Electronic Supplementary Information (ESI)

Genetic Encoding of ε-*N*-L-lactyllysine for Detecting Delactylase Activity in Living Cells

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Supplementary figures

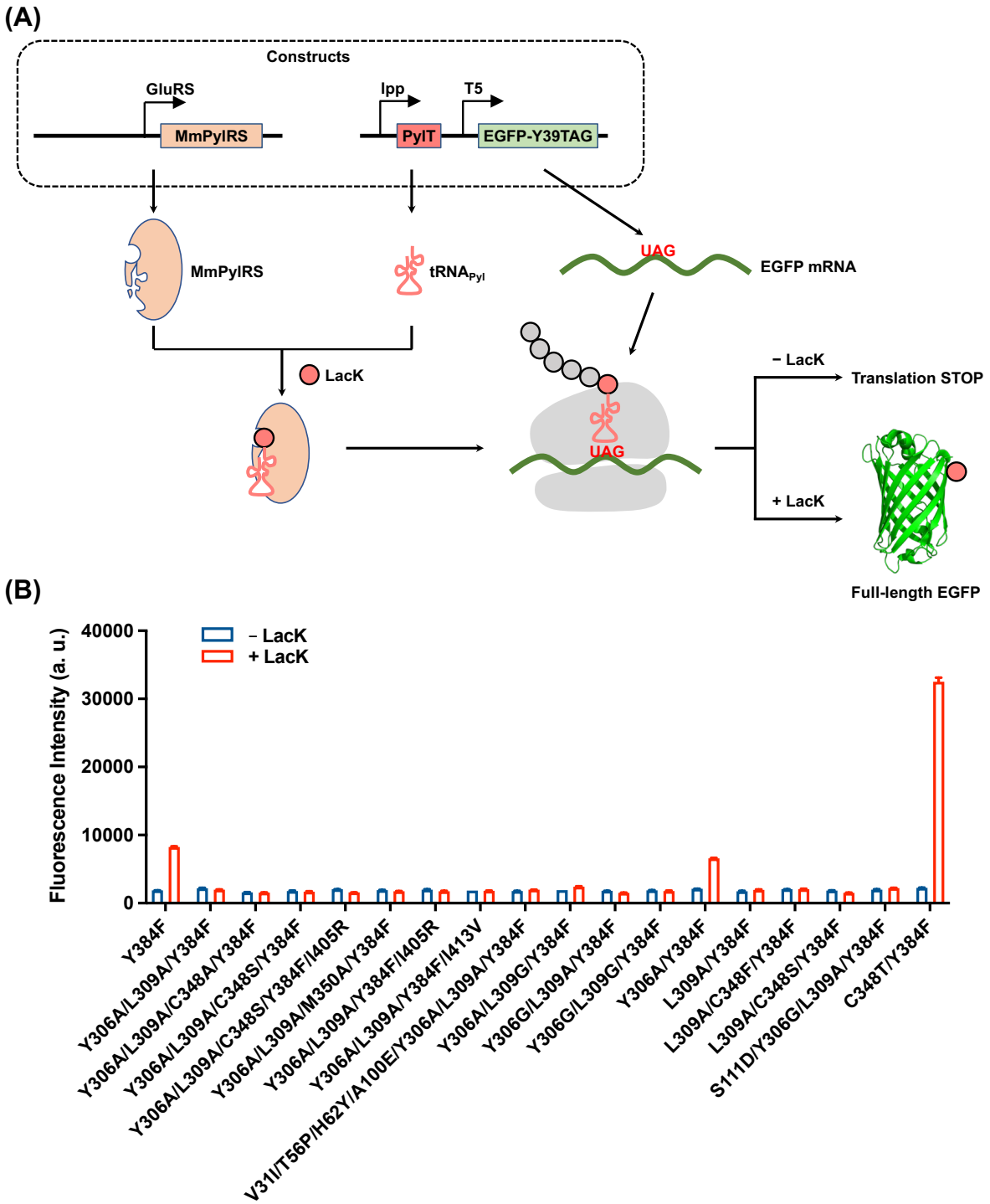


Fig. S1 (A) Schematic of the genetic code expansion technology for site-specific encoding of LacK into proteins (e.g., EGFP-Y39TAG). (B) Screening of MmPylRS mutants for site-specific incorporation of LacK into EGFP-Y39TAG in *E. coli*. The pLX-EGFP-Y39TAG plasmid was co-transformed with individual pBX-MmPylRS variant plasmid into *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium overnight at 37 °C and then inoculated by 1:100 dilution into fresh TB medium. 4 mM LacK was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 10 h at 37 °C. The EGFP fluorescence intensity of individual

bacterial culture was measured on a fluorescence microplate reader and compared with the control culture in the absence of LacK. The fluorescence was normalized to OD_{600}.

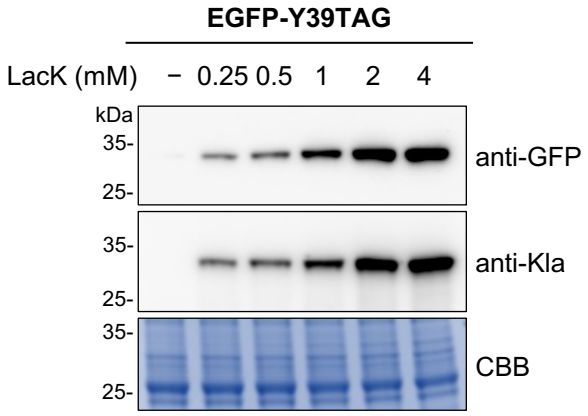


Fig. S2 Concentration-dependent incorporation of LacK into EGFP-Y39TAG in *E. coli*. The pLX-EGFP-Y39TAG plasmid was co-transformed with the pBX-LacKRS plasmid into *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium overnight at 37 °C and then inoculated by 1:100 dilution into fresh TB medium. LacK at different concentrations (from 0 mM to 4 mM) was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, protein expression was induced with 1 mM IPTG. After another 2 h incubation, 20 mM NAM was added and the *E. coli* cells were cultured at 37 °C for 10 h. Cells were harvested and lysed with 4% SDS lysis buffer. The resulting cell lysates were analyzed by western blotting and Coomassie Brilliant Blue (CBB) staining.

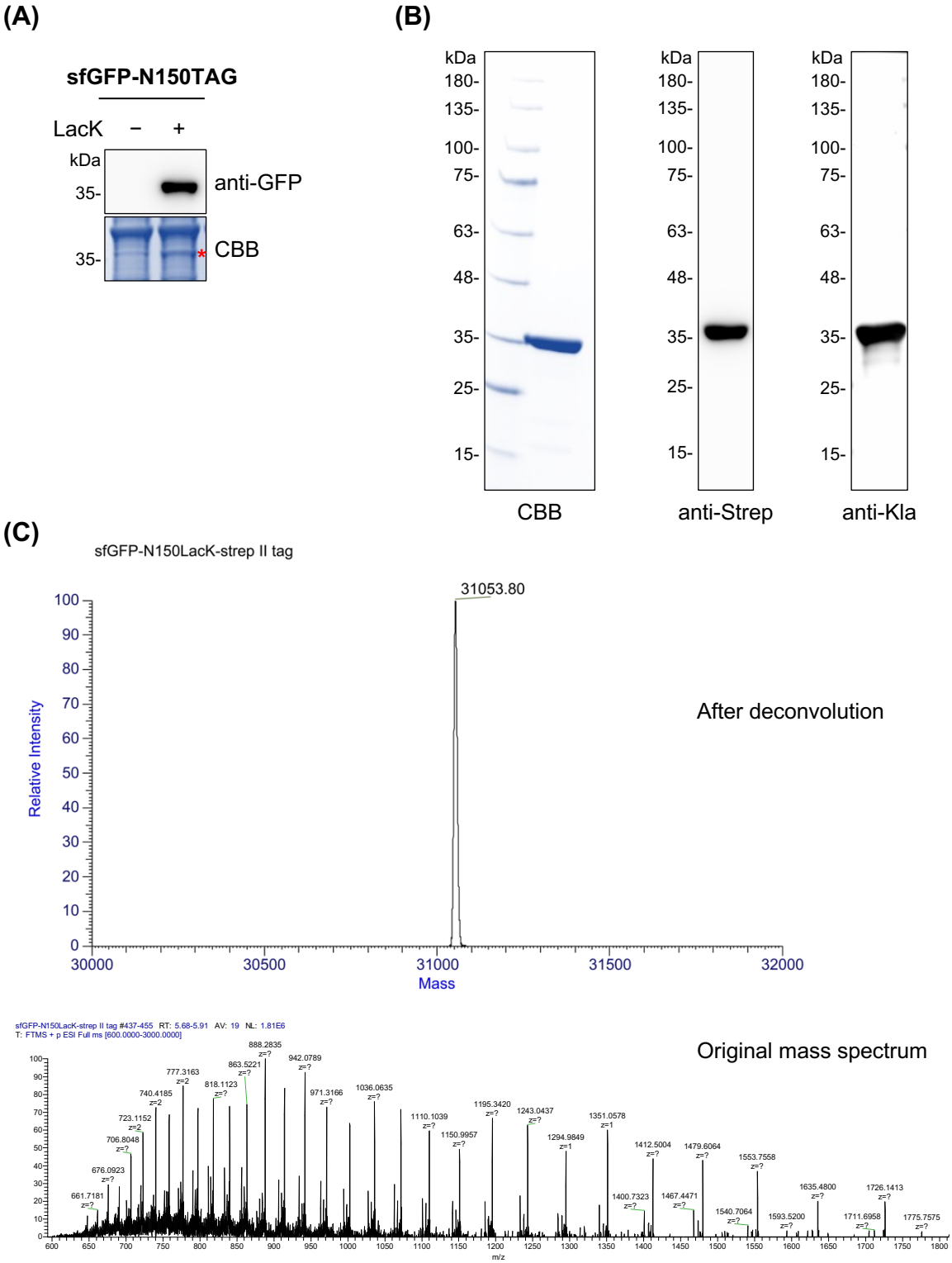


Fig. S3 Site-specific incorporation of LacK into sfGFP-N150TAG in *E. coli*. (A) Expression of sfGFP-N150LacK in *E. coli* examined by western blot, with CBB as the loading control. The red asterisk indicates the sfGFP-N150LacK protein. (B) SDS-PAGE gel and western blotting analyses of purified sfGFP-N150LacK. (C) ESI-MS spectrum of purified sfGFP-N150LacK protein. Calculated mass of sfGFP-N150LacK: 31,053.89 Da; observed mass: 31,053.80 Da. The pLX-sfGFP-N150TAG plasmid was co-transformed with the pBX-LacKRS plasmid into *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium overnight at 37 °C and then inoculated by 1:100 dilution into fresh TB medium. LacK (4 mM) was added

into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, protein expression was induced with 1 mM IPTG. After another 2 h incubation, 20 mM NAM was added and the *E. coli* cells were cultured at 37 °C for 10 h. Cells were harvested and lysed with 4% SDS lysis buffer. The resulting cell lysates were analyzed by western blotting and Coomassie Brilliant Blue (CBB) staining. Alternatively, cells were lysed with a sonic disruptor in Buffer W (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing protease inhibitor cocktails, PMSF, deoxyribonuclease I, and lysozyme. The protein was purified by Strep-Tactin XT Superflow resin and analyzed by ESI-MS.

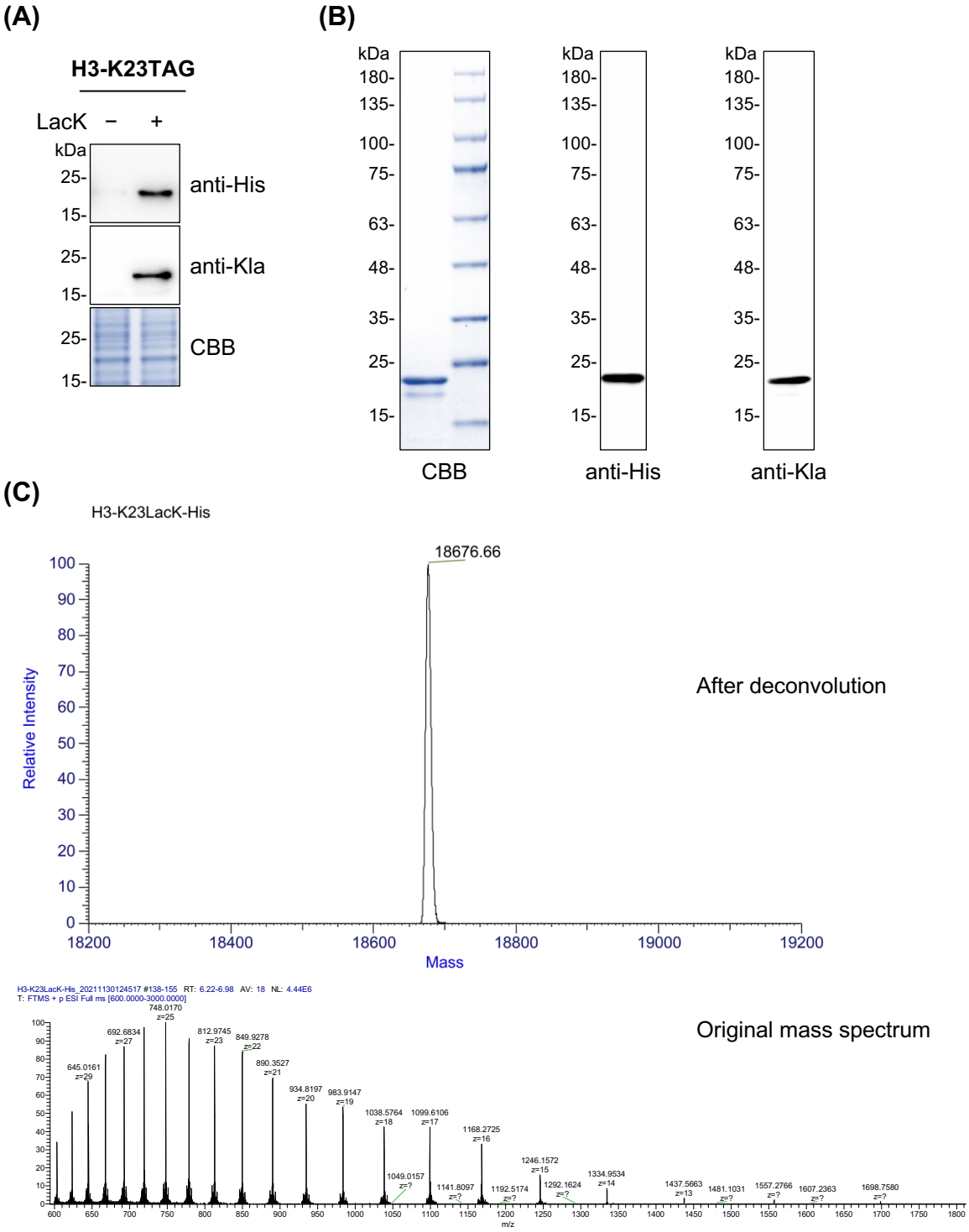


Fig. S4 Site-specific incorporation of LacK into H3-K23TAG in *E. coli*. (A) Expression of H3-K23LacK in *E. coli*. (B) SDS-PAGE gel and western blotting analyses of purified H3-K23LacK. (C) ESI-MS spectrum of purified H3-K23LacK protein. Calculated mass of H3-K23LacK: 18,676.73 Da; observed mass: 18,676.66 Da. The pET22b-H3-K23TAG plasmid was co-transformed with the pUltra-LacKRS plasmid into *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium overnight at 37 °C and then inoculated by 1:100 dilution into fresh TB medium. LacK (4 mM) was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, protein expression was induced with 1 mM IPTG. After another 2 h incubation, 20 mM NAM was added and the *E. coli* cells were cultured at 37 °C

for 10 h. Cells were harvested and lysed with 4% SDS lysis buffer. The resulting cell lysates were analyzed by western blotting and Coomassie Brilliant Blue (CBB) staining. Alternatively, cells were lysed with a sonic disruptor in the binding buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 8.0) containing protease inhibitor cocktails, PMSF, deoxyribonuclease I, and lysozyme. The supernatant was purified by Ni-NTA Sefinose resin and analyzed by ESI-MS.

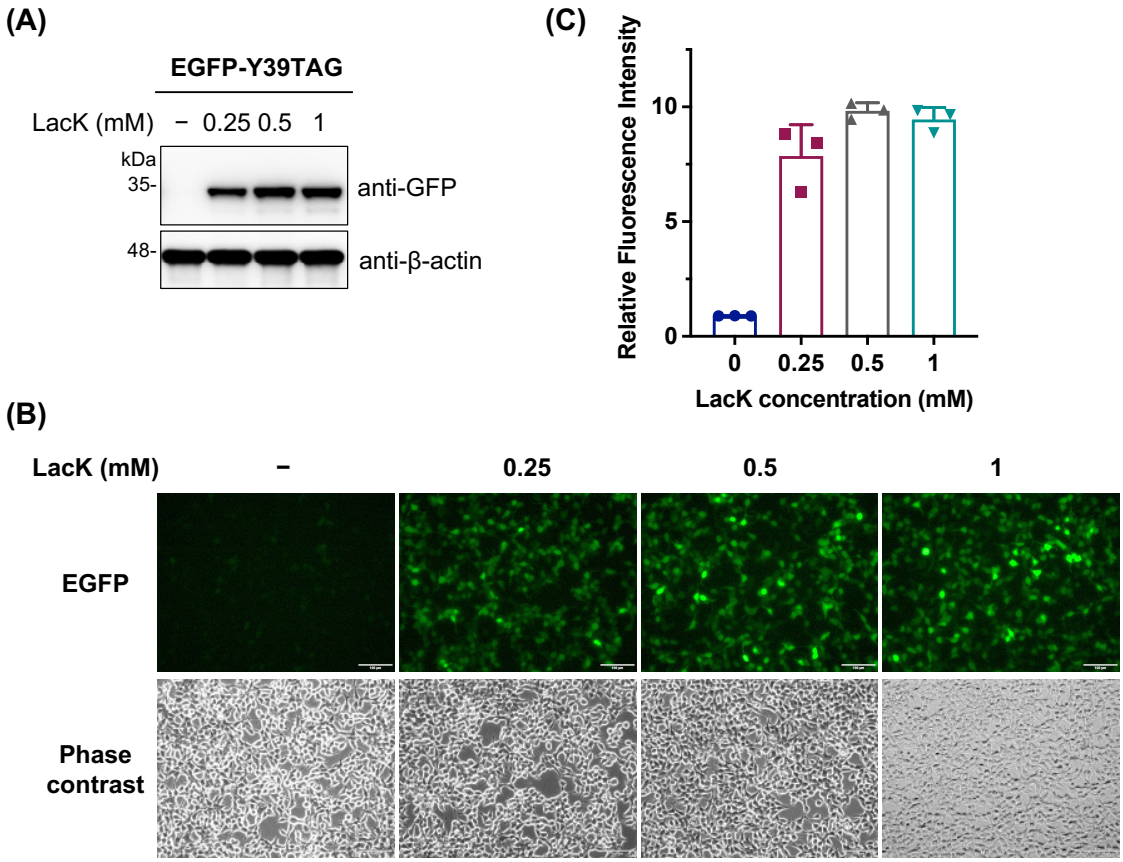


Fig. S5 Site-specific incorporation of LacK into EGFP-Y39TAG in mammalian cells. (A) Western blotting analysis of EGFP-Y39LacK expression in the presence of varying concentrations of LacK in HEK293T cells. (B) Fluorescence imaging of EGFP-Y39LacK expression in the presence of varying concentrations of LacK in HEK293T cells. Scale bars represent 100 μm. (C) Quantification of fluorescence images shown in (B). Data are shown as mean ± standard deviation (*n* = 3 independent experiments). HEK293T cells were transfected with EGFP-Y39TAG and LacKRS in the presence of LacK (from 0 mM to 1 mM) for 24 h and lysed for western blot analysis or analyzed with a fluorescence microscope.

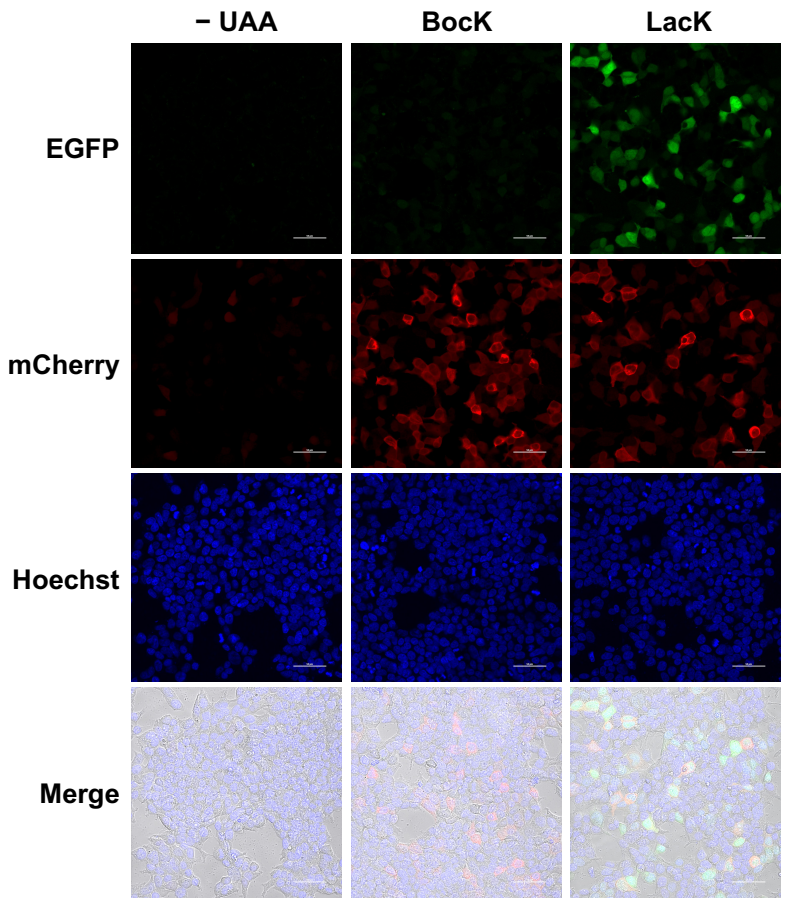


Fig. S6 Fluorescence detection of delactylase activities in live HEK293T cells with EGFP-K85LacK-mCherry using EGFP-K85BocK-mCherry as a negative control. HEK293T cells were transfected with EGFP-K85TAG-mCherry and LacKRS or MmPylRS in the presence of LacK (1 mM) or BocK (0.25 mM), respectively, for 24 h. Nuclei were stained with Hoechst 33342. Scale bars represent 50 μm.

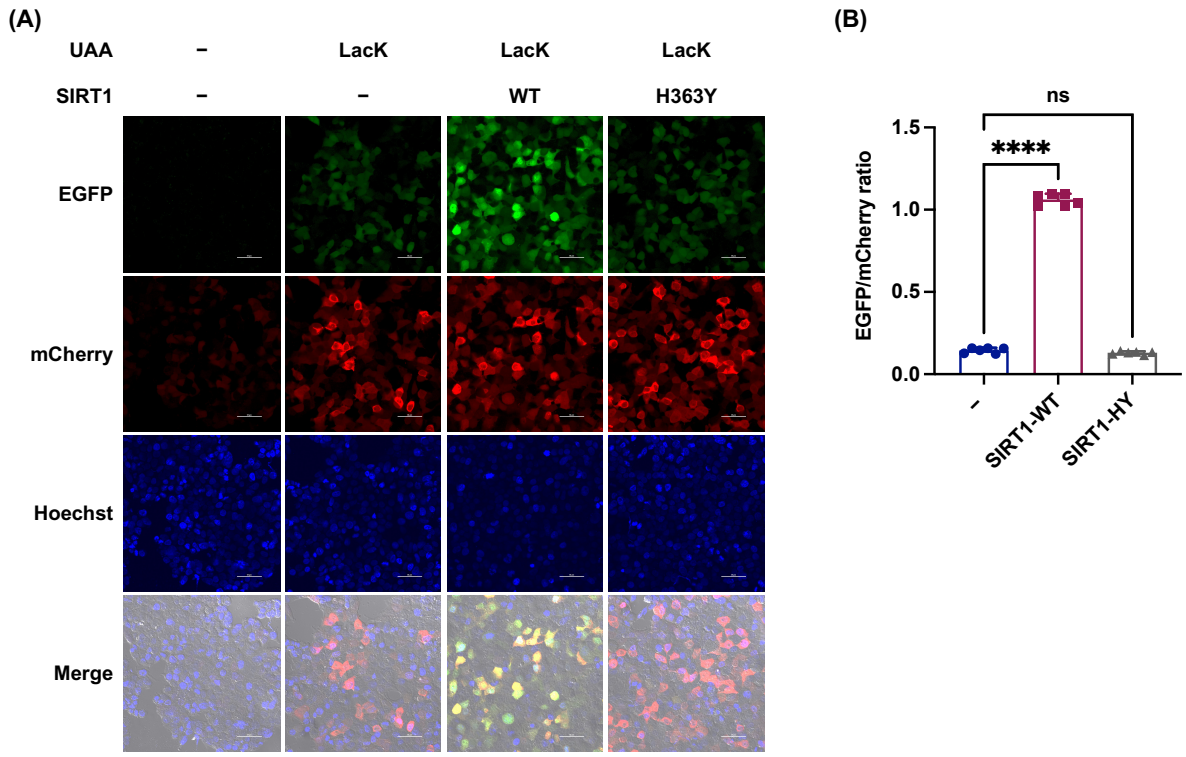


Fig. S7 Fluorescence detection of the delactylase activity of SIRT1 in live HEK293T cells with EGFP-K85LacK-mCherry. (A) HEK293T cells were co-transfected with EGFP-K85TAG-mCherry, LacKRS, and SIRT1 or its inactive mutant (SIRT1-H363Y) in the presence of LacK (1 mM) for 24 h. Nuclei were stained with Hoechst 33342. Scale bars represent 50 μm. (B) Quantification of the EGFP/mCherry fluorescence intensity ratio in images shown in (A). Data are shown as mean ± standard deviation (n = 6 independent experiments). Statistical analysis for multiple comparison was performed using one-way ANOVA. **** $p < 0.0001$, ns $p > 0.05$.

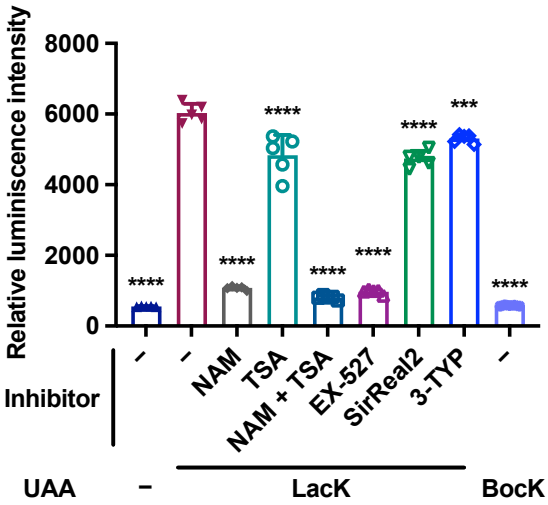


Fig. S8 Luminescence detection of delactylase activities in live HEK293T cells with fLuc-K529LacK. HEK293T cells were transfected with fLuc-K529TAG and LacKRS in the presence of LacK (1 mM), treated with indicated inhibitors (NAM, 10 mM; TSA, 0.4 μM; EX-527, 15 μM; SirReal2, 1 μM; 3-TYP, 1 μM) for 24 h, and analyzed with luminescence intensities. Data are shown as mean intensity ± standard deviation (n = 5 independent experiments). Statistical analysis for multiple comparison with no inhibitor treatment was performed using one-way ANOVA. *** $p < 0.001$, **** $p < 0.0001$. See Fig. 3B in the main text for luminescence images.

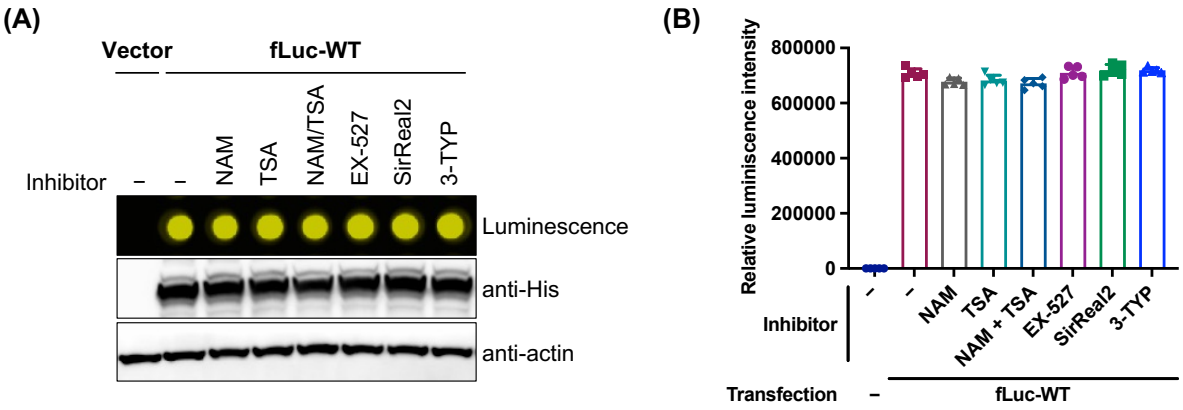


Fig. S9 Examination of the enzymatic activity of wild-type fLuc in the presence of deacylase inhibitors. (A) Luminescence activity and expression of wild-type fLuc in the presence of indicated inhibitors (NAM, 10 mM; TSA, 0.4 μM; EX-527, 15 μM; SirReal2, 1 μM; 3-TYP, 1 μM). (B) Quantification of the luminescence data shown in (A). Data are shown as mean intensity ± standard deviation (n = 5 independent experiments).

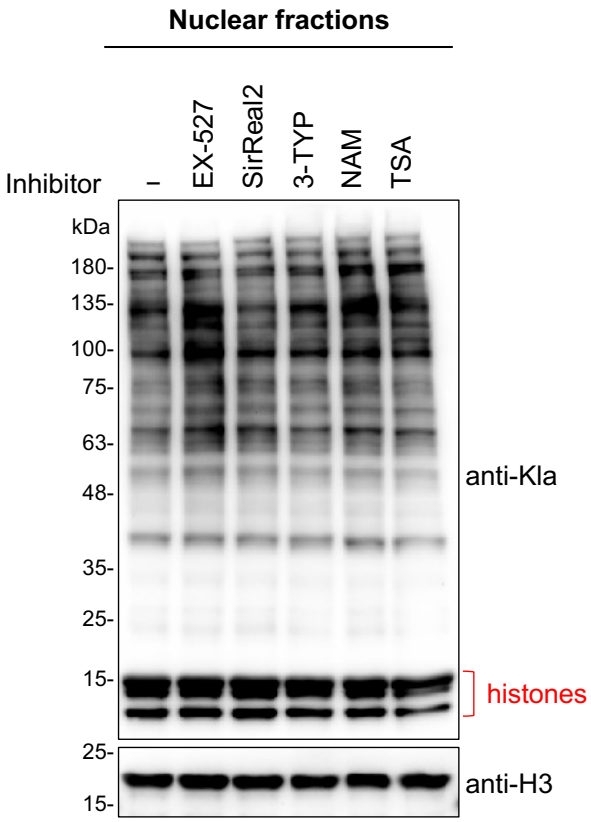


Fig. S10 Western blot analysis of the lactylation levels of nuclear proteins. HEK293T cells were treated with indicated inhibitors (EX-527, 15 μM; SirReal2, 15 μM; 3-TYP, 15 μM; NAM, 10 mM; TSA, 1 μM) for 24 h. Note: the histone lactylation levels were not increased upon TSA treatment,[1] probably due to compensation of the HDAC inhibition after TSA treatment for extended times (24 hours).

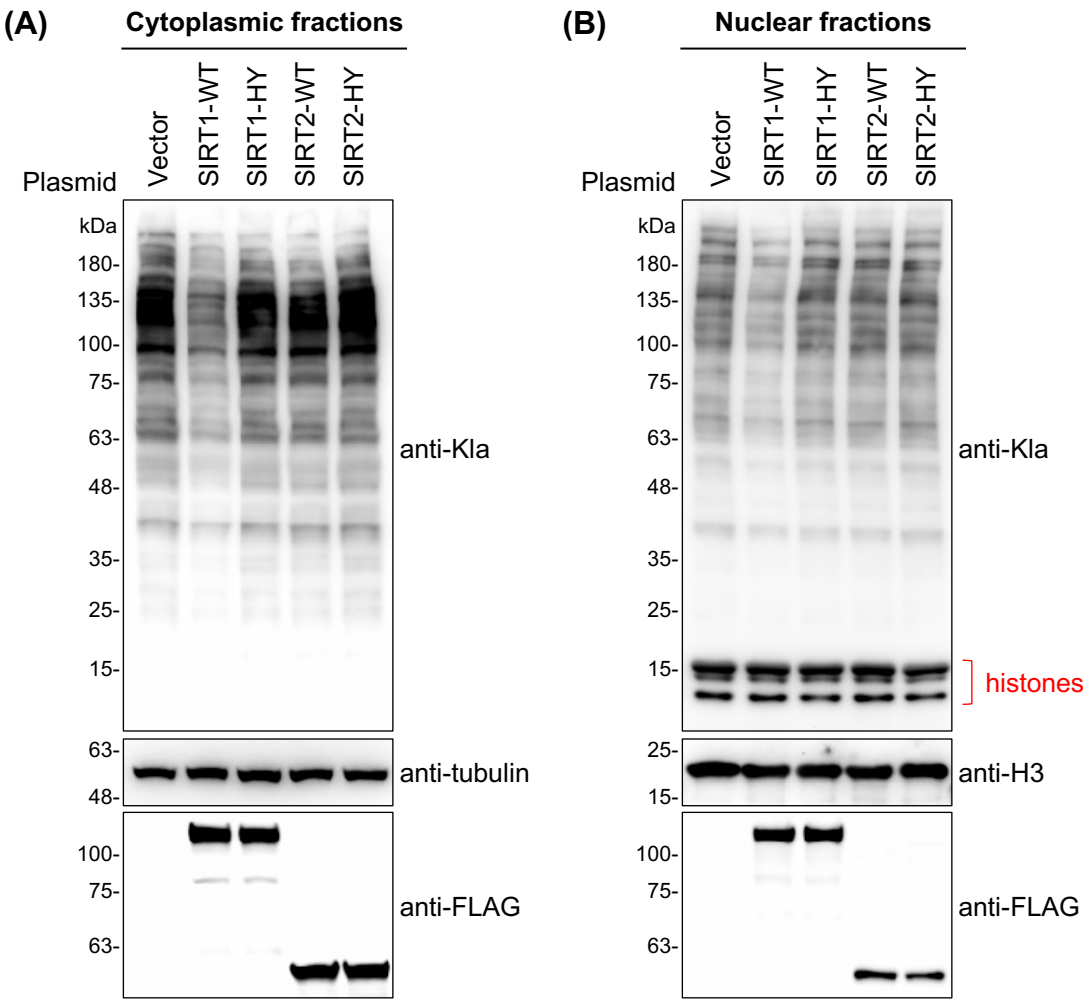


Fig. S11 Western blot analysis of the effects of SIRT1–2 on delactylation. (A) Lactylation levels of cytoplasmic proteins in HEK293T cells transfected with FLAG-tagged SIRT1–2 or inactive mutants (SIRT1-HY and SIRT2-HY). (B) Lactylation levels of nuclear proteins in HEK293T cells transfected with FLAG-tagged SIRT1–2 or inactive mutants (SIRT1-HY and SIRT2-HY).

Fig. 1B

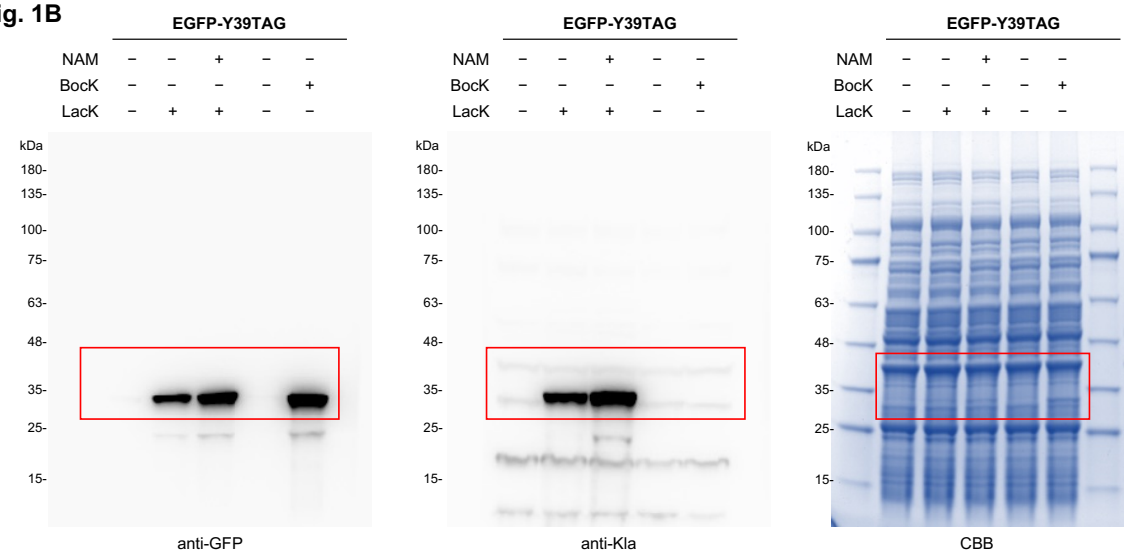


Fig. 1C

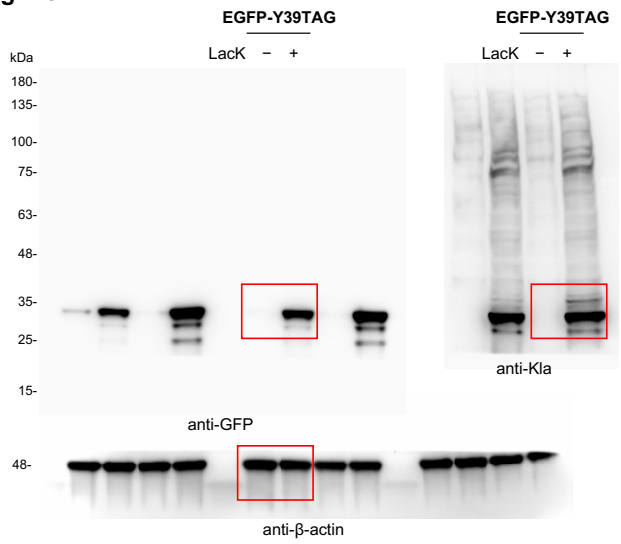


Fig. 3B

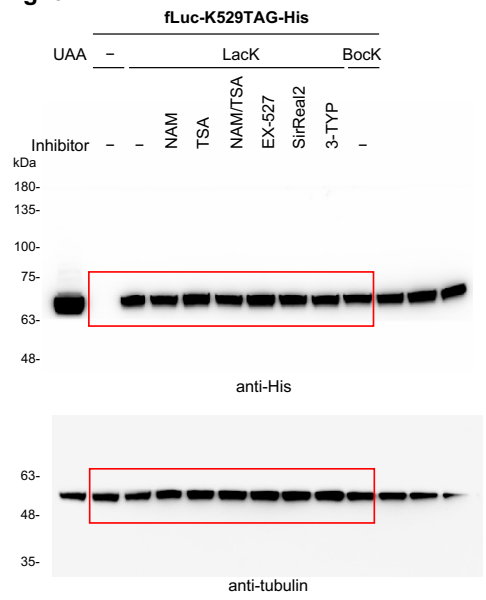


Fig. 4

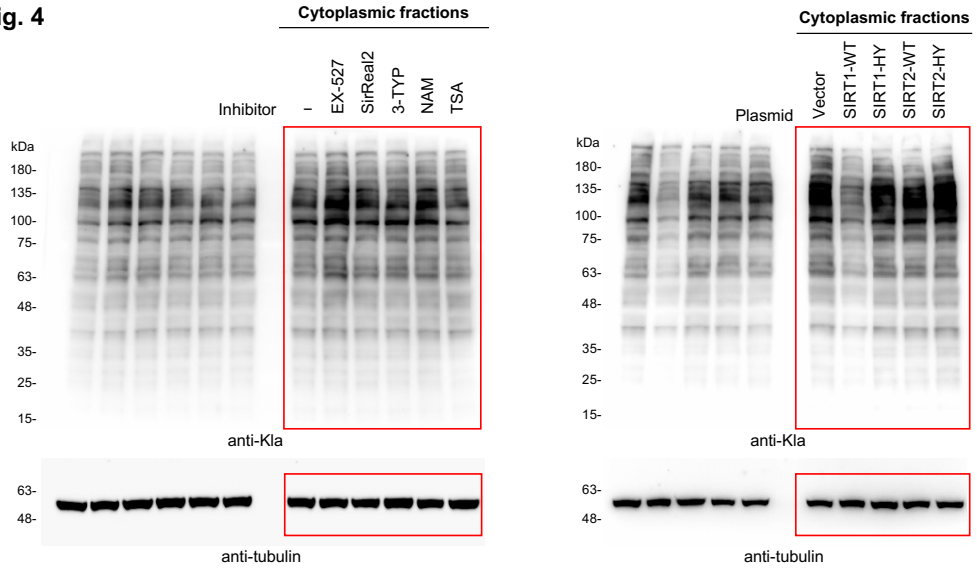


Fig. S12 Full gels and blots shown in the manuscript.

Fig. S2

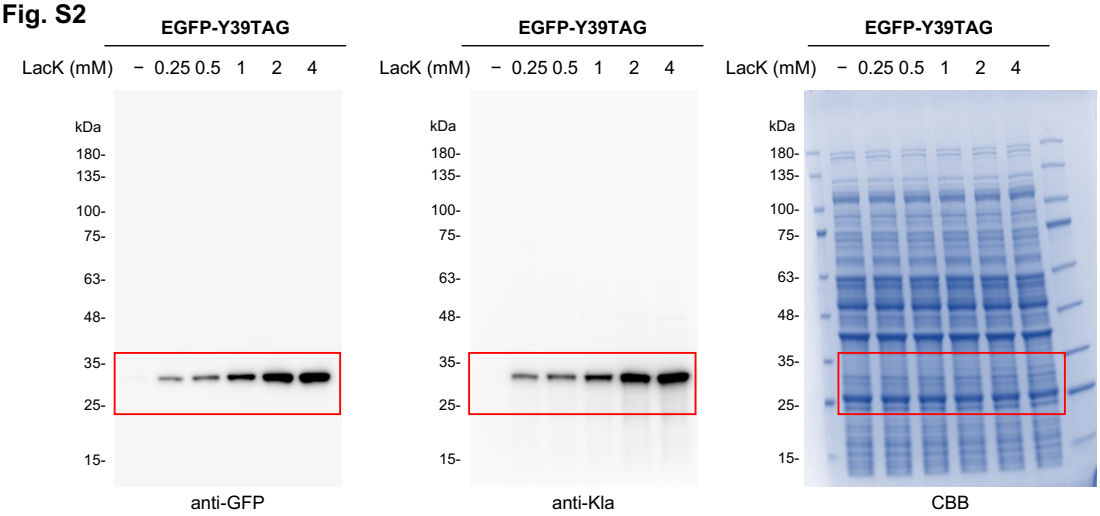


Fig. S3

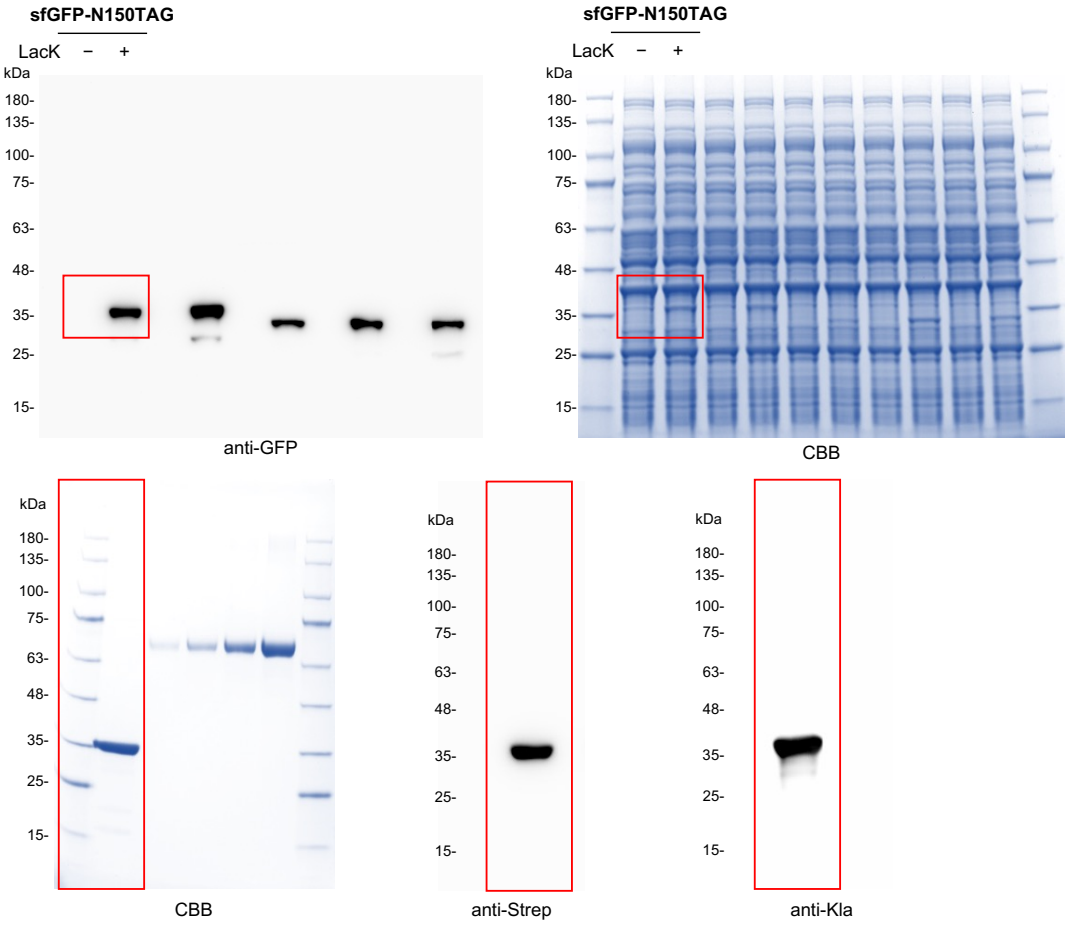


Fig. S13 Full gels and blots shown in Fig. S2–S3.

Fig. S4

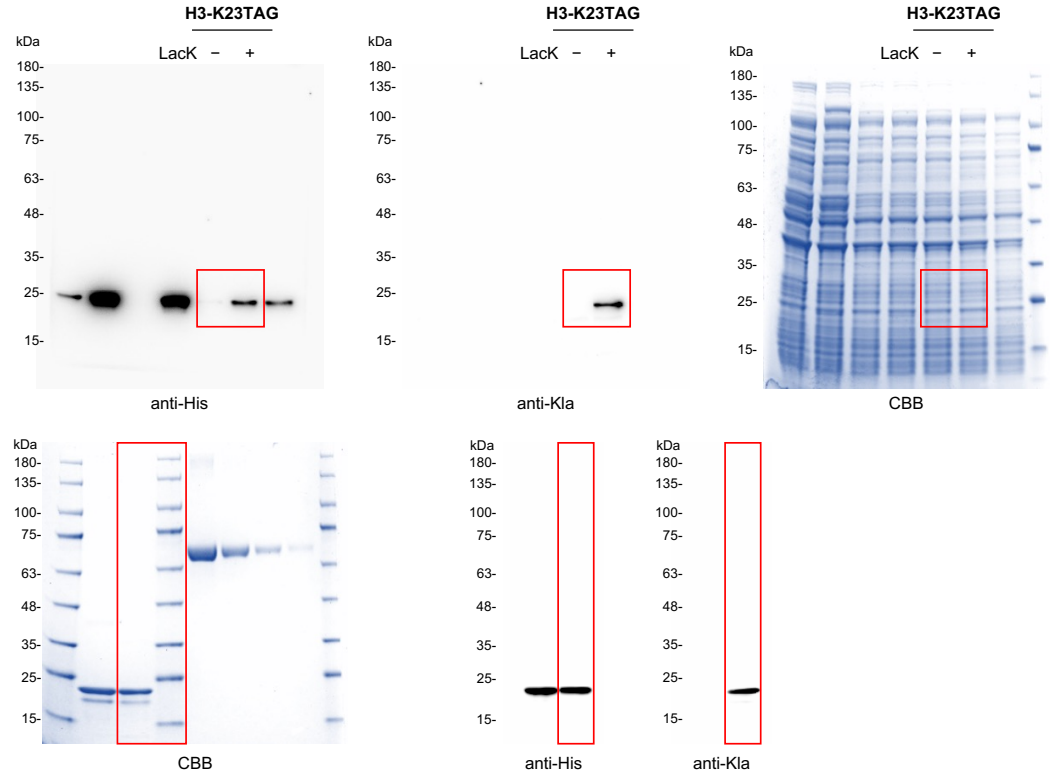


Fig. S5

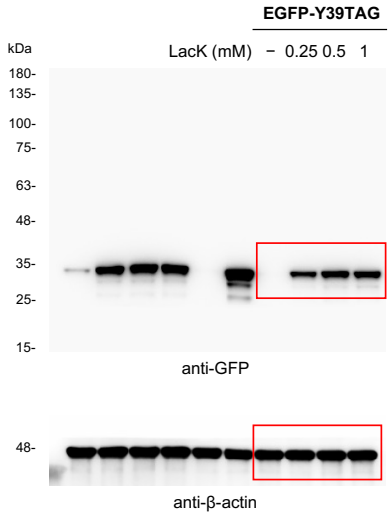


Fig. S9

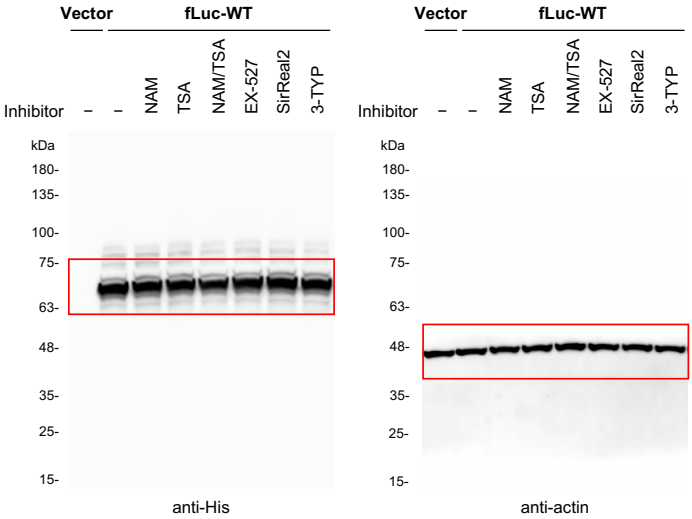


Fig. S14 Full gels and blots shown in Fig. S4, S5, and S9.

Fig. S10

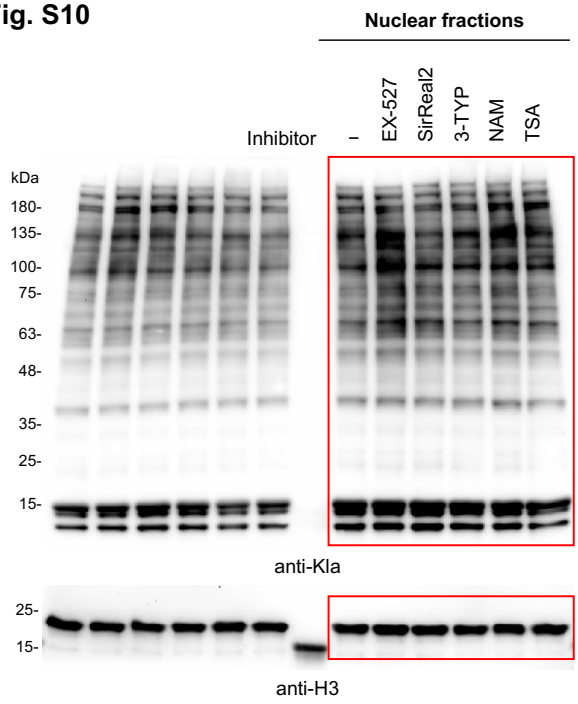


Fig. S11

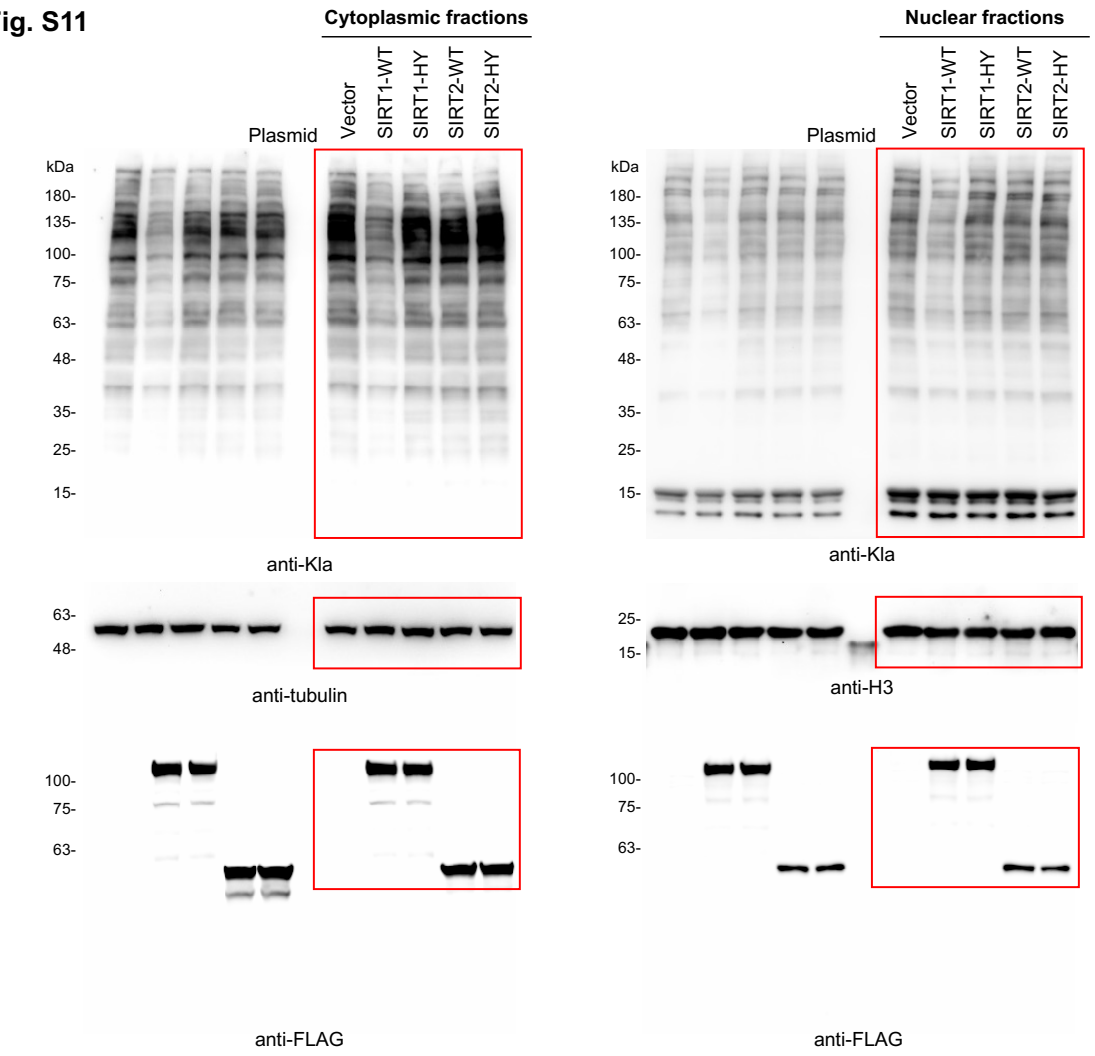


Fig. S15 Full gels and blots shown in Fig. S10–S11.

Supplementary tables

Table S1 Histone deacetylase inhibitors used in this study.

Name	CAS No.	Target	Description	Ref
Nicotinamide (NAM)	98-92-0	HDAC class III (SIRT1–7), IC_{50}s = 50–184 μM.	An pan-inhibitor of HDAC class III (sirtuins, SIRT1–7).	[2]
Trichostatin A (TSA)	58880-19-6	HDAC class I/II (HDAC1–10), IC_{50} = 1.8 nM.	A potent and specific inhibitor of HDAC class I/II (HDAC1–10).	[3]
EX-527 (Selisistat)	49843-98-3	SIRT1, IC_{50} = 38 nM.	A potent and selective SIRT1 inhibitor, with >200-fold selectivity against SIRT2 and SIRT3.	[4]
SirReal2	709002-46-0	SIRT2, IC_{50} = 140 nM.	A potent and selective SIRT2 inhibitor, with very little effect on the activities of SIRT3–5.	[5]
3-TYP	120241-79-4	SIRT3, IC_{50} = 16 nM.	A selective SIRT3 inhibitor, more potent over SIRT1 and SIRT2.	[6]

Experimental procedures

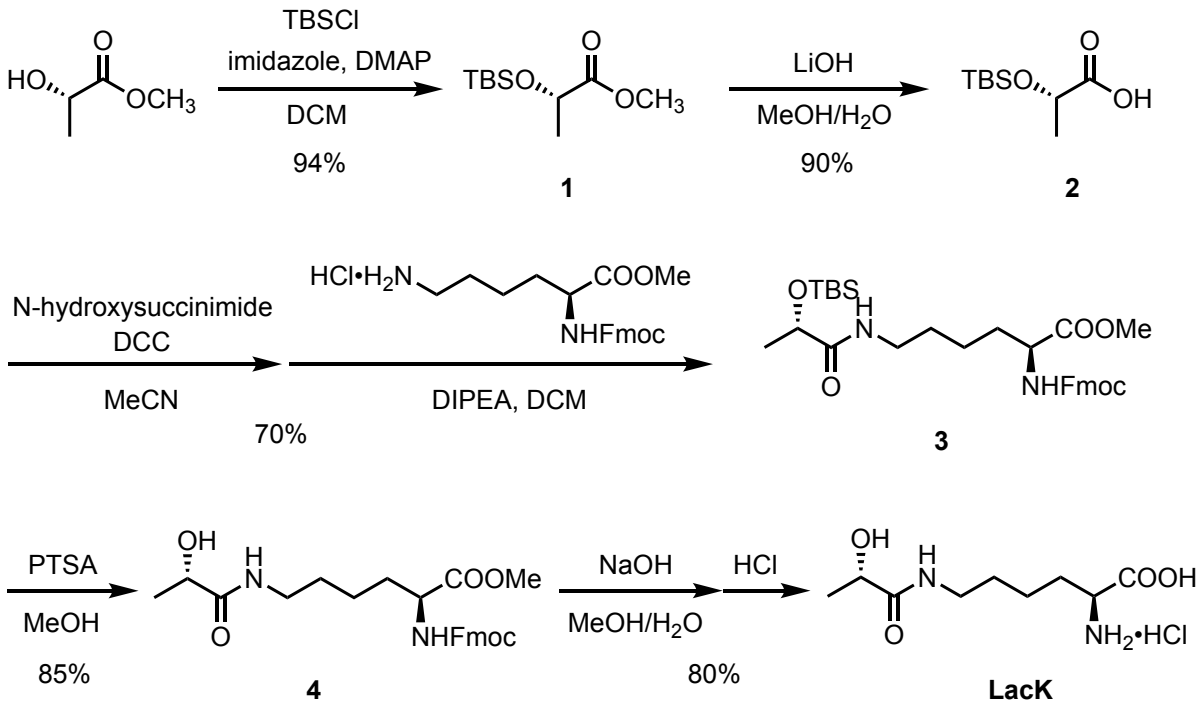
General methods and materials

Unless otherwise noted, chemicals and solvents for organic synthesis were obtained from Bidepharm and were used directly as received without further purification. Chemical reactions were performed in oven-dried round-bottom flasks. TLC was conducted on silica gel 60 GF254 glass plates (Qingdao Haiyang Chemical Co., Ltd), and spots were visualized by staining with potassium permanganate ($KMnO_4$) or phosphomolybdic acid (PMA). Flash column chromatography was performed with silica gel (200-300 mesh, reagent grade) from Accela ChemBio Co., Ltd. Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$, CD_3OD, or $(CD_3)_2SO$ at room temperature on Bruker Avance NMR Spectrometers operating at 300, 400, or 500 MHz for ^1H. Chemical shifts are reported in δ ppm, and coupling constants (*J* values) are reported in Hz. ^1H NMR chemical shifts are calibrated using tetramethylsilane (TMS, δ = 0.00 ppm) in $CDCl_3$ as the internal standard or with the residual solvent peaks of CD_3OD (δ = 3.31 ppm) or $(CD_3)_2SO$ (δ = 2.50 ppm). ^{13}C NMR chemical shifts are calibrated with the residual solvent peaks of $CDCl_3$ (δ = 77.16 ppm), CD_3OD (δ = 49.00 ppm), or $(CD_3)_2SO$ (δ = 39.52 ppm). High resolution mass spectra (HRMS) using the ESI source were recorded with a Q Exactive Focus (Thermo Fisher Scientific) mass spectrometer.

PfuUltra High-Fidelity DNA Polymerase for PCR was obtained from Agilent Technologies. Restriction enzymes and dNTPs were obtained from New England Biolabs. Oligonucleotide primers and gene fragments were synthesized by Tsingke Biotechnology. Site-directed mutagenesis was performed with QuikChange II Site-Directed Mutagenesis Kit (Agilent) with primers designed by Agilent Primer Design Program. Plasmid DNA isolation was carried out with Plasmid Mini Kit (Omega). Polyethylenimine (PEI) was purchased from Polysciences. Protease inhibitor cocktail (cOmplete ULTRA mini Tablets) was purchased from Roche. Isopropyl-β-D-thiogalactoside (IPTG), kanamycin, and chloramphenicol were purchased from Sangon Biotech. N^{ε}-Boc-L-Lysine (BocK) was purchased from Adamas and dissolved in 0.1 M NaOH as the stock solution (2 M). ε-*N*-L-lactyllysine (LacK) was dissolved in ddH_2O as the stock solution (2 M). Optical density (OD) was measured with a UV-Vis spectrophotometer (Techcomp UV-1000). Fluorescence emission intensities were measured on a Biotek Synergy H1 microplate reader. Confocal fluorescence imaging was performed with a Nikon A1R confocal fluorescence microscope. Western blotting analyses were recorded on a Chemidoc MP imaging system (Biorad).

Synthesis of the unnatural amino acid LacK

Scheme S1. Synthesis of LacK



Synthesis of methyl (*S*)-2-((*tert*-butyldimethylsilyl)oxy)propanoate (compound 1)

To a solution of methyl (*S*)-2-hydroxypropanoate (2.1 g, 20 mmol), imidazole (1.6 g, 24 mmol), and 4-dimethylaminopyridine (DMAP; 0.24 g, 2 mmol) in anhydrous CH_2Cl_2 (40 mL) at 0 °C was added tert-butylchlorodimethylsilane (TBSCl; 3.3 g, 22 mmol) slowly. The reaction was stirred at room temperature overnight and quenched with saturated NH_4Cl solution. The mixture was washed with water and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (eluent: petroleum ether/EtOAc = 4/1) to afford compound **1** as a colorless oil (4.1 g, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.32 (q, *J* = 6.7 Hz, 1H), 3.71 (s, 3H), 1.39 (d, *J* = 6.8 Hz, 3H), 0.89 (s, 9H), 0.09 (s, 3H), 0.06 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.53, 68.36, 51.82, 25.68, 21.32, 18.28, -5.02, -5.31.

Synthesis of (*S*)-2-((*tert*-butyldimethylsilyl)oxy)propanoic acid (compound 2)

To a solution of methyl (*S*)-2-((tert-butyldimethylsilyl)oxy)propanoate (compound **1**) (4.0 g, 18 mmol) in MeOH and H_2O (30 mL, MeOH/H_2O = 5/1, v/v) was added LiOH (0.86 g, 38 mmol). The reaction mixture was stirred at room temperature for about 3 hours and concentrated *in vacuo* to remove methanol. The residual aqueous solution was diluted with water (15 mL) and extracted with Et_2O (2 × 15 mL). Then the aqueous phase was acidified with citric acid and extracted with $CHCl_3$ (2 × 15 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated to afford compound **2** (3.3 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.35 (q, *J* = 6.8 Hz, 1H), 1.44 (d, *J* = 6.8 Hz, 3H), 0.92 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.74, 68.42, 25.62, 21.11, 18.05, -4.88, -5.23.

Synthesis of methyl N^2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-N^6-((*S*)-2-((*tert*-butyldimethylsilyl)oxy)propanoyl)-*L*-lysinate (compound 3)

To a solution of (*S*)-2-((*tert*-butyldimethylsilyl)oxy)propanoic acid (compound **2**) (0.73 g, 3.6 mmol) and dicyclohexylmethanediimine (DCC; 0.89 g, 4.3 mmol) in 30 mL CH_3CN was added *N*-hydroxysuccinimide (0.46 g, 4.0 mmol). The mixture was stirred at room temperature for 6 hours and filtered to remove the precipitates. The solvent was then evaporated. The resulting crude intermediate was re-dissolved in 50 mL CH_2Cl_2 followed by addition of *N*, *N*-diisopropylethylamine (DIPEA; 0.52 g, 4.0 mmol) and Fmoc-L-Lys-OMe·HCl (1.0 g, 2.4 mmol). The reaction was then stirred at room temperature for 16 hours. The mixture was washed with aqueous NH_4Cl solution and brine. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: petroleum ether/EtOAc = 1/1) to afford compound **3** (0.95 g, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.61 (d, *J* = 7.2 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 6.71 (s, 1H), 5.46 (d, *J* = 8.1 Hz, 1H), 4.46 – 4.31 (m, 3H), 4.26 – 4.16 (m, 2H), 3.75 (s, 3H), 3.33 – 3.19 (m, 2H), 1.93 – 1.68 (m, 2H), 1.58 – 1.49 (m, 2H), 1.44 – 1.33 (m, 5H), 0.91 (s, 9H), 0.10 (s, 3H), 0.08 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.49, 172.84, 155.93, 143.84, 143.72, 141.25, 127.65, 127.01, 125.07, 119.92, 69.97, 66.95, 53.68, 52.38, 47.11, 38.17, 31.92, 29.29, 25.68, 22.31, 21.91, 17.96, -4.70, -5.37. HRMS (ESI) calcd for $C_{31}H_{45}N_2O_6Si$ $[M + H]^+$ 569.30414, found 569.30426.

Synthesis of methyl *N*^{2}-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*^{6}-((*S*)-2-hydroxypropanoyl)-*L*-lysinate (compound 4)

The mixture of compound **3** (0.8 g, 1.4 mmol) and 4-methylbenzenesulfonic acid monohydrate ($PTSA·H_2O$; 0.053 g, 0.28 mmol) in MeOH (20 mL) was stirred at room temperature until TLC showed the starting material was completely consumed. The reaction mixture was concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (eluent: petroleum ether/EtOAc = 1/2) to afford compound **4** (0.54 g, 85% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 7.1 Hz, 2H), 7.39 (t, *J* = 7.3 Hz, 2H), 7.30 (t, *J* = 7.1 Hz, 2H), 6.80 (t, *J* = 5.5 Hz, 1H), 5.62 (d, *J* = 8.2 Hz, 1H), 4.47 – 4.27 (m, 3H), 4.24 – 4.16 (m, 2H), 3.73 (s, 3H), 3.64 (d, *J* = 4.7 Hz, 1H), 3.24 (dd, *J* = 12.8, 6.4 Hz, 2H), 1.90 – 1.77 (m, 1H), 1.75 – 1.61 (m, 1H), 1.59 – 1.50 (m, 2H), 1.42 – 1.30 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.93, 172.91, 156.13, 143.74, 143.61, 141.20, 127.67, 127.01, 125.01, 119.92, 68.30, 66.98, 53.56, 52.40, 47.03, 38.31, 31.92, 28.82, 22.25, 21.14. HRMS (ESI) calcd for $C_{25}H_{30}N_2O_6Na$ $[M + Na]^+$ 477.19961, found 477.19974.

Synthesis of *N*^{6}-((*S*)-2-hydroxypropanoyl)-*L*-lysine hydrochloride (LacK)

To a solution of compound **4** (0.5 g, 1.1 mmol) in MeOH and H_2O (10 mL, MeOH/H_2O = 7/3, v/v) was added NaOH (0.13 g, 3.3 mmol). The reaction mixture was stirred at room temperature for 8 hours. Solvents were removed under reduced pressure. The solid residue was washed extensively with CH_2Cl_2 and then re-suspended in 2.0 M HCl in 1,4-dioxane (10 mL). The supernatant was discarded and the resulting oily materials were dissolved in MeOH (10 mL) and filtered. The filtrate was evaporated to give the unnatural amino acid LacK as a viscous oil (0.19 g, 80% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 4.11 (q, *J* = 6.8 Hz, 1H), 3.97 (t, *J* = 6.3 Hz, 1H), 3.24 (t, *J* = 6.9 Hz, 2H), 2.05 – 1.84 (m, 2H), 1.65 – 1.55 (m, 2H), 1.54 – 1.39 (m, 2H), 1.34 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.89, 172.27, 69.01, 54.16, 39.43, 31.20, 29.96, 23.27, 21.27. HRMS (ESI) calcd for $C_9H_{19}N_2O_4$ $[M + H]^+$ 219.13393, found 219.13405.

Plasmids and cloning

For *E. coli* transformation, the pBX-MmPylRS plasmid expressing wild-type PylRS from *Methanosarcina mazei* was constructed as previously reported.[7] The plasmid pBX-MmPylRS was mutated at indicated sites with site-directed mutagenesis to produce the PylRS variants. The pLX-EGFP-Y39TAG plasmid containing the *Methanosarcina mazei* $tRNA_{CUA}$-Pyl gene under the *lpp* promoter and *rrnC* terminator and the EGFP-Y39TAG gene was constructed as previously reported.[7] The EGFP-Y39TAG gene containing a *C*-terminal 6*His-tag was under the control of a bacteriophage T5 promoter and t_0 terminator. The superfolder green fluorescent protein (sfGFP) encoding gene fused with an in-frame *C*-terminal Twin-Strep-tag (WSHPQFEKGGGSGGGSGGSAWSHPQFEK) was amplified with PCR from the pBad-sfGFP-150TAG plasmid (a kind gift from Prof. Ryan Mehl; Addgene plasmid # 85483) and used to replace the EGFP-Y39TAG insert in pLX-EGFP-Y39TAG plasmid to create the plasmid pLX-sfGFP-N150TAG. The pET22b-H3-K23TAG plasmid encoding histone H3 with an *N*-terminal pelB signal sequence and a *C*-terminal 6*His-tag was constructed by insertion of the H3 gene fragment (synthesized by Genewiz) into the pET22b vector (Novagen). The pUltra-LacKRS plasmid was modified from pUltra-sY (a kind gift from Prof. Chang Liu; Addgene plasmid # 82417) by replacing the tRNA and aaRS in pUltra-sY with $tRNA_{CUA}$-Pyl and LacKRS, respectively.

For mammalian cell transfection, the pEF1α-FLAG-MmPylRS plasmid was initially developed by Prof. Jason Chin[8] and constructed in the lab previously.[7] The pCMV-EGFP-Y39TAG plasmid was previously developed in the lab.[7, 9] The desired mutations were introduced into the pEF1α-FLAG-MmPylRS and pCMV-EGFP-Y39TAG plasmids to create pEF1α-FLAG-LacKRS and pCMV-EGFP-K85TAG plasmids, respectively. pCMV-EGFP-K85TAG-mCherry plasmid was created by inserting a *C*-terminal mCherry tag from the mCherry2-N1 vector (a kind gift from Prof. Michael Davidson; Addgene plasmid # 54517) into pCMV-EGFP-K85TAG.[7] The firefly luciferase encoding gene was amplified with PCR from the pGL3 vector (Promega) and used to replace the EGFP insert in pCMV-EGFP-K85TAG to create the plasmid pCMV-fLuc. An amber TAG codon was introduced into the pCMV-fLuc plasmid at K529 position by site-directed mutagenesis.[7] The pcDNA3.1-SIRT1-Flag plasmid was purchased from WZ Biosciences. The pEnCMV-SIRT2-Flag plasmid was purchased from ML Biosciences. The inactive mutant plasmids of SIRT1 and SIRT2, i.e., SIRT1-H363Y and SIRT2-H187Y, respectively, were generated by site-directed mutagenesis.

Screening of MmPylRS variants for LacK incorporation

The pLX-EGFP-Y39TAG plasmid was co-transformed with individual pBX-MmPylRS mutant plasmid into the *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C and then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. 4 mM LacK was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, 1 mM IPTG was added into the culture to induce the protein expression. After another 2 h incubation, 20 mM nicotinamide (NAM; Sigma, cat#72340; 50 mM stock solution in ddH_2O) was added to suppress the activity of *E. coli* sirtuins,[10] and the *E. coli* cells were cultured at 37 °C for 10 h. The EGFP fluorescence intensity of individual bacterial culture was measured on a Biotek Synergy H1 microplate reader and compared with the control culture in the absence of LacK.

Incorporation of LacK into proteins in *E. coli*

The desired plasmid of interest containing amber TAG codon was co-transformed with the pBX-LacKRS plasmid into the *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C and then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. 4 mM LacK was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, 1 mM IPTG was added into the culture to induce the protein expression. After another 2 h incubation, 20 mM NAM was added to suppress the activity of *E. coli* sirtuins,[10] and the *E. coli* cells were cultured at 37 °C for 10 h. The cells were harvested and lysed with 4% SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine pH 7.4) at 95 °C for 5 min. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). Finally, the cell lysates were separated on 4-20% SDS-PAGE gels (Genscript Biotech) and analyzed by western blotting and Coomassie Brilliant Blue staining.

For optimization of LacK concentrations in *E. coli*, the pLX-EGFP-Y39TAG plasmid was co-transformed with the pBX-LacKRS plasmid into the E. coli strain BL21 (DE3). The transformed bacteria cells were grown in LB medium with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C and then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. LacK at different concentrations (from 0 mM to 4 mM) was added into the bacterial culture when OD_{600} reached 0.6. EGFP-Y39TAG protein expression was induced and assessed by western blotting and Coomassie Brilliant Blue staining as described above.

Purification of sfGFP-N150LacK and H3-K23LacK from *E. coli*

For purification of sfGFP-N150LacK, the pLX-sfGFP-N150TAG plasmid was co-transformed with the pBX-LacKRS plasmid into the *E. coli* strain BL21 (DE3). The transformed bacteria cells were grown in LB medium with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C and then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. 4 mM LacK was added into the bacterial culture when OD_{600} reached 0.6. After 1 h incubation, 1 mM IPTG was added into the culture to induce the protein expression. After another 2 h incubation, 20 mM NAM was added to suppress the activity of *E. coli* sirtuins,[10] and the *E. coli* cells were cultured at 37 °C for 10 h. The cells were harvested and lysed with a sonic disruptor (Scientz, JY92-IIN) in Buffer W (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing protease inhibitor cocktails (Roche, cat#A52955), PMSF (Sigma, cat#78830), deoxyribonuclease I (DNase; Diamond, cat#B002138-0100), and lysozyme (Sangon Biotech, cat#A610308). The supernatant after centrifugation was then purified by Strep-Tactin XT Superflow resin (IBA Lifescience) according to the manufacturer's protocol and eluted with Buffer BXT (Buffer W with 50 mM biotin). The purified protein was stored at -80 °C.

For purification of H3-K23LacK, the pET22b-H3-K23TAG plasmid was used to transform the *E. coli* strain BL21 (DE3) together with the pUltra-LacKRS plasmid. The H3-K23LacK protein was expressed as described above for sfGFP-N150LacK. The cells were harvested and lysed with a sonic disruptor (Scientz, JY92-IIN) in the binding buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 8.0) containing protease inhibitor cocktails, PMSF, deoxyribonuclease I, and lysozyme. The supernatant after centrifugation was then purified by Ni-NTA Sefinose Resin 6FF (Sangon Biotech, cat#C600033-0025) according to the

manufacturer's protocol and eluted with the elution buffer (binding buffer supplemented with 500 mM imidazole). The purified protein was stored at -80 °C.

Sequences of sfGFP-N150LacK and H3-K23LacK are as follows.

sfGFP-N150LacK sequence (Twin-Strep-tag; Kla):
MVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPV
PWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHKlaVYITADKQKNGIKANFKIRHNVEDGSVQ
LADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYK
GSMDELYKRSAWSHPQFEKGGGSGGGSGGSAWSHPQFEK

H3-K23LacK sequence (pelB signal sequence; 6*His-tag; Kla):
MKYLLPTAAAGLLLLAAQPAMAMARTKQTARKSTGGKAPRKQLATKlaAARKSAPS
TGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSAAIGALQE
ASEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERARSHHHHHH

Mass spectrometry analysis of purified proteins

The purified proteins were desalted with 10 kDa Amicon centrifugal filters (Millipore, cat#MRCPRT010), washed with water, and reconstituted into 0.01% ammonia solution. The resulting sample was then analyzed on a Q Exactive Focus LC-MS/MS system (Thermo Fisher Scientific) equipped with a nano-ESI ionization source. The mass spectra were deconvoluted with BioPharma Finder (Thermo Fisher Scientific).

Mammalian cell culture and transfection

HEK293T cells were obtained from ATCC. Cells were grown in DMEM (Dulbecco's modified Eagle's medium; Corning, cat#10-013-CVR) supplemented with 10% FBS (fetal bovine serum; Corning, cat#35-076-CV) in a humidified incubator at 37 °C with 5% CO_2. For transfection, cells were grown on cell culture dishes or plates to 70% confluence and transfected with indicated plasmids using PEI (Polysciences; ~2.5:1 ratio of PEI/DNA) for 18-24 h in complete cell growth media.

Incorporation of LacK into proteins in mammalian cells

HEK293T cells were seeded on 12-well plates (Corning) and cultured overnight in 1 mL of growth media. The next day cells were co-transfected with the desired plasmid of interest containing an amber TAG codon (0.65 μg per well) and the PylRS plasmid (0.35 μg per well) using PEI (2.5 μg per well) in complete cell growth media in the absence or presence of UAA (1 mM unless otherwise stated). For expression of LacK modified proteins, pEF1α-FLAG-LacKRS was used, while pEF1α-FLAG-MmPylRS was used for incorporation of BocK. After 24 h transfection, the cells were lysed with 4% SDS lysis buffer containing Roche protease inhibitor and benzonase by sonication and vortexing. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). Finally, the cell lysates were separated on 4-20% SDS-PAGE gels (Genscript Biotech) and analyzed by western blotting.

Detection of delactylase activities in living cells by fluorescence

For inhibition of HDACs and sirtuins, HEK293T cells were seeded on poly-D-lysine-coated sterilized 35-mm glass bottom dishes (Jet Bio-Filtration) and cultured overnight in growth media. On the next day cells were co-transfected with plasmids pEF1α-FLAG-LacKRS (0.7 μg per dish) and pCMV-EGFP-K85TAG-mCherry (1.3 μg per dish) using PEI (5 μg per dish) in complete cell growth media in the presence of LacK (1 mM). The cells were treated with different inhibitors, including 10 mM nicotinamide (NAM; Sigma, cat#72340; 50 mM stock solution in ddH_2O), 0.4 μM trichostatin A (TSA; Cell Signaling Technology, cat#9950S; 50 mM stock solution in DMSO), 15 μM selisistat (EX-527; MedChemExpress, cat#HY-15452; 10 mM stock solution in DMSO), 1 μM SirReal2 (MedChemExpress, cat#HY-100591; 10 mM stock solution in DMSO), or 1 μM 3-TYP (MedChemExpress, cat#HY-108331; 10 mM stock solution in DMSO). After 24 h incubation, the cells were washed once with warmed PBS, stained with Hoechst 33342 (Beyotime, cat#C1029), and imaged on a Nikon A1R confocal fluorescence microscope. For the Hoechst channel, the 405 nm laser was used as the excitation, and emission was collected between 425 nm to 475 nm. For the EGFP channel, the 488 nm laser was used as the excitation, and emission was collected between 500 nm to 550 nm. For the mCherry channel, the 561 nm laser was used as the excitation, and emission was collected between 570 nm to 620 nm. In a control experiment, HEK293T cells were co-transfected with plasmids pEF1α-FLAG-MmPylRS and pCMV-EGFP-K85TAG-mCherry in the presence of BocK (0.25 mM) and processed as above.

For overexpression experiments, HEK293T cells were seeded on poly-D-lysine-coated sterilized 35-mm glass bottom dishes (Jet Bio-Filtration) and cultured overnight in growth media. On the next day cells were co-transfected with plasmids pEF1α-FLAG-LacKRS (0.7 μg per dish), pCMV-EGFP-K85TAG-mCherry (1.3 μg per dish), and pCDNA3.1-SIRT1-Flag or its inactive mutant (1 μg per dish) using PEI (7.5 μg per dish) in complete cell growth media in the presence of LacK (1 mM). After 24 h incubation, the cells were washed once with warmed PBS, stained with Hoechst 33342 (Beyotime, cat#C1029), and imaged on a Nikon A1R confocal fluorescence microscope.

For quantification of fluorescence images, at least three fields of view per dish were randomly selected for every fluorescence imaging experiment. The fluorescence intensity of every image was quantified in ImageJ and grouped for statistical analysis. Statistical analyses for multiple comparisons were performed using GraphPad Prism.

Detection of delactylase activities in living cells by luminescence

HEK293T cells were seeded on poly-D-lysine-coated 96-well optical-bottom black plates (Corning) and cultured overnight in 0.2 mL of growth media. On the next day cells were co-transfected with plasmids pEF1α-FLAG-LacKRS (35 ng per well) and pCMV-fLuc-K529TAG (65 ng per well) using PEI (250 ng per well) in complete cell growth media in the presence of LacK (1 mM). The cells were treated with different inhibitors, including 10 mM NAM, 0.4 μM TSA, 15 μM EX-527, 1 μM SirReal2, or 1 μM 3-TYP. After 24 h incubation, luciferase activities were measured using a Bright-Lumi luciferase assay kit (Beyotime, cat#RG051M) on a Biotek Synergy H1 microplate reader. Bioluminescence images were taken on a Chemidoc MP imaging system (Biorad) using the chemiluminescence detection mode.

Detection of delactylase activities in living cells by western blotting

For inhibition of HDACs and sirtuins, HEK293T cells were incubated with different inhibitors (10 mM NAM, 1 μM TSA, 15 μM EX-527, 15 μM SirReal2, or 15 μM 3-TYP) in the presence of 25 mM sodium L-lactate. After 24 h incubation, the cells were washed with warmed PBS and harvested. For overexpression experiments, HEK293T cells were seeded in 6-well plates in growth media. On the next day cells were transfected with plasmids encoding wild-type SIRT1, SIRT1 inactive mutant (H363Y), wild-type SIRT2, or SIRT2 inactive mutant (H187Y). After 8 h transfection, the cells were treated with 25 mM sodium L-lactate in fresh growth media. After 24 h incubation, the cells were washed with warmed PBS and harvested.

For separation of the nuclear and cytoplasmic fractions, the above cell pellets were lysed in ice-chilled Brij lysis buffer (1% Brij 97, 150 mM NaCl, 50 mM triethanolamine, pH 7.4, supplemented with EDTA-free protease inhibitor cocktail) with brief vortexing. The resulting cell lysates were centrifuged at 5,000g for 5 min at 4 °C and the supernatant was collected as the cytoplasmic fractions. The nuclear pellets were gently washed with the above lysis buffer without Brij 97 and further lysed with SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine, pH 7.4, supplemented with benzonase) as the nuclear fractions. Both fractions were centrifuged at 12,000g for 20 min at room temperature to remove debris. Protein concentrations were determined by the BCA assay (Pierce). Cell lysates were normalized with the corresponding lysis buffer to equal protein concentrations. Finally, the cell lysates were separated on 4-20% SDS-PAGE gels (Genscript Biotech) and analyzed by western blotting.

Western blotting

Gels were transferred to nitrocellulose membranes using Biorad Trans-Blot Turbo Transfer System (25 V, 30 min). The membranes were blocked with PBST (0.05% Tween-20 in PBS) containing 5% nonfat milk for 30 min at room temperature and then incubated with primary antibodies at 4 °C overnight. Membranes were washed with PBST three times, incubated with appropriate secondary antibodies, and developed using Biorad Clarity Western ECL substrate. Membranes were imaged with a Chemidoc MP imaging system (Biorad).

Pan anti-lactyllysine (PTM-1401, 1:1000 dilution) was purchased from PTM Biolabs for anti-Kla blots. Anti-H3 (17168-1-AP, 1:2000 dilution), anti-6*His-HRP (HRP-66005, 1:5000 dilution), anti-GFP-HRP (HRP-66002, 1:1000 dilution), anti-α-tubulin-HRP (HRP-66031, 1:5000 dilution), and anti-β-actin-HRP (HRP-60008, 1:5000 dilution) were purchased from Proteintech. Anti-Strep-HRP conjugate (A01742, 1:1000 dilution) was purchased from Genscript. Anti-FLAG M2 antibody (F1804, 1:1000 dilution) was purchased from Sigma. Goat anti-mouse-HRP and anti-rabbit-HRP secondary antibodies (1:10000 dilution) were purchased from Jackson ImmunoResearch Laboratories.

Quantification and statistical analysis

Data were generally presented as mean ± standard deviation determined from biological replicates. The method for determining error bars and significance is indicated in the corresponding figure legends. Statistical analysis was performed with GraphPad Prism 9.

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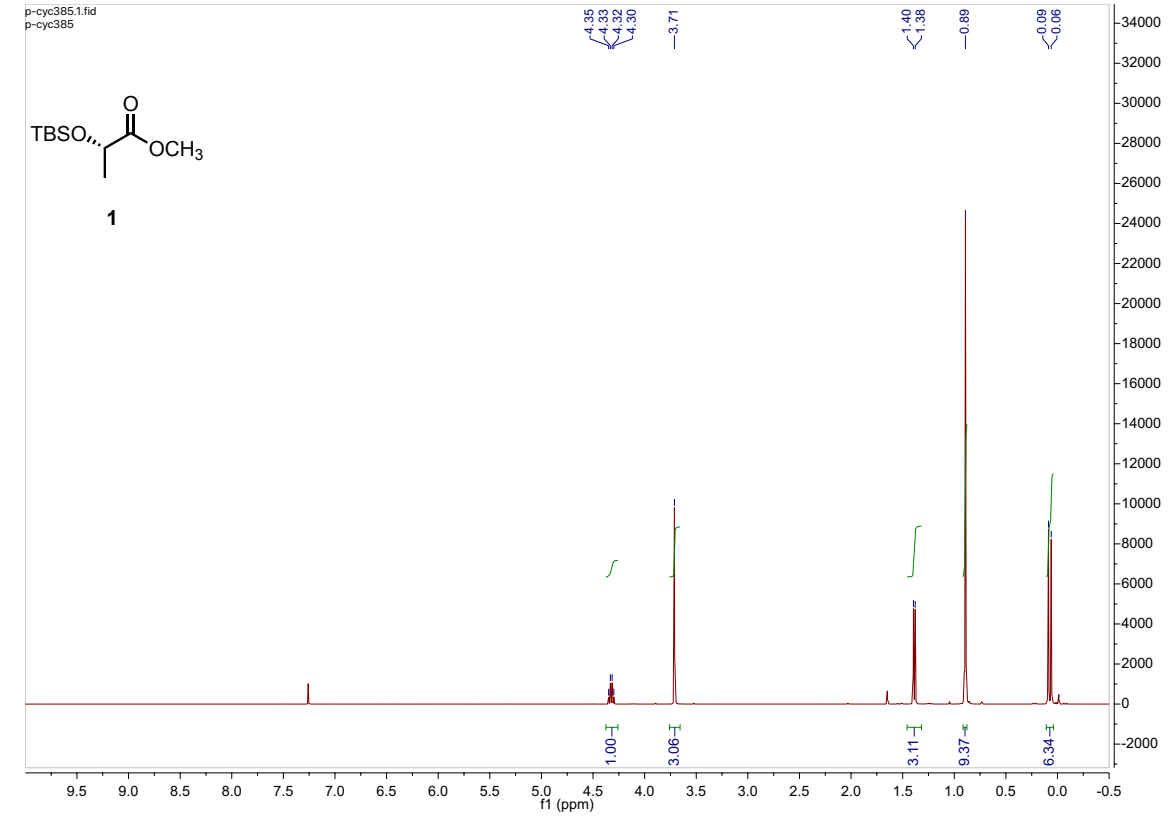
NMR spectra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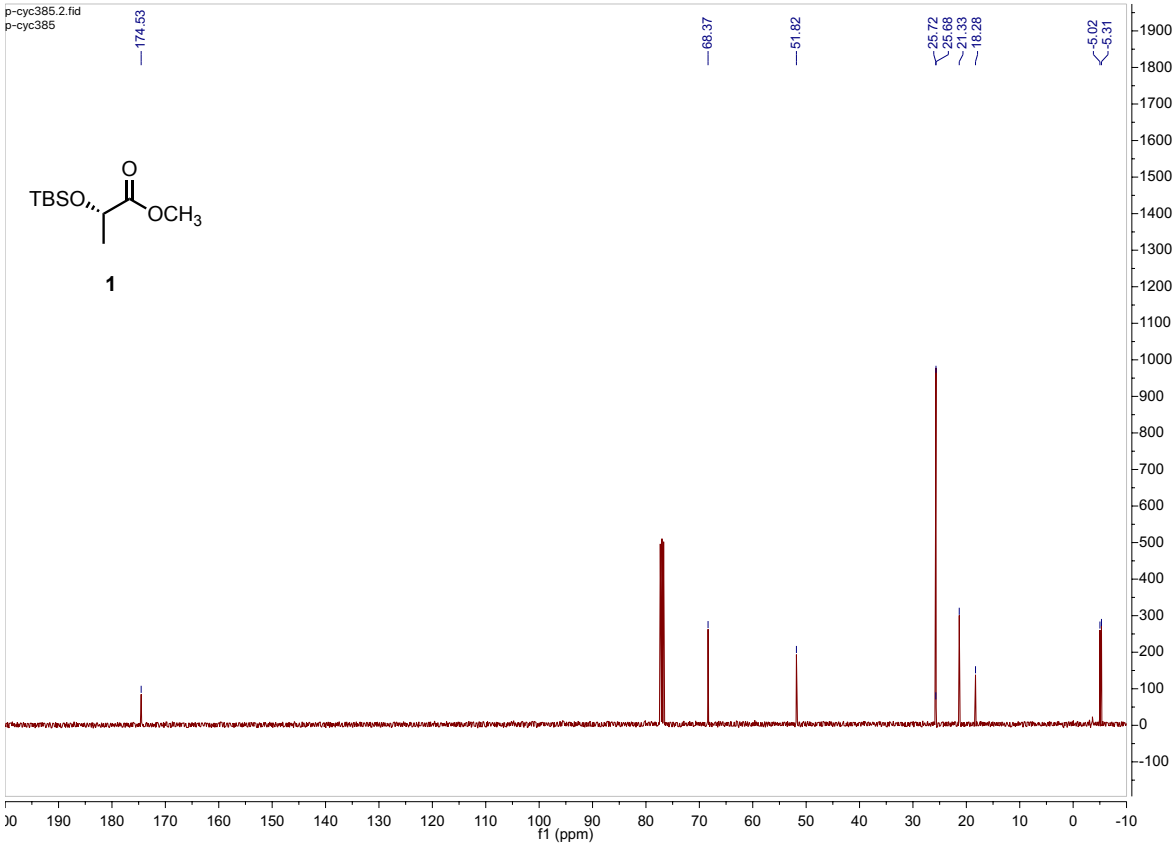
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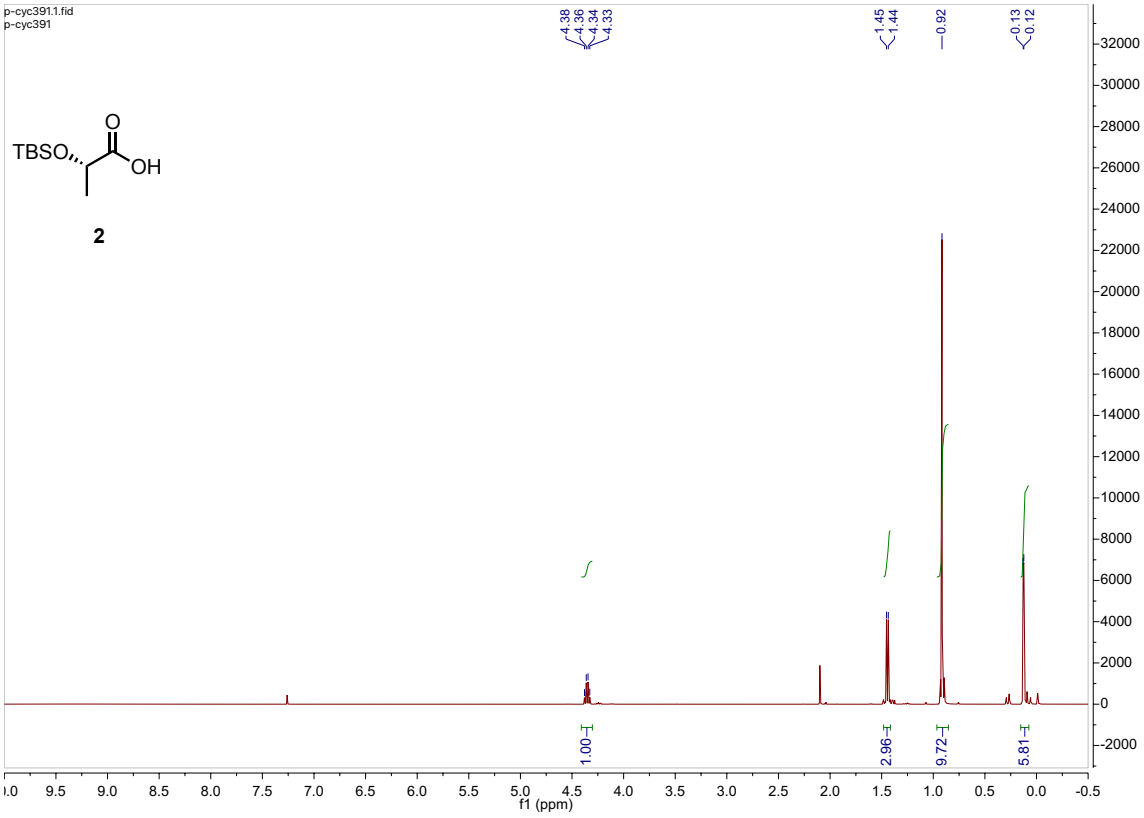
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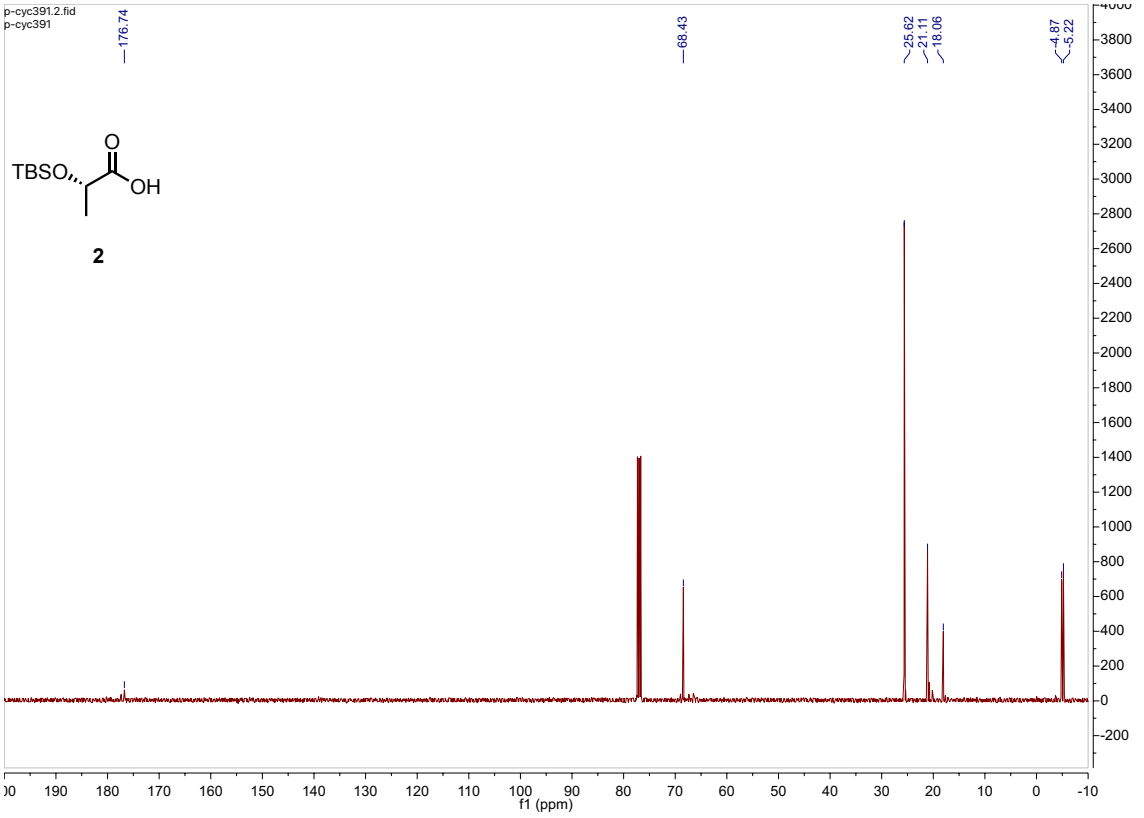
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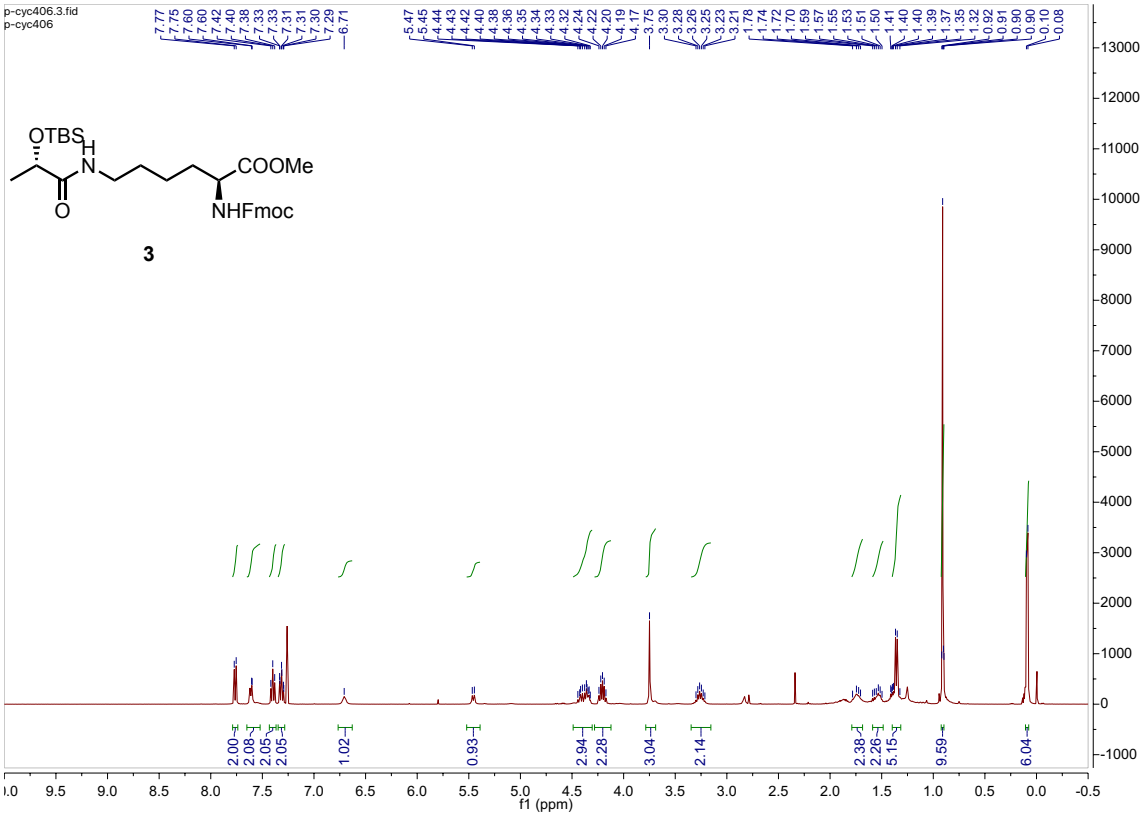
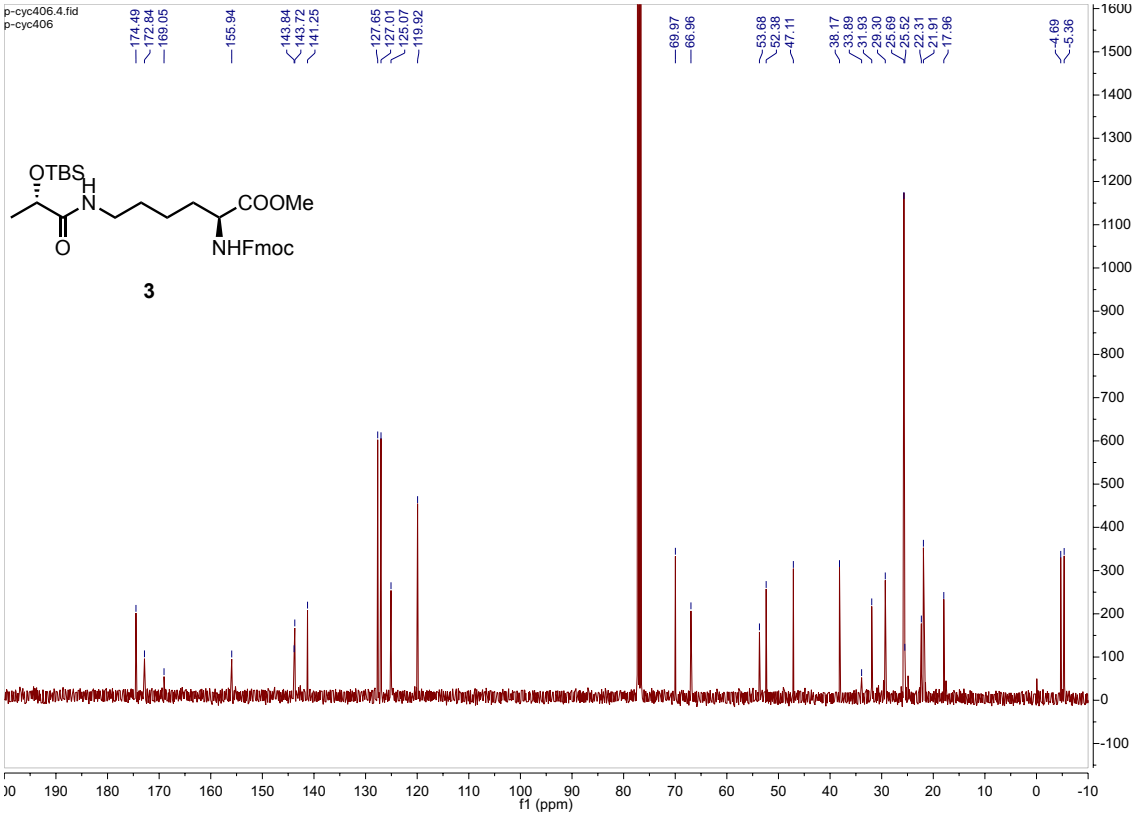
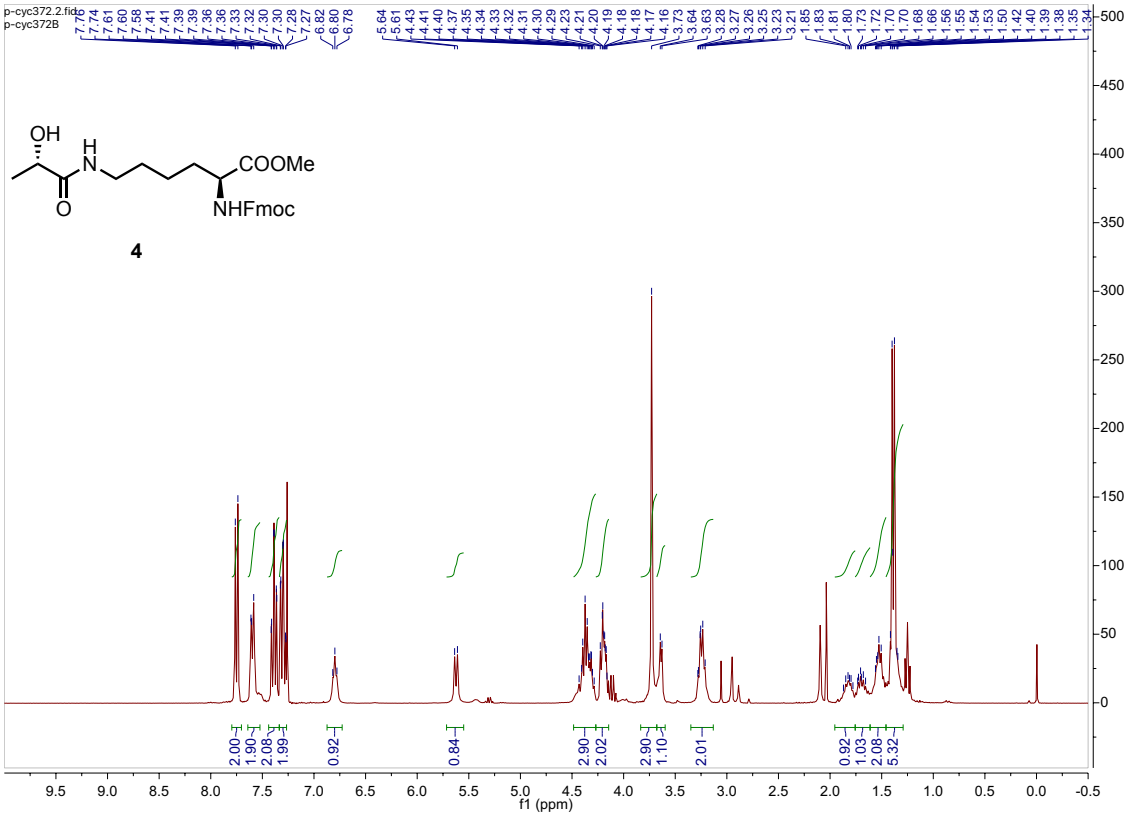
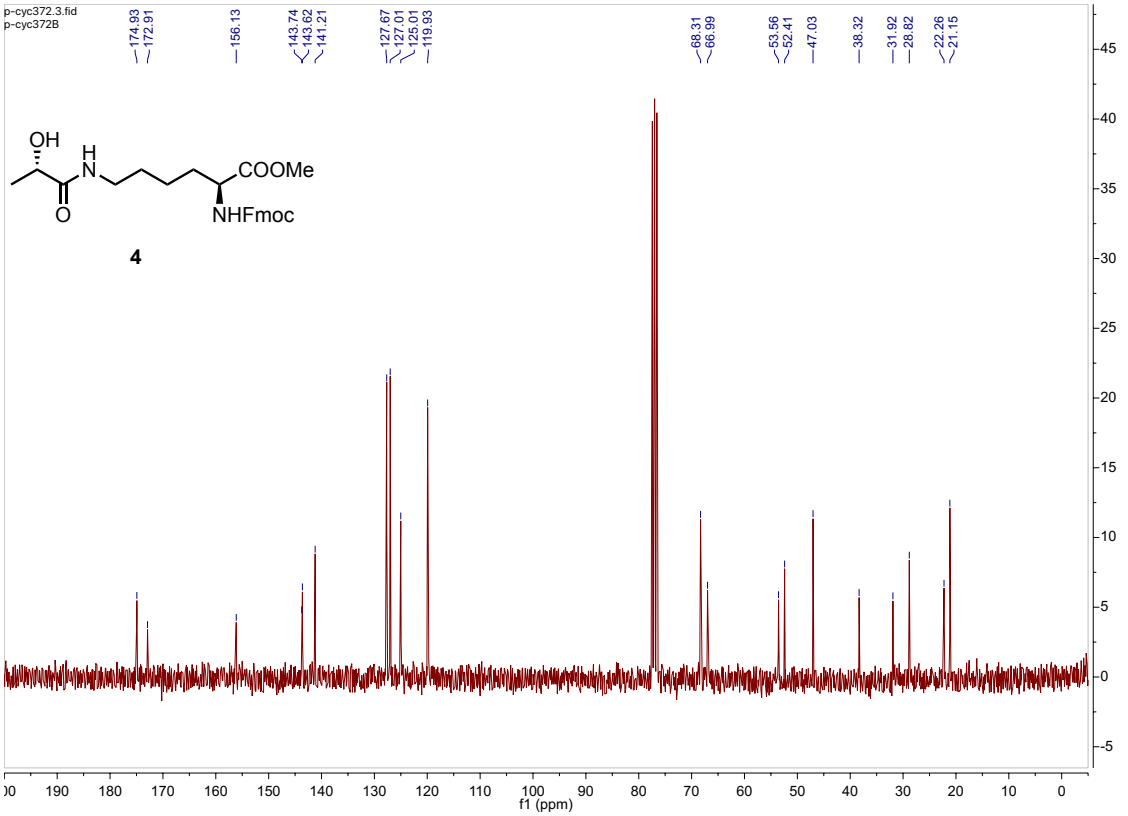
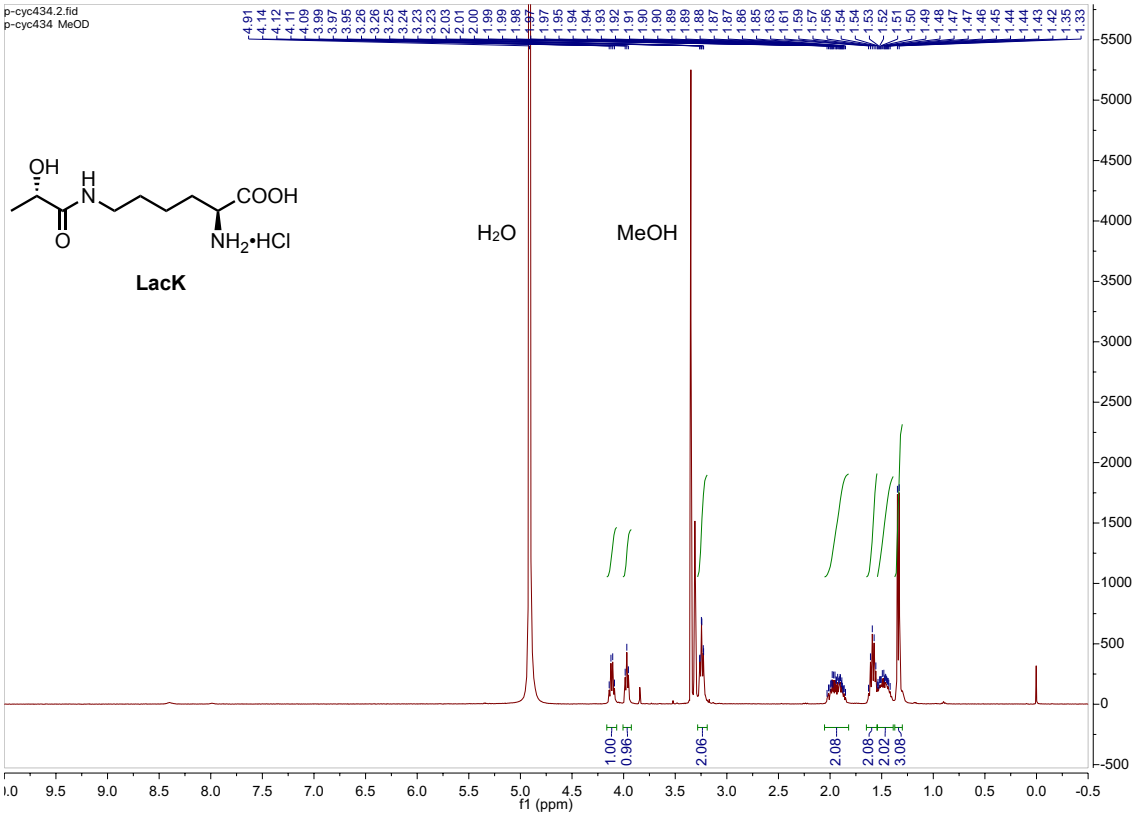
2
f1 (ppm)

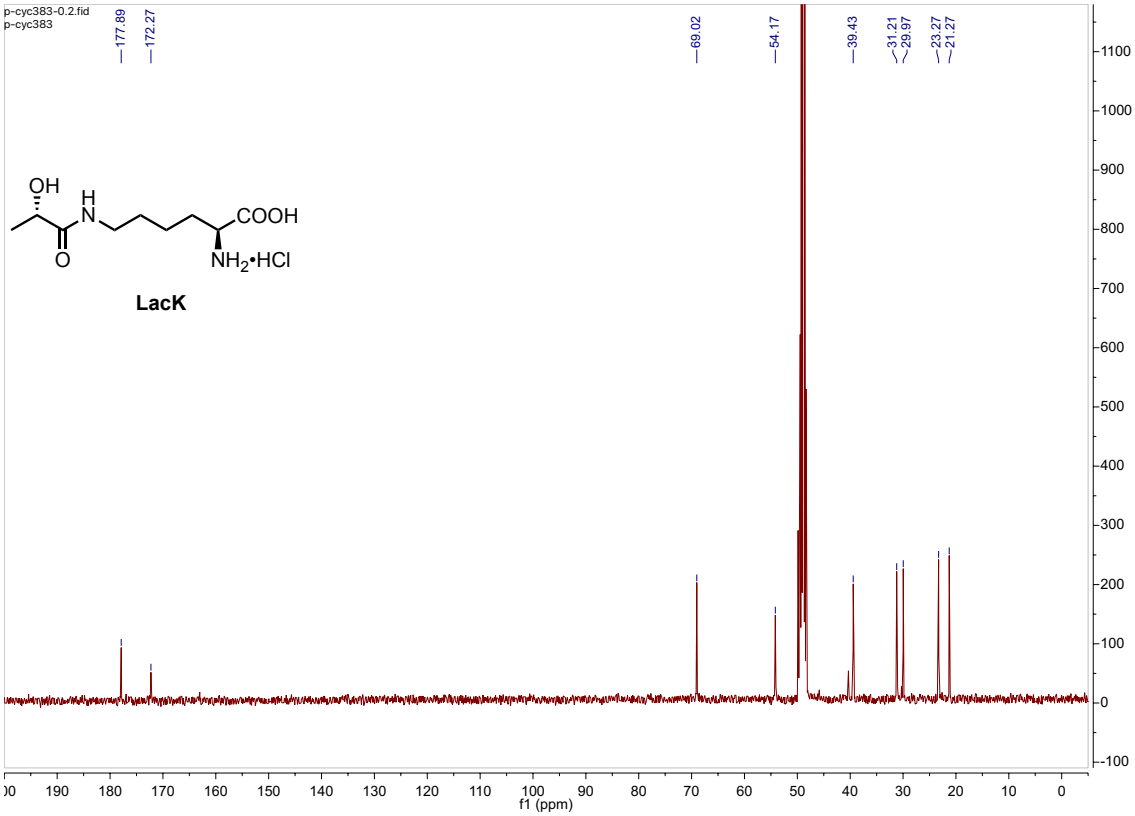

p-cyc406.3.fid
p-cyc406
OTBS
H
N
O
COOMe
NHFmoc
3
f1 (ppm)


p-cyc406.4.fid
p-cyc406
OTBS
H
N
O
COOMe
NHFmoc
3
f1 (ppm)


p-cyc372.2.fid
p-cyc372B
OH
H
N
O
COOMe
NHFmoc
4
f1 (ppm)


p-cyc372.3.fid
p-cyc372B
OH
H
N
O
COOMe
NHFmoc
4
f1 (ppm)


p-cyc434.2.fid
p-cyc434 MeOD
OH
H
N
O
COOH
NH2•HCl
LacK
H2O
MeOH
f1 (ppm)


p-cyc383-0.2.fid
p-cyc383
177.89
172.27
69.02
54.17
39.43
31.21
29.97
23.27
21.27
OH
H
N
O
COOH
NH2•HCl
LacK
f1 (ppm)