Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information:

Miniaturized readout strategy for endogenous histone deacetylase activity

Jan Oliver Jost, Alfred Hanswillemenke and Dirk Schwarzer*

Interfaculty Institute of Biochemistry (IFIB), University of Tübingen; Hoppe-Seyler-Str.4,

72076 Tübingen, Germany

*Corresponding author: dirk.schwarzer@uni-tuebingen.de

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Experimental Section

General methods and reagents: Amino acid derivatives were purchased from GLS (Shanghai, China). Coupling reagents were bought from Merck Novabiochem (Darmstadt, Germany), Tentagel RAM resin from Rapp Polymere (Tuebingen, Germany). Other chemicals were purchased from Sigma Aldrich (Germany) unless stated otherwise. Analytical and preparative HPLC were performed on a Varian ProStar 210 HPLC system with a Nucleosil C18 column (5 µm, 4.6 x 250 mm, Machery-Nagel) or Dynamax C18 column (10 µm, 21.4 x 250 mm, Varian), with 0.1% TFA in water (A) and 80% ACN, 0.1% TFA in water (B), as eluents. All peptides were analyzed by MALDI-MS on a MALDI-TOF spectrometer (Reflex IV, Bruker Daltonics, Bremen, Germany) and by analytical RP-HPLC.

Synthesis of ($^{13}C_6$, $^{15}N_2$)-Fmoc-Lys(Boc)-OH: ($^{13}C_6$, $^{15}N_2$)-H-L-Lys-OH was purchased from Silantes (Munich, Germany). Installation of the protection groups was performed on the basis of an established synthesis scheme:[1] 572 mg (1 eq) of $^{13}C_6$, $^{15}N_2$-Lysine (M=190.65 g/mol) was dissolved in 15 ml $NaHCO_3$ (1 M). After adding 375 mg (0.5 eq) $CuSO_4*H_2O$ and 851 mg (1.3 eq) Boc_2O (solved in 3 ml acetone) the solution was stirred for 24 h at room temperature (RT). The copper complex precipitated from the solution. After 24 h 655 mg Boc_2O was added and the solution was stirred for one more day. Afterwards the copper complex was filtered off, yielding 680 mg (1.21 mmol, 76%) of crude material. Next, the complex was dissolved in 60 mL water and upon addition of 435 mg (3 eq) 8-hydroxyquinoline the copper was chelated and removed by extraction with ethyl acetate (50 mL, 3-times). The aqueous phase was lyophilized yielding 513 mg (2 mmol, 65%) of crude material. ($^{13}C_6$, $^{15}N_2$)-H-L-Lys(Boc)-OH (513 mg) were solved in 15 mL water. The pH was adjusted to 9.5 with 0.3 mL of triethylamine, followed by addition of 742 mg (1.1 eq) of N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) solved in 6 ml ACN. The mixture was stirred for 1 h at RT and then reduced to an oil in vacuum. Afterwards 40 ml of 20% citric acid (w/v) was added and the mixture was extracted 3-times with 50 mL ethyl acetate. The organic phases were combined, dried with $MgSO_4$ and the solvent was removed in vacuum. The residual was solved in 10 mL of 20% (v/v) ACN/H_2O and lyophilized. In total 703 mg of ($^{13}C_6$, $^{15}N_2$)-Fmoc-L-Lys(Boc)-OH (47% yield) were obtained as white powder and could be used for SPPS without further purification.

Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized on a Intavis ResPep XL Synthesizer using TentaGel R Ram (Rapp-Polymere, Tuebingen, Germany) as solid support. Coupling reactions were performed with HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate) as activator, NMM (N-metyhlmorpholine) as base in DMF/NMP as solvent for 45 min. Side chains of amino acids were protected as follows: Arg(Pbf), His(Trt), Lys(Boc), Lys(Mtt), Lys(Ac) and Ser(tBu). Each amino acid was coupled twice with 5-fold molar excess of Fmoc-protected amino acids. The Fmoc-group was removed with 20% piperidine in DMF.

Incorporation of ($^{13}C_6$, $^{15}N_2$)-Fmoc-Lys(Boc)-OH was performed manually by double coupling 2 eq of the building block for 1 h. We chose to incorporate the labeled lysine residues close to the N-termini of peptides in order to keep the number of subsequent reactions low. Isotopic labeled Lys(Ac) was installed on solid support in a two-step process. At first Fmoc-Lys(Mtt)-OH was installed at the respective acetylation sites. Afterwards the Mtt-group was selectively removed with 1% (v/v) TFA/DCM and the resin was treated with ($^{13}C_4$, $^{2}H_6$)-acetic anhydride in presence of 5 eq N,N-diisopropylethylamine .

Peptide cleavage and side-chain deprotection was performed by treating the resin with a cleavage cocktail containing TFA / phenol / triisopropylsilane / H_2O (85 : 5 : 5 : 5) for 4 h. Crude peptides were precipitated with cold diethyl ether, centrifuged and washed with diethyl ether, dissolved in H_2O and lyophilized. Finally, crude peptides were purified by HPLC on a preparative C18 column to approximately 95% purity (see Fig. S8 ESI). Peptide stock solutions were prepared on a gravimetric basis and the concentrations were adjusted by HPLC analysis.

Cloning, expression and purification of HDAC8: The cDNA encoding human HDAC8 was purchased from ImaGenes (Berlin, Germany). At first an internal NdeI restriction site was removed by introducing a silent mutation with site-directed mutagenesis (oligonucleotides: 5'-CTTTGATTGAAGCGTATGCA CTGCATAAG-3' and 5'-GTTTTTCACAGCGTATGGTCCTGATTATG-3'). Afterwards the HDAC8 gene was amplified by PCR (oligonucleotides: 5'-GATCCATATGGAGGAGCCGGAGGAA-3' and 5'-GATCCTCGAGGACC ACATGCTTC-3') and cloned in the pET23 expression vector using restriction sites NdeI and XhoI, resulting in the pET23-HDAC8 expression vector. After transforming *E. coli* BL21(DE3) with the pET23-HDAC8 expression vector the C-terminal His_6-tagged protein was expressed with the EnPresso system (BioSilta, Oulu, Finnland) based on the manufacturers manual. Briefly, 500 mL of EnPresso medium was supplied with 12.5 µL of a $ZnCl_2$ (1 mM) solution and 25 µL of “EnZ I’m” mix. The medium was inoculated 1:100 with a 6 h-pre-culture at 37 °C under gentle shaking (160 rpm), followed by incubation over night. Afterwards, the temperature was reduced to 25 °C, the medium was further supplied with a “booster tablet” and 50 µL “EnZ I’m” mix. Protein production was induced by adding 500 µM IPTG and the culture was incubated for 24 h at 250 rpm. Next, the cells were harvested by centrifugation (10 min at 3,000 x g). The pellet was re-suspended in PBS buffer (pH 8.0) completed with 0.5 mM Na_3VO_4 and lysed with a homogenizer (EmulsiFlex-C5, Avestin, Canada) at 4 °C. The lysate was cleared by centrifugation (20 min at 30,000 x g). Afterwards, the cleared lysate was incubated with 1 mL of Ni-NTA Agarose slurry (Qiagen) for 2 h at 4 °C. The resin was washed with 50 mL PBS, pH 8.0, 4 °C and the proteins were eluted with 300 mM imidazole in HDAC buffer (8 mM KCl, 100 mM NaCl, 10 mM HEPES, pH 8.0). Pure fractions were combined and dialyzed against HDAC buffer containing 10 mM EDTA and subsequently against HDAC buffer supplied with 0.5 mM EDTA. Finally, the proteins were concentrated by centrifugation in a 10 kD MWCO filter device (Amicon Ultra, Merck Millipore, Germany), supplied with 20% (v/v) glycerol and stored at -80 °C until usage.

Expression of PP1α: 700 ml LB medium supplied with 1 mM $MnCl_2$ was inoculated with 5 ml over-night-culture of *E. coli* BL21(DE3) transformed with the pET23-PP1α expression vector, incubated until an OD_{600} of 0.6. Expression was performed over night (20°C) by adding 0.5 mM IPTG. Cells were harvested by centrifugation, resuspended in PP1-buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl completed with protease inhibitor (Roche complete EDTA free)) and lysed with a homogenizer (EmusiFlex-C5, Avestin, Canada).

The lysate was cleared by centrifugation (20 min at 30,000 x g) and afterwards incubated with 1 mL of Ni-NTA Agarose slurry (Qiagen) for 1 h at 4 °C. The beads were washed with PP1-buffer containing 20 mM imidazol. Proteins were eluted with 300 mM imidazole in PP1-buffer in a total volume of 3 mL. All fractions were analyzed by SDS-PAGE. Fractions containing pure protein were combined and imidazole was removed by HiTrap-Desalting on an FPLC-system (Äkta Prime, GE Healtcare). Proteins were concentrated by centrifugation in a 10 kD MWCO filter device (Amicon Ultra, Merck Millipore, Germany), supplied with 20% (v/v) glycerol and stored at -80 °C until usage.

Preparation of HeLa extracts: 1-2 x 10^8 HeLa cells were harvested and suspended in 1.5 mL PBS. Nuclear extracts and cytosolic extracts were prepared according to the method described by Dignam et al.[2] 4-(2-aminoethyl)-benzensulfonylfluorid (AEBSF) was added to all buffers instead of phenylmethylsulfonylfluorid (PMSF). The nuclear and cytosolic fractions were combined to yield HeLa whole-cell extract used in this investigation.

Deacetylation assays: Apo-HDAC8 was supplied with metal ions prior to the reaction: 2 µL of the apo-enzyme (HDAC without metal ion) solution (140 µM) was incubated on ice with 4 µL of 1 mM $CoCl_2$ or $ZnCl_2$ for 30 min. Afterwards the holo-HDAC8 was diluted 50-fold into HDAC buffer.

Deacetylation reactions for MALDI-TOF readout were performed in a volume of 20 µL. At first the peptide substrates were solved in HDAC buffer at a concentration of 20 mM. Afterwards the stock solutions were diluted with HDAC buffer until the desired concentrations were reached (25 to 800 µM final concentration). The reaction was initiated by mixing 10 µL of diluted substrate with 10 µL of diluted holo-HDAC. The reaction mixture was incubated at 25 °C. At selected time points 2 µL of reaction mixture were removed and quenched with 8 µL of stopping solution (6.25 µM trichostatin A/0.1% TFA/H_2O). Afterwards 10 µL of quenched assay were mixed with 10 µL of calibration solution (4 µM isotopic coded reference peptide solved in 0.1% (v/v) TFA/H_2O) and diluted with another 20 µl of 0.1% TFA/H_2O for assays with p53K382ac, p53K373ac and p53K320ac or 80 µl of 0.1% TFA/H_2O for assays with H4K16acK20ac. In total 1 µL of either of these solutions was mixed with 1 µL of matrix solution (5 mg/mL CHCA in 0.1%TFA/ 50%ACN/ H_20) and spotted onto a polished steel MALDI target. MALDI-MS measurements were performed as described below.

The HPLC-readout was performed in the same way but on a 100 µL scale. At selected time points 20 µl of reaction mixture were removed and mixed with 80 µl of stopping solution. Afterwards 50 µL of quenched reaction mixture were analyzed on analytical RP-HPLC. Conversions were calculated from integrated signal intensities of deacetylated products and starting material.

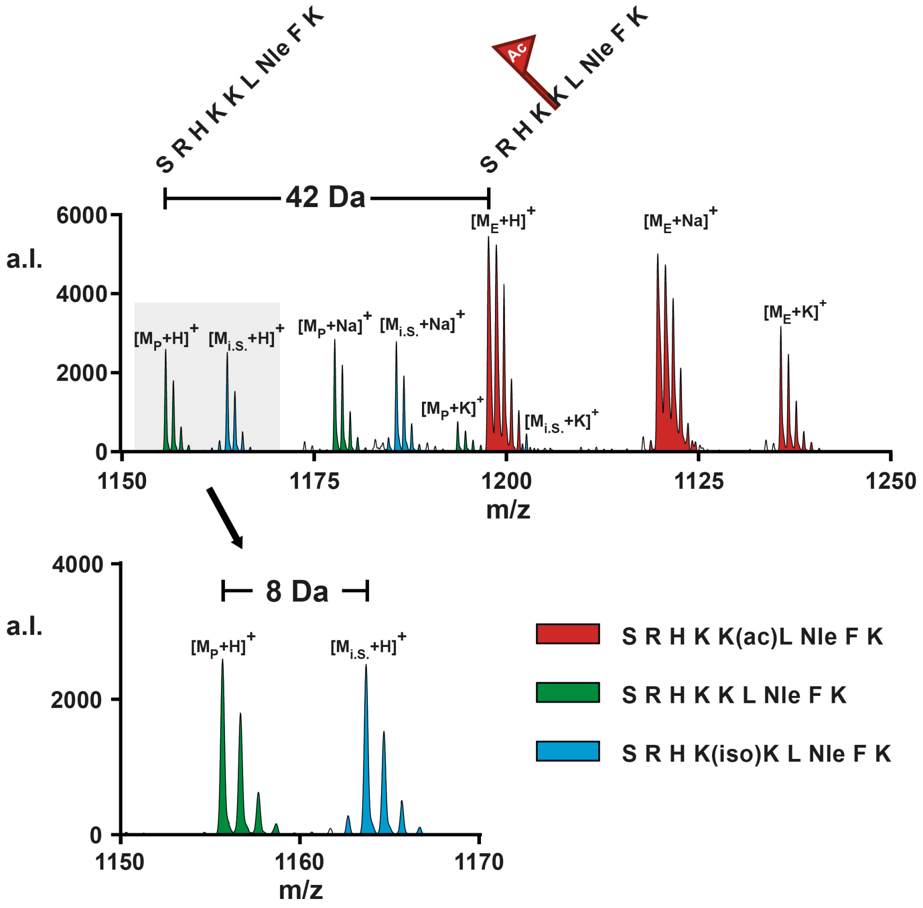
Dephosphorylation assay: The dephosphorylation assays were performed in a similar way as described above. The reactions were performed in a total volume of 40 µL in PP1 reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MnCl_2$) at RT. Reactions were started by mixing 20 µL of PP1α enzyme (200 nM) solved in PP1 reaction buffer and 20 µL of substrate solution resulting in final concentrations of H3S10ph ranging from 2 to 1000 µM. For inhibition studies the reactions were performed at a fixed concentration of H3S10ph (100 µM) and various concentrations of microcystin-LR (1 nM to 1000 nM). The reactions were quenched with 10 µl of stopping solution (5% TFA/ACN). Afterwards 10 µL of calibration solution was added containing H3S10(iso) in PP1 reaction buffer at concentrations ranging from 1 to 50 µM, depending on the amounts of formed product. These assays were analyzed on a prespotted AnchorChip (PAC) MALDI plate (Eppendorf, Germany). The prespotted matrix was equilibrated with 10 µL of 0.1% TFA/H_2O before 1 µL of the quenched assay containing the reference was added to the droplet on the target. The droplet was incubated for 5 min on the prespotted matrix and removed afterwards. The dried spots were analyzed by MALDI-TOF as described below.

MALDI-TOF measurements: Each spot was measured with at least 250 shots with Laser intensity between 35% and 42% on Bruker Reflex IV MALDI-TOF. Analyses of the spectra were performed using MMass 2 software. Spectra were smoothed with 3 runs of the Savitzkay-Golay filter (window size 0.3 m/z) and baseline corrected (precision 97%/ offset 25), before peaks were analyzed. The amount of formed product was determined from the signal intensity of the mono-isotopic $[M+H]^+$ product peak referenced to the mono-isotopic peak of the isotopic-coded reference.

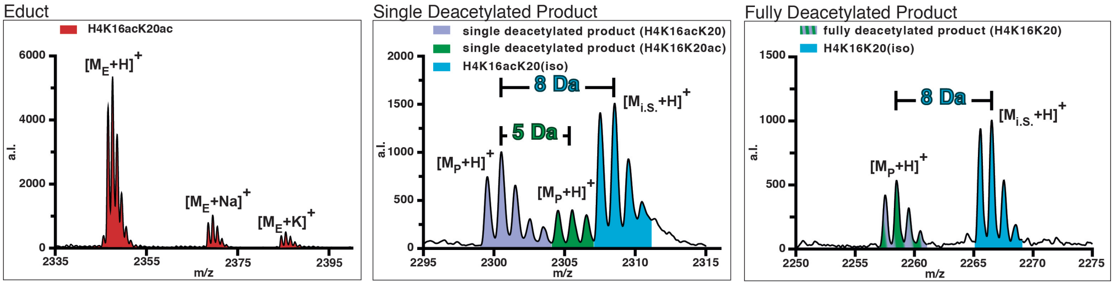
Determination of kinetic parameters: The apparent velocities (V) of product formation were determined from the concentrations of formed products at selected time points. Quantification was performed by referencing the signal intensities to the signal intensities of the isotope-coded references, which were added in defined quantities. Kinetic parameters were determined by plotting the deacetylation rate against substrate concentration. When the plot showed saturation the data set was fitted to the Michaelis-Menten equation (V=(Vmax*[S])/(Km+[S])). For comparing the substrates, the velocity was normalized to the apparent Michaels constant (V/K). When no saturation was observed the data set was fitted to V/[S]=Vmax/Km, assuming [S] << Km. In this case V/K was calculated from the linear slope.

Isolation and on-resin analysis of endogenous HDACs: At first 10 µL of either Protein A-/ or Protein G-agarose slurry was loaded into gel-loading tips. The resin was equilibrated with 200 µL of HDAC buffer. In the following 10 µL of either anti-HDAC1 mouse IgG1 (200 µg/ml, sc-81598, SantaCruz, USA), anti-HDAC2 rabbit IgG (1000 µg/ml, PA1-861, Thermo, Germany), anti-HDAC3 rabbit IgG (1000 µg/ml, PA1-862, Thermo, Germany), anti-HDAC6 rabbit IgG (200 µg/ml, sc-11420, Santa Cruz, USA) or anti-HADC8 rabbit IgG (200µg/ml, sc-11405, SantaCruz, USA) were loaded onto the resin by pipetting, followed by incubation for 2 h at RT. Afterwards the resins were washed with 400 µL of HDAC buffer and incubated with either 20 µL of HeLa whole-cell extract or 20 µL recombinant HDAC8 solution (1.4 µM) over night at 4°C. The resins were rinsed twice with 200 µL HDAC buffer containing BSA (10 µM) and $CoCl_2$ (10 µM). In the following 20 µL of a 200 µM H4K16acK20ac solution was loaded onto the resin and at selected intervals (10 to 90 min) samples of 2 µL were eluted, quenched and analyzed as described above.

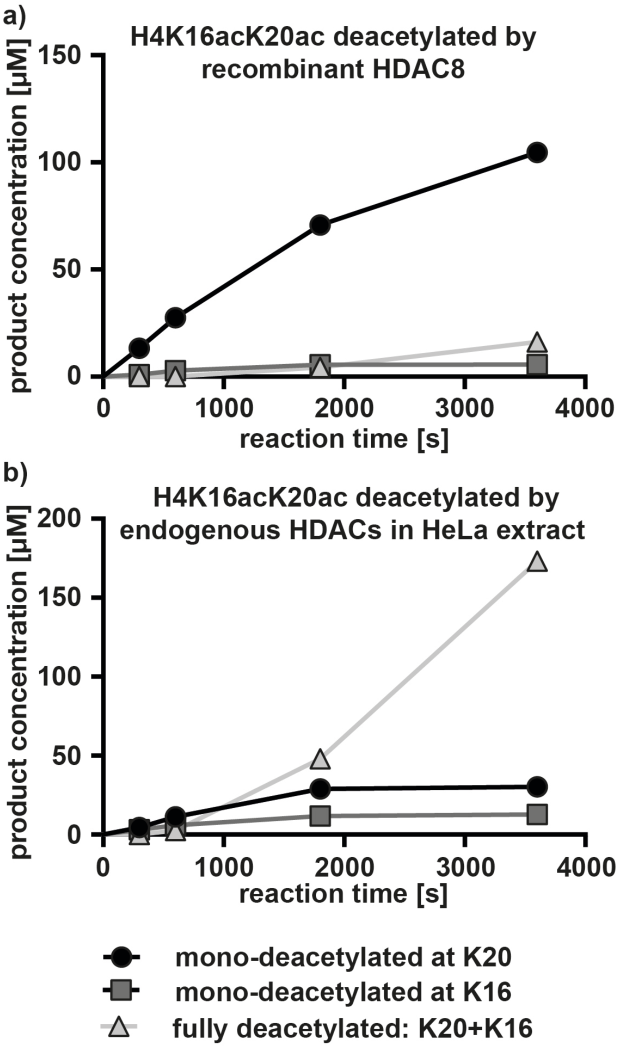
Supporting Figure



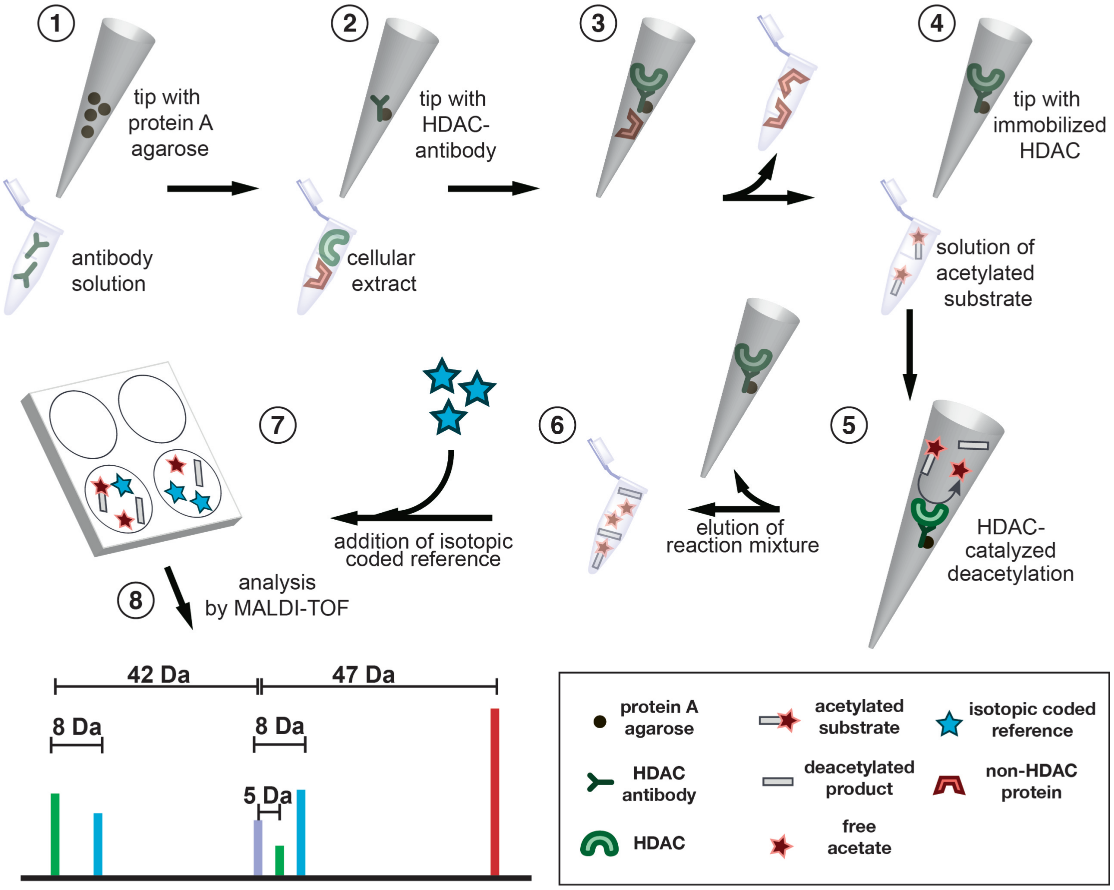
Supporting Figure S1: Representative MALDI-TOF spectrum of p53K382ac deacetylated by recombinant HDAC8. The signals of the protonated species, sodium and potassium adducts are detected. The signals of the deacetylated product are color-coded in green, signals of the isotopic coded reference (p53K382iso) are marked in blue and the acetylated educt (p53K382ac) is marked in red. The concentration of the formed product was determined from the signal intensity of the deacetylated product after normalizing the signal to that of the isotopic coded reference. Nle: norleucine; M_P: signal of product; $M_{i.S.}$: signal of isotopic coded reference; M_E: signal of educt.



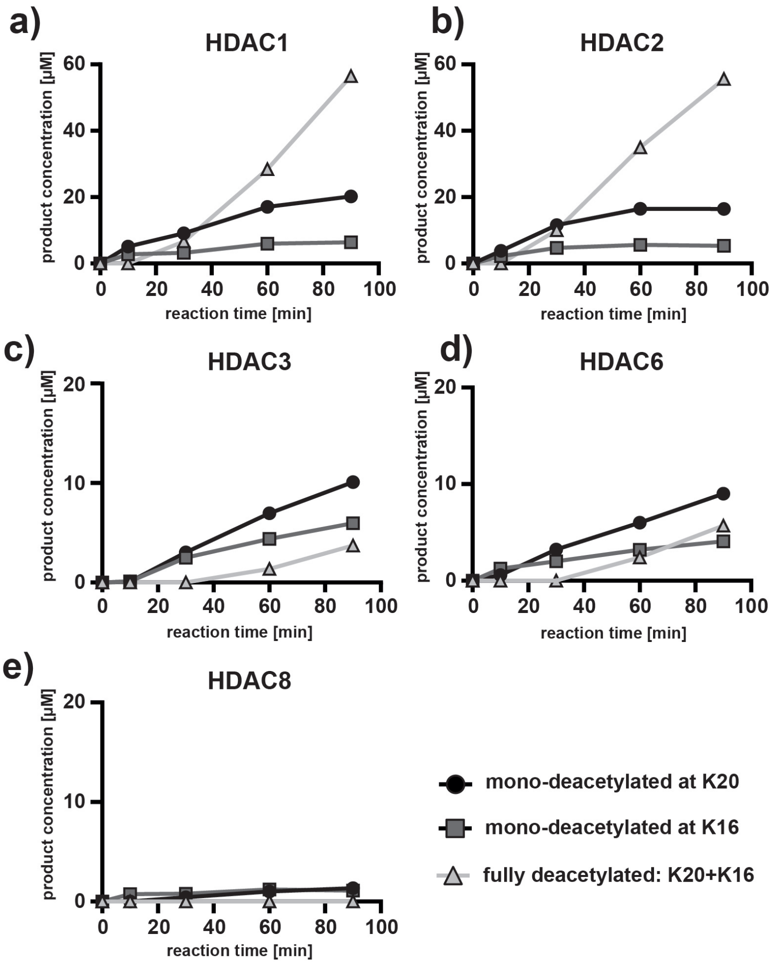
Supporting Figure S2: Representative MALDI-TOF spectrum of HDAC8-catalyzed deacetylation of H4K16acK20ac. The observed product pattern contains three different products: Two single deacetylated peptides (either K16 or K20) and the fully deacetylated product. All of these signals can be distinguished by the isotopic coding strategy. M_P: signal of product; $M_{i.S.}$: signal of isotopic coded reference; M_E: signal of educt.



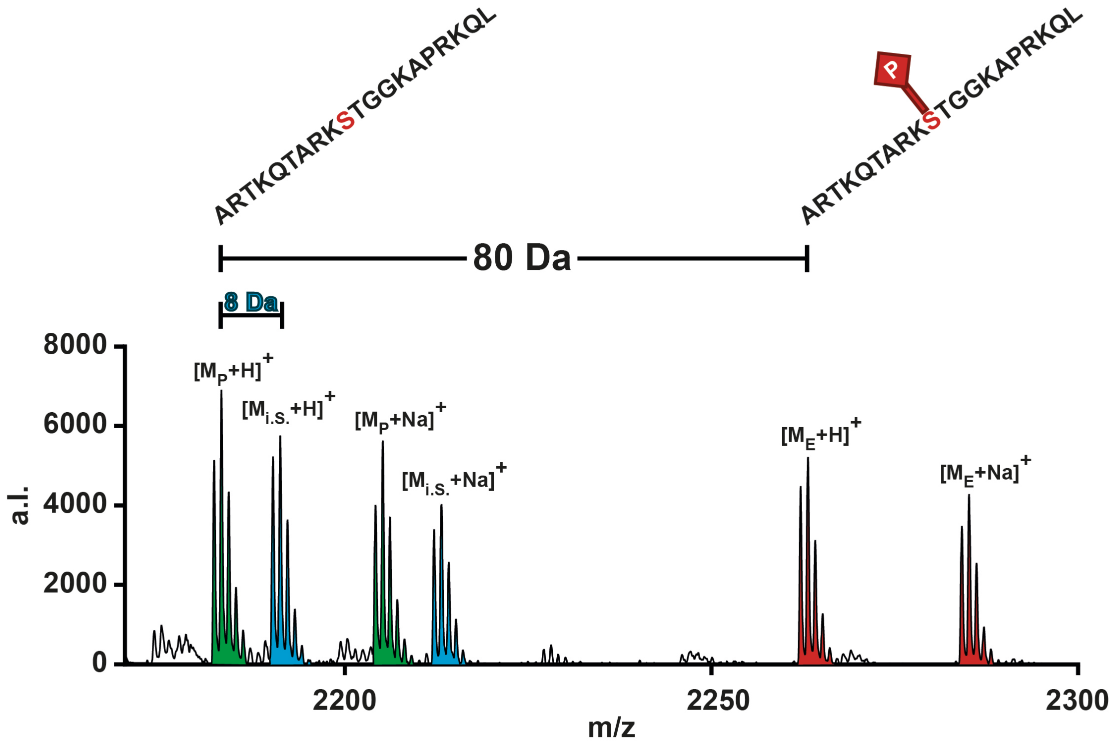
Supporting Figure S3: Progress curves of H4K16acK20ac deacetylation. (a) Deacetylation of H4K16acK20ac by recombinant HDAC8. (b) Deacetylation of H4K16acK20ac in HeLa extracts. All assays were performed at a fixed substrate concentration of 200 µM. Formation of the K20- and K16-mono-deacetylated products as well as the fully deacetylated product (K20+K16) is shown in a time-dependent manner. It should be noted that we did not detect masses indicating degradation of H4K16acK20ac or any of the deacetylated products.



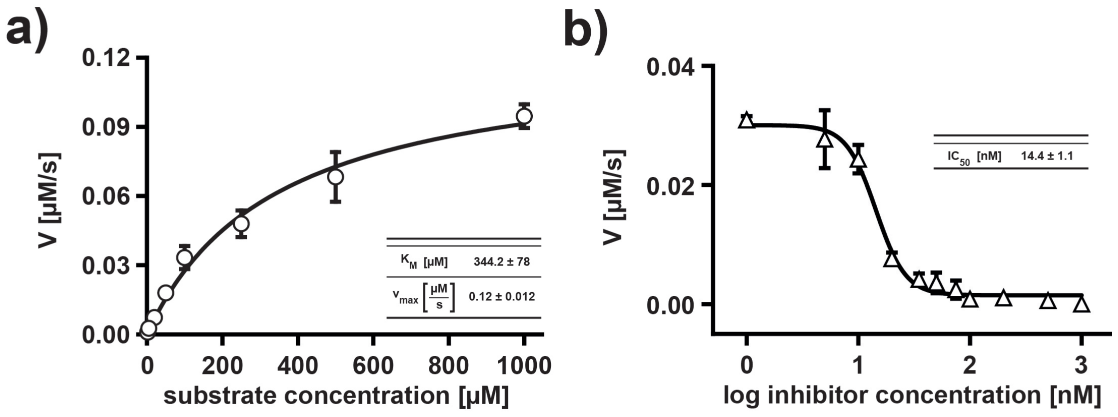
Supporting Figure S4: Overview of experimental procedures for immunoprecipiation and MALDI-TOF analysis of endogenous HDACs. (1) Protein A or protein G agarose is loaded into a barrier-tip followed by immobilzation of the respective HDAC antibody. (2) Pipetting of cellular lysates and elution of non-HDAC proteins (3) results in immobilzed HDACs (4). Loading acetylated substrate peptide (H4H16acK20ac) into the pipette-tip initiates the deacetylation reaction by resin-bound HDACs (5). After elution (6) and mixing with defined amounts of isotopic standard (7) the reaction mixture is spotted onto a MALDI plate, analyzed, and the amounts of formed deacetylated products were determined from the singal intensities (8).



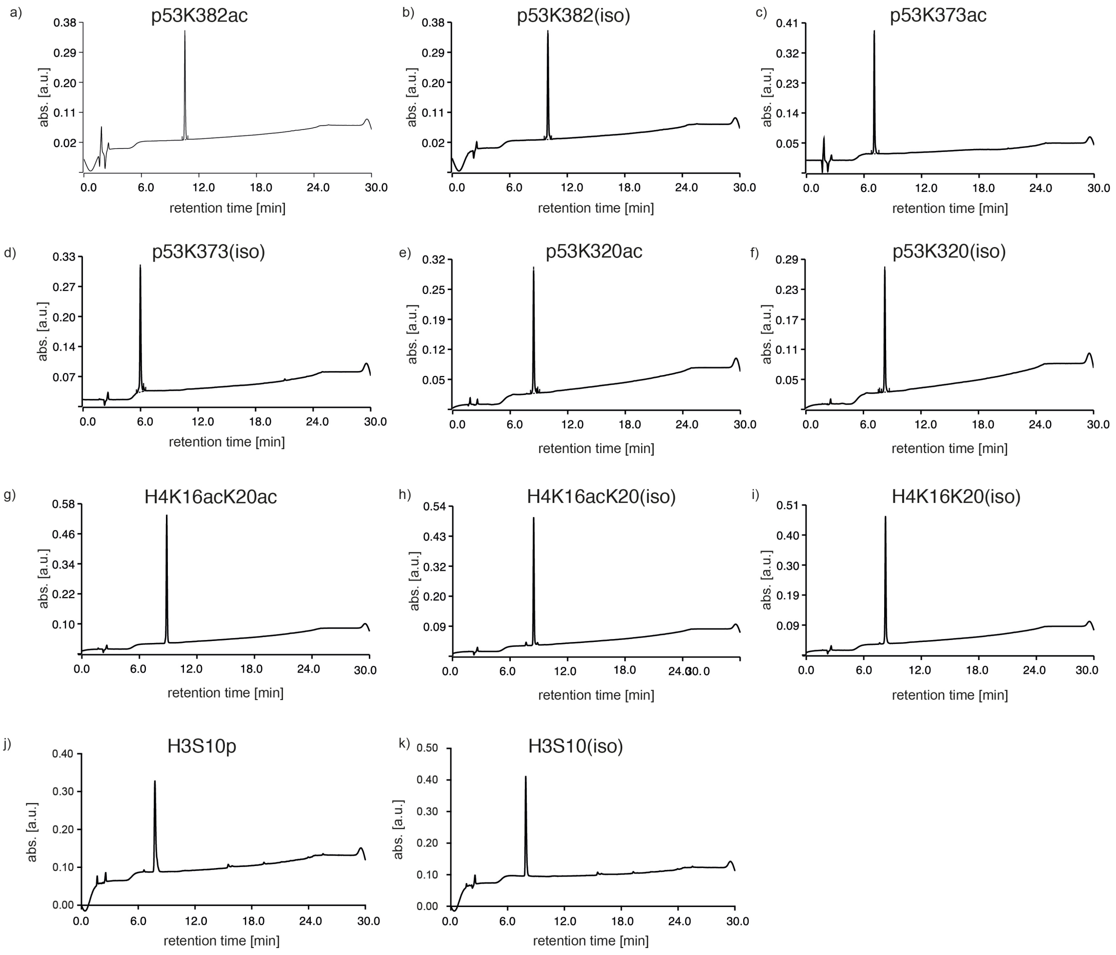
Supporting Figure S5: Progress curves of H4K16acK20ac deacetylation, catalyzed by selected endogenous HDACs immunoprecipitated from HeLa lysates. All assays were performed at a fixed substrate concentration of 200 µM. Formation of the K20- and K16-single deacetylated products as well as the fully deacetylated product (K20+K16) is shown in a time-dependent manner. We analyzed endogenous (a) HDAC1, (b) HDAC2, (c) HDAC3, (d) HDAC6 and (e) HDAC8. The activity observed with endogenous HDAC8 was very low compared to the experiments with the recombinant enzyme, indicating that HDAC8 is a low abundant enzyme in HeLa extracts.



Supporting Figure S6: Representative MALDI-TOF spectrum of PP1α-catalyzed dephosphorylation of H3S10ph. Signals of the protonated species and sodium adducts are detected. The signals of the dephosphorylated product are color-coded in green, signals of the isotopic coded reference (H3S10iso) are marked in blue and the phosphorylated educt (H3S10ph) are marked in red. M_P: signal of product; $M_{i.S.}$: signal of isotopic coded reference; M_E: signal of educt.



Supporting Figure S7: Kinetic investigation of PP1 α–catalyzed dephosphorylation of H3S10ph. (a) Plot of velocity (V) against product concentration. The data were fitted to the Michaelis-Menten equation. (b) Determination of PP1 α inhibition by microcystin-LR (MC). PP1 α–catalyzed dephosphorylation of H3S10ph was measured at a fixed substrate concentration of 100 µM in presence of various concentrations of MC. The IC_{50} value was determined by fitting the data to the following equations: $V = v_{min} + (v_{max}-v_{min})/(1+10^{(\log(IC50)-c(MC))*b})$; with c(MC): concentration of microcystin-LR, v_{min}: velocity at bottom plateau, v_{max}: velocity at top plateau, b: Hill slope.



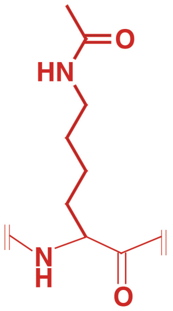
Supporting Figure S8: Analytical HPLC chromatogramms of all synthesized peptides. (a) p53K382ac, (b) p53K382(iso), (c) p53K373ac, (d) p53K373(iso), (e)p53K320ac, (f) p53K320(iso), (g) H4K16acK20ac, (h) H4K16acK20(iso), (i) H4K16K20(iso), (j) H3S10ph, and (k) H3S10(iso).

Supporting Tables

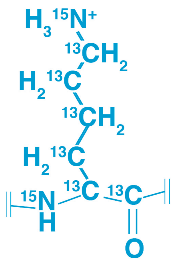
Supporting Table S1: List of all synthesized peptides. Red **K(ac)** and **S(ph)**: modificatified lysine (acetylation) and serine (phosphorylation). Blue **K**: isotopic labled lysine residue. Red/Blue **K(ac)**: acetylated lysine with isotopic labled acetyl-group, the lysine residue itself is not labled. Nle: Norleucine

Peptides	Sequence	Modifications
p53K382ac	S R H K K(ac) L Nle F K	$N\varepsilon$-Ac-Lys(382)
p53K382(iso)	S R H K K L Nle F K	$^{13}C_6$, $^{15}N_2$-Lys(381)
p53K373ac	H L K S K K(ac) G Q S T	$N\varepsilon$-Ac-Lys(373)
p53K373(iso)	H L K S K K G Q S T	$^{13}C_6$, $^{15}N_2$-Lys(372)
p53K320ac	S P Q P K K(ac) K P L D G	$N\varepsilon$-Ac-Lys(320)
p53K320(iso)	H L K S K K G Q S T	$^{13}C_6$, $^{15}N_2$-Lys(320)
H4K16acK20ac	R G K G G K G L G K G G A K(ac) R H R K(ac) V L R	$N\alpha$-Ac-Arg(3); $N\varepsilon$-Ac-Lys(16); $N\varepsilon$-($^{13}C_2$, $^{2}H_3$)Ac-Lys(20)
H4K16acK20(iso)	R G K G G K G L G K G G A K(ac) R H R K V L R	$N\alpha$-Ac-Arg(3); $^{13}C_6$, $^{15}N_2$-Lys(5); $N\varepsilon$-Ac-Lys(16)
H4K16K20(iso)	R G K G G K G L G K G G A K R H R K V L R	$N\alpha$-Ac-Arg(3); $^{13}C_6$, $^{15}N_2$-Lys(5)
H3S10ph	A R T K Q T A R K S(ph) T G G K A P R K Q L	O-Phospho-Ser(10)
H3S10(iso)	A R T K Q T A R K S T G G K A P R K Q L	$^{13}C_6$, $^{15}N_2$-Lys(4)

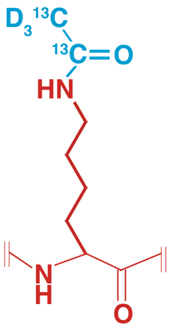
K(ac):



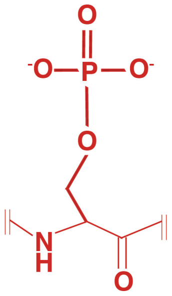
K:



K(ac):



S(ph):



Supporting References:

(1) Dose, A.; Liokatis, S.; Theillet, F.X.; Selenko, P.; and Schwarzer, D. (2011) *ACS Chem. Biol*. **6**, 419-424.

(2) Dignam, J. D.; Martin, P. L.; Shastry, B. S.; and Roeder, R. G. (1983) *Method Enzymol.* ***101***, 582-598.