Supporting Information

Reconstitution of Mammalian Enzymatic Deacylation Reactions in Live Bacteria Using Native Acylated Substrates

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## Supplementary Tables

**Table S1.** Accession numbers and source of cloned genes.

| KDAC name | Accession number | Cloned residues | Source |
|-----------|------------------|----------------|--------|
| SIRT1     | NP_036370.2      | 1-669          | Addgene ID: 13812[^1] |
| SIRT2     | NP_036369.2      | 1-351          | Addgene ID: 13813[^1] |
| SIRT3     | NP_036371.1      | 1-399          | Addgene ID: 13814[^1] |
| SIRT4     | NP_036372.1      | 1-314          | Addgene ID: 13815[^1] |
| SIRT5     | NP_036373.1      | 1-310          | Addgene ID: 13816[^1] |
| SIRT6     | NP_057623.2      | 1-355          | Addgene ID: 13817[^1] |
| SIRT7     | NP_057622.1      | 1-400          | Addgene ID: 13818[^1] |
| HDAC1     | NP_004955.2      | 1-481          | Addgene ID: 13820[^2] |
| HDAC2     | NP_001518.3      | 1-488          | DNASU ID: HsCD00005288 |
| HDAC3     | NP_003874.2      | 1-428          | Addgene ID: 13819[^2] |
| HDAC4     | NP_006028.2      | 1-1084         | Addgene ID: 13821[^3] |
| HDAC5     | NP_005465.2      | 1-1122         | Addgene ID: 13822[^3] |
| HDAC6     | NP_001308154.1   | 1-1215         | Addgene ID: 13823[^3] |
| HDAC7     | NP_001091886.1   | 1-915          | Addgene ID: 13824[^3] |
| HDAC8     | NP_060956.1      | 1-377          | Addgene ID: 13825[^4] |
| HDAC9     | ACE86818.1       | 1-621          | DNASU ID: HsCD00296891 |
| HDAC10    | NP_114408.3      | 1-669          | DNASU ID: HsCD00073643 |
| HDAC11    | NP_079103.2      | 1-347          | DNASU ID: HsCD00082102 |
| NCOR2     | NP_006303.4      | 395-489        | This work |
| SIRT4*    | NP_036372.1      | 33-314         | This work |
| HDAC4*    | NP_006028.2      | 648-1057       | This work |
| HDAC6*    | NP_001308154.1   | 479-835        | This work |
| HDAC7*    | NP_001091886.1   | 483-903        | This work |

All C-terminally Flag-tagged KDACs were cloned into pACYC-Duet expression vector downstream of a T7 inducible promoter. Truncated KDACs are marked by an asterisk.
Figure S1. CobB knockout (ΔCobB) E. coli BL21(DE3) strain. 

A. Sequencing of the cobB gene of E. coli BL21(DE3) knockout strain (reverse complement sequence). Dashed line marks the position of the deleted sequence.

B. Nucleotide sequence of the cobB gene, displaying the edited cobB sequence (Top) and the sequence that was deleted using CRISPR/cas9 editing (bottom). The 203 base-pairs (bp) that were excised from the genomic DNA included 82 bp upstream (−82) and 121 bp downstream (+121) to the first methionine residue (+1), disrupting both promoter and coding regions. The cobB gene is marked with a red underline; first methionine codon (ATG) and opal stop-codon (TGA) of cobB are in bold letters; nucleotide numbers are indicated.

C. PCR amplification of cobB gene area in WT and ΔCobB BL21(DE3) E. coli (expected size: 922 and 721 bp, respectively).

D. Expression of H3 AcK9 peptide in WT and ΔCobB BL21(DE3) E. coli. In WT E. coli, acetylation level was dependent on inhibition of endogenous CobB by the deacetylase inhibitor NAM. In contrast, in ΔCobB E. coli, similar levels of acetylation were found when bacteria were incubated in the presence or absence of NAM, suggesting no deacetylation activity in the ΔCobB strain. Therefore, acetylated proteins can be expressed in ΔCobB BL21(DE3) E. coli strain incubated in media without NAM.
Figure S2. Antibody detection via Western blot of cloned mammalian KDACs that could be expressed in bacteria. Samples normalized by O.D$_{600}$ were prepared as described in Supplementary Methods and analyzed by Western blot using an antibody against the C-terminal Flag-tag. SIRT7 was fused to an N-terminal maltose binding protein since the full-length protein showed no expression or activity, nor did several truncated forms tested (data not shown). Of the 17 expressed KDACs (full-length and truncated versions), HDACs 4, 5 and 7 could not be expressed in bacteria as full-length proteins. Where possible, truncated forms of specific KDACs were expressed, based on previous studies and crystal structures. The KDACs expressed in a truncated form are marked by an asterisk. HDACs 1, 3, 4*, 7* and 11, as well as SIRT4*, displayed no deacetylase activity in bacteria (size markers, in kDa, are indicated on the left side of each membrane). A. Western blot analyses of full-length mammalian HDACs expressed in bacteria. B. Western blot analyses of full-length mammalian sirtuins expressed in bacteria. C. Western blot analyses of truncated KDACs expressed in bacteria.
Figure S3. A. Schematic representation of the DNA elements and coded proteins used in the expression of the interchangeable acylated peptides. The interchangeable peptide construct was initially introduced into pCDF-Duet expression vector downstream of a T7 inducible promoter (dashed box) by means of standard restriction-ligation between 5’ NcoI and 3’ Xhol restriction sites. TAG mutants of different peptides (green), with ∼7 amino acids before and after the TAG codon (red) were cloned between 5’ NcoI and 3’ KpnI restriction sites. The peptide was followed by a flexible Gly-Gly-Ser-Gly linker (purple), maltose binding protein (orange), and a 6×His tag (cyan) at the C-terminal. The interchangeable peptide can be easily replaced with a different sequence, through restriction-ligation of short double-stranded oligos.

B. DNA sequence of the interchangeable acylated peptide substrate (using H3 AcK9 peptide as an example). The construct contains the acylated peptide (green) with a TAG stop codon (red) encoding for acyl-lysine incorporation, and restriction sites (underlined) to enable convenient cloning of different peptides. The peptide was C-terminally fused to a flexible GGSG linker (purple) followed by a maltose binding protein (orange) and a 6×His tag (cyan). The displayed sequence of the H3 AcK9 peptide was derived from the immunogen used to develop the H3 AcK9 antibody used in this work, and therefore it does not follow the ‘traditional’ ∼15 amino acid long peptide, as explained in A.
Figure S4. ncAA-dependent expression of acylated substrates. Indicated substrates were expressed in the presence (+) or absence (−) of acetylated lysine (A and B) or other acyl-lysine derivatives (butyryl lysine, propionyl lysine, or crotonyl lysine, C). Substrate expression levels were evaluated by immunoblotting using antibodies against C-terminal 6×His tag (top panels). Total protein loads were quantified by in-gel fluorescence, following UV-induced reaction with TCE (bottom panels). Normalized expression levels are displayed in graphs.
Figure S5. Co-expression of HDAC3 and NCOR2. HDAC3 was cloned into pACYC-Duet expression vector together with the NCOR2 domain (N-terminally fused to a maltose binding protein), which is known to facilitate HDAC3 activity. The two proteins were cloned under separate, inducible, open reading frames. The graphs depict HDAC3 deacetylation activity toward the four co-expressed acetylated substrates: A. H3 AcK9 peptide. B. H3 AcK18 peptide. C. RelA AcK310 peptide. D. trRelA AcK310. No apparent deacylase activity was observed, even toward H3 AcK18 peptide, which is derived from a known HDAC3 substrate. E. Expression of MBP-NCOR2 in E. coli, detected by Western blot using an anti-MBP antibody.
Figure S6. $^{1}$H-NMR spectrum of propanoyl lysine·TFA (2, 400 MHz, CD$_3$OD)
Figure S7. $^1$H-NMR spectrum of butanoyl lysine·TFA (3, 400 MHz, CD$_3$OD)
Figure S8. $^1$H-NMR spectrum of crotonoyl lysine·TFA (4, 400 MHz, CD$_3$OD)
**Supplementary Methods**

**General**

General chemicals and DNA oligomers were ordered from Sigma Aldrich (Darmstadt, Germany). Primers for Gibson assembly reactions were designed using the online NEBuilder Assembly Tool. The H3 AcK9 peptide fused to MBP and C-terminal 6×His tag (Supplementary Fig. S3) and NCOR2 residues 395–489 were synthesized de-novo by IDT (Coralville, IA). DNA sequencing was performed by the sequencing facility at Ben-Gurion University. Plasmids carrying various genes were ordered from Addgene or DNAsU as detailed in Table S1. pBK vector for expression of evolved acetyl-lysine synthetase was kindly provided by Dr. Jason W. Chin (MRC-LMB, Cambridge, UK). Enzymes for molecular cloning (i.e., restriction, ligation, DNA modifications, Phusion High-Fidelity DNA Polymerase for PCR reactions, and Gibson Assembly kit) were purchased from NEB (Ipswich, MA) and used according to the manufacturer’s instructions. DNA was purified using spin columns from Macherey Nagel (Düren, Germany). Acetyl lysine was purchased from Chem-Impex International Inc. (Wood Dale, IL) and used without further purification. Nicotinamide and SAHA were purchased from Sigma Aldrich (Darmstadt, Germany) and used without further purification. DH10B *E. coli* strain (Life technologies, Carlsbad, CA) was used for molecular cloning and plasmid propagation. BL21(DE3) *E. coli* strain (NEB) was used for protein expression and deacylation assays. During cloning steps, bacteria were grown in liquid LB media or on LB/agar plates supplemented with antibiotics (50 µg/mL kanamycin, 50 µg/mL spectinomycin, 50 µg/mL chloramphenicol). Primary antibodies: anti-6×His (#G020), anti-H3K9 acetyl (#ab61231), anti-H3K18 acetyl (#ab61233), and anti-RelAK310 acetyl (#ab52175) were purchased from Abcam (Cambridge, UK). Primary anti-Flag (#F1804) was purchased from Sigma Aldrich. Secondary antibodies: anti-mouse IgG (#ab7068) and anti-rabbit IgG (#ab92080) were purchased from Abcam (Cambridge, UK).

**Molecular Cloning**

**Cloning of deacetylases**

Deacetylases listed in Table S1 were cloned with a C-terminal Flag-tag downstream of the first T7 promoter of a pACYC-Duet vector (Merck/Novagen, Darmstadt, Germany), using Gibson Assembly Kit (NEB). Sirtuins 1-3, 5-7 and HDAC8 were cloned as full length proteins. SIRT4 and HDAC6 were cloned as truncated proteins. SIRT7 was fused to N-terminal maltose-binding protein (MBP).
Cloning of substrates

Substrates were cloned with a C-terminal 6×His-tag into a pCDF-Duet vector as described in Supplementary Fig. S3. This vector also contained a U25C mutant of pyrrolysine amber suppressor tRNA under constitutive expression,\[^6\] and expression of substrate was controlled by a T7 promoter. The K310-acetylated RelA substrate (residues 1-323, accession number NP_068810.3) was expressed as an N'-MBP fusion protein. All peptide substrates were cloned using a designed interchangeable segment (Supplementary Fig. S3), which was initially ordered as a dsDNA fragment and cloned into the expression vector. Epitopes were constructed by including ∼7 amino acids before and after the acetylated lysine residue, that was encoded by a TAG codon (e.g., the K310-acetylated RelA-peptide is a 15 amino acids long segment derived from the sequence of RelA, spanning amino acids 303-316, with a TAG codon at position 310). The ∼15 amino acid-long peptides were expressed as fusion proteins, with a short C-terminal linker (GGSG) followed by MBP and 6×His-tag (Supplementary Fig. S3). When possible, the amino acid sequence used as the immunogen for creating the primary antibody was used for that peptide (e.g., the K9-acetylated histone 3-peptide is a 13 amino acids long segment spanning amino acids 1-12, with a TAG codon at position 9). Epitopes were ordered as complementary ssDNA oligos that were annealed to a dsDNA and ligated to the vector.

CobB-knockout *E. coli* strain

The CobB-knockout strain of *E. coli* BL21(DE3) was generated according to the method described by Jiang et al. (Supplementary Fig. S1).\[^7\] Briefly, *E. coli* BL21(DE3) cells were first transformed with the temperature sensitive replicating vector pCas (Addgene ID: 62225)\[^7\] for constitutive expression of Cas9 and arabinose-induced expression of λ-Red (required for exonuclease activity and recombination, respectively). The transformed bacteria were then co-transformed with (i) pTargetF vector (Addgene ID: 62226),\[^7\] harboring the specific N20 sequence complementing a part of the CobB locus, and (ii) ∼1000 bp dsDNA fragment (a.k.a. donor DNA) that complements a region in the CobB locus, to facilitate homologous recombination together with excision of ∼200 bp. Cells were cured from pTargetF and pCas plasmids by IPTG induction and incubation at 37°C, respectively. Finally, elimination of the DNA fragment from CobB locus was verified by colony PCR and DNA sequencing (Supplementary Fig. S1). The N20 sequence from CobB locus was identified using the free ATUM CRISPR gRNA design tool (ATUM, Newark, California). Whole BL21(DE3) E. coli genome (NCBI Reference Sequence: NC_012971.2) was used to design primers for sequencing of CobB locus.
Co-expression of acylated substrates and deacetylase assay

Deacylation assay in 2×TY medium

Plasmids containing deacetylase and substrate (100-200 ng DNA each) were co-transformed into 50 µL of heat-shock competent CobB knockout BL21(DE3) E.coli, harboring pBK vector for constitutive expression of evolved AcKRS3 gene (for expression with CroK/ButK/ProK, a pBK vector harboring the wild-type PylRS was used). Cells were recovered for 1 h in 1 mL SOC (37°C, 600 rpm), transferred into 25 mL LB with 25 µg/mL of each antibiotic, and incubated for 16-18 h (37°C, 220 rpm). Cultures were then diluted to OD$_{600}$=0.05 into 25 mL 2×TY medium supplemented with the same concentration of antibiotics and incubated at 37°C (220 rpm). At OD$_{600}$≈0.4, cultures were supplemented with 2 mM CroK, ButK, or ProK, cells were incubated for additional 20 min, and protein expression was induced by adding 0.5 mM of IPTG. Induced cells were incubated at 22°C until cultures reached OD$_{600}$≈3-4.

Deacylation assay in Auto-Induction medium

Auto Induction (AI) medium lacking lysine and glutamine was prepared as described, and filter-sterilized before usage.[8] Transformation and over-night incubation in LB medium were performed as described above for expression in 2×TY medium. Following over-night incubation, cultures were diluted to OD$_{600}$=0.05 into 5 mL of pre-warmed (37°C) AI medium, supplemented with 10 mM AcK, 25 µg/mL chloramphenicol, 25 µg/mL spectinomycin, and 50 µg/mL kanamycin. Cultures were incubated at 37°C (220 rpm) for 5 h, and then temperature was lowered to 22°C and culture was incubated for ∼43 h (typical final OD$_{600}$ values ranged between 6 to 12).

Deacylation assay in 2×TY medium supplemented with deacetylase inhibitors

For this procedure, the same expression protocol was used as described for expression in 2×TY medium. Upon addition of 10 mM AcK (at OD$_{600}$≈0.4), growth media was additionally supplemented with indicated concentration of nicotinamide or SAHA (Figure 3). Following 20 min incubation at 37°C (220 rpm), protein expression was induced with 0.5 mM IPTG. Temperature was lowered to 22°C (220 rpm) and cultures were incubated until OD$_{600}$≈3-4.
Western blot analysis

Following expression, 1 mL of culture normalized to OD$_{600}$=1 (using 1×PBS) and bacterial cells were resuspended in 375 µL of ice-cold 1×PBS and mixed with 125 µL of 4×Laemmli Sample Buffer. Clear lysates were obtained following a 7 min incubation at 95°C with gentle agitation and centrifugation at 4°C (15,000 rpm). Equal volumes from each normalized sample were loaded onto and resolved in 12% SDS-PAGE (50% loading was used with lysates of bacteria not expressing a KDAC). Proteins were then transferred to a 0.2 µm nitrocellulose membrane using semi-dry transfer apparatus (Trans-Blot Turbo System, Bio Rad). Membranes were incubated in 10 mL blocking solution (5% bovine serum albumin in 1×TBST solution) for 1 h at room temperature with gentle agitation, washed with 1×TBST and incubated over-night with primary antibody at 4°C. On the following day, membranes were washed with 1×TBST, incubated with a secondary antibody (1% skim-milk in 1×TBST solution) for 1 h at room temperature, and washed again. Finally, proteins were visualized using ECL reagent (GE Healthcare, Chicago, IL) and bands were quantified using ImageJ software.[9] To quantify total protein load, 0.5% (v/v) 2,2,2-trichloroethanol (TCE) was added to polyacrylamide gels before polymerization, as described by Ladner et al.[10] Following electrophoresis, gels were illuminated for 90 s on a UV transilluminator to induce the reaction between tryptophan residues and TCE. Proteins were illuminated by UV light to produce quantifiable fluorescent bands.

Statistical Analysis

In order to measure the significance in differences in acetylation levels between samples containing active KDACs and a negative control (i.e., acetylation level that is significantly lower than -KDAC), a one-way ANOVA test was performed together with a one-tailed Dunnett post-hoc test. Samples, which showed significantly lower values compared to the control group, were marked by asterisks over their corresponding columns (* p<0.01, ** p<0.001, *** p<0.0001). All statistical analyses were carried out using Statistica 12.0 software (TIBCO, Palo Alto, CA).
Organic synthesis

\[
\begin{align*}
\text{H}_2\text{N} & \overset{1}{\text{COOH}} \quad \text{NHBoc} \quad \overset{O}{\underset{\text{Cl}}{\text{R}}} \quad \text{NaOH, THF} \quad 0.5\text{C}\text{-rt, } 12\text{ h} \\
\text{H}_2\text{N} & \overset{2}{\underset{\text{O}}{\text{R}}} \quad \text{COOH} \quad \text{NHBoc} \quad \overset{TFA}{\text{NH}_2\text{COOH}} \quad \text{TFA} \\
\text{NHBoc} & \text{COOH} \quad \text{NaOH, THF} \quad 0.5\text{C}\text{-rt, } 12\text{ h} \\
\text{NHBoc} & \text{COOH} \quad \text{TFA} \\
\end{align*}
\]

2: \(R= \text{CH}_2\text{CH}_3\)  
3: \(R= \text{CH}_2\text{CH}_2\text{CH}_3\)  
4: \(R= \text{CH}:\text{CHCH}_3\)

\(\alpha\)-Boc-Lys-OH·HCl (0.01 mol, 4 g) was dissolved in 50 mL solution of NaOH 1 M and THF (1:1). The solution was cooled down in an ice-bath and the indicated acyl chloride derivative (1.5 eq) was added dropwise. The reaction mixture was continuously adjusted to pH=9 using 1 M sodium hydroxide and left stirred at room temperature overnight. Reaction progress was monitored by HPLC. Reaction mixture was then acidified to pH=3 and washed with ice-cold Et\(_2\)O (3×70 mL). The organic phase was dried over Na\(_2\)SO\(_4\) and the solvent was evaporated under vacuum, affording viscous oil. The Boc protecting group was removed by TFA at room temperature and the volatiles were removed under vacuum. The oily crude product was dissolved in minimal amount of MeOH and added dropwise into 50 mL of ice-cold Et\(_2\)O. The precipitated white solid was dissolved in water (30 mL) with TFA (0.5 mL) and lyophilized for two days to afford white solid. The purity of the final product was validated by H\(^1\)-NMR (Supplementary Figs. S6–S8). Reaction progress was monitored by HPLC (Alliance e2695 equipped with PDA detector, Waters Corporation, Milford, MA) using a C18 column (XBridge BEH C18, Waters Corporation, Milford, MA) by following the UV absorbance at 195–220 nm under a flow rate of 0.5 mL/min. Products were eluted using a 3 min isocratic aqueous phase followed by linear gradient up to 95% acetonitrile over 9 min. The three final products presented retention time (t\(_R\)) of less than 4 min with good resolution separation from protected-acylated and protected-non-acylated lysine (t\(_R\) of \(\sim 13\) and \(\sim 12\) min, respectively).

Propionyl lysine·TFA 2. \(^1\)H-NMR (400 MHz, CD\(_3\)OD, Supplementary Fig. S6) \(\delta H=3.56\) (t, \(J=7.2, 1\text{H, CH}^{\text{Lys}}\)), 3.18 (dt, \(J=2.8, 6.8, 2\text{H, CH}^{\text{2Lys}}\)), 2.19 (q, \(J=7.6, 2\text{H, CH}^{\text{Pro}}\)), 1.72-1.97 (m, 2H, \(2\text{CH}^{\text{Lys}}\)), 1.38-1.59 (m, 4H, \(2\text{CH}^{\text{2Lys}}\)), 1.12 (t, \(J=7.6, 3\text{H, CH}^{\text{3Pro}}\)) ppm. HPLC t\(_R\) of 2 min.

Butyryl lysine·TFA 3. \(^1\)H-NMR (400 MHz, CD\(_3\)OD, Supplementary Fig. S7) \(H=3.94\) (t, \(J=6.0, 1\text{H, CH}^{\text{Lys}}\)), 3.19 (t, \(J=7.2, 2\text{H, CH}^{\text{2Lys}}\)), 2.16 (t, \(J=8.0, 2\text{H, CH}^{\text{But}}\)), 1.82-2.03 (m, 2H, \(2\text{CH}^{\text{Lys}}\)), 1.37-1.68 (m, 6H, \(2\text{CH}^{\text{2Lys}}, 2\text{CH}^{\text{But}}\)), 0.94 (t, \(J=7.2, 3\text{H, CH}^{\text{3But}}\)) ppm. HPLC t\(_R\) of 4 min.

Crotonyl lysine·TFA 4. \(^1\)H-NMR (400 MHz, CD\(_3\)OD, Supplementary Fig. S8) \(H=6.78\) (dq,
$J=15.3, \ 7.0, \ 1H, \ CH^{Cro}$, 5.92 (dq, $J=15.3, \ 1.6, \ 1H, \ CH^{Cro}$), 3.94 (t, $J=6.4, \ 1H, \ CH^{Lys}$), 3.25 (dt, $J=6.8, \ 1.6, \ 2H, \ CH_{2}^{Lys}$), 1.81-2.05 (m, 2H, CH$_{2}^{Lys}$), 1.84 (dd, $J=7.0, \ 1.6, \ 3H, \ CH_{3}^{Cro}$), 0.36-1.65 (m, 4H, 2CH$_{2}^{Lys}$) ppm. HPLC $t_R$ of 4 min.
Supplementary References

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