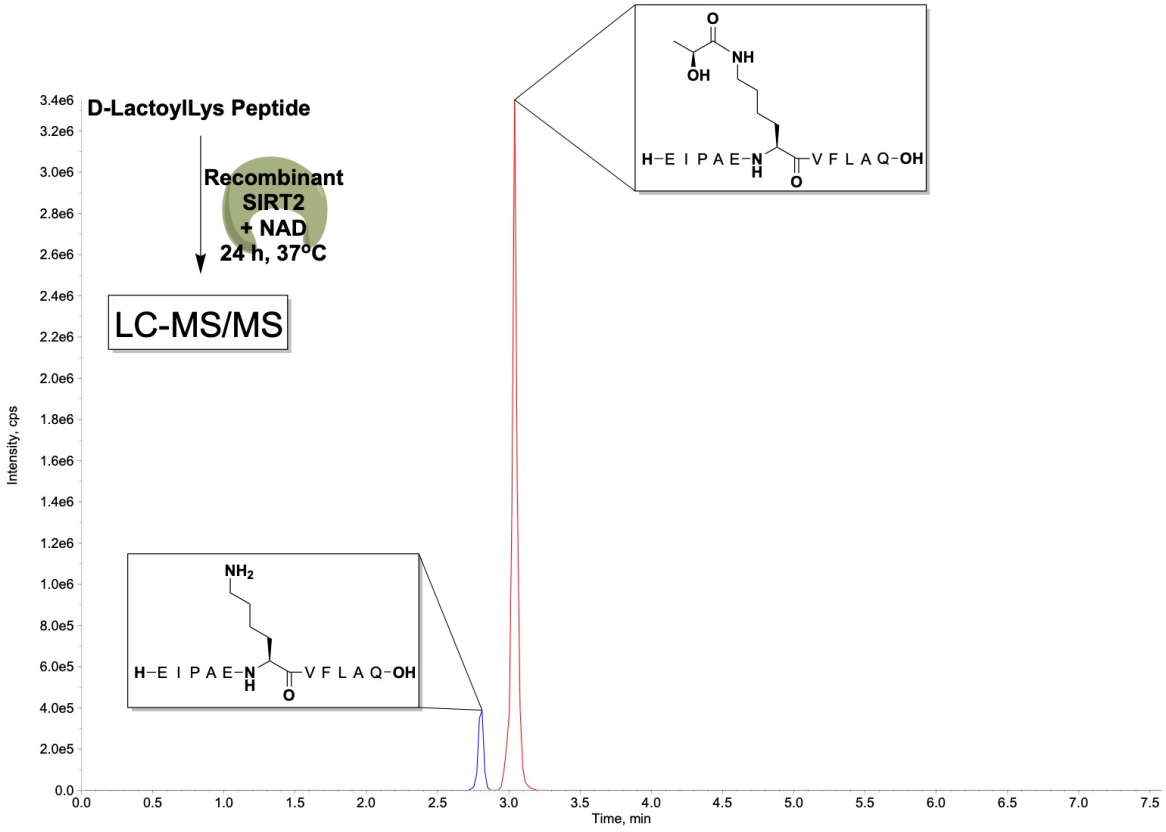

Figure S13. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT2 and NAD+ for 24 hours at 37°C.

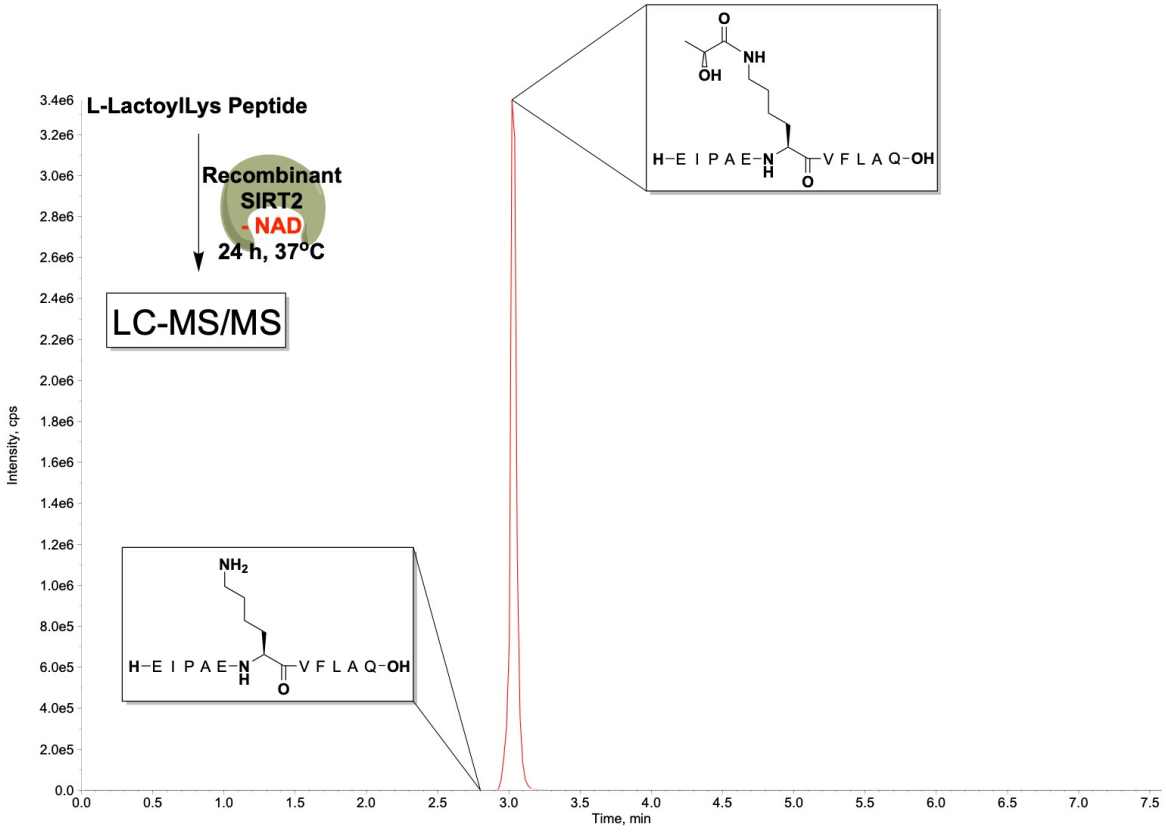

Figure 14. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT2 without NAD+ for 24 hours at 37°C.

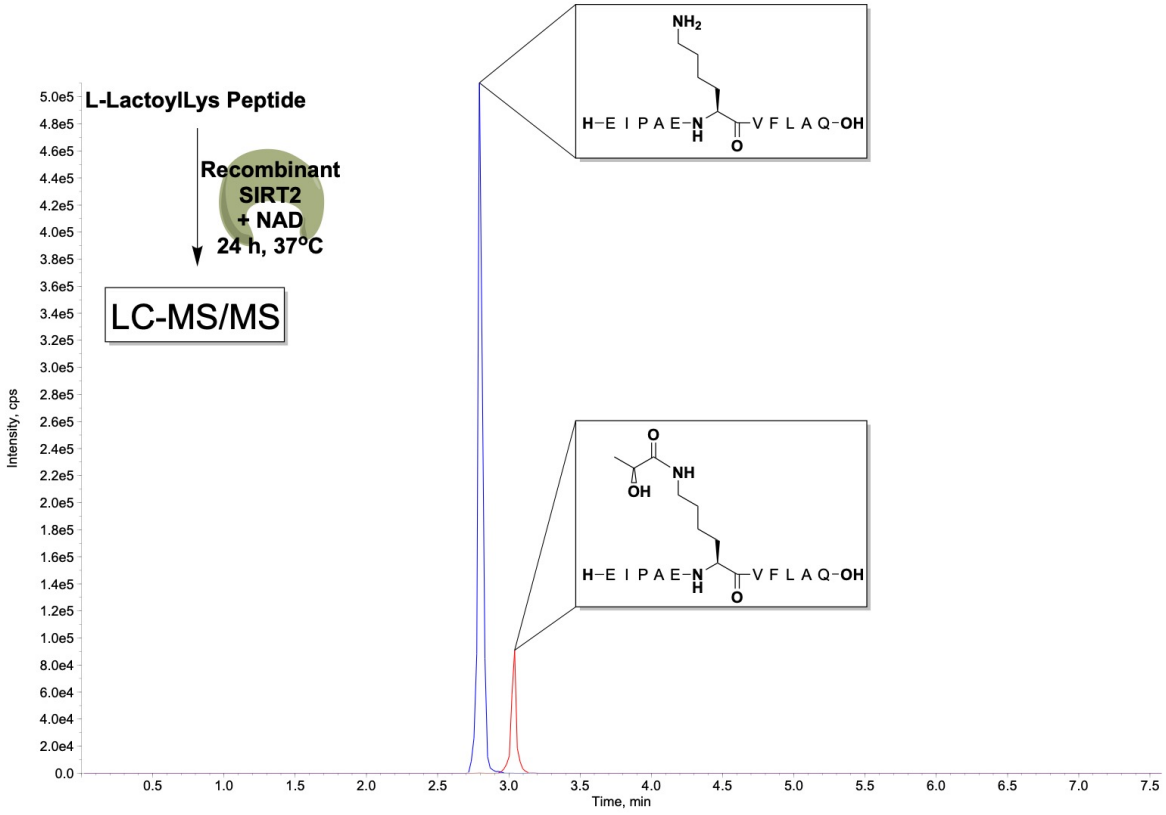

Figure S15. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT2 and NAD+ for 24 hours at 37°C.

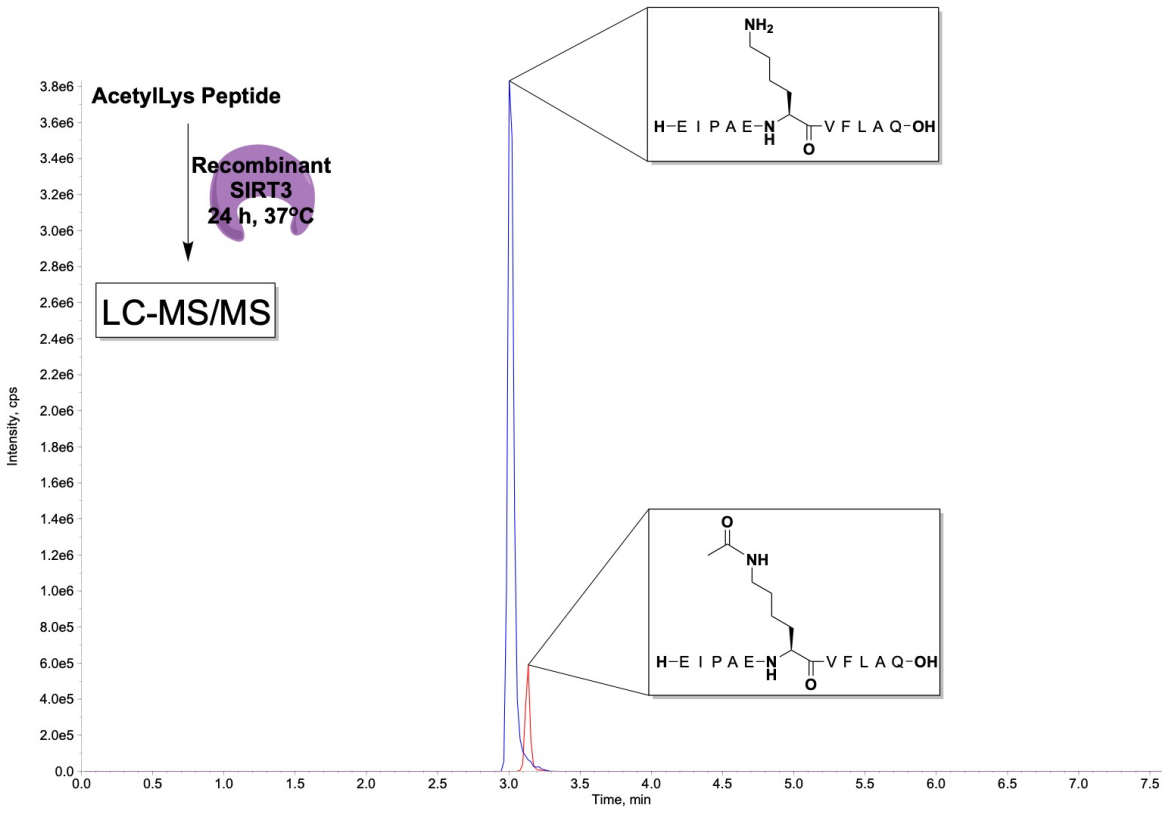

Figure S16. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT3 and NAD+ for 24 hours at 37°C.

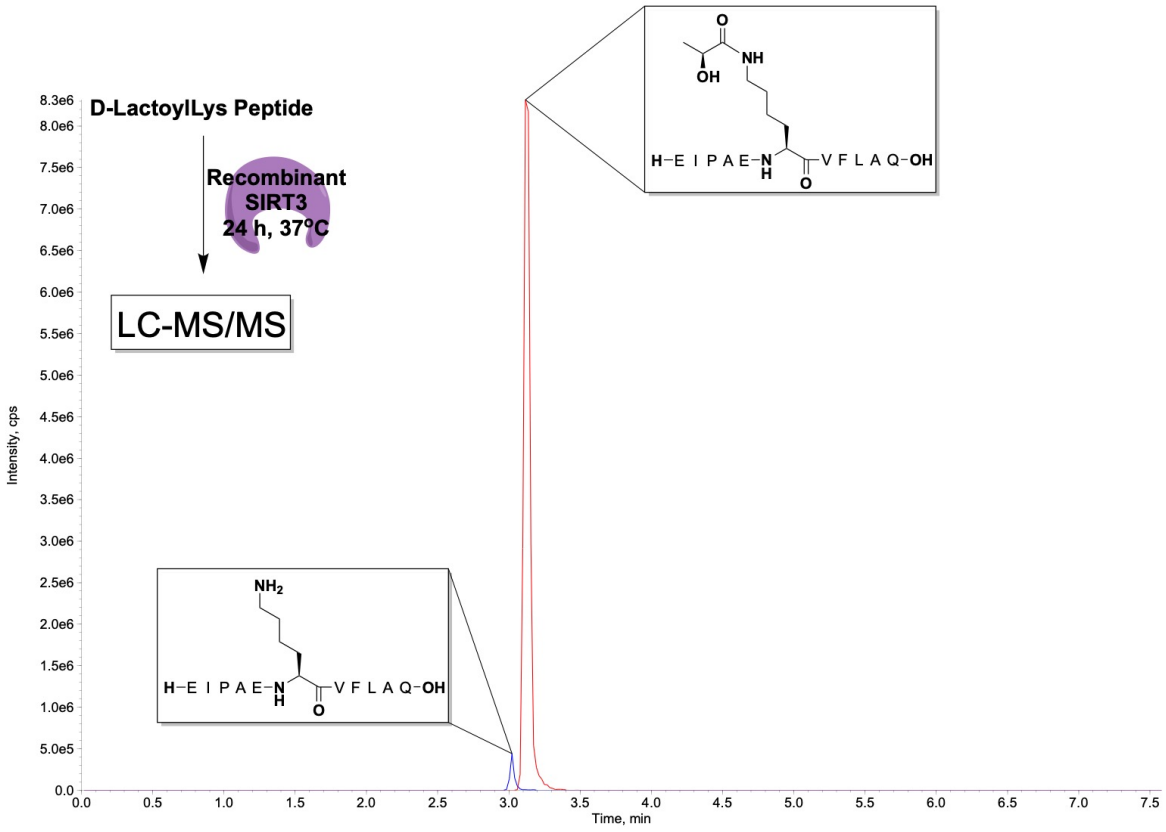

Figure S17. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT3 and NAD+ for 24 hours at 37°C.

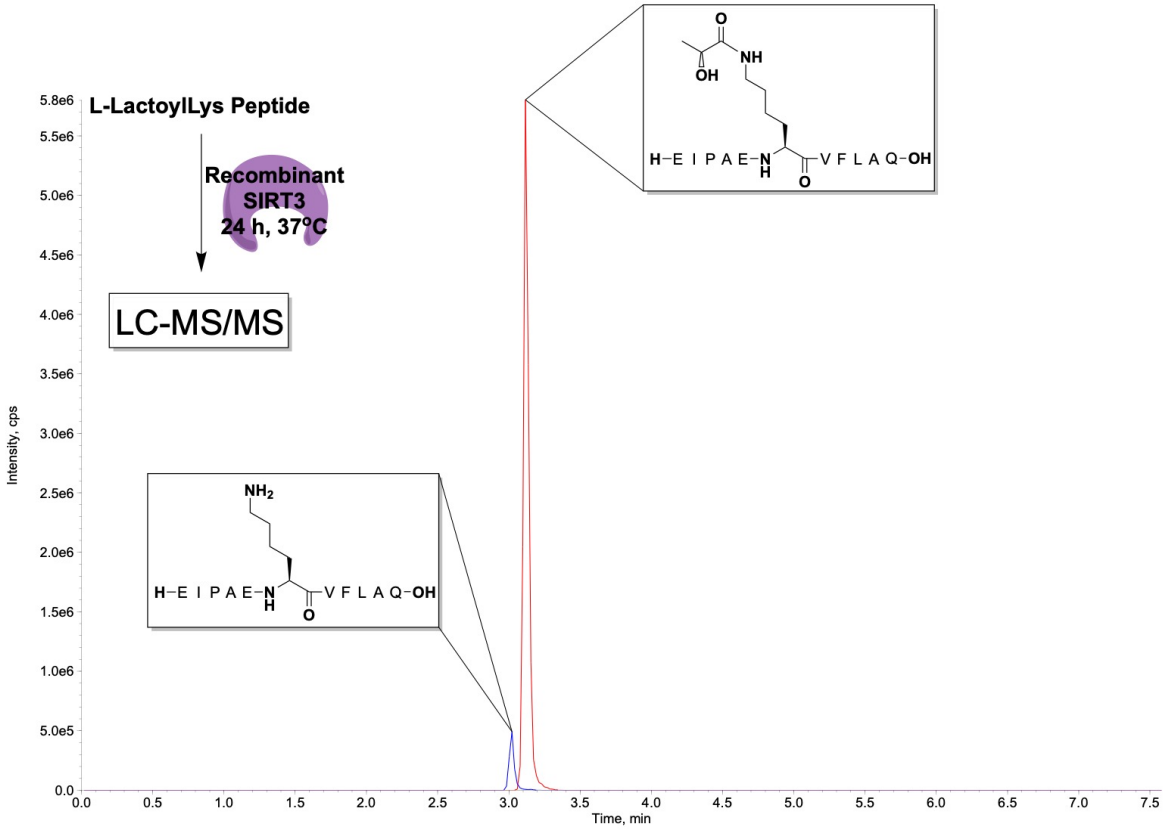

Figure S18. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT3 and NAD+ for 24 hours at 37°C.

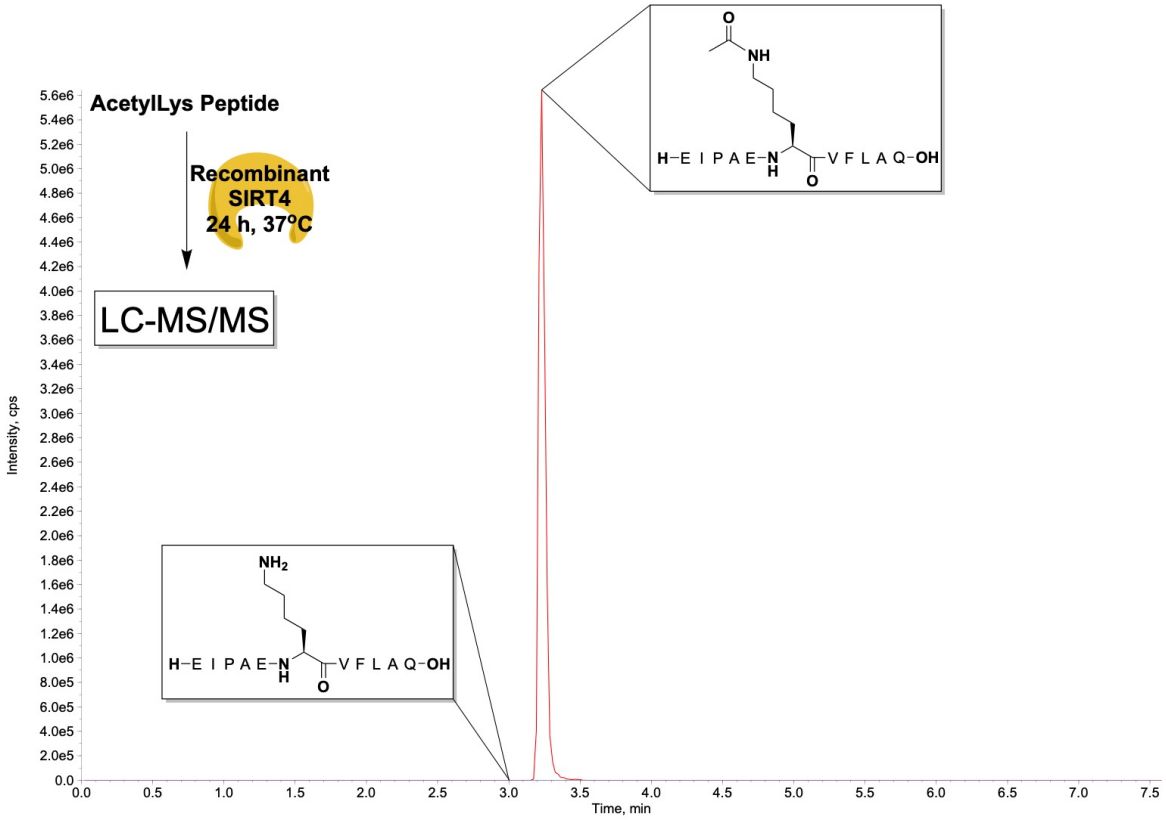

Figure S19. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT4 and NAD+ for 24 hours at 37°C.

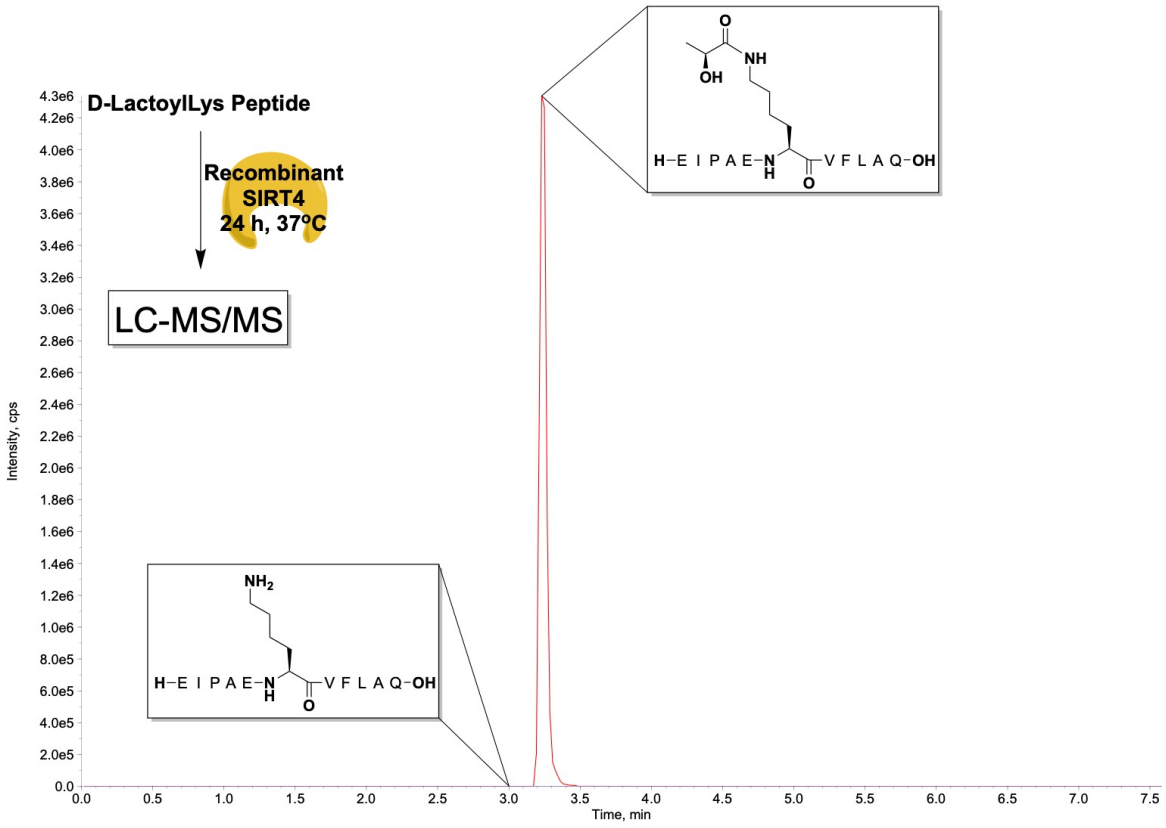

Figure S20. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT4 and NAD+ for 24 hours at 37°C.

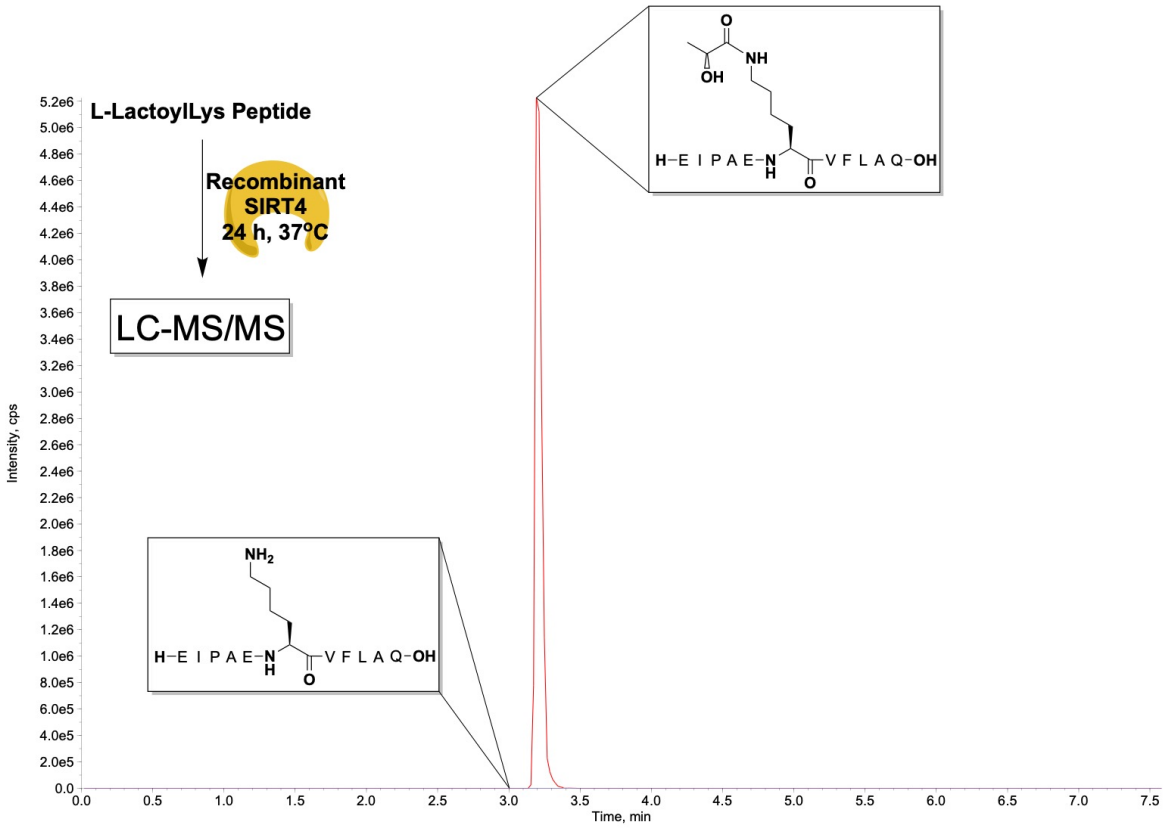

Figure S21. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT4 and NAD+ for 24 hours at 37°C.

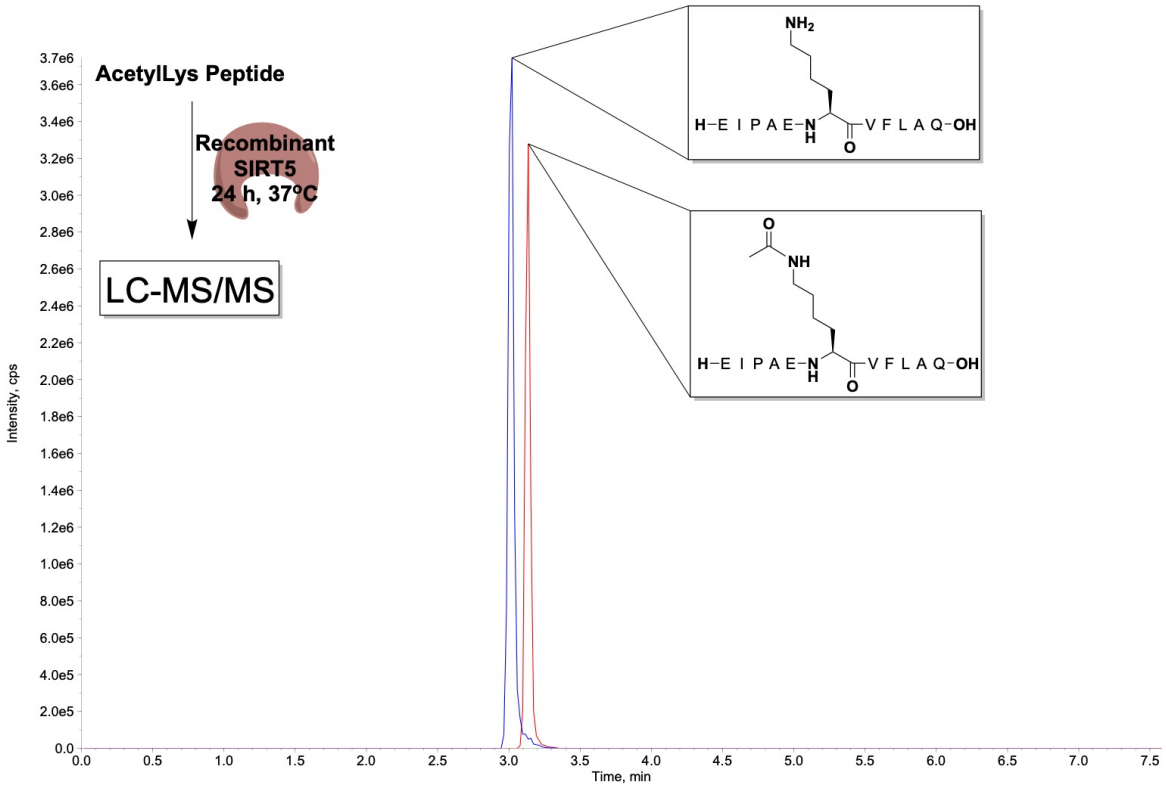

Figure S22. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT5 and NAD+ for 24 hours at 37°C.

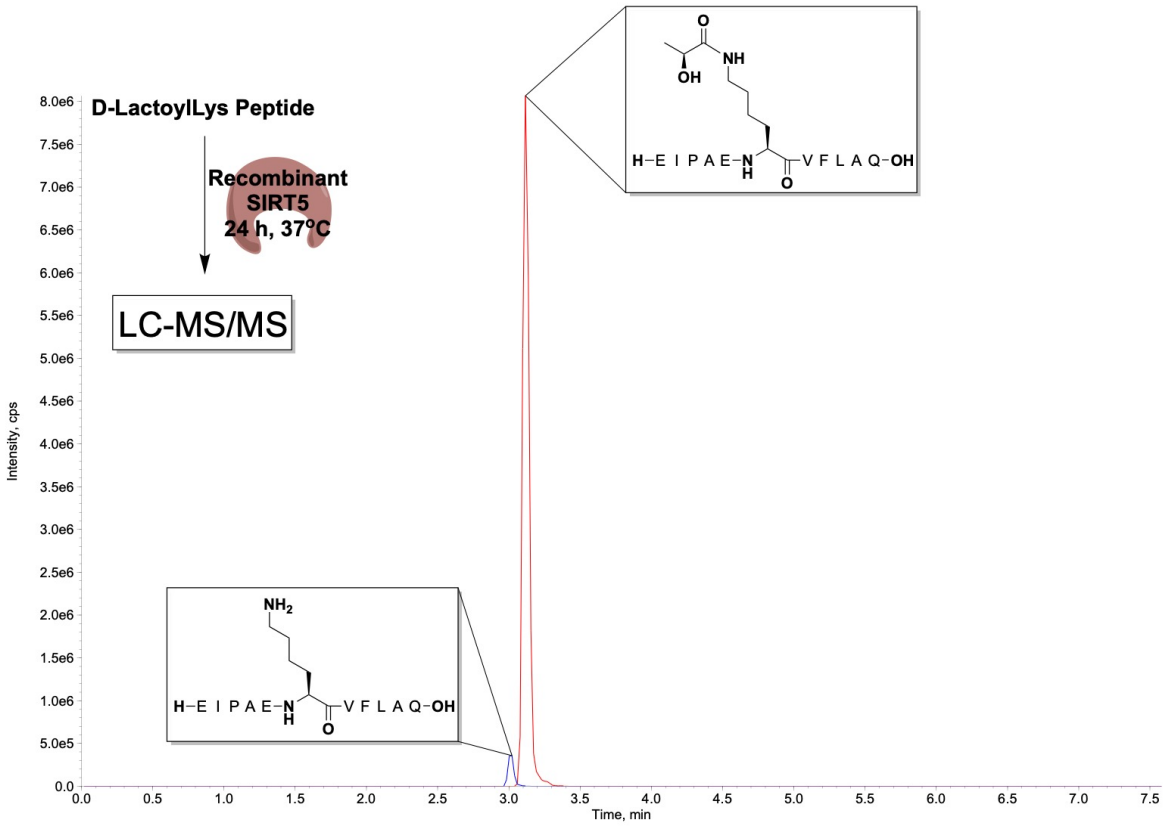

Figure S23. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT5 and NAD+ for 24 hours at 37°C.

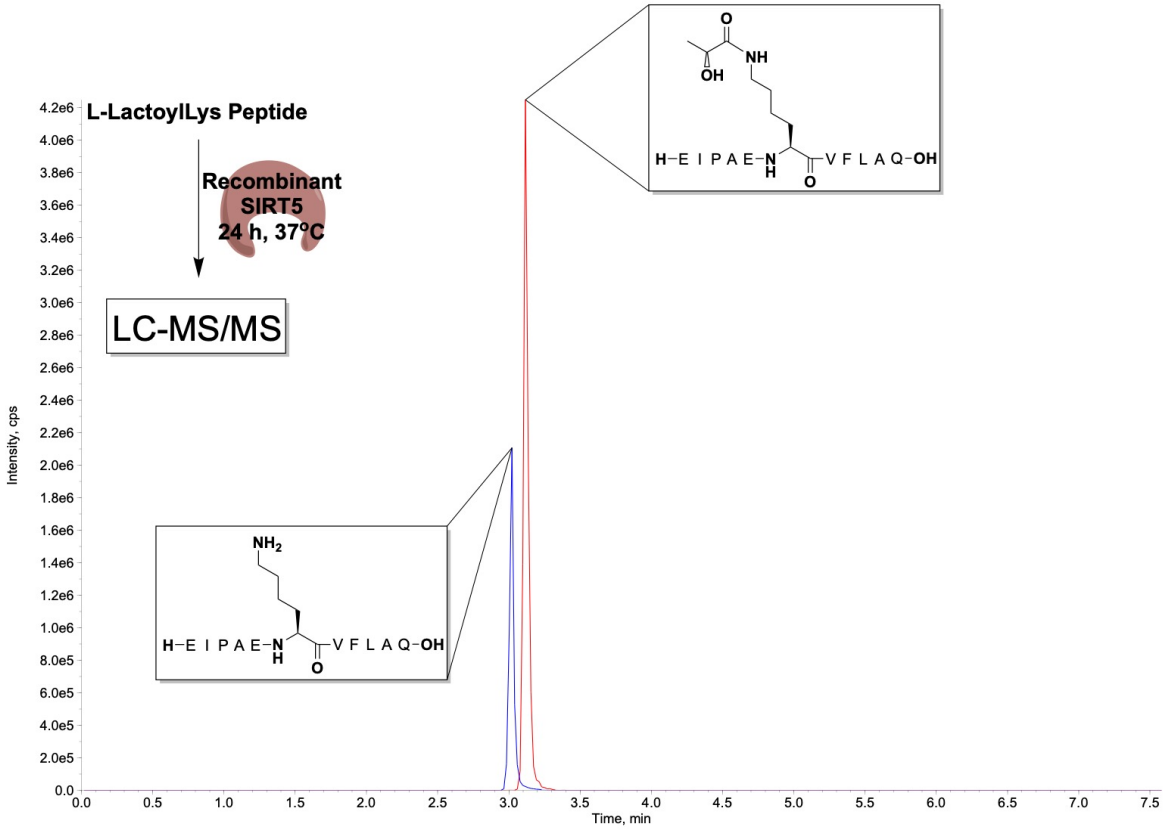

Figure S24. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT5 and NAD+ for 24 hours at 37°C.

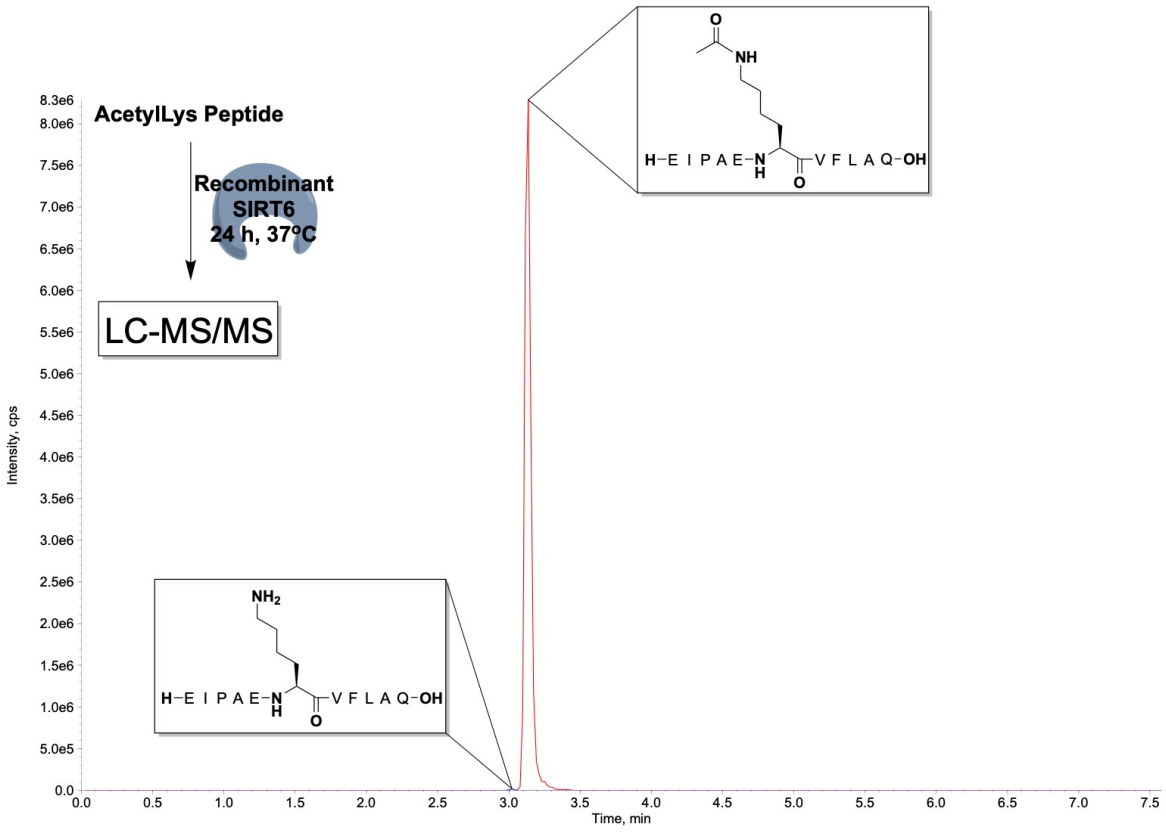

Figure S25. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT6 and NAD+ for 24 hours at 37°C.

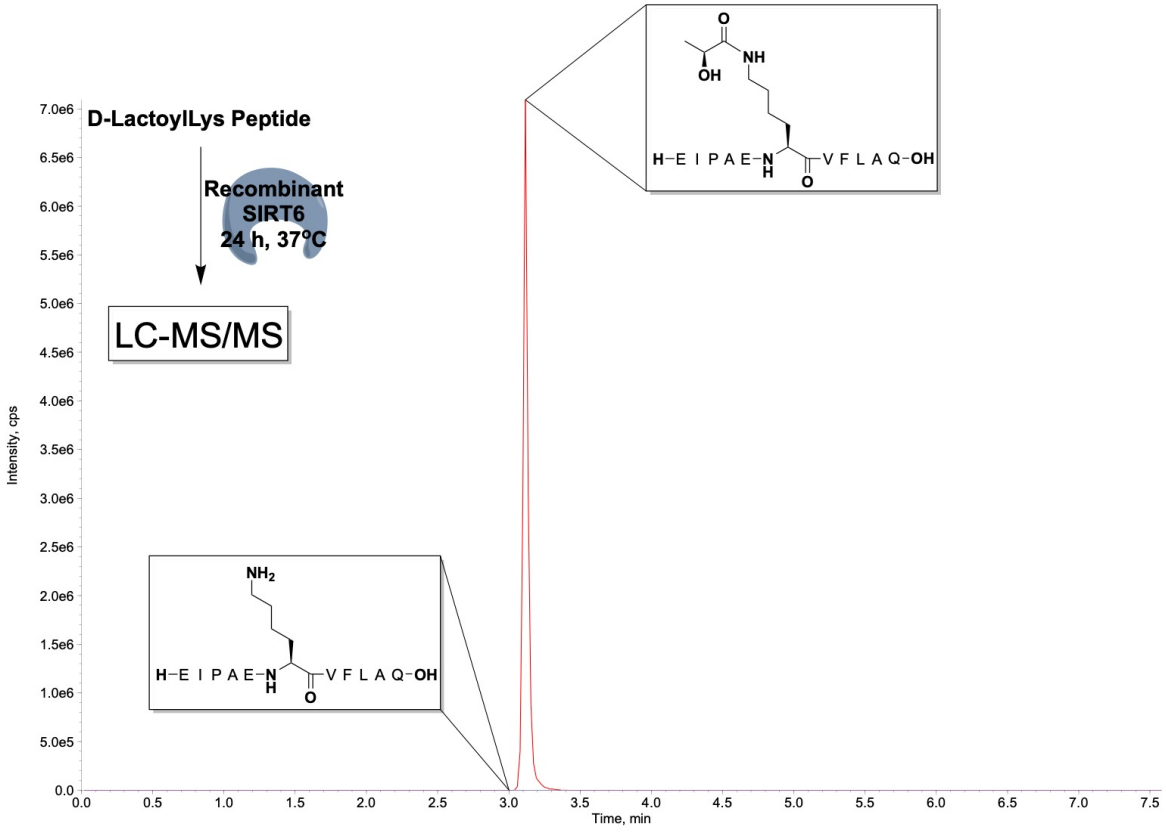

Figure S26. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT6 and NAD+ for 24 hours at 37°C.

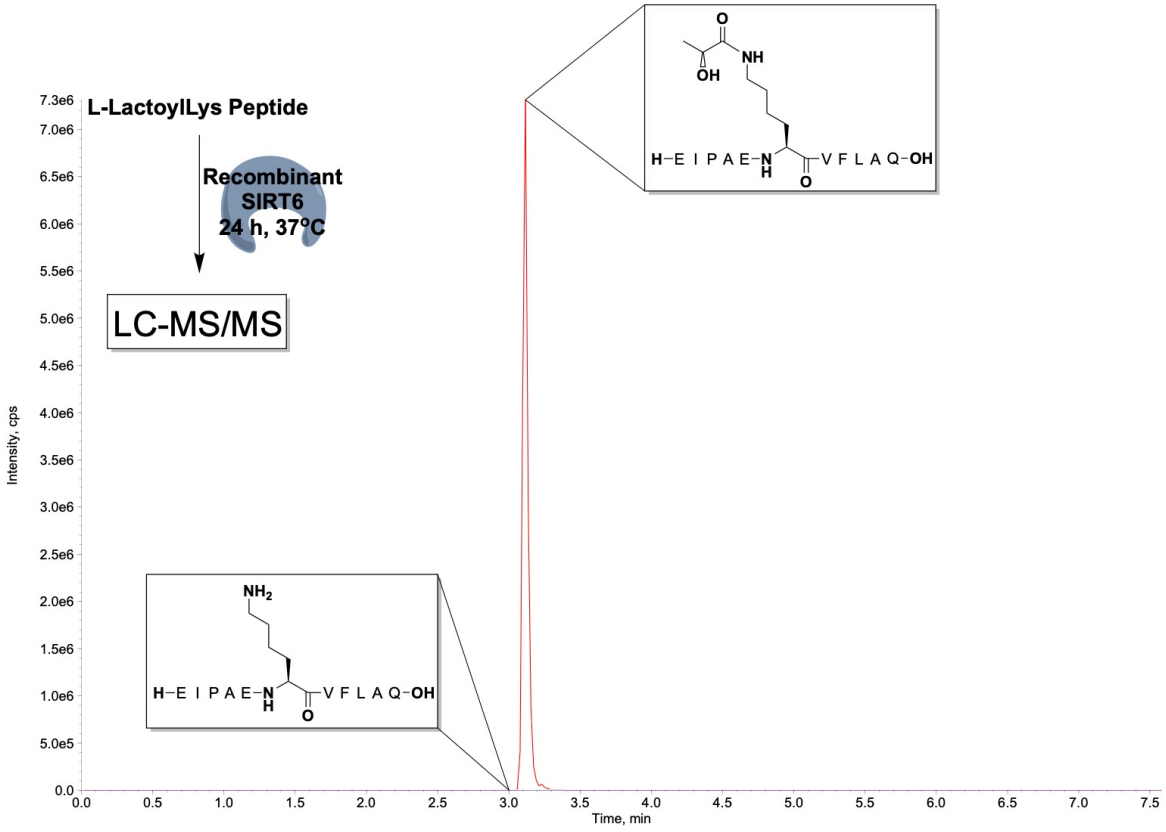

Figure S27. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT6 and NAD+ for 24 hours at 37°C.

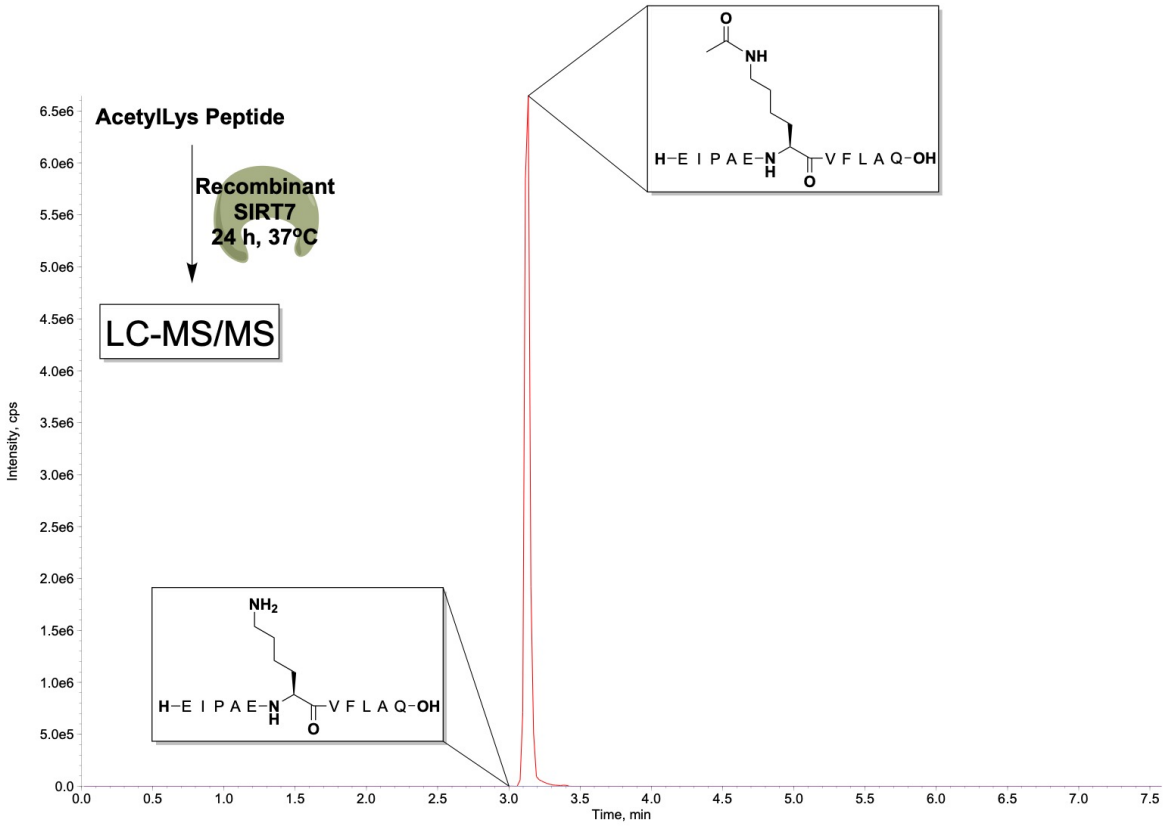

Figure S28. LC-MS/MS chromatograph of acetylated PKM2 peptide incubated with recombinant SIRT7 and NAD+ for 24 hours at 37°C.

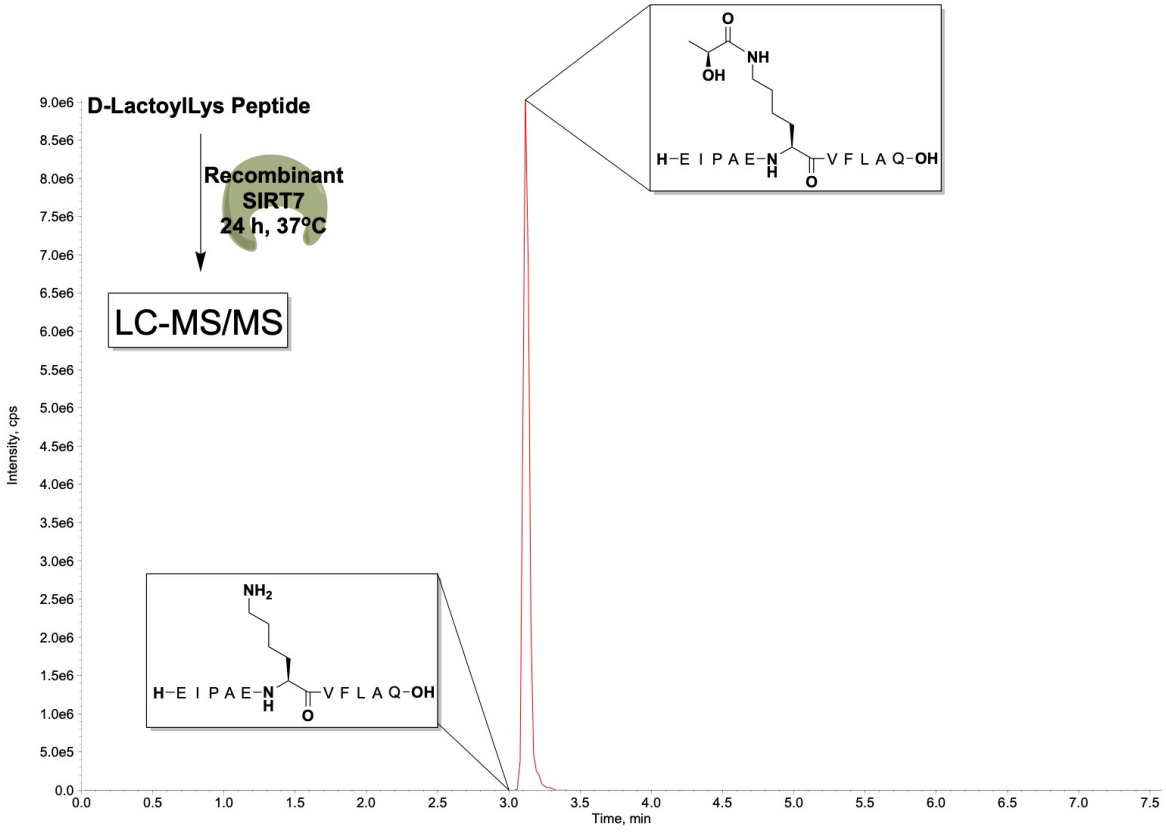

Figure S29. LC-MS/MS chromatograph of D-lactoylated PKM2 peptide incubated with recombinant SIRT7 and NAD+ for 24 hours at 37°C.

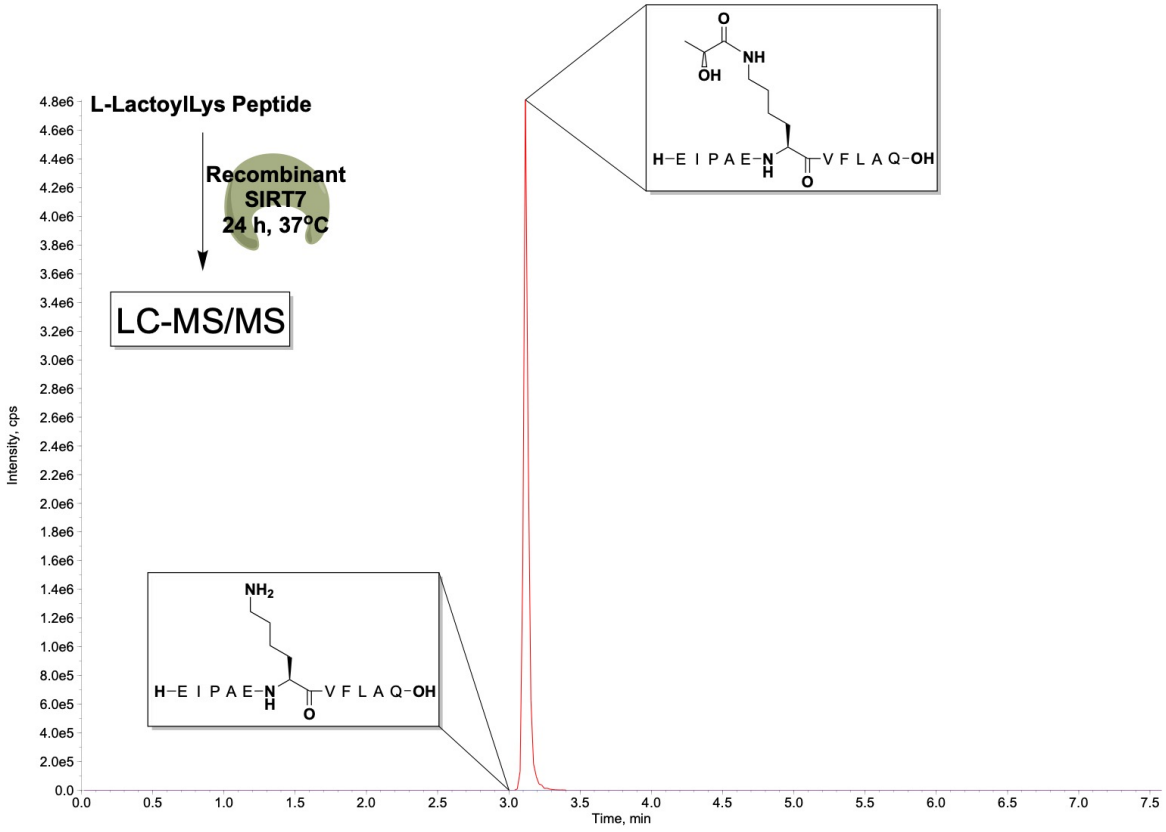

Figure S30. LC-MS/MS chromatograph of L-lactoylated PKM2 peptide incubated with recombinant SIRT7 and NAD+ for 24 hours at 37°C.

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