Histone deacetylase inhibitors correct the cholesterol storage defect in most NPC1 mutant cells

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Running Title: Correction of NPC1 mutants by HDACi
Abstract

Niemann Pick C disease (NPC) is an autosomal recessive disorder that leads to excessive storage of cholesterol and other lipids in late endosomes and lysosomes. The large majority of NPC disease is caused by mutations in NPC1, a large polytopic membrane protein that functions in late endosomes. There are many disease-associated mutations in NPC1, and most patients are compound heterozygotes. The most common mutation NPC1I1061T has been shown to cause endoplasmic reticulum associated degradation of the NPC1 protein. Treatment of patient derived NPC1I1061T fibroblasts with histone deacetylase inhibitors (HDACi) Vorinostat or Panobinostat increases expression of the mutant NPC1 protein and leads to correction of the cholesterol storage. Herein we show that several other human NPC1 mutant fibroblast cell lines can also be corrected by Vorinostat or Panobinostat and that treatment with Vorinostat extends the lifetime of the NPC1I1061T protein. To test effects of HDACi on a large number of NPC1 mutants, we engineered a U2OS cell line to suppress NPC1 expression by shRNA and then transiently transfected these cells with 60 different NPC1 mutant constructs. The mutant NPC1 did not significantly reduce cholesterol accumulation, but approximately 85% of the mutants showed reduced cholesterol accumulation when treated with Vorinostat or Panobinostat.

Key words: Niemann Pick type C, Cellular cholesterol, lipid transport, Inborn errors of metabolism, Drug therapy, Cholesterol trafficking
Introduction

Lipoprotein-derived cholesterol is normally transported out of late endosomes and lysosome (LE/Ly) by a process that requires the NPC1 and NPC2 proteins (1, 2). A current model (3) suggests that cholesterol, which is a product of the hydrolysis of cholesteryl esters by lysosomal acid lipase, is first transferred to NPC2, a cholesterol binding protein. The cholesterol can then be transferred to the N-terminal domain of NPC1, a LE/Ly membrane protein with 13 transmembrane segments (4). This model is supported by recent structural studies of NPC1 (5, 6) and of NPC2 bound to the middle lumenal domain of NPC1 (7). Cholesterol finally leaves the LE/Ly by a process that is not well understood. In cells with mutations in NPC1 or NPC2, cholesterol and other lipids accumulate in the LE/Ly. Cells with these mutations also show multiple defects in lipid and protein trafficking (8, 9).

Defects in NPC1 or NPC2 in humans lead to an autosomal recessive lysosomal storage disease, Niemann-Pick C (NPC) disease, which causes pathology in multiple tissues but especially in the central nervous system (10, 11). A high fraction of patients with NPC disease die of neurological complications before the age of 25 (10). In addition to cholesterol other lipids, including glycosphingolipids, accumulate in the LE/Ly of patient cells. There are currently no effective treatments for NPC disease approved by the U.S. Food and Drug Administration (FDA). Miglustat, an inhibitor of glycosphingolipid synthesis (12), shows some beneficial effects and is approved for use in several countries (13, 14). Treatment of mice and cats with hydroxypropyl-beta-cyclodextrin (HPBCD) has been shown to effectively reduce the storage of cholesterol and other lipids in cells and to ameliorate symptoms (15-17). HPBCD does not cross the blood brain barrier, so in cats and humans it needs to be injected directly into the CNS. Early stage clinical trials of HPBCD have been carried out in humans (18).
In a previous study (19) we showed that HDACi, including Vorinostat (also called suberoylanilide hydroxamic acid or SAHA) and Panobinostat (LBH589), are remarkably effective in correcting the NPC1 phenotype in human fibroblast cells that have an $NPC1^{I1061T}$ mutation. The pharmacological profile was most consistent with the effects being attributed to inhibition of HDACs 1, 2, or 3 (20). Treatment of patient-derived fibroblasts with HDACi reduced the accumulation of cholesterol in lysosomal storage organelles (LSOs) and restored other aspects of cholesterol homeostasis including normal processing of sterol regulatory element-binding protein 2 (SREBP2) and reduction of the expression of low density lipoprotein (LDL) receptors (19, 21). HDACi treatment did not correct the cholesterol storage defect of patient-derived cells expressing $NPC2$ mutations (19), indicating that the HDACi do not bypass the need for the NPC1/NPC2 transport system as HPBCD does (22). This indicated that the HDACi might work by allowing the mutant NPC1 proteins to function sufficiently well to correct the cholesterol transport out of LSOs. Vorinostat and Panobinostat do enter the CNS, although the levels achieved in the brain are much lower than in the plasma (20, 23, 24). Nevertheless, there is some evidence that Vorinostat has effects on tumors in brains (23). Some other HDACi do cross the blood brain barrier more efficiently and have been shown to have neurological effects in animal studies (25).

The mechanism by which HDACi might restore the function of mutant NPC1 proteins has not been determined. It has been observed that there is more rapid degradation of the NPC1$^{I1061T}$ protein as compared to wild type (WT) NPC1 protein, and it was proposed that this is due to enhanced endoplasmic reticulum associated degradation (ERAD) of the mutant protein (26). Treatment of cells expressing NPC1$^{I1061T}$ with HDACi such as Panobinostat or Vorinostat increased the expression of the mutant NPC1 protein (19). Correction of the NPC phenotype.
would require that this mutant protein retains adequate functional capability and that a sufficient amount is delivered to the LE/Ly. Other data are consistent with the hypothesis that some mutant NPC1 proteins can function in LE/Ly if they are delivered to those organelles. Simply overexpressing NPC1\textsuperscript{I1061T} in mutant cells leads to partial correction of the phenotype (26). Some indirect treatments also increase the abundance of NPC1 and lead to correction of the phenotype in cultured cells. These include treatment with ryanodine receptor antagonists (27), treatment with oxysterols that bind to NPC1 (28), or reduced expression of TMEM97, an NPC1-binding protein (29). These studies have indicated that alterations in the proteostasis environment (30-32) by various mechanisms leads to reduced degradation of mutant forms of NPC1. As described herein, we found that treatment of some NPC1 mutant cells with Vorinostat led to a longer lifetime of the NPC1\textsuperscript{I1061T} protein and increased delivery of the protein to LE/Ly.

A mouse knock-in model of NPC1\textsuperscript{I1061T} has been described recently, and mouse embryo fibroblasts from these mice respond to Vorinostat similar to the human NPC1\textsuperscript{I1061T} fibroblasts (33). Another recent study in Npc1\textsuperscript{I1061T} mutant mice, which have a D1005G mutation in the Npc1 protein, reported that a combination therapy with Vorinostat, HPBCD, and polyethylene glycol led to slowed neuronal degeneration and improved lifespan in Npc1\textsuperscript{I1061T} mutant animals (34).

Approximately 95% of NPC cases are due to mutations in the NPC1 protein, and the NPC1\textsuperscript{I1061T} mutation, which occurs in about 15-20% of NPC1 patients, is the most commonly observed mutation (35, 36). However, more than 300 different NPC1 mutations have been observed that are known to be or are likely to be pathogenic (10, 37). It would be very difficult to test drug treatments in hundreds of different human NPC1 mutant fibroblast cell lines, and the large number of compound heterozygous mutations would make it nearly impossible to evaluate the ability of HDACi to correct a specific mutation. In order to evaluate the effectiveness of
HDACi as a potential therapy for NPC patients we developed an efficient screening system using an engineered cell line. Human U2OS osteosarcoma cells were stably transfected with Scavenger Receptor type A (SRA), and the endogenous NPC1 expression in the cells was stably silenced with an shRNA. The U2OS-SRA-shNPC1 cells were then transiently transfected with a bicistronic vector expressing GFP (to identify transfected cells) and one of 60 NPC1 mutations found in patients. This system was used to test the effect of HDACi treatments on multiple NPC1 mutations simultaneously. After treatment with Vorinostat or Panobinostat, a high fraction of NPC1 mutant proteins were effective in reducing cholesterol accumulation. This suggests that HDACi therapy might be effective for a large majority of NPC1 patients. Since Vorinostat and Panobinostat are FDA-approved drugs for treatment of some cancers, and other HDACi have been in large scale clinical trials, HDACi may be considered as a potential therapy for NPC1 disease (20).

Materials and Methods

Reagents

Gibco® McCoy's 5A Medium, Modified Eagle’s Medium (MEM), Fetal Bovine Serum (FBS), Hank’s Balanced Salt Solution (HBSS), penicillin/streptomycin (P/S), Geneticin (G418), AlexaFluor-546, Cy5 Goat anti-Rat (A10525), and Alexa Fluor 546 Goat anti-Rabbit and Alexa Flour 488 Goat anti-Rat antibody, 1.1’-Dioctadecyl-3,3,3’3’-Tetramethyindocarbocyanine perchlorate (DiIC18(3)) were purchased from Invitrogen Life Technologies Corporation (Carlsbad, CA). Effectene Transfection Reagent Kit and DNA purification kit was purchased from QIAGEN Inc. (Valencia, CA). HDACi’s (Vorinostat and Panobinostat) were stocked at 5 mM in Dimethyl Sulfoxide (DMSO) and stored at -20°C. Vorinostat and Panobinostat were a generous gift from Dr. Paul Helquist (University of Notre Dame, South Bend, IN). Acetylated
low-density lipoprotein (AcLDL) was prepared by acetylation of LDL with acetic anhydride (38). AlexaFluor-546 labeled human LDL (LDL-Alexa546) was prepared as described (39, 40). Rabbit polyclonal anti-NPC1 antibody and rabbit polyclonal anti-LAMP1 (lysosomal associated membrane protein 1) antibody (ab24170) were purchased from Abcam (Cambridge, MA). A rat monoclonal anti-NPC1 antibody has been described previously (32). The specificity of the rabbit anti-NPC1 antibody was verified by immunostaining U2OS-SRA-shNPC1 cells, which are stably silenced for NPC1 expression in parallel with wild type U2OS cells. In wild type U2OS cells NPC1 staining was observed in punctate LE/Ly, but no staining was observed in U2OS-SRA-shNPC1 cells indicating that antibody specifically binds to NPC1 protein in cells (Supplemental Figure S1). Similar specificity of labeling was observed for the rat monoclonal anti-NPC1. All other chemicals, including (DMSO, 99% fatty-acid free bovine serum albumin (BSA), filipin, paraformaldehyde (PFA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Chemical (St. Louis, MO). Draq5 was from Biostatus (Leicestershire, UK), Metamorph image-analysis software was from Molecular Devices (Downington, PA).

**Human fibroblast cells**

Human NPC1 fibroblasts GM05659, GM18453 (homozygous *NPC1* mutant *I1061T*), and GM03123 (heterozygous *NPC1* mutations *P237S* and *I1061T*) were from Coriell Institute, (Camden, NJ). (The P237S allele has been found recently to be in linkage with a pathogenic splice mutation that may be responsible for its defect (F.D. Porter, submitted)). Patient derived mutant NPC1 skin fibroblasts were from Forbes Porter’s laboratory at the National Institutes of Health (Bethesda, MD) as listed in Table 1. All human skin fibroblasts were maintained in...
Modified Eagle’s Medium (MEM) supplemented with 10% FBS. For drug treatment cells were maintained in MEM supplemented with 5.5% FBS and 20 mM HEPES. The use of human fibroblasts has been approved by an Institutional Review Board at Weill Cornell Medical College for the research performed.

**U2OS cells stably expressing of SRA (U2OS-SRA)**

Human osteosarcoma U2OS (HTB-96™) cells from ATCC were transfected with murine scavenger receptor type IIA (SRA-II) cDNA [Kind gift from Dr. Monty Krieger (Massachusetts Institute of Technology, Boston, MA)]. Briefly, the 1.0 kb coding region of murine scavenger receptor type II was cut from a larger 4.0 kb plasmid encoding the coding region and the 3’ untranslated region in a pcDNA3.1 expression vector (41). The coding sequence was amplified using polymerase chain reaction (PCR) and inserted into a neomycin resistant pcDNA 3.1 TOPO plasmid using pcDNA 3.1 Directional TOPO Expression kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The insertion of SRA-II in the pcDNA3.1 TOPO plasmid was confirmed by band size of full length plasmid and also after digestion with appropriate restriction enzymes using gel electrophoresis. The pcDNA 3.1 TOPO plasmid encoding SRA-II was purified using Qiagen DNA purification kit (Qiagen Inc. Valencia, CA) and transfected in U2OS cells using Lipofectamine (Invitrogen, Carlsbad, CA) as a transfection reagent. U2OS cells expressing murine SRA-II were grown in McCoy's 5A medium supplemented with 10% FBS, 1% P/S and selection antibiotic G418 (1 mg/ml) in a humidified incubator with 5% CO₂ at 37°C for five passages. To select a population of U2OS cells expressing the SRA-II gene, cells were incubated with DilC18(3) labeled AcLDL and sorted using flow cytometry cell sorting (FACS). Murine macrophage cells J774 were used as a positive
control, and the pool of cells with equivalent Dil intensity, indicative of SRA-II expression, were collected. The cells were expanded, and stock cultures were frozen for future experiments.

**Effect of HDAC inhibitor treatment on human fibroblasts**

The dose dependence of two HDACis (Vorinostat and Panobinostat) was determined after 48 hours treatment of NPC1 fibroblasts from several patients carrying different mutations. The assay is based on the use of filipin, a fluorescent dye that binds to unesterified cholesterol. Human fibroblasts were seeded in 384 well plates at 450 cells/well in growth medium on day one. Four cell lines were seeded in different wells of a plate, and GM03123 fibroblasts were used as a control in each plate. After overnight incubation, 2X concentrated compounds were added at six different doses such that the final concentration ranged from 40 nM to 10 \( \mu \)M for Vorinostat and 5 nM to 1 \( \mu \)M for Panobinostat diluted in growth medium supplemented with 20 mM HEPES buffer and 5.5% FBS. DMSO was used as a control in each plate for each concentration and for each cell line. After 48 hours, the plate was washed with PBS three times, fixed with 1.5% PFA, and stained with 50 \( \mu \)g/ml filipin and nuclear stain Draq5. Measurements were made from 4 wells for each condition in each experiment, and the experiment was repeated three times. Images were acquired on an ImageXpress\textsuperscript{Micro} automatic fluorescence microscope, at four sites per well and analyzed to obtain the LSO compartment ratio, which is a measure of filipin labeling of stored cholesterol (42). A high LSO ratio is associated with high levels of cholesterol in LE/Ly. Removal of this cholesterol leads to reduction in the LSO ratio. The LSO compartment ratio for each concentration was normalized to corresponding DMSO treated control.

**Persistence of Vorinostat effects**
NPC1 human fibroblasts GM03123 and GM18453 were seeded in four different 384 well plates at 450 cells/well in growth medium on day one. After overnight incubation, compounds diluted in growth medium supplemented with 20 mM HEPES buffer and 5.5% FBS were added in three of the four plates to achieve the desired final concentrations. DMSO was used as a control in each plate for each concentration. After 3 days, Vorinostat-supplemented medium was aspirated from each plate and replaced with normal growth medium, and the cells were incubated for additional 0, 1, 2 or 3 days. At the end of each time point cells were stained with filipin, and the LSO ratio was determined. The experiment was performed twice independently. The LSO ratio for each concentration was normalized to corresponding DMSO treated value.

*Stable silencing of NPC1*

A line of NPC1-deficient stable U2OS-SRA cells was generated by silencing the endogenous NPC1 expression with 3’-UTR shNPC1 lentivirus. A Mission shRNA clone was purchased from Sigma against 3’UTR (TRCN000000542) of human NPC1 (pLKO-shNPC1) for knockdown of NPC1 expression. Stable clones of NPC1-deficient cells were selected with 5 µg/ml puromycin for three weeks. Stable U2OS-SRA-shNPC1 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS, 50-units/ml penicillin, 50 µg/ml streptomycin, 5 µg/ml puromycin and 1 mg/ml G418.

*NPC1 expression vector*

cDNA encoding human ΔU3hNPC1-WT construct was kindly provided by Dan Ory (Washington University, St Louis). The NPC1 gene was subcloned into bicistronic retroviral plasmid, pMIEG3 using EcoRI and NotI restriction enzyme sites. pMIEG3 vector was generated
from pMSCVneo vector (Clontech) in which the murine phosphoglycerate kinase (PKG) promoter and the Neomycin resistance (Neo') genes were replaced by IRES (Internal Ribosome Entry Site) and EGFP (enhanced green fluorescent protein) genes. The coexpression of eGFP with the NPC1 gene enables detection of cells expressing the human NPC1 protein by fluorescence microscopy. The WT construct used in this study has four variants as compared to a standard reference NPC1 sequence (37). These are: 387 T>C (Y129Y), 1415 T>C (L472P), 1925 T>C M642T and 2587 T>C (S863P). We found that these variants were also present in the human ΔU3hNPC1-WT construct, which has been used as a wild type construct in previous publications (43). The ΔU3hNPC1-WT was derived from pSV-SPORT/NPC1, which has also been used as a wild type in several previous studies (2, 44-46). Because the sequence differs from the reference NPC1 sequence, we will refer to these as wild type-variant (WT-V). Using WT-V pMIEG3-hNPC1 plasmid as a template, human NPC1 mutants were generated with Quick-Change XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The PMIEG3 plasmid harbors an ampicillin resistance gene as a selection marker. These plasmids were transiently expressed in U2OS-SRA-shNPC1 cells. It should be noted that all of the mutant constructs contain the same variants as WT-V.

Reverse transfection, drug treatment and cholesterol loading of U2OS-SRA-shNPC1 cells

Using Effectene Transfection Reagent Kit, an expression vector containing one of the NPC1 mutants (0.4 µg DNA) was mixed with 3.2 µl enhancer in 100 µl of DNA-condensation buffer (EC) buffer for 5 min. They were then mixed with 4µl Effectene in 100 µl EC buffer, and 5 µl of this mixture was added to each well of 384-well plates using a Perkin Elmer Mini-JANUS liquid dispenser (PerkinElmer, Waltham, MA). The plates were centrifuged at 16 G for 10 min. U2OS-
SRA-shNPC1 cells were prepared at 1.67x10^5 cells/ml in McCoy's 5A medium supplemented with 5% FBS, 1% P/S (medium A), and 5000 cells (30 µl of U2OS-SRA-shNPC1 cells in medium A) were plated in each well of 384-well plates using a Titertek Multidrop 384 model 832 liquid dispenser (Thermo Fisher Scientific Inc., Waltham, MA). Cells were grown with 30 µl medium A in a humidified incubator with 5% CO₂ at 37°C for 24 hours. After 24 hours cells were treated with Vorinostat (10 µM from a 5 mM stock in DMSO) or Panobinostat (50 nM from a 5 mM DMSO stock) for 48 hours. Control cells were treated with equivalent volumes of DMSO. The drugs in DMSO were diluted in medium A. To obtain final concentration of 10 µM for Vorinostat treatment, 30 µl of the premixed 20 µM Vorinostat in medium A was dispensed to each well using a Thermo Multi-Drop liquid dispenser. To obtain final concentration of 50 nM for Panobinostat treatment, 30 µl of the premixed 100 nM Panobinostat in medium A was added into each well of the plates containing cells. After 48 hours of treatment, cells were fixed with 1.5% PFA in PBS and stained with 50 µg/ml filipin in PBS (42). In order to load cells with cholesterol, cells were treated with 50 µg/ml AcLDL in medium A for 2 hours before fixation. All reagent additions, buffer changes and labeling with filipin were carried out robotically using a Thermo Multi-Drop liquid dispenser and a Bio-Tek Elx405 plate washer (Bio-Tek Instruments, Inc., Winooski, VT).

**Fluorescence Microscopy**

An automated ImageXpress Micro imaging system from Molecular Devices equipped with a 300W Xenon-arc lamp from Perkin-Elmer, a Nikon 10X Plan Fluor 0.3 numerical aperture (NA) objective, and a Photometrics CoolSnapHQ camera (1,392 x 1,040 pixels) from Roper Scientific was used to acquire images. Filipin images were acquired using 377/50 nm excitation and 447/60
nm emission filters with a 415 dichroic long-pass filter. GFP images were acquired using 472/30 nm excitation and 520/35 nm emission filters with a 669 dichroic long-pass (DCLP) filter. Filter sets assembled in Nikon filter cubes were obtained from Semrock.

**NPC1 lifetime measurements**

Wild type GM05659 and NPC1<sup>I1061T</sup> mutant GM18453 skin fibroblasts were plated in six well plates at 50,000 cells per well. On day two cells were treated with DMSO or Vorinostat for 48 hours in growth medium supplemented with 5.5% FBS. After 48 hours, cells were switched to cysteine/ methionine free medium supplemented with 2% dialyzed FBS for one hour followed by a one hour incubation with <sup>35</sup>S Cys/Met (150 µCi). In each experiment two wells were used for competition control, to which 10X excess cysteine and methionine as compared to that in normal medium were added. The cells were subsequently washed twice with HBSS and chased in regular growth medium for times between 0-32 hours. Cell lysates were prepared at each time point, after washing cells twice with HBSS and lysing in 500 µl non-denaturing lysis buffer (1% TritonX-100, 50 mM tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 10 mM iodoacetamide, 1 mM PMSF, 2 µg/ml leupeptin). Separately, antibody conjugated beads were prepared combining 50% protein A-Sepaharose bead slurry in ice-cold PBS and polyclonal rabbit antibody to NPC1 (6 µg/ml) and incubating the suspension at 4°C for 16 hours. Beads were washed three times with non-denaturing lysis buffer and resuspended in 500 µl non-denaturing lysis buffer supplemented with 0.1% BSA. The entire cell lysate from each well was incubated with 30 µl of NPC1 antibody conjugated Protein A sepharose beads in 500 µl in non-denaturing buffer for 2 hours at 4°C while mixing on tube rotator. Beads were centrifuged and washed three times with ice cold wash buffer (0.1% w/v triton X-100, 50 mM Tris-Cl, pH 7.4,
300 mM NaCl, 5 mM EDTA, 0.2% sodium azide). Beads were finally resuspended in 50 µl of 4X Laemmli SDS buffer and centrifuged. 40 µl of each supernatant was analyzed by electrophoresis on 4-12% Bis-Tris gels (Nupage NP0321, Life Technologies) followed by drying. Dried gels were exposed to a phosphor imager screen (Amersham Bioscience) overnight. The screen was scanned using a Typhoon Trio PhosphorImager (GE Healthcare), and the images were saved as tiff files. The integrated intensity of the bands at the molecular weight corresponding to NPC1 protein was measured using Metamorph image analysis software. Within each condition, the intensities of bands corresponding to NPC1 protein were first corrected for background by subtracting the intensity at the same position in the competition control lane. Background-corrected values at each time were normalized to the value with no chase.

**Western Blot:** Western blot analysis was performed on wild type and HDACi treated human NPC1 mutant fibroblasts using a primary polyclonal anti-rabbit NPC1 antibody from Abcam. Secondary antibodies were from Pierce.

**Imaging and analysis**

A method described previously (42) was used to quantify the cholesterol accumulation in lysosomal storage organelles. *NPC1* mutant cells show a bright region of filipin labeling near the cell center, corresponding to the sterol-loaded LSOs. The 384-well plates containing cells were imaged for GFP and filipin using a 10X 0.3 NA dry objective on an ImageXpressMicro automatic fluorescence microscope. Each well was imaged at four sites. Images were analyzed using MetaXpress image-analysis software. First, all images were corrected for slightly inhomogeneous illumination as described previously (42). A background intensity value was set as the fifth percentile intensity of each image, and this intensity value was subtracted from each
pixel in the image. At the plating density used in this study, all fields maintain at least 5% of the imaged area cell-free. To identify the cells expressing GFP, a threshold value was chosen for each experiment and was applied to GFP images. Objects containing at least 540 pixels above threshold were selected as transfected cells.

For the GFP-expressing cells, we obtained an LSO ratio, which is the ratio of filipin fluorescence intensity in the brightly labeled center of the cells divided by the area of the cells. The LSO ratio is determined using two thresholds that are applied to the filipin images. A low threshold is set to include all areas occupied by cells. The outlines of cells using the low threshold are similar to cell outlines in transmitted light images. A higher threshold is then set to identify regions brightly stained with filipin in cells. The thresholds were chosen for each experiment. The LSO ratio of transfected cells on a per image basis was determined using the equation below:

\[
\text{LSO Ratio} = \frac{\text{Total intensity above high thresholded filipin intensity in GFP positive cells}}{\text{Number of pixels above low thresholded filipin intensity in GFP positive cells}}
\]

Each NPC1 mutant was transfected in 12 wells of a 384-plate. Six wells were treated with drug 10 µM Vorinostat or 50 nM Panobinostat, and the other six were treated with DMSO as solvent control. WT-V and I1061T NPC1 were used as controls in each plate. There were three parallel plates for each experiment, and three independent experiments for each set of NPC1 mutants. The LSO ratio values for each experiment were normalized to the corresponding value for DMSO-treated I1061T NPC1 transfected cells. Normalized LSO ratio values from three independent experiments were averaged. The LSO ratio value for each NPC1 mutant with each treatment condition was averaged per image from 216 images (four sites × six wells × three
plates × three experiments). In 216 images, there were about 40 to 120 images with GFP positive cells. On average there were three GFP positive cells per image, so 120 to 360 NPC1 expressing cells were averaged to get the LSO ratio value for each NPC1 mutant with each treatment condition. A standard error of mean (SEM) was calculated for each LSO ratio value. A p-value comparing LSO ratio values in DMSO treated cells and drug treated cells was calculated using t-test with two-tailed distribution and two-sample equal variance (homoscedastic) type using Microsoft Excel software.

NPC1 immunolocalization in NPC1I1016T human fibroblasts and U2OS-SRA-shNPC1 cells expressing NPC1I1016T

WT human fibroblasts (GM05659), NPC1I1061T/P237S (GM03123), and NPC1I1061T (GM18453) human fibroblasts were treated with 10µM Vorinostat or DMSO solvent control for 48 hours. Cells were then incubated with 50 µg/ml Alexa546-LDL in MEM growth medium supplemented with 5.5% FBS and 10 µM Vorinostat or DMSO solvent control for 4 hours, rinsed with growth medium and chased in MEM growth medium supplemented with 5.5% FBS for 30 minutes. Cells were washed three times with PBS and then fixed with 1.5% PFA in PBS. For immunostaining cells were permeabilized with 0.5% saponin and 10% goat serum (GS) in PBS for 30 minutes. Cells were incubated with 0.8 µg/ml anti-NPC1 rabbit polyclonal primary antibody for two hours in the presence of 0.05% saponin and 0.5% GS at room temperature, followed by Alexa 488 labeled goat anti-rabbit secondary antibody (1:1000, Life Technologies, Grand Island, NY) for 45 min at room temperature. Finally, cells were washed three times with PBS, and images were acquired using a wide-field microscope with a 63X 1.32 NA oil immersion objective and standard FITC and TRITC filters.
U2OS-SRA-shNPC1 cells were transfected with WT-V or NPC1^{11061T}. As described in Methods, our wild type NPC1 construct has four variants as compared to the reference NPC1 sequence. Thus, we describe it here as WT-V. One day after transfection, cells were treated with 10 µM Vorinostat, 50 nM Panobinostat or DMSO solvent control. After 48 hours treatment, cells were fixed with 1.5% PFA and processed for immunofluorescence as above except that 180 ng/ml anti-NPC1 rat monoclonal antibody (produced in the Balch laboratory) and 1:500 anti-LAMP1 rabbit polyclonal antibody were used followed by 1:1000 dilution of Cy5 goat anti-rat and 1:500 of Alexa Fluor 546 goat anti-rabbit as secondary antibodies. Fluorescence images were acquired with a Zeiss LSM 510 laser scanning confocal microscope (Thornwood, NY) using a 63X 1.4 NA objective (axial resolution 1.0 µm).

Results

Protein lifetime measurements

Treatment with Vorinostat and other HDACi increases the expression of the NPC1^{11061T} protein in human patient-derived fibroblasts (19). Using procedures described in detail in the Methods section, we measured the effect of treatment with Vorinostat on the lifetime of newly synthesized NPC1 protein in fibroblasts with a homozygous NPC1^{11061T} mutation. WT and two sets of GM18453 cells were plated in 6 well plates on day one. On day two WT and one set of GM18453 cells were treated with DMSO and another set of GM18453 cells were treated with Vorinostat for 48 hours. After 48 hours, all cells were incubated in Cys/Met-free media for one hour followed by pulse-labeling for one hour with ^{35}S Cys/Met followed by a chase in complete medium for various times ranging from 0 – 32 hours. Lysates of cells were immunoprecipitated with anti-NPC1 specific antibody (Fig. S1) followed by gel electrophoresis and determination of radioactivity at the molecular weight of NPC1 (210 kDa). As described previously (26), a
substantial fraction of the WT NPC1 is degraded rapidly, and the remainder has a half time of over a day. **Fig. 1A** shows representative bands corresponding to the radiolabeled NPC1 protein from one experiment. For quantification the intensity of the bands were measured for each time and corrected for background by subtracting the intensities for competition control. Corrected intensities in each experiment were normalized to the corresponding initial value. As shown in **Fig. 1B**, nearly all of the NPC1\textsuperscript{I1061T} protein is degraded rapidly in untreated cells. When cells with the NPC1\textsuperscript{I1061T} mutation were pretreated for two days with Vorinostat (10 µM), the protein degradation profile was remarkably similar to that seen for the wild type protein. This indicates that treatment with an HDACi prevents the excessive degradation of the mutant protein.

**HDAC inhibitors lead to correct localization of mutant NPC1**

To see if the treatment with HDACi leads to correct targeting of the mutant protein, we examined the localization of mutant NPC1 protein in GM03123 (P237S & splice mutation, I1061T) (**Fig. 2A**) and in GM18453 (I1061T) (**Fig. 2B**) NPC1 mutant human fibroblasts. The cells were treated with 10 µM Vorinostat or solvent control DMSO for 48 hours. The cells were then incubated with Alexa546 LDL for 3.5 hours followed by a 0.5 hour chase to deliver labeled LDL to LE/Ly (47-49). The cells were fixed, and NPC1 protein was detected by immunofluorescence (**Fig. 2A and B**). Most of the mutant NPC1 protein does not co-localize with Alexa546 LDL in LE/Ly in the DMSO-treated cells. After treatment with Vorinostat, a large fraction of the mutant NPC1 protein localizes in LE/Ly (yellow/orange color in overlays). This indicates that treatment with Vorinostat leads to delivery of the mutant NPC1 to the organelles in which LDL is releasing cholesterol, which must be transported out of the organelles.
Testing the activity of Vorinostat after drug withdrawal

If the effect of HDACi is to improve delivery of mutant NPC1 protein to LE/Ly, the effect should persist after removal of the drug since the NPC1 will remain in the organelles for over a day. To test this, we performed Western blot analysis to determine the expression of NPC1 protein. GM18453 cells were plated in seven dishes. On the next day DMSO was added in one dish, and 10 µM Vorinostat was added in six other dishes. Individual dishes of Vorinostat-treated cells were lysed after 24, 48 and 72 hours. After 72 hours the Vorinostat-containing medium in the remaining three dishes was replaced by fresh growth medium. These dishes of cells were lysed after 24, 48 or 72 hours. All seven lysates were run on SDS-PAGE gel electrophoresis, and Western blot analysis was performed. Shown in Fig. 3A are the bands corresponding to NPC1 protein and the loading control GAPDH for each time point. The levels of expression of the NPC1 protein remained elevated for 2-3 days after drug withdrawal.

To determine if NPC1 is retained in LE/Ly following drug removal, cells were treated with DMSO or 10 µM Vorinostat for 72 hours followed by 72 hours in growth medium without Vorinostat. Cells were fixed and permeabilized followed by immunostaining for NPC1 and LAMP1 (Supplemental Figure S2). In DMSO treated GM18453 fibroblasts, the NPC1 labeling was very weak, and the NPC1 was not in punctate organelles containing LAMP1. After Vorinostat treatment for 72 hours, the intensity of NPC1 labeling was greatly enhanced, and the distribution colocalized well with punctate organelles containing LAMP1 (i.e., LE/Ly). This colocalization persisted for 72 hours after withdrawal of Vorinostat although the NPC1 labeling became somewhat weaker.
To measure the cholesterol storage after drug withdrawal, NPC1 mutant human fibroblasts GM03123 (Fig. 3B) and GM18453 (Fig. 3C) were treated with Vorinostat at varying concentrations for 72 hours. The cells were rinsed and incubated for an additional 0, 1, 2 or 3 days in normal growth medium without Vorinostat. At each time point, cells were fixed with PFA and stained with the cholesterol-binding dye, filipin. We applied a quantitative image analysis method that has been described previously (19, 42) to measure cholesterol storage in the cells. The filipin-labeled cholesterol in LE/Ly is described by a parameter referred to as an “LSO value”, which measures the filipin fluorescence per cell in regions corresponding to the lysosomal storage organelles (see Methods). A high LSO ratio is associated with high levels of cholesterol in LE/Ly. The LSO values were determined relative to the corresponding DMSO control treatment, and the normalized value was plotted as a function of Vorinostat concentration. Figs. 3B and C show that the activity of Vorinostat to reduce stored cholesterol was retained for 2-3 days after withdrawal of drug. The persistence was observed at both 3.3 µM and at 10 µM.

Treatment of several patient-derived human fibroblasts with Vorinostat

We have shown previously that treatment with several HDACi corrects the cholesterol accumulation in homozygous and heterozygous I1061T mutant cell lines (19). To see if Vorinostat would also correct other NPC1 mutations, we tested its effect on cholesterol accumulation in eight lines of patient-derived fibroblasts (Table 1). All of the cell lines responded to Vorinostat, but the dose response varied among the cell lines (Fig. 4A). Two cell lines (NPC1-17 and NPC1-25) had only partial reduction in cholesterol storage after 48 hours Vorinostat treatment, while the NPC1-22 cells were nearly completely corrected by
approximately 1 μM Vorinostat. These results indicate that Vorinostat treatment can correct the NPC1 phenotype in cell lines with several different compound heterozygous mutations. Similar results were obtained with Panobinostat, and NPC1-25 was also only partially responsive to Panobinostat as compared to the other cell lines tested (Fig. 4B). As observed previously (19), high concentrations of Panobinostat lead to higher levels of cholesterol storage. This probably reflects the effects of HDACi treatment on expression of many genes (50).

**Effect of HDACi in cells expressing NPC1 mutants**

To test the effect of HDACi on individual NPC1 mutations, we established a cell line that lacked expression of endogenous NPC1 and expressed scavenger receptor type A (SRA) to allow efficient uptake of lipoprotein-derived cholesterol. We stably transfected SRA into a human osteosarcoma cell line, U2OS cells, that have been used widely in drug screening trials (51, 52). These U2OS-SRA cells were then stably transfected with a plasmid expressing shRNA targeting a non-translated region of the NPC1 mRNA to silence the expression of endogenous NPC1. A library of disease-associated NPC1 mutants was created in a bicistronic vector also expressing GFP to identify the transfected cells. Using reverse transfection, different NPC1 mutants were transfected into individual wells of a 384-well plate. Half of the wells were treated with an HDACi (in DMSO), while the other half were treated with the same amount of DMSO as a solvent control. The NPC1 sequence used in these experiments contains four variants as compared to an NPC1 reference sequence, and we describe this as an NPC-V sequence (see Methods for details). The NPC-V was also used as the basis for constructing the individual mutations that were being analyzed. Vectors expressing WT-V NPC1 and NPC1<sup>1106T</sup> were
included in each plate as controls. A more complete description of the NPC-V variant and its use in previous studies will be published elsewhere.

**Fig. 5** illustrates the screening process. After transfection and drug treatment, cells were fixed and stained with filipin. Untransfected U2OS-SRA-shNPC1 cells incubated with 50 µg/ml AcLDL for 2 hours are brightly labeled with filipin (**Fig. 5A**), reflecting the high levels of unesterified cholesterol stored in the LSOs of these cells (19, 42, 53). Cells transfected with WT-V NPC1 (i.e., those expressing GFP) showed greatly reduced filipin labeling as compared to their untransfected neighbors (**Fig. 5A**). Cells transfected with NPC1\textsuperscript{I1061T} (expressing GFP) showed little, if any, decrease in filipin labeling as compared to their untransfected neighbors. However, treatment of these NPC1\textsuperscript{I1061T} transfected cells with either Vorinostat (10 µM) or Panobinostat (50 nM) for two days greatly reduced the filipin labeling of transfected cells with no discernible effect on the untransfected neighbors. This indicates that the NPC1\textsuperscript{I1061T} protein can effectively remove cholesterol from LSOs in cells treated with an effective dose of an HDACi. These results also show that treatment with an HDACi does not correct the cholesterol storage defect in cells lacking NPC1 expression.

To quantify the relative amount of cholesterol storage under various conditions, we measured the LSO values (19, 42). **Fig. 5B** shows the quantitative analysis of the effects of transfecting U2OS-SRA-shNPC1 with WT-V NPC1 or NPC1\textsuperscript{I1061T}. Expression of the NPC1\textsuperscript{I1061T} mutant only caused a significant decrease in the LSO value in cells treated with an HDACi. No significant effect on the LSO value was seen in untransfected U2OS-SRA-shNPC1 cells treated with the HDACi.

As with the human patient fibroblasts (**Fig. 2**), treatment of U2OS-SRA-shNPC1 cells expressing NPC1\textsuperscript{I1061T} with an HDACi leads to delivery of the mutant NPC1 to LE/Ly labeled...
with antibodies to the abundant lysosome associated membrane protein, LAMP1 (54) (Fig. 6). When U2OS-SRA-shNPC1 cells were transfected with WT-V NPC1 (top row), there was good colocalization of the NPC1 (detected by immunofluorescence) with the LAMP1, indicating that a high fraction of the NPC1 protein is in LE/Ly. When cells were transfected with NPC1I1061T (second row), there was little colocalization of NPC1 with LAMP1, indicating a failure to deliver the mutant NPC1 to LE/Ly. However, if the cells transfected with NPC1I1061T were treated with Vorinostat (third row) or Panobinostat (fourth row), there was significant colocalization of the mutant NPC1 with the LAMP1 as seen by the yellow/orange organelles in the overlays. An untransfected cell lacking GFP expression in the third row does not show labeling with the rat monoclonal NPC1 antibody, verifying the specificity of the antibody (32).

The full length NPC1 protein has 13 transmembrane domains and three large luminal domains. These are clustered into six different groups (Fig. 8). As described in Methods, 60 different NPC1 mutants that have been found in patients were transiently transfected into U2OS-SRA-shNPC1 cells. Using the assay for filipin labeling, we tested the effect of Vorinostat (10 \( \mu \)M) and Panobinostat (50 nM) on cells expressing these 60 different NPC1 mutations (Fig. 7 and Supplemental Fig. S3). With Vorinostat or Panobinostat treatment, 52 of the 60 mutants showed a reduction of the LSO Value with \( p < 0.05 \) (blue bars). (Mutants that showed no change or reduction of LSO value with \( p > 0.05 \) are plotted in red/pink. As expected, the LSO values for WT-V transfected cells were not reduced significantly after HDACi treatment as shown by first set of red bars in each plot.

The results are summarized in Fig. 8. Mutants that showed a statistically significant (\( p < 0.05 \)) response to Vorinostat are listed in black, and nonresponsive mutants are listed in red. Over 85% of the mutations showed a statistically significant response to either of the HDACi
treatments. Some of the mutants that were not corrected to a statistically significant level had low starting cholesterol, which made it more difficult to demonstrate an effect. Expression of these mutants may have had some corrective effect even before HDACi treatment. In general the data shown in Fig. 7 indicate that there is not a strong correlation between the LSO value before treatment and the correction seen after HDACi treatment. After HDACi treatment, many of the mutants that showed high cholesterol storage before treatment were corrected to nearly the level in WT-V transfectants.

A full listing of the mutants and the statistical significance of their responses is provided in Supplemental Table S1. To see if correction depended on the severity of the initial cholesterol accumulation, we compared the LSO values of DMSO-treated and Vorinostat or Panobinostat treated cells. It can be seen that most of the mutations with the highest initial cholesterol storage were corrected.

Discussion

Niemann-Pick C disease is a devastating inherited disorder with no US FDA approved treatment. More than 300 disease causing mutations have been found in NPC1 (10, 37), and most of these are mis-sense mutations in this large membrane protein. As part of a screen for compounds that would correct the cholesterol storage defect in patient-derived fibroblasts, we found that several HDACi compounds were included among the hits (19). The pharmacological profile of the effective inhibitors suggested that HDACs 1, 2, or 3 were the most relevant targets (19, 20). Although increased expression of the NPC1I1061T protein was observed following treatment with HDACi, the mechanism of correction and the applicability to a variety of mutations were not determined.
It has been shown previously that nearly half of the wild type NPC1 protein is degraded by an ERAD pathway, and essentially all of the newly synthesized NPC1^{I1061T} protein is degraded by ERAD (26). In this study we found that treatment of cells with Vorinostat causes the stability of the NPC1^{I1061T} protein to be indistinguishable from the wild type protein. The longer-lived fraction of both wild type and HDACi-treated NPC1^{I1061T} are degraded with a halftime of over one day. We also observed that the NPC1^{I1061T} protein is correctly delivered to LE/Ly that contain endocytosed LDL, so after exit from the ER a significant amount of the mutant protein is trafficked to the correct organelle. The effect of HDACi treatment on cholesterol storage in LSOs persists for 2-3 days after withdrawal of the drug, which is consistent with the long lifetime of the NPC1^{I1061T} protein after it has passed the ER quality control system. The long term effect of the HDACi after removal from the medium may be useful in designing clinical protocols since drug may only have to be administered intermittently.

HDACi treatments corrects the cholesterol storage in cells expressing a large majority of NPC1 mutations

In order to test the effect of HDACi treatment on a large number of mutants, we used a reverse transfection strategy that could be used for screening in multiwell plates (55). The U2OS-SRA-shNPC1 cells were developed for this purpose. The stable expression of SRA was valuable since it allowed us to deliver a large amount of cholesterol to the cells via AcLDL, which binds to SRA and is internalized by receptor-mediated endocytosis (56). Cells with defects in the NPC1 protein were unable to clear this bolus of cholesterol from the LE/Ly.

It was shown previously that very high levels of over-expression of the NPC1^{I1061T} protein can correct the cholesterol storage defect in cells, presumably because a small fraction of
the protein gets into the LE/Ly (26). We did not measure expression levels of the various mutants, but we did find that most mutants did not correct the cholesterol storage in the absence of drug treatment. We observed that more than 85% of the NPC1 mutants could be corrected for their cholesterol accumulation by treatment with either Vorinostat or Panobinostat. This indicates that more than 90% of patients would be expected to have at least one allele that is susceptible to HDACi treatment. Mechanistically, this suggests that the large majority of NPC1 mis-sense mutations can be functional if they are delivered to the correct organelles.

The analysis of the mutants that can and cannot be corrected provides some insight into the requirements for a functional NPC1 protein. One caveat is that these assays were carried out in a high throughput screening system, and they have not been individually verified in detail. However, analysis of the data provides some measures of quality control. First, it should be noted that there is a good general correspondence between the mutations that were affected by Vorinostat and those affected by Panobinostat. Some of the mutants that were listed as corrected by one drug but not the other were only statistically significant at \( p < 0.05 \) (Supplementary Table 1), suggesting that these might have been only partially corrected by one or both HDACi.

**Mutations resistant to HDACi treatment**

The lack of effect of both Vorinostat and Panobinostat is consistent with the lack of an effect of HDACi treatment on cells lacking NPC1 expression. One of the non-corrected mutations (L80V) is in the N-terminal domain, which is responsible for cholesterol binding. The leucine at position 80 is close to the cholesterol binding pocket (4). NPC2 binds to the first lumenal loop (Group 3 in **Fig. 8**) of NPC1, and it has been shown that mutations R404Q and R518Q interfere with this binding (57). Neither of these mutations was corrected by Vorinostat, and R518Q is not corrected by Panobinostat. It has been shown that NPC1\(^{R518Q}\) goes to punctate
organelles in a distribution consistent with LE/Ly without drug treatment (57). The P691S mutation is in the sterol sensing domain of NPC1 (35). It has been shown that this mutation is ineffective in promoting cholesterol transport even though it is delivered correctly to LE/Ly (58). It is noteworthy that all of the mutations in Group 5 can be rescued by both Vorinostat and Panobinostat. Apparently this segment of NPC1 can tolerate many mutations without losing the ability to export cholesterol from LE/Ly.

Mechanism of correction by HDACi

The mechanism by which HDACi treatment rescues the function of mutant NPC1 proteins will require further mechanistic studies. One possibility is that HDACi treatment alters the proteostatic environment (30-32). This would allow more of the mutant protein to escape ERAD, as is illustrated for NPC1I1061T in Fig. 1. It has been reported that HDACi treatment can increase expression of some protein chaperonins (59) or directly modulate the activity of chaperones by altering their acetylation status (60). As discussed in the Introduction, treatments that would be expected to enhance NPC1 folding in the ER such as chemical chaperones or treatments that alter ER chaperone activity have been shown to correct the storage defect in some NPC1 mutant cell lines (27-29).

An indirect mechanism for overcoming the cholesterol accumulation caused by mutations in NPC1 is consistent with findings that patients with identical mutations can have different times to initial onset and disease severity (10). This suggests that other factors in the genetic background can alter the degree of loss of function. It is not known if this is related to higher levels of correct targeting of mutant NPC1 protein in the tissues of less affected individuals.
Our data suggest that the vast majority of NPC1 patients carry mutations that could be corrected by HDACi treatment. Vorinostat and Panobinostat are FDA-approved drugs for treatment of some cancers, and HDACi have been used in clinical trials for many other diseases (20). Vorinostat is generally well tolerated as a cancer therapeutic agent (61). Several other HDACi have been in large scale clinical trials. Since the most important pathologies of NPC disease are related to neuronal cell dysfunction and death (10), penetration of an HDACi into the brain will be essential for effective treatment. Some HDACi have excellent penetration into the brain and have been used in animal and human studies for treatment of neurological diseases (20). The results described herein support the investigation of HDACi as single or combination therapies (34) for NPC1 disease.

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Table 1: List of mutations in NPC1 patient fibroblasts

| Sample | Mutation Details |
|--------|------------------|
| GM03123 | Heterozygous I1061T/ P237S |
| GM18453 | Homozygous I1061T |
| NPC1-2 | V1165M/ 3741-44 del ACTC |
| NPC1-5 | I1061T/ R1186G |
| NPC1-16 | P887L/ 3741-44 del ACTC |
| NPC1-17 | I1061T, 10 bp deletion in exon 19 at codon 962=fs(exon19) |
| NPC1-19 | 1920delG/ IVS9-1009G>A |
| NPC1-22 | R978C, IVS21-2 A>G |
| NPC1-25 | N701K/ C2979 dupA |
| NPC1-31 | G46V/ L491P |
Fig. 1: NPC1 protein lifetime measurements. After 48 h treatment with 10 μM Vorinostat (or DMSO solvent control), GM05659 (WT) or GM18453 (NPC1^{I1061T}) fibroblasts were incubated for one hour with Cys/ Met free culture medium supplemented with [^{35}S] Cys/Met followed by a 0–32 h chase in normal culture medium. At the end of each chase time, cells were lysed, and cell lysates were immunoprecipitated (IP) with anti-NPC1 antibody. Protein antibody conjugates were resuspended in 50 μl of 4X Laemml SDS buffer, vortexed and centrifuged 40 μl was loaded on 4-12% Bis-Tris gels. Protein samples were separated by SDS-PAGE, and the densitometry values corresponding to radiolabeled NPC1 were measured. For each experiment the values were normalized to the zero chase time value. A. Immunoprecipitated NPC1 bands as observed on Phosphorimager screen. Left lanes for each condition show (0h+Competition control) which were used to correct for background noise. Duplicate samples for each time points in each experiment were set. Images shown are from one of five independent experiments. For each condition and cell type samples were run on three separate gels as outlined by yellow bounding boxes. Images shown were from the gels run on the same day in parallel B. Plot of the data are shown for solvent-treated GM05659 cells (blue diamonds), solvent-treated GM18453 cells (red squares), and Vorinostat-treated GM18453 cells (green triangles). Each data point is representative of ten values from five independent experiments ± SEM.
Fig. 2: Vorinostat rescues the localization of NPC1 in NPC1 mutant fibroblasts. GM03123 A. or GM18453 B. cells were plated in poly-D-lysine coated cover-slip bottom dishes in MEM growth medium supplemented with 5.5% FBS. On day 2 cells were treated with DMSO or 10µM Vorinostat and incubated for 48 hours. For the last 4 hours, cells were incubated with 50 μg/ml Alexa546 labeled LDL followed by a 30 minute chase in normal medium. Cells were fixed with 2% PFA, permeabilized and immunostained for NPC1 with a rabbit anti-NPC1 antibody. Images were acquired on Zeiss LSM880 confocal microscope using a 63X objective. Images shown are maximum intensity projections. In B the region outlined in the Overlay images is shown at higher magnification in the inset. Scale bar = 10 µm.
Fig. 3: Persistent effect of HDACi treatment in NPC1 mutant human fibroblasts.

A. Immunoblot of cell lysates from GM18453 cells treated for 24, 48 or 72 hours with 10 µM Vorinostat, followed by incubation for an additional 24, 48 or 72 hours in the absence of Vorinostat. Lysates were blotted with rabbit anti-NPC1 antibody and anti-GAPDH. Bands at 190 and 210 kDa represent glycosylated NPC1 protein. GM03123 B. or GM 18453 C. Cells were treated with Vorinostat at varying concentrations for 72 hours. The cells were then incubated for an additional 0, 1, 2 or 3 days without Vorinostat in normal growth medium. At the end of each time point cells were stained with filipin, and the LSO value was measured to determine the relative amount of stored cholesterol. Data for each cell line are from two independent experiments, and each data point is obtained using 48 images. Each data point is normalized to its corresponding DMSO-treated condition, so the value of one represents no effect. Error bars: SEM.
Fig. 4. Dose dependent effect of Vorinostat and Panobinostat on multiple patient derived NPC1 mutant cell lines. NPC1 mutant human fibroblasts were treated with A. Vorinostat. or B. Panobinostat for 48 hours followed by fixation, staining with filipin and imaging using the ImageXpress Micro automatic fluorescence microscope. DMSO was used as a solvent control. Images were analyzed to obtain the LSO value as a measure of cholesterol accumulation. Data were normalized to the corresponding DMSO treated cells. Data for each cell line are averages of three independent experiments totaling 60 images (5 wells x 4 sites x 3 experiments). Error bars: SEM.
**Fig. 5.** **Illustration of the screening system.**

**A. Representative images of transfected cells.** 24 hours after transfection with a bicistronic vector containing eGFP plus WT-V-NPC1 or NPC1\textsuperscript{I1061T}, U2OS-SRA-shNPC1 cells were treated with Vorinostat (10 µM), Panobinostat (50 nM), or DMSO solvent control for 48 hours. Cells were treated with 50 µg/ml AcLDL for the final 2 hours, fixed with PFA, and stained with filipin. Images were acquired on a Leica wide-field microscope using standard GFP and A4 filters. Transfection with wild type NPC1 reduces the cholesterol accumulation, but transfection with NPC1\textsuperscript{I1061T} does not unless they are treated with an HDACi.

**B. Quantification of filipin in transfected cells.** Cholesterol accumulation in LSO of GFP positive cells was measured based on filipin fluorescence, and the LSO values are shown. As described in Methods, images were obtained from 216 fields in three experiments for each condition. Between 40-120 of these images had GFP-labeled cells, and the LSO value was obtained for each of these images. In each experiment the LSO values were normalized to the solvent-treated control. These normalized LSO values were used for statistical analysis. Error bars: SEM. **p < 0.01.
Fig. 6. Vorinostat and Panobinostat rescue localization of NPC1<sup>I1061T</sup> in U2OS-SRA-shNPC1 cells.

WT-V NPC1 or NPC1<sup>I1061T</sup> was expressed in U2OS-SRA-shNPC1 cells using a bicistronic vector also encoding eGFP. Starting one day later, wells transfected with NPC1<sup>I1061T</sup> were treated with 10 µM Vorinostat, 50 nM Panobinostat or DMSO solvent control for 48 hours. GFP serves as a marker of transfected cells. NPC1 protein was detected by immunofluorescence using anti-NPC1 Rat monoclonal primary antibody and Cy5 Goat anti-rat secondary antibody. Lysosomes were identified by using anti-LAMP1 rabbit polyclonal antibody and Alexa Fluor 546 goat anti-rabbit secondary antibody. The co-localizations of NPC1 protein and LE/Ly are shown in yellow. Scale bar = 10 µm.
Group 1 - Vorinostat Treatment

Group 2 - Vorinostat Treatment

Group 3 - Vorinostat Treatment

Group 4 - Vorinostat Treatment

Group 5 - Vorinostat Treatment

Group 6 - Vorinostat Treatment
Fig. 7. Quantification of effects of Vorinostat on cholesterol accumulation in NPC1 mutants.

The effect of 10 μM Vorinostat was tested on 60 different NPC1 mutations from five segments of the NPC1 protein as illustrated in Figure 8. DMSO was used as a solvent control. Filipin fluorescence images of the transfected cells were analyzed to obtain an LSO value as explained in methods. Data represent averages ± SEM from 15-25 images. Each image includes about 1-5 transfected cells. Dark blue (DMSO treated) and light blue bars (Vorinostat treated) for each group represent mutants that showed reduction in LSO values with p<0.05, and dark red (DMSO treated) and pink bars (Vorinostat treated) represent mutants that showed no significant reduction in LSO value. Statistical significance was measured by t-test with two-tailed distribution and two-sample equal variance (homoscedastic) type using Microsoft Excel software.
Figure 8. List of NPC1 mutants tested with Vorinostat and Panobinostat

60 NPC1 mutants were treated with Vorinostat (10 µM) or Panobinostat (50 nM) as described in Fig. 7. The mutants are separated into 6 groups by their locations in the NPC1 protein structure. Mutants that showed a statistically significant (p < 0.05) response to Vorinostat are listed in black, and nonresponsive mutants (p > 0.05) are in red.