Compromised Structure and Function of HDAC8 Mutants Identified in Cornelia de Lange Syndrome Spectrum Disorders

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—— SUPPORTING INFORMATION ——
METHODS

Reagents. Most chemicals used for buffers or crystallization were purchased from Fisher or Sigma. The HDAC inhibitor 4-(dimethylamino)-N-[7-(hydroxyamino)-7-oxoheptyl]benzamide (M344) was obtained from Sigma, and the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) were obtained from Cayman Chemical. Inhibitors were used without any further purification.

Expression and Purification of HDAC8 Mutants. Mutations in human HDAC8 were introduced into a previously described HDAC8-6His-pET20b construct using QuikChange site-directed mutagenesis (Agilent Genomics). Forward and reverse primers for PCR mutagenesis are listed in Table S1. DNA sequencing at the University of Pennsylvania Perelman School of Medicine confirmed the incorporation of the desired mutations. Recombinant HDAC8 mutants C153F, A188T, I243N, T311M, H334R, A188T/Y306F, I243N/Y306F, and H334R/Y306F were expressed in BL21(DE3) Escherichia coli cells and purified according to a previously published procedure, with minor modifications. Briefly, 50 mL cultures (LB media supplemented with 100 µg/L ampicillin) were grown overnight and used to inoculate 1 L flasks (minimal media supplemented with 100 µg/L ampicillin). Typically, 6 liters were expressed for each mutant, except for T311M (12 liters). Cells were grown at 37°C until OD600 ~ 0.5, at which point the temperature was lowered to 18°C. After 30 minutes, cells were induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.4 mM final concentration) and zinc chloride (100 µM final concentration), and grown overnight at 18°C. The cells were pelleted by centrifugation and kept at -80°C until purification. After thawing, cells were resuspended in ~ 50 mL of lysis buffer (50 mM Tris (pH 8.0), 500 mM KCl, 5% glycerol, 3 mM β-mercaptoethanol (BME), and 115 µM phenylmethanesulfonyl fluoride), and lysed by sonication on ice. Cell debris was pelleted by centrifugation. The supernatant was loaded onto an affinity column (Talon resin, Clontech Labs) preequilibrated with 50 mM Tris (pH 8.0), 500 mM KCl, 5% glycerol, and 3 mM BME. The
column was washed with the same buffer supplemented with 10 mM imidazole, and the protein was eluted with 200 mM imidazole. Fractions containing the protein were pooled, concentrated to below 10 mL, and further purified by size exclusion chromatography in 50 mM Tris (pH 8.0), 150 mM KCl, 5% glycerol, and 1 mM dithiothreitol (DTT) using a HiLoad 26/60 Superdex column (GE Healthcare Life Sciences) to yield pure protein (over 95% as indicated by SDS-PAGE analysis). The protein was concentrated to 7–12 mg/mL. Protein concentrations were determined from the absorbance at 280 nm using the calculated extinction coefficient $\varepsilon = 50,240$ M$^{-1}$ cm$^{-1}$ for all the single mutants except C153F ($\varepsilon = 50,180$ M$^{-1}$ cm$^{-1}$), and $\varepsilon = 48,960$ M$^{-1}$ cm$^{-1}$ for all the double mutants.$^2$

**Enzyme Activity Assays.** The Fluor-de-Lys tetrapeptide assay substrate Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin (BML-KI178-0005, Enzo Life Sciences) was used to measure the catalytic activities of HDAC8 mutants.$^3$ Deacetylation of the substrate by HDAC8 is followed by the cleavage of the amide bond linking the C-terminal 7-amino-4-methylcoumarin (AMC) to the peptide backbone by a protease developer, resulting in a fluorescence shift. Activity assays were run at 25°C in assay buffer (25 mM Tris (pH 8.2), 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl) and contained 150 µM tetrapeptide substrate with the following enzyme concentrations: 0.5 µM (wild-type), 0.75 µM (H334R), 1.5 µM (A188T, I243N) or 3 µM (T311M, C153F), in a final volume of 50 µL. After 30 min, reactions were quenched by the addition of the same volume of a developing solution containing 200 µM M344 (a known inhibitor of HDAC8) and the commercial Developer II (BML-KI176-1250, Enzo Life Sciences) in assay buffer. After 45 min, reaction solution samples (100 µL) were transferred to a 96-well plate and the fluorescence of product AMC was measured using a Fluoroskan plate reader (excitation = 355 nm, emission = 460 nm). All assays were run in triplicate.

Heat inactivation studies were performed using a 10 µM enzyme stock solutions at 37°C for 15, 30, or 60 min after which the enzymes were diluted to the desired concentration for
activity assay. Activity assays after heat inactivation were performed at 25°C under the same conditions as described above. All assays were run in triplicate.

The HDAC8 activator\textsuperscript{4} TM-2-51 was the generous gift of Prof. D.K. Srivastava, North Dakota State University. Activity assays with TM-2-51 were performed under slightly different conditions. Briefly, the assay buffer contained 150 µM tetrapeptide substrate, 0, 1, 10 or 100 µM TM-2-51, 2% DMSO, and the following enzyme concentrations: 0.1 µM (wild-type), 0.15 µM (H334R), 0.4 µM (A188T, I243N), 1.5 µM (T311M) or 3.0 µM (C153F). Reaction time, developer, developing time, and fluorescence reading were the same as described above. All assays were run in triplicate.

\textbf{Thermostability Assay.} The thermostabilities of HDAC8 mutants were assessed using a thermal shift assay.\textsuperscript{5} Assay mixtures contained 5 µM HDAC8 mutant, 0 or 50 µM M344, 25 mM HEPES (pH = 7.5), 150 mM KCl, 500 µM TCEP, and SYPRO orange dye (S6650, Life Technologies) at a 5X final concentration. HDAC8 enzymes were incubated with or without M344 for 45 min at 4°C before the addition of SYPRO orange. Since the M344 stock solution was prepared in DMSO, the corresponding amount of DMSO was added to each enzyme for assays in the absence of inhibitor. 20 µL of each mixture was transfered in a 96-well plate (MicroAmp fast 96-well reaction plate, Applied Biosystems). The plate was sealed (MicroAmp adhesive film, Applied Biosystems) and incubated in a real-time polymerase chain reaction instrument (StepOnePlus, Applied Biosystems) for 1 min at 20°C followed by a 1°C increase per minute up to 90°C. During the thermal scan, fluorescence was monitored using a predefined filter (ROX). Protein unfolding induces an increase in SYPRO orange fluorescence, which was used to monitor thermal denaturation. Melting temperatures ($T_m$) were designated as the inflection point by fitting the initial portion of the curve (up to its maximum) with a Boltzmann equation.\textsuperscript{5} All assays were run in triplicate.
Crystallization and Structure Determination of HDAC8 Mutants. Crystals of HDAC8 mutant-inhibitor complexes were prepared by cocrystallization at 21°C (except C153F, at 4°C) in sitting drops using the vapor diffusion method. In general, a 500 nL drop containing 5 mg/mL HDAC8 mutant, 50 mM Tris (pH 8.0), 150 mM KCl, 5% glycerol, 1 mM DTT, 2 mM inhibitor and 0.03 M glycyglyclyglycine was added to a 500 nL drop of precipitant solution and equilibrated against a 100 µL reservoir of precipitant solution. C153F HDAC8 was cocrystallized with inhibitor SAHA using a precipitant solution of 100 mM Tris (pH 8.0), 17% PEG 8,000 (Hampton Research), and 4 mM TCEP. A188T HDAC8 was cocrystallized with inhibitor M344 using a precipitant solution of 100 mM BisTris (pH 6.5), 20% PEG 8,000, and 4 mM TCEP. I243N HDAC8 was cocrystallized with inhibitor SAHA using a precipitant solution of 100 mM BisTris (pH 6.5), 18% PEG 8,000, and 4 mM TCEP. T311M HDAC8 was cocrystallized with inhibitor TSA using a precipitant solution of 100 mM bicine (pH 8.5) and 15% PEG 10,000 (Hampton Research). H334R HDAC8 was cocrystallized with inhibitor M344 using a precipitant solution of 100 mM imidazole (pH 7.0), 20% PEG 8,000, and 4 mM TCEP.

Double mutants complexed with the tetrapeptide assay substrate Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin (Enzo Life Sciences) were cocrystallized in similar fashion. Briefly, a 500 nL drop containing 4.5 mg/mL HDAC8 mutant, 2.5 mM substrate, 0.03 M glycyglyclyglycine, 50 mM Tris (pH 8.0), 76.4 mM KCl, 68.5 mM NaCl, 2.5% glycerol, 0.5 mM MgCl₂, and 0.5 mM DTT was added to a 500 nL drop of precipitant solution and equilibrated against a 100 µL reservoir of precipitant solution. A188T/Y306F HDAC8 was cocrystallized at 21°C with substrate using a precipitant solution of 100 mM Tris (pH 8.0), 8% PEG 35,000 (Hampton Research), and 4 mM TCEP. I243N/Y306F HDAC8 was cocrystallized at 4°C with substrate using a precipitant solution of 100 mM Tris (pH 8.0), 15% PEG 10,000 (Hampton Research), and 4 mM TCEP. H334R/Y306F HDAC8 was cocrystallized at 21°C with substrate
using a precipitant solution of 100 mM Tris (pH 8.0), 17.5% PEG 400 (Hampton Research), and 4 mM TCEP.

Typically, crystals appeared within 1–2 days for each mutant. Crystals were flash-cooled in liquid nitrogen after transfer to a cryoprotectant solution consisting of precipitant solution supplemented with 20–30% glycerol. X-ray diffraction data were collected on beamline X29 at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory, New York). Data collection statistics are presented in Table S2. Data were indexed, integrated and scaled using HKL2000.6

Structures were solved by molecular replacement using PHENIX7 with the atomic coordinates of the H143A HDAC8–tetrapeptide substrate complex (PDB accession code 3EWF) less substrate, ion, and solvent used as a search probe for rotation and translation function calculations. Refinement and manual model adjustment were performed with PHENIX and COOT respectively.7,8 Refinement of the molecular replacement solution obtained for the A188T variant failed: R factors would not decrease below 0.35 even after model rebuilding and several refinement cycles. Non-crystallographic pseudotranslation (c/2 along the c axis) was diagnosed based on a Patterson peak at (0, 0, 0.5) that was 63% the height of the origin peak. Origin correction was performed using the space group validation program Zanuda (online server, York Structural Biology Laboratory, http://www.ysbl.york.ac.uk/YSBLPrograms/).9 The corrected solution was used to successfully complete refinement.

Certain segments in each structure (the N-terminus, the C-terminus, and/or a portion of the L2 loop) appeared to be disordered and were accordingly excluded from each final model as follows: C153F HDAC8, M1-S13 (monomers A and B), H385-H389 (monomer A), I378-H389 (monomer B); A188T HDAC8, M1-S13 (monomers A, B, C, and D), G86-I94 (monomer A), G86-E95 (monomer C), V377-H389 (monomers A and C), H384-H389 (monomers B and D); I243N HDAC8, M1-S13 (monomers A and B), D87-E95 (monomer A), E379-H389 (monomers A and
B); T311M HDAC8, M1-S13 (monomers A and B), D88-D89 (monomer B), E379-H389 (monomer A), I378-H389 (monomer B); H334R HDAC8, M1-Q12, E379-H389; A188T/Y306F, M1-S13 (monomers A and B), I378-H389 (monomer A), E380-H389 (monomer B); I243N/Y306F, M1-S13 (monomer A), M1-Q12 (monomer B), I378-H389 (monomers A and B); and H334R/Y306F HDAC8, M1-Q12, I378-H389. Likewise, side chains of residues that were completely disordered were excluded from the model:

- C153F HDAC8, monomer A: D73; monomer B: D88, K370
- A188T HDAC8, monomer A: L14; monomer B: L14; monomer C: L14, K60; monomer D: Q84, E85
- I243N HDAC8, monomer A: K58, E85, E106, Q363; monomer B: K36, K81, Q84, D87, D89, E238, Q253
- T311M HDAC8, monomer A: K33, K36, K52, K60, K81, E85, D101, E358; monomer B: K33, K60, Q84, D92, D101, K132, E238, K249, Q363, K374
- H334R: D88, D89, E170, E238, E358, K370
- I243N/Y306F HDAC8, monomer A: E106, K221; monomer B: L31, E85, K221
- H334R/Y306F HDAC8: K60, D89, E170, Q253, K258, K325, K370, K374

Parts of some ligands were disordered as judged by a lack of electron density; the occupancies of corresponding atoms were set to zero:

- A188T/Y306F HDAC8, tetrapeptide-coumarin substrate: Ac-Arg (monomer A and B) and the acetyl group of the acetyl-lysine that does not bind to the Zn$^{2+}$ ion (monomer A)

Complete refinement statistics are recorded in Table S2.
**Table S1.** Primers used to generate HDAC8 mutants

| Mutation | 5’-3’ sequence |
|----------|----------------|
| C153F    | forward: GCT TCT GGT TTC ttt TAC CTG AAC GAT GCC G<br>reverse: CGG CAT CGT TCA GGT Aaa aGA AAC CAG AAG C |
| A188T    | forward: GAT GGT GTT GAA GAC acg TTC AGT TTC ACC TCC<br>reverse: GGA GGT GAA ACT GAA cgt GTC TTC AAC ACC ATC |
| I243N    | forward: GAT GAA AAA TAT TAC CAG aac TGT GAA AGT GTA CTA<br>reverse: TAG TAC ACT TTC ACA gtt CTG GTA ATA TTT TTC ATC |
| T311M    | forward: GGT TAC AAC CTG GCG AAC atg GCT CGC TGC TGG ACC TAC<br>reverse: GTA GGT CCA GCA GCG AGC cat GTT CGC CAG GTT GTA ACC |
| H334R    | forward: GCT CTG AGA TCC CAG ATc gtG AGT TTT TCA CAG C<br>reverse: GCT GTG AAA AAC TCa cgA TCT GGG ATC TCA GAG C |
| Y306F    | forward: GGT GGC GGT GGT ttt AAC CTG GCG AAC ACT G<br>reverse: CAG TGT TCG CCA GGT Taa aAC CAC CGC CAC C |
### Table S2. Data collection and refinement statistics

| HDAC8 mutant-ligand complex | C153F-SHA | A188T-M344 | I243N-SHA | T311M-TSA | H334R-M344 | A188T/Y306F-substrate | I243N/Y306F-substrate | H334R/Y306F-substrate |
|-----------------------------|-----------|------------|-----------|-----------|-------------|------------------------|------------------------|------------------------|
| **Unit cell**               |           |            |           |           |             |                        |                        |                        |
| space group symmetry        | P2₁       | P2₁       | P2₁       | P2₁       | P2₁;2₁     | P2₁;2;2₁             | P2₁;2;2₁              | P2₁;2₁                 |
| a, b, c (Å)                 | 53.5, 84.8, 95.0 | 98.4, 82.6, 105.6 | 53.0, 84.1, 94.4 | 51.2, 83.1, 94.3 | 80.7, 80.7, 107.4 | 82.8, 97.8, 105.5 | 51.8, 85.1, 94.4 | 80.4, 80.4, 106.1 |
| α, β, γ (deg)               | 90, 99.3, 90 | 90, 102.2, 90 | 90, 98.6, 90 | 90, 95.9, 90 | 90, 90, 120 | 90, 90, 90 | 90, 97.5, 90 | 90, 90, 120 |
| **Data Collection**         |           |            |           |           |             |                        |                        |                        |
| wavelength (Å)              | 1.075     | 1.075     | 1.075     | 1.075     | 1.075       | 1.075                 | 1.075                 | 1.075                 |
| resolution limits (Å)       | 50.0 - 2.24 | 50.0 - 1.92 | 50.0 - 2.37 | 50.0 - 2.87 | 50.0 - 1.98 | 50.0 - 1.76 | 50.0 - 2.05 | 50.0 - 2.31 |
| total/unique reflections    | 301764/40281 | 384791/124361 | 148877/33016 | 79148/17775 | 368336/28649 | 574658/85478 | 373088/49982 | 214558/17922 |
| R<sub>merge</sub><sup>a</sup> | 0.130 (0.711) | 0.088 (0.609) | 0.108 (0.308) | 0.120 (0.484) | 0.079<sup>b</sup> | 0.091 (0.725) | 0.115 (0.807) | 0.082<sup>c</sup> |
| I/σ(I)<sup>d</sup>         | 13.7 (3.0) | 10.7 (2.0) | 12.2 (4.9) | 11.4 (3.0) | 27.3 (2.0) | 19.5 (3.0) | 16.7 (2.4) | 26.9 (2.0) |
| redundancy                  | 7.5 (7.2) | 3.1 (3.0) | 4.5 (4.5) | 4.5 (4.2) | 12.9 (11.0) | 6.7 (6.6) | 7.5 (7.0) | 12.0 (11.5) |
| completeness (%)<sup>e</sup> | 100.0 (100.0) | 98.0 (96.7) | 99.8 (99.4) | 98.6 (93.2) | 99.9 (99.8) | 100.0 (100.0) | 98.3 (95.4) | 100.0 (100.0) |
| **Refinement**              |           |            |           |           |             |                        |                        |                        |
| reflections used in refinement/test set | 40252/2015 | 124299/6219 | 32999/2369 | 17755/1800 | 28629/2062 | 85386/4274 | 49953/2532 | 17891/1814 |
| R<sub>cryst</sub><sup>f</sup> | 0.178 | 0.175 | 0.170 | 0.178 | 0.177 | 0.167 | 0.181 | 0.204 |
| R<sub>free</sub><sup>f</sup> | 0.210 | 0.215 | 0.217 | 0.234 | 0.205 | 0.194 | 0.212 | 0.242 |
| protein atoms<sup>g</sup>   | 5760 | 11490 | 56210 | 5639 | 2883 | 5872 | 5733 | 2624 |
| water molecules<sup>h</sup> | 438 | 1221 | 180 | 33 | 137 | 626 | 385 | 49 |
| ligand molecules<sup>i</sup> | 2 | 4 | 2 | 2 | 1 | 2 | 2 | 1 |
| Zn<sup>2+</sup> ions<sup>j</sup> | 2 | 4 | 2 | 2 | 1 | 2 | 2 | 1 |
| K<sup>+</sup> ions<sup>j</sup> | 4 | 8 | 4 | 4 | 2 | 4 | 4 | 2 |
Mg\(^{2+}\) ions \(F\) glycerol molecules

|        | 1 | 2 | 3 | 1 | 2 | 1 |
|--------|---|---|---|---|---|---|
| R.m.s. Deviations |
| bonds (Å) | 0.003 | 0.005 | 0.002 | 0.002 | 0.003 | 0.006 | 0.002 | 0.002 |
| angles (*) | 0.7 | 0.9 | 0.7 | 0.6 | 0.8 | 1.1 | 0.7 | 0.6 |
| dihedral angles (*) | 12 | 13 | 12 | 13 | 14 | 13 | 11 | 11 |

Ramachandran Plot (%)

|        | 1 | 2 | 3 | 1 | 2 | 1 |
|--------|---|---|---|---|---|---|
| allowed | 89.7 | 91.0 | 91.0 | 90.7 | 91.1 | 90.5 | 90.3 | 90.1 |
| additionally allowed | 10.3 | 9.0 | 9.0 | 9.3 | 8.9 | 9.5 | 9.7 | 9.9 |

PDB accession code

|        | 4QA0 | 4QA1 | 4QA2 | 4QA3 | 4QA4 | 4QA5 | 4QA6 | 4QA7 |
|--------|------|------|------|------|------|------|------|------|

\(^a\) Values in parentheses refer to the highest shell of data.  
\(^b\) \(R_{\text{merge}} = \sum |l_h - \langle l \rangle_h|/\sum l_h\), where \(\langle l \rangle_h\) is the average intensity calculated from replicate reflections.  
\(^c\) \(R_{\text{merge}}\) values higher than 1.000 (for the highest shell resolution) are reported as 0.000 by HKL2000. Given the high redundancy for the outer shell of these data sets, \(R_{\text{pim}}\) is a more appropriate measure of the data quality than \(R_{\text{merge}}\). \(R_{\text{pim}} = 0.024 (0.369)\) and 0.029 (0.482) for H334R and H334R/Y306F data sets, respectively.  
\(^d\) \(R_{\text{cryst}} = \sum |F_o| - |F_c|/\sum |F_o|\) for reflections contained in the working set. \(|F_o|\) and \(|F_c|\) are the observed and calculated structure factor amplitudes, respectively.  
\(^e\) \(R_{\text{free}} = \sum |F_o| - |F_c|/\sum |F_o|\) for reflections contained in the test set aside during refinement.  
\(^f\) Per asymmetric unit.  
\(^g\) Calculated with PROCHECK version 3.4.4.
Table S3. Activation data shown in Figure 7

| Activity (nmol product•µmol enzyme⁻¹•min⁻¹)ᵃ | TM-2-51 concentration (µM) | Wild type | C153F | A188T | I243N | T311M | H334R |
|---------------------------------------------|---------------------------|----------|-------|-------|-------|-------|-------|
| 0                                           |                           | 1200 ± 60| 32 ± 2| 460 ± 20| 510 ± 10| 83 ± 6| 1100 ± 90|
| 1                                           |                           | 1630 ± 80| 39 ± 3| 570 ± 50| 620 ± 30| 94 ± 4| 1450 ± 90|
| 10                                          |                           | 2330 ± 70| 42 ± 3| 740 ± 50| 900 ± 50| 130 ± 10| 2330 ± 90|
| 100                                         |                           | 2800 ± 100| 45 ± 4| 1120 ± 50| 1420 ± 70| 210 ± 10| 2840 ± 80|

ᵃ All measurements made in triplicate and reported as mean ± standard deviation
Figure S1. C153F HDAC8. (a) Simulated annealing omit map (contoured at 3.0σ) showing the Phe153 side chain in the C153F HDAC8-SAHA complex (monomer A). Atomic color codes are as follows: C = yellow (protein) or tan (SAHA), N = blue, O = red, water molecules = red spheres. (b) Simulated annealing omit map (contoured at 3.0σ) showing SAHA binding in the active site of C153F HDAC8 (monomer A, same color code). In the wild-type HDAC8-SAHA complex (PDB accession code 1T69), the benzamide carbonyl group of SAHA forms a hydrogen with Asp101 (which presumably must be protonated to accommodate this interaction). In C153F HDAC8, however, the amide group of SAHA is flipped so that the NH benzamide group interacts with Asp101 through a bridging hydrogen bonded water molecule.
**Figure S2.** Simulated annealing omit map (contoured at 2.5σ) showing the Met311 side chain in monomer A of the T311M HDAC8-TSA complex. Atomic color-codes are as follows: C = yellow, N = blue, O = red, S = green.
**Figure S3.** Comparison of the T311M HDAC8-TSA complex (yellow, Zn\(^{2+}\) = yellow sphere; TSA: C = tan, N = blue, O = red, monomer A) and the wild-type HDAC8-TSA complex (blue, Zn\(^{2+}\) = blue sphere; TSA: C = gray, N = blue, O = red) (PDB 1T64, monomer A). While two TSA molecules are observed to bind in the active site of the wild-type enzyme, structural changes in the active site of T311M HDAC8 block the binding of a second TSA molecule.
Figure S4. Comparison of the A188T HDAC8-M344 complex (yellow, C = yellow, O = red, K$^+$ = yellow sphere, monomer B) with the wild-type HDAC8-M344 complex (blue, Na$^+$ = blue sphere; PDB accession code 1T67). Selected residues are indicated; the hydrogen bond between the Thr188 hydroxyl group and the backbone carbonyl of Gly184 is shown as a black dashed line. The A188T substitution causes a ~0.4 Å shift in Phe70 and its associated loop, which in turn causes a slight shift of helix B2. While the L2 loop is disordered in the wild-type HDAC8-M344 complex, it becomes ordered in monomers B and D (but remains partially disordered in monomers A and C) of the A188T HDAC8-M344 complex.
**Figure S5.** Simulated annealing omit map (contoured at 3.0σ) showing the partially disordered tetrapeptide substrate bound in the active site of the A188T/Y306F HDAC8-substrate complex (monomer A). Atomic color codes are as follows: C = yellow (protein) or tan (substrate), N = blue, O = red, Zn$^{2+}$ = yellow sphere, water molecule = red sphere. Metal coordination and hydrogen bond interactions are shown as solid black and dashed black lines, respectively. Also superimposed is the Y306F HDAC8-substrate complex (C = blue (protein) or gray (substrate), Zn$^{2+}$ = blue sphere, water molecule = orange sphere; PDB 2V5W).
Figure S6. Comparison of the I243N HDAC8-SAHA complex (yellow, C = yellow, N = blue, O = red, monomer B) with the wild-type HDAC8-SAHA complex (blue; PDB accession code 1T69). Selected residues are indicated. The I243N substitution in the hydrophobic core of the protein causes 0.3–1.6 Å shifts in helix H3.
**Figure S7.** I243N HDAC8. (a) Active site comparison (monomer B) of the I243N HDAC8-SAHA complex (yellow, Zn$^{2+}$ = yellow sphere, SAHA = tan; monomer A = green) with the wild-type HDAC8-SAHA complex (blue, Zn$^{2+}$ = blue sphere, SAHA = gray; PDB accession code 1T69). Water molecules observed in the mutant enzyme active site appear as red spheres. In the wild-type HDAC8-SAHA complex, the benzamide carbonyl group of SAHA forms a hydrogen with Asp101 (which presumably must be protonated to accommodate this interaction). In I243N HDAC8, however, the amide group of SAHA is flipped so that the NH benzamide group is directed toward Asp101; however, the N---O separation is too long (> 4 Å) for a hydrogen bond interaction. Additionally, the benzamide carbonyl group of SAHA group interacts with a network of hydrogen bonded solvent molecules that in turn interact with His180, the backbone NH group of Phe208, and the backbone carbonyl group of Pro273 in the adjacent monomer. (b) Simulated annealing omit map (contoured at 3.0σ) showing SAHA binding in the active site of I243N HDAC8 (monomer B, color-coded as in (a)).
Figure S8. Comparison of substrate binding in the I243N/Y306F HDAC8-substrate complex (C = yellow (protein) or tan (substrate), N = blue, O = red, Zn$^{2+}$ = yellow sphere) and the Y306F HDAC8-substrate complex (C = blue (protein) or gray (substrate), N = blue, O = red, Zn$^{2+}$ = blue sphere) (PDB 2V5W, monomer A). Water molecules are indicated as red or orange spheres, respectively. Metal coordination and hydrogen bond interactions are shown as solid black and dashed black lines, respectively. The simulated annealing omit map (contoured at 3.0σ) shows a nearly fully-ordered tetrapeptide substrate bound in the active site of I243N/Y306F HDAC8.
Figure S9. Comparison of the H334R/Y306F HDAC8-substrate complex (yellow) and the Y306F HDAC8-substrate complex (blue; PDB accession code 2V5W, monomer A); the substrate is Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin and is shown as a stick-figure with tan or gray C atoms, respectively.
**Figure S10.** Comparison of substrate binding in the H334R/Y306F HDAC8-substrate complex (C = yellow (protein) or tan (substrate), N = blue, O = red, Zn$^{2+}$ = yellow sphere) and the Y306F HDAC8-substrate complex (C = blue (protein) or gray (substrate), N = blue, O = red, Zn$^{2+}$ = blue sphere) (PDB 2V5W, monomer A). Water molecules are indicated as red or orange spheres, respectively. Metal coordination and hydrogen bond interactions are shown as solid black and dashed black lines, respectively. The simulated annealing omit map (contoured at 3.0σ) shows a nearly fully-ordered tetrapeptide substrate bound in the active site of H334R/Y306F HDAC8.
Figure S11. Time dependent heat inactivation of HDAC8 activity (expressed as nmol product·µmol enzyme⁻¹·min⁻¹).
Figure S12. Chemical structures of inhibitors used in this study. The common feature of each inhibitor is a hydroxamate group that targets metal coordination interactions with the active site zinc ion, which is a standard design motif for HDAC8 inhibitors.
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