Supplementary Methods and Results

Reduced Histone Deacetylase 7 Activity Restores Function to Misfolded CFTR in Cystic Fibrosis

Darren M. Hutt1*, David Herman2*, Ana P. C. Rodrigues3, Sabrina Noel4, Joseph M. Pilewski5, Jeanne Matteson1, Ben Hoch2, Wendy Kellner1, Jeffery W. Kelly6,7, Andre Schmidt8, Philip J. Thomas8, Yoshihiro Matsumura9, William R. Skach9, Martina Gentzsch10, John R. Riordan11, Eric J. Sorscher12, John R. Yates III1,14, Tsukasa Okiyoneda13, Gergely L. Lukacs13, Raymond A. Frizzell4, Gerard Manning3, Joel M. Gottesfeld2 and William E. Balch1,2,14,15#

Departments of Cell Biology1, Department of Molecular Biology2, Department of Chemical Physiology14, The Institute for Childhood and Neglected Diseases15, Department of Chemistry6 and Skaggs Institute of Chemical Biology7 at The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA, 92037 USA; Razavi Newman Center for Bioinformatics, Salk Institute for Biological Studies, La Jolla, CA, 92037 USA; Department of Cell Biology and Physiology4 and Division of Pulmonary, Allergy and Critical Care Medicine5, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; Molecular Biophysics, University of Texas Southwestern Medical Center, 6001 Forest Park Lane, Dallas, TX 75390; Department of Biochemistry and Molecular Biology, Oregon Health and Sciences University, Portland, OR 97239; Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599; Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27510; Department of Cell Biology and Physiology, University of Alabama at Birmingham, Birmingham, AL 35294; Department of Physiology, McGill University, Montreal, QC, H3G1Y6 Canada13.

*Contributed equally

# To whom correspondence should be directed: webalch@scripps.edu
SUPPLEMENTAL METHODS

Reagents and Cells. HDACi (SAHA) was purchased from Cayman Chemical, Scriptaid was purchased from Biomol, MS-275 was purchased from Axxora, and TSA from Sigma, and were dissolved (1-50 mM) in DMSO. Stock solutions of forskolin (10 mM) (Sigma) and genistein (50 mM) (Sigma) were prepared in either DMSO or pure ethanol. Amiloride (10 mM) (Sigma) was dissolved in water and CFTR_{inh-172} in DMSO (stock solution, 10 mM) was obtained from the CF Foundation corrector panel. siRNA were obtained from Ambion. RNA extraction kits were obtained from Qiagen. 3G11 and M3A7 antibodies used for immunoblotting were obtained from the Cystic Fibrosis Consortium (http://www.cftrfolding.org/index.htm). Other reagents were obtained as indicated below.

siRNA-mediated silencing of HDACs. ΔF508 expressing CFBE41o- or wild-type corrected (WT-HBE41o-) (where indicated in the Results) cells were plated in 12-well dishes (immunoblot) or 60 mm (iodide efflux) and grown to 60-70% confluence. Silencing of individual HDACs was performed using 50 nM of validated siRNA for the indicated HDACs (Ambion) and RNAi-max (Invitrogen) as per manufacturers directions. Cells were cultured overnight in the serum-free Opti-MEM containing transfection complexes and subsequently washed and cultured for an addition 48hrs in the presence of growth medium. Cells reached confluency 24 h prior to processing for CFTR immunoblot analysis or iodide efflux assays.

qRT-PCR. qRT-PCR was performed using the iScript One-Step RT-PCR kit with SYBR green (Bio-Rad) and the following primers for CFTR: 5' - GTGGCTGCTTCTTTGGTTGT-3' and 5' - CGAACTGCTGCTGGTGATAA-3'. RNA was standardized by quantification of GUS mRNA using primers 5' - CTCATTGTGAGTTCGGTTGATT-3' and 5' - CCGAGTGAAGATTTGCCCCCCCTTTT-3', and all values are expressed relative to GUS.
Statistical analysis was performed on three independent qRT-PCR experiments for each RNA sample, and error bars shown in the figures represent SEM.

**HDAC assays.** Deacetylation assays were based on a homogenous fluorescence release assay, using the synthetic substrate Acetyl-Lys(Ac)-AMC. In short, purified recombinant HDAC enzymes were incubated with serially-diluted inhibitors at various concentrations, in HDAC buffer (Tris-HCl buffer (pH 8.0), 137 mM NaCl, 1 mmol/L MgCl$_2$, 2.7 mmole/L KCl). Acetyl-Lys(Ac)-AMC substrate was added and incubated for an hour. Addition of a developer solution containing trypsin (5 mg/ml) cleaved deacetylated AMC substrate to release flourogenic 4-methylcoumarin-7amide (MCA). Fluorescence is measured using a 96 well-plate reader with excitation/emission wavelengths of 370 nm and 470 nm respectively. The IC$_{50}$ was determined by fitting the data using a nonlinear regression analysis with variable slope. Assays were performed by Reaction Biology Corp. (Malvern, PA).

**CFTR transport assays.** Where indicated, either ΔF508 expressing CFBE41o- lung cells were cultured in the presence or absence of the indicated reagent in 12 well Sarstedt dishes, harvested, lysed and the processing of CFTR from the band B to the band C glycoform detected by SDS-PAGE and immunoblotting using either monoclonal 3G11 or M3A7 as previously described$^1$.

**Quantitation of CFTR glycoforms.** Immunoblot exposures shown in the Results were selected to allow visualization of CFTR recovery under identical protein loads in same SDS-PAGE for all treatments. Given the dynamic range, quantitation of the band B and C glycoforms was made by analysis of band intensities that were in the linear range. In brief, the x-ray films were exposed for increasing time and the different exposures were quantified using a FluroChemSP (Alpha Inotech) densitometer/software package. Where
band B and C were quantified from different exposures, an internal reference was used to normalize the signal intensity.

**Iodide efflux Assay.** Cells were incubated for 1 h at room temperature with loading buffer (136 mM Nal, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 20 mM Hepes, 11 mM glucose). Cells were subsequently washed with efflux buffer (136 mM NaNO₃, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 20 mM Hepes, 11 mM glucose) to remove excess iodide. Cells were stimulated for 4 min with 10 μM forskolin and 50 μM genistein in efflux buffer to activate CFTR and subsequently washed out in efflux buffer for an additional 6 min. Samples were collected every min and analyzed for iodide content using an iodide selective electrode. Iodide concentration was assessed by comparison to a standard curve and values normalized as fold-stimulation relative to pre-stimulation point. In the case of CFInh172, the compound was present in all buffers following the iodide loading step.

**Ussing chamber conductance assay cell culture.** CFBE41o- cells stably expressing ΔF508 CFTR² via lentivirus transduction³ were cultured on permeable supports (Costar Transwell filters, 0.33 cm², 0.4-μm pore) for 6-10 days as described previously⁴. The filters were coated with plating medium containing 1% collagen (PureCol, Sigma), 1% fibronectin (BD biosciences, Franklin Lanes, NJ) and 98% serum free medium. DF/DF-HBE cells were cultured from excess pathological tissue obtained from ΔF508 homozygous patients following lung transplantation and organ donation under a protocol approved by the University of Pittsburgh Investigational Review Board, as previously described⁵. DF/DF-HBE cells were cultured on human placental collagen-coated Transwell filters (above) as previously described⁵,⁶ and used for experiments following 4–6 weeks of culture at an air-liquid interface.
Transepithelial recordings. Short-circuit currents ($I_{sc}$) were measured as previously described. In brief, cells cultured on filter supports were mounted in modified Ussing chambers, and the cultures were continuously short-circuited with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance, $R_t$, was measured periodically from the current required to apply a 2.5-mV bipolar voltage pulse. $R_t$ was calculated from Ohm's law. The basolateral bathing Ringer solution was composed of 120 mM NaCl, 25 mM NaHCO$_3$, 3.3 mM KH$_2$PO$_4$, 0.8 mM K$_2$HPO$_4$, 1.2 mM MgCl$_2$, 1.2 mM CaCl$_2$, and 10 mM glucose. The NaCl concentration of the apical bathing solution was reduced by replacing NaCl with equimolar Na-gluconate. The chambers were maintained at 37°C and gassed continuously with a mixture of 95% O$_2$, 5% CO$_2$ which fixed the pH at 7.4. Following a 5-min equilibration period, the baseline $I_{sc}$ was recorded. Sodium currents were blocked by addition of the sodium channel blocker amiloride (10 µM) to the apical solution. Subsequently, the cAMP agonist, forskolin (10 µM, Sigma), the CFTR potentiator genistein (50 µM, Sigma), and the CFTR channel blocker CFTRInh-172 (10 µM; Calbiochem, San Diego, CA) were added sequentially to determine cAMP-stimulated CFTR currents.

Cell surface labeling of CFTR. BHK-21 cells stable expressing ΔF508 CFTR containing an extracytoplasmic epitope tag were plated at 40,000 cells/well in Corning 96 well Cell Bind plates and cultured in DMEM/F12 (50:50) medium at 37°C / 5% CO$_2$ atmosphere for 24 h. Compounds to be tested were added to media at the indicated final concentrations and incubation continued for a further 24 h. Detection employed a primary antibody recognizing the external epitope (anti-HA11) and a fluorescently labeled secondary antibody. Briefly, following exposure to compounds, medium was removed and cells washed with PBS before and after fixation with 2% PFA for 20 min at room temperature. Following a 1 h incubation in blocking solution (1% BSA plus 5%
normal goat serum in PBS), primary antibody was added at a 1:500 dilution and incubation continued for 2 h with gentle mixing. Primary antibody solution was removed and cells washed twice with PBS prior to incubation with secondary antibody at a 1:1000 dilution for 1 h. Syto 60 nuclear stain (Molecular Probes) was also present during the secondary incubation. After removal of this solution and PBS washing, plates were scanned on a Li-Cor IR Fluorescence scanner at 800 nm to detect labeled secondary antibody bound to the cell surface and 700 nm to detect the nuclear stain. The latter signal was used to normalize for any variation in cell number.

**Surface density of CFTR.** Surface density of CFTR was measured in using the CFBE-L-ΔF508 cell line stably expressing ΔF508-CFTR containing an HA epitope in the extracellular domain in CFBE41o- cells. Cell surface density was measured by an anti-HA antibody ELISA as described above.

**Adenoviral expression of extope-ΔF508 (ΔF508extope) CFTR in HBE cultures.** Adenoviral vectors for expression of ΔF508extope CFTR were generated by the UNC Cystic Fibrosis Center Molecular Biology Core and the UNC Vector Core Facility. Human excess donor lungs and excised recipient lungs were obtained at the time of lung transplantation from portions of main stem or lumbar bronchi. Airway epithelial cells were harvested by enzymatic digestion as described under a protocol approved by the UNC medical school institutional review board. To express ΔF508extope CFTR in primary HBE cultures, cells were seeded on collagen-coated Millicell culture plate inserts and maintained at an air-liquid interface for 40 days. The well-differentiated cultures were then transduced with adenoviral vector. 30 h after infection cultures were treated with 0.1 μM TSA, 5.0 μM SAHA, 5 μM ScriptAid, 1 μM MS-275 or correctors compounds C3 (VRT-325) and C4 (Corr-4a) (10 μM each; provided by the Cystic Fibrosis Foundation [http://cftrfolding.org/CFFTReagents.htm]) for 46 h at 37°C or incubated without
correctors at 27°C. For detection of apical ΔF508extope, primary HBE cultures were labeled with anti-HA antibody HA11 (Covance) from the apical side for 45 min and frozen culture sections were prepared. Sections were fixed with 4% PFA, permeabilized with 0.1 % Triton X-100, blocked with 1% BSA, and 10% normal goat serum in PBS and incubated with goat anti-mouse IgG Alexa Fluor 488 conjugate. Nuclei were stained with TO-PRO-3 iodide. Cells were examined using a Zeiss LSM 510 confocal laser scanning microscope. Scale bar=10 µm.

**Microarray analysis.** Raw expression data files were obtained for three siHDAC7 treatment replicates and three siSCR control replicates with the Affymetrix Human Genome U133 Plus 2.0 Array. All microarray analysis was performed with Bioconductor\(^\text{12}\). Standard data quality validation as suggested by Affymetrix was carried out with the ‘simpleaffy’ package, followed by ‘affyPLM’, which identified no problematic chips. The raw data were preprocessed according to the GC-RMA method\(^\text{13}\) (implemented in ‘gcrma’), which performs probe sequence based background adjustment, quantile normalization, and utilizes a robust multi-chip average to summarize information into single expression measurements for each probeset (see Supplemental Fig. S7 ‘expression siHDAC1/7 versus siSCR’). Prior to statistical testing, the data were submitted to a non-specific filter (via the package ‘genefilter’) that removed probesets without Entrez Gene IDs and with an expression interquartile range smaller than 0.5. To identify genes that were significantly differentially expressed between conditions, linear modeling and empirical Bayes analysis was performed using the ‘limma’ package\(^\text{14}\). Limma computes an empirical Bayes adjustment for the t-test (moderated t-statistic), which is more robust than the standard two-sample t-test comparisons. To correct for multiple testing Benjamin and Hochberg’s method to control for false discovery rate was used\(^\text{15}\). Probesets with an adjusted p-value of 0.01 or
smaller were considered differentially expressed (Supplemental Fig. 7 ‘comparison siHDAC1/7 versus siSCR’).

**Pathway enrichment analysis**

3333 genes (4402 probesets) were differentially expressed in response to HDAC7 silencing. These were mapped to 3141 objects within the MetaCore platform\(^\text{16}\). All four CFTR-relevant MetaCore Cystic Fibrosis pathways involved in CFTR metabolic stability were selected for enrichment analysis: CFTR Folding and Maturation; Regulation of CFTR Degradation; CFTR Traffic; and Regulation of CFTR Activity. The probability of each pathway to include the observed number of differentially expressed objects was calculated using the hypergeometric distribution:

\[
P(r,n,R,N) = \frac {R \binom {N-R} {n-r}} {n \binom N n},
\]

where \(n\) is the number of objects in the pathway, \(N\) the total number of objects, \(R\) the number of differentially expressed objects in response to HDAC7 silencing and \(r\) the number of differentially expressed objects that are part of the pathway. Additional probesets mapped to CFTR-related pathways involved in bacterial pathogenesis and the immune response (unpublished).

**Network construction**

Objects and interactions in GeneGo Cystic Fibrosis pathways were used to build a network that captures the effect of HDAC7 silencing on all genes known to influence CFTR.
**In Vitro Translation**

CFTR protein was translated at 25°C for 2 hours in reaction containing 20 μg/mL CFTR RNA, 40% (v/v) nuclease-treated rabbit reticulocyte lysate, canine pancreas microsomes (3 OD280), 1 mM ATP, 1 mM GTP, 12 mM creatine phosphate, 40 μM each of 19 essential amino acids, except methionine, 1 μCi/mL of Trans[35S]-label (MP Biomedicals, Solon, OH), 40 μg/mL creatine kinase, 0.1 mg/mL bovine calf tRNA, 0.1 U/mL RNase inhibitor, 10 mM Tris-acetate (pH 7.5), 100 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT. SAHA (5 μM) or 0.2% (v/v) DMSO (vehicle) was added at the start of translation. Aliquots of reaction at indicated time were subjected to SDS-PAGE (7%) and autoradiography.

**Intact cell glucocerebrosidase (GC) enzyme activity assay.**

The intact cell GC activity assay was performed as previously17. Briefly, approximately 10⁴ L444P GC cells were plated in each well of a 96-well plate (100 μl per well) overnight to allow cell attachment. Medium was replaced with fresh medium containing 1 mM SAHA every day for 5 days and plates were incubated at 37 °C. The medium was then removed and monolayers washed with PBS. The assay reaction was started by the addition of 100 ml of 3 mM 4-methylumbelliferyl b-D-glucoside in 0.2 M acetate buffer (pH 4.0) to each well. Plates were incubated at 37 °C for 2 h and the reaction was stopped by the addition of 150 ml of 0.2 M glycine buffer (pH 10.8) to each well. Liberated 4-methylumbelliferone was measured (excitation 365 nm, emission 445 nm) using a SpectraMax Gemini plate reader (Molecular Device, Sunnyvale, CA). Control experiments to evaluate the extent of non-specific non-lysosomal GC activity were performed by adding coniduritol B epoxide to the assay reaction. GC activities measured were normalized to corresponding DMSO vehicle control treated cells.

**Chromosomal Immunoprecipitation**
CFBE41o- cells were treated with 0.01% DMSO (v/v) or 5 mM SAHA for 24hrs prior to crosslinking with 1% paraformaldehyde at 37°C. Cells were harvested by scraping in Buffer A (10mM Hepes pH 8, 10mM EDTA, 0.5mM EGTA and 0.25% Triton X-100) and incubated for 10 minutes at 4°C in a 15ml conical tube. Cells were pelleted by centrifugation at 500xg and resuspended in Buffer B (10mM Hepes pH 8, 200mM NaCl, 1mM EDTA, 0.5mM EGTA and 0.01% Triton X-100) and subsequently incubated for 10 minutes at 4°C. Cells were again pelleted as above and resuspended in ChIP buffer (25mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitor (Sigma). Lysate was aliquoted to 2ml tubes and sonicated on ice for 7 pulses of 30 seconds at 100% output with 60 seconds on ice between pulses. The crosslinked, soluble chromatin was obtained by collecting the supernatant from a 15 minutes centrifugation at 4°C at maximum speed. The sample is pre-cleared with Protein A/G for 2 hrs at 4°C prior to incubation with anti-acetylated histone H3 or anti-RNA polymerase II antibodies and subsequent antibody pull down with Protein A/G for 2 hrs each at 4°C. The beads are collected by centrifugation at 1500xg for 2 minutes and sequentially washed with ChIP buffer, Wash Buffer 1 (25mM Tris-HCl pH 8, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), Wash Buffer 2 (10mM Tris-HCl pH 8, 250mM LiCl, 1% NP40, 1% Sodium deoxycholate, 1mM EDTA) and TE Buffer (10mM Trish-HCl pH 8, 1mM EDTA). The bound material is eluted with 1% SDS, 100mM sodium carbonate for 10 minutes at RT. The crosslinking is reversed by the addition of NaCl to a final concentration of 200mM and heating at 65°C overnight. The protein content is removed by proteinase K digestion for 1 hr at 42°C in 35mM Tris-HCl pH 6.5, 10mM EDTA and 40µg/ml proteinase K. The DNA is ethanol precipitated, resuspended in H₂O and subjected to qPCR for Sar1b using the following primer pairs (forw: 5’GCATGGATGAGACCTGTCCT-3’; rev: 5’-TAGAAGGCACCTTTTAGGG-3’).
**Thermal denaturation of NBD1-CFTR**

ΔF508 NBD1-CFTR was purified as previously described\(^1\). Thermal denaturation was measured by monitoring at 300 nm turbidity (aggregation) of 5 µM NBD1-CFTR in Buffer M (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl\(_2\), 65 µM ATP, 0.5 % DMSO, pH 7.6), in presence or absence of SAHA or 2 mM ATP as indicated in the legend. Turbidity was measured every 0.5 °C, rate of temperature increase was 0.5 °C/min. Melting Temperature (T\(_M\)) was determined by taking the second derivative.

**REFERENCES CITED**


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. S1. Chemical structure of compounds utilized or discussed in the manuscript.

Supplemental Fig. S2. a, CFBE41o- cells were treated with 0.2, 1 or 5 µM of SAHA, Scriptaid or MS-275 for 24 h at 37°C. Immunoblot analysis of 10 µg of total protein is shown for each compound tested and the subsequent analysis is shown below (mean ± SEM, n = 3). b, RT-PCR analysis of CFTR mRNA following treatment of CFBE41o- cells with 0.1 µM TSA, 5 µM SAHA, 1 µM Scriptaid and 5 µM MS-275. Data is shown as fold change of the ratio of CFTR mRNA to glucoronidsase (GUS) mRNA relative to vehicle treated control (mean ± SEM, n = 3). c, Immunoblot analysis of 15 µg CFBE41o- cell lysates following treatment with the indicated HDACi as in b in the absence or presence of 50 µM actinomycin D (Act. D). CFTR was detected using the M3A7 antibody. d, RT-PCR analysis of CFTR mRNA following treatment with 5 µM SAHA for the indicated time. Data is shown as fold change of the ratio of CFTR mRNA to glucoronidsase (GUS) mRNA relative to vehicle treated control (mean ± SEM, n = 3).

Supplemental Fig. S3. SAHA does not affect CFTR synthesis or stability of NBD1 directly. a. In vitro translation of CFTR for the indicated time in the presence of 5 µM SAHA or 0.2% (v/v) DMSO (vehicle). Samples were subjected to SDS-PAGE (7%) and autoradiography and full length CFTR is shown. b. Thermal denaturation of NBD1-CFTR: Black, Control; red, + 2 mM ATP; green, + 5 µM SAHA; yellow, + 50 µM SAHA. Relative turbidity as a function of temperature. Traces are from a representative experiment. Inset Tm averages (mean ± SD, n = 3).
**Supplemental Fig. S4. Chaperone profiling.** a. Representative Western blots of the indicated chaperone proteins levels following treatment of CFBE41o- cells with 0.1 µM TSA, 5 µM SAHA, 1 µM Scriptaid and 5 µM MS-275. Controls of CFBE41o- cells cultured at physiological (37°C) and permissive temperature (30°C) as well as HBE41o-cells are shown. b. Quantification of cellular chaperones relative to actin loading control was performed for CFBE41o- cells treated with the indicated in a.

**Supplemental Fig. S5. SAHA did not increase L444P glucoceribrosidase (GC) enzyme activity in Gaucher patient-derived fibroblasts.** L444P GC fibroblasts were treated with 1 µM SAHA for 5 d before the intact cell GC enzyme activity assay was performed. The drug treated-GC activity data was normalized to the DMSO vehicle control (mean ± SD; n = 3).

**Supplemental Fig. S6.** CFBE41o- cells were treated with 10 or 30 µM of Sirtuin1 Inhibitor 3, Splitomycin or 0.1% DMSO (v/v) for 24 h at 37°C. Immunoblot analysis of 15 µg of total protein is shown for CFTR (M3A7) for each compound tested.

**Supplemental Fig. S7.** a, b, Ussing chamber tracings from CFBE41o- treated with 0.5 µM TSA (a) or 10 µM SAHA (b). c, Ussing chamber tracings from primary DF/DF-HBE cells harvested from patients treated as described in the legend of Fig. 6a.

**Supplemental Fig. S8.** a, qRT-PCR analysis of HDAC expression levels following siRNA-mediated knockdown of the indicated HDAC isoform in CFBE41o- cells. b, Analysis of CFTR mRNA levels following silencing of HDACs 1,2,3 and 7 in CFBE41o-cells. In both panels, data is expressed as a percent of siScramble (siScr) control (mean ± SD; n = 3).
Supplemental Fig. S9. Chaperone profiling. a. Representative Western blots of the indicated chaperone proteins levels following siRNA knockdown of HDAC1-10 in CFBE41o- cells. b. Quantification of cellular chaperones relative to actin loading control was performed for siHDAC1 and siHDAC7 treated CFBE41o- cells. (mean ± SEM, n = 3).

Supplemental Fig. S10. Grouping-oriented layout of the CFTR-related network of genes whose expression was altered in response to HDAC7 knockdown in CFBE41o- cells. Genes are shown as ovals colored according to their moderated t-statistic. Thicker node outlines indicate greater than 2-fold expression changes. Non-differentially expressed genes that provide a path between a differentially expressed gene and CFTR are shown in grey.

Supplemental Fig. S11. Chromosomal immunoprecipitation of acetylated histone H3 (upper) or RNA polymerase II (lower) and qPCR analysis of the associated Sar1b DNA from CFBE410- cells treated with 5 µM SAHA.

Supplemental Table S1. Table showing IC50 values for HDACs 1-10 for the indicated HDACi (upper). The tested concentration (µM) for in vivo analysis is indicated in the lower panel. The values represent a single point (n = 1) for each concentration.

Supplemental Table S2. Microarray data for CFBE41o- cells following HDAC1 and 7 silencing. Excel spreadsheet containing processed expression data and gene expression changes in observed in response to HDAC1 or 7 silencing. Analysis was performed as described in the Supplemental Methods.
Suppl. Figure 3
Suppl. Figure 4
Suppl. Figure 5
Suppl. Figure 6

DMSO 10 µM 30 µM

ΔF508

C
B

Sirt1 Inh III

Splitomycin
Suppl. Figure 7
Suppl. Figure 8
Suppl. Figure 9
Suppl Figure 11
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Suppl. Table 1