**Vorinostat, a Histone Deacetylase Inhibitor, as a
Candidate Therapy to Treat Liver Pathology in a
Niemann-Pick type C Disease Mouse Model**

By

Natalie Hammond

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Abstract

Niemann-Pick type C (NPC) disease is a rare neuro-visceral, lysosomal storage disorder for which no effective therapy yet exists. A recessive mutation in the late endosomal/lysosomal cholesterol transport genes *NPC1* (95%) or *NPC2* (5%) are the causative factors which leads to an accumulation of unesterified cholesterol and sphingolipids in the late endosome/lysosome. It is a build-up of these lipids that, in the majority of cases, ultimately leads to premature death prior to adolescence. In recent years, an imbalance of histone acetylation in a yeast model of NPC disease and subsequently an increased expression of histone deacetylase genes in NPC patient fibroblasts relative to healthy controls was discovered. This led to the finding that Vorinostat (suberoylanilide hydroxamic acid (SAHA); Zolinza®) a histone deacetylase inhibitor (HDACi) drug, rescued unesterified cholesterol accumulation in NPC patient fibroblasts. From these findings in NPC patient fibroblasts, a Phase I clinical trial testing the efficacy of orally-administered Vorinostat in adult NPC disease patients commenced in 2014; however, the therapeutic efficacy of Vorinostat in a whole animal model of NPC disease has not been investigated and is thus unknown. In this thesis, the therapeutic efficacy of intra-peritoneal administered 150 mg/kg Vorinostat in the *Npc1^{nmf164}* mouse was explored. This internationally approved HDACi reduced liver disease by decreasing lipid accumulation without increasing expression of NPC1; however, the treatment did not delay weight loss, onset of ataxia and premature death, possibly due to insufficient concentrations penetrating through the blood brain barrier. Transcriptome analysis suggested Vorinostat improved liver disease in a pleiotropic manner, not surprising given the epigenetic nature of HDACi at the gene expression level. Overall, the results herein are of particular importance to the current clinical trial where the therapeutic efficacy is being investigated without any knowledge of efficacy in an animal of NPC disease.

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Abbreviations

2,5-DHB	2,5-dihydroxybenzoic acid
ac-H3	Acetylated-histone-H3
ALT	Alanine transaminase
AUC	Area under the curve
BBB	Blood brain barrier
BMP	Bis(monoacylglycero) phosphate
CNS	Central nervous system
CCL3	Chemokine (C-C Motif) ligand 3
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
FDA	Food and Drug Administration
GM2	Ganglioside monosialic 2
GM3	Ganglioside monosialic 3
HAT	Histone acetyltransferase
HDACi	Histone deacetylase inhibitor
HDL	High density lipoprotein
HPB-CD	2-hydroxypropyl-beta-cyclodextrin
HSP70	Heat shock protein 70
i.p.	Intra-peritoneal
LE/Ly	Late endosome/ lysosome
LDL	Low density lipoprotein
LMP	Lysosomal membrane protein
LSD	Lysosomal storage disease
MALDI-TOF MS	Matrix-assisted laser desorption ionisation mass spectrometer
MGD	Mouse Genome Database
NPC	Niemann-Pick type C
P14	14 days post-partum

PANTHER	Protein ANalysis Through Evolutionary Relationships
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PEG	Polyethylene glycol 400
SM	Sphingomyelin
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
TNF-α	Tumor necrosis factor-alpha
TSA	Trichostatin A
WEBGESTALT	WEB-based Gene SeT AnaLysis Toolkit

Chapter 1. Literature Review

1.1 The lysosome

Lysosomes are organelles whose primary role is in degradation and recycling of extracellular or intracellular macromolecules presented to it through endocytosis, phagocytosis or autophagy (Saftig and Klumperman 2009). Two important components of the lysosomal machinery include lysosomal membrane proteins (LMPs) and lysosomal hydrolases (Appelqvist, Waster et al. 2013). LMPs are present in the lysosomal membrane, and function in membrane trafficking, cellular distribution and exocytosis. These proteins are heavily glycosylated as a protective measure against degradation from lysosomal hydrolases present inside the lysosome (Appelqvist, Waster et al. 2013). The catabolic activity of the lysosomal hydrolases is dependent on an acidic lysosome interior (pH 4.6-5.0), maintained *via* an H⁺ ATPase pump which transfers protons from the cytosol to the lysosomal lumen (Ohkuma, Moriyama et al. 1982). Once the macromolecules are broken down the building blocks are recycled back into the cell (Appelqvist, Waster et al. 2013). Lysosomes are the terminal station for numerous cellular processes involved in cellular homeostasis, including endocytosis, autophagy, apoptosis and phagocytosis (Fig.1).

1.1.1 Functions of the lysosome

Endocytosis is the process by which the plasma membrane internalises extracellular cargo into the cell (Luzio, Pryor et al. 2007). Once internalised, they are termed early endosomes and function largely in cargo sorting. Early endosomes mature into late endosomes which fuse with the lysosome where extracellular cargo is consequently degraded (Appelqvist, Waster et al. 2013). Autophagy is the cellular process involving recycling of damaged proteins and organelles and intracellular cytoplasmic components in times of stress or in the presence of aggregated proteins (Saftig and Klumperman 2009). Three types of autophagy exist, including chaperone mediated autophagy, microautophagy and macroautophagy (Levine and Kroemer 2008). Chaperone mediated autophagy, as the name implies functions with chaperone proteins in complex with target proteins containing the specific KFERQ motif, and delivers it to the lysosomal membrane (Platt, Boland et al. 2012). Here, it gains entry via interaction with a LMP known as Lamp-2 receptor. Microautophagy is not well characterized, but briefly, it involves direct pinocytosis of cytosolic material at the

Figure 1: Functions of the lysosome. Lysosomes function primarily in degradation and recycling. An H^+ ATPase pump transfers protons from the cytosol to the lysosomal lumen thus maintaining an acidic interior for the organelle's catabolic activity. Macromolecules are presented to the lysosome through a number of different processes. **1.** Endocytosis begins with the internalisation of extracellular cargo into the cell (early endosome). After maturing into a late endosome, it next fuses with the lysosome (endolysosome) and degradation takes place. **2.** Autophagy consists of three pathways: (i) microautophagy involves pinocytosis of macromolecules at the lysosomal membrane; (ii) chaperone-mediated autophagy involves macromolecules specifically tagged for degradation which are delivered to the lysosome with the help of chaperone proteins; and (iii) macroautophagy, which begins with the generation of a new membrane (phagophore) that uptakes macromolecules for degradation, this matures into an autophagosome which either fuses with an endosome (amphisome) or straight to the lysosome (autolysosome) for degradation. **3.** Apoptosis involves the release of lysosomal hydrolases into the cytosol which breaks down cellular components. **4.** Phagocytosis begins with the uptake of foreign particle from outside the cell (phagosome), which fuses with a lysosome for degradation (phagolysosome). **5.** Degraded macromolecules are released from the lysosome to the outside of the cell through exocytosis. Figure adapted from Saftig and Klumperman (2009).

lysosomal membrane (Schultz, Tecedor et al. 2011). Macroautophagy, hereon referred to simply as autophagy, is the most characterized type of autophagy. It is initiated when cytoplasmic contents are captured in a newly generated membrane, termed phagophore. This matures into an autophagosome which can either fuse to an endosome (termed amphisome) followed by the lysosome (Sarkar, Carroll et al. 2013), or fuse straight to a lysosome termed autolysosome, cargo is then degraded into macromolecules and recycled into the cytosol (Appelqvist, Waster et al. 2013). Apoptosis is programmed cell death, arising from the release of lysosomal hydrolases from the lysosomal lumen into the cytosol. This results in the indiscriminate breakdown of important cellular components (Boya and Kroemer 2008). Phagocytosis begins when cells engulf foreign particles which mature into a phagosome. For

degradation, the phagosome fuses with the lysosome, termed phagolysosome (Luzio, Pryor et al. 2007). Broken down macromolecules are released into the outside of the cell through exocytosis.

1.2 Lysosomal Storage Disease (LSD)

As outlined above, numerous cellular functions rely on functional lysosomes to maintain cellular homeostasis. Thus when mutations arise in lysosomal genes, profound disturbances result. When LMPs or catabolic enzymes are genetically defective, this leads to inhibition of other enzymes and ultimately resulting in aberrations in normal cell functions and secondary substrate accumulation (Schultz, Tecedor et al. 2011). There are over 50 known lysosomal storage diseases (LSDs) caused by the reduced activity or absence of a lysosomal related gene (Parenti, Andria et al. 2015). Although independently LSDs are rare, collectively prevalence is $\sim 1/8000$ (Poupětová, Ledvinová et al. 2010). Mutations in lysosomal related genes do not only lead to LSDs, but are also observed in cancer and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Levine and Kroemer 2008). The degree of disease severity and age of onset of LSDs depend on the degree of residual function of the defective protein. With null or near null mutations, disease is often severe and present in early utero or early infancy; reduced function is often less severe and juvenile/adult onset is common (Vitner, Platt et al. 2010).

Neurodegeneration and visceral pathology are common in LSDs, but not all exhibit pathology in these organs (Platt, Boland et al. 2012). Although different macromolecules accumulate depending on the mutation, many of the LSDs share commonalities in clinical features, cellular aberrations and biochemical characteristics – all LSDs are caused by specific accumulating macromolecules inside the lysosome (Platt, Boland et al. 2012). Therefore, if a specific pathway that regulates several LSDs can be identified, this pathway may be a candidate target to treat these LSDs, where currently limited therapeutics are available (Parenti, Andria et al. 2015).

Current therapeutic interventions for LSDs include: enzyme replacement therapy, substrate reduction therapy, pharmacological chaperone therapy and gene therapy (Platt, Boland et al. 2012). The main objective of these therapies is to either

Figure 2: Therapeutic targets for LSDs. Therapeutic interventions to treat LSDs aim to either increase the level of mutated enzyme/protein or reduce the accumulating toxic substrate. **1.** Enzyme replacement therapy replaces defective enzymes with wild-type enzyme. **2.** Substrate reduction therapy inhibits the biosynthetic pathway of the accumulating substrate. **3.** Pharmacological chaperone therapy involves molecules that help mutated protein fold. **4.** Gene therapy involves replacing the defective gene with a wild-type copy. Figure adapted from Parenti *et al.* (2015).

increase the level of the mutated enzyme/protein or reduce the accumulating toxic substrate. Fig.2 outlines where in the pathway these therapies target (Parenti, Andria *et al.* 2015). Enzyme replacement therapy involves the replacement of defective enzymes, with a wild-type enzyme endocytosed into macrophages. In the 1990s, enzyme replacement therapy showed promising efficacy in Type 1 Gaucher's disease (Barton, Brady *et al.* 1991) and is now available for treating Fabry disease, Pompe disease and Mucopolysaccharidosis I, II and VI (Parenti, Andria *et al.* 2015). Limitations of this approach however, is the limited blood brain barrier (BBB) penetration with intravenous injection, due to the large molecular size of the recombinant enzymes (Schultz, Tecedor *et al.* 2011). Further, treatments of this kind can only be used for disorders where a mutation is present in an enzyme, like the diseases mentioned above, as opposed to a disease that results due to a mutation in a protein (*e.g.*, Niemann-Pick type C disease). Substrate reduction therapy functions to inhibit steps of the biosynthetic pathway of the accumulating substrate (Schultz, Tecedor *et al.* 2011). Miglustat, is an example of a substrate reducing therapy that is approved to treat patients with Gaucher's disease. This therapy functions by inhibiting

glucosylceramide synthase and thus glycosphingolipid biosynthesis (Ficicioglu 2008). Pharmacological chaperone therapy involves the use of small-molecule ligands that function to help mutated, disease-causing protein to fold efficiently and escape degradation by quality control systems; thus the mutant protein is partially rescued (Parenti 2009). Additionally this therapy has also showed promising efficacy in preclinical *in vitro* and *in vivo* studies when combined with enzyme replacement therapies, enhancing the stability of wild-type recombinant enzymes (*e.g.*, Gaucher's disease) (Parenti, Andria et al. 2015). Lastly, gene therapy involves delivering a wild-type copy of the defective gene so to increase its activity in the cell (Futerman and van Meer 2004). Because LSDs are monogenic, the idea of gene therapy is promising however a limitation of this therapy is that delivery of functional genes involves using viral vectors as carriers, a method that may cause harmful side effects (Schultz, Tecedor et al. 2011). Clinical trials are in progress using gene therapy in Pompe disease, Fabry disease and Gaucher's disease (Parenti, Andria et al. 2015).

1.3 Niemann-Pick type C disease

1.3.1 History and classification

Niemann-Pick type C (NPC) disease is a member of a small group of LSDs collectively termed Niemann-Pick disease – named after Albert Niemann and Ludwig Pick from early research conducted in the 1920s (Vanier 2010). Forty years later, Crocker and Farber revealed the heterogeneous presentation among cases of Niemann-Pick disease; primarily there was variability in age of onset and levels of sphingomyelin accumulation in diseased tissue. From here Crocker put forward a classification system, separating Niemann-Pick into four subtypes: type A, type B, type C and type D (Vanier and Millat 2003). Type A has severe neurological deterioration and immense sphingomyelin storage abnormalities in the viscera and brain. Type B is similar but disease primarily affects visceral organs, with little involvement of the central nervous system (CNS). Type C differs from subtype A and B in that its main offending lipid is unesterified cholesterol, as opposed to sphingomyelin. In addition, both the viscera and CNS are affected in type C. Type D, now grouped within type C, was previously thought to be a genetic variant of NPC1 unique to persons in Nova

Scotia, but this variant was later recovered elsewhere in the world (Greer, Riddell et al. 1998).

1.3.2 Disease description

NPC disease is a fatal autosomal recessive, monogenic disorder caused by mutations in *NPC1* (95% of cases) or *NPC2* (5% of cases) (Vanier 2010). It is characterised by aberrations in intracellular cholesterol transport that result in accumulations of unesterified cholesterol and sphingolipids in the late endosome/lysosome (herein referred to as LE/Ly) (Mengel, Klunemann et al. 2013). Like the other LSDs, NPC disease is rare, arising in approximately 1/120,000 live births, however this estimate is thought to be unfitting due to difficulty in diagnosis (Vanier 2010).

1.3.3 Signs, symptoms and life expectancy

NPC disease is neuro-visceral and highly heterogeneous in clinical presentation, showing major pathology in the brain, liver, spleen and occasionally in the lung (Patterson, Mengel et al. 2013). For severe cases of disease, sufferers die at birth or within six months from visceral organ failure. More commonly, sufferers will develop visceral disease with neurological disease progressing ultimately causing death (Vanier 2010). Like clinical presentation, life expectancy varies significantly between individuals wherein life expectancy is between 10-25 years of age (Vanier 2010).

1.3.4 Diagnosis

The first step taken for diagnosing NPC disease is the examination of medical history and detection of characteristic signs and symptoms (*e.g.*, combined splenomegaly, ataxia and vertical supranuclear gaze palsy) (Vanier 2010). However the heterogeneous presentation of the disease means diagnosis is troublesome and often undetected for years where it becomes too late to reverse the neurological damage that has occurred. To confirm diagnosis, genetic testing (*e.g.*, DNA sequencing to identify mutations in *NPC1* or *NPC2*) and biochemical tests (*e.g.*, filipin staining of patient fibroblasts to detect unesterified cholesterol accumulation) are undertaken (Mengel, Klunemann et al. 2013). DNA sequencing and filipin staining are currently the primary laboratory diagnostic tools to detect NPC disease (Tangemo, Weber et al. 2011). Additionally, the rarity of NPC disease contributes to the difficulty in diagnosis;

doctors may not come across such a disease in their lifetime, whereas symptoms like splenomegaly are common.

1.3.5 NPC1 and NPC2 protein

NPC1 is a transmembrane protein (1278 amino acids, 170-190 kDa) in LE/Ly (Gelsthorpe, Baumann et al. 2008); NPC2 is a small soluble protein in LE/Ly (151 amino acids, 18 kDa) (Zampieri, Bianchi et al. 2014). It is theorized that NPC1 and NPC2 work as a tag-team duo in unesterified cholesterol egress (Infante, Wang et al. 2008) (Fig.3). First, to get to the lysosome, low density lipoprotein (LDL) enters the cell by binding to low density lipoprotein receptor (LDLR) present on the cell surface where it is subsequently endocytosed to the lysosome via vesicles (Brown and Goldstein 1979). In the lysosome, lysosomal acid lipase acts to hydrolyse cholesteryl ester to form unesterified cholesterol, which is bound by NPC2 and transferred to NPC1 in the membrane where it is ultimately transported out of the lysosome (Kwon, Abi-Mosleh et al. 2009). Unesterified cholesterol may then be transported to the plasma membrane for structural purposes, the endoplasmic reticulum (ER) for re-esterification, or used for bile acid synthesis (Vance and Karten 2014). However, in

Figure 3: NPC2 and NPC1 hand-off model. Exogenous cholesterol in the form of LDL particles (containing cholesteryl ester) enters the cell through binding to its receptor LDLR, and subsequently endocytosed into the LE/Ly. Once inside, acid lipase hydrolyses cholesteryl ester to unesterified cholesterol. NPC2 binds to unesterified cholesterol and transfers it to NPC1 where it then may exit the lysosome. Figure adapted from Kwon *et al.* (2009).

the case of NPC disease, mutations in *NPC1* or *NPC2* prevent the egress of cholesterol from the lysosome thus resulting in the cellular hallmark of the disease, the lysosomal accumulation of unesterified cholesterol in the LE/Ly.

1.4 Molecular pathology of NPC disease

1.4.1 Mutations in *NPC1* and *NPC2*

Mutations in *NPC1* (95%) or *NPC2* (5%) are the causative factors which lead to unesterified cholesterol and sphingolipid accumulation in the LE/Ly in a tissue-specific manner (Loftus, Morris et al. 1997). Up to the year 2008, 234 variants in *NPC1*, and 17 variants in *NPC2* had been described in an online database (database no longer exists) (Runz, Dolle et al. 2008). As seen in Fig.4 majority of mutations in *NPC1* are missense mutations (~70%) (Vanier and Millat 2003); which commonly occur in the cysteine-rich domain. I1061T is reported to be the most common mutation, accounting for 20% of the alleles. The G992R/W variant is common in Europe while the P1007A variant is predominantly observed in the Nova Scotian population (Vanier and Millat 2003). Numerous studies have been undertaken whereby either functional *NPC1* protein replaced mutated protein in tissue specific regions, or alternatively functional *NPC1* protein was knocked-down and phenotypes relating to this knock-down were observed. Importantly, mutations in *NPC1* in neurons specifically, account for neurodegeneration; as opposed to other major cell types in the brain (astrocytes and microglia) (Vance and Karten 2014). Consistent with this, expression of functional *NPC1* in neurons in NPC diseased mice, prevented neurodegeneration (Loftus, Erickson et al. 2002, Lopez, Klein et al. 2011). Moreover when *NPC1* was inhibited in Purkinje cells specifically, mice displayed ataxia but did not undergo weight loss or premature death (Elrick, Pacheco et al. 2010). In the liver, lipid filled macrophage (foam) cells and liver enzymes (*e.g.*, alanine aminotransferase, (ALT)) decreased when functional *NPC1* was provided to cells (Lopez, Klein et al. 2012). These results back the idea that mutations in the *NPC1* protein are responsible for causing disease and thus disease can be rescued when the functional protein is supplied to the tissue.

Figure 4: Mutations in *NPC1*. Over 234 variants have been found in *NPC1*, majority of these are missense mutations and occur in the cysteine-rich domain. I1061T is the most commonly occurring mutation in patients. G992R/W is common in Europe and P1007A is common in the Nova Scotian population. The blue arrow points to the location of the D1005G variant seen in the *Npc1^{nmf164}* mouse model. Figure modified from Runz *et al.* (2008).

1.4.2 Unesterified cholesterol accumulation

There are two ways a cell can acquire cholesterol: 1) endocytosis of exogenous LDL via LDLR or 2) through endogenous synthesis in the ER (Vance and Karten 2014). In NPC disease, the sequestration of unesterified cholesterol in the LE/Ly means cholesterol delivered to the ER falls below a critical threshold and consequently cholesterol synthesis and LDLR is increased to compensate for this reduction (Liscum and Faust 1987). Therefore, pathologies observed in NPC disease are not only produced from the accumulation of unesterified cholesterol in the LE/Ly but also the reduced delivery to the ER, which leads to the increased expression of genes involved in cholesterol synthesis and LDL uptake (Liscum and Faust 1987).

The accumulation of unesterified cholesterol occurs in specific tissues. The liver undergoes significant unesterified cholesterol accumulation in diseased models with an 8- to 10-fold increase in mouse models and a 2- to 3-fold increase in humans (Walkley and Vanier 2009). This is largely a consequence of ~80% of circulating LDL being cleared by the liver (Vance and Karten 2014). Unesterified cholesterol accumulation in the brain appears to not show the same intense build-up as seen in

the viscera, thought to be because of demyelination which occurs as neurodegeneration progresses (Xie, Burns et al. 2000). Additionally, unesterified cholesterol has been reported to be increased by 2- to 5-fold in the spleen, lung and kidney in NPC diseased mice (Xie, Turley et al. 1999). As expected, an accumulation of unesterified cholesterol will result in reduced levels of cholesteryl ester (Mengel, Klunemann et al. 2013).

1.4.3 Additional lipid accumulation

Additional lipids that accumulate in NPC disease include various sphingolipids including sphingomyelin, sphingosine and sphinganine, as well as the gangliosides GM2 and GM3, and the glycolipids glucosylceramide and lactosylceramide. In addition, the phospholipid bis(monoacylglycero) phosphate (BMP) accumulates. Like cholesterol accumulation, the pattern of accumulation of additional lipids is tissue specific. In visceral tissue, all of these additional lipids accumulate at variable concentrations. In contrast, sphingomyelin and BMP do not abundantly accumulate in the brain (Vanier 2010). The main lipids to accumulate in the human brain are GM2 and GM3 (10- to 20-fold increase) (Walkley and Vanier 2009); additionally increases in glucosylceramide, lactosylceramide and sphingosine are also observed in the brain.

1.4.4 Autophagy

Autophagy is defective in NPC disease; however the specific step that is defective is disputed. One theory is that lipid accumulation increases autophagic flux and substrate accumulation therefore leading to increased autophagosomes (Elrick, Yu et al. 2012). Alternatively, it has been suggested that the autophagy pathway is blocked, where autophagosomes cannot fuse with amphisomes, thus degradation cannot efficiently occur (Sarkar, Carroll et al. 2013), again resulting in increased autophagosomes. Interestingly, other neurodegenerative diseases (*e.g.*, Alzheimer's disease, Parkinson's disease and Huntington's disease) display decreased levels of autophagy with disease leading to protein aggregation and ultimately neurodegeneration (Pacheco and Lieberman 2008). By activating autophagy in these neurodegenerative diseases, aggregates causing disease may be cleared. Thus it is clear that levels of autophagy must be tightly controlled, with excessive levels as well as decreased levels leading to disease.

1.4.5 Apoptosis

Apoptosis is dysregulated in NPC disease based on increased expression of apoptotic related genes. Tumour necrosis factor – alpha (TNF- α) is a multifunctional gene involved in both inflammation and apoptotic signalling cascades and has been shown to be significantly up-regulated in the liver with NPC disease (Rimkunas, Graham et al. 2009). Likewise, apoptosis has been observed in the brain with levels of TNF- α mRNA increased by 30- to 50-fold at late stage disease. Further, pro-apoptotic genes (*e.g.*, caspase-8, FADD, TNFp66, TNFp55) were also correlated with NPC disease progression (Wu, Mizukami et al. 2005). Prior to neurodegeneration, the apoptotic markers did not differ from wild-type, conversely these genes were significantly up-regulated at the onset of neurodegeneration compared to wild-type mice (Wu, Mizukami et al. 2005). This result suggested apoptosis was a significant regulator of neurodegeneration. However, when diseased mice were treated with Minocycline, a drug that inhibits apoptosis, neurodegeneration was not subdued (Erickson and Bernard 2002). Conversely, treatment with Imatinib a drug that inhibits c-Abl, the gene that regulates the transcription factor (p73) that induces transcription of pro-apoptotic genes, neurodegeneration decreased (Alvarez, Klein et al. 2008). Therefore apoptosis is dysregulated with disease progression; however treatments targeting apoptosis directly have conflicting results with regards to neurodegeneration rescue.

1.4.6 Immune response

A number of phenotypes involved in immune response are clearly expressed in NPC disease, such as macrophages and the expression of chemokine (C-C Motif) ligand 3 (CCL3) and TNF- α . Questions regarding their contribution to disease have been thoroughly debated to decipher whether inflammation causes the pathogenic cascade in disease or whether inflammation is merely a secondary offence (Lopez, Klein et al. 2012). To test these theories, macrophages, an inflammation factor increased in NPC disease, were specifically depleted in the liver by chemical treatment; however no improvement in liver disease was achieved (*e.g.*, ALT levels remained increased). A reduction in ALT levels in the liver was only observed when NPC diseased mice were provided with a functional NPC1 protein (Lopez, Klein et al. 2012). In Sandhoff disease, a LSD that also accumulates gangliosides, neurodegeneration was delayed in a mouse

model where pro-inflammatory cytokine (CCL3) was deleted (Wu and Proia 2004). However when trialled in NPC diseased mice, no improvement was seen (Lopez, Klein et al. 2012) . Therefore it is likely that inflammation is a secondary effect.

1.5 Model organisms of NPC disease

1.5.1 Human cell lines

The ideal models for demonstrating human NPC disease are human cells derived from patients. These can be skin fibroblasts or hepatic and neuronal cells derived from these fibroblasts. Cell lines of both peripheral and neuronal origin are available for modelling NPC disease; results using these models have gained great insight into biological and biochemical defects in NPC disease. Pentchev *et al.* were the first to observe a defect in cholesterol processing, specifically a defect in cholesterol esterification, in human NPC diseased fibroblasts (Pentchev, Comly et al. 1985). Additionally, they observed abnormal cholesterol storage in human NPC diseased fibroblasts using the filipin stain (Pentchev, Comly et al. 1985). Another noteworthy finding using human NPC diseased fibroblasts was the discovery by Liscum and colleagues, that down-regulation of cholesterol synthesis and LDLR activity by LDL is delayed in diseased fibroblasts (Liscum and Faust 1987). Further, movement of cholesterol from lysosomes to other sites (*e.g.*, plasma membrane) is impaired (Liscum, Ruggiero et al. 1989). Many studies have been conducted in peripheral cells, however neuronal cell models are lacking in comparison, due to the difficulty in obtaining live human neurons from affected patients. Additionally results from peripheral cell models may not be well extrapolated to neural disease as the pattern of lipid accumulation is tissue/organ specific (Walkley and Vanier 2009). Therefore seeing this need for neuronal NPC models, several laboratories have developed different ways of engineering neuronal models; this has been made possible with advances in human stem cell biology (Ordonez, Roberts et al. 2012, Bergamin, Dardis et al. 2013). An advantage of this model is the ability to generate a NPC-like model from control non-diseased cells with treatment of cells with U18666, a class II amphiphilic amine that mimics NPC disease by inducing cholesterol sequestration in LE/Ly (Vance and Karten 2014). This is beneficial especially due to the rarity of this disease. A major disadvantage of cell lines however is their inability to mimic natural

organs or tissue; it is therefore necessary to compile information gained *in vitro* with *in vivo* models (Lopez and Scott 2013).

With mutations in the human *NPC1* gene being fatal and its display of evolutionary conservation from yeast to humans (Lopez and Scott 2013), researchers hypothesize that the molecular defects in humans would be conserved in lower eukaryotic organisms. Thus NPC disease models can give great insight into how mutations lead to disease, how affected proteins function and possible therapeutic avenues to explore for curing human disease. A range of spontaneous and engineered NPC disease models exist: *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (worm), *Drosophila melanogaster* (fruit fly), *Mus musculus* (mouse) and *Felis catus* (cat).

1.5.2 Yeast models

NCR1 is the yeast orthologue of human *NPC1*. Interestingly, deletion of *NCR1* does not give the typical disease phenotype (sterol accumulation), proposed to be due to a redundant pathway of sphingolipid recycling (Malathi, Higaki et al. 2004). The use of yeast to model many human diseases has been established primarily due to the 90 minute generation time of yeast and genome-wide mutant libraries of yeast strains. Munkacsı and colleagues manipulated the yeast model of NPC disease and conducted genome-wide analyses to identify 13 genetic modifiers of NPC disease (Munkacsı, Chen et al. 2011). Here it was found that individually deleting 13 genes resulted in significant growth inhibition of the *NCR1*-deficient yeast strain model. Two of these genes were of great interest as they encoded for proteins in the histone acetyltransferase (HAT) complex, *EAF1* and *YAF9 eaf1*; this suggested an imbalance of histone acetylation with disease. This discovery using the *NCR1* yeast model led onto investigations of histone deacetylase inhibitors as a possible therapy to treat NPC disease. In 2005, *NPC2* the yeast orthologue of human *NPC2* was discovered; *npc2* Δ is not essential for yeast viability and does not show the typical sterol accumulation (Berger, Vanderford et al. 2005). Interestingly, yeast *NPC2* expressed in *NPC2*-deficient human fibroblasts rescues unesterified cholesterol accumulation (Berger, Vanderford et al. 2005).

1.5.3 Worm models

The worm model of NPC disease was identified by the *C. elegans* Genome Project; F02E8.6 and F09G8.4 are two human *NPC1* orthologs now referred to as *ncr-1* and *ncr-2*, respectively. Frameshift mutations in *ncr-1* and the double mutant with two mutations in *ncr-1* and *ncr-2* showed that when grown in a cholesterol deficient environment, newly hatched worms died at the first or second larvae stage (Sym, Basson et al. 2000). This suggests the *NPC1* orthologue in the worm functions in the uptake and metabolism of cholesterol. Additional defects were also observed with reduced progeny, a developmental delay (*ncr-1* only), and inappropriate dauer larva formation (double mutant *ncr-1* and *ncr-2* only). Results therefore suggested that defects in cholesterol metabolism also lead to developmental defects. Nonetheless, this model, like yeast, is valuable for genomic analyses to identify genetic and chemical suppressors to identify drug targets for human disease.

1.5.4 Fruit fly models

There are two homologous *NPC1* genes in the fruit fly, *npc1a* and *npc2a*. The *npc1a* mutant exhibits premature developmental arrest and cholesterol accumulation similar to that seen in human patients (Huang, Suyama et al. 2005). However a drawback of this model was that no significant neurodegeneration was present in the diseased fruit flies. Unique among the different NPC model organisms is that the fruit fly contains a family of eight *NPC2* orthologues referred to as *npc2a-h*. Huang and colleagues found that a fly with mutations in *npc2a* and *npc2b* displayed apoptotic neurodegeneration, therefore becoming a potential new fly model of this neurodegenerative disease (Huang, Warren et al. 2007).

1.5.5 Mouse models

Mouse models are important in the development of novel drugs and are required for Food and Drug Administration (FDA) approval before the completion of human clinical trials. There are numerous mouse models of NPC disease both spontaneous and genetically engineered 1) The *Npc1*^{-/-} mouse model has a spontaneously occurring mutation in the nih allele of the *NPC* gene, resulting in a complete lack of functional *NPC1* protein. It is the most commonly used NPC mouse model with a significantly shortened lifespan (~60-80 days), attributable to an overwhelming display of neurodegeneration (Pentchev, Comly et al. 1986). Other

pathologies observed include: weight loss, ataxia, Purkinje cell loss and lipid accumulation; disease hallmarks are comparable to those seen in human disease. 2) The *Npc1^{nmf164}* mouse model is spontaneously occurring with a D1005G amino acid substitution mutation in the nmf164 allele. This leaves a partially functional NPC1 protein, with a lifespan of ~112 days and an overall less severe form of NPC disease than the *Npc1^{-/-}* (Maue, Burgess et al. 2012). The low expression level of NPC1 protein in this model, makes it amendable to proteostatic therapies, however evidence showing that this mutation effects protein folding is yet to be established (Praggastis, Tortelli et al. 2015). The location of the D1005G mutation in the cysteine rich region flanks mutations reported in NPC patients; NPC1(S1004L), NPC1(P1007A), and NPC1(P1007L). Of note, little research has been undertaken in this mouse model (Maue, Burgess et al. 2012, Alam, Getz et al. 2014) and molecular pathologies are yet to be completely characterised. 3) The *Npc1^{I1061T}* knock-in mouse model was genetically engineered for the use of testing proteostatic therapies. Mutations in I1061T are common in human patients (20% diseased alleles) (Runz, Dolle et al. 2008) and when knocked-in to mice, the expected protein misfolding and subsequent degradation is observed (Praggastis, Tortelli et al. 2015). This partially functional protein, gives the model a lifespan of 125 days. Like the *Npc1^{nmf164}* model: weight loss, ataxia, Purkinje cell loss and lipid accumulation are less severe than the *Npc1^{-/-}* model. 4) The *Npc1^{pf/pf}* model engineered by Xie *et al.* has two mutations in the N-terminal domain of NPC1(P202A/F203A) which cause a disease phenotype comparable to the *Npc1^{-/-}* model with a lifespan of ~84 days (Xie, Brown et al. 2011). 5) Rimkunas *et al.* developed a liver-specific model of NPC disease through an antisense oligonucleotide injection that blocked NPC1 expression in the liver. With this treatment, mice displayed typical NPC liver disease pathology: lipid accumulation, elevated liver enzymes and foam cells and did not undergo weight loss and neurodegeneration (Rimkunas, Graham et al. 2008). This is economically valuable in respect to the homozygous recessive nature of the disease seen in the spontaneous mouse models above. 6) A cerebellum specific model was created by Erick *et al.* (2010) through gene targeting where NPC1 was deleted in Purkinje cells of the cerebellum alone. Mice here displayed ataxia however interestingly did not lose weight and die prematurely,

therefore suggesting the importance of other brain regions in NPC disease pathology (Elrick, Pacheco et al. 2010).

1.5.6 Cat models

The naturally occurring NPC cat model has a missense mutation in amino acid C955S (Lowenthal, Cummings et al. 1990, Brown, Thrall et al. 1994). This mutation results in a reduced lifespan (24 weeks) relative to healthy cats (15 years) (Vite, Bagel et al. 2015). Abnormalities recorded in this model are phenotypically, morphological and biochemically similar to human NPC disease: progressive neurodegeneration and ataxia, low birth weight, hepatosplenomegaly, increase in liver enzymes, foam cells, abnormal accumulation of lipids and lower amounts of cholesteryl ester. A spontaneous mutation in feline NPC2 model has also recently been reported (Zampieri, Bianchi et al. 2014), although this mutant has not been investigated beyond the initial identification of the mutation. Unlike the mouse models that are each used in many laboratories around the world, there is only one laboratory at the University of Pennsylvania that maintains and investigates the cat model of NPC disease.

1.6 Therapies for NPC disease

1.6.1 Arimoclomol in NPC disease

Arimoclomol is a small molecule drug developed by Orphazyme (Garber 2013). This drug is a recombinant version of human heat shock protein 70 (HSP70) that has reduces pathology in a number of neurodegenerative disease models by binding to the lipid, BMP, resulting in acid sphingomyelinase activity and additionally helps to maintain proper protein folding (Kirkegaard, Roth et al. 2010, Kalmar, Lu et al. 2014). In the *Npc1*^{-/-} mouse, Arimoclomol has shown to delay onset of ataxia, reduce lipid accumulation and increase lifespan (<http://www.nnpdf.org/Orphazyme.html>). Interestingly, since this investigation involved the null mouse model, the reduction in NPC disease pathology is not through the molecular chaperone function of Arimoclomol, suggesting Arimoclomol may be therapeutic in missense mutant mouse models as well as NPC patients with either null or missense mutations. Recently, Arimoclomol has been approved for a human clinical trial that has passed Phase I and

is currently recruiting NPC patients for Phase II trials (<https://www.clinicaltrials.gov/ct2/show/NCT02435030>).

1.6.2 Miglustat in NPC disease

Miglustat, is a substrate reduction therapy that reversibly inhibits one of the first steps in glycosphingolipid synthesis, glucosylceramide synthase (Platt, Neises et al. 1994). It was initially investigated in NPC disease given that it is approved in the United States by the FDA to treat Gaucher's disease, a related LSD (Elstein, Hollak et al. 2004). Miglustat is especially effective for Gaucher's disease as its target is upstream of the accumulating substrate, glucosylceramide (Ficicioglu 2008). An appealing ability of Miglustat is its competence to penetrate the BBB therefore making it a promising therapy for treating neurodegenerative diseases (Patterson, Vecchio et al. 2007). With the idea that even though cholesterol storage defects are the main culprit for disease, gangliosides occur as a secondary defect to cholesterol and thus may also cause pathology. By decreasing ganglioside accumulation, a decrease in lysosome congestion improves efflux from lysosomes and thus decreases pathology (Zervas, Somers et al. 2001).

The ability of Miglustat to decrease disease pathology was first discovered in mouse and cat NPC models where improvements in ganglioside accumulation in the brain, longevity and onset of ataxia were observed with treatment (Zervas, Somers et al. 2001). Later, studies in the cat NPC model however saw no improvements with treatment in lipid accumulation in the liver or the liver function enzyme, ALT (Stein, Crooks et al. 2012). Therefore this increase in longevity is due to improvements in neurological disease alone, having no beneficial effect in the liver. In patients, treatment with Miglustat for one year saw improvements or stabilisation in a number of neurological deficits such as improvements in horizontal saccadic eye movement, dysphagia, cognitive tests and slowed ambulation deterioration (Patterson, Vecchio et al. 2007). Miglustat has been internationally approved to treat NPC disease in Europe, South Korea, Brazil, Russia and Australia; however it was not approved by the FDA in the United States due to insufficient data proving its efficacy.

1.6.3 Allopregnanolone in NPC disease

With the observation that the neurosteroid allopregnanolone was decreased in NPC diseased mice, treatment using a by-product-replacement therapy was proposed. Allopregnanolone is involved in neuronal growth, survival and deterioration (Griffin, Gong et al. 2004). When dissolved in a vehicle of 2-hydroxypropyl-beta-cyclodextrin (HPB-CD) and given as a single injection 7 days post-partum (P7) or multiple injections starting at P7, lifespan was doubled and neurodegeneration was delayed (Griffin, Gong et al. 2004). However, the efficacy of Allopregnanolone was scrutinized, where it was found that the beneficial effects initially thought to be from Allopregnanolone were in fact improvements caused by its HPB-CD vehicle. When administered with another vehicle (DMSO), longevity and ataxia showed no improvement, thus suggesting the therapeutic effects were primarily due to the vehicle HPB-CD (Davidson, Ali et al. 2009).

1.6.4 HPB-CD in NPC disease

Cyclodextrins are naturally-occurring cyclic oligosaccharides in a toroid structure with a hydrophobic cavity and a hydrophilic exterior, thus allowing it to complex with otherwise insoluble hydrophobic compounds (Liu 2012). This unique property has meant cyclodextrin is commonly used as a delivery vehicle for other drugs. HPB-CD is a nontoxic synthetic derivative of cyclodextrin and has shown promising therapeutic efficacy in NPC disease. Functionally HPB-CD is initially internalised into the LE/Ly where it works to extract sequestered cholesterol out into the cytosol in an NPC1- and NPC2- independent manner. Studies conducted by Davidson *et al.* found HPB-CD administered at P7 delayed ataxia and weight loss, reduced GM accumulation and extended the lifespan of HPB-CD treated diseased mice. Additionally, the liver showed improvements in pathology where lipid accumulation was near normal (Davidson, Ali et al. 2009). However when treatment was delayed until 49 days post-partum (P49), efficacy of the drug in the brain was diminished (Liu, Ramirez et al. 2010). At P7, the BBB thought to still be forming, therefore HPB-CD is able to cross and improve pathology in the brain. Older aged mice however have a BBB that is completely formed, therefore HPB-CD penetration and subsequent efficacy of the drug is reduced. Additionally, the large size of HPB-CD (1.396 kDa), makes penetrating the BBB difficult.

In 2009, the FDA approved an Investigation New Drug protocol to trial HPB-CD in two NPC diseased patients (<http://www.nnpdf.org/cyclodextrin.html>); HPB-CD had been approved previously as an internationally approved drug vehicle. An Investigation New Drug protocol allows the sponsor of the drug to distribute the drug to clinical investigators for use in human clinical trials; it is one of the first steps in the drug review process. This protocol was initially undertaken on twins suffering from the disease, whom were administered a weekly intravenous infusion of the drug and later expanded to other NPC patients. Although results suggested improvements in visceral pathology, ataxia and swallowing, overall neurological decline was still proceeding due to its inability to penetrate the BBB at sufficient concentrations. Thus improved drug delivery was necessary for rescue of neurodegeneration. Promising results from NPC mice receiving a direct infusion into the CNS with a intracerebroventricular injection via a surgically implanted Ommaya reservoir prevented neurodegeneration (Liu 2012). Similar results of direct administration into the CNS, were also seen in the cat model of NPC disease (Vite, Bagel et al. 2015). These results paved the way for similar experiments conducted in humans.

In January 2013, the FDA approved an application for Phase I safety and efficacy tests for intracerebroventricular injections through an Ommaya reservoir. This trial initially was tested in three NPC diseased patients. Two months following the initiation of this trial however, bacterial infections at the Ommaya reservoir site were reported. Fears of the infection spreading into the brain were apparent. In September 2013, HPB-CD administered via a lumbar intrathecal infusion in replace of the intracerebroventricular injection begun and trials are ongoing. Potential hearing-loss side effects have since been reported (<https://clinicaltrials.gov/ct2/show/NCT01747135>).

1.6.5 Vorinostat in NPC disease

The use of HDACi as a potential therapy for disease, was first recognised when screening for potential anticancer agents where HDACi agents induced tumour cell differentiation (Falkenberg and Johnstone 2014). Three HDACi now have FDA approval for cancer treatment: Panobinostat (for multiple myeloma), Vorinostat and Romidepsin (for T-cell lymphoma) (West and Johnstone 2014). The potential of HDACi

to treat other diseases has now expanded into a number of neurodegenerative diseases: Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and NPC disease (Falkenberg and Johnstone 2014). How disease is rescued in these diseases is not through one specific pathway but multiple pathways, a result that is not surprising given that inhibiting histone deacetylases (HDAC) has been reported to change the expression of 2-10% of the genome (Dokmanovic, Clarke et al. 2007, Chittur, Sangster-Guity et al. 2008).

HDACi were first identified as a therapeutic target to treat NPC disease via the use of the HDACi valproic acid to treat neural stem cells derived from *Npc1*^{-/-} mice. Treatment with valproic acid showed to recover defective cholesterol metabolism in neural stem cells from NPC diseased mice (Kim, Lee et al. 2007). This study was not followed up until 2011 when a yeast genomic analysis independently identified Vorinostat (suberoylanilide hydroxamic acid; SAHA; Zolinza®), as a candidate therapy (Munkacsi, Chen et al. 2011) and a separate study investigated numerous HDACi (Vorinostat, CI-994, LBH589, Trichostatin A (TSA), thiophene benzamide and PCI-34051) to treat NPC disease (Pipalia, Cosner et al. 2011). The latter studies found in NPC patient fibroblasts that HDACs are transcriptionally up-regulated, HDAC4 and HDAC5 were up-regulated most significantly (Munkacsi, Chen et al. 2011), and Vorinostat treatment reduced intracellular cholesterol accumulation by inhibiting these enzymes. Pipalia *et al.* (2011) proposed that the reduction in cholesterol accumulation was as a result of Vorinostat increasing the expression of the mutant NPC1 protein by a chaperone mechanism that improves the folding of the improperly folded mutant protein. The ability of Vorinostat to penetrate the BBB after visceral administration is debated (Hanson, La et al. 2013), but has shown to rescue neurodegeneration in a Huntington's disease mouse model when administered orally in a vehicle of HPB-CD (Hockly, Richon et al. 2003, Mielcarek, Benn et al. 2011). In 2014, as a consequence of these studies and clinical trials that resulted in the approval of Vorinostat to treat cutaneous T-cell lymphoma, the requirement for an Investigation New Drug application was waived and Vorinostat Phase I safety and efficacy trials in adult NPC patients began (<https://clinicaltrials.gov/ct2/show/NCT02124083>). Nonetheless, data testing HDACi

treatment in NPC animal models still is essential in determining the efficacy in whole body NPC models.

1.7 Aim and hypotheses

It is clear that there is an urgent need for a therapeutic treatment capable of reducing NPC disease hallmarks in the brain and the liver. Several treatments are promising, but each has significant drawbacks. Miglustat effectively treats neurodegeneration and extends lifespan of the *Npc1*^{-/-} mouse model of NPC disease, but does not impact the liver (Stein, Crooks et al. 2012). HPB-CD also effectively treats neurodegeneration and extends lifespan of the *Npc1*^{-/-} mouse model of NPC disease as well as being therapeutic in the liver (Davidson, Ali et al. 2009), yet it does not penetrate the BBB, thus it must be administered via a lumbar intrathecal infusion in the current clinical trial (<https://clinicaltrials.gov/ct2/show/NCT01747135>). Vorinostat is an FDA-approved HDACi to treat cutaneous T-cell lymphoma, and has shown to reduce cholesterol and sphingolipid accumulation in NPC patient fibroblasts (Munkacsı, Chen et al. 2011, Pipalia, Cosner et al. 2011). Given the urgency to identify a treatment for this rare disease, a Vorinostat clinical trial is ongoing without any demonstration that Vorinostat is therapeutic in a mouse model of NPC disease (<https://clinicaltrials.gov/ct2/show/NCT02124083>). Thus it is essential that results gained *in vitro* in NPC patient fibroblasts are translated to *in vivo* NPC animal models. The overall aim of this thesis is to conduct the first investigation of Vorinostat in a whole animal model of NPC disease. The specific aims of this thesis are the following: 1) to determine the therapeutic efficacy of Vorinostat in *Npc1*^{nmf164} mice and 2) to determine a mechanism of action of Vorinostat that potentially mediates the progression of NPC disease pathology in the *Npc1*^{nmf164} mouse. It is hypothesized that treatment with Vorinostat will function in a pleiotropic manner to reduce lipid accumulation, rescue liver disease and neurodegeneration, and extend the lifespan of *Npc1*^{nmf164} mice.

Chapter 2. Investigation of the potential to use Vorinostat as a therapy to treat NPC disease

2.1 Introduction

Niemann-Pick type C (NPC) disease is a rare neuro-visceral, lysosomal storage disorder (LSD) for which no effective therapy yet exists. A recessive mutation in the late endosomal/lysosomal (LE/Ly) cholesterol transport genes *NPC1* (95%) or *NPC2* (5%) are the causative factors which leads to an accumulation of unesterified cholesterol and sphingolipids in the LE/Ly; this build-up causes a range of molecular pathologies primarily in the brain and viscera which ultimately leads to premature death at 10-25 years of age (Vanier 2010). Because an NPC patient's life is significantly shortened, time is the enemy of many scientific researchers and families of sufferers with the disease. Therefore development of a new non-internationally approved drug is undesirable, given the 10-15 years that it takes for a novel drug to gain approval. A promising therapy that is already FDA approved to treat an unrelated disease is Vorinostat (suberoylanilide hydroxamic acid (SAHA); Zolinza®), a histone deacetylase inhibitor (HDACi), which is approved to treat cutaneous T-cell lymphoma. However, the therapeutic efficacy of Vorinostat in a whole animal model of NPC disease has not been investigated and is thus unknown.

HDACi were originally identified as promising cancer treatments, which induced tumour cell differentiation (Falkenberg and Johnstone 2014). Treatment has since expanded into a number of other diseases such as those that affect the central nervous system (CNS): Alzheimer's disease, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis (Falkenberg and Johnstone 2014). The primary mechanism by which these diseases are rescued with HDACi treatment is variable, not surprising with their ability to alter gene expression of 2-10% of the genome (Dokmanovic, Clarke et al. 2007, Chittur, Sangster-Guity et al. 2008). A potential mechanism that in this thesis is explored is intervention at the proteostatic level, specifically the activation of chaperone-mediated refolding pathways.

Vorinostat has been shown to rescue neurodegeneration in a mouse model of Huntington's disease, thus it is capable of penetrating the blood brain barrier (BBB) when administered orally in a vehicle of 2-hydroxypropyl-beta-cyclodextrin (HPB-CD) (Hockly, Richon et al. 2003, Mielcarek, Benn et al. 2011). In recent years, Vorinostat was discovered as a potential therapy to treat NPC disease after Munkacsy *et al.*

discovered an imbalance of histone acetylation in a yeast model of disease and subsequently an increase in histone deacetylase (HDAC) genes in NPC patient fibroblasts relative to healthy controls (Munkacsi, Chen et al. 2011). Inhibiting these HDAC proteins using an HDACi treatment rescued unesterified cholesterol accumulation in NPC patient fibroblasts (Munkacsi, Chen et al. 2011, Pipalia, Cosner et al. 2011). Pipalia *et al.* suggested the reduction of lipid accumulation with Vorinostat treatment is through a chaperone-mediated mechanism, induced by the HDACi, leading to proper folding of NPC1 and consequently elevated expression of NPC1 (Pipalia, Cosner et al. 2011). From these findings in NPC patient fibroblasts, a Phase I clinical trial testing the efficacy of orally-administered Vorinostat in adult NPC disease patients begun in 2014 (<https://clinicaltrials.gov/ct2/show/NCT02124083>). This was an exciting yet risky venture without any evidence that Vorinostat is therapeutic in an animal model of NPC disease.

In this investigation, the therapeutic efficacy of 150 mg/kg Vorinostat 5x/wk via i.p. injection in the *Npc1^{nmf164}* mouse was explored. This FDA-approved HDACi reduced liver disease by decreasing lipid accumulation; however, the treatment did not delay weight loss, onset of ataxia and premature death, possibly due to insufficient concentrations penetrating through the BBB. Transcriptome analysis suggested Vorinostat functions in a pluripotent manner to reduce liver disease; cholesterol biosynthesis was here identified to be targeted by Vorinostat, reducing gene expression levels. Thus, Vorinostat is a promising therapy to treat NPC disease in the viscera, however improved penetration into the CNS is needed for the potential rescue of neurodegeneration. Overall, the results herein are of particular importance to the current clinical trial where the therapeutic efficacy is being investigated without any knowledge of efficacy in an animal of NPC disease.

2.2 Methods

2.2.1 Mouse maintenance and breeding

Research was conducted in compliance with Victoria University Animal Ethics Committee. Mice were subjected to a controlled 12 h light/dark cycle and had unlimited access to mouse chow (Diet 86, Sharpes Stockfeed) and fresh water. Bedding (Fort Richards) was changed every 7 d and sterilised shredded paper was provided as environmental stimuli. The *Npc1*^{nmf164} strain on a C57BL/6J background was generated through chemical mutagenesis at Jackson Laboratories and subsequently characterised (Maue, Burgess et al. 2012).

Breeding cages contained one male and one-two female heterozygous *Npc1*^{+/nmf164} mice. Mice generated by these breeding pairs were used for drug efficacy studies wherein wild-type mice were used as controls and the *Npc1*^{nmf164} mice were used as a model of NPC disease given the D1005G missense mutation in the NPC1 protein. Both male and female mice were used in combination in all drug efficacy experiments and ended at the P60 time-point (early-stage disease) with the exception of body weight analysis in which continued to P90 (late-stage disease).

2.2.2 Genotype determination

Ear-tagging, gender and genotype identification of mice was completed at 14 days post-partum (P14). Genomic DNA was extracted from 2 mm of tail tissue cut from the tip of the tail with a razor blade and stored in a 1.5 mL tube. Tail tissue was dissolved in 300 µL of 50 mM NaOH and incubated on a heat block for 50 min at 98°C. Samples were vortexed and returned to a more neutral pH with 30 µL of 1 M Tris-HCl (pH 8) followed by centrifugation at 16,000 xg for 6 min. The supernatant was used as template in a polymerase chain reaction (PCR) to amplify and digest a 641 bp region in the *NPC1* gene as previously described (Maue, Burgess et al. 2012). The PCR was conducted in 25 µL reactions (Table 1) on a TC-5000 PCR Thermal Cycler (Techne). Primers for genotyping were previously used by Maue *et al.* (2012).

NPC1nmf164 F

5'- TGAATGGCTTGAGTCTGTGC – 3'

NPC1nmf164 R

5'- GGGGTAGCAGGGATTAGAAC-3'

Cycling conditions began with an initial denaturation step at 94°C for 10 min, followed by 36 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 5 min. PCR products were electrophoresed on a 1% agarose in 1X TAE running buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA and adjusted to 1 L with ddH₂O), visualised under UV light with the Alpha Imager® Mini (Cell Biosciences), and confirmed via comparison with 1 Kb+ DNA ladder (Thermo Fisher, 10787018).

Table 1: PCR components

Reaction Component	Volume (µL)/Reaction
ddH ₂ O	15.375
10x Ex Taq PCR Buffer	2.5
dNTPs (2.5 mM)	4
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
Takara Ex Taq	0.125
DNA	1
Total Volume	25

PCR products were then digested with *Bst*II to distinguish mice void of any mutation (wild-type mice), mice with one NPC1 mutation (heterozygotes), and mice with two NPC1 mutations (homozygotes). Restriction digests were conducted in 31 µL reactions (Table 2), incubated at 37°C for 3 h, electrophoresed in a 2% agarose gel, and visualised under UV light with the Alpha Imager® Mini.

Table 2: Restriction digest components

Reaction Component	Volume (µL)/Reaction
ddH ₂ O	18
10X Buffer O	2
BstEII (10 units/µL)	1
PCR Product	10
Total Volume	31

2.2.3 Evaluation of growth and disease progression

Mice were examined daily and weighed weekly to monitor growth and disease progression. Weight measures initiated at time of weaning 21 days post-partum (P21) and continued weekly until termination at either 60 days post-partum (P60), 90 days

post-partum (P90), or 30% weight loss from maximum weight. Ataxia onset, visible to the naked eye was recorded.

2.2.4 Intra-peritoneal (i.p.) administration

Mice were weaned into treatment groups at P21 where drug treatment begun and continued until P60 (early-stage disease) or P90 (late-stage disease). In experiments conducted previously by Munkacsy *et al.* (unpublished data), an i.p. injection of 150 mg/kg Vorinostat was found to be the maximum non-toxic dose in wild-type mice and thus was used here for drug efficacy analysis in the *Npc1^{nmf164}* mouse model. Here Vorinostat was administered via i.p. with 150 mg/kg 5x/wk from P21 to P60 or P21 to P90 for longevity tests. The four treatment groups consisted of: wild-type + vehicle, wild-type + Vorinostat, *Npc1^{nmf164}* + vehicle and *Npc1^{nmf164}* + Vorinostat. Administration via i.p. injection with 150 mg/kg of Vorinostat (LC Laboratories) + 10% DMSO (Sigma) + 45% PEG400 (Sigma) in ddH₂O (herein referred to as Vorinostat) or equivalent volume of 10% DMSO + 45% PEG400 in ddH₂O alone (herein referred to as vehicle); this drug formulation was previously described in a study that injected Vorinostat in a mouse model of cancer (Palmieri, Lockman et al. 2009). To aid in dissolving drug in solvent, the mixture was heated for 30 s at 37°C and vortexed for 30 s. A 1 ml syringe (Becton Dickinson) and 27-gauge needle (Becton Dickinson) was used to pierce the peritoneum of the lower abdomen where an appropriate volume according to weight was dispensed (*e.g.*, 15 gram mouse administered with 150 µL).

2.2.5 Time-point analysis of histone acetylation

For time-point analysis, P60 wild-type mice were given a one-time-only i.p. injection of 150 mg/kg of Vorinostat. Next, mice were dissected (using protocol below) at varying time-points: 3 h, 6 h, 12 h and 24 h post-drug treatment. Mice at 0 h were not administered with 150 mg/kg Vorinostat and used as a control.

2.2.6 Collection of serum and tissues

Upon termination at P60 or P90, experimental mice, used in all efficacy tests or unless otherwise stated, were given their last treatment 6 h before dissection. Prior to dissection, the mice were weighed and administered with a lethal dose of ketamine (100 mg/kg) (Provet) and xylazine (10 mg/kg) (Provet) via i.p. injection. Once sedated

(5-10 min), mouse paws were pinned with ventrum up and 70% ethanol was sprayed over the body to prevent contamination of tissue with hair. Using forceps and surgical scissors, an incision was made to the lower left-side of the abdomen. Surgical scissors were used to cut through the diaphragm and rib cage in order to expose the beating heart. Forceps were then used to hold the heart in place where a 1 mL syringe rinsed in 0.5 M EDTA and a 27-gauge needle each were used to pierce the right ventricle and whole blood was collected (300-400 μ L). For saline perfusion, a 50 mL syringe and 27-gauge needle containing 40 mL of 0.9% saline solution (0.9% NaCl in ddH₂O) was used to pierce the left ventricle and saline was dispensed in order to remove blood from organs. Liver tissue and cerebrum were collected, snap frozen on dry ice, and stored at -80°C.

2.2.7 Serum analysis

Mice were not fasted prior to blood collection. Whole blood was collected by cardiac puncture and incubated at room temperature for 30 min to clot. Samples were then centrifuged 1,711 xg for 10 min at room temperature, and serum (upper phase) was collected (200–250 μ L) into a clean tube, then stored at -80°C until analysis. Samples were sent on ice to New Zealand Veterinary Pathology (NZVP) for analysis of the following metabolites: alanine aminotransferase (ALT), total cholesterol, high density lipoprotein (HDL) and triglycerides. Low density lipoprotein (LDL) was calculated using the Friedewald equation [$\text{LDL} = \text{Total cholesterol} - \text{HDL cholesterol} - (\text{Triglyceride}/2.17) \text{ mmol/L}$] (Friedewald, Levy et al. 1972).

2.2.8 Lipidomics

2.2.8.1 Lipid extraction

Sphingomyelin (SM) and phosphatidylcholine (PC) were extracted using a modified Bligh and Dyer method (Bligh and Dyer 1959). Frozen tissue was homogenized using a hand-held glass Dounce in 2 mL of chloroform: methanol (1:2) (Thermo Fisher). 650 μ L of this homogenate was transferred to a 1.5 mL tube for immediate lipid extraction and the remainder was stored at -80°C. Next, 300 μ L of chloroform and 200 μ L of 1 M KCl was added to the homogenate. Samples underwent a 'break phase' which consisted of vortex for 30 s and incubation on ice for 1 min, repeated two times. To separate phases, samples were centrifuged at 9,300 xg for 2

min at 4°C. The lower phase (containing chloroform and lipids) was transferred to a new tube and stored on ice while a second 'break phase' was repeated after addition of 400 µL chloroform. The lower phase from this step was pooled with the first. Samples were dried using a Centrivap Concentrator (Labconco), re-suspended in 40 µL chloroform: methanol (1:2), and stored at -80°C until analysis with mass spectrometer.

2.2.8.2 Matrix-assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS and MS/MS)

Matrix to conduct MALDI-TOF MS and MS/MS was prepared by dissolving 10 mg/ml of 2,5-dihydroxybenzoic acid (2,5-DHB) (matrix) in a 1:1 solution of acetonitrile (Thermo Fisher) and ddH₂O, and vortexed until powder was fully dissolved. The matrix was mixed with the lipid extract at a ratio of 1:100 where 1 µL of mixture was spotted onto a 384-Opti-TOF plate and dried with a warm stream of air. Liver lipids were analysed on the AB Sciex TOF/TOF™ 5800 system. Firstly to calibrate machine, calibration mix 1 (904.45 m/z, 1296.69 m/z, 1570.67 m/z and 2093.08 m/z) (SCIEX) was used in positive mode and the laser was fired at 5800 with a mass range of 900–4000 m/z and focus range of 2000 m/z. Calibration was accepted when a minimum of four peaks were successfully matched with a mass tolerance of 0.2 m/z. To gain spectra of samples in positive mode, the laser was fired at 5800 with a mass range of 100–1000 m/z and focus range 500 m/z. MS/MS was used for further fragmentation of SM (725.6 m/z) and PC (758.6 m/z). Spectra were analysed using Data Explorer® Software (Applied Biosystems) where area under the curve (AUC) was used to determine amount of SM in each sample (Angelini, Vitale et al. 2012) normalised to PC. Eight replicates for each sample were run to ensure an accurate read.

2.2.9 Western blot analysis

2.2.9.1 Total protein extraction

For total protein extraction, 1 mL of ice cold RIPA buffer (Sigma) containing protease inhibitor (Roche) was added to a hand-held glass Dounce homogenizer with a half lobe of frozen liver tissue and homogenized with 20-25 strokes. Homogenate was transferred to a clean 1.5 mL tube, incubated on ice for 30 min, centrifuged at 16,000 xg for 30 min at 4°C, and the upper phase containing total protein was transferred to a new tube and stored at -80°C.

2.2.9.2 Histone extraction

Histones were extracted using the *EpiQuik*TM Total Histone Extraction Kit (EpiGentek). A half lobe of frozen liver tissue was homogenized using a hand-held glass Dounce homogenizer, and histones were extracted according to the manufacturer's protocol. Histone extracts were stored at –80°C until analysis of acetyl-histone-H3 (ac-H3) by Western blot analysis and enzyme-linked immunosorbent assay (ELISA).

2.2.9.3 Protein quantification

Total proteins and histones were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher) following the manufacturer's protocol. Samples were diluted 1:4 with ddH₂O and the absorbance was measured at 600 nm on the EnSpire® 2300 Multimode Reader (Perkin Elmer).

2.2.9.4 Protein denaturation

Protein samples were diluted with ddH₂O to equal concentrations and denatured with 12 µL of 5X Laemmli sample buffer (60 mM Tris-Cl pH 6.8 (Formedium), 2% sodium dodecyl sulfate (SDS) (Invitrogen), 10% glycerol (Invitrogen) and 0.01% bromophenol blue (Sigma)) and 10% β-mercaptoethanol (Sigma) to give a final volume of 60 µL. Samples were then vortexed for 15 s and centrifuged at 2,600 xg for 30 s before denaturing at conditions specific for each protein of interest (Table 3). Denatured protein was stored at -80°C for future use.

Table 3: Denaturing conditions

Protein	Time (min)	Temperature (°C)
NPC1	30	4
LC3	10	37
ac-H3	10	37

2.2.9.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE using a Bis-Tris separating gel (1.5 M Tris HCl pH 8.8, 200 µL 10% SDS, 100 µL ammonium persulfate (APS) (Sigma) and 10 µL tetramethylethylenediamine (TEMED) (Sigma)); for volumes of ddH₂O and 30% acrylamide (Bio-Rad), (see Table 4) and a 4% Bis-Tris stacking gel (6.1 mL ddH₂O, 1.33

mL 30% acrylamide, 2.5 mL 0.5 M Tris-HCl pH 6.8, 100 μ L 10% SDS, 1.33 mL 30% acrylamide, 50 μ L APS and 10 μ L TEMED). The quantity of total protein extract added to SDS-PAGE was protein-specific (Table 4). The gel was run with 2 μ L of Precision Plus Protein Dual Colour (Bio-Rad) at 110 V for 1-2 h in SDS running buffer (1 g SDS, 3.3 g Tris Base and 14.41 g glycine dissolved in 1 L ddH₂O).

Table 4: SDS-PAGE conditions

Protein	% Separating Gel	ddH ₂ O (mL)	30% Acrylamide (mL)	Protein (μ g)
NPC1	7.5	9.7	5	30
LC3	15	4.69	10	30
ac-H3	15	4.69	10	5

2.2.9.6 Membrane transfer

Separated proteins were transferred onto a 0.2 μ m polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Bio-Rad transfer system. Firstly, membrane was normalised in methanol for 2 min and rinsed in ice-cold transfer buffer (3.03 g Tris Base, 14.4 g glycine (Life Technologies), 200 mL methanol (Thermo Fisher), adjusted to 1 L with ddH₂O). Transfer was run at room temperature with an ice-block at either 100 V for 1 h for larger protein (NPC1) or 40 V for 2.25 h for smaller protein (LC3, acetyl-histone-H3).

2.2.9.7 LC3 and ac-H3 membrane probing

Membranes were blocked with 1X PBS (8 g NaCl (Thermo Fisher), 0.2 g KCl (Thermo Fisher), 1.44 g Na₂HPO₄ (Thermo Fisher), 0.24 g KH₂PO (Thermo Fisher) dissolved in 1 L ddH₂O, pH 7.4) containing 1% non-fat milk for 1 h on a rocker at 4°C, and then rinsed with 1X PBS for 10 min three times. The primary antibody, rabbit-polyclonal anti-LC3 (Novus Biologicals, 1:1000) or rabbit-polyclonal anti-ac-H3 (Millipore, 1:1000) in 1X PBS containing 1% non-fat milk, was then incubated with the membrane overnight (15-20 h) at 4°C on a rocker. The membrane was then washed for 10 min three times in 1X PBS followed by incubation with the secondary antibody anti-rabbit Cy5 (GE Healthcare, 1:2000) in 1X PBS containing 1% non-fat milk for 1.5 h at 4°C in the dark, and then rinsed 3 times for 10 min in 1X PBS.

2.2.9.8 NPC1 membrane probing

The membrane was blocked with 1X TBST (8.8 g NaCl, 0.2 g KCl, 3 g Tris Base, 1% Tween20 (Sigma) dissolved in 1 L ddH₂O, pH 7.4) containing 5% non-fat milk for 1 h on a rocker at room temperature, and then rinsed with 1X TBST for 10 min three times. The primary rabbit-monoclonal anti-NPC1 (Abcam, 1:1000) in 1X TBST containing 5% non-fat milk was then incubated with the membrane overnight on the rocker at 4°C. Then the membrane was washed with 1X TBST for 10 min three times, followed by incubation with the secondary anti-rabbit Cy5 (GE Healthcare, 1:2000) in 1X TBST containing 5% milk for 2 h at 4°C in the dark, and then rinsed 3 times for 10 min in 1X TBST.

2.2.9.9 Housekeeping membrane probing

After probing membranes for the protein of interest, mouse anti-actin (Sigma, 1:1000) was used to probe the membrane in a buffer appropriate for the experimental protein of interest (*i.e.*, 1X TBST containing 5% non-fat milk for NPC1 or 1X PBS containing 1% non-fat milk for LC3 and ac-H3) for 2 h at 4°C on the rocker in the dark. The membrane was then rinsed in 1X TBST or PBS for 10 min three times, probed with the secondary anti-mouse Cy5 (GE Healthcare, 1:1000) for 1.5 h at 4°C with agitation in the dark, and rinsed with 1X TBST or PBS for 10 min three times.

2.2.9.10 Blot visualisation and analysis

All western blots were visualised using a fluorescent image analyser (Fujifilm FLA-5100) and quantified by using densitometry software (ImageJ) (Schindelin, Arganda-Carreras et al. 2012) where experimental bands (NPC1, LC3 and ac-H3) were normalised to the housekeeping protein, actin.

2.2.10 mRNA analysis

2.2.10.1 RNA extraction and quantification

Total RNA was extracted from a half lobe of frozen liver using Trizol (Life Technologies) and the PureLink® RNA Mini kit (Life Technologies) following the manufacturer's instructions and a motor cordless homogeniser (Kimble Chase) with pellet pestles (Sigma). RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) where samples were diluted 1:10 in ddH₂O.

Both concentration (ng/μL) and purity (260/280) was recorded in duplicate for each sample. RNA was then diluted to equal concentrations (80 ng/μL) with RNase-free ddH₂O water and stored at -80°C.

2.2.10.2 Primers

Primers were chosen from previously published papers or designed by PrimerBank (Wang, Spandidos et al. 2012) (Supplementary Table 1). All primer pairs were verified for specificity and spanning of one intro using PrimerBlast (Ye, Coulouris et al. 2012). Lyophilized primers were re-suspended at 100 μM in 1X TE (10 mM Tris-HCl pH 8 and 0.1 mM EDTA) and subsequently diluted in water to 10 μM for qRT-PCR analyses.

2.2.10.3 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Table 5: qRT-PCR components

Reaction Component	Volume (μL)/Reaction
Power SYBR® Green RT-PCR Mix (2X) (Life Technologies)	10
Forward Primer (10 μM) (Macrogen)	0.2
Reverse Primer (10 μM) (Macrogen)	0.2
RT Enzyme Mix (125X) (Life Technologies)	0.16
RNase-free ddH₂O	8.44
RNA Template (80 ng/μL)	1
Total Volume	20

The Power SYBR® Green RNA-to-CT™ kit (Life Technologies) was used to measure mRNA levels in the liver in 20 μL reactions (Table 5). Reactions were set up following manufactures instructions in an RNase-free environment. Samples were run on a CFX Connect Real-Time PCR Detection System (Bio-Rad) with specific thermal cycling conditions (Table 6) in triplicate and normalised to GAPDH using the 2^{-ΔCT} method for analysis (Schmittgen and Livak 2008).

Table 6: qRT-PCR thermo cycling conditions

Stage	Temperature (°C)	Time
Holding	48	30 min
Holding	95	10 min
Cycling (40 cycles)	95	15 s
	60	1 min
Melt Curve (in 0.5°C increments)	65 to 95	5 s

2.2.11 ac-H3 ELISA

The *EpiQuik*TM Total Histone H3 acetylation kit (EpiGentek) was used to measure the activity of ac-H3 in liver samples. As stated above, total histones were extracted from a half liver lobe using the *EpiQuik*TM Total Histone Extraction Kit (EpiGentek) and quantified using the Pierce BCA protein assay (Thermo Fisher). To measure activity of ac-H3, the protocol provided by manufacturer was followed with the modification of starting with 20 ng rather than the recommended 50-200 ng. The concentration of ac-H3 was quantified using absorbance measured at 450 nm on the EnSpire[®] 2300 Multimode Reader (Perkin Elmer) and the standard curve from the positive control provided with the kit.

2.2.12 Phosphorylated AMP-activated protein kinase (AMPK) α ELISA

To measure phosphorylated AMPK α in mouse liver tissue, the AMPK α pThr172 ELISA kit (Abcam) was used in a colorimetric assay (OD was measured at 450 nm on EnSpire[®] 2300 Multimode Reader) according to the manufacturer's instructions with the modification that absorbance of TMB Development Solution was not read, instead blue colour development was stopped with 100 μ L stop solution (1M HCl) when colour of standards was visually different. Proteins were extracted using extraction buffer provided in the kit and a glass Dounce homogenizer. Protein was quantified using the Pierce BCA Protein Assay kit (Thermo Fisher) and extracts were stored at -80°C.

2.2.13 RNA-Seq Analysis

RNA samples derived from liver tissue underwent RNA-Seq analysis at the Australian Genome Research Facility. In brief, RNA-Seq libraries were prepared using TruSeq RNA v2 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 (v2.0.13). Transcripts were assembled utilizing reference-based annotation with Cufflinks (v2.2.1). Gene expression was normalized and quantified as fragments per kb of exon per million fragments mapped (FPKM). Differential Gene Expression analysis of Vorinostat-treated relative to vehicle-treated was calculated using log₂ ratios (fold change) and statistically evaluated using edgeR. Functions of differentially expressed genes were classified using Protein ANalysis Through Evolutionary Relationships (PANTHER) (Mi, Muruganujan et al. 2013) and Mouse Genome Database (MGD) (Eppig, Blake et al. 2015). WEB-based Gene SeT ANalysis Toolkit (WEBGESTALT) was used to identify pathways enriched among candidate genes (Wang, Duncan et al. 2013).

2.3 Results

2.3.1 *Npc1^{nmf164}* mice were generated by crossing heterozygous mice

The *Npc1^{nmf164}* mouse model used in this investigation has a missense mutation in NPC1(D1005G); amino acid 1005 is in the cysteine-rich domain of NPC1 along with many other mutations, most notably three different missense mutations in NPC patients in amino acid 1007 (S1004L, P1007A, P1007L). This specific model displays disease similar to that seen in humans with lipid accumulation, hepatosplenomegaly, neurodegeneration, weight loss, ataxia and premature death (Maue, Burgess et al. 2012); therefore it is a good model to test the therapeutic efficacy of Vorinostat. Specific to this investigation, the low expression levels of NPC1 in this mouse model is necessary to test Vorinostat as it is hypothesized to function at the proteostatic level, by increasing chaperone activity thus increasing NPC1 expression (Pipalia, Cosner et al. 2011).

To obtain *Npc1^{nmf164}* mice to assess the therapeutic efficacy of Vorinostat, breeding cages were set up containing one male and one female heterozygote. When offspring reached P14, genotypes were determined using a restriction digest analysis (Maue, Burgess et al. 2012). A 641 bp product was amplified using PCR (Fig.5A) and

Figure 5: Genotype determination. **A.** A representative gel displays PCR product run on a 1% agarose gel electrophoresis and visualised under UV light at 641 bp/43 bp. **B.** A representative gel shows PCR product digested using *BstEII* enzyme, run on a 2% gel electrophoresis and viewed under UV light: wild-type (641/43 bp), heterozygous mice (641/363/278/43 bp) and homozygous *Npc1^{nmf164}* mice (363/278/43 bp).

digested using the *Bst*II enzyme (Fig.5B). Wild-type mice were identified with two bands (641/43 bp), heterozygous mice were identified with four bands (641/363/278/43 bp) and homozygous *Npc1^{nmf164}* mice were identified with three bands (363/278/43 bp). Once genotyping was complete, offspring were separated into experimental groups at weaning age (P21), where treatment begun.

2.3.2 Vorinostat treatment rescues lipid accumulation and improves liver disease in *Npc1^{nmf164}* mice

Liver lipid accumulation is a primary hallmark of NPC disease proposed to cause liver cell damage (Beltroy, Liu et al. 2007). Vorinostat treatment has previously been shown to reduce cholesterol accumulation in NPC human fibroblasts (Munkacsi, Chen et al. 2011, Pipalia, Cosner et al. 2011). Lipids that accumulate include: cholesterol, sphingomyelin, sphingosine, sphinganine, glucosylceramide, lactosylceramide, GM2, GM3 and BMP. Here, mass spectrometry analysis on the MALDI-TOF MS and MS/MS was used to quantify and identify lipids in Vorinostat-treated mouse liver samples compared to vehicle-treated mouse liver samples. Of note only SM was successfully quantified as other lipids of interest were not successfully identified using the MALDI-TOF MS and MS/MS method. Therefore other methods need to be pursued to accurately determine the effect of Vorinostat on lipid concentration. With this method SM (SM 16:0 (+ Na⁺)) at 725.6 m/z was first normalised to a housekeeping lipid PC (PC 16:0) at 758.6 m/z (Fig.6A). MS/MS was used for identification of lipids, peaks for SM were observed at 666.5 m/z, 542.5 m/z (Fig.6B) and 184 m/z and for PC at 184 m/z (Fig.6C). Quantification was calculated using normalised AUC; results suggested a typical lipid accumulation phenotype (Maue, Burgess et al. 2012), with an 81.8% increase in SM compared to wild-type ($p = 0.014$). Vorinostat treatment decreases SM accumulation in *Npc1^{nmf164}* mice by 39.8% relative to vehicle-treated *Npc1^{nmf164}* mice ($p = 0.014$) (Fig.6D). SM was additionally normalised to total lipid and a similar reduction in SM in Vorinostat-treated *Npc1^{nmf164}* mice was observed (27.5%, $p = 0.022$).

Figure 6: Vorinostat reduces SM accumulation in the liver of *Npc1*^{nmf164} mice. **A.** Representative image from MALDI-TOF MS of SM (725 m/z) and PC (758 m/z) spectrum captured in positive mode at a laser intensity of 5800. **B.** Representative image of the identification of SM using MALDI-TOF MS/MS. SM is fragmented at 666.5 m/z, 542.5 m/z and 184 m/z. **C.** Representative image of the identification of PC using MALDI-TOF MS/MS. PC fragmented at 184 m/z. **D.** Wild-type and *Npc1*^{nmf164} mice were treated with either Vorinostat or vehicle – treatment from P21 to P60, at which mice were dissected and liver collected. SM and PC was extracted and measured on the MALDI-TOF MS. AUC was used for quantification, where SM was then normalised to PC (housekeeping lipid) and expressed at Mol %. Each sample was read in replicates of eight and averaged across the run. SM, sphingomyelin. Error bars are S.E. Wild-type + vehicle (n = 3, white); wild-type + Vorinostat (n = 4, white with stripes); *Npc1*^{nmf164} + vehicle (n = 5, grey); *Npc1*^{nmf164} + Vorinostat (n = 8, grey with stripes). *, $p < 0.05$, Student's t-test.

This result is encouraging as it suggests that Vorinostat reduces lipid accumulation in the liver and has the potential to rescue liver disease. To test if this reduction in lipids owing to Vorinostat treatment would rescue liver cell damage, serum analysis was undertaken where ALT was measured. ALT is an enzyme released into the blood when liver cells are damaged and is commonly used as a biomarker for liver disease. It was observed that in vehicle-treated *Npc1^{nmf164}* mice, ALT was significantly increased (73.9%, $p = 0.0003$) relative to vehicle-treated wild-type, similar to that seen human and animal NPC models (Rimkunas, Graham et al. 2008, Lopez, Klein et al. 2012). Vorinostat-treated *Npc1^{nmf164}* mice displayed a significant reduction (25.7%, $p = 0.0496$) in ALT levels relative to vehicle-treated *Npc1^{nmf164}* mice (Fig.7A).

Additional serum analysis observed total cholesterol, HDL, LDL and triglycerides. Total cholesterol quantitates in serum appeared significantly increased (27.1%, $p = 0.032$ in vehicle-treated *Npc1^{nmf164}* mice compared to vehicle-treated wild-

Figure 7: Vorinostat improves liver function and lowers total cholesterol in the serum of *Npc1^{nmf164}* mice. Wild-type and *Npc1^{nmf164}* mice were treated with either Vorinostat or vehicle treatment from P21 to P60, at which non-fasted mice were dissected and serum was collected. Liver function was assessed through **A.** Alanine aminotransferase (ALT) and expressed as International Units/Litre (IU/L) **B.** Total cholesterol **C.** HDL cholesterol and **D.** Triglycerides were measured and expressed as mmol/L. Error bars are S.E. Wild-type + vehicle (n = 5, white); wild-type + Vorinostat (n = 5, white with stripes); *Npc1^{nmf164}* + vehicle (n = 4, grey); *Npc1^{nmf164}* + Vorinostat (n = 5, grey with stripes). *, $p < 0.05$, Student's t-test.

type, this decreased with Vorinostat-treated *Npc1^{nmf164}* mice by 22.0% ($p = 0.04$) (Fig.7B). No significant elevations or reductions were seen between treatment groups of HDL and triglycerides (Fig.7C-D). LDL, calculated using the Friedewald equation (Friedewald, Levy et al. 1972) showed a significant increase in vehicle-treated *Npc1^{nmf164}* mice compared to vehicle-treated wild-type (71.7%, $p = 0.02$) which was reduced significantly with Vorinostat treatment in *Npc1^{nmf164}* mice (45.6%, $p = 0.05$).

2.3.3 Vorinostat treatment does not delay weight loss or extend lifespan of *Npc1^{nmf164}* mice

It is the accumulation of lipids in the brain that leads to the loss of neurons and Purkinje cells, which ultimately result in neurodegeneration in NPC disease that is observed through weight loss, ataxia and premature death (Elrick, Pacheco et al. 2010). Here, weight loss was used as a biomarker for neurodegeneration to determine if i.p.-administered Vorinostat penetrated the fully formed BBB and delayed neurodegeneration in *Npc1^{nmf164}* mice. Body weight measured weekly from P21–P90 showed vehicle-treated *Npc1^{nmf164}* mice ceased gaining weight at 9 weeks (Fig.8) a result that is comparable to that in the initial characterization of the *Npc1^{nmf164}* mouse (Maue, Burgess et al. 2012). The Vorinostat treatment did not delay weight loss of the *Npc1^{nmf164}* mice, suggesting neurodegeneration was still proceeding despite the Vorinostat treatment. Additionally, longevity of Vorinostat-treated *Npc1^{nmf164}* mice was not increased compared to vehicle-treated *Npc1^{nmf164}* mice, where all *Npc1^{nmf164}*

Figure 8: Vorinostat treatment does not delay weight loss in *Npc1^{nmf164}* mice. Mice were weighed weekly from P21 to P90. Error bars are S.E.

mice died between 10-14 weeks, a result that is comparable to that in the initial characterization of the *Npc1^{nmf164}* mouse (Maue, Burgess et al. 2012), further suggesting neurodegeneration is not rescued with the Vorinostat treatment.

Ataxia is a prominent phenotype in the *Npc1^{nmf164}* mice at 10-13 weeks that occurs as a result of loss of Purkinje cells in the cerebellum (Maue, Burgess et al. 2012). The onset of ataxia that was visible to the naked eye was recorded in this investigation. It was observed that onset of ataxia was not delayed in Vorinostat-treated *Npc1^{nmf164}* mice relative to vehicle-treated *Npc1^{nmf164}* mice in that both groups exhibited ataxia 12 weeks. It must be noted that quantitative assessments of motor degeneration were not undertaken (*e.g.*, rotarod, footprint analysis), albeit the expectation would be that there would also not be a difference using these quantitative methods.

2.3.4 Lipid accumulation in the liver is reduced by an NPC1-independent mechanism

It was previously demonstrated in NPC patient fibroblasts that Vorinostat has a chaperone effect on the NPC1 protein wherein the treatment increased proteins levels of NPC1 (Pipalia, Cosner et al. 2011). Here NPC1 was measured via Western blotting and qRT-PCR in Vorinostat-treated *Npc1^{nmf164}* mice compared to vehicle-treated *Npc1^{nmf164}* mice. The vehicle-treated *Npc1^{nmf164}* mice express only 19% of NPC1 protein relative to vehicle-treated wild-type mice (Fig.9A,B) This is a statistically significant reduction ($p = 5.76 \times 10^{-8}$) and is a result that is comparable to the previously reported 10-15% of NPC1 in *Npc1^{nmf164}* mice (Maue, Burgess et al. 2012). Surprisingly, the levels of NPC1 protein did not increase with Vorinostat treatment in the Vorinostat-treated *Npc1^{nmf164}* mice compared to the vehicle-treated *Npc1^{nmf164}* mice ($p = 0.43$). To test if Vorinostat affected NPC1 gene expression and to further understand measurements at the protein level, NPC1 at the mRNA level by qRT-PCR was measured. No difference was observed within or between treatment groups (Fig.C). Therefore, improvements in liver disease in this investigation are occurring through an NPC1-independent mechanism.

Figure 9: Vorinostat does not improve pathology through an increased expression of NPC1. **A.** NPC1 protein expression was assessed through Western blot analysis. Total protein extracted from P60 liver samples and denatured at 4°C for 30 min. Representative image of 30 µg of protein, run on a 7.5% Bis-Tris gel and transferred onto a 0.22 µm PVDF membrane. NPC1 antibody (1:1000) in 5% milk TBST and actin antibody (1:1000). **B.** NPC1 (180-200 kDa) protein blot quantified using densitometry software (ImageJ) and normalised to actin (42 kDa). Error bars are S.E. Wild-type + vehicle (n = 7, white); wild-type + Vorinostat (n = 7, white with stripes); *Npc1^{nmf164}* + vehicle (n = 7, grey); *Npc1^{nmf164}* + Vorinostat (n = 7, grey with stripes). *, $p < 0.05$, Student's *t*-test. **C.** mRNA expression of NPC1 was measured using qRT-PCR and quantified using the $2^{-\Delta CT}$ method, normalised to GAPDH. Primers are as stated in Appendix Table 1. Error bars are S.D. Wild-type + vehicle (n = 3, white); wild-type + Vorinostat (n = 3, white with stripes); *Npc1^{nmf164}* + vehicle (n = 3, grey); *Npc1^{nmf164}* + Vorinostat (n = 3, grey with stripes). *, $p < 0.05$, Student's *t*-test.

2.3.5 Vorinostat does not regulate autophagy in the liver

Autophagy is increased in the liver of the *Npc1^{-/-}* mouse (Elrick, Yu et al. 2012). In contrast, autophagy has not been investigated in the *Npc1^{nmf164}* mouse. Here levels of autophagy were measured in *Npc1^{nmf164}* mice and compared to wild-type mice as well as Vorinostat-treated mice. A well-established Western blot analysis (Mizushima and Yoshimori 2007) measuring levels of LC3-II, a protein that is specifically localised to autophagosomes, was used for detection. Vehicle-treated *Npc1^{nmf164}* mice show increased levels of LC3-II compared to vehicle-treated wild-type mice ($p = 0.021$), although LC3-II levels in Vorinostat-treated *Npc1^{nmf164}* mice were not altered compared to vehicle-treated *Npc1^{nmf164}* mice ($p = 0.096$) (Fig.10A, B). These results indicate that autophagy is increased in *Npc1^{nmf164}* mice, but this autophagy defect is not regulated by Vorinostat.

Figure 10: Vorinostat does not regulate LC3-II levels. **A.** LC3-II protein expression was assessed through Western blot analysis. Total protein extracted from P60 liver samples and denatured at 37°C for 10 min. Representative image of 30 µg of protein, run on a 15% Bis-Tris gel and transferred onto a 0.22 µm PVDF membrane. LC3-II antibody (1:1000) in 1% milk 1X PBS and actin antibody (1:1000). **B.** LC3-II (14 kDa) protein blot quantified using densitometry software (ImageJ) and normalised to actin (42 kDa). Error bars are S.E. Wild-type + vehicle (n = 7, white); wild-type + Vorinostat (n = 7, white with stripes); *Npc1*^{nmf164} + vehicle (n = 7, grey); *Npc1*^{nmf164} + Vorinostat (n = 7, grey with stripes). *, $p < 0.05$, Student's t-test.

2.3.6 Vorinostat did not increase ac-H3 in the liver

It is well established that ac-H3 expression is increased with HDAC inhibition in cell culture (Lu, Yang et al. 2011). To determine if i.p.-administration of 150 mg/kg Vorinostat increases ac-H3, western blotting and ELISA assays were undertaken to measure changes in protein expression and activity in the liver. Given that previous studies in mouse models of cancer showed 200 mg/kg Vorinostat increased ac-H3 six hours after the i.p. injection (Yin, Ong et al. 2007), ac-H3 was measured six hours after the last injection. Levels of ac-H3 were detected with a single band of 15 kDa. Quantification of this band did not identify any change in Vorinostat-treated mice compared to vehicle-treated mice for either *Npc1*^{nmf164} or wild-type mice (Fig.11A,B). Interestingly, a comparison between genotypes indicated decreased levels of ac-H3 in vehicle-treated *Npc1*^{nmf164} mice compared to vehicle-treated wild-type mice ($p = 0.034$).

To measure activity of ac-H3, a colorimetric ELISA analysis was conducted on the same samples used in the Western blot analysis of ac-H3 protein levels. The standard curve in this assay is able to detect ac-H3 activity between 1 – 100 ng/µL. In

agreement with the Western blot measurements of ac-H3 levels, there was no difference in ac-H3 activity in Vorinostat-treated mice compared to vehicle-treated mice for either *Npc1^{nmf164}* or wild-type mice (Fig.11C). In contrast to the Western blot measurements of ac-H3 levels, there was no difference between vehicle-treated *Npc1^{nmf164}* mice compared to vehicle-treated wild-type mice ($p = 0.45$).

Figure 11: Vorinostat does not increase ac-H3 levels in the liver 6 hours after administration. **A.** ac-H3 protein expression was assessed through Western blot analysis. Histone protein extracted from P60 liver samples and denatured at 37°C for 10 min. Representative image of 5 μg of histone protein, run on a 15% Bis-Tris gel and transferred onto a 0.22 μm PVDF membrane. ac-H3 antibody (1:1000) in 1% milk 1X PBS and actin antibody (1:1000) as loading control. **B.** ac-H3 (15 kDa) protein blot quantified using densitometry software (ImageJ) and normalised to actin (42 kDa). **C.** Activity of ac-H3 was measured through ELISA analysis, using the same protein samples as Western blot analysis. Error bars are S.E. Wild-type + vehicle (n = 5, white); wild-type + Vorinostat (n = 5, white with stripes); *Npc1^{nmf164}* + vehicle (n = 5, grey); *Npc1^{nmf164}* + Vorinostat (n = 5, grey with stripes). *, $p < 0.05$, Student's t-test.

To determine if the six hour time point was too early or late to detect changes in histone acetylation, a time-point experiment in wild-type mice through Western blot analysis was performed to measure ac-H3 levels at 0, 3, 6, 12 and 24 hours. There was no significant difference between any treatment groups (Fig. 12A, B). These results of not detecting changes in histone acetylation with 150 mg/kg Vorinostat are in line with a previous study that tested various doses and did not detect increased acetylation of histones until 200 mg/kg was administered (Hockly, Richon et al. 2003).

Figure 12: Vorinostat does not increase ac-H3 levels at any time-point. **A.** ac-H3 protein expression at differing time-points after Vorinostat injection was assessed through Western blot analysis. Histone protein was extracted from age-matched wild-type liver samples and denatured at 37°C for 10 min. 5 µg of histone protein, run on a 15% Bis-Tris gel and transferred onto a 0.22 µm PVDF membrane. ac-H3 antibody (1:1000) in 1% milk 1X PBS and actin antibody (1:1000) as loading control. **B.** ac-H3 (15 kDa) protein blot quantified using densitometry software (ImageJ) and normalised to actin (42 kDa). Error bars are S.E. Wild-type no injection (n = 3, white); wild-type + Vorinostat, 3 hours post-injection (n = 3, white with stripes); wild-type + Vorinostat, 6 hours post-injection (n = 3, white with stripes); wild-type + Vorinostat, 12 hours post-injection (n = 3, white with stripes); wild-type + Vorinostat, 24 hours post-injection (n = 3, white with stripes). *, $p < 0.05$, Student's t-test.

2.3.7 Vorinostat transcriptionally regulates genes involved in cholesterol homeostasis

Trichostatin A (TSA), an HDACi, has previously been shown to transcriptionally regulate the expression levels of genes involved in cholesterol homeostasis (Chittur, Sangster-Guity et al. 2008, Nunes, Moutinho et al. 2013). Thus qRT-PCR was used to measure a subset of genes involved in the synthesis, uptake, esterification, and efflux of cholesterol in livers of Vorinostat-treated *Npc1^{nmf164}* mice (Fig.13A-H). Specifically, the mRNA analyses included the following genes: *cholesterol synthesis*, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR); *cholesterol uptake*, low density lipoprotein receptor (LDLR); *cholesterol esterification*, sterol O-acyltransferase (SOAT2) and lysosomal acid lipase (LIPA); *regulation of cholesterol efflux*, the ATP-binding cassette transporters (ABCA1 and ABCG1); *cholesterol efflux*, Niemann-Pick type C2 (NPC2); and one of the genes involved in the *conversion of cholesterol to bile acid*, cytochrome

P450 7A1 (CYP7A1). The expression levels of HMGCR was significantly decreased by 2.7-fold in Vorinostat-treated *Npc1^{nmf164}* mice compared to vehicle-treated *Npc1^{nmf164}* ($p = 0.02$). Levels of HMGCR were also nearly significantly decreased in Vorinostat-treated wild-type mice compared to vehicle-treated mice ($p = 0.060$). No difference was seen due to genotype (Fig.13A). The expression of LDLR was down-regulated with Vorinostat treatment by 1.7-fold in *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice ($p = 0.044$). No difference between Vorinostat-treated wild-type and vehicle-treated wild-type mice ($p = 0.372$). A genotype difference was evident with LDLR expression increased by 1.89-fold in vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type ($p = 0.013$) (Fig.13B). The expression of SOAT2 was unaltered with Vorinostat treatment in both *Npc1^{nmf164}* and wild-type mice. SOAT2 expression however was decreased in vehicle-treated *Npc1^{nmf164}* mice (1.7-fold, $p = 0.024$) (Fig.13C). Expression of LIPA with Vorinostat treatment was unchanged in *Npc1^{nmf164}* mice compared to vehicle-treated mice. Conversely in wild-type mice, Vorinostat treatment increased LIPA expression relative to control (1.56-fold, $p = 0.001$). A difference between genotype was seen, where vehicle-treated *Npc1^{nmf164}* had increased expression of LIPA compared to vehicle-treated wild-type (3.5-fold, $p = 1.56 \times 10^{-5}$) (Fig.13D). The expression of ABCG1 was not regulated with Vorinostat treatment in *Npc1^{nmf164}* mice, however in wild-type mice Vorinostat treatment down-regulated ABCG1 expression (2.5-fold, $p = 0.008$). ABCG1 expression was elevated in vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type (1.43-fold, $p = 0.054$) (Fig.13F). Expression of NPC2 was not regulated by Vorinostat treatment in either *Npc1^{nmf164}* or wild-type mice. However vehicle-treated *Npc1^{nmf164}* mice displayed significantly increased NPC2 expression compared to vehicle-treated wild-type (4.7-fold, $p = 0.016$) (Fig.13G). Expression of ABCA1 and CYP7A1 saw no difference between treatment or genotype groups (Fig.E and H, respectively). These results indicate that Vorinostat is transcriptionally regulating genes involved in synthesis and uptake but not cholesterol esterification, efflux nor the conversion of cholesterol to bile acid in *Npc1^{nmf164}* mice.

Figure 13: Vorinostat regulates genes involved in cholesterol homeostasis. mRNA expression of cholesterol homeostasis genes were measured using qRT-PCR and quantified using the $2^{-\Delta CT}$ method, normalised to GAPDH. Primers are as stated in Appendix Table 1. Vehicle-treated wild-type mice were assigned a baseline expression of 1. **A.** Synthesis gene (HMGCR) **B.** Uptake gene (LDLR) **C-D.** Esterification genes (SOAT2 and LIPA) **E-F.** Regulation of efflux genes (ABCA1 and ABCG2) **G.** Efflux gene (NPC2) **H.** Metabolism in the liver gene (CYP7A1) Error bars are S.D. Wild-type + vehicle (n = 3, white); wild-type + Vorinostat (n = 3, white with stripes); *Npc1*^{nmf164} + vehicle (n = 3, grey); *Npc1*^{nmf164} + Vorinostat (n = 3, grey with stripes). *, $p < 0.05$, Student's t-test.

2.3.8 HMGCR expression is not down-regulated through an up-regulation of phosphorylated AMPK α mechanism

Here, it was determined that Vorinostat reduced HMGCR expression; this can be a consequence of phosphorylated AMPK α activity. To determine if Vorinostat is modulating HMGCR expression via phosphorylated AMPK α , ELISA was undertaken to monitor its levels upon Vorinostat treatment. Previous work had shown an up-regulation of phosphorylated AMPK α with other HDACi treatments (Gao, Yin et al. 2009). Here, results show that the levels of phosphorylated AMPK α were not significantly different between genotype or treatment groups (Fig.14). Thus HMGCR is down-regulated in this investigation through an alternative mechanism, independent of the phosphorylation- dephosphorylation mechanism of AMPK.

Figure 14: Phosphorylated AMPK α is not regulated by Vorinostat. To measure phosphorylated AMPK α , ELISA analysis was conducted on liver protein extracts. Error bars are S.E. Wild-type + vehicle (n = 3, white); wild-type + Vorinostat (n = 3, white with stripes); *Npc1*^{nmf164} + vehicle (n = 4, grey); *Npc1*^{nmf164} + Vorinostat (n = 5, grey with stripes). *, $p < 0.05$, Student's t-test.

2.3.9 RNA transcriptome sequencing identifies that Vorinostat is pleiotropically modulating hundreds of genes that include cholesterol biosynthetic genes

Given that Vorinostat is an HDACi that is an established epigenetic drug, RNA transcriptome sequencing was conducted to determine a mechanism of action on a genome-wide scale. Specifically, a differential gene expression analysis on liver samples derived from Vorinostat-treated *Npc1*^{nmf164} mice (n=7) compared to vehicle – treated *Npc1*^{nmf164} mice (n=7) at P60 after treatments at 5 days per week starting at P21. Here 14,516 transcripts were measured, which is comparable to previous

Figure 15: Genome-wide RNA-Seq analysis compares expression levels of 14,516 transcripts in the liver of Vorinostat-treated vs vehicle-treated *Npc1^{nmf164}* mice. A. Volcano plot of log₂-fold change between *Npc1^{nmf164}* + vehicle (n = 7) and *Npc1^{nmf164}* + Vorinostat (n = 7) in 14,516 transcripts. Blue line marks threshold that was accepted for further analysis (> 0.6 log₂-fold change, $p < 0.05$). **B.** Out of the 14,516 transcripts measured, 844 were significantly altered; 603 up-regulated (blue) and 241 down-regulated (red).

transcriptome analyses of mouse livers (Dokmanovic, Clarke et al. 2007, Chittur, Sangster-Guity et al. 2008). Of the 14,516 transcripts, 844 transcripts (5.9% of the transcriptome) had a gene expression significantly altered with Vorinostat treatment (log₂-fold change > 0.6, $p = 0.05$), of which 603 (71.4%) transcripts were up-regulated and 241 (28.6%) were down-regulated (Fig.15A, B).

To decipher functions of transcripts that are significantly regulated with the Vorinostat treatment, the 844 transcripts were assigned to a diversity of cellular processes using PANTHER (Mi, Muruganujan et al. 2013). These biological processes include: cellular process (20.7%), localization (8.7%), apoptotic process (1.9%), reproduction (1%), biological regulation (11.3%), response to stimulus (9%), developmental process (7.4%), multicellular organismal process (4.8%), locomotion (0.9%), biological adhesion (2%), metabolic process (22.7%), immune system process (6.1%) and cellular component organization or biogenesis (3.5%) (Fig.16A). Likewise, a diversity of molecular functions were identified by PANTHER including transporter activity (8.8%), translation regulator activity (0.3%), protein binding transcription factor activity (1.4%), enzyme regulator activity (5.9%), catalytic activity (32.3%),

Figure 16: Significantly altered transcripts were assigned using PANTHER to A. Biological process and B. Molecular function.

receptor activity (9.5%), nucleic acid binding transcription factor activity (6.2%), antioxidant activity (0.1%), structural molecule activity (5%) and binding (30.5%) (Fig.16B).

To further examine the significantly regulated transcripts, gene expression changes were further analysed using heat map analysis (Fig.17A, B). To assemble these diverse processes and functions in the context of NPC disease and Vorinostat, transcripts were next assigned to cellular functions that would be expected to change with NPC disease or with treatment with an HDACi like Vorinostat. MGD (Eppig, Blake et al. 2015) was used to assign the 844 transcripts to a function. A subset of these are displayed for each function, some transcripts are seen in multiple functional groups: cytoskeleton (11/55), proteolysis/ubiquitination (16/68), lipid metabolism (31/121), endosomal/lysosomal (13/35), cell death (8/42) and gene expression (16/137) (Fig.17B).

Figure 17: Vorinostat regulates the transcription of genes involved in a variety of processes. **A.** Relative expression levels of 844 transcripts significantly regulated by Vorinostat treatment ($> 0.6 \log_2$ -fold change, $p < 0.05$). **B.** 116 genes assigned to functional groups by MGD identified functions associated with NPC disease (endosomal/lysosomal and lipid metabolism) or HDACi (gene expression, proteolysis, cytoskeleton and cell death). *Npc1^{nmf164}* + vehicle ($n = 7$) and *Npc1^{nmf164}* + Vorinostat ($n = 7$) mice. The scale bar (applies to **A** and **B**) indicates proportional read counts with red and white shade representing, down-regulated and up-regulated transcripts, respectively.

From these groups, genes previously shown to change in expression upon HDACi treatment were identified. Amyloid beta precursor-like protein 1 (APLP1) was up-regulated after Vorinostat treatment (Claerhout, Lim et al. 2011), here APLP1 increased by 2.56 log₂-fold ($p = 1.95 \times 10^{-19}$). The aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL), also referred to as BMAL1, is regulated by HDAC3 (Yin and Lazar 2005). Here ARNTL is increased by 2.59 log₂-fold ($p = 1.18 \times 10^{-7}$), thought to be caused by Vorinostat inhibition of HDAC3. The high-mobility group A2 (HMGA2) protein was previously shown to be down-regulated with TSA treatment (Ferguson, Henry et al. 2003); here we observe a down-regulation of HMGA2 of 2.2 log₂-fold ($p = 0.013$). Additionally, the cyclin D1 (CCND1) protein has been previously shown to decrease in expression by class I and II HDACi, apicidin (Yang, Dahl et al. 2013); here Vorinostat decreased CCND1 1.34 log₂-fold ($p = 1.8 \times 10^{-6}$). Lastly, the GATA-binding transcription factor (GATA1) has previously been shown to increase in expression with Vorinostat treatment (Marks and Breslow 2007); here treatment resulted in an up-regulation of 3.6 log₂-fold ($p = 0.0001$).

Genes previously observed to be elevated in NPC diseased models were observed (Reddy, Ganley et al. 2006, Alam, Getz et al. 2014). Here Vorinostat treatment down-regulated these genes; lysozyme 1 (LYZ1) (1.06 log₂-fold, $p = 0.0004$), solute carrier family 23 member 3 (SLC23A3) (1.7 log₂-fold, $p = 0.009$) and oxysterol binding protein-like-3 (OSBPL3) (1.40 log₂-fold, $p = 2.86 \times 10^{-7}$) suggesting these NPC disease biomarkers are potentially decreasing to wild-type levels upon due to Vorinostat treatment.

Interestingly, 'lipid metabolism' transcripts saw genes involved in lipid synthesis to be down-regulated: fatty acid 2-hydroxylase (FA2H) (2.61 log₂-fold, $p = 0.0006$), MID1 interacting protein 1 (MID1IP1) (0.91 log₂-fold, $p = 9.77 \times 10^{-5}$) HMGCR (0.94 log₂-fold, $p = 0.003$), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) (-0.68 log₂-fold, $p = 0.02$), lanosterol synthase (LSS) (-0.68 log₂-fold, $p = 0.002$), isopentenyl-diphosphate delta isomerase (IDI1) (0.67 log₂-fold, $p = 0.01$), mevalonate (MVD) (0.65 log₂-fold, $p = 0.004$), mevalonate kinase (MVK) (0.62 log₂-fold, $p = 0.002$), lanosterol 14 alpha-demethylase (CYP51) (0.6 log₂-fold, $p = 0.003$) and sterol regulatory element binding transcription factor 1 (SREBF1) (0.64 log₂-fold, $p = 0.22$). Genes involved in lipid metabolism were up-regulated after treatment: cytochrome

P450, family 8, subfamily B, polypeptide 1 (CYP8B1) (1.1 log₂-fold, $p = 0.0004$), cytochrome P450, family 46, subfamily A, polypeptide 1 (CYP46A1) (1.55 log₂-fold, $p = 0.00012$) and fatty acid binding protein 3 (FABP3) (2.27 log₂-fold, $p = 0.045$).

The matrix metalloproteinase (MMP) family of enzymes has recently shown to be dysregulated in *Npc1*^{-/-} mice relative to wild-type (Liao, Wang et al. 2015); specifically MMP9 was found to be down-regulated relative to wild-type mice. Interestingly, results from this analysis saw MMP9 to be up-regulated upon Vorinostat treatment (1.54 log₂-fold, $p = 2.13 \times 10^{-6}$). Additionally, other members of this family were found to be changed upon Vorinostat treatment in this investigation (MMP8, MMP17 and MMP15), suggesting MMPs are regulated by Vorinostat.

To determine if there is functional enrichment among the 844 genes, WEBGESTALT (Wang, Duncan et al. 2013) was used to identify 10 pathways that were statistically over-represented in the 844 gene set (Table 7): GPCRs Class A Rhodopsin-like ($p = 7.86 \times 10^{-6}$), cholesterol biosynthesis ($p = 2.66 \times 10^{-5}$), non-odorant GPCRs ($p = 7.54 \times 10^{-5}$), chemokine signalling pathway ($p = 0.0004$), peptide GPCRs ($p = 0.0004$), amino acid metabolism ($p = 0.0019$), DNA replication ($p = 0.0036$), leptin insulin overlap ($p = 0.0042$), metapathway biotransformation ($p = 0.0137$) and matrix metalloproteinase ($p = 0.0127$). Of particular interest was the cholesterol biosynthesis pathway significantly enriched with seven of the 16 genes down-regulated upon Vorinostat treatment (HMGCS1, HMGCR, MVK, MVD, IDI1, LSS and CYP51) ($p = 2.66 \times 10^{-5}$).

Table 7: WEBGESTALT enriched pathways

Pathway	Reference genes	Significantly regulated genes	p value
GPCRs, Class A Rhodopsin-like	72	16	7.86×10^{-6}
Cholesterol Biosynthesis	16	7	2.66×10^{-5}
Non-odorant GPCRs	132	21	7.54×10^{-5}
Chemokine signalling pathway	160	22	0.0004
Peptide GPCRs	23	7	0.0004
Amino Acid metabolism	103	15	0.0019
DNA Replication	41	8	0.0036
Leptin Insulin Overlap	18	5	0.0042
Metapathway biotransformation	127	15	0.0137
Matrix Metalloproteinases	23	5	0.0127

2.3.10 qRT-PCR analysis validated Vorinostat reducing cholesterol biosynthesis genes

To validate the RNA-Seq result of Vorinostat regulating cholesterol biosynthesis transcripts, qRT-PCR analysis was conducted (Fig.18A-G). In these analyses, all four treatment groups were included so to determine if Vorinostat regulates these cholesterol biosynthesis genes in wild-type and *Npc1^{nmf164}* mice as well as to determine if these genes are differentially regulated in vehicle-treated wild-type and *Npc1^{nmf164}* mice. First, the expression of HMGCS1 was down-regulated by 1.6-fold in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice ($p = 0.0005$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in HMGCS1 expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type (4.8-fold, $p = 2.06 \times 10^{-5}$) (Fig.18A). Second, the expression of HMGCR was down-regulated in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice (2-fold, $p = 0.0002$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in HMGCR expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type (2-fold, $p = 0.016$) (Fig.18B). Third, the expression of MVK was reduced with Vorinostat treatment in both *Npc1^{nmf164}* (2.41-fold, $p = 0.0065$) and wild-type (1.77-fold, $p = 0.003$) mice compared to vehicle control. A difference due to genotype in MVK expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type mice (4.4-fold, $p = 0.0018$) (Fig.18C). Fourth, the expression of MVD was down-regulated in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice (2.79-fold, $p = 0.0034$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in MVD expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type mice (5.1-fold, $p = 0.0034$) (Fig.18D). Fifth, the expression of IDI1 was down-regulated in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice (2.72-fold, $p = 0.008$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in IDI1 expression was observed between vehicle-treated

Npc1^{nmf164} compared to vehicle-treated wild-type mice (3.68-fold, $p = 0.00275$) (Fig.18E). Sixth, the expression of LSS is down-regulated in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice (2.45-fold, $p = 0.02$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in LSS expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type mice (4.69-fold, $p = 0.004$) (Fig.18F). Lastly, the expression of CYP51 is down-regulated in Vorinostat-treated *Npc1^{nmf164}* compared to vehicle-treated *Npc1^{nmf164}* mice (1.9-fold, $p = 0.045$). No difference was observed between Vorinostat-treated wild-type mice compared to vehicle-treated wild-type. A difference due to genotype in CYP51 expression was observed between vehicle-treated *Npc1^{nmf164}* compared to vehicle-treated wild-type mice (2.15-fold, $p = 0.038$) (Fig.18G). Collectively, these results suggest that cholesterol biosynthetic genes are elevated in *Npc1^{nmf164}* mice at P60. Upon treatment with Vorinostat, these genes are down-regulated to wild-type levels.

Figure 18: qRT-PCR validated reduction in cholesterol biosynthetic genes in Vorinostat-treated *Npc1*^{nmf164} mice. mRNA expression of cholesterol biosynthetic genes was measured using qRT-PCR and quantified using the $2^{-\Delta CT}$ method, normalised to GAPDH. Primers are as stated in Appendix Table 1. Vehicle-treated wild-type mice were assigned a baseline expression of 1. **A.** HMGCS **B.** HMGCR **C.** MVK **D.** MVD **E.** IDI1 **F.** LSS and **G.** CYP51. Error bars are S.D. Wild-type + vehicle (n = 3, white); wild-type + Vorinostat (n = 3, white with stripes); *Npc1*^{nmf164} + vehicle (n = 3, grey); *Npc1*^{nmf164} + Vorinostat (n = 3, grey with stripes). *, $p < 0.05$, Student's t-test.

2.4 Discussion

NPC disease is a fatal neuro-visceral LSD, for which no therapy exists. The disorder primarily affects children, consuming their lives at 10-25 years of age (Vanier 2010). Because lifespan is significantly shortened, an FDA-approved drug where toxicity tests are already complete is desirable since patients do not have the time to wait the 10-15 years required to identify, develop and approve a new drug. Treatment of NPC patient fibroblasts with the FDA-approved HDACi, Vorinostat, was previously found to decrease cholesterol accumulation (Munkacsy, Chen et al. 2011, Pipalia, Cosner et al. 2011). The aim of this investigation was to test the *in vivo* therapeutic efficacy of Vorinostat in the *Npc1^{nmf164}* mouse model of NPC disease (*Npc1^{nmf164}*); no animal studies in any other animal model have been published at the time of submission of this thesis. Here it was found that Vorinostat down-regulated expression of cholesterol biosynthetic genes and decreased lipid accumulation in liver tissue and in the serum, thus alleviating liver function defects in the *Npc1^{nmf164}* mouse model. However, disease progression observed through weight loss, ataxia and premature death was not delayed. These results suggest that Vorinostat was therapeutic in the liver but not the brain as it was incapable of rescuing neurodegeneration.

2.4.1 Lipid analysis

It is well established that SM accumulates progressively in human patients affected with NPC disease (Vanier 2010). This accumulation is conserved in the *Npc1^{nmf164}* mouse at P60 (Maue, Burgess et al. 2012). Encouragingly in this investigation, SM accumulation at P60 in the *Npc1^{nmf164}* mouse was significantly reduced with the Vorinostat treatment by 39.8% relative to the vehicle control. This rescue, albeit promising, is inferior to the nearly complete rescue in the *Npc1^{-/-}* mouse model treated with HPB-CD that showed wild-type SM levels (Davidson, Ali et al. 2009). Given both studies started injections at P21, it appears that HPB-CD is a more potent regulator of SM metabolism than Vorinostat.

The measurement of SM was conducted using MALDI-TOF MS and MS/MS. Unfortunately out of the many lipids that accumulate in NPC disease, only SM was successfully identified through MALDI-TOF MS/MS analysis. This was an unexpected

result given the modified Bligh and Dyer lipid extraction protocol that was used is sufficient for the extraction of unesterified cholesterol, SM and PC (Petkovic, Vocks et al. 2005). For example, unesterified cholesterol, commonly detected at 386 m/z or 369 m/z ($M + H - H_2O$) using this method (Schiller, Zschornig et al. 2001) was detected in MALDI-TOF MS but was not confirmed using MALDI-TOF MS/MS. It is possible that the matrix used (2,5-DHB) for analysis had too many matrix-related low mass peaks corresponding to it, making identification of unesterified cholesterol problematic (Yang and Fujino 2015). An additional limitation is the quantification of lipids using MALDI-TOF MS. Quantification can be difficult as crystallisation of the analyte and matrix does not form a uniform layer over the target surface, therefore signals from the analyte may be variable (Duncan, Roder et al. 2008). To overcome this issue in this investigation, each sample was spotted in replicates of eight and an average was calculated from these replicates. Although it is thought this limitation was overcome, lipid analysis on a second mass spectrometer would further assess the extent to which the limitation was overcome. Quantification of an experimental lipid can be accomplished by comparing (normalising) the experimental lipid to protein content, tissue weight or housekeeping lipid. In this investigation, two of these methods were used; normalisation to total lipid (sum of area under all peaks) and normalisation to PC, a housekeeping lipid (PC). Results using both methods indicated SM was decreased in the livers of Vorinostat-treated *Npc1^{nmf164}* mice.

With the observation that SM is reduced upon Vorinostat treatment, alongside the RNA-Seq result that suggests Vorinostat targets the cholesterol biosynthesis pathway, sphingolipid biosynthesis genes were additionally observed. No differences in any genes involved in sphingolipid biosynthesis were observed.

2.4.2 Serum analysis

The liver is one of the primary organs affected in NPC disease, thus ALT, an enzyme released into the blood when liver cells are damaged, is commonly elevated in both human NPC patients and animal models (Beltroy, Liu et al. 2007). Impressively, Vorinostat treatment resulted in significant reductions (25.7%) in ALT levels compared to vehicle-treated *Npc1^{nmf164}* mice in this investigation. It is plausible that a more

dramatic rescue could occur with Vorinostat treatment starting earlier than P21 or continuation of Vorinostat treatment beyond P60.

Total cholesterol and LDL are commonly elevated in the serum in NPC disease (Rimkunas, Graham et al. 2008) This investigation demonstrated that these hallmarks are conserved in *Npc1^{nmf164}* mice, and also demonstrated that the Vorinostat treatment reduced total cholesterol and LDL levels in the serum by 22% and 46%, respectively. In comparison to the other promising therapeutics, HPB-CD also significantly reduces ALT and total cholesterol levels in the NPC feline model (Vite, Bagel et al. 2015) while Miglustat does not impact liver disease, thus ALT and total cholesterol levels remain elevated in the serum with Miglustat treatment (Stein, Crooks et al. 2012).

Similar to ALT, alkaline phosphatase (ALP) is an enzyme that is released into the blood in liver and bone disease. In this investigation, measurements for ALP levels were sought however EDTA was used to pre-rinse tools, invalidating the ALP result (Imafuku, Meguro et al. 2002). Nonetheless, the RNA-Seq transcriptome results identified that Vorinostat decreased expression of the transcript *ALPL*, which encodes the enzyme regulating ALP levels. This suggests that ALP levels would be reduced in Vorinostat-treated *Npc1^{nmf164}* mice. Also, the targeted qRT-PCR analysis of cholesterol homeostasis genes identified that the expression of the cholesterol uptake gene *LDLR*, the primary factor for amount of LDL cholesterol in plasma, was down-regulated by Vorinostat in *Npc1^{nmf164}* mice by 1.7-fold compared to vehicle-treated *Npc1^{nmf164}* mice ($p = 0.044$). This decrease in *LDLR* expression upon Vorinostat treatment parallels results gained with serum analysis where it was found that LDL cholesterol decreased by 45.6% in Vorinostat-treated mice relative to vehicle control.

A limitation for serum analysis conducted here is that mice were not fasted. Fasting mice (12-14 hours) is important for triglyceride estimation, because triglyceride levels remain high after a meal. Confoundingly, the Friedewald equation, used here to calculate LDL quantities, assumes fasting triglyceride (Nigam 2011); thus, LDL calculated here is likely to be underestimated and must be considered with caution.

2.4.3 Disease progression

To measure disease progression in NPC mice, age at which weight and life was lost was measured to assess the therapeutic efficacy of Vorinostat. Here Vorinostat did not delay weight loss or death in treated mice relative to vehicle control. In comparison, HPB-CD and Miglustat are both capable of delaying weight loss and extending lifespan (Davidson, Ali et al. 2009). This is thought to be due to the efficacy of these drugs in the brain, where both treatments are capable of reducing accumulating lipids (*e.g.*, gangliosides) in the brain. This explanation would be in agreement with previous reports that i.p.-administered Vorinostat is poor at penetrating the BBB (Hanson, La et al. 2013).

Purkinje cells are the main cell type in the cerebellum, a region of the brain involved in motor-coordination, and are one of the main cell types in the brain to be affected by NPC disease (Vance and Karten 2014). Purkinje cell loss leads to progressive ataxia in NPC patients and animal models of NPC disease (Maue, Burgess et al. 2012). Although no quantitative tests measuring ataxia were undertaken herein, ataxia visual to the naked eye was recorded and the onset of ataxia was at comparable time-points in Vorinostat-treated and vehicle-treated *Npc1^{nmf164}* mice, thus suggesting Vorinostat is not ameliorating neurodegeneration.

2.4.4 Chaperone mechanism of Vorinostat

HDACi's have previously been shown to function through a chaperone mechanism, helping mutated proteins refold efficiently, thus bypassing degradation (Hutt, Herman et al. 2010). Vorinostat treatment in NPC patient fibroblasts or mouse embryonic fibroblasts containing an I1061T mutation, the most commonly occurring mutation among NPC patients, resulted in reduction of cholesterol accumulation via enhanced expression of NPC1 (Pipalia, Cosner et al. 2011, Praggastis, Tortelli et al. 2015). Gelsthorpe and colleagues have shown that the *NPC1^{I1061T}* mutation is targeted for degradation by the endoplasmic reticulum-associated protein degradation (ERAD) system due to improper protein folding (Gelsthorpe, Baumann et al. 2008). Therefore, the working model suggests that Vorinostat had helped mutated *NPC1^{I1061T}* fold and escape ERAD targeted degradation. It was theorized that Vorinostat would function to improve folding in other NPC1 mutations like the *NPC1^{D1005G}* mutation in the *Npc1^{nmf164}* mouse model, a mutation which flanks

mutations reported in NPC patients; NPC1(S1004L), NPC1(P1007A), and NPC1(P1007L). However, results gained in this investigation in the *Npc1^{nmf164}* mouse model wherein Vorinostat did not increase NPC1 expression at the protein or mRNA level suggest that the chaperone efficacy of Vorinostat is limited to cell culture in which Vorinostat maintains its concentration during incubation whereas in *in vivo*, Vorinostat is rapidly metabolised in a 90-minute half-life (Marks and Breslow 2007). It is also plausible that the increase in *NPC1^{I1061T}* with Vorinostat treatment (Pipalia, Cosner et al. 2011) is mutation-specific and unable to sufficiently improve folding of other diseased alleles. If this is the case, determining the therapeutic efficacy of Vorinostat across the ~300 mutations that exist is essential to evaluate Vorinostat as a candidate therapy (Vanier 2010).

Furthermore, the difference in half-life of the NPC1 protein between mutations may cause for a false negative where a change in NPC1 expression occurs but then decreased back to its basal level before dissection. Wild-type NPC1 has a half-life of 36 hours, while conversely the half-life of NPC1(I1061T) is 6.5 hours (Gelsthorpe, Baumann et al. 2008). Considering the lifespan of the NPC1(I1061T) and NPC1(D1005G) mouse models, the I1061T model has a longer lifespan (16-18 weeks) compared to the NPC1(D1005G) model (10-14 weeks). Therefore, it is plausible that the half-life of the NPC1(D1005G) is less than 6.5 hours half-life of NPC1(I1061T). If so, it is possible that Vorinostat could have increased NPC1, then due to its abbreviated half-life, decreased back to its basal level before mice were dissected at 6 hours after the last Vorinostat injection. To fully investigate the possibility of a chaperone mechanism of Vorinostat in the *Npc1^{nmf164}* mouse model, it is critical to determine the half-life of NPC1(D1005G).

Ideally, a therapy to treat NPC disease would specifically target NPC1 (in patients with an NPC1 mutation) or NPC2 (in patients with an NPC mutation). Such therapies would be logical given that these are the only defective genes that confer the monogenic NPC disease. A drug that targets NPC1 would specifically act to improve protein folding of NPC1 and theoretically result in fewer side effects than a drug that targets multiple proteins. HPB-CD, the most promising therapy currently in clinical trial (<https://clinicaltrials.gov/ct2/show/NCT01747135>), does not work

through a NPC1-dependent mechanism, rather it targets cholesterol itself and thus elicits side-effects such as hearing loss (Ward, O'Donnell et al. 2010).

Nonetheless, it is proposed that in this investigation, Vorinostat does not function in a NPC1-dependent mechanism but instead functions pleiotropically, where numerous pathways are targeted and together function to improve some but not all disease hallmarks. An NPC1-independent mechanism proposed here is further strengthened by comparing the degree of ALT rescue in the results herein to another study in which NPC1 was knocked-down in the liver (Sayre, Rimkunas et al. 2010). When functional NPC1 was restored in that study with an antisense oligo, ALT levels improved by 78% compared to the 25% rescue that was observed in this investigation. Thus a more striking improvement in ALT levels in this investigation would be expected if Vorinostat was functioning in a NPC1-dependent manner.

2.4.5 Expected drug targets of Vorinostat

With the opposing functions of HDAC and HAT enzymes, it is well-established that inhibition of HDAC enzymes with an HDACi leads to increased levels of HAT enzymes (Lu, Yang et al. 2011). Specifically, Yin *et al.* found that 100 mg/kg of i.p. administered Vorinostat increased ac-H3 in the brain, 6 hours after administration (Yin, Ong et al. 2007). However in this investigation, no increase in ac-H3 in the liver was observed six hours after i.p.-administered 150 mg/kg Vorinostat. Additional time-points in wild-type mice were conducted to examine different time-points with the idea that perhaps six hours was too early or too late to observe increased ac-H3 in the liver. Surprisingly, no difference in ac-H3 was observed at any time-point relative to no injection. This result is in agreement where 100 mg/kg Vorinostat in DMSO administered through a single i.p. injection did not see increases in acetylation in the spleen, and it was only when Vorinostat was administered through a subcutaneous injection at 200 mg/kg was there an increase in H2B and H4 acetylation two hours post-injection (Hockly, Richon et al. 2003).

Even though no changes in the established drug target, ac-H3, were observed in this study, whether it is because of the concentration or administration method, the NPC liver hallmarks of increased SM accumulation in the liver as well as increased ALT and LDL levels in the serum were still rescued with the drug regime administered here

which is a promising result. It remains unresolved whether optimising the drug regime herein to conditions where changes in acetylation are detected will further enhance the therapeutic effects recorded in this investigation.

Importantly, Vorinostat did show transcriptional changes in genes other than histones, that are established targets of Vorinostat: APLP1 (Claerhout, Lim et al. 2011), a transcriptional target of p53 whose activity may be down-regulated by HDAC -1, 2 and 3 (Juan, Shia et al. 2000); ARNTL has been shown to be up-regulated when HDAC3 is inhibited (Yin and Lazar 2005); HMGA2 (Ferguson, Henry et al. 2003) expression has been reported to be down-regulated when HDAC1 and HDAC2 are inhibited (Lee, Jung et al. 2011); CCND1 (Yang, Dahl et al. 2013) and GATA1 (Marks and Breslow 2007) expression has also been reported to be regulated with Vorinostat treatment. These changes are promising, suggesting that Vorinostat is having an effect at the transcriptome level, thus changing the expression of known targets.

2.4.6 Autophagy

The *Npc1*^{-/-} mouse and NPC patient fibroblasts have previously been observed to show disrupted flux in the autophagy pathway where autophagosomes cannot fuse with amphisomes, resulting in increased levels of LC3-II (Sarkar, Carroll et al. 2013). Previous literature has found that this block can be overcome through treatment with an autophagy inducer drug where amphisome formation is bypassed and autophagosomes fuse directly with lysosomes leading to degradation (Maetzel, Sarkar et al. 2014). Vorinostat has been shown in recent years to induce autophagy in a histone acetylation-dependent manner (Gammoh, Lam et al. 2012, Dupere-Richer, Kinal et al. 2013). However in this investigation, no increase in LC3-II levels was observed with Vorinostat treatment, therefore it is suspected that autophagy is not further induced. The absence of an increase in ac-H3 parallels this result, as an increase in autophagy was expected to be histone acetylation dependent. A notable finding from this investigation however, was the significant increase in LC3-II relative to wild-type that was observed in the *Npc1*^{nmf164} mouse model, a finding that has not yet been reported but was predicted from NPC patients and the null mouse model of NPC disease.

Using LC3-II to quantify autophagy must be approached with caution as LC3-II levels reflect autophagosome formation but do not guarantee complete autophagic flux (Mizushima and Yoshimori 2007). Therefore, additional work is needed to characterise the autophagic defect in the *Npc1^{nmf164}* mouse model. Determining whether an increase in LC3-II is due to a block in the pathway or an increase in autophagic flux can be tested by collecting cells from Vorinostat-treated and vehicle-treated wild-type and *Npc1^{nmf164}* mice at time of dissection. Subsequently treating these cells in cell culture with a lysosomal protease inhibitor such as bafilomycin and examining LC3-II levels through Western blot analysis will help to decipher between the two possible scenarios. In bafilomycin treated *Npc1^{nmf164}* cells, if LC3-II is further increased compared to non-bafilomycin treated *Npc1^{nmf164}* cells, the defect is likely to be an increase in autophagic flux. Conversely if no further increase is observed, a defect in fusion is likely (Mizushima and Yoshimori 2007).

RNA-Seq transcriptome analysis identified gene expression modifications in cell death genes with Vorinostat treatment. The 844 significantly regulated genes classified into functional groups using MGD, identified 42 genes to be involved in cell death; of these 42, five were involved in autophagy specifically and the remaining in apoptosis. These five transcripts classified as being involved in autophagy include: S100A8, S100A9, XBP1, SRPX and 5330417C22Rik. Interestingly, S100 calcium-binding protein A8 (S100A8) and S100 calcium-binding protein A9 (S100A9) have previously been reported to function together to induce autophagy in cell lines, increasing LC3-II levels after incubation with S100A8/A9 (Ghavami, Eshragi et al. 2010). In this investigation S100A8 was up-regulated by 1.89 log₂ fold ($p = 9.9 \times 10^{-7}$) and S100A9 by 1.93 log₂ fold ($p = 2.4 \times 10^{-7}$), thus suggesting that autophagy levels are increased with Vorinostat treatment.

2.4.7 Cholesterol homeostasis

RNA transcriptome sequencing, identified 844 transcripts that were significantly changed upon treatment with Vorinostat (0.6 FC log₂, $p = 0.05$), which is approximately 5.9% of the liver transcriptome, similar to the transcriptional regulation of 2-10% of transcriptomes seen in other HDACi studies (Dokmanovic, Clarke et al. 2007, Chittur, Sangster-Guity et al. 2008). In this investigation of the liver

transcriptome of *Npc1^{nmf164}* mice, genes regulated by Vorinostat were explored and the analysis identified cholesterol biosynthesis as significantly enriched pathway regulated with this drug regime. Seven of the 16 cholesterol biosynthetic genes were down-regulated in the RNA-Seq analysis and subsequently validated with qRT-PCR. This finding is in agreement with previous reports that HDACi treatment down-regulated cholesterol biosynthetic genes in HepG2 (liver cell line) (Chittur, Sangster-Guity et al. 2008) and SH-SY5Y (neuronal cell line) (Chittur, Sangster-Guity et al. 2008, Nunes, Moutinho et al. 2013). HMGCR is the primary regulator of cholesterol biosynthesis and its activity is controlled by a number of different mechanisms (Burg and Espenshade 2011). Firstly, HMGCR may be regulated through a phosphorylation-dephosphorylation mechanism. The AMP-activated protein kinase (AMPK) which is activated upon phosphorylation, phosphorylates HMGCR thereby decreasing the activity of the enzyme. AMPK is made up of three subunits; α -subunit is the catalytic subunit and the β -, γ - subunits are the regulatory subunits. In this investigation, levels of phosphorylated AMPK- α measured through ELISA analysis were found to be non-regulated with Vorinostat treatment. Previous literature reported an up-regulation of AMPK and subsequent down-regulation of HMGCR upon HDACi treatment (Gao, Yin et al. 2009). HMGCR may also be regulated through regulating gene expression (Burg and Espenshade 2011). Sterol regulatory element-binding factor (SREBF) genes code for transcription factors that regulate the expression of genes involved in lipid homeostasis. SREBF-1c regulates transcription of fatty acid synthesis, SREBF-2 regulates transcription of cholesterol synthesis, and SREBF-1a regulates the transcription of fatty acid synthesis and cholesterol synthesis (Sakakura, Shimano et al. 2001). RNA-Seq did not indicate between -1a or 1c. Surprisingly, in this investigation at the mRNA level, SREBF-2 expression was unchanged upon Vorinostat treatment, while SREBF-1 expression was down-regulated with treatment (0.64 log₂ FC, $p = 0.022$). Nunes *et al.* (2013) who also observed significant down-regulation of cholesterol biosynthetic genes with HDACi treatment, found no significant difference in SREBF-2 at the mRNA level, but did at the protein level (Nunes, Moutinho et al. 2013), a result that is potentially at play here. Therefore it is possible that SREBF-1 is targeted by Vorinostat and is responsible for the down-regulation of cholesterol biosynthesis genes seen here. Additionally, the transcription factors from the SREBF

family, can be inhibited by insulin-induced gene (INSIG), therefore down-regulating the expression of cholesterol biosynthetic genes (Burg and Espenshade 2011). In this thesis, INSIG2 expression is increased in Vorinostat-treated *Npc1^{nmf164}* mice (0.6 log₂-fold, $p = 0.03$), while INSIG1 expression is unchanged. Lastly, HMGCR can be regulated through the rate of degradation. INSIG is again at play here however functioning non-transcriptionally; by binding to the autocrine motility factor (AMFR), ubiquitination can be initiated and HMGCR degraded (Burg and Espenshade 2011). In this thesis, AMFR gene expression is unchanged with Vorinostat treatment. Therefore results in this thesis suggest that Vorinostat targets the SREBF transcription factor and therefore down-regulates the expression of genes involved in cholesterol biosynthesis.

The RNA-Seq analysis also identified that CYP46A1 was up-regulated (1.55 log₂ FC, $p = 0.0001$) in the livers of Vorinostat-treated *Npc1^{nmf164}* mice relative to vehicle-treated *Npc1^{nmf164}* mice. In addition to being the major regulator of cholesterol homeostasis in the brain converting cholesterol into 24(S)-hydroxycholesterol, CYP46A1 is also expressed in the liver, colon, kidney and testis to name a few (Uhlen, Fagerberg et al. 2015). This finding in the livers of *Npc1^{nmf164}* mice is in agreement with a recent report that CYP46A1 was up-regulated in SH-SY5Y cells with HDACi drugs (Nunes, Milagre et al. 2010, Nunes, Moutinho et al. 2013). Given Vorinostat did not significantly up-regulate the cholesterol efflux genes ABCA1, APOE and NPC1, like that was observed with TSA treated SH-SY5Y cells (Nunes, Moutinho et al. 2013), the transcriptome results suggest Vorinostat is targeting multiple genes involved in cholesterol metabolism that collectively contribute to the lipid reduction observed in this investigation in the liver and serum of *Npc1^{nmf164}* mice. Figure 19 summarizes these validated gene expression changes in cholesterol biosynthetic genes upon Vorinostat treatment.

Figure 19: Working model – Vorinostat down-regulates the expression of cholesterol biosynthetic genes. WEBGESTALT identified cholesterol biosynthesis to be enriched in RNA-Seq analysis and further validation through qRT-PCR analysis. Yellow increased expression and red decreased expression. Figure adapted from Chittur *et al.* (2008).

2.4.8 Conclusion

To conclude, NPC disease is a rare neuro-visceral, LSD for which no effective therapy yet exists. Lipid accumulation caused by defects in the cholesterol transport genes (*NPC1* (95%) or *NPC2* (5%)) are the causative factors (Vanier 2010). The multitude of pathologies that result from these mutations, primarily occur in the liver and the brain and ultimately lead to premature death at 10-25 years of age (Vanier 2010). Because an NPC patient's life is significantly shortened, time is the enemy of many scientific researchers and families of sufferers with the disease. Thus with the discovery that Vorinostat, a FDA-approved HDACi, was capable of lowering lipid accumulation in fibroblasts from NPC-diseased patients, early approval of a Phase I clinical trial, testing the efficacy of orally-administered Vorinostat in adult NPC disease patients was granted. Until now, the therapeutic efficacy of Vorinostat in a NPC diseased, whole body animal model, has not been reported. In this thesis, Vorinostat reduced liver disease in the *Npc1^{nmf164}* mouse by decreasing lipid accumulation; however, the treatment did not delay weight loss, onset of ataxia and premature death. Targeted and unbiased transcriptome analysis found Vorinostat functions in a pleiotropic manner, independent of the suspected NPC1 chaperone mechanism, and significantly regulates the expression of 844 genes. Of particular interest, were the seven out of 16 cholesterol biosynthetic genes that after additional validation studies remained to be significantly reduced with Vorinostat treatment. Therefore if these results were translated to human patients it is expected that Vorinostat has the potential to treat visceral disease, however due to its lack of BBB penetration, will not prevent neurodegeneration and thus delay death.

Chapter 3. Future Directions

3.1 Future directions

In this thesis, experiments were conducted to demonstrate that Vorinostat ameliorated selected hallmarks of NPC disease and subsequently the potential mechanisms behind the rescue of disease hallmarks were investigated. Experiments conducted here however were limited to the one year of thesis research and thus did not comprehensively assess disease rescue by Vorinostat. Additional experiments for future investigations are discussed below.

3.1.1 Measuring extent of neurodegeneration

In this investigation, the assessment of neurodegeneration is limited to weight loss, ataxia visual to the naked eye, and lifespan. Additional assessments that measure neurodegeneration in a NPC mouse model are discussed here. As neurodegeneration progresses with NPC disease, ataxia progressively worsens due to the increase in Purkinje cell death in the cerebellum. Ataxia can be measured quantitatively through a number of analyses (*e.g.*, rotarod and footprint analysis). Rotarod analysis involves initially training mice (before the onset of symptoms) to walk on a rotating rod at a controlled speed, and the average time it takes for a mouse to fall off the rotating rod is recorded (Deacon 2013). This method of analysis has been used in analyses of the *Npc1*^{-/-} mouse to measure the extent of ataxia (Elrick, Pacheco et al. 2010, Elrick, Yu et al. 2012). *Npc1*^{-/-} displayed significant ataxia by P58; a late stage symptom, with death occurring between P60-P80 in this null model (Elrick, Yu et al. 2012). In *Npc1*^{nmf164} mice, rotarod analysis initiated before the onset of ataxia (*e.g.*, P40) is not expected to differ from wild-type controls, however by P90, when Purkinje cells are noticeably decreased relative to wild-type (Maue, Burgess et al. 2012), mice are expected to fall from the rotarod before wild-type mice as a result of poor motor coordination. If Vorinostat-treated *Npc1*^{nmf164} mice stay on the rotarod for an extended time compared to vehicle-treated *Npc1*^{nmf164} mice, it would suggest that Purkinje cell death is delayed and Vorinostat is therapeutic in the brain. A second test that measures ataxia is footprint analysis; this involves painting the paws of a mouse (*e.g.*, hind-paws painted blue and fore-paws painted red) and monitoring the paint imprinted on white paper as the mice walk down a narrow enclosed runway lined with white paper (Heck, Zhao et al. 2008). By measuring the stride length (distance between blue and red

print), ataxia can be quantitatively measured (Loftus, Erickson et al. 2002). Smaller stride lengths are expected from mice suffering from ataxia, compared to longer strides from wild-type mice. In the *Npc1^{nmf164}* mouse, it is predicted that by the P90 time-point, mice would display decreased stride length compared to wild-type mice, therefore suggesting diseased mice have undergone significant neurodegeneration.

To complement the visual and quantitative analyses of ataxia, histological analysis of the loss of Purkinje cells may be assessed to determine the extent of neurodegeneration in the *Npc1^{nmf164}* mouse. Here immunohistochemistry can be performed on cerebellum sections probed with an antibody specific for calbindin, a Purkinje cell-specific protein (Reid, Sakashita et al. 2004). In *Npc1^{nmf164}* mice, a significant loss of Purkinje cells is expected at P60–P90 (Maue, Burgess et al. 2012), a time-point fitting for the P60 mice investigated in this thesis. However, a delay in Purkinje cell loss would not have been expected in the Vorinostat-treated *Npc1^{nmf164}* mice compared to the vehicle-treated *Npc1^{nmf164}* mice because the Vorinostat treatment did not delay weight loss, ataxia, or time of death.

Investigating the accumulation of lipids in the brain would also be necessary to further assess neurodegeneration since lipid accumulation is a primary cause of neurodegeneration in NPC disease. This can be accomplished using LC/MS to measure the major lipids that accumulate in the brain in NPC disease (the gangliosides GM2 and GM3, glucosylceramide, lactosylceramide and sphingosine) (Walkley and Vanier 2009). Cholesterol and sphingomyelin however do not overtly accumulate in the brain (Xie, Turley et al. 1999). It would be expected with the treatment used in this thesis, that Vorinostat would not reduce lipid levels in the brain because of the poor BBB permeability of i.p.-administered Vorinostat in a vehicle of 10% DMSO and 45% PEG-400.

3.1.2 Improving BBB penetration

In this thesis, Vorinostat did not delay neurodegeneration. Therefore, it is suggested that i.p.-administered Vorinostat in a vehicle of 10% DMSO + 45% PEG-400 struggles to penetrate the BBB and is insufficient to increase histone acetylation in the brain. Trialling other methods of administration is thus necessary to improve delivery of Vorinostat to the brain. For each method of administration, expression levels of ac-

H3 may be used as an indicator of BBB penetration and the suspected mechanism of action of Vorinostat.

A sub-cutaneous injection may be performed as an alternative to i.p.-administration; this administration method has previously been shown to increase acetylation of histone proteins in the spleen and brain of a Huntington's disease mouse model injected with 200 mg/kg Vorinostat in a solvent of 1.8% HPB-CD (Hockly, Richon et al. 2003). A problem that may arise using this method with the drug regime used in this thesis however is the large volume that needs to be administered five times per week from P21 to P60, making it uncomfortable and potentially toxic for the mouse.

Alternatively, oral administration of 100 mg/kg Vorinostat in a solvent of 1.8% HPB-CD may be trialled in the *Npc1^{nmf164}* mouse; this administration has previously been found to delay neurodegeneration in a mouse model of Huntington's disease (Hockly, Richon et al. 2003, Mielcarek, Benn et al. 2011). Because 5% HPB-CD has been shown to extend the lifespan of the *Npc1^{-/-}* mouse (Davidson, Ali et al. 2009) and HPB-CD is currently in clinical trials for NPC disease, the dose of HPB-CD would need to be carefully optimised to test non-therapeutic as well as therapeutic doses of HPB-CD. Determining whether a synergistic effect exists between HPB-CD and Vorinostat is intriguing, particularly with the results of this thesis demonstrating some efficacy in the serum and liver. Another combination treatment worthy of investigation in the future is the combination of Miglustat and Vorinostat. As previously mentioned, Miglustat has shown improvements in neurodegeneration, however is unable to be therapeutic in visceral organs (Hockly, Richon et al. 2003, Mielcarek, Benn et al. 2011). With the improvement of liver disease upon Vorinostat in this investigation, the combination of these two therapies is intriguing.

Injection directly into the brain is another method that may be considered for increasing Vorinostat levels in the brain. This method was used to successfully ameliorate cognitive defects in a mouse model of Alzheimer's disease (Peleg, Sananbenesi et al. 2010). Problems with this type of administration however is that it is an invasive technique requiring surgery, therefore number of injections may be limited (once per week in animal models and once per month in humans). This delivery dynamic has already been dealt with in NPC patients in the HPB-CD clinical trial where

the drug was initially administered through an Ommaya pump in the brain to increase levels reaching the brain, however bacterial infections arose. A new administration method into the spinal cord that avoided bacterial infections but compromised the amount of HPB-CD reaching the brain was instead continued. In summary, it is important to decipher if levels of Vorinostat can be increased in the brain by altering the method of administration and dosing regimen, especially with the current clinical trial testing the efficacy of orally-administered Vorinostat in adult NPC patients.

3.1.3 Additional liver disease measurements

In this thesis, sphingomyelin (SM) was found to significantly decrease upon Vorinostat treatment. Unfortunately, the many other lipids that also accumulate in the liver in NPC disease were not successfully identified using the MALDI-TOF MS/MS instrument. Further lipidomic analysis is thus necessary to fully determine the extent of lipid rescue. Other mass spectrometers would be useful for the detection of these lipids, such as liquid chromatography-mass spectrometry (LC/MS) to measure the diversity of sphingolipids that accumulate as well as gas chromatography-mass spectrometry (GC/MS) to measure the levels of unesterified cholesterol that accumulate in the liver of NPC disease patients and mouse models.

The P60 time-point that liver disease was investigated in this thesis is an early time point in the presentation of disease symptoms by the *Npc1^{nmf164}* mouse. It would be interesting to examine Vorinostat-treated mice with a more severe stage of disease (*e.g.*, late-stage disease at P90) to determine whether Vorinostat treatment would further increase the rescue that was observed at P60 or whether it is a saturated effect (*i.e.*, rescue in P90 is equal to rescue in P60).

To complement the quantification of unesterified cholesterol in the whole liver and brain using mass spectroscopy, fluorescent microscopy should be conducted in future experiments to visualise the distribution of unesterified cholesterol in these tissues. This is critical given that the mass spectrometry may not detect a change in the total levels, while the fluorescent microscopy may detect a change in the intracellular distribution. Filipin, a fluorescent stain specific for unesterified cholesterol, is a common tool to diagnose NPC disease in skin fibroblasts derived from patients but can also be used on tissue sections (Tangemo, Weber et al. 2011). It

is expected that filipin stained tissue in *Npc1^{nmf164}* mice (e.g., liver or brain sections) will display increased fluorescence, indicating an increased presence of unesterified cholesterol. In this thesis, given the reduction of total cholesterol and LDL in the serum analysis, it is expected that unesterified cholesterol will also decrease with treatment as indicated with a decrease in fluorescence in the liver and brain. Hematoxylin- and eosin-stained liver sections may also be conducted to identify lipid accumulation with the observation of foam cells. Foam cells are commonly reported in NPC patients and animal models, including the *Npc1^{nmf164}* mouse model in which foam cells are evident between P60-P90 (Maue, Burgess et al. 2012). Given that a reduction in lipids upon Vorinostat treatment is observed in this thesis, a decrease in the number of foam cells is also expected.

3.1.4 Further investigation of the potential for Vorinostat-mediated chaperone folding of NPC1

It is not yet established whether the missense mutation in the *Npc1^{nmf164}* mouse leads to a misfolded protein, and whether this mutated protein is then capable of refolding with additional chaperone mediated mechanisms from Vorinostat treatment. It is thus critical to quantify the half-life of the NPC1 protein in the *Npc1^{nmf164}* mouse in order to determine if Vorinostat increases that half-life in a chaperone-mediated fashion. These analyses can be conducted in cells derived from the *Npc1^{nmf164}* mouse or the whole body *Npc1^{nmf164}* mouse itself. It is possible that Vorinostat does correct the particular mutation at D1005G mutation in *Npc1^{nmf164}* cells or mouse; however the result observed *in vitro* in cells may not be well replicated *in vivo* due to the additional elimination features of whole body animals. Experiments to investigate the D1005G mutation would mimic the experiments that have been used to characterise 6.5-hour half-life of NPC1 caused by the I1061T mutation (Gelsthorpe, Baumann et al. 2008), with the addition of Vorinostat thus to determine if Vorinostat extends the half-life of NPC1 caused by the D1005G mutation. It is possible that there is a confounder in the 20% of wild-type NPC1 expressed in the *Npc1^{nmf164}* mouse that was determined in this thesis. It is plausible that 20% of normal levels is too low of a level to be rescued by a chaperone mechanism (i.e., the protein is so misfolded that it cannot be correctly refolded). Heterozygous mice with one allele displaying the D1005G mutation could also be observed. NPC1 expression level in these

heterozygous mice is predicted to be 50% of wild-type, compared to the 19% seen in *Npc1^{nmf164}* mice. It would be interesting to treat heterozygous mice with Vorinostat to determine if NPC1 levels exceed 50% of wild-type levels. Even though no increase in treated mice was seen in *Npc1^{nmf164}* mice in this thesis, the mutation present on one allele (in heterozygous mice) may cause a less intense disruption in protein folding that is amenable to being refolded with Vorinostat treatment.

3.1.5 Establishing cholesterol biosynthesis as a target of Vorinostat

It was demonstrated in this thesis that Vorinostat down-regulated expression of cholesterol biosynthetic genes in the livers of *Npc1^{nmf164}* mice. To fully determine if cholesterol biosynthesis is a target of Vorinostat, the proteins encoded by the down-regulated genes should be observed at the protein level by Western blot analysis. Given the observed down-regulation at the mRNA level, it is expected that these cholesterol biosynthetic genes will also be decreased in expression at the protein level. However, it is possible that changes seen at the mRNA level are not additionally observed at the protein level. Statins are an established family of drugs that specifically inhibit HMGCR, the first and rate-limiting step in cholesterol biosynthesis. An interesting experiment would be to compare a statin drug and Vorinostat in primary hepatocyte cells derived from *Npc1^{nmf164}* mice for their efficacy to reduce cholesterol levels and down-regulate cholesterol biosynthetic genes. If Vorinostat proves as good as or better than a statin drug, the potential of Vorinostat to be used in heart disease would be intriguing and warrant examination of cholesterol levels in human cancer patients currently taking Vorinostat.

3.1.6 Further characterisation of the pleiotropic mechanism

In this thesis, Vorinostat is shown to function in a pleiotropic manner significantly changing the expression of 844 genes. Vorinostat regulated genes, other than genes involved in cholesterol biosynthesis, must be further investigated to determine their relevance in the rescue of liver disease in *Npc1^{nmf164}* mice. Preliminary results of a few of these 844 genes have been validated through qRT-PCR analysis in *Npc1^{nmf164}* mice only and these were selected based on association with liver disease. Firstly, perilipin 4 (PLIN4) was shown by RNA-Seq to be significantly down-

regulated (1.3 log₂-fold, $p = 1.44 \times 10^{-5}$), and qRT-PCR validated this result where Vorinostat down-regulated PLIN4 expression 5.9-fold. Interestingly, it has been demonstrated that *Plin4*^{-/-} mice exhibit reduced lipid accumulation in the heart (Chen, Chang et al. 2013). However, PLIN4 expression is found mainly in white adipose tissue and is absent in brown adipose tissue and the liver of wild-type mice (Chen, Chang et al. 2013). Because of its detection in RNA liver samples analysed in this investigation, it is suggested that PLIN4 is abnormally increased in the liver of *Npc1*^{nmf164} mice. Expression of PLIN4 in wild-type mice must be confirmed to validate this increase in NPC diseased mice. Nonetheless, a reduction of PLIN4 in the liver of Vorinostat-treated *Npc1*^{nmf164} mice is an intriguing result potentially regulating lipid accumulation.

ARNTL, previously reported as being regulated by HDAC3 (Yin and Lazar 2005), was up-regulated in RNA-Seq by 2.6 log₂ fold ($p = 1.18 \times 10^{-7}$), and qRT-PCR analysis validated the RNA-Seq result with a 7.1-fold increase ($p = 0.02$) of ARNTL relative to vehicle-treated *Npc1*^{nmf164} mice. Interestingly, the *Arntl*^{-/-} mice exhibit increased LDL levels in the serum (Shimba, Ogawa et al. 2011), thus it would be interesting to directly examine LDL levels in *Npc1*^{nmf164} mice overexpressing ARNTL. If ARNTL is a major contributor to the pleiotropic mechanism observed in this thesis, then the prediction would be that ARNTL overexpression would reduce LDL levels in *Npc1*^{nmf164} mice. Furthermore, it would be interesting to measure HDAC3 at the protein level in Vorinostat-treated *Npc1*^{nmf164} mice to determine if HDAC3 is down-regulated with Vorinostat treatment.

Chapter 4. References

Alam, M. S., M. Getz, S. Yi, J. Kurkewich, I. Safeukui and K. Haldar (2014). "Plasma signature of neurological disease in the monogenetic disorder Niemann-Pick Type C." J Biol Chem **289**(12): 8051-8066.

Alvarez, A. R., A. Klein, J. Castro, G. I. Cancino, J. Amigo, M. Mosqueira, L. M. Vargas, L. F. Yevenes, F. C. Bronfman and S. Zanlungo (2008). "Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann-Pick type C disease." Faseb j **22**(10): 3617-3627.

Angelini, R., R. Vitale, V. A. Patil, T. Cocco, B. Ludwig, M. L. Greenberg and A. Corcelli (2012). "Lipidomics of intact mitochondria by MALDI-TOF/MS." J Lipid Res **53**(7): 1417-1425.

Appelqvist, H., P. Waster, K. Kagedal and K. Ollinger (2013). "The lysosome: from waste bag to potential therapeutic target." J Mol Cell Biol **5**(4): 214-226.

Barton, N. W., R. O. Brady, J. M. Dambrosia, A. M. Di Bisceglie, S. H. Doppelt, S. C. Hill, H. J. Mankin, G. J. Murray, R. I. Parker, C. E. Argoff and et al. (1991). "Replacement therapy for inherited enzyme deficiency--macrophage-targeted glucocerebrosidase for Gaucher's disease." N Engl J Med **324**(21): 1464-1470.

Beltroy, E. P., B. Liu, J. M. Dietschy and S. D. Turley (2007). "Lysosomal unesterified cholesterol content correlates with liver cell death in murine Niemann-Pick type C disease." J Lipid Res **48**(4): 869-881.

Bergamin, N., A. Dardis, A. Beltrami, D. Cesselli, S. Rigo, S. Zampieri, R. Domenis, B. Bembi and C. A. Beltrami (2013). "A human neuronal model of Niemann Pick C disease developed from stem cells isolated from patient's skin." Orphanet J Rare Dis **8**: 34.

Berger, A. C., T. H. Vanderford, K. M. Gernert, J. W. Nichols, V. Faundez and A. H. Corbett (2005). "Saccharomyces cerevisiae Npc2p is a functionally conserved homologue of the human Niemann-Pick disease type C 2 protein, hNPC2." Eukaryot Cell **4**(11): 1851-1862.

Bligh, E. G. and W. J. Dyer (1959). "A rapid method of total lipid extraction and purification." Can J Biochem Physiol **37**(8): 911-917.

Boya, P. and G. Kroemer (2008). "Lysosomal membrane permeabilization in cell death." Oncogene **27**(50): 6434-6451.

Brown, D. E., M. A. Thrall, S. U. Walkley, D. A. Wenger, T. W. Mitchell, M. O. Smith, K. L. Royals, P. A. March and R. W. Allison (1994). "Feline Niemann-Pick disease type C." Am J Pathol **144**(6): 1412-1415.

Brown, M. S. and J. L. Goldstein (1979). "Receptor-mediated endocytosis: insights from the lipoprotein receptor system." Proc Natl Acad Sci U S A **76**(7): 3330-3337.

Burg, J. S. and P. J. Espenshade (2011). "Regulation of HMG-CoA reductase in mammals and yeast." Prog Lipid Res **50**(4): 403-410.

Chen, W., B. Chang, X. Wu, L. Li, M. Sleeman and L. Chan (2013). "Inactivation of Plin4 downregulates Plin5 and reduces cardiac lipid accumulation in mice." Am J Physiol Endocrinol Metab **304**(7): E770-779.

Chittur, S. V., N. Sangster-Guity and P. J. McCormick (2008). "Histone deacetylase inhibitors: a new mode for inhibition of cholesterol metabolism." BMC Genomics **9**: 507.

Claerhout, S., J. Y. Lim, W. Choi, Y. Y. Park, K. Kim, S. B. Kim, J. S. Lee, G. B. Mills and J. Y. Cho (2011). "Gene expression signature analysis identifies vorinostat as a candidate therapy for gastric cancer." PLoS One **6**(9): e24662.

Davidson, C. D., N. F. Ali, M. C. Micsenyi, G. Stephney, S. Renault, K. Dobrenis, D. S. Ory, M. T. Vanier and S. U. Walkley (2009). "Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression." PLoS One **4**(9): e6951.

Deacon, R. M. (2013). "Measuring motor coordination in mice." J Vis Exp(75): e2609.

Dokmanovic, M., C. Clarke and P. A. Marks (2007). "Histone deacetylase inhibitors: overview and perspectives." Mol Cancer Res **5**(10): 981-989.

Duncan, M. W., H. Roder and S. W. Hunsucker (2008). "Quantitative matrix-assisted laser desorption/ionization mass spectrometry." Brief Funct Genomic Proteomic **7**(5): 355-370.

Dupere-Richer, D., M. Kinal, V. Menasche, T. H. Nielsen, S. Del Rincon, F. Pettersson and W. H. Miller, Jr. (2013). "Vorinostat-induced autophagy switches from a death-promoting to a cytoprotective signal to drive acquired resistance." Cell Death Dis **4**: e486.

Elrick, M. J., C. D. Pacheco, T. Yu, N. Dadgar, V. G. Shakkottai, C. Ware, H. L. Paulson and A. P. Lieberman (2010). "Conditional Niemann-Pick C mice demonstrate cell autonomous Purkinje cell neurodegeneration." Hum Mol Genet **19**(5): 837-847.

Elrick, M. J., T. Yu, C. Chung and A. P. Lieberman (2012). "Impaired proteolysis underlies autophagic dysfunction in Niemann–Pick type C disease." Human Molecular Genetics **21**(22): 4876-4887.

Elstein, D., C. Hollak, J. M. Aerts, S. van Weely, M. Maas, T. M. Cox, R. H. Lachmann, M. Hrebicek, F. M. Platt, T. D. Butters, R. A. Dwek and A. Zimran (2004). "Sustained therapeutic effects of oral miglustat (Zavesca, N-butyldeoxynojirimycin, OGT 918) in type I Gaucher disease." J Inherit Metab Dis **27**(6): 757-766.

Eppig, J. T., J. A. Blake, C. J. Bult, J. A. Kadin and J. E. Richardson (2015). "The Mouse Genome Database (MGD): facilitating mouse as a model for human biology and disease." Nucleic Acids Res **43**(Database issue): D726-736.

- Erickson, R. P. and O. Bernard (2002). "Studies on neuronal death in the mouse model of Niemann-Pick C disease." J Neurosci Res **68**(6): 738-744.
- Falkenberg, K. J. and R. W. Johnstone (2014). "Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders." Nat Rev Drug Discov **13**(9): 673-691.
- Ferguson, M., P. A. Henry and R. A. Currie (2003). "Histone deacetylase inhibition is associated with transcriptional repression of the Hmga2 gene." Nucleic Acids Res **31**(12): 3123-3133.
- Ficicioglu, C. (2008). "Review of miglustat for clinical management in Gaucher disease type 1." Therapeutics and Clinical Risk Management **4**(2): 425-431.
- Friedewald, W. T., R. I. Levy and D. S. Fredrickson (1972). "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge." Clin Chem **18**(6): 499-502.
- Futerman, A. H. and G. van Meer (2004). "The cell biology of lysosomal storage disorders." Nat Rev Mol Cell Biol **5**(7): 554-565.
- Gammoh, N., D. Lam, C. Puente, I. Ganley, P. A. Marks and X. Jiang (2012). "Role of autophagy in histone deacetylase inhibitor-induced apoptotic and nonapoptotic cell death." Proc Natl Acad Sci U S A **109**(17): 6561-6565.
- Gao, Z., J. Yin, J. Zhang, R. E. Ward, R. J. Martin, M. Lefevre, W. T. Cefalu and J. Ye (2009). "Butyrate improves insulin sensitivity and increases energy expenditure in mice." Diabetes **58**(7): 1509-1517.
- Garber, K. (2013). "Orphazyme." Nat Biotechnol **31**(3): 189.
- Gelsthorpe, M. E., N. Baumann, E. Millard, S. E. Gale, S. J. Langmade, J. E. Schaffer and D. S. Ory (2008). "Niemann-Pick type C1 I1061T mutant encodes a functional protein that is selected for endoplasmic reticulum-associated degradation due to protein misfolding." J Biol Chem **283**(13): 8229-8236.
- Ghavami, S., M. Eshragi, S. R. Ande, W. J. Chazin, T. Klonisch, A. J. Halayko, K. D. McNeill, M. Hashemi, C. Kerkhoff and M. Los (2010). "S100A8/A9 induces autophagy and apoptosis via ROS-mediated cross-talk between mitochondria and lysosomes that involves BNIP3." Cell Res **20**(3): 314-331.
- Greer, W. L., D. C. Riddell, T. L. Gillan, G. S. Girouard, S. M. Sparrow, D. M. Byers, M. J. Dobson and P. E. Neumann (1998). "The Nova Scotia (type D) form of Niemann-Pick disease is caused by a G3097-->T transversion in NPC1." Am J Hum Genet **63**(1): 52-54.
- Griffin, L. D., W. Gong, L. Verot and S. H. Mellon (2004). "Niemann-Pick type C disease involves disrupted neurosteroidogenesis and responds to allopregnanolone." Nat Med **10**(7): 704-711.

Hanson, J. E., H. La, E. Plise, Y.-H. Chen, X. Ding, T. Hanania, E. V. Sabath, V. Alexandrov, D. Brunner, E. Leahy, P. Steiner, L. Liu, K. Searce-Levie and Q. Zhou (2013). "SAHA Enhances Synaptic Function and Plasticity In Vitro but Has Limited Brain Availability In Vivo and Does Not Impact Cognition." PLoS ONE **8**(7): e69964.

Heck, D. H., Y. Zhao, S. Roy, M. S. LeDoux and L. T. Reiter (2008). "Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors." Hum Mol Genet **17**(14): 2181-2189.

Hockly, E., V. M. Richon, B. Woodman, D. L. Smith, X. Zhou, E. Rosa, K. Sathasivam, S. Ghazi-Noori, A. Mahal, P. A. Lowden, J. S. Steffan, J. L. Marsh, L. M. Thompson, C. M. Lewis, P. A. Marks and G. P. Bates (2003). "Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease." Proc Natl Acad Sci U S A **100**(4): 2041-2046.

Huang, X., K. Suyama, J. Buchanan, A. J. Zhu and M. P. Scott (2005). "A Drosophila model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis." Development **132**(22): 5115-5124.

Huang, X., J. T. Warren, J. Buchanan, L. I. Gilbert and M. P. Scott (2007). "Drosophila Niemann-Pick type C-2 genes control sterol homeostasis and steroid biosynthesis: a model of human neurodegenerative disease." Development **134**(20): 3733-3742.

Hutt, D. M., D. Herman, A. P. Rodrigues, S. Noel, J. M. Pilewski, J. Matteson, B. Hoch, W. Kellner, J. W. Kelly, A. Schmidt, P. J. Thomas, Y. Matsumura, W. R. Skach, M. Gentzsch, J. R. Riordan, E. J. Sorscher, T. Okiyonedo, J. R. Yates, 3rd, G. L. Lukacs, R. A. Frizzell, G. Manning, J. M. Gottesfeld and W. E. Balch (2010). "Reduced histone deacetylase 7 activity restores function to misfolded CFTR in cystic fibrosis." Nat Chem Biol **6**(1): 25-33.

Imafuku, Y., S. Meguro, K. Kanno, H. Hiraki, U. Nemoto, R. Hata, K. Takahashi, Y. Miura and H. Yoshida (2002). "The effect of EDTA contaminated in sera on laboratory data." Clin Chim Acta **325**(1-2): 105-111.

Infante, R. E., M. L. Wang, A. Radhakrishnan, H. J. Kwon, M. S. Brown and J. L. Goldstein (2008). "NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes." Proc Natl Acad Sci U S A **105**(40): 15287-15292.

Juan, L. J., W. J. Shia, M. H. Chen, W. M. Yang, E. Seto, Y. S. Lin and C. W. Wu (2000). "Histone deacetylases specifically down-regulate p53-dependent gene activation." J Biol Chem **275**(27): 20436-20443.

Kalmar, B., C. H. Lu and L. Greensmith (2014). "The role of heat shock proteins in Amyotrophic Lateral Sclerosis: The therapeutic potential of Arimoclomol." Pharmacol Ther **141**(1): 40-54.

Kim, S. J., B. H. Lee, Y. S. Lee and K. S. Kang (2007). "Defective cholesterol traffic and neuronal differentiation in neural stem cells of Niemann-Pick type C disease improved

by valproic acid, a histone deacetylase inhibitor." Biochem Biophys Res Commun **360**(3): 593-599.

Kirkegaard, T., A. G. Roth, N. H. Petersen, A. K. Mahalka, O. D. Olsen, I. Moilanen, A. Zylicz, J. Knudsen, K. Sandhoff, C. Arenz, P. K. Kinnunen, J. Nylandsted and M. Jaattela (2010). "Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology." Nature **463**(7280): 549-553.

Kwon, H. J., L. Abi-Mosleh, M. L. Wang, J. Deisenhofer, J. L. Goldstein, M. S. Brown and R. E. Infante (2009). "Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol." Cell **137**(7): 1213-1224.

Lee, S., J. W. Jung, S. B. Park, K. Roh, S. Y. Lee, J. H. Kim, S. K. Kang and K. S. Kang (2011). "Histone deacetylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging." Cell Mol Life Sci **68**(2): 325-336.

Levine, B. and G. Kroemer (2008). "Autophagy in the Pathogenesis of Disease." Cell **132**(1): 27-42.

Liao, G., Z. Wang, E. Lee, S. Moreno, O. Abuelnasr, M. Baudry and X. Bi (2015). "Enhanced expression of matrix metalloproteinase-12 contributes to Npc1 deficiency-induced axonal degeneration." Exp Neurol **269**: 67-74.

Liscum, L. and J. R. Faust (1987). "Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts." J Biol Chem **262**(35): 17002-17008.

Liscum, L., R. M. Ruggiero and J. R. Faust (1989). "The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts." J Cell Biol **108**(5): 1625-1636.

Liu, B. (2012). "Therapeutic potential of cyclodextrins in the treatment of Niemann-Pick type C disease." Clin Lipidol **7**(3): 289-301.

Liu, B., C. M. Ramirez, A. M. Miller, J. J. Repa, S. D. Turley and J. M. Dietschy (2010). "Cyclodextrin overcomes the transport defect in nearly every organ of NPC1 mice leading to excretion of sequestered cholesterol as bile acid." J Lipid Res **51**(5): 933-944.

Loftus, S. K., R. P. Erickson, S. U. Walkley, M. A. Bryant, A. Incao, R. A. Heidenreich and W. J. Pavan (2002). "Rescue of neurodegeneration in Niemann-Pick C mice by a prion-promoter-driven Npc1 cDNA transgene." Hum Mol Genet **11**(24): 3107-3114.

Loftus, S. K., J. A. Morris, E. D. Carstea, J. Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. A. Rosenfeld, D. A. Tagle, P. G. Pentchev and W. J. Pavan (1997). "Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene." Science **277**(5323): 232-235.

Lopez, M. E., A. D. Klein, U. J. Dimbil and M. P. Scott (2011). "Anatomically defined neuron-based rescue of neurodegenerative Niemann-Pick type C disorder." J Neurosci **31**(12): 4367-4378.

Lopez, M. E., A. D. Klein, J. Hong, U. J. Dimbil and M. P. Scott (2012). "Neuronal and epithelial cell rescue resolves chronic systemic inflammation in the lipid storage disorder Niemann-Pick C." Hum Mol Genet **21**(13): 2946-2960.

Lopez, M. E. and M. P. Scott (2013). "Genetic dissection of a cell-autonomous neurodegenerative disorder: lessons learned from mouse models of Niemann-Pick disease type C." Dis Model Mech **6**(5): 1089-1100.

Lowenthal, A. C., J. F. Cummings, D. A. Wenger, M. A. Thrall, P. A. Wood and A. de Lahunta (1990). "Feline sphingolipidosis resembling Niemann-Pick disease type C." Acta Neuropathol **81**(2): 189-197.

Lu, J., C. Yang, M. Chen, D. Y. Ye, R. R. Lonser, R. O. Brady and Z. Zhuang (2011). "Histone deacetylase inhibitors prevent the degradation and restore the activity of glucocerebrosidase in Gaucher disease." Proc Natl Acad Sci U S A **108**(52): 21200-21205.

Luzio, J. P., P. R. Pryor and N. A. Bright (2007). "Lysosomes: fusion and function." Nat Rev Mol Cell Biol **8**(8): 622-632.

Maetzel, D., S. Sarkar, H. Wang, L. Abi-Mosleh, P. Xu, Albert W. Cheng, Q. Gao, M. Mitalipova and R. Jaenisch (2014). "Genetic and Chemical Correction of Cholesterol Accumulation and Impaired Autophagy in Hepatic and Neural Cells Derived from Niemann-Pick Type C Patient-Specific iPS Cells." Stem Cell Reports **2**(6): 866-880.

Malathi, K., K. Higaki, A. H. Tinkelenberg, D. A. Balderes, D. Almanzar-Paramio, L. J. Wilcox, N. Erdeniz, F. Redican, M. Padamsee, Y. Liu, S. Khan, F. Alcantara, E. D. Carstea, J. A. Morris and S. L. Sturley (2004). "Mutagenesis of the putative sterol-sensing domain of yeast Niemann Pick C-related protein reveals a primordial role in subcellular sphingolipid distribution." J Cell Biol **164**(4): 547-556.

Marks, P. A. and R. Breslow (2007). "Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug." Nat Biotechnol **25**(1): 84-90.

Maue, R. A., R. W. Burgess, B. Wang, C. M. Wooley, K. L. Seburn, M. T. Vanier, M. A. Rogers, C. C. Chang, T. Y. Chang, B. T. Harris, D. J. Graber, C. A. Penatti, D. M. Porter, B. S. Szwegold, L. P. Henderson, J. W. Totenhagen, T. P. Trouard, I. A. Borbon and R. P. Erickson (2012). "A novel mouse model of Niemann-Pick type C disease carrying a D1005G-Npc1 mutation comparable to commonly observed human mutations." Hum Mol Genet **21**(4): 730-750.

Mengel, E., H. H. Klunemann, C. M. Lourenco, C. J. Hendriksz, F. Sedel, M. Walterfang and S. A. Kolb (2013). "Niemann-Pick disease type C symptomatology: an expert-based clinical description." Orphanet J Rare Dis **8**: 166.

Mi, H., A. Muruganujan, J. T. Casagrande and P. D. Thomas (2013). "Large-scale gene function analysis with the PANTHER classification system." Nat Protoc **8**(8): 1551-1566.

Mielcarek, M., C. L. Benn, S. A. Franklin, D. L. Smith, B. Woodman, P. A. Marks and G. P. Bates (2011). "SAHA decreases HDAC 2 and 4 levels in vivo and improves molecular phenotypes in the R6/2 mouse model of Huntington's disease." PLoS One **6**(11): e27746.

Mizushima, N. and T. Yoshimori (2007). "How to interpret LC3 immunoblotting." Autophagy **3**(6): 542-545.

Munkacsy, A. B., F. W. Chen, M. A. Brinkman, K. Higaki, G. D. Gutierrez, J. Chaudhari, J. V. Layer, A. Tong, M. Bard, C. Boone, Y. A. Ioannou and S. L. Sturley (2011). "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." J Biol Chem **286**(27): 23842-23851.

Nigam, P. K. (2011). "Serum Lipid Profile: Fasting or Non-fasting?" Indian Journal of Clinical Biochemistry **26**(1): 96-97.

Nunes, M. J., I. Milagre, M. Schnekenburger, M. J. Gama, M. Diederich and E. Rodrigues (2010). "Sp proteins play a critical role in histone deacetylase inhibitor-mediated derepression of CYP46A1 gene transcription." J Neurochem **113**(2): 418-431.

Nunes, M. J., M. Moutinho, M. J. Gama, C. M. Rodrigues and E. Rodrigues (2013). "Histone deacetylase inhibition decreases cholesterol levels in neuronal cells by modulating key genes in cholesterol synthesis, uptake and efflux." PLoS One **8**(1): e53394.

Ohkuma, S., Y. Moriyama and T. Takano (1982). "Identification and characterization of a proton pump on lysosomes by fluorescein-isothiocyanate-dextran fluorescence." Proc Natl Acad Sci U S A **79**(9): 2758-2762.

Ordóñez, M. P., E. A. Roberts, C. U. Kidwell, S. H. Yuan, W. C. Plaisted and L. S. Goldstein (2012). "Disruption and therapeutic rescue of autophagy in a human neuronal model of Niemann Pick type C1." Hum Mol Genet **21**(12): 2651-2662.

Pacheco, C. D. and A. P. Lieberman (2008). "The pathogenesis of Niemann-Pick type C disease: a role for autophagy?" Expert Rev Mol Med **10**: e26.

Palmieri, D., P. R. Lockman, F. C. Thomas, E. Hua, J. Herring, E. Hargrave, M. Johnson, N. Flores, Y. Qian, E. Vega-Valle, K. S. Taskar, V. Rudraraju, R. K. Mittapalli, J. A. Gaasch, K. A. Bohn, H. R. Thorsheim, D. J. Liewehr, S. Davis, J. F. Reilly, R. Walker, J. L. Bronder, L. Feigenbaum, S. M. Steinberg, K. Camphausen, P. S. Meltzer, V. M. Richon, Q. R. Smith and P. S. Steeg (2009). "Vorinostat inhibits brain metastatic colonization in a model of triple-negative breast cancer and induces DNA double-strand breaks." Clin Cancer Res **15**(19): 6148-6157.

Parenti, G. (2009). "Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics." EMBO Mol Med **1**(5): 268-279.

Parenti, G., G. Andria and A. Ballabio (2015). "Lysosomal storage diseases: from pathophysiology to therapy." Annu Rev Med **66**: 471-486.

Patterson, M. C., E. Mengel, F. A. Wijburg, A. Muller, B. Schwierin, H. Drevon, M. T. Vanier and M. Pineda (2013). "Disease and patient characteristics in NP-C patients: findings from an international disease registry." Orphanet J Rare Dis **8**: 12.

Patterson, M. C., D. Vecchio, H. Prady, L. Abel and J. E. Wraith (2007). "Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study." Lancet Neurol **6**(9): 765-772.

Peleg, S., F. Sananbenesi, A. Zovoilis, S. Burkhardt, S. Bahari-Javan, R. C. Agis-Balboa, P. Cota, J. L. Wittnam, A. Gogol-Doering, L. Opitz, G. Salinas-Riester, M. Dettenhofer, H. Kang, L. Farinelli, W. Chen and A. Fischer (2010). "Altered histone acetylation is associated with age-dependent memory impairment in mice." Science **328**(5979): 753-756.

Pentchev, P. G., M. E. Comly, H. S. Kruth, S. Patel, M. Proestel and H. Weintraub (1986). "The cholesterol storage disorder of the mutant BALB/c mouse. A primary genetic lesion closely linked to defective esterification of exogenously derived cholesterol and its relationship to human type C Niemann-Pick disease." J Biol Chem **261**(6): 2772-2777.

Pentchev, P. G., M. E. Comly, H. S. Kruth, M. T. Vanier, D. A. Wenger, S. Patel and R. O. Brady (1985). "A defect in cholesterol esterification in Niemann-Pick disease (type C) patients." Proc Natl Acad Sci U S A **82**(23): 8247-8251.

Petkovic, M., A. Vocks, M. Muller, J. Schiller and J. Arnhold (2005). "Comparison of different procedures for the lipid extraction from HL-60 cells: a MALDI-TOF mass spectrometric study." Z Naturforsch C **60**(1-2): 143-151.

Pipalia, N. H., C. C. Cosner, A. Huang, A. Chatterjee, P. Bourbon, N. Farley, P. Helquist, O. Wiest and F. R. Maxfield (2011). "Histone deacetylase inhibitor treatment dramatically reduces cholesterol accumulation in Niemann-Pick type C1 mutant human fibroblasts." Proc Natl Acad Sci U S A **108**(14): 5620-5625.

Platt, F. M., B. Boland and A. C. van der Spoel (2012). "The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction." J Cell Biol **199**(5): 723-734.

Platt, F. M., G. R. Neises, R. A. Dwek and T. D. Butters (1994). "N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis." J Biol Chem **269**(11): 8362-8365.

Poupětová, H., J. Ledvinová, L. Berná, L. Dvořáková, V. Kožich and M. Elleder (2010). "The birth prevalence of lysosomal storage disorders in the Czech Republic:

comparison with data in different populations." Journal of Inherited Metabolic Disease **33**(4): 387-396.

Praggastis, M., B. Tortelli, J. Zhang, H. Fujiwara, R. Sidhu, A. Chacko, Z. Chen and C. Chung (2015). "A murine Niemann-Pick C1 I1061T knock-in model recapitulates the pathological features of the most prevalent human disease allele." **35**(21): 8091-8106.

Reddy, J. V., I. G. Ganley and S. R. Pfeffer (2006). "Clues to Neuro-Degeneration in Niemann-Pick Type C Disease from Global Gene Expression Profiling." PLoS ONE **1**(1): e19.

Reid, P. C., N. Sakashita, S. Sugii, Y. Ohno-Iwashita, Y. Shimada, W. F. Hickey and T. Y. Chang (2004). "A novel cholesterol stain reveals early neuronal cholesterol accumulation in the Niemann-Pick type C1 mouse brain." J Lipid Res **45**(3): 582-591.

Rimkunas, V. M., M. J. Graham, R. M. Crooke and L. Liscum (2008). "In vivo antisense oligonucleotide reduction of NPC1 expression as a novel mouse model for Niemann Pick type C- associated liver disease." Hepatology **47**(5): 1504-1512.

Rimkunas, V. M., M. J. Graham, R. M. Crooke and L. Liscum (2009). "TNF- α plays a role in hepatocyte apoptosis in Niemann-Pick type C liver disease." J Lipid Res **50**(2): 327-333.

Runz, H., D. Dolle, A. M. Schlitter and J. Zschocke (2008). "NPC-db, a Niemann-Pick type C disease gene variation database." Hum Mutat **29**(3): 345-350.

Saftig, P. and J. Klumperman (2009). "Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function." Nat Rev Mol Cell Biol **10**(9): 623-635.

Sakakura, Y., H. Shimano, H. Sone, A. Takahashi, N. Inoue, H. Toyoshima, S. Suzuki and N. Yamada (2001). "Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis." Biochem Biophys Res Commun **286**(1): 176-183.

Sarkar, S., B. Carroll, Y. Buganim, D. Maetzel, A. H. Ng, J. P. Cassady, M. A. Cohen, S. Chakraborty, H. Wang, E. Spooner, H. Ploegh, J. Gsponer, V. I. Korolchuk and R. Jaenisch (2013). "Impaired autophagy in the lipid-storage disorder Niemann-Pick type C1 disease." Cell Rep **5**(5): 1302-1315.

Sayre, N. L., V. M. Rimkunas, M. J. Graham, R. M. Crooke and L. Liscum (2010). "Recovery from liver disease in a Niemann-Pick type C mouse model." J Lipid Res **51**(8): 2372-2383.

Schiller, J., O. Zschornig, M. Petkovic, M. Muller, J. Arnhold and K. Arnold (2001). "Lipid analysis of human HDL and LDL by MALDI-TOF mass spectrometry and $(31)\text{P}$ -NMR." J Lipid Res **42**(9): 1501-1508.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein,

K. Eliceiri, P. Tomancak and A. Cardona (2012). "Fiji: an open-source platform for biological-image analysis." Nat Methods **9**(7): 676-682.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Schultz, M. L., L. Tecedor, M. Chang and B. L. Davidson (2011). "Clarifying lysosomal storage diseases." Trends Neurosci **34**(8): 401-410.

Shimba, S., T. Ogawa, S. Hitosugi, Y. Ichihashi, Y. Nakadaira, M. Kobayashi, M. Tezuka, Y. Kosuge, K. Ishige, Y. Ito, K. Komiyama, Y. Okamatsu-Ogura, K. Kimura and M. Saito (2011). "Deficient of a clock gene, brain and muscle Arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation." PLoS One **6**(9): e25231.

Stein, V. M., A. Crooks, W. Ding, M. Prociuk, P. O'Donnell, C. Bryan, T. Sikora, J. Dingemanse, M. T. Vanier, S. U. Walkley and C. H. Vite (2012). "Miglustat improves purkinje cell survival and alters microglial phenotype in feline Niemann-Pick disease type C." J Neuropathol Exp Neurol **71**(5): 434-448.

Sym, M., M. Basson and C. Johnson (2000). "A model for niemann-pick type C disease in the nematode *Caenorhabditis elegans*." Curr Biol **10**(9): 527-530.

Tangemo, C., D. Weber, S. Theiss, E. Mengel and H. Runz (2011). "Niemann-Pick Type C disease: characterizing lipid levels in patients with variant lysosomal cholesterol storage." J Lipid Res **52**(4): 813-825.

Uhlen, M., L. Fagerberg, B. M. Hallstrom, C. Lindskog, P. Oksvold, A. Mardinoglu, A. Sivertsson, C. Kampf, E. Sjostedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A. Szgyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P. H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen and F. Ponten (2015). "Proteomics. Tissue-based map of the human proteome." Science **347**(6220): 1260419.

Vance, J. E. and B. Karten (2014). "Niemann-Pick C disease and mobilization of lysosomal cholesterol by cyclodextrin." J Lipid Res **55**(8): 1609-1621.

Vanier, M. T. (2010). "Niemann-Pick disease type C." Orphanet J Rare Dis **5**: 16.

Vanier, M. T. and G. Millat (2003). "Niemann-Pick disease type C." Clin Genet **64**(4): 269-281.

Vite, C. H., J. H. Bagel, G. P. Swain, M. Prociuk, T. U. Sikora, V. M. Stein, P. O'Donnell, T. Ruane, S. Ward, A. Crooks, S. Li, E. Mauldin, S. Stellar, M. De Meulder, M. L. Kao, D. S. Ory, C. Davidson, M. T. Vanier and S. U. Walkley (2015). "Intracisternal Cyclodextrin Prevents Cerebellar Dysfunction and Purkinje Cell Death in Feline Niemann-Pick type C1 disease." Science translational medicine **7**(276): 276ra226-276ra226.

- Vitner, E. B., F. M. Platt and A. H. Futerman (2010). "Common and uncommon pathogenic cascades in lysosomal storage diseases." J Biol Chem **285**(27): 20423-20427.
- Walkley, S. U. and M. T. Vanier (2009). "Secondary lipid accumulation in lysosomal disease." Biochim Biophys Acta **1793**(4): 726-736.
- Wang, J., D. Duncan, Z. Shi and B. Zhang (2013). "WEB-based GENE SeT AnaLysis Toolkit (WebGestalt): update 2013." Nucleic Acids Res **41**(Web Server issue): W77-83.
- Wang, X., A. Spandidos, H. Wang and B. Seed (2012). "PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update." Nucleic Acids Res **40**(Database issue): D1144-1149.
- Ward, S., P. O'Donnell, S. Fernandez and C. H. Vite (2010). "2-hydroxypropyl-beta-cyclodextrin raises hearing threshold in normal cats and in cats with Niemann-Pick type C disease." Pediatr Res **68**(1): 52-56.
- West, A. C. and R. W. Johnstone (2014). "New and emerging HDAC inhibitors for cancer treatment." J Clin Invest **124**(1): 30-39.
- Wu, Y. P., H. Mizukami, J. Matsuda, Y. Saito, R. L. Proia and K. Suzuki (2005). "Apoptosis accompanied by up-regulation of TNF-alpha death pathway genes in the brain of Niemann-Pick type C disease." Mol Genet Metab **84**(1): 9-17.
- Wu, Y. P. and R. L. Proia (2004). "Deletion of macrophage-inflammatory protein 1 alpha retards neurodegeneration in Sandhoff disease mice." Proc Natl Acad Sci U S A **101**(22): 8425-8430.
- Xie, C., D. K. Burns, S. D. Turley and J. M. Dietschy (2000). "Cholesterol is sequestered in the brains of mice with Niemann-Pick type C disease but turnover is increased." J Neuropathol Exp Neurol **59**(12): 1106-1117.
- Xie, C., S. D. Turley, P. G. Pentchev and J. M. Dietschy (1999). "Cholesterol balance and metabolism in mice with loss of function of Niemann-Pick C protein." Am J Physiol **276**(2 Pt 1): E336-344.
- Xie, X., M. S. Brown, J. M. Shelton, J. A. Richardson, J. L. Goldstein and G. Liang (2011). "Amino acid substitution in NPC1 that abolishes cholesterol binding reproduces phenotype of complete NPC1 deficiency in mice." Proc Natl Acad Sci U S A **108**(37): 15330-15335.
- Yang, M. and T. Fujino (2015). "Ammonium Ion Exchanged Zeolite for Laser Desorption/Ionization Mass Spectrometry of Phosphorylated Peptides." Int J Anal Chem **2015**: 513761.
- Yang, Q., M. J. Dahl, K. H. Albertine, R. Ramchandran, M. Sun and J. U. Raj (2013). "Role of histone deacetylases in regulation of phenotype of ovine newborn pulmonary arterial smooth muscle cells." Cell Prolif **46**(6): 654-664.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T. L. Madden (2012). "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction." BMC Bioinformatics **13**: 134-134.

Yin, D., J. M. Ong, J. Hu, J. C. Desmond, N. Kawamata, B. M. Konda, K. L. Black and H. P. Koeffler (2007). "Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor: effects on gene expression and growth of glioma cells in vitro and in vivo." Clin Cancer Res **13**(3): 1045-1052.

Yin, L. and M. A. Lazar (2005). "The orphan nuclear receptor Rev-erb α recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene." Mol Endocrinol **19**(6): 1452-1459.

Zampieri, S., E. Bianchi, C. Cantile, R. Saleri, B. Bembi and A. Dardis (2014). "Characterization of a spontaneous novel mutation in the NPC2 gene in a cat affected by Niemann Pick type C disease." PLoS One **9**(11): e112503.

Zervas, M., K. L. Somers, M. A. Thrall and S. U. Walkley (2001). "Critical role for glycosphingolipids in Niemann-Pick disease type C." Curr Biol **11**(16): 1283-1287.

Chapter 5. Appendix

Appendix Table 1: Primers

Genes		Sequence (5' -3')	Reference
ABCA1	F:	CGTTTCGGGGAAGTGCTCTA	Liu et al. 2009
	R:	GCTAGAGATGACAAGGAGGATGGA	
ABOG1	F:	GCTGTGCGTTTTGTGCTGT	Liu et al. 2009
	R:	TGCAGCTCCAATCAGTAGTCTAA	
ARNTL	F:	GGCTGTTGAGCAGCATGAAAAC	PrimerBlast
	R:	GCTGCOCTGAGAATTAGGTGTT	
CYP51	F:	CCTGGATGGAGGTTTTACCC	PrimerBlast
	R:	TCTCTCGATGGGCTCTATCC	
CYP7A1	F:	AGCAACTAAACAACTGCGAGTACTA	Liu et al. 2009
	R:	GTCCGGATATTCAAGGATGCA	
GAPDH	F:	TCCATGACAACTTTGGCATTG	Alam et al. 2012
	R:	CAGTCTTCTGGGTGGCAGTGA	
HMGCR	F:	CTTGTGGAATGCCTTGATGTTG	Liu et al. 2009
	R:	AGCCGAAGCAGCAGCATGAT	
HMGCS	F:	GCCGTGAACTGGGTGAA	Liu et al. 2009
	R:	GCATATATAGCAATGTCTCTGCAA	
IDI1	F:	AAAGCCGAGTTGGGAATACCC	PrimerBlast
	R:	ACCATCAGATTGGGCTTGT	
LDLR	F:	GAGGAACTGGCGGCTGAA	Liu et al. 2009
	R:	GTGCTGGATGGGGAGGTCT	
LIPA	F:	TCCCTCAGAGTGGGATGCT	Xie et al. 2011
	R:	GCGCAAAGCTCCTTCATGAT	
LSS	F:	GCGGCTGTGGATGCT	PrimerBlast
	R:	AGGTAGCGAAGCCGCCA	
MVD	F:	CCGGTCAACATCGCAGTTATC	PrimerBlast
	R:	TTGTGGTCGTTTTAGCTGGT	
MVK	F:	GGTGTGGTCGGAACCTCC	PrimerBlast
	R:	CCTTGAGCGGGTTGGAGAC	
NPC1	F:	AACCGTGACACTGCAGGACAT	Xie et al. 2011
	R:	CTCATAATGGTGCAGTTCTTGTGT	
NPC2	F:	TGGCGCTGTGTCTGTGT	Xie et al. 2011
	R:	TCGGGTCTGATTTCTGTTCTTC	
PLIN4	F:	GTGTCCACCAACTCACAGATG	PrimerBlast
	R:	GGACCATTCCTTTGCAGCAT	
SOAT2	F:	GOCTCCGCCGAGATGCT	Liu et al. 2009
	R:	GTAGTTGGAGAAGGAAGTCGAGTTC	