An “Exacerbate-reverse” Strategy in Yeast Identifies Histone Deacetylase Inhibition as a Correction for Cholesterol and Sphingolipid Transport Defects in Human Niemann-Pick Type C Disease*(*5*)

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Niemann-Pick type C (NP-C) disease is a fatal lysosomal lipid storage disorder for which no effective therapy exists. A genome-wide, conditional synthetic lethality screen was performed using the yeast model of NP-C disease during anaerobiosis, an auxotrophic condition that requires yeast to utilize exogenous sterol. We identified 12 pathways and 13 genes as modifiers of the absence of the yeast NPC1 ortholog (NCR1) and quantified the impact of loss of these genes on sterol metabolism in ncr1Δ strains grown under viable aerobic conditions. Deletion of components of the yeast NuA4 histone acetyltransferase complex in ncr1Δ strains conferred anaerobic inviability and accumulation of multiple sterol intermediates. Thus, we hypothesize an imbalance in histone acetylation in human NP-C disease. Accordingly, we show that the majority of the 11 histone deacetylase (HDAC) genes are transcriptionally up-regulated in diseases (1). Two strikingly conserved genes encode proteins that when defective result in NP-C7 disease, a fatal pediatric neurodegenerative disease caused by a lysosomal accumulation of cholesterol and sphingolipids. The clinical hallmark of NP-C disease, for individuals that survive past the neonatal stage, is progressive dementia with markedly reduced executive function and global cognitive impairment, typically culminating in loss of life before adolescence. NP-C disease is conferred by mutations in either the NPC1 gene (95% of cases) or NPC2 gene (5% of cases). NPC1 encodes an ~13-pass lysosomal transmembrane domain protein, and NPC2 encodes a soluble low molecular weight luminal protein in the lysosome. Both proteins possess lipid-binding domains and have enigmatic functions as putative lipid transporters or chaperones (2, 3). NPC2 likely “hands off” cholesterol to NPC1 (4), but the interactions of this pathway before and after this event are unknown. As a result, treatments are currently experimental and limited to the suspected targets, i.e. modulating the metabolism of cholesterol (5) or sphingolipids (6). Approved therapies for NP-C disease, particularly in the United States, are thus limited to palliative treat-

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ment of the symptoms, and there is a striking clinical need for a novel intervention in what is an invariably fatal disease.

There is no doubting the conservation of NP-C disease across evolution. The mammalian and yeast NPC orthologs are interchangeable to the extent that the yeast orthologs of NPC1 and NPC2 complement lesions in the corresponding genes in cultured mammalian cells (7, 8). There is 35% amino acid sequence identity between the human and yeast NPC1 orthologs, and both proteins localize to the limiting membranes of the lysosomal (vacuolar)/endosomal systems. Moreover, 43% of missense mutations and 15% of missense polymorphisms in NPC1 in NP-C patients are at evolutionarily conserved residues (9). Together, these data suggest an evolutionarily conserved function is disturbed in NPC patients. Model organisms thus have the potential to identify conserved modifiers of the disease. Investigation of the function of NP-C disease orthologs is active in many model systems, including murine, feline, nematode, insect, and yeast (reviewed in Ref. 10). Given the NP-C disease phenotypes of lysosomal lipidosis and neurodegeneration, the ideal modifier in terms of a treatment strategy would be one with the capacity to impact multiple aspects of lipid transport and be amenable to compounds that cross the blood-brain barrier.

Here, we report the results of an unbiased genome-wide screen to identify genetic modifiers of NP-C disease. We exploited the yeast model of NP-C disease (7), a strain in which NCR1 (a functional and structural ortholog of human NPC1) is deleted ([nrr1Δ]). Our aim was to identify genes and pathways that exacerbate lethality of the yeast model of NP-C disease and then pharmacologically manipulate the orthologous pathways in the opposite direction as a potential therapeutic strategy in human cells. We present this “exacerbate-reverse” strategy to find new targets for disease intervention. Using this approach, we identify histone deacetylase inhibition as a potential therapy for NP-C disease and in particular identify suberoylanilide hydroxamic acid (SAHA) as a Food and Drug Administration-approved, brain-penetrative, lead candidate.

**EXPERIMENTAL PROCEDURES**

**Anaerobic Synthetic Genetic Arrays**—All yeast strains used in this study were derived from BY4741. The query strain for all synthetic genetic array analyses was Y3656 (MATa can1Δ::MFA1pr-HIS3-MFa1pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 lys2Δ0 (11)) with the addition of nrr1Δ::NAT8, which was introduced by PCR-mediated gene disruption (12). Synthetic genetic array analysis was performed as described previously (11, 13). Growth of each double mutant was quantitatively assessed with Anaero Packs (Mitsubishi). To validate interactions, we introduced by PCR-mediated gene disruption (12). Synthetic genetic array analysis was performed as described previously (11), we generated 5,500 haploid double mutants with the parental single mutants and BY4741 in the presence and absence of oxygen.

**Gene Annotations**—Biological processes of interacting yeast genes were derived from the on-line Saccharomyces Genome Database. Orthologous human proteins and complexes were identified using Princeton Protein Orthology Database (43).

**Sterol Profiles**—Liquid cultures (100 ml) of the indicated yeast strains were grown to an A600 of 1.0 in YPD, pelleted, and frozen at −80 °C. Sterols were separated and quantified by GC-MS as described previously (15).

**Cell Culture**—All fibroblasts were cultured in DMEM supplemented with 10% FBS, 2 mMEDTA, and 50 μg/ml gentamicin in a humidified incubator at 37 °C with 5% CO2. Human control fibroblasts (GM06114) were obtained from Coriell Cell Repositories. The NPC-26, NPC-2, and NPC-29 mutant human fibroblasts were obtained from the National Institutes of Health; these fibroblasts were derived from unrelated patients with genetically distinct compound heterozygous missense mutations in NPC1. The NPC-J4 fibroblast line was obtained from a skin biopsy of a Japanese patient homozygous for the NPC1 H510P allele (16).

**Drugs**—SAHA (Cayman), MC1568 (Selleck), MGCD0103 (Selleck), and trichostatin A (Sigma) were applied to fibroblasts for 18 h. Concentrations used were previously described to achieve global (SAHA, trichostatin A) or class-specific (MC1568, MGCD0103) inhibition of HDAC genes.

**Cholesterol and Sphingolipid Trafficking**—Control and NPC1-deficient human fibroblasts were assessed for trafficking of cholesterol and sphingolipids. Cholesterol and the glycosphingolipid globotriosylceramide (GL-3) accumulation were monitored using filipin (Polysciences) or verotoxin, respectively, as described previously (17, 18). The trafficking of lactosylceramide was monitored using BODIPY-LacCer; cells in a glass bottom dish (Iwaki, Japan) were washed with Hanks’ buffered saline solution, incubated with 5 μM BODIPY-LacCer with BSA complex (Molecular Probes) for 30 min at 4 °C, and incubated with DMEM 10% FBS at 37 °C for 30 min as described in the manufacturer’s protocol. For detection of ganglioside GM1, cells in glass bottom dish were washed with DMEM, 25 mM Hapes, pH 7.4, and 0.01% BSA, incubated with 20 nM Alexa Fluor 555-conjugated cholera toxin subunit B (Molecular Probes) for 1 h at 37 °C, and fixed with 4% paraformaldehyde (7). Images were obtained sequentially using confocal laser microscopy, and quantification analysis was performed using ImageJ64 or MetaVue (17).

**Esterification of LDL-derived Cholesterol**—Human fibroblasts were assessed for esterification of LDL-derived cholesterol in 4 h as described previously (19).

**Gene Expression**—Quantitative real time PCR was performed using a MyiQ machine (Bio-Rad) and SYBR Green chemistry (Bio-Rad) to measure gene expression. The sequences of the primers used in these determinations are listed in supplemental Table S1. The mRNA levels were expressed relative to GAPDH and were calculated by the ΔΔCT method (20).

**RESULTS**

**Genome-wide Screen for Modifiers of NP-C Disease in Yeast**—To identify genetic targets with NP-C disease-modifying potential, we performed a genome-wide screen for synthetic lethality in the yeast model of NP-C disease. Using synthetic genetic array methodology (11), we generated ~5,500 haploid double mutants of nrr1Δ with deletions in ~4,800 nonessential genes or knockdown alleles (21) of ~700 essential genes (corresponding in total to ~92% of the yeast genome). Under standard aer-
Histone Acetylation and Lipid Homeostasis

| gene        | aerobic (normal) | anaerobic (sterol auxotrophy) |
|-------------|-----------------|-------------------------------|
| control     |                 |                               |
| ncr1Δ       |                 |                               |
| eaf1Δ       |                 |                               |
| ncr1Δ eaf1Δ |                 |                               |
| yaf9Δ       |                 |                               |
| ncr1Δ yaf9Δ |                 |                               |
| pah1Δ       |                 |                               |
| ncr1Δ pah1Δ |                 |                               |
| tir3Δ       |                 |                               |
| ncr1Δ tir3Δ |                 |                               |
| ctk1Δ       |                 |                               |
| ncr1Δ ctk1Δ |                 |                               |
| chs5Δ       |                 |                               |
| ncr1Δ chs5Δ |                 |                               |
| bem2Δ       |                 |                               |
| ncr1Δ bem2Δ |                 |                               |
| kgd1Δ       |                 |                               |
| ncr1Δ kgd1Δ |                 |                               |
| pho80Δ      |                 |                               |
| ncr1Δ pho80Δ|                 |                               |
| sec66Δ      |                 |                               |
| ncr1Δ sec66Δ|                 |                               |
| pmp3Δ       |                 |                               |
| ncr1Δ pmp3Δ |                 |                               |
| dbf2Δ       |                 |                               |
| ncr1Δ dbf2Δ |                 |                               |
| sin4Δ       |                 |                               |
| ncr1Δ sin4Δ |                 |                               |

The interaction screen was clearly successful in that it identified several pathways already associated with lipid metabolism and/or NP-C disease. For example, PHO80 encodes a regulatory component of the yeast cyclin-dependent kinase PHO85, of which the mammalian ortholog (CDK5) is dysregulated in the murine model of NP-C disease (22). Similarly, the CHS5 gene encodes a component of a Golgi to plasma membrane transport pathway that is also defective in the transport of cholesterol in NP-C disease (23). Among the remaining 11 genes, PAH1 encodes a key enzyme (phosphatidic acid phosphohydrolase) in glycerophospholipid metabolism (24), and BEM2 has been previously implicated in sterol uptake (25). Nine of the 13 genes are conserved in humans and thus may be important in modifying NPC1 disease pathogenesis. Notably, deletion of either EAF1 or YAF9, both of which are components of the NuA4 histone acetyltransferase complex, confer anaerobic inviability to ncr1Δ strains (Fig. 1). Compellingly, this was the only instance of multiple interactions in our dataset that are components of the same complex. Interestingly, mutations in EAF1 or YAF9 have enlarged vacuoles (26), the equivalent of the mammalian lysosome in yeast. EAF1 encoded activity has also been shown to regulate Golgi-vacuole vesicle-mediated transport (26).

Sterol Metabolism in the Yeast NP-C Model Is Impacted by Modifier Gene Deletions—The anaerobic inviability of the double mutants indicates a genetic interaction, putatively dependent on exogenous sterol utilization. Inviability in the screen may be a consequence of alterations in fatty acid metabolism due to unsaturated fatty acid auxotrophy also associated with anaerobiosis. We therefore examined aerobic growth of the double mutants in the presence of fatty acids and sterols, a state

| Gene   | Biological process            | Orthologous human protein-complex |
|--------|--------------------------------|----------------------------------|
| EAF1   | Histone acetyltransferase      | p400                             |
| YAF9   | Histone acetyltransferase      | GAS41                            |
| PAH1   | Phosphatidic acid hydrolase    | Lipins                           |
| TIR3   | Unknown                        | None                             |
| CTK1   | Phosphorylation of RNA polymerase II | CDK13                        |
| CHS5   | Golgi to plasma membrane transport | Neurofilament H                |
| BEM2   | Cytoskeleton organization      | ARHGAP24                         |
| KGD1   | 2-Oxoglutarate metabolism in TCA cycle | OGDH                        |
| PHO80  | Cyclin-dependent protein kinase | CDK5 complex                      |
| SEC66  | Protein targeting and import   | SEC63 complex                     |
| PMP3   | Regulation of membrane potential | None                           |
| DBF2   | Stress-response kinase         | None                             |
| SIN4   | Transcription from RNA polymerase II promoter | None                          |

FIGURE 1. Deletion of 13 genes confers significant growth defects to the ncr1Δ yeast model of NP-C disease as a consequence of sterol auxotrophy. Haploid double deletions were derived from a cross of ncr1Δ and the indicated haploid single gene deletions. 5-Fold dilutions (left to right) of saturated, aerobically grown cultures of the single mutants and their derived double mutant were grown at 30 °C aerobically on SCD and anaerobically on SCD + 20 μg/ml ergosterol + 0.5% Tween 80 for 3 days.
that permits uptake and metabolism of the fatty acids but not sterol. The growth of all double mutants was normal under this condition (data not shown), a result that further implicates sterols as the toxic metabolites in the screen. To assess the impact of the 13 NCR1 modifiers on sterol levels in ncr1Δ/H9004 strains, we used GC-MS to quantify endogenous levels of sterols in the 13 double deletions and a control strain under aerobic conditions in which the double deletions are viable. Compared with the control, three double deletions (ncr1Δ eaf1Δ, ncr1Δ sin4Δ, and ncr1Δ pah1Δ) displayed a 300% increase in at least one sterol intermediate (Table 2). Only ncr1Δ eaf1Δ strains exhibited an increase in more than one sterol. We further quantified the sterol accumulation in ncr1Δ eaf1Δ cells, this time relative to the parental strains. ncr1Δ eaf1Δ strains exhibited statistically significant increases in the intracellular accumulation of squalene, lanosterol, zymosterol, and fecosterol and a significant

**TABLE 2**

Aerobically viable sterol profiles of the 13 anaerobically sensitive interactions distinguish three double mutants with >300% increases in intracellular accumulation of sterols

Cells of the control strain and all double mutants indicated in Fig. 1 were grown in triplicate under standard aerobic conditions in YPD at 30 °C to 100 OD units. Sterols were extracted as described and measured by GC-MS. The data are expressed here for each double mutant as a percentage relative to levels in the control strain.

| Percent sterol relative to control | Squalene | Lanosterol | 4,4-Dimethylzymosterol | Zymosterol | Fecosterol | Episterol + Ergosta-5–7-dien-3β-ol | Ergosterol |
|-----------------------------------|----------|------------|------------------------|------------|------------|-----------------------------------|------------|
| Control                           | 100.00   | 100.00     | 100.00                 | 100.00     | 100.00     | 100.00                            | 100.00     |
| ncr1Δ eaf1Δ                      | 411.95   | 343.50     | 102.83                 | 81.04      | 142.41     | 101.39                            | 76.83      |
| ncr1Δ yaf9Δ                      | 83.12    | 85.16      | 27.88                  | 85.86      | 160.54     | 65.92                             | 102.06     |
| ncr1Δ pah1Δ                      | 216.30   | 84.96      | 335.85                 | 92.59      | 97.48      | 98.48                             | 100.00     |
| ncr1Δ tir3Δ                      | 168.92   | 117.95     | 98.02                  | 88.91      | 130.31     | 70.06                             | 109.04     |
| ncr1Δ ctk1Δ                      | 160.38   | 87.85      | 119.07                 | 71.29      | 84.61      | 132.47                            | 97.89      |
| ncr1Δ chs5Δ                      | 136.78   | 73.54      | 98.26                  | 102.77     | 91.45      | 123.44                            | 93.37      |
| ncr1Δ ben2Δ                      | 111.99   | 87.67      | 73.74                  | 107.01     | 160.80     | 95.66                             | 89.45      |
| ncr1Δ kgd1Δ                      | 116.76   | 165.44     | 203.08                 | 114.59     | 116.45     | 125.14                            | 88.83      |
| ncr1Δ pho80Δ                     | 74.31    | 111.19     | 76.25                  | 100.29     | 118.95     | 97.22                             | 95.95      |
| ncr1Δ sec66Δ                     | 215.20   | 129.25     | 107.31                 | 91.59      | 94.24      | 102.12                            | 98.70      |
| ncr1Δ pmp33Δ                     | 203.23   | 119.36     | 94.26                  | 110.48     | 126.92     | 114.15                            | 92.42      |
| ncr1Δ dbf2Δ                      | 159.74   | 62.26      | 125.85                 | 141.56     | 207.83     | 86.23                             | 76.98      |
| ncr1Δ sin4Δ                      | 529.54   | 183.34     | 117.19                 | 141.56     | 207.83     | 86.23                             | 76.98      |

**FIGURE 2.** Sterol metabolism is disturbed in yeast ncr1Δ eaf1Δ cells. A, analysis of ncr1Δ eaf1Δ strains identifies a bottleneck in aerobic sterol synthesis with increased intracellular accumulation of ergosterol precursors and decreased ergosterol. Cells were grown in triplicate under aerobic conditions in YPD at 30 °C to 100 OD units. Sterol biosynthetic intermediates were measured by GC-MS and are expressed as a percentage of total sterols. *, p < 0.05, two-tailed Student's t test comparison of ncr1Δ eaf1Δ cells with control, ncr1Δ, or eaf1Δ strains. B, sensitivity of ncr1Δ eaf1Δ to fluconazole and nystatin. 5-Fold dilutions (left to right) of saturated, aerobically grown cultures were grown aerobically at 30 °C for 2 days in the presence of the indicated drug.
decrease in ergosterol relative to the single mutant strains (Fig. 2A).

We further characterized ncr1Δ eaf1Δ cells by examining growth in the presence of two drugs that target sterol metabolism. The aerobic growth of ncr1Δ eaf1Δ strains in the presence of fluconazole or nystatin was markedly impaired relative to control or single deletion parental strains (Fig. 2B). Fluconazole is an inhibitor of lanosterol demethylation. The growth defect due to fluconazole therefore suggests that ncr1Δ eaf1Δ cells, which already display increased lanosterol and decreased ergosterol, cannot tolerate further sterol imbalance. By contrast, nystatin is a polyene antibiotic that binds ergosterol in membranes. The growth defect in response to nystatin, despite the lower levels of total ergosterol in ncr1Δ eaf1Δ cells relative to the single mutants or control, suggests that ncr1Δ eaf1Δ membranes may be more permeable to nystatin. Alternatively, ergosterol in ncr1Δ eaf1Δ strains may be mis-transported to a nystatin-sensitive compartment (e.g. the vacuole (27)) and thus manifest as elevated sensitivity.

Global Up-regulation of Histone Deacetylase Genes in NP-C-deficient Human Fibroblasts—The anaerobic inviability and sterol accumulation of ncr1Δ eaf1Δ strains suggest the surprising interpretation that the activity of histone acetylases is key to cholesterol homeostasis in NP-C disease. Moreover, the histone acetyltransferase encoded by EAF1, and the complex in which it resides, is conserved in humans. We therefore hypothesized that an imbalance in histone acetylation contributes to human NPC1 disease pathophysiology. The relative activities of HDACs are maintained in a tightly controlled cellular equilibrium in part by transcriptional regulation of the deacetylases (28). Therefore, we used quantitative real time PCR to measure expression of the 11 HDAC genes (HDAC1–11) in fibroblasts relative to the single mutants or control, suggests that ncr1Δ eaf1Δ membranes may be more permeable to nystatin. Alternatively, ergosterol in ncr1Δ eaf1Δ strains may be mis-transported to a nystatin-sensitive compartment (e.g. the vacuole (27)) and thus manifest as elevated sensitivity.

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HDAC Inhibition in Human Fibroblasts Ameliorates Multiple Biochemical Hallmarks of NP-C Disease—Having demonstrated global HDAC dysregulation in NP-C fibroblasts, we
sought to determine whether these cells could be normalized by HDAC inhibition. To inhibit HDAC, we used SAHA (Vorinostat, Zolinza®) because it is a potent global inhibitor of histone deacetylation (28). Furthermore, SAHA has been shown to cross the blood-brain barrier (30) and is Food and Drug Administration-approved to treat cutaneous T-cell lymphoma, a critical incentive given the short life span of NP-C patients who cannot wait for the development and approval of a new drug. A 24-h treatment of 5 μM SAHA in NP-C fibroblasts had the expected outcome for several known transcriptionally regulated targets of this drug (Fig. 4) (28). Many HDAC genes are transcriptionally regulated by HDAC enzymatic inhibitors such as SAHA (28). Strikingly, after a 24-h treatment of 5 μM SAHA, the expression of seven of the nine up-regulated HDAC genes in NPC-26 fibroblasts were significantly restored toward control levels (Fig. 5).

We reasoned that inhibiting HDAC might reverse the cellular and biochemical phenotypes of NP-C disease. We characterized the effect of 2.5, 5.0, and 10.0 μM SAHA on the trafficking of LDL-derived cholesterol by evaluating filipin fluorescence in human fibroblasts. We determined that a dose of 5 μM SAHA significantly ameliorated filipin accumulation in NPC-26 cells. After 24 h of treatment with 5 μM SAHA, the lysosomal accumulation of cholesterol in NPC-26 or NPC-J4 was significantly reduced by 50–60% compared with untreated NP-C mutant cells (Fig. 6A). Significant reductions in filipin fluorescence were also observed for NPC-2 and NPC-29 (data not shown). We then examined the effect of 5 μM SAHA in NPC-26 cells on the esterification of LDL-derived cholesterol, a defect that is also a biochemical hallmark of NP-C disease (19). Following a 24-h treatment with 5 μM SAHA, esterification of LDL-cholesterol (added at 20 h) was significantly increased by ~200% (Fig. 6B) to a value that approximated esterification in untreated controls, which were also elevated by SAHA treatment.

The NP-C lipidosis was also typified by aberrant lysosomal/endosomal accumulation of sphingolipids such as monosialotetrahexosylganglioside (GM1 (31)) and trihexosylceramide (GL-3) or defective transport of fluorescent sphingolipid analogs such as 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene lactosylceramide (BODIPY-LacCer (32)). We evaluated remediation of cholesterol and sphingolipid transport in NPC1 mutant fibroblasts (NPC-26 and NPC-J4) cultured with inhibitors of the HDACs. Treatment with 5 μM SAHA significantly reduced the accumulation of GL-3 by ~30% compared with untreated NP-C cells (Fig. 7A). Similarly, 5 μM SAHA completely cleared GM1 from the lysosomal compartment in the NPC-J4 NPC1 mutant line as assessed by Alexa Fluor 555-cholera toxin subunit B fluorescence (Fig. 7B). Finally, we assessed the transport of a fluorescent analog of lactosylceramide (BODIPY-LacCer) in NPC-J4 cells treated with SAHA. BODIPY-LacCer accumulates in the Golgi of normal cells but is retained in the lysosomal/endosomal compartment of NPC1 mutants (32), a localization that was reversed following treatment with SAHA (Fig. 7C). Similarly, treating the NPC-J4 mutant fibroblast line with another global HDAC inhibitor, 300 nM trichostatin A (TSA), produced a striking restoration of cholesterol and sphingolipid trafficking (supplemental Fig. S1).

To elucidate the mechanism by which SAHA- or TSA-mediated HDAC inhibition ameliorates NP-C disease phenotypes, we next determined whether treatment with isoform-specific HDAC inhibitors can rescue the lysosomal accumulation of cholesterol in fibroblasts. Using MC1568 (33), an inhibitor of

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only HDAC class IIa genes (HDAC4, -5, -7, and -9), and MGCD0103 (34), an inhibitor of class I and class IV HDAC genes (HDAC1–3 and -11, respectively), we assessed sterol accumulation in the NPC-26 mutant fibroblast line. Like SAHA, the HDAC class-specific inhibitors MC1568 and MGCD0103 (5 μM) also reduced filipin accumulation in NPC-26 compared with untreated cells after 24 h of treatment (Fig. 8). MC1568 and MGCD0103 reduced filipin accumulation by 28 and 41% compared with untreated cells, values that are significant but markedly below the 60% impact of global HDAC inhibitors such as SAHA and TSA.

DISCUSSION

The identification of genetic or physical interactions underlying NP-C disease remains elusive. For example, both proteins defective in this disease are clearly associated with binding and transport of the same molecule, cholesterol, but have yet to be shown to interact at any level. NPC1 likely encodes a transporter that directly or indirectly affects the status of multiple lipids. We hypothesized that NPC1 and its orthologs would display numerous genetic interactions. We approached this problem using the yeast model of NP-C disease and identified 13 interacting loci that potentially identify modulators of the syndrome. Our premise is that an unbiased identification of genetic modifiers provides a means to identify previously unsuspected pharmacological targets to treat NP-C disease. The focus of this study emanates from two model systems separated by over 2 billion years of evolution. The genetic interaction of yeast NCR1 with EAF1, encoding a histone acetylase, conferred altered sterol metabolism and was manifested in human NP-C fibroblasts as a striking HDAC imbalance; the majority of HDAC genes are up-regulated in NP-C fibroblasts derived from patients. Pharmacological intervention in this pathway in several human NP-C lines by inhibiting HDAC cor-
rected multiple intracellular lipid transport defects associated with NP-C disease. Consequently, we propose that SAHA, an Food and Drug Administration-approved agent, provides a novel point of entry into rapidly treating this devastating disorder. Moreover, we consider this as proof-of-concept that unbiased identification of genetic modifiers is a means to identify pharmacological targets to treat NP-C disease.

How does the activity of transcriptional regulators such as histone acetylases/deacetylases impact sterol homeostasis? The impact of HDAC inhibition on lipid accumulation in NP-C-deficient fibroblasts was not due to enhanced expression of NPC1, the chaperone HSP90, or RAB9 a genetic modifier that has been shown to ameliorate lipid accumulation in NP-C fibroblasts upon overexpression (35). Interestingly, SAHA treatment did induce expression of NPC2 in control and NPC1 mutant fibroblasts relative to untreated fibroblasts (data not shown), indicating NPC2 is a transcriptional target of SAHA. Increased expression of NPC2 in NPC1 mutant cells is established but does not however alter disease phenotypes (36). The amelioration of NPC disease phenotypes in the absence of increased expression of the NPC1 gene is in agreement with the use of valproate (a relatively nonspecific inhibitor of HDAC,
glycogen synthase kinase 3, and ion channel transport) to rescue cholesterol accumulation in neural stem cells derived from an NPC1-null mouse (37). The histone-independent effects of SAHA, valproate, and other HDAC inhibitors are well established (28), raising the possibility that salvage of lipid transport may be unrelated to transcriptional regulation. Thus, the mechanism by which HDAC inhibitors correct lipid homeostasis in the human NP-C fibroblasts remains to be determined.

Our demonstration of greater rescue of cholesterol regulation with the global HDAC inhibitor than with isoform-specific HDAC inhibitors (MC1568 and MGCD0103) suggests this pathway is additively regulated by several HDAC genes, a prediction consistent with the global up-regulation of multiple HDAC genes observed here in human NP-C-deficient cells. HDAC4 was up-regulated in human NPC1 mutant cells and markedly responsive to SAHA suggesting HDAC4 may be a contributor to lipid homeostasis in NP-C disease. Interestingly, HDAC4 activity is critical to neuronal survival (38) achieving its impact via both histone and non-histone targets of HDAC genes. The major neurological pathology of NP-C disease is the loss of Purkinje cells in the cerebellum. HDAC6 and -11 are the most highly expressed HDAC genes in Purkinje cells (39), and our findings would suggest an HDAC11-specific inhibitor is a better target for NP-C disease.

The global inhibitor SAHA is Food and Drug Administration-approved and crosses the blood-brain barrier, critical parameters to NP-C patients who can ill afford to wait for development of a new drug. Despite this promise, we should be skeptical until drugs such as SAHA have been tested in the model organism and then pharmacologically manipulating the orthologous pathways in the opposite direction as a therapy in human cells could be utilized in any model organism. Proof of this concept is provided by our findings and a study published during the revision of this manuscript (41) that HDAC inhibition corrects defective subcellular lipid transport, the phenomena that precedes neurodegeneration in NP-C disease.
31. Taniguchi, M., Shinoda, Y., Ninomiya, H., Vanier, M. T., and Ohno, K. (2001) *Brain Dev.* **23**, 414–421
32. Choudhury, A., Sharma, D. K., Marks, D. L., and Pagano, R. E. (2004) *Mol. Biol. Cell* **15**, 4500–4511
33. Duong, V., Bret, C., Altucci, L., Mai, A., Duraffourd, C., Loubersac, J., Harmand, P. O., Bonnet, S., Valente, S., Maudelonde, T., Cavailles, V., and Boulle, N. (2008) *Mol. Cancer Res.* **6**, 1908–1919
34. Fournel, M., Bonfils, C., Hou, Y., Yan, P. T., Trachy-Bourget, M. C., Kalita, A., Liu, J., Lu, A. H., Zhou, N. Z., Robert, M. F., Gillespie, J., Wang, J. I., Ste-Croix, H., Rahil, J., Lefebvre, S., Moradei, O., Delorme, D., Macleod, A. R., Besterman, J. M., and Li, Z. (2008) *Mol. Cancer Ther.* **7**, 759–768
35. Walter, M., Davies, J. P., and Ioannou, Y. A. (2003) *J. Lipid Res.* **44**, 243–253
36. Storch, J., and Xu, Z. (2009) *Biochim. Biophys. Acta* **1791**, 671–678
37. Kim, S. J., Lee, B. H., Lee, Y. S., and Kang, K. S. (2007) *Biochem. Biophys. Res. Commun.* **360**, 593–599
38. Majdzadeh, N., Wang, L., Morrison, B. E., Bassel-Duby, R., Olson, E. N., and D’Mello, S. R. (2008) *Dev. Neurobiol.* **68**, 1076–1092
39. Southwood, C. M., Peppi, M., Dryden, S., Tainsky, M. A., and Gow, A. (2007) *Neurochem. Res.* **32**, 187–195
40. Alvarez, A. R., Klein, A., Castro, J., Cancio, G. L., Amigo, J., Mosquera, M., Vargas, I. M., Yévenes, L. F., Bronfman, F. C., and Zanlungo, S. (2008) *FASEB J.* **22**, 3617–3627
41. Pipalia, N. H., Cosner, C. C., Huang, A., Chatterjee, A., Bourbon, P., Farley, N., Helquist, P., Wiest, O., and Maxfield, F. R. (2011) *Proc. Natl. Acad. Sci. U.S.A.*, in press
42. Reddy, J. V., Ganley, I. G., and Pfeffer, S. R. (2006) *PLoS ONE* **1**, e1943
43. Heinicke, S., Livstone, M. S., Lu, C., Oughtred, R., Kang, F., Angioli, S. V., White, O., Botstein, D., and Dolinski, K. (2007) *PLoS ONE* **2**, e766
44. Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L., Toufighi, K., Mostafavi, S., Prinz, J., St Onge, R. P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F. J., Alizadeh, S., Bahr, S., Brost, R. L., Chen, Y., Kokol, M., Deshpande, R., Li, Z., Lin, Z. Y., Liang, W., Marback, M., Paw, J., San Luis, B. J., Shuteriqi, E., Tong, A. H., van Dyk, N., Wallace, I. M., Whitney, J. A., Weirauch, M. T., Zhong, G., Zhu, H., Houry, W. A., Budnho, M., Ragibizadeh, S., Papp, B., Pál, C., Roth, F. P., Giaever, G., Nislow, C., Troyanskaya, O. G., Bussey, H., Bader, G. D., Gingras, A. C., Morris, Q. D., Kim, P. M., Kaiser, C. A., Myers, C. L., Andrews, B. J., and Boone, C. (2010) *Science* **327**, 425–431