**Supporting Materials and Methods**

**Expression and Purification.** The histone deacetylase (HDAC) 8 gene (1–377 residues) (GenBank accession no. AF230097; purchased from Stratagene) was PCR-amplified to insert a factor Xa cleavage site (Ile-Glu-Gly-Arg), a Gly-Ser linker, and a hexa-histidine tag at the C terminus and subcloned in pET21b (Novagen), essentially as described in ref. 1. Overexpression was performed by induction of mid-log phase BL21 DE3 *Escherichia coli* cells with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and in the presence of 100 μM $ZnCl_2$ for 22 h at 18°C in minimal medium supplemented with 10% casamino acids. Harvested bacteria were resuspended in lysis buffer (50 mM Tris•HCl, pH 8.0/3 mM $MgCl_2$/150 mM KCl/5% glycerol/0.25% Nonidet P-40/1 mM 2-mercaptoethanol/complete EDTA-free protease inhibitor mixture tablets) and lysed with a cell disruptor (Constant Systems, Daventry, England). Lysate was incubated with DNase I (5 units/ml) in the presence of 10 mM $MgCl_2$ and clarified by centrifugation at 35,000 × *g* for 1 h. The supernatant was then loaded into a Ni-NTA resin column preequilibrated with lysis buffer without Nonidet P-40. HDAC8 was eluted with 100 mM imidazole, then dialyzed against buffer A-Q (50 mM Tris•HCl, pH 8.0/50 mM KCl /5% glycerol/10 μM $ZnCl_2$/1 mM DTT) and loaded onto a 6-ml Resource Q column (Amersham Pharmacia) preequilibrated with buffer A-Q. The protein was eluted at 250 mM KCl by using a linear gradient and then loaded onto a G75 26/60 gel filtration column (Amersham Pharmacia) equilibrated in 50 mM Tris•HCl (pH 8.0), 150 mM KCl, 5% glycerol, and 1 mM DTT (gel filtration buffer). The protein was analyzed by electrospray ionization-MS and confirmed to be >95% pure, with an experimental mass of 43,180 Da.

Full-length cDNAs for HDAC1 and HDAC3 with FLAG-epitope sequences were subcloned into a pCDNA3 vector (Invitrogen) as well as Gal4 DBD fused to the nuclear receptor corepressor (N-CoR) N-terminal region (P.G., unpublished data). HeLa cells were transiently transfected with pCDNA3 constructs encoding flagged-HDAC1 or flagged-HDAC3 plus GAL4-N-CoR. For affinity purification, whole-cell extracts were prepared, incubated with M2-agarose beads (Sigma), and eluted in a buffer containing 50

mM Hepes (pH 7.4), 5% glycerol, 100 mM KCl, 0.01% Triton X-100, and 0.1 mg/ml FLAG peptide. All purification steps were performed at 4°C.

**CD Spectroscopy.** Spectra were collected by using a Jasco J-710 spectropolarimeter (Easton, MD) equipped with a temperature-controlling device in a quartz cell with a path length of 0.1 cm. Before experiments, HDAC8 was dialyzed in 5 mM Tris•HCl (pH 8.0), 0.5% glycerol, 0.1 mM DTT, and either 15 mM KCl (buffer 1) or 15 mM NaCl (buffer 2). The protein concentration was 5 μM. Spectra were recorded at 12°C in the far-UV from 250 to 190 nm and were the average of five scans. For thermal denaturation experiments, a temperature scan from 12 to 54°C was performed at 222 nm. Thermal melts were reversible. Superimposable folding and unfolding curves were observed, and >90% of the signal was regained upon cooling.

**Data Collection.** Crystals were first equilibrated for 10–20 min in 25 mM Tris•HCl (pH 8.0), 50 mM Mes (pH 5.3), 75 mM KCl, 20% polyethylene glycol 6000, 10% glycerol, 0.5 mM Tris-2-carboxyethyl-phosphine, and 50 μM inhibitor, then flash-cooled in liquid nitrogen, increasing polyethlene glycol concentration up to 40%. Data were collected at 100 K by using synchrotron radiation on beamline ID14-H3 at the European Synchrotron Radiation Facility, Grenoble, France. Data were processed with MOSFLM (2) and scaled with SCALA (3). Data collection statistics are summarized in Table 2.

**Structure Determination and Refinement.** The structure was solved by molecular replacement with the program AMORE (4) by using a truncated homology model, residues 63–320 of HDAC8, based on the crystal structure of archeobacterial homolog of eukaryotic deacetylases (HDLP) in complex with trichostatin A (TSA) (5) (PDB ID code 1C3R). Rotation and translation function calculations in the range 12.0–4.0 Å yielded two independent solutions, which had, after rigid body fitting in AMORE , an *R* factor of 0.51 and a correlation coefficient of 0.32. Rigid-body refinement then was carried out in CNX (Accelrys, San Diego) in the range 50.0–3.0 Å. At this stage, phase improvement by twofold averaging and phase extension to 2.5 Å was carried out in CNX by using a monomer mask calculated from the HDLP structure. The resulting maps were of good

quality and allowed us to build most of the model by using QUANTA 2000 (Accelrys). After several cycles of refinement with CNX and manual building, including placement of the inhibitor, $Zn^{2+}$, and $K^{+}$ ions, and solvent molecules, the refinement converged (Table 2 and Fig. 7). The starting homology model and the final crystallographic model have an overall rms deviation (rmsd) of 1.8 Å for the $C^{\alpha}$ trace (residues 63–320) with the largest deviations in all loop regions. The final model encompasses residues 13–374 of human HDAC8 (377 residues). Residues 85–94, located in a solvent-exposed loop, were disordered and therefore excluded from the final model. Acetyllysine, taken from PDB ID code 1E6I, was manually docked in the active site, and energy was minimized by using CNX. Figures were generated with PYMOL (DeLano Scientific, San Carlos, CA). The program ESPRIPT (6) was used to generate Fig. 4.

**Short Interfering RNA (siRNA) Transfection and Cell-Growth Assay.** A549 human lung carcinoma cells were cultured in F12K Nutrient Mixture (GIBCO), HeLa human cervical carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO), and HCT-116 colorectal carcinoma cells were cultured in McCoy's 5A medium (GIBCO). All media were supplemented with 10% FBS, 2 mM L-glutamine, 100 units of penicillin, and 100 μg/ml streptomycin. siRNAs were designed against nucleotides 216–234 and 674–692 of HDAC8 mRNA (refseq no. NM_018486, Dharmacon Research). We transfected 60% confluent cells with 50 nM siRNAs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Silencing was assayed by TaqMan analysis 24 h after transfection: Total RNA was extracted by using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantitative RT-PCR of HDAC8 mRNA was performed in triplicate on 150 ng of total RNA per well by using One-Step RT-PCR Master Mix (Applied Biosystems) with the following set of primers and probe: sense, 5'-AGATGGCCACCTTCCACACT-3', 400 nM; antisense, 5'-TGATCATCATCGCCCTCTTG-3', 400 nM; and probe (FAMTAMRA), 5'-ATGCTTATCTGCAGCATCTCCAGAAGGTCA-3', 200 nM. Normalization was performed on the same amount of template by amplification of GAPDH with the corresponding PreDeveloped TaqMan Assay Reagents (Applied Biosystems) in triplicate. Detection was performed with an ABI Prism 7900HT Sequence Detection System

(Applied Biosystems). For the growth assay, 2,500 cells per well were replated in 96-well Cytostar-T scintillating microplates in triplicate, in the presence of 80 nCi (1 Ci = 37 GBq) of [methyl-$^{14}$C]thymidine (Amersham Pharmacia). Labeled thymidine incorporation was measured up to 72 h after transfection with a TOP Count NXT Microplate Scintillation and Luminescence Counter (Packard).

**Activity Assay.** $^{3}$H-labeled acetylated histones were prepared essentially as described in ref. 7. Deacetylase activities of individual HDACs were assayed by incubating the recombinant enzymes with 10,000–15,000 dpm of $^{3}$H-labeled acetylated HeLa histones in a total volume of 100 μl of activity buffer (50 mM Hepes, pH 7.4/5% glycerol/13 mM KCl/0.01% Triton X-100/100 μg/ml BSA) at 23°C for 2 h. Protein concentrations were chosen to ensure ≤15% substrate conversion (25 nM HDAC8, 2 nM HDAC1, and 1 nM HDAC3/N-CoR). Reaction mixtures were quenched with 20 μl of stop solution (0.5 M HCl/1 M acetic acid) and extracted with 1 ml of ethyl acetate. Released $^{3}$H acetic acid and the residual $^{3}$H acetylated substrate were measured by scintillation counting of the organic and aqueous phases, respectively. The 50% inhibitory concentrations ($IC_{50}$) were calculated at increasing inhibitor concentrations by multiparameter logistic fitting of the experimental data.

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