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Supporting Information

Evolved, Selective Erasers of Distinct Lysine Acylations**

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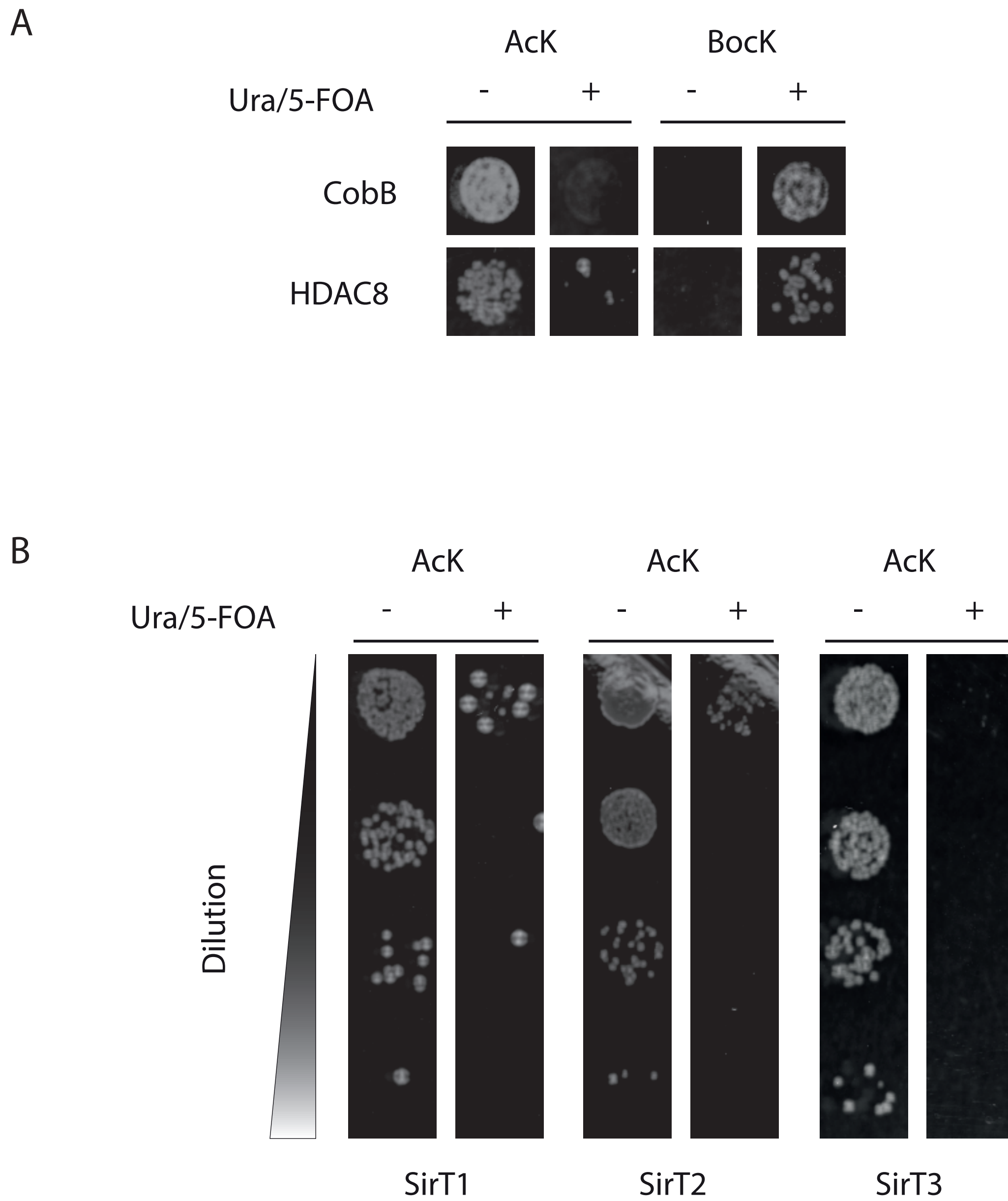
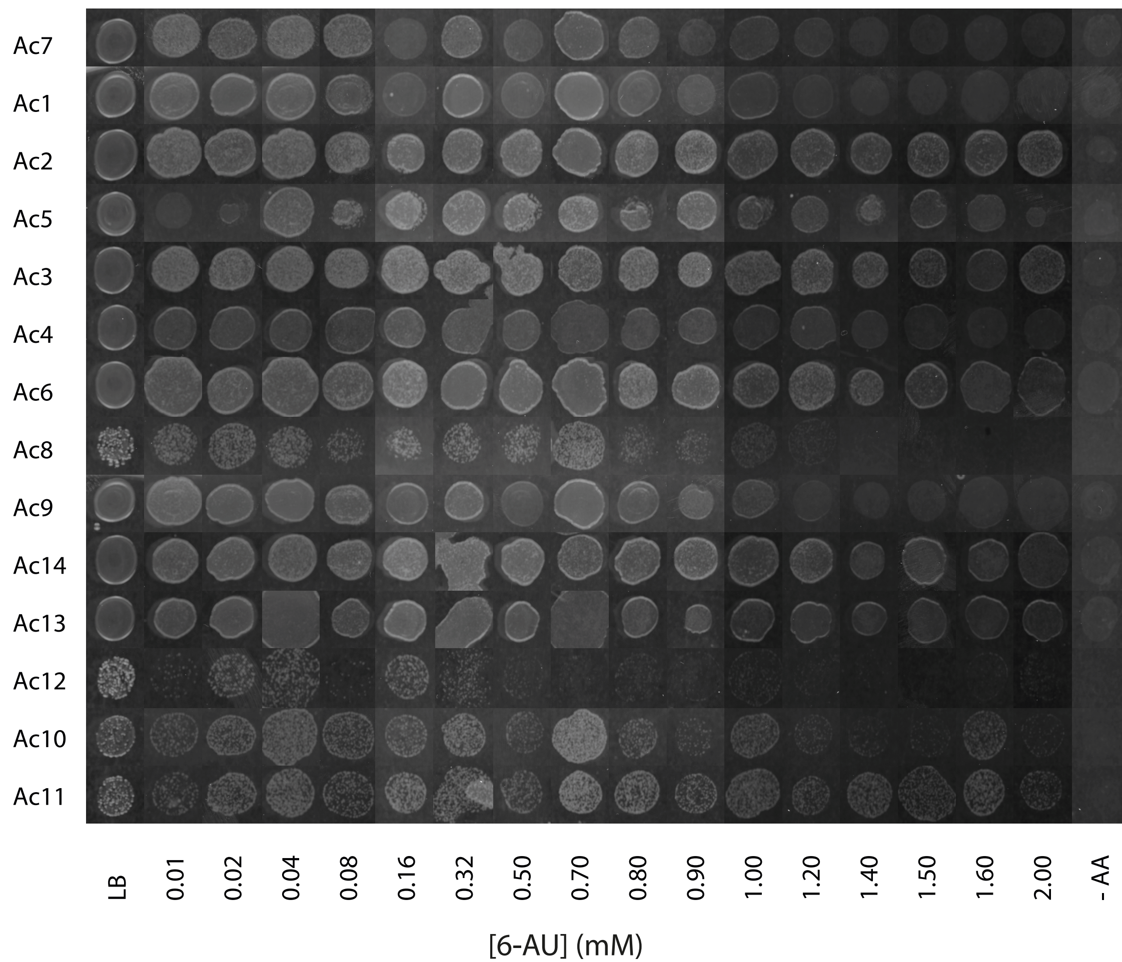
Author Contributions

M.S. Investigation: Lead; Writing—Original Draft: Supporting; Writing—Review & Editing: Supporting
P.N. Investigation: Supporting; Writing—Original Draft: Supporting; Writing—Review & Editing: Supporting
M.E. Investigation: Supporting
R.G. Formal analysis: Supporting; Methodology: Supporting
H.N. Supervision: Lead; Writing—Original Draft: Lead; Writing—Review & Editing: Lead.

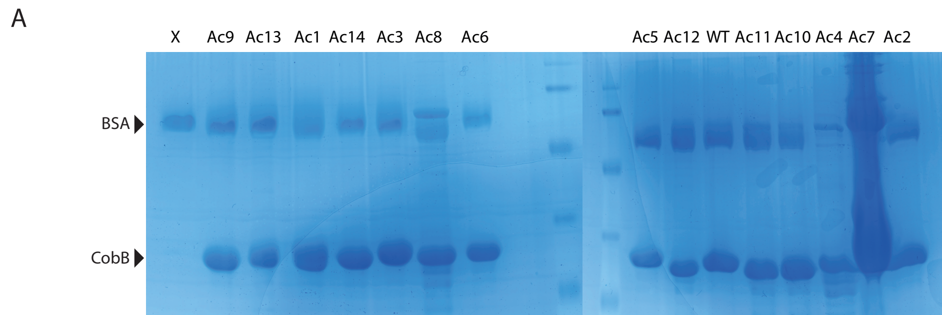


Supplementary Figure 1: Selection of KDACs using Ura3 modified on K93. A) DH10B ΔpyrF ΔcobB expressing CobB or HDAC8 and either Ura3 K93ac or Ura3 K93BocK were grown on minimal medium with or without uracil and 0.1% 5-FOA. B) DH10B ΔpyrF ΔcobB expressing one of three human Sirtuins and Ura3 K93ac were grown under conditions as in A.

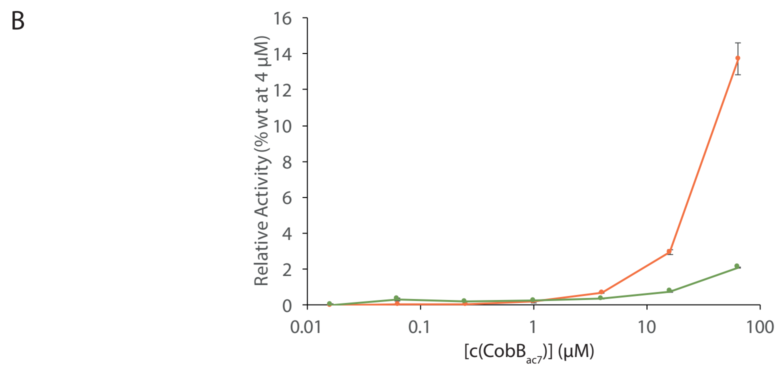


Supplementary Figure 2: Growth of CobB variants in the presence of 6-AU. *E. coli* expressing Ura3 K93ac as sole source of OMP decarboxylase and one of the CobB variants identified in the selection for acetyl-specificity were challenged to grow on selection plates in the presence of increasing concentrations of 6-AU. The growth was compared to growth on LB plates, no growth is observed on minimal medium without addition of acetyl lysine (-AA).

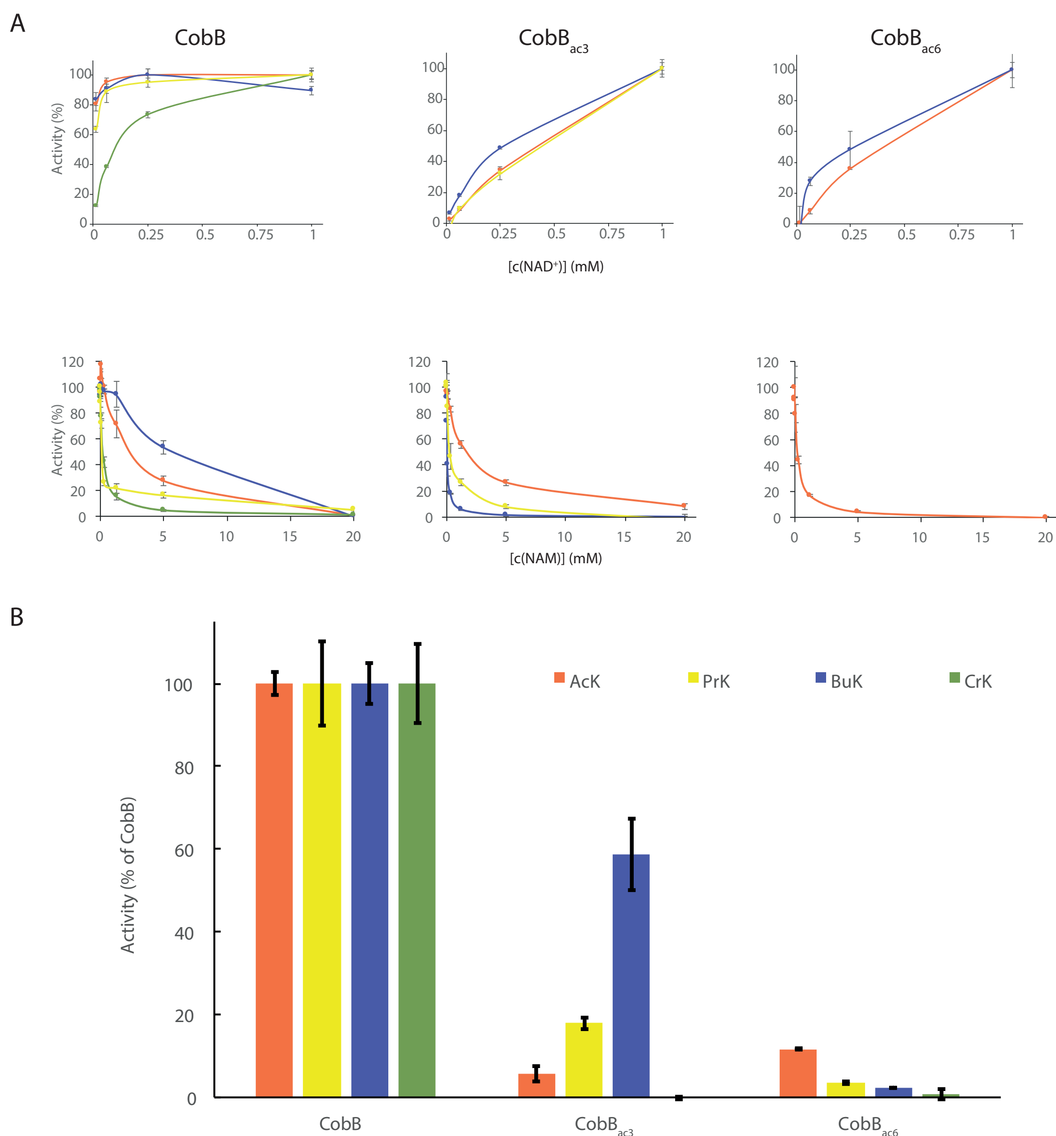
A



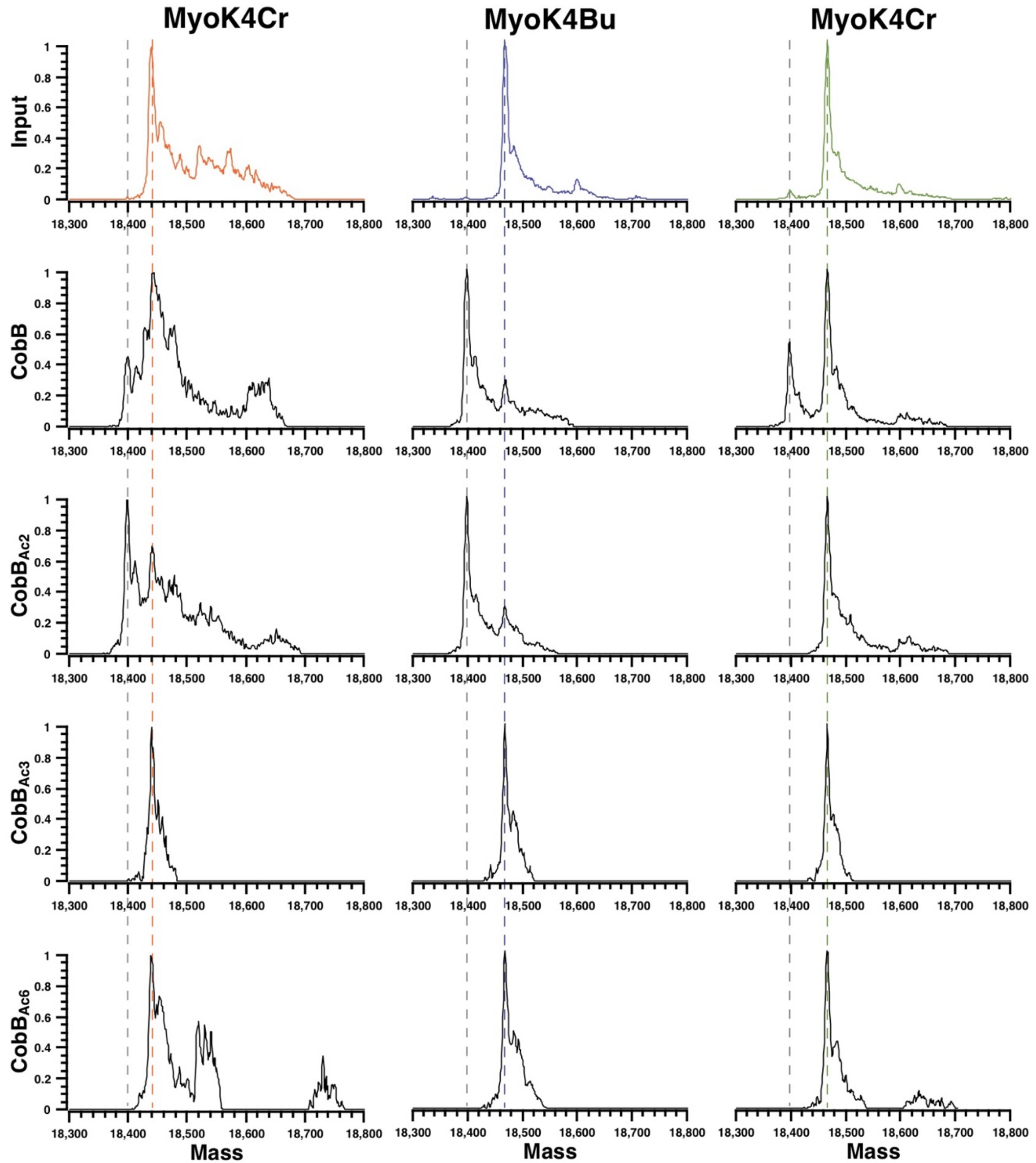
B



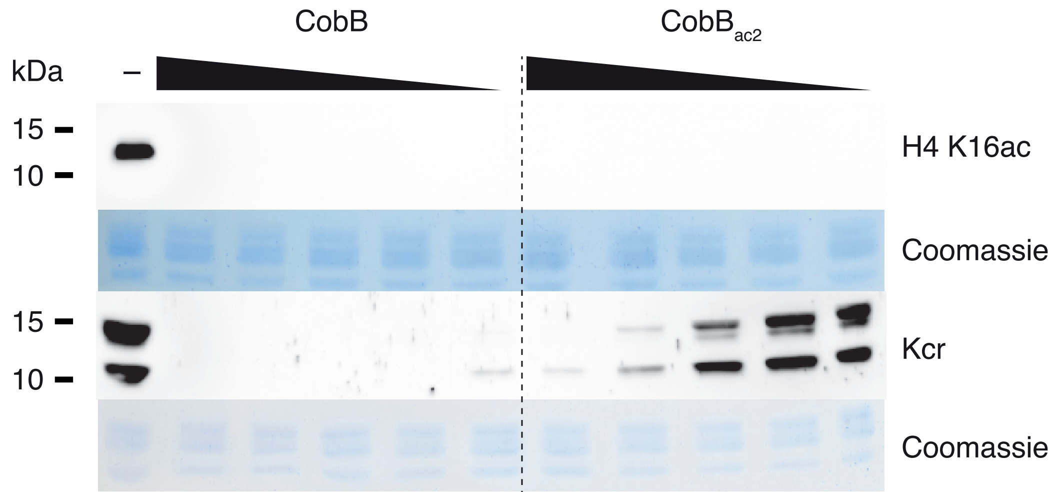
Supplementary Figure 3: Loading control of in vitro luciferase assay in figure 2B and titration of CobB$_{ac7}$. A) The SDS-PAGE shows CobB proteins used in figure 2B. BSA was present during the deacylation reaction. B) Activity of CobB$_{ac7}$ towards FLuc K529ac (orange) and K529cr (green) relative to CobB wild-type at 4 µM was measured separately.



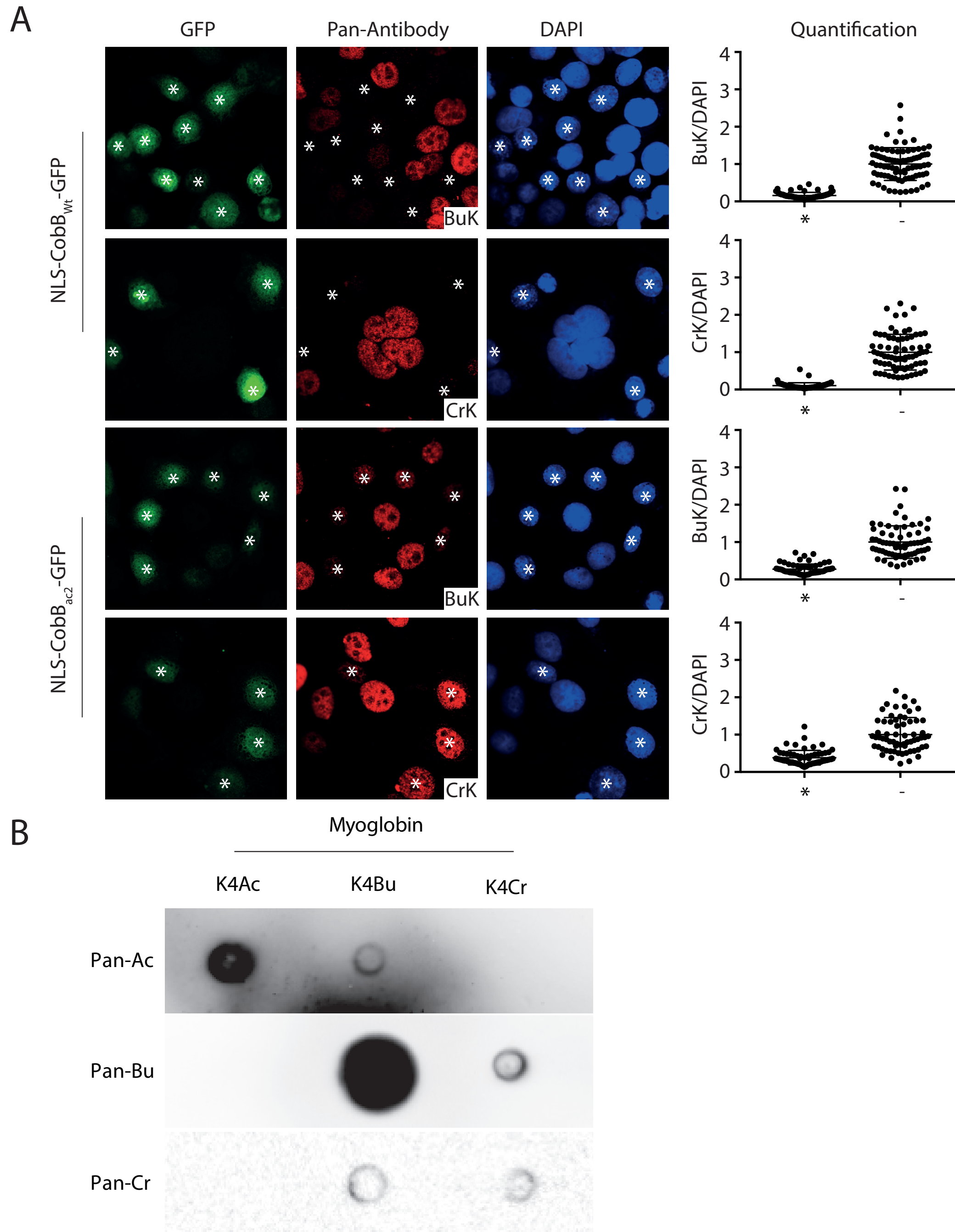
Supplementary Figure 4: Catalytic rates of acetyl-selective CobB variants in dependence of NAD$^+$, NAM concentration and FLuc acylation. A) FLuc with the indicated modification was incubated with CobB (64 nM), CobB$_{ac3}$ (2 µM) or CobB$_{ac6}$ (2 µM) and luminescence assayed in endpoint format. 2 mM NAD$^+$ was present in the NAM titrations. B) CobB variants were assayed on purified FLuc carrying the indicated modification on K529. Experiments were performed in triplicates, error bars are standard deviation of the mean.



Supplementary Figure 5: LC-ESI-MS analysis of Myoglobin proteins before and after incubation with CobB variants. Dotted lines mark the expected mass of the free (grey) or modified lysine (colored). No reaction was observed for $CobB_{ac3}$ (32 μM) and $CobB_{ac6}$ (16 μM) after 2 h incubation at 30°C by ESI-MS in positive ion mode.

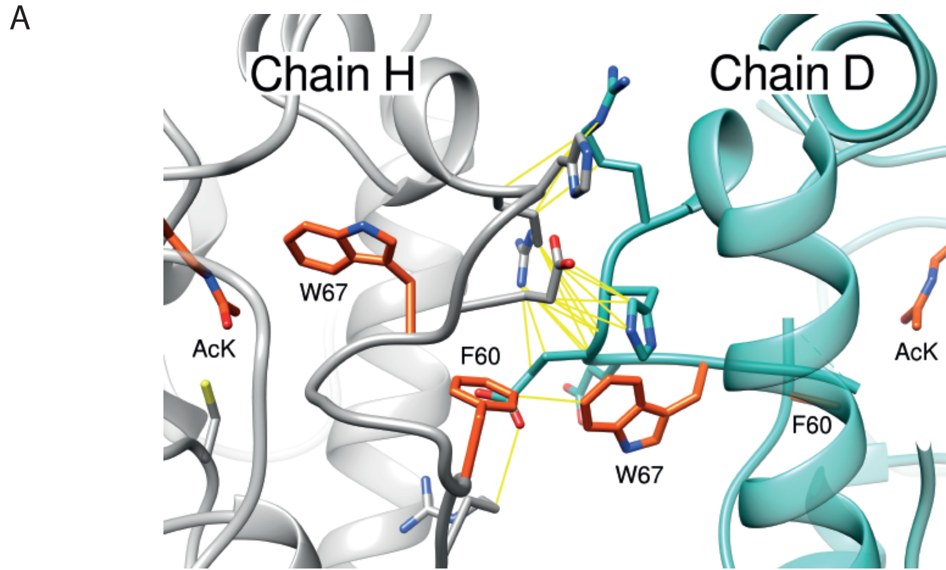


Supplementary Figure 6: Deacylation of histones with CobB and variant CobB$_{ac2}$. Purified human histones were incubated with CobB or CobB$_{ac2}$ (63 nM to 1 µM) for 2 h at 30°C in the presence of 2 mM NAD^+. The modification state of the histones was analysed by Western blot using anti-H4 K16ac and anti-crotonyl-lysine antibodies**.**



Supplementary Figure 7: Detection of nuclear butyrylation and crotonylation and determining the selectivity of the used antibodies. A) NLS-CobB$_{wt}$-GFP and NLS-CobB$_{ac2}$-GFP were expressed in HeLa cells for 24 h and cells stained by immunofluorescence for lysine butyrylation or crotonylation (no treatment with crotonate). The change of the nuclear acylation level was quantified in transfected (*) and untransfected (-) cells (n >= 50) as ratio of the antibody signal divided by the DAPI fluorescence and normalized to the average ratio of the untransfected cells. Shown are all individual measurements with the standard deviation of the mean. B) Dot-blot spotted with 200 ng spotted myoglobin carrying an acetyl, butyryl or crotonyl group at position 4 to test the selectivity of different pan-acyl antibodies.

A



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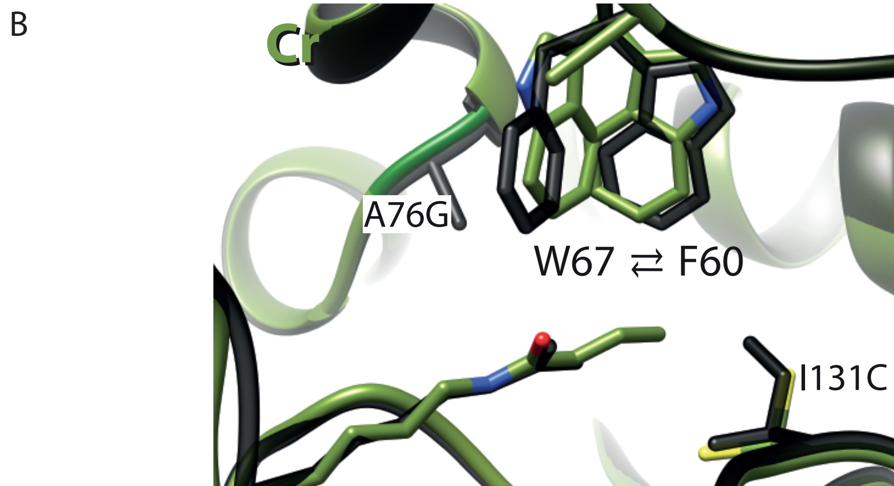
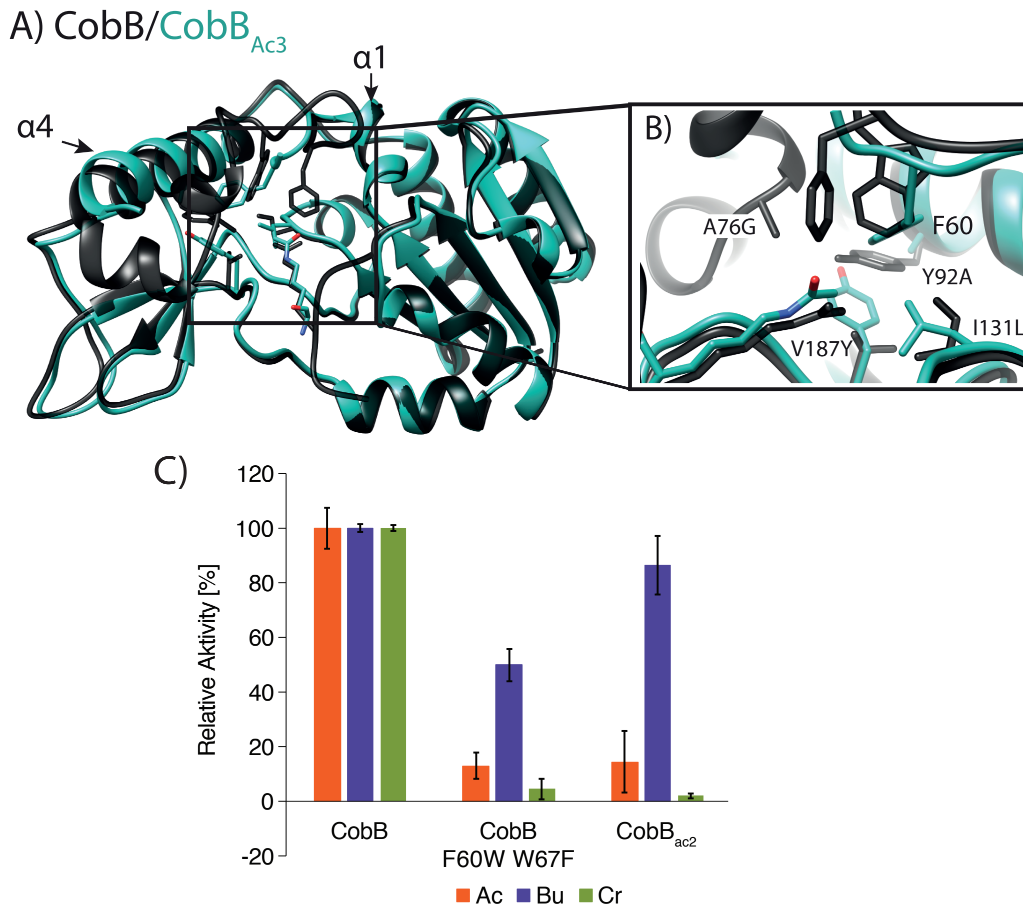
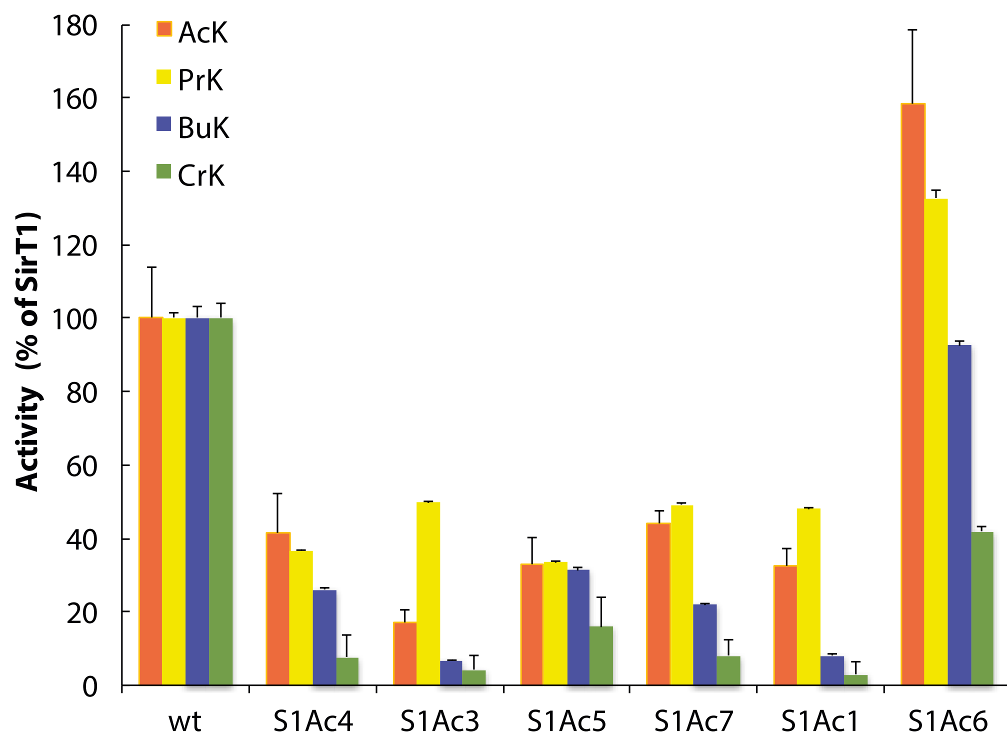


Supplementary Figure 8: Omitted chains from overlay of CobB and CobB$_{ac2}$ A) Crystal chains omitted from overlay of CobB$_{ac2}$ acetyl bound state (Figure 3C), due to contact (yellow lines) between the substrate binding loops of both chains. Acetyl-lysine, W60 and F60 of both chains (H, grey, D, opal) are highlighted in orange. B) Second molecule (Chain A) of CobB$_{ac2}$ in unit cell of H4 K16cr co-crystal. Chain A shows a mixture of conformations with W67 switching positions between the alternative and native state; F60 unresolved.



Supplementary Figure 9: Structure of CobB$_{ac3}$ and activity of the CobB F60W W67F mutant. A) Overlay of H4 K16ac bound structures of CobB (black) and CobB$_{ac3}$ (A76G, Y92A, I131L, V187Y, opal). CobB$_{ac3}$ shows major distortions of cofactor binding loop and helix α2 and α3 following residue F60 until the beginning of helix α4. B) Close-up of the active site. The 4 mutations in CobB$_{ac3}$ allow F60 to bind the hydrophobic pocket previously held by W67. C) Selectivity of designed mutant (2 μM**)** towards different lysine modifications relative to CobB and CobB$_{Ac2}$ measured by Firefly luciferases KDAC assay for acetyl- (Ac) butyryl- (Bu) and crotonyl-lysine (Cr). Swapping F60 with W67 in CobB leads to acyl selectivity comparable to CobB$_{ac2}$. Error bars are standard deviations of the means of three biologically independent replicates performed in triplicates.



Supplementary Figure 10: Acyl selectivity of SirT1 variants. SirT1 variants assayed on FLuc K529ac/pr/bu/cr. Error bars are standard deviations of the means of three replicates.

Table S1: CobB variants selected for AcK and against CrK cleavage.

Mutant	*A76*	*Y92*	*R95*	*I131*	*V187*	*Other*	*n*
$CobB_{ac1}$	C	S	G	V	C		*8*
$CobB_{ac2}$	G			C		V161A	*7*
$CobB_{ac3}$	G	A		L	Y		*4*
$CobB_{ac4}$	L	A	E	L	A	P36S, V45A, A203T	*4*
$CobB_{ac5}$	L	K	W	L	I		*5*
$CobB_{ac6}$	L	S	S	L	W		*4*
$CobB_{ac7}$	S	G	K		L	S206P	*18*
$CobB_{ac8}$	S	I	A		V		*3*
$CobB_{ac9}$	L	G	M	M	P		*2*
$CobB_{ac10}$	L	A		L	P		*1*
$CobB_{ac11}$	L		V	M			*1*
$CobB_{ac12}$	L	M	T	V	Y	E103G, V161A	*1*
$CobB_{ac13}$	L	W	N	W	Q	E79G, K192R, M202I	*1*
$CobB_{ac14}$	V	V	N	W	Q	Q105R, D172G	*1*
SUM							*60*

Table S2: Data collection and refinement statistics

	$CobB_{ac3}$-H4K16Ac	**CobB-H4K16Ac**	**CobB-H4K16Bu**	**CobB-H4K16Cr**	**$CobB_{ac2}$-H4K16Bu**
Space group	C121 (5)	C2221(20)	P41212 (92)	P41212 (92)	P212121 (19)
Cell parameters (Å)	90.25, 79.10, 39.90	131.10, 131.26, 58.54	92.73 92.73 58.62	93.96, 93.96, 58.54	60.49, 92.44, 94.45
(°)	90, 110.77, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Wavelength (Å)	0.978	1	1	1	1
Resolution (Å)	50-1.6 (1.66-1.60)	50-1.6 (1.66-1.60)	50-1.35 (1.38-1.35)	50-2.3 (2.38-2.3)	50-1.95 (2.02-1.95)
Unique reflections	34254 (2536)	66812 (4841)	57070 (4152)	12129 (881)	39338 (2868)
Multiplicity	6.5 (6.9)	13.0 (13.2)	25.2 (25.6)	25.6 (25.7)	13.1 (13.2)
CC 1/2	99.8 (86.3)	99.9 (64.4)	97.8 (77.5)	99.9 (81.6)	99.9 (75.3)
Completeness (%)	98.9 (99)	100 (99.5)	100 (99.9)	100 (100)	100 (100)
I/σ_I	17.6 (3.1)	18.8 (1.6)	19.0 (1.6)	23.9 (2.5)	18.0 (1.7)
R_{merge} (%)	10.1 (70.4)	6.7 (158.1)	9.0 (261)	9.8 (164.1)	7.9 (163.7)
R_{work}/R_{free} (%)	17.9 / 20.5	17.2/18.7	16.9/18.8	21.1/25.0	19.7/21.5
Wilson B ($Å^2$)	14.7	27.8	18.4	53	39.3
Mean isotropic B factor ($Å^2$)	24.4	36.27	25.5	59.1	47.9
Bond length r.m.s.d. (Å)	0.010	0.008	0.012	0.003	0.004
Bond angle r.m.s.d. (°)	1.290	1.16	1.42	0.76	0.861
Ramachandran favoured (outliers)	100 (0)	100 (0)	98.0 (0)	99 (0)	98.6 (0)
Number of atoms	3773	7800	4129	1931	4020
No H	1771	3707	2224	0	0
Water	220	294	287	27	187
Ligand (CIF)	Acetyl-Lysine (aly)	Acetyl-Lysine (aly)	Butyryl-Lysine (btk)	Crotonyl-Lysine (kcr)	Butyryl-Lysine (btk)
Pdb accession code	6RXS	6RXJ	6RXK	6RXL	6RXO

Highest resolution shell in parentheses

Table S2: cont.

	$CobB_{ac2}$-H4K16Ac	**$CobB_{ac2}$-H4K16Cr**	**$CobB_{ac2}$-H4K16Cr-Int3-36h**	**$CobB_{ac2}$-H4K16Cr-Int3-16h**
Space group	P212121 (19)	P212121 (19)	P1211 (4)	C121 (5)
Cell parameters (Å)	92.30, 95.38, 168.83	57.56, 91.89, 95.90	61.38, 94.07, 94.06	132.9, 132.95, 57.99
(°)	90, 90, 90	90, 90, 90	90, 90.03, 90	90, 90.04, 90
Wavelength (Å)	1	1	1	1
Resolution (Å)	50-1.92 (1.99-1.92)	50-1.8 (1.86-1.80)	50-1.7 (1.76-1.70)	50-2.0 (2.07-2.00)
Unique reflections	114176 (8343)	47854 (3481)	117292 (8604)	62989 (5013)
Multiplicity	12.9 (12.4)	12.6 (12.4)	6.7 (6.9)	6.9 (7.0)
CC 1/2	99.8 (67.9)	99.9 (83.5)	100 (63.9)	100 (68.5)
Completeness (%)	100 (100)	100 (99.9)	100 (99.9)	99.9(100)
I/σ_I	13.9 (2.1)	15.3 (2.1)	20.1 (1.3)	19.6 (1.8)
R_{merge} (%)	12.0 (133.0)	8.5 (134.4)	4.7 (142.5)	4.3 (109.0)
R_{work}/R_{free} (%)	20.4/24.3	19.2/23.4	19.9/22.8	22.3 /25.9
Wilson B ($Å^2$)	32.3	30.7	32.9	48
Mean isotropic B factor ($Å^2$)	41.6	38.9	44.1	59.6
Bond length r.m.s.d. (Å)	0.010	0.007	0.007	0.004
Bond angle r.m.s.d. (°)	1.300	1.120	1.380	1.04
Ramachandran favoured (outliers)	99 (0.2)	99.0 (0.2)	97.0 (0.1)	97.0 (0)
Number of atoms	12180	4166	8316	8133
No H	0	0	0	0
Water	879	335	582	235
Ligand (CIF)	Acetyl-Lysine (aly)	Crotonyl-Lysine (kcr)	Cr-Int.3 (2I3)	Cr-Int.3 (2I3)
Pdb accession code	6RXM	6RXP	6RXQ	6RXR

Highest resolution shell in parentheses

Materials and Methods

Plasmids

pPylT-URA3

pMyo4TAG-PylT-based plasmid [1] contains p15A origin, tetracycline resistance, PylT of *Methanosarcina barkeri* and URA3 under arabinose promotor inserted by Nco I/Xho I restriction sites.

pPylT-URA3-K93TAG-PylS, pPylT-URA3-K93TAG-AcKRS3

Based on pPylT-URA3 plasmid. TAG codon was introduced at the position of K93 by QuickChange mutagenesis. Either the *Methanosarcina barkeri* PylS or AcKRS3 [2] were introduced downstream of URA3 via Xho I/Kpn I restriction sites.

pCDF-PylT-FLuc(opt)His6-K529TAG

The gene for Firefly Luciferase codon-optimized for expression in *E. coli* and containing an amber codon replacing the codon for Lys-529 as well as a C-terminal His_6-Tag was custom synthesized by Genscript and cloned into Nco I/Xho I of pCDF-PylT [2].

pBK-AcKRS3opt

(expressing acetyl-lysyl-tRNA synthetase with mutations improving tRNA binding) was generated from pBK-AcKRS3 by three rounds of QuickChange mutagenesis introducing mutations V31I, T56P, H62Y and A100E [3].

pBK-His_6-CobB

His_6-CobB was amplified from pBAD-CobB-H3-H4-Hat1 [4] with Bgl II/Stu I sites and cloned into BamH I/Stu I sites of pBK-PylS [1].

pBK-His_6-HDAC8

His_6-HDAC8 gene was custom synthesized by Invitrogen. It was amplified from pMA-T backbone with Nco I/Xba I sites and cloned sticky/blunt ended into Nco I/Stu I sites downstream of the Ara-BAD promotor of pBK-CobB, replacing the CobB gene.

pBK-His_6-SirT1cat

His_6-SirT1cat (encoding aa 225-664 of SirT1) was amplified from pRSF-Duet1-His6-SirT1 [5] with Nco I/Xba I sites and cloned into Nco I and Xba I sites of pBK-His6-hsHDAC8.

pBK-His_6-TEV-SirT2cat and pBK-His_6-TEV-SirT3cat

The catalytic domain of SirT2 (aa 56-356) and SirT3 (aa 118-399) were amplified from pGEX-T5S-TEV-SirT2/3 [5] with Nco I/Xba I sites, His_6-Tag and TEV protease cleavage site and cloned into pBK-His6-hsHDAC8 using the Nco I and Xba I sites.

pCDF-His_6-Sirt1NCatC

For expression, the mutations were transferred from pBK-His_6-Sirt1cat to pCDF-His_6-Sirt1NCatC containing a N- and C-terminal extended SirT1 (158-665). The mutations were transferred using restriction cloning in Bgl II and Hind III sites.

pCRISPR-CobB-PAM, pCRISPR_spec-PyrF-PAM

New spacer sequences were introduced in pCRISPR (#Addgene 42875). As CobB spacer, the following oligos were used 5’-aaa cTG GAA AAA CCA AGA GTA CTC GTA CTG ACA Gg-3’ and 5’-aaa acC TGT CAG TAC GAG TAC TCT TGG TTT TTC CA-3’.

For PyrF the oligos 5’-AAA CAA GGT CGG CAA AGA GAT GTT TAC ATT GTT TG-3’ and 5’-AAA ACA AAC AAT GTA AAC ATC TCT TTG CCG ACC TT-3’ were used.

Also, the antibiotic resistance marker of the plasmid was changed to spectinomycin by restriction cloning using Xho I/Spe I sites.

Strains

DB6656 was obtained from CGSC (CGSC#: 6868), DH10B was purchased from ThermoFisher. DH10B *ΔpyrF ΔcobB* was created using CRISPR/Cas9: The genes encoding PyrF and CobB were inactivated in *E. coli* DH10B by inserting several stop codons shortly after the start codon of the respective gene in two sequential rounds of genome engineering using CRISPR/Cas9 and editing oligos 5'-CAC GCT GTT GAA GTT CGC GCA CAA ACT GTT AAG CTT ACA ATG TAA ACA TCT CTT TGC CGA GGT TC-3' (pyrF) and 5'-AGG TAC GAA TAC CTG ATT CCG CAG AAA TTt aag ctt aTG TCA GTA CGA GTA CTC TTG GTT TTT CCA-3' (cobB) following established protocols [6]. Successful mutagenesis was confirmed by colony PCR and restriction digest analysis of a simultaneously introduced Hind III site. Plasmids were cured with 60 µg/mL promazine [7].

Expression of KDACs

CobB: *E. coli* DH10B Δ*pyrF* Δ*cobB* pPylT-URA3 was transformed with the respective pBK plasmids for CobB wild-type or mutant. An overnight culture (ONC) in 10 mL LB-medium (50 µg/mL kanamycin, 15 µg/mL tetracycline) was prepared. The ONC was used to inoculate 1 L LB medium (50 µg/mL kanamycin, 15 µg/mL tetracycline) and cells were grown to an OD_{600} of 0.3. The temperature was reduced to 30°C 1 h before expression was induced by addition of arabinose to a final concentration of 0.2%. Cells were harvested by

centrifugation after 16 h (20 min, 6000 rpm, 4°C). The cell pellets were washed with PBS and stored at –20°C.

SirT1: BL21(DE3) cells were transformed with wild-type or mutant pCDF-His_6-SirT1-NcatC plasmid. An ONC was prepared in 10 mL LB (50 µg/mL spectinomycin) and incubated at 37°C while agitating at 200 rpm. The expression culture was inoculated with the ONC and grown at 37°C and 200 rpm to an OD_{600} of 0.8. The expression was induced by addition of 0.5 mM IPTG and the culture shifted to 18°C. The cells were harvested by centrifugation the next day, washed with PBS and stored at –20°C.

Purification of KDACs

a) CobB variants: Cell pellets were thawed on ice and resuspended in 25 mL Ni-NTA wash buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 20 mM imidazole, 1 mM DTT) supplemented with lysozyme (~0.5 mg/mL), DNase (1 mg) and protease inhibitor (1 mM PMSF). Cells were lysed using a pneumatic cell disintegrator, debris removed by centrifugation (20 min, 20,000 rpm, 4°C) and HisPur^{TM} Ni^{2+}-NTA resin (2 mL in 50 mL solution) was added to the supernatant. After 1 h at 4°C the suspension was loaded on a plastic column (BioRad, Munich) and washed with 40 mL Ni-NTA wash buffer. Protein was eluted in 4 mL Ni-NTA wash buffer supplemented with 200 mM imidazole. The elutate was concentrated and the buffer exchanged to gelfiltration buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM DTT) before loading on a HILoad^{TM} 26/70 Superdex^{TM} 200 size-exclusion chromatography column (GE healthcare, UK) preequilibrated with gel filtration buffer. Fractions containing protein were analyzed on a SDS-PAGE, pooled and

concentrated in a microfiltrator (Amicon Ultra-15 Centrifugal Unit, 10 kDa, Merck Millipore). The protein was aliquoted, flash frozen in liquid nitrogen and stored at –80°C. b) SirT1 variants: The cell pellet was resuspended in wash buffer (20 mM Tris pH 8, 200 mM NaCl, 20 mM Imidazole) supplemented with 0.2 mM PMSF, lysozyme (0.5 mg/mL) and DNAse (ca 1 mg). The protein was purified as above with the modification of using a Superdex 75 gel filtration column with 20 mM Tris pH 8, 50 mM NaCl, 10 mM DTT.

Library creation

The active site mutant library was designed based on the CobB crystal structure 1S5P [8]. The codons for A76, Y92, R95, I131, V187 were replaced by NNK codons in three rounds of inverse PCR using the Expand™ High Fidelity PCR System (Roche). One PCR reaction contained 1x Buffer 2, 0.2 mM dNTPs, 1 μM forward and reverse primer, 100 ng pBK-CobB plasmid DNA, 3.5 U Expand Polymerase in a 50 μL volume and run with the following program: 1 min 95°C; 10x [20 s 95°C; 30 s 65°C (-1°C/cycle); 5 min 68°C]; 25x [20 s 95°C; 30 s 55°C; 5 min 68°C]; 5 min 68°C; hold 4°C.

The PCR reaction was purified using the QIAquick PCR Purification Kit (Qiagen) after digestion of the template plasmid with 20 U Dpn I (NEB) for 1 h at 37°C. The purified fragments were digested with 80 U HF-Bsa I (NEB) for 2 h and again purified. The digested PCR product was circularized by T4 DNA ligase in a total volume of 500 μL 1x T4 DNA Ligase Reaction Buffer containing 100 μL digested PCR product and 10 kU T4 DNA Ligase (NEB). The reaction was incubated overnight at 16°C and precipitated with ethanol. The DNA was dissolved in 10 μL water and used to electroporate MAX Efficiency™ DH10B™ Competent Cells (ThermoFisher). Transformation efficiency was determined

from the colony count of a dilution series (10^{-4} to 10^{-7}) on LB agar plates (50 μg/mL kanamycin) for every round of mutagenesis. Coverage was calculated using the GLUE-IT web tool and was estimated to cover for 99.6% of all possible amino acid combinations in the final round with 10^8 transformants [9]. The plasmid DNA was isolated from the mutant pool (Plasmid Maxi Kit, QIAGEN).

The SirT1 mutant library was created in the same manner as the CobB library. Positions A313, I316, I347, F366L and I411 were mutated to NNK in 3 rounds of mutagenesis. The final transformation step had a transformation efficiency of 1.8 x 10^7 covering 85% of all possible variants [9].

Design of the KDAC selection system

To set up the selection strategy for CobB (Figure 1B), *E. coli* DB6656 (Δ*pyrF*) were transformed with pPylT-URA3, pPylT-URA3-K93TAG-PylS or pPylT-URA3-K93TAG-AcKRS3 and grown overnight in LB-medium (15 μg/mL tetracycline). The cells were replicated in tenfold dilutions with M9 medium (10^0 to 10^{-7}) onto M9 selection plates (M9 minimal medium, 0.2% arabinose, 1% glycerol, 0.1 mM tryptophan, 50 μg/mL kanamycin, 15 μg/mL tetracycline, ± 0.1 mM uracil, ± 10 mM acetyl-lysine and 1 mM boc-lysine, ± 0.1% 5-fluoroorotic acid). Plates were imaged after incubation for 48 h at 37°C.

To adapt the selection system to HDAC8, SirT1, SirT2 and SirT3, *E. coli* DH10B Δ*pyrF* Δ*cobB* was transformed with either pPylT-URA3, pPylT-URA3-K93TAG-PylS or pPylT-URA3-K93TAG-AcKRS3 and with either pBK-His_6-HDAC8, pBK-His_6-SirT1cat, pBK-His_6-TEV-SirT2cat or pBK-His_6-TEV-SirT3cat. Transformants were grown overnight in 4 mL LB (50 μg/mL kanamycin, 15 μg/mL tetracycline) and replicated in a tenfold dilution

series onto M9 selection plates (M9 minimal medium, 0.2% arabinose, 1% glycerol, 0.4% glucose, 0.1 mM tryptophan, 80 mg/L valine, 80 mg/L isoleucine, 80 mg/L leucine, 50 μg/mL kanamycin, 15 μg/mL tetracycline, ± 0.1 mM uracil, ± 10 mM acetyl-lysine and ± 0.1% 5-fluoroorotic acid). Plates were imaged after incubation for 48 h at 37°C.

Library selection

Freshly prepared electrocompetent *E. coli* DH10B Δ*pyrF* Δ*cobB* containing pPylT-URA3-K93TAG-PylS or pPylT-URA3-K93TAG-AcKRS3 were electroporated with the CobB or SirT1 mutant library with an efficiency to cover >95% of the possible diversity and incubated at 37°C overnight. Before plating the cells, the pool was diluted 1:50 in 50 mL LB (50 μg/mL kanamycin, 15 μg/mL tetracycline) and incubated for 3 h at 37°C and 200 rpm. After 3 h the cells were pre-incubated with the amino acid (10 mM acetyl-lysine or 1 mM for other modified lysine) and 0.2% arabinose. After 3 h, 1 mL cells were harvested by centrifugation (8000 rpm, 2 min) and washed twice with 1 mL PBS to remove the amino acid and uracil. The washed cells were resuspended to an OD_{600} of 1 in PBS. 100 μL cell suspension was plated on the selection plate (positive selection: M9 selection plate +amino acid, –uracil, –5-FOA or negative selection: M9 selection plate +amino acid, +uracil, +5-FOA) and a control plate (M9 selection plate, –amino acid, –uracil, –5-FOA). The petri dishes were incubated for at least 48 h at 37°C. Colonies were scraped off the plate, plasmid DNA was isolated and used to transform *E. coli* cells for subsequent selections. To obtain CobB mutants with preference for deacetylation over decrotonylation, three rounds of selection on the active site library were performed, starting with a positive selection for deacetylation followed by negative selection against decrotonylation and a final positive

selection for deacetylation. Single colonies were picked from the third-round selection plate, grown in LB (1 mL, 50 μg/mL kanamycin), plasmid DNA was isolated, retransformed in DH10B by heat shock, and once more plasmid DNA isolated and sequenced.

Activity plate assay using 6-Azauridine (6-AU)

Individual plasmid isolates were used to transform *E. coli* DH10B Δ*pyrF* Δ*cobB* containing pPylT-URA3-K93TAG-AcKRS3 and grown in 1 mL LB (50 μg/mL kanamycin, 15 μg/mL tetracycline) in a 96 well block. Cells were replicated using a pin head replicator onto LB agar plates (50 μg/mL kanamycin, 15 μg/mL tetracycline), M9 selection plates and selection plates containing increasing amounts of 6-AU (positive selection: M9 selection plate ±10 mM acetyl-lysine, –uracil, –5-FOA, + 0.01-2 mM 6-AU). Plates were imaged after 48 h.

Purification of Firefly Luciferase K529$_{mod}$

The luciferase was prepared essentially as published [10] but modified for other acylation. In short, *E. coli* BL21 DE3 RIL were transformed with plasmids pCDF-PylT-FLuc(opt)His6-K529TAG and pBK-AcKRS3opt (for FLuc K529ac) or pBK-PylS [1] (other acylations). Cells were grown in LB medium in the presence of antibiotics (50 μg/ml spectinomycin and 50 μg/ml kanamycin) to an OD_{600} of 0.3 at 37°C. Then, cells were shifted to 30°C, and after 1 h protein expression was induced by the addition of 1 mM IPTG, modified-lysine (acetyl-lysine 10 mM, others 1 mM) and 50 mM NAM. After a 16 h incubation at 30°C, cells were harvested, washed with PBS and suspended in Ni-wash

buffer (20 mM Tris/HCl pH 8, 10 mM imidazole, 200 mM NaCl, 10 mM DTT, 2 mM PMSF, 0.5x Roche Protease Inhibitor cocktail), supplemented 20 mM NAM and lysozyme. Cells were lysed using a pneumatic cell disintegrator and debris were removed by centrifugation (20 min, 50,000 g, 4°C). The supernatant was incubated with 500 µl Ni-NTA-beads for 2 h with agitation at 4°C, then beads were washed with 30 ml Ni-NTA wash buffer and bound proteins eluted in Ni-NTA wash buffer supplemented with 200 mM imidazole. Eluates were dialysed against storage buffer (20 mM Tris pH 8, 50 mM NaCl, 10 mM DTT) and used as substrate in KDAC assays in appropriate dilution (s. below).

Luciferase-based KDAC assay

The luciferase assay was essentially set up as published [10] but modified for measurement of different acylation. In short, typical endpoint deacetylation reactions contain: 5 µL diluted Firefly Luciferase K529mod (Final dilution: 1:1500 Ac, 1:500 Bu, 1:125 Cr and Pr), 2 mM NAD^+, 2 µM KDAC in 50 µl KDAC buffer (25 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA). The reactions were run for 2 h at 30°C. Luciferase activity was then assayed by addition of an equal volume of a mixture containing 40 mM Tricine pH 7.8, 200 µM EDTA, 7.4 mM $MgSO_4$, 2 mM $NaHCO_3$, 34 mM DTT, 0.5 mM ATP and 0.5 mM luciferin [11]. Luminescence was quantified using a FluoStar Omega Microplate Reader (BMG Labtech).

Sirtuin reaction on histone extract and Western blot analysis

Isolation of histones: HEK293 $GnTI^-$ cells were grown in Freestyle medium supplemented with 2% FBS in an orbital shaker at 37°C with 8% CO_2 to a density of 2-3 × 10^6 cells/ml.

Then, cells were incubated for further 8 h at 37°C with 8% CO_2 before addition of 10 mM sodium butyrate to inhibit deacylation. Following the addition, the flask was shifted to 30°C with 8% CO_2 while shaking at 130 rpm. Cells were harvested 40 h after addition of butyrate, resuspended in lysis buffer (25 mM Tris pH 8, 300 mM NaCl, 10% glycerol, 1 mM TCEP, 1 mM EDTA, 5 µg/ml AEBSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin) and passed through a microfluidizer. Cell debris were collected by centrifugation at 15.000 g for 5 min at 4°C. The histones were extracted from the cell debris following the acidic extraction protocol of Sechter et al. [12]. After TCA precipitation the histones were suspended in water at a concentration of 1 mg/mL and used as substrate stock.

Histone deacylation assay: Each reaction mix contained 250 ng histone extract, Sirtuin (64 nM to 4 µM) and 2 mM NAD^+ in KDAC buffer (25 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA). The reaction mix was incubated for 2 h at 30°C and stopped by adding 10 µL 3x SDS-Sample Buffer and boiled at 90°C for 10 min. 5 µL were loaded on a 15% SDS-PAGE and histone modifications analysed by Western blot. The H4 K16ac modification was detected using a monoclonal antibody (Abcam, ab109463, 1:15000 in 3% skim milk/TBS) and lysine crotonylation was detected using a pan-specific anti-CrK antibody (PTM Biolabs, PTM501, 1:2000 in 5% % skim milk/TBST).

Demodification reactions of myoglobin K4mod

Modified myoglobin $K4_{mod}$ was expressed and purified according to published protocols [1]. Demodification reactions contained 50 µL of 40 mM Tris pH 8, 50 mM NaCl, 1 mM DTT, 0.1 mM $ZnCl_2$, 3 mM NAD^+, 30 µM myoglobin $K4_{mod}$ and purified CobB (4 µM

wildtype, 16 µM $CobB_{ac2}$, 48 µM $CobB_{ac3}$ and 36 µM $CobB_{ac6}$). The reactions ran for 16 h at 30°C and were stopped by chilling at 4°C. 12 µL of each reaction mix was injected on a HPLC (Agilent 1100, Agilent Technologies) and desalted by C4 column. The proteins were eluted with a gradient of 20-80% acetonitrile in water (0.1% TFA) and analyzed by ESI-MS (LCQ Advantage MAX, Thermo Finnigan). Protein mass was calculated by MagTran and myoglobin peaks were normalized on maximal peak intensity [13].

Dot blot

200 ng acylated myoglobin $K4_{mod}$ (Acetylated, Butyrylated or Crotonylated) was spotted on a nitrocellulose membrane. The membranes were incubated with blocked buffer for 2h (panKac-101: 5% BSA/TBS, panKcr-501: 5% % skim milk/TBST, panKbu-301: 5% BSA/TBST, PTM Biolabs), followed by overnight incubation with the antibodies diluted in blocking buffer (panKac-101 1:2000, panKcr-501 1:2000 and panKbu-301 1:2000).

Immune fluorescence

HeLa cells were maintained in 5% CO_2, 37°C in DMEM+GlutaMAX™-I medium (Gibco life technologies, by Thermo Fisher Scientific, 10569-010) supplemented with 10% heat-inactivated fetal calf serum. For microscopy, Hela cells were seeded in 6-well plates on glass coverslips to a 1.5 $x10^5$ cells/ml density 24 h prior to transient transfection of CobB-GFP constructs with Lipofectamine 2000 (Thermo Fisher Scientific). As indicated in the text, cells were either fixed 24 h p. transfection (or after additional treatment with 2 mM sodium crotonate pH 7.2 for 2 h) for 15 min with 4% paraformaldehyde. Staining with panKacetyl, panKcrotonyl and panKbutyryl antibodies in blocking buffer (3% BSA, 0.1%

TritonX-100 in PBS; antibody dilution 1:1000, panKac-101, panKcr-501, panKbu-301 PTM Biolabs) lasted 2 h at 37°C and the secondary antibody anti-rabbit-Cy3 conjugate (1:1000 Jackson Immuno Research,111-165-144) together with 1 µg/ml DAPI was done for 1 h at 37°C. Thereafter, the coverslips were dried, mounted and analyzed by LSM (Zeiss LSM800/510, 40x or 63x oil objective).

Crystallization of CobB mutants

The crystallization of CobB, $CobB_{ac2}$ and $CobB_{ac3}$ were set up as reported for CobB [8]. Crystals were obtained for CobB and $CobB_{ac2}$ after 1 day from hanging drop crystallization with 100 mM Bis-Tris, 30 mM HCl, 22% PEG 3350 ($CobB_{ac2}$ 23% Ac and Cr, 24% Bu) as precipitant and 10 mg/mL CobB (10.5 mg/mL $CobB_{ac2}$) preincubated with 1 mM H4 $K16_X$ (X: Ac, Bu, Cr) peptide. Crystals were flash frozen in liquid nitrogen with 20% PEG400 as cryoprotectant containing 1 mM H4 $K16_X$ (X: Ac, Bu, Cr).

Crystals from $CobB_{ac3}$ were obtained after 1 week from hanging drop crystallization with 100 mM Bis-Tris, 10 mM HCl, 22% PEG 3350 as precipitant and 7.2 mg/mL $CobB_{ac3}$ preincubated with 1 mM H4 $K16_{Ac}$ peptide. Crystals were flash frozen in liquid nitrogen with 20% glycerol as cryoprotectant containing 1 mM H4 $K16_{Ac}$.

The $CobB_{ac2}$-H4$K16_{Cr}$-NAD^+ complex was obtained after 16 h hanging drop crystallization with 100 mM Bis-Tris, 20 mM HCl, 22% PEG 3350 as precipitant and 11.1 mg/mL $CobB_{ac2}$ preincubated on ice with 1 mM NAD^+ and 1 mM H4 $K16_{Cr}$ peptide. Crystals were flash frozen in liquid nitrogen with 20% PEG400 as cryoprotectant containing 1 mM NAD^+ and 1 mM H4 $K16_{Cr}$.

For soaking, crystals of $CobB_{ac2}$-$H4K16_{Cr}$ were transferred using a nylon loop in a 1 µL hanging drop supplemented with 1 mM $H4K16_{Cr}$ peptide and 1 mM NAD^+ and incubated for 2 h or 36 h. Crystals were flash frozen in liquid nitrogen and 20% PEG400 as cryoprotectant containing 1 mM NAD^+ and 1 mM H4 $K16_{Cr.}$

Data collection was performed at beamline PXII X10SA at the Suisse Light Source, Villigen, Switzerland. The data was processed with XDS and scaled using XSCALE [14]. Molecular replacement was performed with Phaser [15] and the data was refined using Refmac5 [16] initially and Phenix.refine [17] in the final steps. The ligand geometry file was created using JLigand [18] for coordinates and PRODRG [19] for calculation of restraints. Model building was done with Coot [20].

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