Normalization of Hepatic Homeostasis in the Npc1<sup>nmf164</sup> Mouse Model of Niemann-Pick Type C Disease Treated with the Histone Deacetylase Inhibitor Vorinostat*<sup>a,b</sup>  

Received for publication, December 2, 2016, and in revised form, December 21, 2016. Published, JBC Papers in Press, December 28, 2016, DOI 10.1074/jbc.M116.770578

Andrew B. Munkaci<sup>a,b,1,2</sup>, Natalie Hammond,<sup>a</sup> Remy T. Schneider,<sup>a</sup> Dinindu S. Senanayake,<sup>a</sup> Katsumi Higaki,<sup>c,d</sup> Kirill Lagutin,<sup>d</sup> Stephen J. Bloor,<sup>d</sup> Daniel S. Ory,<sup>c</sup> Robert A. Maue,<sup>e</sup> Fannie W. Chen,<sup>f</sup> Antonio Hernandez-Ono,<sup>h</sup> Nicole Dahlson,<sup>f</sup> Joyce J. Repa,<sup>f</sup> Henry N. Ginsberg,<sup>i</sup> Yiannis A. Ioannou,<sup>j</sup> and Stephen L. Sturley<sup>k</sup>

From the<sup>a</sup>School of Biological Sciences and <sup>b</sup>Centre for Biodiscovery, Victoria University of Wellington, Wellington 6012, New Zealand, the<sup>c</sup>Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, Yonago 683-8503, Japan, the<sup>d</sup>Callaghan Innovation, Lower Hutt 5040, New Zealand, the<sup>e</sup>Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110, the<sup>f</sup>Department of Physiology and Neurobiology and the Department of Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire 03755, the<sup>g</sup>Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, New York 10029, the<sup>h</sup>Departments of Physiology and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390, and the<sup>i</sup>Department of Medicine and the<sup>j</sup>Department of Genetics and Development, Columbia University Medical Center, New York, New York 10032

Edited by Dennis R. Voelker

Niemann-Pick type C (NP-C) disease is a fatal genetic lipidosi s for which there is no Food and Drug Administration (FDA)- approved therapy. Vorinostat, an FDA-approved inhibitor of histone deacetylases, ameliorates lysosomal lipid accumulation in cultured NP-C patient fibroblasts. To assess the therapeutic potential of histone deacetylase inhibition, we pursued these <i>in vitro</i> observations in two murine models of NP-C disease. Npc1<sup>nmf164</sup> mice, which express a missense mutation in the <i>Npc1</i> gene, were treated intraperitoneally, from weaning, with the maximum tolerated dose of vorinostat (150 mg/kg, 5 days/week). Disease progression was measured via gene expression, liver function and pathology, serum and tissue lipid levels, body weight, and life span. Transcriptome analyses of treated livers indicated multiple changes consistent with reversal of liver dysfunction that typifies NP-C disease. Significant improvements in liver pathology and function were achieved by this treatment regimen; however, NPC1 protein maturation and levels, disease progression, weight loss, and animal morbidity were not detectably altered. Vorinostat concentrations were &gt;200 μM in the plasma compartment of treated animals but were almost 100-fold lower in brain tissue. Apolipoprotein B metabolism and the expression of key components of lipid homeostasis in primary hepatocytes from null (Npc1<sup>−/−</sup>) and missense (Npc1<sup>nmf164</sup>) mutant mice were altered by vorinostat treatment, consistent with a response by these cells independent of the status of the <i>Npc1</i> locus. These results suggest that HDAC inhibitors have utility to treat visceral NP-C disease. However, it is clear that improved blood-brain barrier penetration will be required to alleviate the neurological symptoms of human NP-C disease.

Histone deacetylase (HDAC)<sup>5</sup> inhibitors were initially identified as treatments for a variety of proliferative diseases, including T-cell lymphoma and other cancers (1). The mechanism of efficacy primarily reflects changes in the transcriptional program of rapidly growing cells and, in some cases, results in altered expression of 2–10% of the genome (2, 3). Subsequently, intervention at the histone acetylation/deacetylation axis of gene expression has been successfully applied to many unrelated diseases (4). Neurological disorders such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and Friedrich’s ataxia have all been found to respond to HDAC inhibitors (4–6). The primary mechanism by which HDAC inhibitors influence these diseases remains undetermined and may well be multivariate. In some disorders (e.g. cystic fibrosis), the treatment is associated with
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alterations at the proteostatic level due in part to activation of chaperone-mediated refolding pathways (7).

Niemann-Pick type C (NP-C) disease is a rare lysosomal storage disorder typified by defective subcellular transport of cholesterol and sphingolipids as well as premature demise due to central nervous system (largely cerebellar) impairment (8). Autosomal recessive mutations in the NPC1 and NPC2 genes confer 95 and 5% of NP-C disease cases, respectively. Both genes are strikingly conserved throughout evolution (from yeast to humans), although their precise functions remain elusive (9, 10). We have proposed that variation in the activity of additional genes influences NP-C disease severity, and these loci represent novel therapeutic interventions (11). To identify such NP-C disease modification pathways, we have used a model system-based approach in the yeast Saccharomyces cerevisiae. Our “exacerbate-reverse” strategy successfully identified 13 genetic loci whose individual absence conferred complete inviability to the yeast deletion of the NPC1 ortholog conditional upon sterol loading. This approach identified the status of histone acetylation as a key component of lipid accumulation in yeast and in cultured human fibroblasts that are deficient in the NP-C pathways (11). We and others have demonstrated that histone deacetylase inhibition by a variety of pharmacological agents normalizes lipid homeostasis in rapidly growing immortalized cell lines derived from patients with NP-C disease (11–13). These studies have progressed to the extent that the FDA-approved HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid, Zolinza®) is the focus of a clinical trial for safety and tolerability in adult NP-C patients (ClinicalTrials.gov number NCT02124083).

Multitissue lipid accumulation and neurodegeneration define NP-C disease. The aberrant subcellular lipid storage is clearly associated with transcriptional dysregulation of numerous genes, including sterol regulatory element-binding protein targets, such as HMG-CoA reductase (HMGCR) and the low density lipoprotein receptor (LDLR) (14, 15). Any candidate intervention that can restore lipid homeostasis in cultured cells, however, must also be tested for an impact on NP-C disease pathology in an in vivo model. This is particularly critical given the challenge of predicting whether results in cell culture will be conserved in human trials (16). Here we describe the impact of vorinostat in a murine model of NP-C disease caused by a missense mutation in the Npc1 gene. We show that this FDA-approved HDAC inhibitor normalizes hepatic transcriptional regulation of cholesterol, corrects apolipoprotein B homeostasis, and improves liver function and pathology, thus raising the possibility that this intervention may treat visceral NP-C disease. With the regimens used here, however, there was no accompanying improvement in mortality or morbidity, probably due to the poor bioavailability of this drug to brain tissues. Overall, these studies establish HDAC inhibition as an attractive approach for treatment of NP-C disease while emphasizing the need for candidate inhibitors to overcome the blood-brain barrier.

Results

NPC1 Missense Variants Can Be Refolded—In the Npc1<sup>1005G</sup> mouse, NP-C disease arises from a missense mutation at codon 1005 in Npc1 that resembles several NPC1 missense mutations that cause NP-C disease in humans (17). Specifically, the murine aspartic acid-to-glycine NPC1<sup>1005G</sup> variant is contextually similar to the NPC1<sup>1004L</sup>, NPC1<sup>1007A</sup>, and NPC1<sup>1007L</sup> variants associated with the juvenile form of human NP-C disease. The Npc1<sup>1005G</sup> mouse model presents with a less severe form of the disease than the Npc1<sup>1005G</sup> murine model but nevertheless exhibits an abbreviated life span, progressive weight loss, neurological impairment, and cellular lipid accumulation compared with control littermates (17).

In contrast to the Npc1<sup>1005G</sup> mutant mouse model, Npc1<sup>1005G</sup> mice exhibit low but detectable levels of presumably functionally impaired NPC1 protein (17). To specifically determine whether this phenotype is due to misfolding and premature degradation of the NPC1<sup>1005G</sup> variant, fibroblasts derived from the Npc1<sup>1005G</sup> mouse were treated with glycerol, a chemical chaperone known to stabilize misfolded proteins (18). Glycerol increased expression levels of the NPC1<sup>1005G</sup> protein compared with untreated cells (Fig. 1) and in addition promoted its maturation to a higher molecular weight form. The properties of the NPC1<sup>1005G</sup> protein were then compared with those of NPC1<sup>1006T</sup>, a previously described misfolded NPC1 variant associated with the most common form of human NP-C disease (18, 19). NPC1-deficient human osteosarcoma U2OS cells expressing the NPC1<sup>1006T</sup> protein were also treated with glycerol. Expression levels of the NPC1<sup>1006T</sup> protein were also increased with glycerol treatment (Fig. 1), consistent with previous studies (18). To elucidate the possible contributions of lysosomal and proteasomal degradation to levels of the NPC1<sup>1005G</sup> protein, the Npc1<sup>1005G</sup> fibroblasts were treated with 3-methyladenine (an autophagy inhibitor) and two inhibitors of the proteasome, MG132 and lactacystin. Each of these treatments increased expression and maturation of the NPC1<sup>1005G</sup> protein (Fig. 1), implicating lysosomal and proteasomal pathways in the degradation of misfolded forms of the NPC1<sup>1005G</sup> protein.

Vorinostat Administration in a Misfolded Protein Mouse Model of NP-C Disease—To investigate the role of vorinostat as an NP-C therapeutic in vivo, we first assessed toxicity of this
HDAC inhibitor in the Npc1\textsuperscript{nmf164} NP-C murine model following i.p. administration in a vehicle of 45% PEG 400 + 10% DMSO (hereafter called PEG/DMSO). This protocol was used previously to deliver vorinostat across the blood-brain barrier and inhibit the metastasis of triple-negative breast cancer to the brain (20). 21-day-old, asymptomatic Npc1\textsuperscript{nmf164} homozygous mutant and WT mice were injected daily with 50, 100, 150, or 200 mg/kg vorinostat for 7 days. All mice were viable after 7 days; however, we observed significant weight loss in mice treated with 200 mg/kg vorinostat. This is in agreement with 200 mg/kg being toxic in control mice as well as in a mouse model of Huntington’s disease (5). Using the maximum tolerated dose and treatment regimen, we determined whether HDAC inhibition with vorinostat can impact NP-C disease progression. Control (WT) and Npc1\textsuperscript{nmf164} mice were treated with 150 mg/kg/day vorinostat or the PEG/DMSO vehicle, 5 times/week from P21, an age where lipid accumulation already exists in the liver and brain (17). Treatment continued until P60, at which point disease symptoms (i.e. weight loss, motor function deficits) are detectable in untreated Npc1\textsuperscript{nmf164} animals (17).

Transcriptional Modulation in Response to Vorinostat—The lysosomal sequestration of free cholesterol and other bioactive lipids in NPC1-deficient animals has striking and chronologically early consequences in terms of gene regulation. Multiple components of lipid homeostasis and inflammation are transcriptionally up-regulated, and this dysregulation is rapidly normalized by treatment with the lipid chelator, 2-hydroxypropyl-\(\beta\)-cyclodextrin (14, 15). To test whether these pathways respond to vorinostat in the liver of Npc1\textsuperscript{nmf164} mice as well as to comprehensively explore the impact of vorinostat on hepatic gene expression in these animals, we performed RNA-Seq analysis on RNA isolated from the livers of vorinostat- and vehicle-treated Npc1\textsuperscript{nmf164} mice. The transcripts were mapped to the mouse mm10 genome assembly, normalized, and quantified. Approximately 16–18 million reads/sample were obtained. Expression levels of 14,516 genes were measured, a number comparable with the 14,326 genes measured previously in the murine liver transcriptome (21). Expression of 844 genes (5.9% of the transcriptome) was significantly regulated (>0.6 log\textsubscript{2}-fold change, \(p < 0.05\)) by vorinostat treatment, and of these 844 genes, 603 (71.4%) were up-regulated and 241 (28.6%) were down-regulated (Fig. 2a, b, and supplemental Table S1). These genes were classified within an extensive array of Gene Ontology processes (Fig. 2a). Notable cellular aspects and processes relevant to NP-C disease and HDAC inhibition include lipid metabolism, inflammation, and histone-mediated gene expression (Fig. 2c).

Critically, the vorinostat-responsive genes include those known to be modulated by HDAC inhibitors in previous studies. For example, the GATA-binding transcription factor Gata1, a hallmark vorinostat-responsive gene (2, 3, 22), was up-regulated 3.6 log\textsubscript{2}-fold (\(p = 0.0001\)) in vorinostat-treated Npc1\textsuperscript{nmf164} mice compared with PEG/DMSO-treated Npc1\textsuperscript{nmf164} mice (Fig. 2c). As observed previously (23, 24), early growth response-1 (Egr1) and amyloid \(\beta\) precursor-like protein 1 (Aplp1) were elevated 1.2 log\textsubscript{2}-fold (\(p = 5.67 \times 10^{-3}\)) and 2.56 log\textsubscript{2}-fold (\(p = 1.95 \times 10^{-15}\)), respectively, after vorinostat treatment (Fig. 2c). Transcription of the aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL, also referred to as BMAL1) is repressed by HDAC3 (25) and was elevated 2.59 log\textsubscript{2}-fold (\(p = 1.18 \times 10^{-7}\)) after treatment with vorinostat (Fig. 2c). Likewise, we determined that vorinostat reduced transcription of the minichromosome maintenance protein-2 (MCM2) by 0.67 log\textsubscript{2}-fold (\(p = 0.0015\)) and the TATA-box-binding protein TAF6L by 0.56 log\textsubscript{2}-fold (\(p = 0.004\)) compared with PEG/DMSO-treated mice (Fig. 2c), consistent with the previously observed down-regulation of these genes by HDAC inhibitors (26, 27). Additionally, the cyclin D1 (Ccdn1) gene has been shown to decrease in expression by HDAC inhibition (28); here vorinostat reduced Ccdn1 expression 1.34 log\textsubscript{2}-fold (\(p = 1.8 \times 10^{-6}\)) (Fig. 2c). It is clear from these responses that a variety of HDAC isoforms, and thus their transcriptional targets, were affected by the treatment protocol in place, as would be anticipated for the impact of a global HDAC inhibitor like vorinostat.

To determine the therapeutic potential of vorinostat, we investigated the overlap of vorinostat-mediated gene expression and candidate biomarkers previously shown to be transcriptionally dysregulated in NP-C disease. Lyz1 (lysozyme 1) transcript levels are up-regulated in the liver and brain of the Npc1\textsuperscript{1+/−} mouse (29), and plasma lysozyme activity is increased in the Npc1\textsuperscript{1+/−} mouse (30). Vorinostat treatment reduced hepatic expression of LYZ1 by 1.1 log\textsubscript{2}-fold (\(p = 4.37 \times 10^{-5}\)) in Npc1\textsuperscript{nmf164} mice compared with vehicle control (Fig. 2c). Likewise, the sterol regulatory element-binding factor (SREBF1) is a transcription factor that regulates cholesterol synthesis and is up-regulated in NPC1-deficient hepatocytes (31), and Srebfl expression was reduced with the vorinostat treatment 0.64 log\textsubscript{2}-fold (\(p = 0.02\)) compared with vehicle-treated Npc1\textsuperscript{nmf164} mice (Fig. 2c). In addition, the genes encoding oxysterol-binding protein OSBPL3 and the perilipin PLIN4 were up-regulated in independent studies of the liver of the Npc1\textsuperscript{1+/−} mouse (29, 32), and we show here that the vorinostat treatment reduced expression of Osbpl3 and Plin4 by 1.4 log\textsubscript{2}-fold (\(p = 2.86 \times 10^{-7}\)) and 1.3 log\textsubscript{2}-fold (\(p = 1.44 \times 10^{-5}\)), respectively, in Npc1\textsuperscript{nmf164} mice (Fig. 2c).

Similarly, several transcriptional pathways associated with inflammation (a characteristic of chronic NP-C disease) were normalized by treatment of the Npc1\textsuperscript{nmf164} mouse with vorinostat. For example, expression levels of four genes (metalloproteinase matrix-15 (Mmp15), CD27 antigen (Cd276), and histocompatibility antigens H2-Ab1 and H2-Eb1) that were consistently shown to be up-regulated in Npc1\textsuperscript{1+/−} mice (29, 32) were down-regulated in vorinostat-treated Npc1\textsuperscript{nmf164} mice compared with vehicle-treated Npc1\textsuperscript{nmf164} mice (Fig. 2c). Expression levels of three additional pro-inflammatory CD antigen genes (Cd8a, Cd79a, and Cd74) were similarly reduced by vorinostat (Fig. 2c). Two S100a genes (S100a8 and S100a9) that are targets of NFkB and critical components of the immune response to liver damage (33) were up-regulated with vorinostat treatment (Fig. 2c). The cathelicidin antimicrobial peptide gene (Camp) is required for inflammatory response regulation; expression of CAMP was up-regulated in two studies of the Npc1\textsuperscript{1+/−} mouse (29, 32) and down-regulated in the Npc1\textsuperscript{nmf164} mice treated with vorinostat. These results suggest that vori-
nositat activates an anti-inflammatory response as a consequence of NP-C disease in the Npc1<sup>nmf164</sup> mouse.

**Transcriptional Modulation of Cholesterol Metabolism**—We used the Web Gene Ontology Enrichment Analysis Tool Kit (34) to identify pathways statistically overrepresented within the 844 vorinostat-regulated genes in the liver transcriptome of Npc1<sup>nmf164</sup> mice. In addition to amino acid metabolism (p = 0.0323), four pathways associated with an anti-inflammatory response were enriched: GPCRs class A rhodopsin-like (p = 0.0008), GPCRs non-odorant (p = 0.0026), GPCRs peptide (p = 0.0323), and chemokine signaling pathway (p = 0.0082). Of particular relevance to NP-C disease, the enrichment analysis revealed that genes critical to the biosynthesis of cholesterol were down-regulated (p = 0.0014) (Fig. 2c). qRT-PCR analysis for seven key genes (Hmgcs1, Hmgcr, Mvk, Mvd, Idi1, Lss, and Cyp51) was conducted on sets of three randomly selected mice from the RNA-Seq analysis (Fig. 3, a–g). Vehicle- and vorinostat-treated WT mice were included to determine whether vo-
Vorinostat regulates these cholesterol biosynthesis genes independent of the status of the NPC1 pathway.

Our analyses suggest that the cholesterol biosynthetic pathway is significantly up-regulated in the Npc1<sup>nmf164</sup> model, presumably in response to the lysosomal accumulation of cholesterol in association with reduced free cholesterol in regulatory pools such as the ER. Indeed, expression levels of Hmgcs1, Hmgcr, Mvk, Mvd, Idi1, Lss, and Cyp51 were significantly up-regulated in the mutant mice relative to WT (Fig. 3, a–g), ranging from an increase of 2-fold to an increase of 5.1-fold. Impressively, vorinostat treatment significantly decreased the expression of all seven genes toward WT levels, with fold change ranging from 1.6- to 2.8-fold. Moreover, these changes were only identified in Npc1<sup>nmf164</sup> mice; expression levels of these seven genes in WT mice were not affected by vorinostat treatment. In addition, we measured expression of Ldlr, a gene that is strongly up-regulated in NP-C disease (14, 15); LDLR was up-regulated 1.9-fold in vehicle-treated Npc1<sup>nmf164</sup> compared with vehicle-treated Npc1<sup>nmf164</sup> mice (p = 0.044) (Fig. 3h). Collectively, these results have three major implications. First, cholesterol homeostatic genes are elevated in Npc1<sup>nmf164</sup> mice at P60. Second, vorinostat does not regulate expression of cholesterol metabolism genes in WT mice. Third, and most importantly, upon treatment with vorinostat, eight genes critical to cholesterol homeostasis and NP-C disease were down-regulated to WT levels.

**Lipid Metabolism Is Fundamental to the Vorinostat Interactome**—To identify the core genes in the vorinostat interactome, we used Phenolyzer (35) to visualize gene-gene interactions, protein-protein interactions, and transcriptional regulation among the 844 genes in the vorinostat interactome (Fig. 2a). This analysis distinguished Cyp27b1, Hmgcr, Pnpla3, and Chka as the core hubs in the vorinostat interactome, each with >20 interactions within the interactome (Fig. 4). Cyp27b1...
Vorinostat Improves Liver Health—To determine whether the transcriptional regulation of cholesterol metabolism by vorinostat has functional consequences, we measured serum levels of alanine aminotransferase (ALT), an enzyme that is detectably altered in chronic liver disease wherein overexpression confers hepatic steatosis (37). Chka encodes choline kinase α, one of two mammalian enzymes that catalyze the phosphorylation of choline to phosphocholine in the biosynthesis of the major membrane phospholipid, phosphatidylcholine (38). The hubs of the vorinostat interactome are all associated with lipid metabolism; specifically, our identification of Hmgcr as a mediator of vorinostat-mediated down-regulation of cholesterol biosynthesis as well as a key hub in the vorinostat interactome indicates that regulation of cholesterol biosynthesis is fundamental to the impact of vorinostat on NP-C disease.

Thin section H&E staining of fixed liver tissue revealed the marked accumulation of lipid-laden cells in untreated Npc1<sup>nmf164</sup> mice at P60 (Fig. 5e), characteristic of lipid accumulation at this stage of the disease in this mouse model (17). Treatment of Npc1<sup>nmf164</sup> mice with vorinostat convincingly and significantly reduced the prevalence of lipid-laden cells by 48.9% (p < 0.001) and elevated the presence of healthy hepatocytes, relative to vehicle controls (Fig. 5e). Taken together, the transcriptional normalization of hepatic cholesterol homeostatic genes and the reductions in serum cholesterol and ALT levels (liver function) in conjunction with improved liver histopathology indicate a marked recovery from visceral NP-C disease due to vorinostat treatment.

To determine whether the vorinostat-mediated changes in liver health correlate with changes in total lipid accumulation in vivo, we quantified levels of free cholesterol, sphingomyelin, sphingosine, glucosylceramide, and lactosylceramide using GC/MS and LC/MS. These lipids are biochemical hallmarks of late stage NP-C disease in the liver (8). Because the status of these lipids has not been reported for the Npc1<sup>nmf164</sup> mouse at P60, we first determined that these lipids were significantly increased in vehicle-treated Npc1<sup>nmf164</sup> mice compared with vehicle-treated WT mice (Fig. 6, a–e). In accordance with the Npc1<sup>1<sup>–/–</sup></sup> and Npc1<sup>I1061T</sup> mouse models of NP-C disease (14, 19, 40), the Npc1<sup>nmf164</sup> mice exhibit increased levels of these lipids relative to WT mice (Fig. 6, a–e). Vorinostat did not detectably alter lipid levels in WT or Npc1<sup>nmf164</sup> mice (Fig. 6, a–e). To further investigate a hallmark lipid using an independent method, we assessed free cholesterol levels using fluorescent microscopy. Filipin, a fluorescent stain for unesterified cholesterol, is a key tool used in the diagnosis of NP-C patients (8). As expected, we observed increased filipin fluorescence in vehicle-treated Npc1<sup>nmf164</sup> mice compared with vehicle-treated WT mice (Fig. 6, f and g). However, filipin fluorescence...
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Vorinostat Treatment Improves Liver Function in Npc1<sup>nmf164</sup> Mice—Animals were treated with either vehicle (PEG/DMSO) or 150 mg/kg vorinostat from P21 to P60, at which point serum was harvested from sacrificed animals. Liver function was evaluated via levels of ALT expressed as mmol/liter. Relative to vehicle-treated WT mice, vehicle-treated Npc1<sup>nmf164</sup> mice significantly lost weight between 10 and 13 weeks (p = 0.0002), a result consistent with prior characterization of the Npc1<sup>nmf164</sup> mouse (17). There was no delay in the weight loss of vorinostat-treated Npc1<sup>nmf164</sup> mice at any point during the treatment (Fig. 7a); treated and untreated animals lost weight and exhibited ataxia to similar levels from 9 to 10 weeks until death. Therapies that have delayed weight loss have also extended life span (14, 42); thus, the absence of change with vorinostat treatment would predict that this intervention protocol does not extend life span. Indeed, vorinostat-treated Npc1<sup>nmf164</sup> mice survived 94 ± 7 days (n = 8), a time range that is not different than the 94 ± 4-day life span of untreated Npc1<sup>nmf164</sup> mice.

Penetration of the Blood-Brain Barrier by Vorinostat—Despite its beneficial effect on the liver, the lack of effect of vorinostat treatment on weight and life span of the Npc1<sup>nmf164</sup> mouse suggests that the drug fails to reach a concentration in the brain required to reverse the overall impact of the disease. Previously, a concentration of 5–10 μM vorinostat was described as sufficient to limit lipid accumulation in cultured NP-C patient fibroblasts (11–13). To determine the drug concentration achieved in the brain, we quantified vorinostat in plasma and brain of treated Npc1<sup>nmf164</sup> mice at P21 (Fig. 7b). The levels of vorinostat in the plasma at 0.5, 1, 2, and 4 h after injection were 222.29, 17.39, 3.01, and 0.97 μM, respectively, and were below the minimum detection level of the assay at 4 h (Fig. 7c). These results indicate a half-life of 5.8 ± 0.2 min. Our findings of rapid clearance and <5% brain penetration, compared with plasma, in Npc1<sup>nmf164</sup> mice are in accordance with observations in normal mice and in a murine model of Huntington’s disease (43, 44).

Improved Liver Function Is Independent of Npc1 Expression—To investigate the mechanism by which vorinostat reduced ALT levels, we measured Npc1 mRNA and protein levels to test the hypothesis that vorinostat elevated expression of the NPC1<sup>D1005G</sup> variant in the Npc1<sup>nmf164</sup> mouse. To determine
whether vorinostat up-regulated Npc1 mRNA expression, we measured mRNA levels of NPC1 by quantitative real-time PCR (Fig. 8a). Expression of Npc1 was not affected in either genotype with vorinostat treatment, although Npc1 mRNA expression was modestly elevated in vehicle-treated Npc1nmf164 compared with vehicle-treated WT mice (p = 0.066). Quantification of Western blotting analysis of liver protein extracts from treated and untreated animals (n = 7/group) indicated that the vehicle-treated Npc1nmf164 mouse expressed 19% of the amount of Npc1 expressed by vehicle-treated WT mice (p = 5.76 × 10^{-7}; Fig. 8, b and c), consistent with previous reports that the Npc1nmf164 mouse expressed 10–15% of WT levels of NPC1 (17). However, our analysis indicated equivalent steady state levels of NPC1 protein in vorinostat-treated liver and vehicle control-treated livers. The mRNA levels of Npc2 were 4.7-fold higher (p = 0.016) in vehicle-treated Npc1nmf164 compared with vehicle-treated WT mice, a result that has been reported previously for the Npc1−/− null and the Npc1pf/pf missense mutant mouse models of NP-C disease (45, 46). However, there was no significant change in expression of Npc2 in vorinostat-treated Npc1nmf164 compared with vehicle-treated Npc1nmf164 mice (Fig. 8d). These results suggest that the improvements in lipid metabolism and liver function occur independently of the concentration of the NPC1D1005G protein and are not a consequence of vorinostat regulating the NPC1/NPC2 pathways at protein or mRNA levels.

Vorinostat Does Not Increase Maturation of the NPC1D1005G Protein—To further understand the lack of impact of vorinostat on expression of the NPC1D1005G protein in the liver of Npc1nmf164 mice, we investigated maturation of the NPC1D1005G protein in the secretory pathway via two glycosidase digestion assays that have been used previously to characterize the NPC1I1061T protein (18, 19). First, we used endoglycosidase H (Endo H), an enzyme that removes immature high mannose N-linked glycans from proteins. Second, we used peptide:N-glycosidase F (PNGase F), an enzyme that removes all N-linked glycan residues regardless of the glycan modification. In livers of vehicle-treated WT mice, the NPC1 protein is glycosidase-sensitive represented by a mature species at 225 kDa that is reduced to two deglycosylated species at 200 and 150 kDa in response to the Endo H and PNGase F treatments, respectively (Fig. 8e). These results indicate that the wild-type NPC1 protein is glycosylated in the ER and further matured in the Golgi. Conversely, the NPC1D1005G protein in both vehicle-
and vorinostat-treated \textit{Npc1}^{\text{11005G}}} mice is predominantly a single species at ~150 kDa in undigested, Endo H-treated, and PNGase F-treated samples, with electrophoretic mobility similar to that of PNGase F-treated WT protein (Fig. 8e). These results indicate that the majority of \textit{Npc1}^{\text{D1005G}} protein in livers of \textit{Npc1}^{\text{11005G}}} mice lacks glycosylation and does not exit the ER as a result of misfolding. This defect was not corrected by vorinostat treatment.

\textbf{Vorinostat Normalizes Apolipoprotein B Metabolism in \textit{Npc1} Null and Missense Mutant Hepatocytes—Inhibition of histone deacetylation results in differential expression of 2–10% of the genome (2, 3). In disorders such as cystic fibrosis, the success of vorinostat treatment was, at least in part, due to activation of chaperone-mediated refolding pathways (7). Thus far, our data suggest that vorinostat is therapeutically via a mechanism that does not require functional NPC1 (Figs. 3 and 8). To directly test whether residual levels of NPC1 protein impact the response to vorinostat treatment, we compared primary hepatocytes prepared from \textit{Npc1}^{\text{11005G}}} and \textit{Npc1}^{\text{11005G}}} mice. In contrast to the \textit{Npc1}^{\text{11005G}}} mice that produce low levels of NPC1 protein, the \textit{Npc1}^{\text{11005G}}} model expresses a null variant of the \textit{Npc1} gene arising from a transposon insertion at the 5’ end of the gene (47).

Metabolic labeling of primary hepatocytes with \textit{35}S\textit{methionine} followed by immunoprecipitation was used to quantify newly synthesized and secreted isoforms of apolipoprotein B (apoB100 and apoB48). These proteins are major exporters of lipid from the liver (48). As described previously (31), the synthesis of apoB100 by hepatocytes derived from \textit{Npc1}^{\text{11005G}}} mice was elevated in response to the lipid accumulation underlying NP-C disease. Similarly, secretion of newly synthesized apoB100 was dramatically increased (507.3%, \( p = 0.006 \)), and apoB48 was marginally increased (18.8%, \( p = 0.0007 \)) in \textit{Npc1}^{\text{11005G}}} hepatocytes compared with control hepatocytes from WT littermates (Fig. 9, a–c). Despite the absence of the NPC1 protein in these cell lines, the ex vivo treatment of 10 \mu M vorinostat strikingly reduced secretion of apoB100 by 76% (\( p = 0.01 \)) to control levels (Fig. 9, a–c). Interestingly, secretion of apoB48 was slightly elevated in vorinostat-treated \textit{Npc1}^{\text{11005G}}} hepatocytes compared with vehicle-treated \textit{Npc1}^{\text{11005G}}} hepatocytes (23.5%, \( p = 0.046 \)). ApoB metabolism was also aberrant in primary hepatocytes from \textit{Npc1}^{\text{11005G}}} mice expressing the \textit{Npc1}^{\text{D1005G}}} missense mutant. Unlike the null mutant hepatocytes, secretion levels of apoB100 and apoB48 were significantly reduced by 57.2% (\( p = 0.04 \)) and 69.6% (\( p = 0.002 \)), respectively, in vehicle-treated \textit{Npc1}^{\text{11005G}}} hepatocytes compared with vehicle-treated control hepatocytes (Figs. 9, d–f). Similar to the response of \textit{Npc1}^{\text{11005G}}} hepatocytes to vorinostat, secretion of newly synthesized apoB48 levels in media was significantly increased (413.7%, \( p = 0.0008 \)) in vorinostat-treated \textit{Npc1}^{\text{11005G}}} hepatocytes compared with vehicle-treated mutant cells (Fig. 9, d–f). Clearly, vorinostat modulated the homeostasis of apoB in both null \textit{Npc1}^{\text{11005G}}} and missense \textit{Npc1}^{\text{11005G}}} hepatocytes, further demonstrating that the mechanism of action of this drug is independent of NPC1.

To further investigate the efficacy of vorinostat in \textit{Npc1}^{\text{11005G}}} and \textit{Npc1}^{\text{11005G}}} primary hepatocytes, we measured expression...
of several genes integral to lipid metabolism (Npc1, Hmgcr, Abcg1, and Apob) and the cellular response to misfolded proteins (CHOP). The expression of Npc1 was significantly increased by 91% (p = 0.016) in vehicle-treated Npc1<sup>nmf164</sup> cells compared with vehicle-treated control hepatocytes, and vorinostat did not impact these expression levels (Fig. 10a). As expected, expression of Npc1 was significantly reduced (81.4%, p = 0.009) in vehicle-treated Npc1<sup>−/−</sup> compared with vehicle-treated WT hepatocytes, and interestingly, albeit probably not functional, this was significantly up-regulated following treatment with vorinostat (Fig. 10a). The expression of Apob was significantly reduced by 50.1% (p = 0.0009) in Npc1<sup>nmf164</sup> hepatocytes compared with WT hepatocytes and was markedly elevated by vorinostat treatment beyond control levels in both Npc1<sup>nmf164</sup> (p = 0.0007) and Npc1<sup>−/−</sup> hepatocytes (p = 0.04) (Fig. 10b). The transcription factor, CCAAT/enhancer-binding protein homologous protein (CHOP), an indicator of ER stress that has been associated with disrupted apoB metabolism (49), was significantly increased in Npc1<sup>nmf164</sup> hepatocytes (111.6%, p = 0.0006) compared with WT hepatocytes (Fig. 10c). In contrast, the expression of Chop was significantly reduced in Npc1<sup>−/−</sup> hepatocytes by 40.4% (p = 0.03) compared with control hepatocytes. This dysregulation was reduced further in both mutant cell types by vorinostat treatment (49.9%, p = 0.0003 in Npc1<sup>−/−</sup> cells; 23.7%, p = 0.001 in Npc1<sup>nmf164</sup> cells) (Fig. 10c). These results suggest that vorinostat transcription-
regulates efflux to HDL, in

demonstration of increased expression of Npc1

47.5% (H9252/H11546/H11002/H11005/H11021/H11001), results that are in agreement with the previous demonstration of increased expression of Hmgcr, a transporter that regulates efflux to HDL, in Npc1−/− hepatocytes (50). Vorinostat treatment significantly increased Abcg1 expression but only in Npc1m164 hepatocytes (92.7%, p = 0.001), a mechanism consistent with observations on the anti-atherogenic effects of HDAC inhibition (51).

**Discussion**

The identification of FDA-approved drugs to treat NP-C disease remains elusive; in nearly 2 decades of effort since the molecular isolation of the NPC1 gene (52), only 2-hydroxypropiol-β-cycloexdrin, the excipient of a failed neurosteroid treatment (53), has progressed to a Phase 3 clinical trial. As an alternate strategy, we applied unbiased, genome-wide, genetic screens in yeast and identified histone acetylation as a negative modifier of the NP-C disease pathway (11). Subsequently, we and others translated this finding by demonstrating the efficacy of HDAC inhibitors to reduce lipid accumulation in NP-C patient fibroblasts (11–13). We hypothesized that this therapeutic effect would be conserved in vivo and that the pathway to regulatory approval of vorinostat for this orphan disease would be relatively rapid and unencumbered. In the present study, we tested the impact of intraperitoneal injection of vorinostat in the Npc1m164 mouse model of NP-C disease. We carefully reviewed toxicity and administration of vorinostat and used a concentration and dosing regimen that was the maximum tolerated dose in this animal. This regimen exceeded that used to treat murine models of cancer (the equivalent of a human dose of 400 mg/day). We were successful in that vorinostat treatment was therapeutic for visceral NP-C disease, as evidenced by normalized liver lipid homeostasis at the transcriptional level and marked improvement of liver pathology and function. However, aspects of disease progression such as morbidity, mortality, and neurodegeneration were not impacted, due, at least in part, to ineffective brain penetration.

We interpret these findings as a very promising step toward applying HDAC inhibitors as therapeutics for this disease; however, the manner by which HDAC inhibitors may act remains to be determined. HDAC inhibitors impact expression of the genome in a global fashion, with immense capacity to simultaneously alter numerous metabolic pathways. Indeed, our results show that vorinostat altered the expression of ~6% of the liver transcriptome of Npc1m164 mice, a result consistent with previous reports of vorinostat regulating 2–10% of the genome (2, 3). Most notably, we detected transcriptional regulation of genes involved in cholesterol homeostasis that correlate with reversal of NP-C disease. The Hmgs1, Hmgcr, Mvd, Idi1, Lss, Cyp51, and Ldlr genes were repressed by vorinostat only in the Npc1m164 mice, reflecting the successful treatment of hepatic NP-C disease as well as a therapeutic mechanism that is independent of Npc1. Interestingly, HDAC inhibitors have also been shown to repress expression of cholesterol homeostatic genes in U18666A-treated SH-SY5Y human neuroblastoma cells (54). HDAC inhibitors can further impact expression of the genome by directing recovery of misfolded mutated proteins via the induction of chaperone pathways (7). In Npc1m164 mice, the NPC1D1005G variant protein persisted as an unglycosylated species that could be chased through the secretion pathway into a fully glycosylated mature species either by a refolding agent, such as glycerol, or by protection from degradation. However, our studies demonstrate that this is not the predominant mechanism by which vorinostat impacts NP-C disease in these animals.

Despite the overall transcriptional responsiveness of the Npc1m164 missense mutant mouse, the vorinostat treatment protocol used here did not detectably alter hepatic expression of Npc1 at either mRNA or protein levels or in terms of its maturation through the secretion pathway. Moreover, when we compared apoB homeostasis in primary hepatocytes from both missense (Npc1m164) and null (Npc1−/−) mice, we concluded that vorinostat normalized both genotypes, relative to their own age-, gender-, and background-matched controls. Interestingly, apoB levels were significantly increased in Npc1−/− hepatocytes (Fig. 10d), consistent with previously demonstrated transcriptional repressions of apoB homeostasis in murine models of cancer (the equivalent of a human dose of 400 mg/day). We were successful in that vorinostat treatment was therapeutic for visceral NP-C disease, as evidenced by normalized liver lipid homeostasis at the transcriptional level and marked improvement of liver pathology and function. However, aspects of disease progression such as morbidity, mortality, and neurodegeneration were not impacted, due, at least in part, to ineffective brain penetration.

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ground in the context of Npc1 missense mutations (e.g. different lipidomic profiles in NPC1

\textsuperscript{H1061T} mice in the BALB/c and C57BL/6 backgrounds) (19). Similarly, there is precedence for varying effects of the \textasciitilde 300 disease-causing mutations in NPC1 on the clinical progression of NP-C disease (8, 55–57). Regarding the efficacy of vorinostat, the extent of misfolding and feasibility of refolding will be critical to decipher for different mutations in NPC1. Our results suggest that vorinostat would be therapeutic in the liver of all NP-C patients regardless of mutation because the mechanism underlying improvement was predominantly independent of Npc1 expression.

It is clear that the bioavailability and stability of vorinostat represent a significant but, we believe, surmountable hurdle to further progress. We determined that i.p. administration and a PEG/DMSO vehicle result in vorinostat levels at <5 \textmu M in the brain, 30 min after injection, a result in stark contrast to the 60 \textmu M concentration in plasma at the same juncture. Concentrations of 5 \textmu M are necessary to reduce lipid accumulation with 24-h treatment in cell culture (11–13). Given the 12-min half-life of vorinostat in rats and dogs (58) compared with the effectively infinite half-life in cell culture, it is plausible that >5 \textmu M is necessary to reduce lipid accumulation in the murine brain. Interestingly, using the PEG/DMSO vehicle and the 150 mg/kg dose in this study, i.p. administered vorinostat reduced the tumor size in the brain of a mouse model of brain cancer (20), a result that was attributed to a disrupted blood-brain barrier in brain cancer models (44). It is widely accepted that the murine and feline models of NP-C disease do not have a compromised blood-brain barrier (59, 60). The blood-brain barrier could be circumvented by a variety of strategies. Rescue of neurodegenerative symptoms in mouse models of Huntington’s disease have been accomplished by dissolving vorinostat in 2-hydroxypropyl-\textbeta-cyclodextrin (5, 43). This is interesting, because although 2-hydroxypropyl-\textbeta-cyclodextrin is a compound not known to cross the blood-brain barrier, perhaps it improves blood-brain barrier penetration of vorinostat in the Huntington’s disease studies. Intriguingly, 2-hydroxypropyl-\textbeta-cyclodextrin alone, when administered directly into the CNS, is itself a promising therapeutic to treat NP-C disease (14, 42) and is currently being evaluated in a clinical trial. Indeed, Alam et al. (61) recently demonstrated a striking synergy (\textasciitilde 2-fold life extension, relative to 2-hydroxypropyl-\textbeta-cyclodextrin monotherapy) when treating Npc1\textsuperscript{Hnmf164} mice (in a BALB/c background) by combining high concentrations of vorinostat with therapeutic doses of 2-hydroxypropyl-\textbeta-cyclodextrin in a PEG/DMSO vehicle. Although the confounding effect of using already therapeutic doses of 2-hydroxypropyl-\textbeta-cyclodextrin and possibly PEG/DMSO remains to be determined, it is apparent that the pharmacokinetics of vorinostat can be improved.

In summary, NP-C disease is a fatal pediatric neurodegenerative disease due to lysosomal accumulation of cholesterol and

FIGURE 10. Transcriptional response of Npc1\textsuperscript{Hnmf164} and Npc1/H1546/H1546 hepatocytes following treatment with vorinostat. Hepatocytes were isolated from livers of Npc1\textsuperscript{Hnmf164} and Npc1/H1546/H1546 mice; incubated for 24 h in DMEM + 10% FBS with 10 \textmu M vorinostat or DMSO; and assessed for Npc1, Apob, Chop, Hmgcr, and Abcg1 gene expression by qRT-PCR relative to cyclophilin (CYCLO) and appropriate WT controls using primers described under “Experimental Procedures.” All groups are n = 3 with data shown as mean \pm S.D. (error bars) * p < 0.05, Student’s t test.
sphingolipids. Currently, there is no effective FDA-approved therapy to treat NP-C disease. Here we have translated prior results in a yeast model of NP-C disease and NP-C patient fibroblasts (11) to the Npc1<sup>nmf164</sup> mouse model of NP-C disease, wherein we report that a marked improvement in liver function was accompanied by normalized expression of key hepatic homeostatic genes in vorinostat-treated mice. With these data, we provide proof of principle that HDAC inhibition has the potential to be therapeutic in vivo in an animal model of NP-C disease. Indeed, the potential of vorinostat to ameliorate peripheral NPC disease in adults is currently the object of a Phase 1/2 clinical trial (ClinicalTrials.gov number NCT02124083).

Our results indicate that vorinostat treatment limits the aberrant up-regulation of cholesterol biosynthesis and uptake that arises from lysosomal sequestration of cholesterol in NP-C disease. Consequently, this markedly improves the visceral pathophysiology of this disease. With improved blood-brain barrier penetration, we propose that vorinostat and other HDAC inhibitors may prove useful for treatment of the neurological aspects of NP-C disease.

**Experimental Procedures**

**Animals**—Animal husbandry and experiments were approved by the Columbia University and Victoria University animal ethics committees. Animals were exposed to alternating 12-h periods of dark and light and were fed a standard diet, ad libitum, until dissection. Npc1<sup>nmf164</sup> mutant and WT littermates (C57BL/6 background) were generated by crossing heterozygous Npc1<sup>nmf164</sup> males and females. Npc1 genotyping was conducted by restriction digests of PCR amplicons as described (17). Mice were i.p. administered with vorinostat (LC Laboratories) in 10% DMSO and 45% PEG 400, as described previously (20), from P21 to P60 (for all in vivo experiments except the body weight/life span experiments, which were continued to P90). Disease progression was monitored in terms of weekly weight gain from P21 to P90. For all experiments except the pharmacokinetic assay, Npc1<sup>nmf164</sup> mutant mice and WT mice were anesthetized 6 h after the last injection with an i.p. administered injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), serum was collected, and animals were transcardially perfused with 0.9% saline solution. Following perfusion, half of the cerebrum and liver were removed and immediately frozen at −80 °C for genetic and biochemical analysis with the other half fixed in 4% paraformaldehyde for histological analysis.

**Primary Hepatocytes and Cell Culture**—Primary hepatocytes were isolated from age- and background-matched control, Npc1<sup>nmf164</sup> (C57BL/6 background), and Npc1<sup>−/−</sup> (BALB/c background, generously provided by X. Huang and S. Walkley) mice (62). Filtered, washed cells were plated into collagen-coated 6-well plates at a density of 500,000 viable cells/well in DMEM + 10% FBS. Fibroblasts derived from Npc1<sup>nmf164</sup> mutant mice and wild-type littermates or NPC1-deficient human osteosarcoma U2OS cells expressing the NPC1<sup>11061T</sup> protein were generated and maintained as described previously (63).

**ApoB Metabolism**—For steady-state apoB labeling and immunoprecipitation, primary hepatocytes were incubated in methionine-free DMEM for 1 h and then labeled with [35S]methionine in methionine-free DMEM for 2 h (64). Cells were collected in 200 μl of lysis buffer (62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM benzamide, 5 mM EDTA, 100 units/ml apro tinin, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, and 10 mM HEPES, pH 8.0) and boiled with sample buffer for 5 min. Tri- chloroacetic acid total precipitable 35S counts of the cell lysate were determined and equal radiolabeled intracellular and medium protein was processed by immunoprecipitation (antilipoprotein B; Calbiochem) and separated on 4% SDS-poly acrylamide gels. The gels were dried under vacuum at 65 °C for 2 h and exposed to X-ray films to visualize apoB100 and apoB48.

RNA-Seq Analysis—Total RNA was extracted from livers of Npc1<sup>nmf164</sup> vehicle control and vorinostat-treated mice (7 animals/group) using the PureLink RNA minikit (Life Technologies) and processed for RNA-Seq analyses at the Australian Genome Research Facility. RNA-Seq libraries were prepared using the TruSeq RNA version 2 kit (Illumina) and quantified using Bioanalyzer (Agilent). The prepared libraries were sequenced using an Illumina HiSeq 2000 (100-bp single end reads). The reads were mapped to the Mus musculus genome (Build version mm10) using TopHat (version 2.0.13). Transcripts were assembled utilizing reference-based annotation with Cufflinks (version 2.2.1). Gene expression was normalized and quantified using trimmed mean of M values. Differential gene expression analysis of vorinostat-treated mice relative to vehicle-treated mice was calculated using log<sub>2</sub> ratio (-fold change) and statistically evaluated using edgeR. The data set has been deposited in the NCBI GEO database. Gene ontology processes of differentially expressed genes were classified using PANTHER (protein analysis through evolutionary relationships) and MGD (Mouse Genome Database) (65, 66). The web-based Gene Set Analysis Toolkit (34) was used to conduct enrichment analyses of functions and pathways. Phenolyzer (35) was used to identify core components of the vorinostat interactome.

qRT-PCR—Gene expression was measured using qRT-PCR. For validation of RNA-Seq results, 80 ng of total RNA was converted to mRNA using the Power SYBR Green RNA-to-CT kit (Applied Biosystems) and quantified using SYBR Green chemistry and a CFX Connect real-time PCR detection system (BioRad). For characterization of primary hepatocytes, RNA was isolated from cells with TRIzol (Invitrogen) and was used as a template in qRT-PCR using an Applied Biosystems 7900HT sequence detection system. The mRNA levels were calculated by the 2−ΔΔCT method (67) using the following 5′ to 3′ primer sequences: Abcg1 (forward, GCTGGTGCATTGTGCTGTT; reverse, TGCAGCTCAATCAGTATGCTCAA) (14); ApoB (forward, CTGGGGTCACAGCAGTT; reverse, TCAC- CAGTTTCTGCTTTTTG) (68); Chap (forward, CACCCA-CCTGAAACGACAC; reverse, GGTGAAAGGA- GGACTCA) (69); Cyclo (forward, TGGAGACACCAAGACACACA; reverse, TGCGGGAGTCGACAATGAT) (70); Cyp51 (forward, CTCGGATGGAGTTTTACC; reverse,
TCTCTCGATGGGCTCTATCC); Gapdh (forward, TGGCGCTGATTTCTGGT; reverse, TGGGCTGATTTCTGGT); Hmger (forward, CTTGTGGATGCTCTTGAT; reverse, AGGCGAGCAACATGAT) (14); Hmges (forward, GCCGTGCTCAGTGCT; reverse, AGTGAGGAAACC GGCAA); Mvd (forward, CCGTGCACATCGCAGTAT; reverse, TTGGTGCTGTTTTTAGCTGGT); Mvk (forward, GGTGTGGTGACACTTC; reverse, CCTGAGCGGTTGAGGAC); Npc1 (forward, AACCCTGACACTGCAGCAG; reverse, CTCAATATGCTGAGTTCTTTGTGTG) (46); and Npc2 (forward, TGGCGCTGTGTGCTGTG; reverse, TCGGCTGTCATTGGTTCTCTC) (46). Primers without references were designed using Primer-BLAST (71).

Immunoblotting and Deglycosylation—Frozen liver was homogenized in radioimmuno precipitation assay buffer with the addition of protease inhibitors (Roche Applied Science) and centrifuged at 16,000 × g. Protein concentration was measured using the BCA protein assay (Bio-Rad). For deglycosylation, protein extracts were combined with glycoprotein denaturant buffer (5% SDS, 0.4 M DTT), denatured at 65 °C for 10 min, and 1000 units of Endo H (New England Biolabs) or GlycoBuffer 3 (New England Biolabs) with 1% Nonidet P-40 and 1% Triton X-100 to prepare lipids by a modified Bligh and Dyer extraction procedure (72). Free cholesterol, sphingomyelin, sphingosine, glucosylceramide, and lactosylceramide were measured using LC/MS as described previously (73, 74) modified for use with a Shimadzu 8040 LC/MS system, an APCI source with MRM and a Waters Acuity BEH C18 1.7-μm, 2.1 × 150-mm column. Standards (Avanti Polar Lipids) were used for quantification, and all measurements were normalized to liver weight. Subcellular accumulation of free cholesterol in liver sections was visualized using fluorescence of filipin as described previously (42) and quantified using densitometry software (ImageJ).

NPC1 Protein Refolding—Cells were incubated in the presence and absence of glycerol, 3-methyladenine, MG132, or lactacystin for 20 h at 37 °C, lysed in 100 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% IGEPEAL CA-630 for 15 min at 4 °C, and centrifuged for 5 min at 14,000 rpm at 4 °C. Total cell lysates were denatured at 70 °C for 10 min, subjected to SDS-PAGE through a 3–8% Tris acetate precast gel (Invitrogen), transferred to nitrocellulose membrane (Protran), and probed using primary antibodies against NPC1 (Abcam ab134113 or Abcam ab36983) or actin antibody (Sigma). Membranes were probed with goat anti-mouse or goat anti-rabbit secondary antibodies (GE Healthcare), visualized using ECL plus (GE Healthcare) with a fluorescent image analyzer (Fuji FLA-5100), and quantified using densitometry software (ImageJ).

Pharmacokinetics—Vorinostat (150 mg/kg) was administered as described above with one injection in Npc1mungtrash mice. Plasma and brain were collected at 30, 60, 120, and 240 min after the injection. Vorinostat levels were measured using LC-MS/MS as described previously (43).

Acknowledgments—We are grateful for the support of Rodney Rothstein and Michael Shalesanski in the final stages of this study.
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