Autophagy and tau in Niemann-Pick type C disease

by

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I dedicate this to the two loves of my life, Sam and Tobin, for their unique ability to give me their full support and keep me grounded, all at the same time.

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Table of Contents

List of Figures

Figure

Abstract

Niemann-Pick type C disease (NPC) is an autosomal recessive lipid storage disorder characterized by disrupted sphingolipid and cholesterol trafficking that produces cognitive impairment, ataxia and death, often in childhood. Most cases are caused by loss of function mutations in the *Npc1* gene. We first demonstrated that NPC1 deficient primary human fibroblasts and *npc1 -/-* mice showed increased autophagy, a bulk protein degradation pathway that has been implicated in the pathogenesis of several neurodegenerative disorders. Autophagy due to NPC1 deficiency was associated with increased expression of Beclin-1, and siRNA knock-down of Beclin-1 decreased the rate of protein degradation. Our data defined a critical role for Beclin-1 in the activation of autophagy in NPC.

Efficient protein degradation by autophagy requires trafficking along microtubules. NPC is characterized by the accumulation of hyperphosphorylated and aggregated tau, a microtubule binding protein implicated in the regulation of intracellular transport. To determine whether these changes in tau contribute to NPC pathogenesis, we decreased tau expression in cellular and mouse models of NPC. In NPC1 deficient primary human fibroblasts, siRNA knock-down of tau decreased autophagy. NPC1/tau double null mutant mice died significantly

earlier and were generated in significantly smaller litters than NPC1 single null mutants, demonstrating a genetic interaction between *Mapt* and *Npc1*. Surviving double null mutants exhibited an enhanced systemic phenotype that included mild facial dysmorphia, kyphosis and an abnormal, toe-walking gait. Our data established that tau modifies the severity of the NPC phenotype through a loss-of-function mechanism that does not involve neurofibrillary tangle formation or tau protein aggregation, and is associated with impaired activation of autophagy.

The study of NPC pathogenesis has been limited by the lack of mammalian model systems in which *Npc1* gene expression can be manipulated in a cell type or temporally controlled manner. To address this problem, in the final part of my thesis I used gene targeting to generate a conditional null mutant mouse in which *Npc1* exon 9 is flanked by *loxP* sites. Studies utilizing these mice are expected to provide insights into the mechanism of neurodegeneration in NPC and lipid trafficking in the CNS.

Chapter 1

Introduction

1.1 Background

The sphingolipid-storage diseases are a collection of ~40 genetically distinct disorders caused by inherited deficiencies of lysosomal hydrolytic activities or lipid transport that result in intracellular accumulations of cholesterol and lipids in the endosomal–lysosomal network. These disorders have an aggregate prevalence of approximately 1 in 8000 births worldwide (1). Among this group is Niemann–Pick type C disease (NPC, MIM 257220), an autosomal recessive, progressive neurovisceral disorder characterized by abnormal cholesterol trafficking and by intracellular accumulations of unesterified cholesterol and glycosphingolipids in late endosomes and lysosomes (2-8). These lipid accumulations cause a multifaceted toxic response in the liver, spleen and brain. NPC is caused by loss of function mutations in either *NPC1* (9) or *NPC2* (10); the protein products of these genes mediate proper intracellular lipid transport through pathways that are incompletely understood.

NPC patients develop symptoms over a wide range of ages (11), and there is no strict correlation between disease-causing mutations and the severity of the clinical phenotype (4, 12). While patients may initially exhibit systemic findings such as hepatosplenomegaly or obstructive jaundice, all eventually develop neurological and/or psychiatric symptoms, the severity of which is inversely associated with lifespan (13, 14). Patients presenting with neurological symptoms during the first two years of life, classified as the most extreme, infantile cases of NPC, demonstrate delayed motor development and hypotonia, and typically die within their first 5 years (15). The classical form of NPC, which encompasses ~60–70% of cases, presents between the ages of 3 and 15 years. Parents of these patients are often the first to notice troublesome signs of the disease, such as loss of speech or increasing clumsiness. The disorder progresses to cause a constellation of neurological symptoms that may include cerebellar ataxia, dysarthria, dysphagia, cataplexy, seizures, dystonia, vertical gaze palsy, progressive dementia and death by 8–25 years (11). Adult-onset cases of NPC are well documented although much less common, and their unusual clinical presentation often initially leads to misdiagnosis. Most adults with NPC develop symptoms in their second or third decades of life, although onset can occur as late as the mid-sixth decade (16). These adult patients develop symptoms similar to the classical form of juvenile NPC and usually die when in their late 30s or 40s. At the current time, there are no effective treatments available to patients with this devastating disorder.

1.2 NPC pathology and biochemical defects

While heterogeneity characterizes the clinical presentation of NPC, there is less pathological heterogeneity at the biochemical level. The vast majority of cases show prominent accumulations of unesterified cholesterol, sphingolipids and complex gangliosides in late endosomes and lysosomes of most cells types of the body (2-4, 11). However, it should be noted that a subset of patients with certain mutations demonstrate much less lipid storage (17), and central nervous system (CNS) pathology is accompanied by particularly prominent storage of sphingolipids and complex gangliosides.

1.2.1 Histopathology

The progression of NPC in the periphery is characterized by an enlargement of the liver and spleen that results from the presence of lipid-laden macrophages, termed foam cells. Kupffer cells in the liver and splenic macrophages, the latter predominantly in the red pulp, exhibit marked cytoplasmic vacuolization due to an accumulation of cholesterol, phospholipids and glycolipids.

Impairment of lipid trafficking also affects the CNS and results in neuron loss throughout the brain (18). The occurrence of swollen neuronal cell bodies in many brain regions, including cortex, basal ganglia, thalamus, cerebellum and brainstem is also characteristic of NPC and reflects lipid accumulation within late endosomes and lysosomes. The cell body of these neurons and their axonal processes also contain granular material, which by electron microscopy appears

as membrane-bound polymorphous bodies containing loosely packed lamellae or as dense osmiophilic inclusions. Additional neuronal pathology includes the formation of ectopic dendrites, swelling of proximal axons to so-called meganeurites, and the presence of axonal spheroids – changes indicative of a neuroaxonal dystrophy (18). Like several other neurodegenerative diseases, NPC is classified as a tauopathy due to the presence of intracellular aggregates of the microtubule-binding protein tau, which are biochemically identical to aggregates in Alzheimer disease (19). The role of tau pathology in NPC pathogenesis is explored in experiments described in Chapter 3. These changes in the NPC brain are accompanied by gliosis in grey and white matter, microglial activation and cerebral atrophy.

1.2.2 Biochemistry

Lipid-trafficking defects within the NPC brain reflect defects in the pathway by which cholesterol and other lipids arrive in neurons and are sorted intracellularly. Studies in normal mice have demonstrated that cholesterol accumulation in the CNS markedly increases during the first three weeks of life, a period of late development and myelination (20, 21). Lipoprotein associated cholesterol from the periphery does not cross the blood–brain barrier to enter the CNS, even during this early phase of brain development, and therefore de novo synthesis is required to meet the needs of both the developing and mature nervous system (22-24). Neurons and other CNS cell types obtain the cholesterol they require though endogenous synthesis or by uptake of cholesterol, perhaps in the form of lipoprotein particles, produced and released within the nervous

system, possibly by astrocytes (25). Once target cells internalize these particles, unesterified cholesterol and companion lipids are trafficked from the endosomal–lysosomal system to compartments such as the Golgi complex and endoplasmic reticulum for processing and to serve as substrates for further reactions (26). Derivatives of cholesterol include oxysterols, which signal a decrease in the de novo synthesis of cholesterol and therefore provide negative feedback (27, 28).

In NPC fibroblasts, lipoprotein cholesterol particles are internalized without disruption but become entrapped within trafficking vesicles, particularly late endosomes and lysosomes (Fig. 1.1) (29-31). The insufficient efflux of cholesterol and lipids out of these structures causes a twofold problem: unesterified cholesterol, sphingolipids and complex gangliosides accumulate within vesicles in the cytoplasm of the cell, while there is a simultaneous paucity of cholesterol and other essential lipids in organelles to which these compounds were originally destined. Decreased cholesterol in the endoplasmic reticulum, Golgi complex and other internal organelles causes deleterious effects on a multitude of processes dependent on proper membrane composition (32). Disrupted trafficking also results in a paucity of substrate for further synthetic reactions. For example, decreased availability of unesterified cholesterol impairs production of oxysterols, which may lead to increased cholesterol synthesis and an exacerbation of cholesterol accumulation in NPC cells (33).

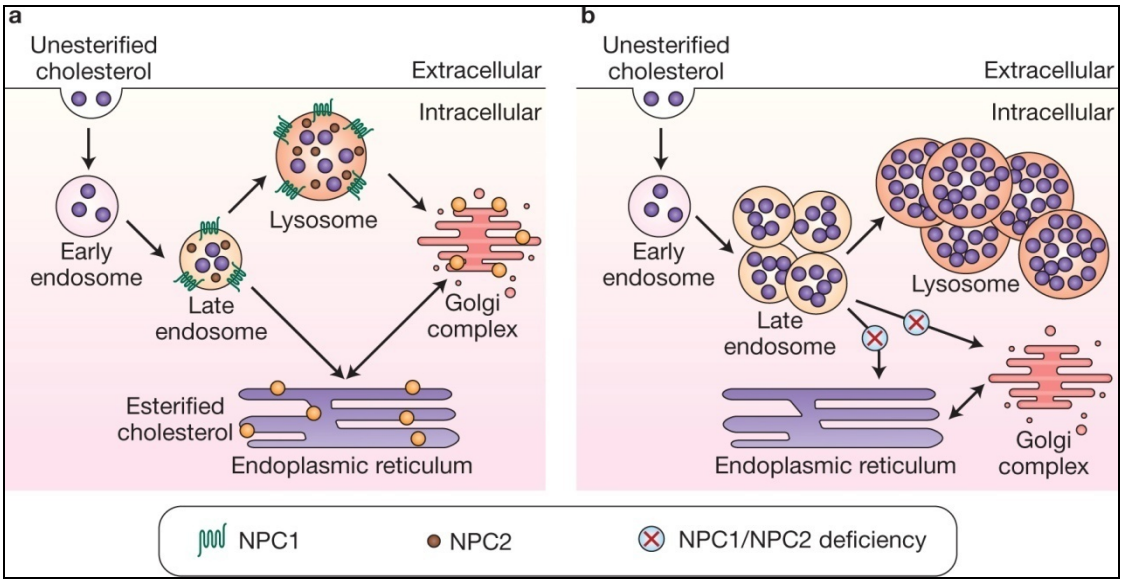
1.3 Genetics of NPC

Almost a century has passed since the original reports by Albert Niemann and Ludwick Pick described patients with hepatosplenomegaly and rapidly progressive brain damage. Since then, biochemical and genetic studies have helped define the classification of the Niemann–Pick disease group. Niemann-Pick types A and B disease (MIM 257200 and 607616), distinct from NPC, are caused by loss of function mutations in the acid sphingomyelinase gene, encoding an enzyme that metabolizes sphingomyelin to ceramide and phosphocholine. Although fibroblasts from NPC patients may show reduced sphingomyelinase activity in culture, this reduction is due to an imbalanced lipid and cholesterol environment, and removal of cholesterol from the media recovers enzymatic activity (34). By contrast, NPC fibroblasts exhibit an accumulation of unesterified cholesterol indicative of a defect in cholesterol trafficking. The major complementation group for NPC (11) was mapped to the proximal long arm of chromosome 18, and mutations in the *NPC1* gene were found in ~95% of patients with this disease (9). The remaining 5% of NPC patients were subsequently found to have loss of function mutations in a separate gene, *NPC2* (10).

The *NPC1* gene encodes a multipass transmembrane protein, NPC1, which is localized to late endosomes and lysosomes (35, 36). The encoded protein is predicted to contain a sterol-sensing domain, similar to that found in

Figure 1.1 Lipid trafficking defects in Niemann–Pick type C disease

(a) Under normal conditions, lipoprotein cholesterol particles enter the endosomal network after binding to cell-surface receptors. Unesterified cholesterol and companion lipids are trafficked from the endosomal–lysosomal system to the Golgi complex, endoplasmic reticulum and other intracellular compartments. (b) Loss of function of NPC1 or NPC2 inhibits the egress of unesterified cholesterol and sphingolipids from late endosomes and lysosomes, resulting in their accumulation at these sites and a paucity of these lipids at the intracellular compartments to which they were destined. Abbreviations: ER, endoplasmic reticulum; NPC, Niemann–Pick type C.



regulators of cholesterol metabolism and in Patched [PTCH1], a receptor for the secreted signaling protein Hedgehog [SHH] (9, 37). Experimental evidence indicates that NPC1 is involved in the efflux of low-density lipoprotein (LDL)-derived unesterified cholesterol from late endosomes and lysosomes to other intracellular compartments (30, 31). Multiple disease-causing mutations within the *NPC1* coding region have been identified that lead to its loss of function (38). The NPC1 protein is conserved within the animal kingdom, and loss of function mutations in orthologous genes in yeast (39), worms (40), flies (41), mice (42),

cats (43) and dogs (44) disrupt lipid trafficking. Introduction of the *S. cerevisiae* *NPC1* orthologue, *ncrp1,* into Chinese hamster ovary cells deficient in NPC1 is sufficient to reverse lipid trafficking defects associated with *NPC1* deficiency, demonstrating that this protein is functionally conserved from yeast to mammals (39).

The *NPC2* gene encodes a small, soluble protein that is localized to lysosomes, but is also secreted (10, 45). Loss of function mutations in this gene result in a disease phenotype that is clinically and biochemically indistinguishable from the disease caused by *NPC1* mutations. The NPC2 protein binds cholesterol, and structural analysis has identified a hydrophobic pocket that serves as a putative cholesterol-binding domain (46). Disease-causing mutations in *NPC2* that affect amino acid residues surrounding this pocket impair cholesterol binding (47). Analysis of *Npc1–Npc2* double mutant mice demonstrates that the disease phenotype is similar to that occurring in *Npc1* or *Npc2* single mutants, providing genetic evidence that the NPC1 and NPC2 proteins function in the same pathway to regulate intracellular lipid transport (48).

1.4 NPC1-deficient mice

1.4.1 Phenotype

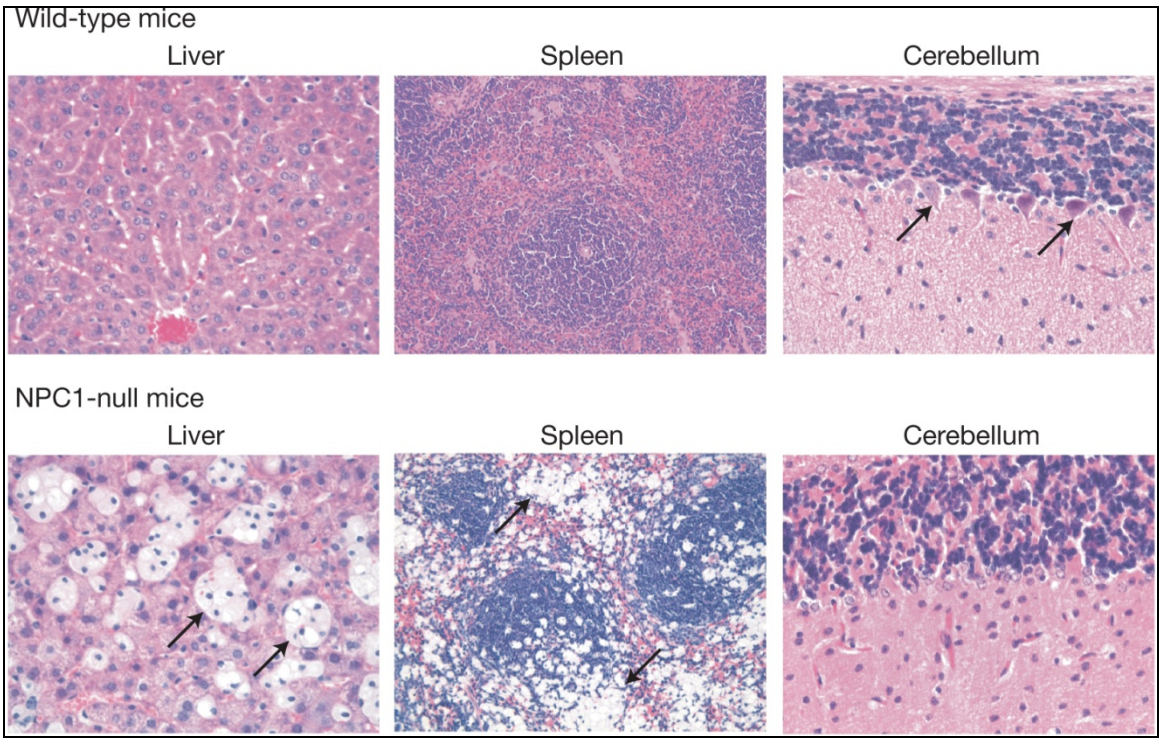
The most commonly used mammalian model of NPC, BALB/cNctr-*Npc1*^{m1N}/J mice (Jackson Laboratory #003092), contains a transposon insertion into *Npc1* exon 9 (49). Animals homozygous for this insertional mutation fail to

express functional NPC1 protein and exhibit a degenerative phenotype that begins around 7 weeks (49, 50). The severity of the phenotype progresses with age and includes weight loss, tremors, ataxia, and death by 10–12 weeks. The liver and spleen contain an abundance of foam cells, reminiscent of the histopathological changes seen in humans with this disorder (Fig. 1.2). Recently, in vivo antisense knockdown of *NPC1* expression only in the liver of normal mice has been shown to recapitulate the hepatic pathology associated with NPC, generating a novel model to examine liver dysfunction associated with disease independent of CNS involvement (51)

Global loss of *NPC1* expression leads to CNS pathology including vacuolated neurons that accumulate glucosylceramide, lactosylceramide, complex gangliosides and cholesterol (52-54), abnormally swollen axons, and decreased myelination in the cerebral white matter (55). A prominent feature of CNS pathology is loss of Purkinje cells, the major neuronal cell type transmitting output from the cerebellum (56). These highly specialized neurons are responsible for timing and smoothness of movements, and lesions within the cerebellum can lead to ataxia. Loss of Purkinje cells is initially apparent in the cerebellar vermis by 7 weeks. Progressive Purkinje cell loss proceeds from the anterior to posterior vermis, yet largely spares lobules IX and X, even in mice with end-stage disease (56). Interestingly, 2-year-old *Npc1*$^{+/-}$ mice also exhibit mild Purkinje cell loss, suggesting that haploinsufficiency for NPC1 may have adverse consequences in the aging brain (57).

Figure 1.2 Niemann–Pick type C disease pathology

Foamy macrophages are abundant in the liver and spleen of NPC1-deficient mice (examples at arrowheads). In the brain, loss of cerebellar Purkinje cells is a prominent pathological feature; these large, pyramidal-shaped neurons normally reside at the interface of the molecular layer and granular cell layer of the cerebellar folia (examples at arrowheads). These cells die in NPC1-deficient mice and are replaced by reactive astrocytes. Abbreviation: NPC, Niemann–Pick type C.



1.4.2 Lipid-trafficking defects

NPC1-deficient mice have been used to explore how lipid-trafficking defects lead to progressive neurodegeneration. Attention has focused on the role of glycosphingolipids, including complex gangliosides such as GM2, which accumulate in NPC neurons (54); although involved in normal brain development, these do not normally accumulate in the adult brain. Their presence in the NPC brain has been implicated in pathogenesis, including the

formation of ectopic dendrites that branch from swollen axons (18). These abnormally long and inappropriately located processes may contribute to neuronal dysfunction. Based on this notion, on indications that some glycosphingolipids are toxic, and on findings that cholesterol accumulation in the NPC brain is ganglioside-dependent (58), glycosphingolipid synthesis has emerged as a therapeutic target in NPC and related sphingolipid-storage disorders (59-61).

Trafficking defects also lead to diminished production of cholesterol derivatives including neurosteroids, which normally help mediate brain development, growth and differentiation (62). A single injection of the neurosteroid allopregnanolone, whose production is significantly decreased in NPC mice, is sufficient to promote Purkinje cell survival and delay disease phenotype (63). Although initial evidence suggested that this beneficial effect was mediated via signaling from the $GABA_A$ receptor (class A receptor for γ-aminobutyric acid), more recent studies indicate that it occurs by activation of the pregnane X receptor (PXR) or liver X receptor (LXR) (64). These steroid receptors are ligand-activated transcription factors that bind the oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol (27, 28). Pathways activated by the receptors promote efflux of cholesterol from late endosomes and lysosomes, and suppress endogenous cholesterol synthesis. Exogenous application of either 25-hydroxycholesterol or 27-hydroxycholesterol is sufficient to correct sterol defects in NPC1-deficient human fibroblasts and to mobilize cholesterol from the late endosomal/lysosomal compartment (65). LXR agonists also promote

cholesterol efflux from the NPC mouse brain, slow neurodegeneration and prolong life (66), suggesting that activation of these pathways is a promising therapeutic strategy.

1.4.3 Cell-autonomous cell death

Defects in lipid trafficking that occur in NPC lead to cell-autonomous neuronal death. This was established by an analysis of chimeric mice that express functional NPC1 protein in only a subset of cells (67). In this model, NPC1-deficient Purkinje cells died, even when neighboring cells harbored normal levels of NPC1 protein. Conversely, Purkinje cells with functional NPC1 were spared, even in an NPC1-deficient environment. Examination of NPC1-deficient Purkinje cells in mutant mice revealed evidence of autophagy, suggesting that this pathway is activated in the NPC brain and may play a role in pathogenesis.

1.5 Autophagy

Autophagy is a conserved cellular pathway that mediates the turnover of damaged or aged macromolecules and organelles. Three types of autophagy have been described: microautophagy, chaperone-mediated autophagy and macroautophagy (68). For the purposes of this review, we discuss only macroautophagy and refer to it simply as ‘autophagy’.

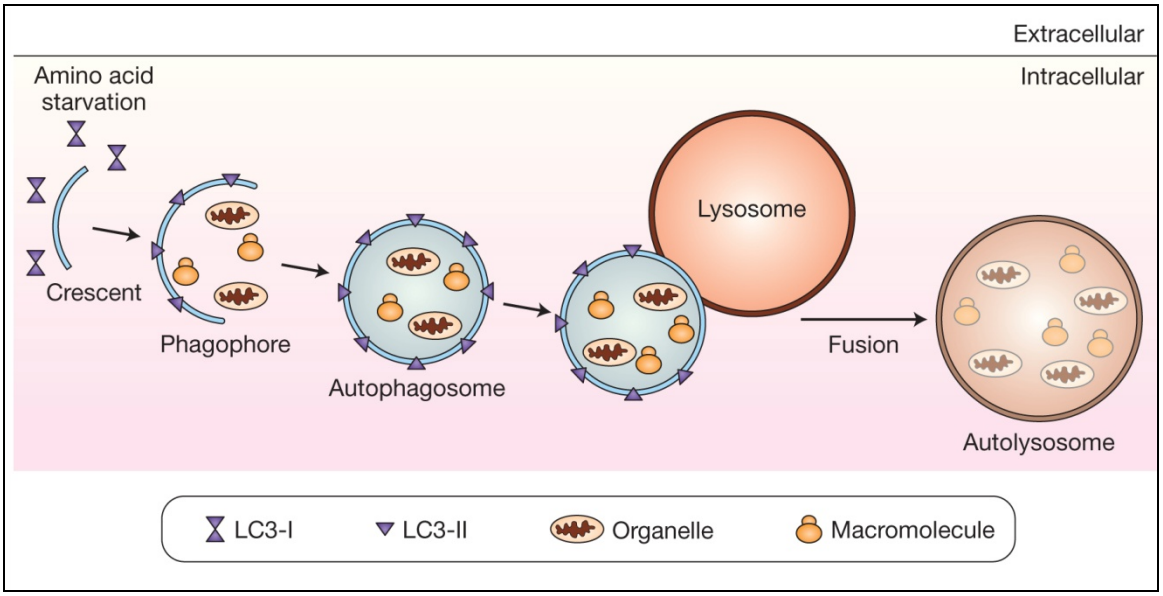
At basal levels, autophagy is a house-keeping mechanism for the constitutive degradation of long-lived proteins and other macromolecules

inaccessible to the ubiquitin–proteasome pathway. Regulated degradation is governed by activation of autophagy-related genes (*ATG*s) (69). Autophagy begins with the generation of an isolation membrane – a crescent-shaped double membrane originating from either smooth ER or another subcellular location that has yet to be defined (70). The isolation membrane surrounds cargo targeted for degradation (Fig. 1.3). During the engulfment process, two ubiquitin-like conjugation reactions are required for progression through autophagy: the formation of an ATG12–ATG5–ATG16 complex; and the conjugation of ATG8 (also known as microtubule-associated protein 1 light chain 3 protein; LC3) to phosphatidylethanolamine (71). This latter step is commonly used as a biochemical marker for the induction of autophagy (72, 73). Following engulfment, mature autophagosomes fuse with lysosomes to enable degradation of contents and recycling of their components, a process indicative of flux through the autophagic pathway (74).

Autophagy can be induced above basal levels by several stimuli, including amino acid starvation or cellular stress. This induction is mediated by signaling through the mammalian target of rapamycin (mTOR) or through a complex of the class III phosphoinositide 3-kinase (PI3K) and Beclin-1 (BECN1) (75). mTOR is a negative regulator of autophagy and its chemical inhibition by rapamycin relieves this blockade (75, 76). The PI3K–Beclin-1 complex is necessary for the induction of autophagy and can activate autophagy independently of mTOR inhibition (77).

Figure 1.3 The autophagic pathway

Autophagy occurs at low, basal levels and can be induced by multiple stimuli, including amino acid starvation or mTOR inhibition. It begins with the formation of an isolation membrane – a crescent-shaped sequestering membrane also termed a phagophore – and the induction of a ubiquitin-like conjugation reaction that couples soluble LC3-I with phosphatidylethanolamine. Lipidated LC3-II studs the phagophore membrane as it engulfs macromolecules, organelles and other cargo destined for degradation. Mature autophagosomes fuse with lysosomes to form autolysosomes, thereby degrading cargo and enabling components to be recycled. Not shown is the fusion of some autophagosomes with endosomes to form so-called amphisomes, another vesicle that fuses with lysosomes to facilitate cargo degradation. Abbreviations: mTOR, mammalian target of rapamycin; LC3, microtubule-associated protein 1 light chain 3 protein (also known as ATG8).



Autophagy is necessary for normal CNS development and function, and has been implicated in the pathogenesis of a diverse array of neurodegenerative diseases, including protein-aggregation disorders such as Alzheimer (78), Parkinson (79), and Huntington disease (80), as well as the sphingolipid-storage disorders (81). For age-related diseases associated with protein misfolding,

inducing autophagy in model systems enhances the clearance of misfolded or mutant proteins that are not efficiently degraded by the proteasome. This enhancement of protein clearance abrogates disease phenotypes (82), indicating that autophagy may have beneficial effects in some disease processes. Conversely, genetic deletion in mouse CNS of either of two autophagy related genes – *Atg5* (83) or *Atg7* (84) – leads to neurodegeneration and the accumulation of ubiquitinated protein aggregates, demonstrating that basal autophagy contributes significantly to protein quality control and cell survival in the nervous system.

Although the induction of autophagy may alleviate disease in some instances, in other situations autophagy can have deleterious effects. In mouse embryonic fibroblasts deficient in apoptosis – the classic programmed cell death pathway – because they lack the apoptotic effectors BAX and BAK, application of death stimuli such as staurosporin or etoposide triggers an apoptosis-independent cell death pathway (85). Further genetic manipulation of these cells through RNA interference knockdown of two autophagy regulators – *Atg5* and *Becn1* – inhibited cell death, demonstrating the occurrence of a death program mediated by autophagy. Contributions from autophagic cell death have been implicated in several processes, including normal development and disease (68, 86).

1.5.1 Autophagy in NPC and other lysosomal storage disorders

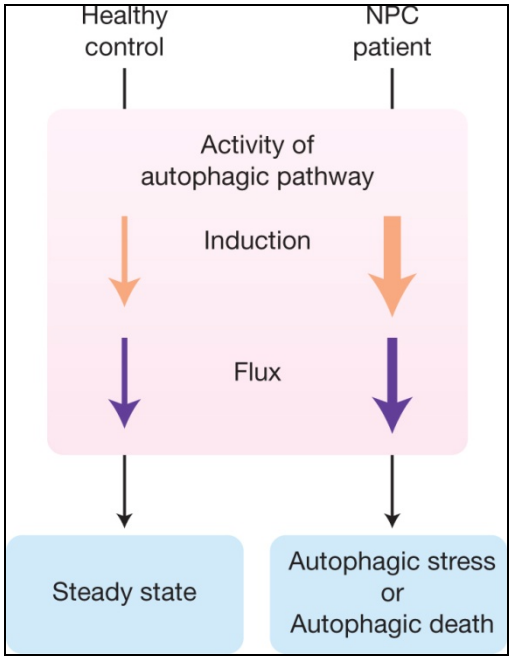
Induction of autophagy in NPC1-deficient mice was first reported by Ko et al., who observed frequent autophagic vacuoles in Purkinje cells of mutant mice accompanied by increased accumulation of lipidated LC3 in cerebellar lysates (67). Subsequent independent findings (87, 88), including our own work presented in Chapter 2, have confirmed autophagy induction in the NPC brain, and extended this observation to additional pathologically affected organs, such as liver, and to primary human fibroblasts from NPC patients. Flux through the autophagic pathway is also increased in NPC1-deficient cells, as confirmed by monitoring the degradation of long-lived proteins and by establishing that inhibition of lysosomal proteases increases lipidated LC3 levels in mutant cells (88); in contrast, inhibition of lysosome function does not alter LC3 levels in pathological situations characterized by impaired autophagosome–lysosome fusion (74). The induction of autophagy in NPC1 deficiency is not mediated by signaling through the AKT–mTOR–S6K1 pathway, but rather by the complex of Beclin-1 and class III PI3K (88). Beclin-1 expression is mildly elevated in NPC1-deficient mouse tissue and human fibroblasts, and Beclin-1 knockdown by small interfering RNA decreases long-lived protein degradation (88). Induction of autophagy and increased Beclin-1 expression are similarly observed in NPC2-deficient primary human fibroblasts and in control cells treated with U18666A (88-90), a compound known to induce an accumulation of unesterified cholesterol. By contrast, Gaucher disease fibroblasts, which traffic sphingolipids normally (26), show wild-type levels of Beclin-1 and lipidated LC3 , suggesting

that autophagy induction is sensitive to alterations in these lipids (88). We envision that the complex of Beclin-1 and class III PI3K senses changes due to the dysregulation of lipid trafficking in NPC and other disorders and activates autophagy.

Although NPC1 deficiency is characterized by elevated induction and flux through the autophagic pathway, ubiquitinated proteins accumulate in the mutant mouse brain. These ubiquitinated proteins can be visualized in cerebellar tissue of NPC1-deficient mice by immunohistochemistry (56). Fractionation studies indicate that much of this material is in the endosomal–lysosomal compartment (87). The paradoxical accumulation of ubiquitinated proteins in the setting of increased autophagic degradation suggests that flux though this pathway is not sufficient to handle the quantity of proteins targeted for degradation (Fig. 1.4). Situations where induction and flux are disproportionate to each other may lead to autophagic stress, a possible mediator of neuronal dysfunction and a precursor to cell death (91).

Figure 1.4 Autophagic induction and flux in Niemann–Pick type C and other lysosomal storage disorders.

In healthy cells, macromolecules are efficiently targeted for degradation by autophagy in a process characterized by proportionate induction and flux through the pathway. This steady-state equilibrium is disrupted in Niemann–Pick type C (NPC) disease and other lysosomal storage diseases. In NPC, our working model holds that sphingolipid-trafficking defects alter membrane lipid composition and induce autophagy through the activity of the complex of class III phosphoinositide 3-kinase and Beclin-1. This induction can be measured experimentally by an accumulation of LC3-II in NCP1- or NPC2-deficient protein lysates. The enhanced induction of autophagy occurs coordinately with a more modest increase in autophagic flux, resulting in the accumulation of ubiquitinated proteins in tissues from NPC patients and animal models. Disproportionate induction and flux through the autophagic pathway can lead to cell stress (so-called 'autophagic stress') and may trigger cell death, with dire consequences for NPC patients. Abbreviations: LC3, microtubule-associated protein 1 light chain 3 protein (also known as ATG8; NPC, Niemann–Pick type C.



Impaired flux through the autophagic pathway has also been observed in other lysosomal storage disorders, including Pompe disease (92), mucolipidosis type IV (93), multiple sulphatase deficiency and mucopolysaccharidosis type IIIA (94). Mouse models of many of these disorders exhibit increased lipidated LC3, an accumulation of enlarged autophagosomes, and impaired autophagosome–lysosome fusion resulting in decreased substrate degradation. A similar block of autophagosome–lysosome fusion has been documented in a mouse model of juvenile neuronal ceroid lipofucinosis, a degenerative disorder caused by mutations in a novel, multipass transmembrane protein localized to late endosomes and lysosomes (95). These data suggest that impaired flux through the autophagic pathway is common to many lysosomal storage diseases, and that an imbalance between induction and flux may contribute to the pathology of this diverse array of disorders by causing autophagic stress or triggering autophagic cell death.

1.6 Clinical implications and outstanding research questions

Current therapeutic options for patients with the sphingolipid-storage disorders are limited (see Table 1 for a summary of NPC therapeutic strategies). For diseases caused by deficiencies of lysosomal hydrolases, ideal therapies would replace the deficient enzymes; however, administration presents a significant challenge. Intravenous infusion of active enzymes has yielded some success in Gaucher disease, Fabry disease, mucopolysaccharidosis I and

glycogen-storage disease type II (96). Yet, this approach treats only the systemic and not CNS disease manifestations because of limited blood–brain barrier permeability. Similarly, bone-marrow transplantation is effective at reducing systemic but not neurological symptoms in NPC (97), as is the case for most, but not all, sphingolipid-storage disorders (98). An alternative treatment strategy is substrate-reduction therapy, which aims to reduce accumulating material in endosomes and lysosomes based on the notion that excess substrate contributes to pathogenesis. In the case of NPC, reduction of intracellular

Table 1. NPC therapeutic targets and strategies

Mechanism of action	Therapeutic	Refs
Current and former strategies		
Introduction of functional *NPC1* or *NPC2*	Bone marrow transplantation	97
Substrate reduction therapy		
Reduction of intracellular cholesterol	Cholestyramine, lovastatin and nicotinic acid	99, 100
Reduction of glycosphingolipid accumulation	Miglustat (N-butyldeoxynojirimycin)	61
Possible future strategies		
Activation of endogenous pathways that promote cholesterol efflux	LXR or PXR agonist	63, 64, 65, 66
Chemical modulation of autophagy	Rapamycin derivatives or novel autophagy inhibitors	67, 87, 88

Abbreviations: LXR, liver X receptor; NPC, Niemann–Pick type C; PXR, pregnane X receptor

cholesterol by a combination of cholestyramine, lovastatin and nicotinic acid was able to lower liver and blood levels of cholesterol, but had little effect on disease progression (99, 100). For disorders characterized by neurodegeneration and the accumulation of glycosphingolipids in the CNS, small-molecule inhibitors of glycosphingolipid synthesis have attracted considerable attention. Iminosugars such as miglustat (*N*-butyl-deoxynojirimycin) are reported to act through this mechanism, delay symptom onset and prolong survival in mouse models of NPC (60) and Sandhoff disease (101). A recently completed clinical trial of miglustat in

a small number of NPC patients similarly yielded a mild, encouraging effect after one year of treatment (61).

Despite these various treatment strategies, only modest clinical improvements have been achieved and new therapeutic approaches are needed. The recently described relationship between autophagy and NPC may lead to novel therapeutic targets. A critical, unanswered question is whether activation of this pathway in NPC patients is detrimental and promotes cell death, or is compensatory and promotes cell survival. To resolve this issue, autophagy will need to be modulated in NPC model systems and effects on disease phenotype established. If it is determined that increasing autophagy is beneficial, then administration of compounds like rapamycin would be an interesting therapeutic strategy. Rapamycin, in the form of Rapamune© (also termed sirolimus), has been tested in humans as an immune suppressant for organ-transplant patients (102). Alternatively, if autophagy is found to enhance the NPC phenotype, small-molecule inhibitors of this pathway may provide therapeutic benefit. A search for compounds that specifically modulate the activity of the autophagic pathway in the CNS will likely yield important reagents for investigational studies of disease mechanisms, and may provide lead compounds for the development of new treatments.

The focus of this thesis is to investigate the pathogenesis of NPC. In Chapter 2, we demonstrate a robust activation of autophagy in mice and human fibroblasts deficient in *Npc1,* which we propose to be a protective response. In Chapter 3, we demonstrate that siRNA knockdown of *MAPT* in NPC1 deficient

fibroblasts disrupts the autophagic pathway and that deletion of endogenous tau in mice causes the exacerbation of the NPC phenotype, most likely due to a diminished capacity of the autophagic pathway. Finally, in Chapter 4 we report on the generation of a *Npc1* conditional mouse model, an important resource which will allow the examination of cell type responsibility in NPC pathogenesis.

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Chapter 2

Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects

2.1 Abstract

Niemann-Pick C disease is an autosomal recessive lipid storage disorder characterized by a disruption of sphingolipid and cholesterol trafficking that produces cognitive impairment, ataxia and death, often in childhood. Most cases are caused by loss of function mutations in the *Npc1* gene, which encodes a protein that localizes to late endosomes and functions in lipid sorting and vesicle trafficking. Here we demonstrate that NPC1 deficient primary human fibroblasts, like *npc1*$^{-/-}$ mice, showed increased autophagy as evidenced by elevated LC3-II levels, numerous autophagic vacuoles and enhanced degradation of long-lived proteins. Autophagy due to NPC1 deficiency was associated with increased expression of Beclin-1 rather than activation of the Akt-mTOR-p70 S6K signaling pathway, and siRNA knockdown of Beclin-1 decreased long-lived protein degradation. Induction of cholesterol trafficking defects in wild type fibroblasts by

treatment with U18666A increased Beclin-1 and LC3-II expression, while treatment of NPC1 deficient fibroblasts with the sphingolipid-lowering compound *N*B-DGJ failed to alter the expression of either Beclin-1 or LC3-II. Primary fibroblasts from patients with two other sphingolipid storage diseases characterized by sphingolipid trafficking defects, NPC2 deficiency and Sandhoff disease, also showed elevated Beclin-1 and LC3-II levels. In contrast, Gaucher disease fibroblasts, which traffic sphingolipids normally, showed wild type levels of Beclin-1 and LC3-II. Our data define a critical role for Beclin-1 in the activation of autophagy due to NPC1 deficiency, and reveal an unexpected role for lipid trafficking in the regulation of this pathway in patients with several sphingolipid storage diseases.

2.2 Introduction

The sphingolipid storage diseases encompass a group of ~40 genetically distinct disorders that result from inherited deficiencies of lysosomal hydrolytic activities or lipid transport. These disorders occur with a collective frequency of 1 in 8000 live births (1), and are often associated with devastating neurodegeneration. Among this group is Niemann-Pick C (NPC) disease, an autosomal recessive disorder of lipid trafficking that produces cognitive impairment, ataxia and death, most often in childhood (2). NPC disease is characterized by the accumulation of unesterified cholesterol and sphingolipids in late endosomes and lysosomes. 95% of NPC disease patients have loss of function mutations in the *Npc1* gene (3). The encoded multipass transmembrane protein contains a sterol-sensing domain (4) and functions in late endosomes to

promote lipid sorting and vesicular trafficking (5-8) through mechanisms that are incompletely understood.

Mice deficient in NPC1, which reproduce the pathology and lipid trafficking defects of NPC disease, arose from a spontaneous mutation in the *Npc1* gene (*npc1 -/-* mice) (9). Similar defects occur in chimeric mice that lack functional NPC1 in only some cells (10). In both cases, NPC1 deficiency leads to the activation of macroautophagy (hereafter referred to as autophagy) in the cerebellum, a process by which cytoplasmic proteins and organelles are sequestered within autophagosomes and are targeted for degradation by lysosomes (11). This regulated and evolutionarily conserved pathway enables recycling of limited or damaged cellular constituents to promote cell survival. However, in other instances, robust activation of autophagy leads to cell death.

Here we have used *npc1 -/-* mice and primary human fibroblasts deficient in NPC1 to explore the mechanism by which autophagy is induced in NPC disease. Our data demonstrate that enhanced basal autophagy in NPC1 deficiency is mediated by increased expression of Beclin-1 rather than by activation of the Akt-mTOR-p70 S6K pathway. We further demonstrate that lipid trafficking defects caused by pharmacological treatment or by human disease gene mutations occurring in other sphingolipid storage diseases also up-regulate Beclin-1 and result in increased autophagy. Our findings establish Beclin-1 as a critical regulator of autophagy in several sphingolipid storage diseases.

2.3 Materials and Methods

2.3.1 Materials

Mouse samples were from age and sex matched BALB/cNctr-*Npc1*^{m1N}/J (Jackson Laboratories stock number 003092) and wild type littermates. Human fibroblasts from age and sex matched donors were from Coriell Cell Repositories (control cells GM00038C, NPC1 deficient GM03123A, NPC2 deficient GM17910, Gaucher disease GM10915, Sandhoff disease GM11707). Filipin, rapamycin, E64d, pepstatin A, anti-FLAG M2 monoclonal antibody, G418 and U18666A were from Sigma. *N*B-DGJ was from Toronto Research Chemicals. β-tubulin and Beclin-1 (BECN1) antibodies were from Santa Cruz Biotechnology. phospho-Akt (Ser473), total Akt, phospho-p70 S6K (Thr412) and total p70 S6K antibodies were from Upstate. phospho-mTOR (Ser2448) and total mTOR antibodies were from Cell Signaling. GAPDH antibody was from Abcam. anti-LC3 antibody was a gift from Dr. Tamotsu Yoshimori. Secondary HRP conjugated goat anti-rabbit and goat anti-mouse antibodies were from BioRad.

2.3.2 Cell culture

Fibroblasts were maintained at 37°C, 5% CO_2 in MEM with Earle's salts and non-essential amino acids (Gibco), supplemented with 15% FBS (Atlanta Biologicals) and 10 µg/mL penicillin, 10 µg/mL streptomycin and 2 mM glutamine (referred to as complete MEM medium).

2.3.3 Western blot analysis

Cells were harvested, washed with PBS and lysed in RIPA buffer containing c*O*mplete Protease Inhibitor Cocktail (Roche Diagnostics) and 0.1% β-mercaptoethanol. Liver and cerebellar samples were homogenized in this same buffer using a motor homogenizer. Lysates were precleared by centrifugation at 15,000 *g* for 10 min at 4^{o}C. Samples were electrophoresed through either a 10% SDS-polyacrylamide gel or a 4-20% tris-glycine gradient gel (Cambrex) then transferred to Immunobilon-P (Millipore) or nitrocellulose membranes (BioRad) using a semidry transfer apparatus. Immunoreactive proteins were detected by chemiluminescence (PerkinElmer). Western blots depicted show representative results from one of three experiments.

2.3.4 Electron microscopy

Cells were fixed in suspension with 4% glutaraldehyde in 0.1 M calcodylate buffer, pH 7.3, overnight at 4^{0}C, and then post-fixed for 1 hour at room temperature in 2% osmium tetraoxide in 0.1 M cacodylate. After dehydration with graded ethanols and propylene oxide, cells were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed on a Philips 400T transmission electron microscope.

2.3.5 Monodansylcadaverine staining

Cells were seeded in 6 well plates at equal density in complete MEM medium for 24 hours and then stained with MDC as previously described (15) . Briefly, cells

were incubated with 0.05 mM MDC in PBS for 10 minutes, then harvested, washed with PBS and collected in 10 mM Tris-HCl, pH 8.0, with 0.1% Triton X-100. Fluorescence of incorporated intracellular MDC (excitation wavelength 390 nm, emission filter 527 nm) was measured by an Ascent Fluroskan microplate reader. To normalize for cell number, a final concentration of 0.2 μM ethidium bromide was added to the suspension and DNA fluorescence was measured (excitation wavelength 544 nm, emission filter 590 nm).

2.3.6 pCMV-mNPC1-3xFLAG expression construct and stable line

The mouse NPC1 cDNA was amplified from pBS-KS(+)mNPC1 (gift from Dr. William Pavan) and an Asp718 site was added at the 3'end. An intermediate construct was generated by ligation of a 1 kB Asp718/EcoR1 fragment from the mNPC1 PCR product to the p3XFLAG-CMV-14 vector (Sigma) after Asp718/EcoR1 digestion. The final expression vector was generated by ligation of a 3 kB EcoR1 fragment from the pBS-KS (+) mNPC1 plasmid to the EcoR1-linearized intermediate construct. All amplified regions and cloning sites were verified by restriction digestion and sequencing.

The plasmid was electroporated into NPC1 deficient human fibroblasts with the Amaxa kit for normal human dermal fibroblasts (adult) using program U-23 on the Amaxa Nucleofector. Cells were plated in complete MEM medium for 2 days, and then selected with 1 mg/mL G418 in complete MEM medium for 1 week. Cells were allowed to recover from G418 selection for 3 days and were harvested for western blot.

2.3.7 Measurement of long-lived protein degradation

Degradation of long-lived proteins was determined using the published method (18) with minor modifications. Cells were seeded in 12 well plates in complete MEM for 24 hours and allowed to grow to 70-80% confluency. After washing with HBSS, cells were labeled with 2 μCi/mL ^{3}H leucine (Amersham) in complete MEM medium. After 48 hours of labeling, cells were washed with HBSS and incubated with complete MEM supplemented with 2.8 mM leucine. Aliquots of medium were collected at the indicated times, 20% TCA was added and samples were stored at 4°C. Upon acquisition of all samples, BSA (final concentration 3 mg/mL) was added, samples were incubated at 4°C for 1 hour and then centrifuged at 15,000 *g* for 5 minutes at 4°C. Supernatants were collected, the pellets were washed twice with cold 20% TCA, then all supernatants were pooled and the total radioactivity was measured by scintillation counting. After the last time point was collected, cells were washed with PBS and incubated for one hour at 37°C in 0.1 N NaOH/0.1% Na deoxycolate. An aliquot of the solubilized cells was used to determine total protein concentration (BioRad). Relative proteolysis was determined by normalizing TCA soluble radioactivity in the medium to protein concentration from the solubilized cells.

2.3.8 siRNA knockdown of Beclin-1

Fibroblasts growing in complete MEM media were collected using the Reagent Pack subculture reagent kit (BioWhitaker), resuspended in Nucleofector solution (human dermal fibroblast Nucleofector kit, Amaxa) and mixed with 1.5 μg ON-

TARGET*plus* SMART pool human BECN1 (NM_003766) siRNA (L-010552-00-0005) or siCONTROL*plus* non-targeting pool (D-001810-10-05) (Dharmacon). The suspension was electroporated using program U-23 on the Amaxa Nucleofector. Cells were plated in 12 well dishes and 4 hours later were used to measure protein degradation. Alternatively, cell lysates were collected 24 after electroporation for western blot.

2.3.9 Filipin Staining

Cells were seeded in chamber slides and incubated in complete MEM medium for 24 hours. After washing with PBS, cells were fixed in 3% paraformaldehyde for 1 hour at room temperature, washed with PBS and incubated with 1.5 mg/mL glycine in PBS for 10 minutes at room temperature. Cells were then stained for 2 hours with 0.05 mg/mL filipin in PBS supplemented with 10% FBS at room temperature. Staining was visualized by fluorescence microscopy using the UV filter set on a Zeiss Axioplan 2 imaging system

2.4 Results

2.4.1 NPC1 deficiency increases basal autophagy.

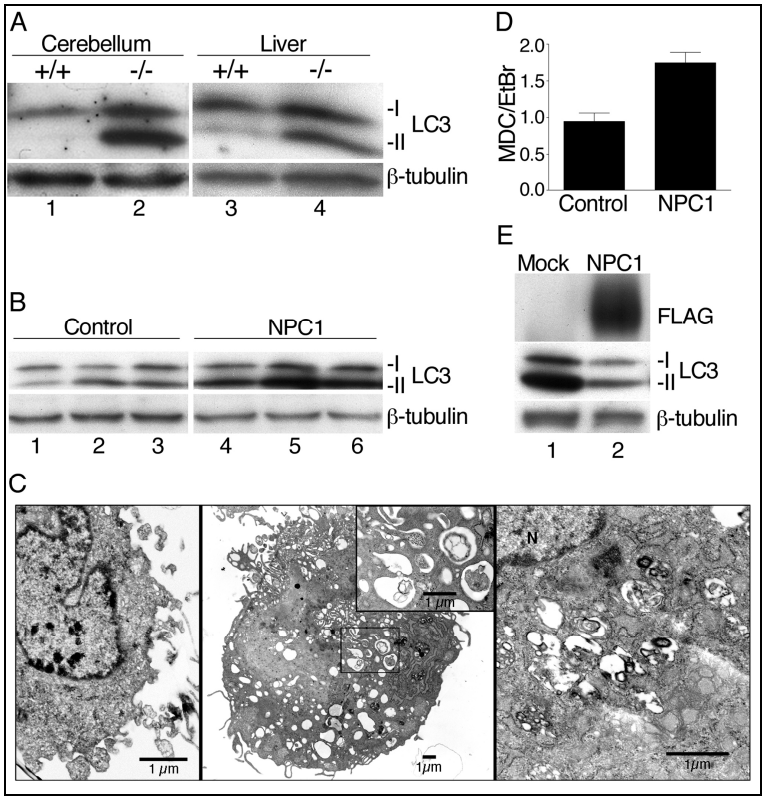
We first sought to determine whether elevated levels of autophagy occur specifically in the central nervous system of NPC1 deficient (*npc1-/-*) mice or also occur in other organs that exhibit pathology. To accomplish this, we used the microtubule associated protein 1 light chain 3 (LC3) as a marker of autophagy.

This protein is modified from its LC3-I cytosolic form to a more rapidly migrating, lipid conjugated LC3-II form associated with autophagosome membranes when autophagy is induced (12, 13). Cerebellar and liver lysates from 6-week old *npc1-/-* mice had elevated levels of LC3-II compared to wild type littermates (Fig. 2.1, A) demonstrating that NPC1 deficiency increased autophagy in both organs. Similarly, primary human fibroblasts deficient in NPC1 expressed higher total LC3 and LC3-II levels than control fibroblasts (Fig. 2.1, B). This difference was observed in untreated cells and following starvation or rapamycin treatment, indicating that basal levels of autophagy were increased by NPC1 deficiency and that pathways leading to its further activation were intact in mutant cells.

High levels of basal autophagy in NPC1 deficient fibroblasts were confirmed by transmission electron microscopy (Fig. 2.1, C). This analysis demonstrated frequent autophagic vacuoles containing rough ER and other cytoplasmic contents in mutant but not in wild type fibroblasts. Similarly, staining with monodansylcadaverine (MDC), a dye that preferentially incorporates into autophagic vacuoles (14, 15) , was significantly higher in NPC1 deficient than control fibroblasts (Fig. 2.1, D). To determine whether exogenous NPC1 could decrease autophagy in mutant fibroblasts, we stably expressed NPC1 protein in null cells. We observed decreased total LC3 and LC3-II levels in pooled NPC1 transfected cells (Fig. 2.1, E) demonstrating that exogenous NPC1 diminished levels of basal autophagy.

Figure 2.1 Increased autophagy in NPC1 deficient mice and human fibroblasts.

(A) Cerebellar (lane 1, 2) and liver (lane 3, 4) lysates from 6 week old wild type (lane 1, 3) and *npc1* -/- (lane 2, 4) mice were examined by western blot for expression of LC3 (top) and β-tubulin (bottom). (B) Lysates from control (lanes 1-3) and NPC1 deficient (lanes 4-6) human fibroblasts were collected from untreated cells (lanes 1, 4), following 2 hr starvation (lanes 2, 5) or 24 hr rapamycin (1 μM) treatment (lanes 3, 6). LC3 (top) and β-tubulin (bottom) were visualized by western blot. (C) Electron micrographs of control human fibroblast (*left panel*) with infrequent cytoplasmic vacuoles, and NPC1 deficient fibroblasts (*middle and right panels*) with frequent vacuoles. *Middle* panel shows low and high (*inset*) magnification images of membrane bound vacuoles with cytoplasmic contents consistent with autophagosomes or autolysosomes. *Right* panel shows autophagic vacuoles and mutilamellar bodies. Scale bars in lower right. N is nucleus. (D) MDC staining of control and NPC1 deficient human fibroblasts normalized to cell number (mean +/- SD). P < 0.02 by unpaired Student's *t* test. (e) Lysates from NPC1 deficient human fibroblasts stably expressing FLAG-tagged NPC1 protein (lane 2) or empty vector (lane 1) were examined by western blot for expression of NPC1 (anti-FLAG, top), LC3 (middle) and β-tubulin (bottom).



We next sought to determine whether the autophagic pathway was intact in NPC1 deficient cells. LC3-II is degraded following fusion of autophagosomes with lysosomes (16). Failure to complete this step, as seen in other lysosomal storage diseases (17), increases LC3-II levels yet renders them resistant to further elevation upon inhibition of lysosomal proteases (16). Treatment of both wild type and NPC1 deficient fibroblasts with lysosomal protease inhibitors E64d and pepstatin A similarly increased LC3-II levels, consistent with the notion that fusion of autophagosomes to lysosomes was intact in mutant cells (Fig. 2.2, A). This conclusion was supported by the additive effect of concurrent induction of autophagy by rapamycin and inhibition of lysosomal proteases by E64d and pepstatin A.

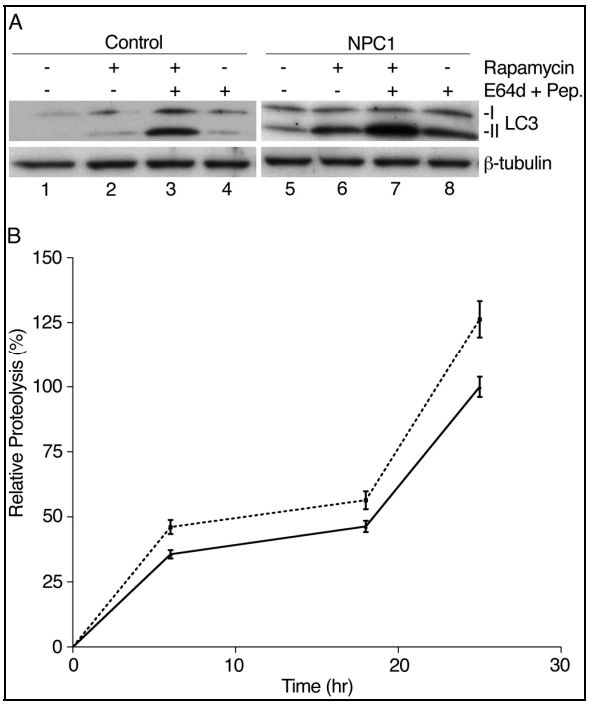


Figure 2.2 The autophagic pathway is intact in NPC1 deficiency

(A) Lysates from control (lanes 1-4) and NPC1 deficient (lanes 5-8) human fibroblasts were collected from untreated cells (lanes 1, 5) or following 24 hr treatment with rapamycin (1 μM) or E64d (10 μg/mL) plus pepstatin A (10 μg/mL), as indicated. Expression of LC3 (top) and β-tubulin (bottom) were determined by western blot. (B) Degradation of long-lived proteins in control (solid line) and NPC1 deficient (dashed line) human fibroblasts. Data (mean +/- SEM) are reported relative to control cells at 24 hr. P = 0.0006 at 6 hr, 0.01 at 12 hr and 0.005 at 24 hr by unpaired Student's *t* test.

As an independent confirmation that the autophagic pathway was intact in NPC1 deficient cells, we measured the degradation of long-lived proteins (18) . This assay provides a functional readout since autophagy is the major pathway through which many of these proteins are degraded (19, 20) . Wild type and NPC1 deficient fibroblasts were labeled with ^{3}H-leucine for 48 hrs, then washed and re-fed, and trichloroacetic acid (TCA) soluble radioactive counts were measured in the medium after 6, 12 and 24 hrs (Fig. 2.2, B). Significantly higher

levels of proteolysis were detected by NPC1 deficient fibroblasts at all points, demonstrating increased protein turnover in cells that also exhibited enhanced autophagy.

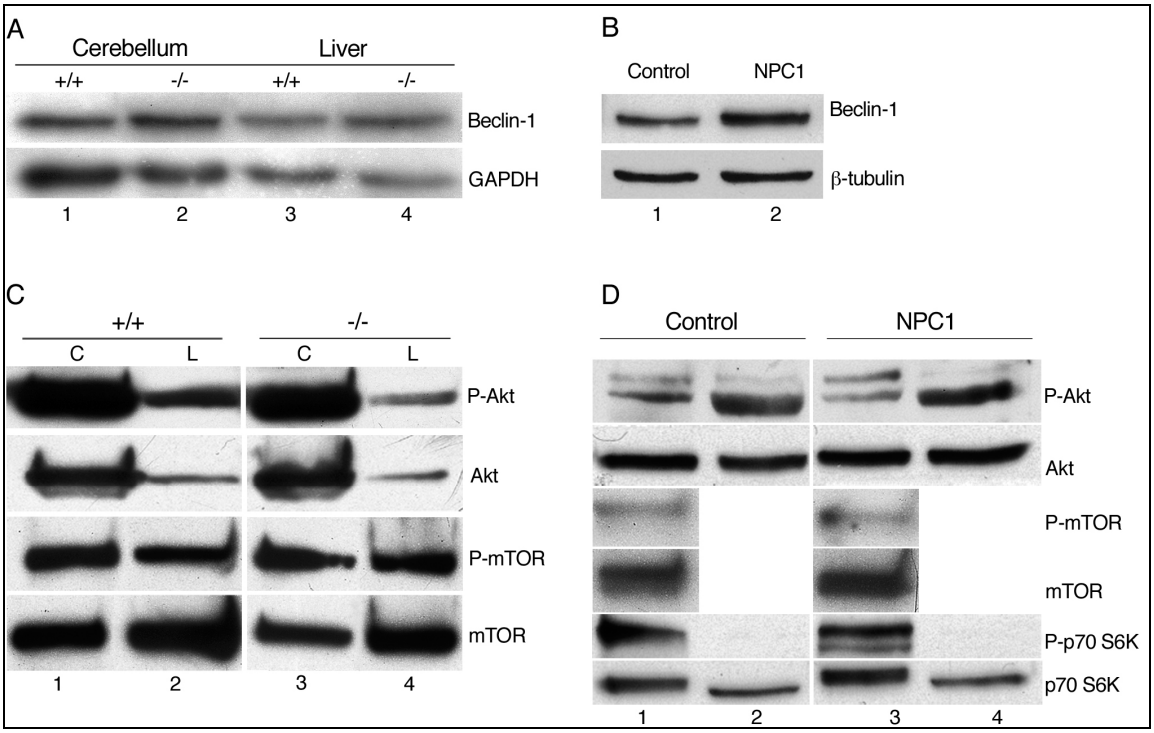
2.4.2 Beclin-1 mediates increased levels of autophagy in NPC1 deficiency.

The induction of autophagy is achieved through either the dephosphorylation of mTOR or the activation of the Beclin-1 pathway (11) . To determine which of these was preferentially activated in NPC1 deficiency, protein lysates from mutant mice and fibroblasts were examined by western blot (Fig. 2.3). Beclin-1, an evolutionarily conserved protein that is part of the Class III PI3K complex that participates in autophagosome formation (21) , was expressed at mildly increased levels in both cerebellum and liver of *npc1 -/-* mice compared to wild type littermates (Fig. 2.3, A). Increased Beclin-1 expression was also observed in NPC1 deficient fibroblasts (Fig. 2.3, B) demonstrating that up-regulation of Beclin-1 occurred in response to NPC1 deficiency in mice and cells.

In contrast, our analyses did not reveal activation of the mTOR pathway as a consequence of NPC1 deficiency. No alteration in the phosphorylation of mTOR, its regulator Akt or its target p70 S6K was detected in *npc1 -/-* mice or NPC1 deficient fibroblasts (Fig. 2.3, C and D). We did, however, observe stimulation of the mTOR pathway in NPC1 deficient fibroblasts as evidenced by starvation-induced Akt dephosphorylation or rapamycin-induced p70 S6K dephosphorylation, confirming that this pathway was intact in mutant cells.

Figure 2.3 NPC1 deficiency causes Beclin-1 up-regulation but not mTOR activation.

(A) Cerebellar (lane 1, 2) and liver (lane 3, 4) lysates from wild type (lane 1, 3) and *npc1 -/-* (lane 2, 4) mice were examined by western blot for expression of Beclin-1 (top) and GAPDH (bottom). (B) Lysates from untreated control (lane 1) and NPC1 deficient (lanes 2) human fibroblasts were analyzed by western blot for expression of Beclin-1 (top) and β-tubulin (bottom). (C) Cerebellar (C) and liver (L) lysates from wild type (lanes 1, 2) and npc1 -/- (lanes 3, 4) mice were probed for phosphorylated-Akt, total Akt, phosphorylated-mTOR and total mTOR and visualized by western blot. (D) Lysates from control (lanes 1, 2) and NPC1 deficient (lanes 3, 4) fibroblasts were collected from untreated cells (lanes 1, 3) or from cells following 2 hr starvation (lanes 2, 4; Akt rows) or 24 hr rapamycin (1 μM) treatment (lanes 2, 4; p70 S6K rows). Expression of phosphorylated-Akt (upper band = specific), total Akt, phosphorylated-mTOR, total mTOR, phosphorylated-p70 S6K and total p70 S6K were visualized by western blot.



These data demonstrated that NPC1 deficiency did not activate the Akt-mTOR-p70 S6K signaling pathway in cell culture or in mice.

To determine the extent to which Beclin-1 up-regulation mediated enhanced basal autophagy in NPC1 deficient fibroblasts, we used pooled, targeted siRNAs to specifically knockdown Beclin-1 expression (Fig. 2.4, A). Treatment with Beclin-1 siRNAs significantly decreased degradation of long-lived proteins in NPC1 deficient but not wild type fibroblasts (Fig. 4, B). We conclude that activation of Beclin-1 and not the mTOR pathway mediated increased basal autophagy in NPC1 deficiency.

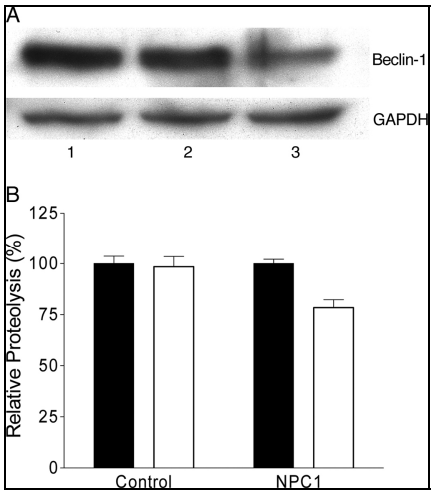


Figure 2.4 siRNA knockdown of Beclin-1 is sufficient to decrease basal autophagy in NPC1 deficient fibroblasts.

(A) Lysates from mock (lane 1), non-targeted siRNA (lane 2) and Beclin-1 siRNA (lane 3) transfected NPC1 deficient fibroblasts were analyzed by western blot for expression of Beclin-1 (top) and GAPDH (bottom). (B) Degradation of long-lived proteins in control and NPC1 deficient human fibroblasts following transfection with non-targeted siRNA (black bars) or Beclin-1 siRNA (white bars). Data (mean +/- SEM) are reported relative non-targeted siRNA transfected cells at 24 hr. Relative proteolysis is significantly decreased in NPC1 deficient cells (P = 0.018 by unpaired Student's *t* test) but not in controls (P = 0.435).

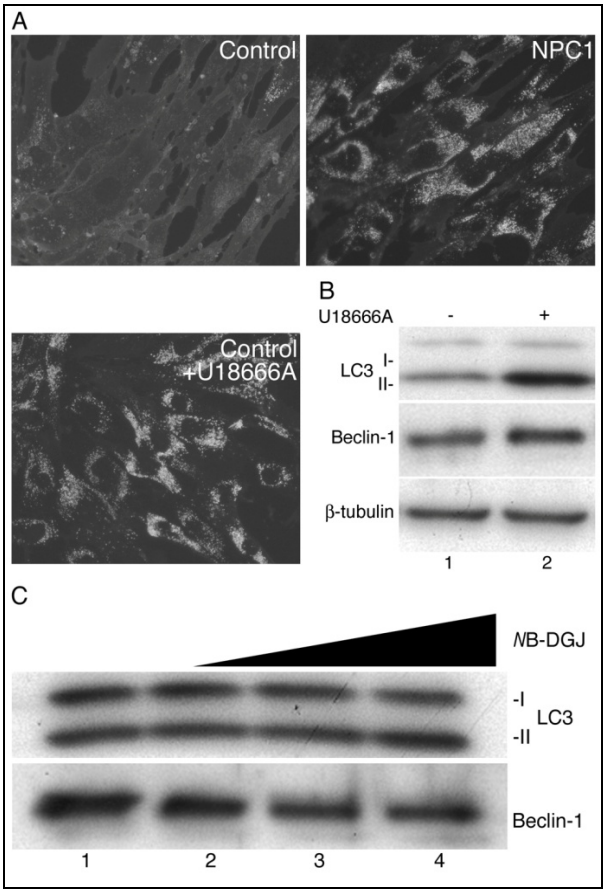
2.4.3 Defects in lipid trafficking lead to up-regulation of Beclin-1.

NPC disease is characterized by the accumulation of both unesterified cholesterol and glycosphingolipids in late endosomes and lysosomes. This trafficking defect creates a functional deficit of cholesterol in other intracellular compartments (22-24). It was recently demonstrated that autophagy is responsive to intracellular cholesterol levels (25, 26). We therefore treated control fibroblasts with U18666A, a compound known to induce an accumulation of unesterified cholesterol similar to that occurring in NPC1 deficiency (27) (Fig. 2.5, A). U18666A treatment of control fibroblasts resulted in increased expression of both LC3-II and Beclin-1 (Fig. 2.5, B), suggesting that cholesterol trafficking defects were sufficient to activate autophagy and increase Beclin-1 expression.

To evaluate the effect of glycosphingolipids on the induction of autophagy, NPC1 deficient cells were treated with the imino sugar *N*-butyl-deoxygalactonojirimycin (*N*B-DGJ). Although this compound decreases glycosphingolipid levels in NPC1 deficient cells (28), *N*B-DGJ treatment did not alter LC3-II or Beclin-1 expression (Fig. 2.5, C). These findings support the notion that elevated basal autophagy in NPC1 deficient cells was primarily a consequence of altered lipid trafficking rather than glycosphingolipid accumulation.

Figure 2.5 Unesterified cholesterol accumulation increases Beclin-1 and LC3-II expression.

(A) Filipin staining of unesterified cholesterol in untreated control and NPC1 deficient fibroblasts, and control fibroblasts after 24 hr treatment with U18666A (1 µg/ml). (B) Lysates from untreated (lane 1, 2) and U18666A-treated (1 µg/ml, 24 hr) control fibroblasts were analyzed for expression of LC3 (top), Beclin-1 (middle) and β-tubulin (bottom) by western blot. (C) Lysates from untreated NPC1 deficient fibroblasts (lane 1) or following treatment with *N*B-DGJ (50 µM) for 1, 3 or 5 days (lanes 2-4) were analyzed for expression of LC3 (top) and Beclin-1 (bottom) by western blot.



To further explore the relationship between lipid trafficking defects, Beclin-1 expression and autophagy, we used a panel of primary human fibroblasts derived from patients with several sphingolipid storage diseases. NPC2 deficiency, which results in a clinical and biochemical phenocopy of NPC1 deficiency and causes ~5% of NPC disease (29), resulted in elevated basal levels of Beclin-1 and LC3-II (Fig. 2.6, A and B). Sandhoff disease fibroblasts, which traffic sphingolipids abnormally (30-32), similarly exhibited elevated basal Beclin-1 and starvation-induced LC3-II levels (Fig. 2.6, A and B). In contrast, Gaucher disease fibroblasts, which lack sphingolipid trafficking defects (30-32), showed wild type basal levels of Beclin-1 and starvation-induced LC3-II expression (Fig. 2.6, A and B). Taken together, these analyses revealed an unexpected contribution of lipid trafficking defects to the regulation of autophagy by altering the expression of Beclin-1.

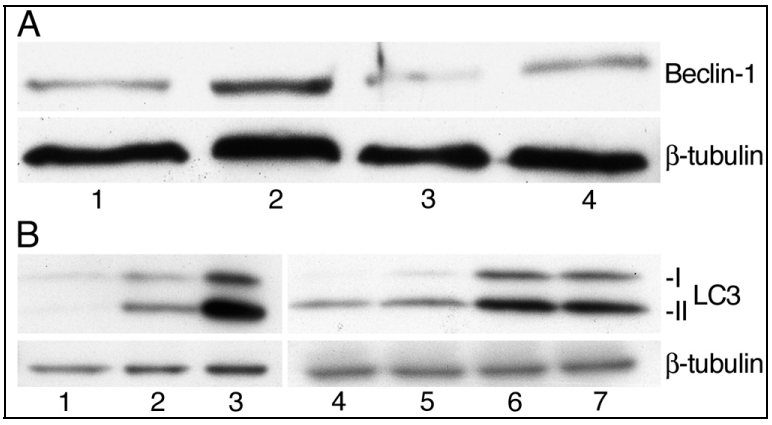


Figure 2.6 Other sphingolipidoses, which are characterized by lipid trafficking defects, display increased Beclin-1 expression.

(A) Lysates from untreated control fibroblasts (lane 1), or from patients with NPC2 deficiency (lane 2), Gaucher disease (lane 3) and Sandhoff disease (lane 4) were analyzed for expression of Beclin-1 (top) and β-tubulin (bottom) by western blot. (B) Lysates were collected from untreated control (lane 1), NPC1 deficient (lane 2) and NPC2 deficient (lane 3) fibroblasts, and from 2 hr serum starved control (lane 4), Gaucher disease (lane 5), NPC1 deficient (lane 6) and Sandhoff disease (lane 7) fibroblasts. Expression of LC3 (top) and β-tubulin (bottom) were determined by western blot.

2.5 Discussion

The studies reported here help define the relationship between defects in lipid trafficking, autophagy and disease. Cholesterol and sphingolipid accumulations in late endosomes and lysosomes of NPC1 deficient cells are biochemical hallmarks of NPC disease. This disruption of lipid trafficking is associated with the induction of autophagy. Prompted by recent reports

demonstrating increased autophagy following depletion of intracellular cholesterol (25, 26), we explored the mechanism of autophagy up-regulation using primary human fibroblasts and mice deficient in NPC1. Our data establish that NPC1 deficiency leads to increased basal autophagy as evidenced by elevated LC3-II levels, frequent autophagic vacuoles, increased MDC staining and enhanced degradation of long-lived proteins. This induction of autophagy is associated with increased expression of Beclin-1 rather than activation of the Akt-mTOR-p70 S6K pathway. Down-regulation of Beclin-1 by siRNA decreased the degradation of long-lived proteins in NPC1 deficient but not wild type fibroblasts, demonstrating a critical role for Beclin-1 in the regulation of basal autophagy rates.

Beclin-1 is the mammalian ortholog of yeast Atg6, and is a cytosolic protein that is part of the Class III PI3K machinery that participates in autophagosome formation (21). In addition to its role as a regulator of autophagy, Beclin-1 interacts with the anti-apoptotic protein Bcl-2, providing an intriguing link between pathways controlling autophagy and cell death (33) . The interaction of Beclin-1 with Bcl-2 inhibits autophagy, while Beclin-1 expression in the absence of Bcl-2 binding potently induces autophagy (33). Thus, induction of Beclin-1 expression in Niemann-Pick C disease may simultaneously affect autophagy and cell survival. Interestingly, pharmacologic disruption of cholesterol trafficking, which we show induces autophagy, also triggers apoptosis in cultured neurons (34, 35). However, a clear role for apoptosis in Niemann-

Pick C neuropathology has not yet been demonstrated (36) and autophagy may influence cell viability through other mechanisms.

Beclin-1 expression was increased by pharmacological treatments that cause an accumulation of unesterified cholesterol and by human disease gene mutations that disrupt lipid trafficking. Treatment of wild type fibroblasts with U18666A and examination of primary cells from patients with NPC2 deficiency and Sandhoff disease revealed increased Beclin-1 levels, consistent with the notion that expression of this autophagy regulator is elevated in cells with defective cholesterol or sphingolipid transport. In contrast, Gaucher disease fibroblasts both traffic sphingolipids normally (30-32) and express wild type levels of Beclin-1 and LC3-II. Although Beclin-1 levels were elevated in Sandhoff disease fibroblasts, basal LC3-II levels were not increased (data not shown) and expression beyond wild type levels was detected only after serum starvation. We conclude that Beclin-1 up-regulation is necessary but not sufficient to increase basal autophagy. The enhanced response of Sandhoff disease fibroblasts to serum starvation suggests that elevated Beclin-1 levels prime cells for autophagy induction in response to additional signals.

Our observations indicate that the Beclin-1/Class III PI3K complex is a critical regulator of autophagy in several sphingolipid storage diseases. Activation of this pathway may promote cell survival by generating building blocks that are otherwise limiting, as is well characterized during periods of amino acid deprivation (11), or by facilitating the removal of damaged organelles or toxic proteins. We favor a model in which enhanced basal autophagy in NPC1

deficient cells acts in this manner to promote cell survival. This notion is consistent with recently characterized *Atg5* and *Atg7* null mice in which suppression of autophagy leads to neurodegeneration (37-39). Autophagy has also been implicated in non-apoptotic cell death, and its robust activation in starved or rapamycin treated cells may lead to cellular demise. This type of cytotoxicity has been demonstrated in *bax/bak* deficient cells that undergo caspase-independent, autophagic cell death following etopiside treatment (40) . Our data suggest that modulating activity of the Beclin-1/Class III PI3K complex and altering rates of autophagy may be a promising therapeutic approach for several sphingolipid storage diseases.

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Chapter 3

Tau deletion exacerbates the phenotype of Niemann-Pick type C disease mice and implicates autophagy in disease pathogenesis

3.1 Abstract

Hyperphosphorylation and aggregation of the microtubule binding protein tau characterizes a diverse array of neurodegenerative disorders. Most of these lack mutations in the encoding *MAPT* gene, and the role of tau pathology in pathogenesis remains controversial. Among these disorders is Niemann-Pick type C disease (NPC), a lysosomal storage disease characterized by progressive neurodegeneration and premature death, most often caused by an inherited deficiency in the intracellular lipid trafficking protein NPC1. Neurofibrillary tangles appear in the brains of NPC patients and hyperphosphorylated tau accumulates in *Npc1 -/-* mouse brains. To determine the extent to which tau pathology affects NPC pathogenesis, we generated *Npc1 -/-* mice deficient in tau. Here we show that NPC1/tau double null mutants are generated in markedly smaller litters and die significantly earlier than single null mutants, demonstrating a genetic interaction between *Mapt* and *Npc1* that affects fertility and lifespan. Surviving double null mutants exhibit an enhanced systemic phenotype including mild facial dysmorphia, kyphosis, penile prolapse and an abnormal, toe-walking gait. Immunohistochemical analysis of livers from 10-week old double null mutants

reveals an accumulation of LC3-positive autophagic vacuoles. Autophagy, the conserved pathway of bulk protein degradation, is dependent upon movement along microtubules and is up-regulated in NPC. We show that siRNA knockdown of *MAPT* in NPC1-deficient fibroblasts reduced LC3-II levels and decreased long-lived protein degradation, a measure of flux through the autophagic pathway. Our data establish that tau modifies the severity of the NPC phenotype through a loss-of-function mechanism that is independent of tau protein aggregation, and suggest that impaired autophagy contributes this effect.

3.2 Introduction

Niemann-Pick type C disease (NPC) is an autosomal recessive sphingolipid storage disorder characterized by severe, progressive neurodegeneration, hepatosplenomegaly and premature death (1). Most cases are caused by loss-of-function mutations in *NPC1* (2), which encodes a multipass transmembrane protein that functions in late endosomes and lysosomes to facilitate intracellular lipid trafficking, possibly by acting as a cholesterol efflux pump (3-6). In the absence of functional NPC1, glycosphingolipids, complex gangliosides and unesterified cholesterol accumulate within cells, demonstrating a critical role for this protein in lipid sorting.

Children with NPC are among the youngest patients to develop neurofibrillary pathology, similar to that which occurs in Alzheimer disease and the frontotemporal dementias (7, 8). In these disorders, the brain accumulates hyperphosphorylated species of the microtubule-associated protein tau (9). The

tau protein is encoded by six alternatively spliced transcripts derived from the *MAPT* gene and directly binds microtubules through three or four C-terminal tubulin-binding domains (10, 11). Experimental evidence suggests that a primary function of tau is to stabilize polymerized microtubules (12). Hyperphosphorylation dissociates tau from microtubules and allows for its self-aggregation into paired helical filaments (PHFs). These PHFs have the ability to form larger aggregates termed neurofibrillary tangles, a pathologic hallmark of many tauopathies, and these or other, soluble tau species can act through a toxic gain-of-function mechanism to impair neuronal survival (9). The diminished association of hyperphosphorylated tau with microtubules also leads to a loss-of-function that is presumably detrimental to neuronal health due to its impact on axonal transport processes (13), however the significance of this mechanism in disease has been uncertain. Emerging data additionally suggest novel roles for tau in the regulation of anterograde and retrograde trafficking that are separate from microtubule stabilization (14, 15). Remarkably, mutant mice which lack endogenous tau (*Mapt -/-* mice) exhibit no overt phenotype (13), likely due to functional redundancy with other microtubule associated proteins. Clearly, our current understanding of the normal function of tau and its role in neurodegenerative diseases is incomplete.

In *Npc1 -/-* mice, tau is hyperphosphorylated at multiple sites in a similar manner to human tauopathies, although neurofibrillary tangles do not form (16, 17). Cyclin-dependent kinase inhibitors reduce tau phosphorylation and attenuate the phenotype of *Npc1 -/-* mice (18), suggesting a role for tau in

disease pathogenesis. However, it remains unknown whether this therapeutic effect is mediated by increased availability of functional tau protein, through decreased formation of toxic tau species, or through actions on a different substrate. To define the role of tau in NPC pathogenesis, we generated NPC1 null mice that lack endogenous tau. Here we show that tau deletion exacerbates the phenotype of NPC mice, resulting in smaller litter size, an enhanced systemic phenotype and early death. Furthermore, we demonstrate that tau knockdown in a cellular model of NPC diminishes macroautophagy (hereafter referred to as autophagy), a highly conserved pathway by which cytoplasmic proteins and damaged organelles are sequestered within autophagic vacuoles and targeted to lysosomes for degradation (19). Prior work has implicated this pathway in the pathogenesis of several neurodegenerative disorders (20-23), including NPC (24-26). Functional autophagy is dependent upon trafficking along microtubules (27, 28), and its impairment is sufficient to cause neurodegeneration in mice (29, 30). Our data establish that tau deletion exacerbates the NPC phenotype in mice, and suggest impaired autophagy contributes to this effect.

3.3 Materials and Methods

3.3.1 Materials

Human fibroblasts from age- and sex-matched donors were from Coriell Cell Repositories (control cells, GM00038C and NPC1-deficient, GM03123A). All mouse samples were from age- and sex-matched BALB/cNctr-*Npc1*^{m1N}/J (Jackson Laboratories stock number 003092) and wild type littermates that were

backcrossed onto C57BL/6J for five generations. Anti-LC3 antibody was from Novus Biologicals. GAPDH antibody was from Abcam. Hsp90 antibody was from Santa Cruz. Anti-hsp40 and an antibody specific for the stress inducible form of hsp70 (SPA-812) were from Stressgen. Anti-20S proteasome was from Calbiochem. The p62 (C-terminal) antibody was from American Research Products. HRP conjugated secondary antibodies were from Biorad (goat anti-rabbit and goat anti-mouse) and Zymed (rabbit anti-guinea pig). MG132 was from Sigma.

3.3.2 Cell culture

Fibroblasts were maintained at 37°C, 5% CO_2 in MEM with Earle's salts and non-essential amino acids (Gibco), supplemented with 15% FBS (Atlanta Biologicals) and 10 µg/ml penicillin, 10 µg/ml streptomycin and 2 mM glutamine (referred to as complete MEM medium).

3.3.3 siRNA knockdown of *MAPT*

Fibroblasts growing in complete MEM media were collected using the Reagent Pack subculture kit (BioWhitaker), resuspended in Nucleofector solution (human dermal fibroblast Nucleofector kit, Amaxa) and mixed with 1.5 µg ON-TARGETplus SMART pool human MAPT (NM_016841) siRNA (L-012488-00-0005) or siCONTROLplus-plus non targeting pool (D-001810-10-05) (Dharmacon). The suspension was electroporated using the program U-23 on the Amaxa Nucleofector. Cells were plated in 6-well dishes and total RNA was collected 72 h later to measure *MAPT* mRNA expression level by TaqMan.

Alternatively, cell lysates were collected 72 h after electroporation for western blotting.

3.3.4 Gene expression analysis

Total RNA isolated from cells with Trizol (Invitrogen) served as a template for cDNA synthesis using the High Capacity cDNA Archive Kit from Applied Biosystems. Gene-specific primers and probes labeled with a fluorescent reporter dye and quencher were purchased from Applied Biosystems. TaqMan assays were performed using 5 ng aliquots of cDNA. Replicate tubes were analyzed for the expression of 18S ribosomal RNA (rRNA) using a VIC-labeled probe. Threshold cycle (Ct) values were determined by an ABI Prism 7900HT Sequence Detection System and relative expression levels were calculated using the standard curve method of analysis.

3.3.5 Measurement of long-lived protein degradation

Degradation of long-lived proteins was determined as previously described (25) with minor modifications. Cells were pooled after electroporation and then seeded in 6-well plates in complete MEM for 4 h. The cells were then washed with HBSS and labeled with 2 µCi/ml ^{3}H-leucine (Amersham) in complete MEM. After 48 h of labeling, cells were washed with HBSS and incubated in complete MEM supplemented with 2.8 mM leucine. Aliquots of medium were collected at the indicated times and added to 20% TCA, and then BSA was added to a final concentration of 3 mg/ml. All samples were stored at 4°C for at least 1 hour, and then centrifuged at 10,000*g* for 5 min at 4°C. Supernatants were collected, the

pellets were washed twice with cold 20% TCA, then all supernatants for each sample were pooled and the total radioactivity was measured by scintillation counting. Pellets were dissolved in cell lysis buffer (0.1 N NaOH/0.1% Na deoxycolate) and radioactivity was measured by scintillation counting. Cells were washed with PBS thrice and incubated for 1 h at 37°C in cell lysis buffer. An aliquot of the solubilized cells was used to determine total protein concentration (BioRad) and another aliquot was used to count the total radioactivity in the cells by scintillation counting. Relative proteolysis was determined by calculating TCA soluble radioactivity in the medium relative to total radioactivity present in the well. Relative proteolysis was normalized to protein concentration from the solubilized cells.

3.3.6 Western blot analysis

Cells were harvested, washed with PBS, lysed in RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics) and 0.1% β-mercaptoethanol, and sonicated. Liver, cerebellar and telencephalon samples were homogenized with the same buffer using a motor homogenizer. Lysates were precleared by centrifugation at 15,000*g* for 10 min at 4°C. For analysis of p62 levels in the pellet, protein concentrations were determined for crude lysates and sample concentrations normalized. Samples were centrifuged at 15, 000*g* for 10 min at 4°C and pellets were resuspended in 1x loading buffer. All samples were electrophoresed through either 10% SDS-polyacrylamide gels or 4-20% Tris-glycine gradient gels (Cambrex) and then transferred to nitrocellulose membranes (BioRad) using a semidry transfer apparatus. Immunoreactive

proteins were detected by chemiluminescence (PerkinElmer). Western blots depicted show representative results from one of three experiments unless otherwise stated.

3.3.7 Footprint analysis

Animals were trained to walk across an 83 cm long platform (7 cm wide) until the mice were able to consistently walk down the center of the path. Forepaws and hindpaws were painted maize and blue, respectively, with nontoxic paint and animals walked over white paper. Images represent at least two mice per genotype.

3.3.8 Functional proteasome assay

Proteasome activity in mouse liver lysates was determined using the 20S Proteasome Assay Kit, SDS-Activation Format (K-900) from Boston Biochem. Previously frozen liver samples were homogenized in sample buffer (2.5 mM Tris-HCl, pH 7.5/0.1 mM EDTA, pH 8.0/0.1 mM NaN_3) and supernatants were collected after spinning at 15,000*g* for 30 minutes at 4°C. 100 µg of soluble protein was added to a 96 well plate and brought to a final volume of 60 µl with sample buffer. SDS-activation buffer was added to all samples and allowed to equilibrate for 5 minutes at room temperature. Substrate solution (Suc-LLVY-AMC) was then added and fluorescent AMC counts released every 5 minutes for 1 hour at 37°C (excitation wavelength 390, emission filter 510) were measured by an Ascent Fluroskan microplate reader. Each sample had a companion well with the same level of protein, pretreated with 1 mM MG132 (a potent

proteasome inhibitor) for 10 minutes at room temperature to block the proteasome and control for non-specific degradation of the substrate. Data represent the rate of AMC release per minute in samples subtracted from companion MG132-treated wells.

3.4 Results

3.4.1 NPC1/tau double null mutant mice demonstrate an exacerbated phenotype

To determine the extent to which tau loss-of-function affects the NPC phenotype *in vivo*, we generated NPC1/tau double null mutant mice (*Npc1* -/-; *Mapt* -/-). The first indication of a genetic interaction between *Mapt* and *Npc1* came from breedings to generate these mice, when we noted the occurrence of significantly smaller litters when both parental genotypes were *Npc1 +/-; Mapt -/-*. While *Npc1* heterozygotes deficient in tau generated litters that averaged ~4 pups, the presence of one or two functional tau alleles in both parents resulted in significantly larger litters (Fig. 1A). This affect was not solely attributable to *Mapt* deficiency, as *Npc1 +/+*; *Mapt -/-* mice generated litters that averaged ~8 pups, similar to wild type (Fig 1C). We considered the possibility that smaller litters generated by *Npc1 +/-; Mapt -/-* mice reflected a selective loss of double null mutant pups. However, analysis of the *Npc1* null allele frequency distribution in pups from *Npc1 +/-*; *Mapt -/-* breedings showed that there was no significant decrease in the number of *Npc1 -/-* pups generated when compared with litters generated from mice with one or two functional tau alleles (Fig. 1B). These

results indicate that small litter size was not due to the specific loss of NPC1/tau double null mutants, but rather suggest that it was attributable to other affects on fertility or fetal survival.

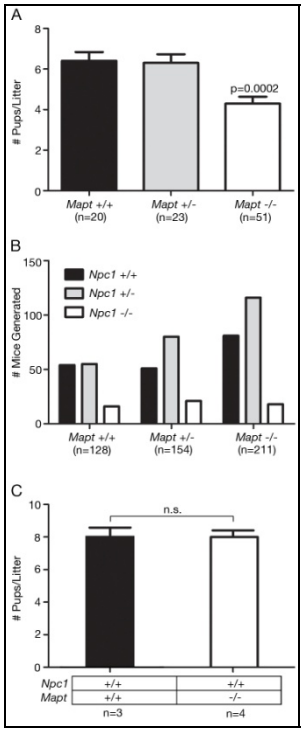


Figure 3.1 *Mapt* deletion decreases litter size of NPC1-deficient mice.

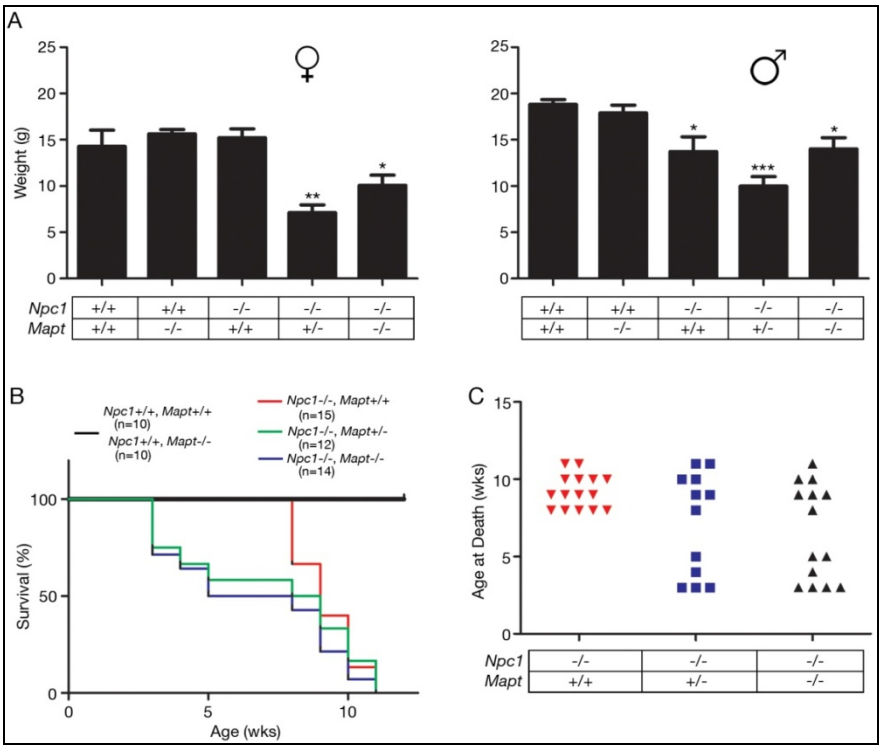
(A) Average litter size (mean +/- SEM) generated by *Npc1 +/-* parents, stratified by parental *Mapt* genotype. *Npc1 +/-*; *Mapt -/-* crosses generated fewer pups on average (p=0.0002 by ANOVA with Newman-Keul's Multiple Comparison Test) than parents with at least one functional *Mapt* allele. N = number of litters. (B) *Npc1* null allele distribution among generated pups, stratified by parental *Mapt* genotype. No significant deficit of *Npc1 -/-; Mapt -/-* mice was observed when data were analyzed by Chi-squared analysis (p=0.1924). N = number of mice. (C) Litter size (mean +/- SEM) of wild type (black bar) and *Mapt -/-* (white bar) mice. N = number of litters; n. s. = not significant (p>0.05) by unpaired Student's *t* test.

The double null mutant mice that were successfully generated displayed a markedly more severe phenotype than NPC1 single null mutants. Young female NPC1 null mutants that were haploinsufficient or deficient in tau weighed significantly less than NPC1 single null mutants (Fig 3.2, A, left panel). Additionally, both male and female double null mutants died earlier than NPC1 single null mutants, which succumb to disease between 8 - 12 weeks in our colony. In contrast, approximately half of the NPC1/tau double null animals died by 5 weeks (Fig. 3.2, B and C). Mice haploinsufficient for *Mapt* (*Npc1 -/-*; *Mapt +/-*) also demonstrated this robust shift in the survival curve, establishing that even partial loss of tau expression was detrimental to the survival of NPC mice. Interestingly, this exacerbation of the weight and death phenotypes associated with NPC1 deficiency occurred in the complete absence of tau, excluding tau hyperphosphorylation or PHF formation as a disease mechanism.

While the severity of the phenotype exhibited by double null mutants varied, many of the animals (approximately half) exhibited mild facial dysmorphia (shortened snout), kyphosis (Fig. 3.3, A) and an abnormal tip toe walking gait (Fig. 3.3, B and C), which has never been documented in NPC1 single null mutants. Footprint analysis clearly showed the tip toe walking phenotype due to the inability of double null mutants to press their paw pads down on the paper (Fig. 3.3, C). Additionally, almost all of the double null mutant males that survived to at least 5 weeks developed penile prolapsed (Fig. 3.3, D). This constellation of features establishes that tau deletion exacerbates the systemic phenotype of NPC1-deficient mice.

Figure 3.2 Decreased weight and early death of NPC1/tau double null mutant mice.

(A) Weight (mean +/- SEM) of 5 week old female (left panel) and male (right panel) mice. A significant difference between single (Npc1 -/-; Mapt +/+) and double (Npc1 -/-; Mapt -/-) null mutant mice was observed only in females, while all Npc1 -/- males demonstrated decreased weights when compared to controls. *, ** and * signify p-values of <0.05, <0.01 and <0.001, respectively, by ANOVA with Newman-Keul's Multiple Comparison Test. (B) Survival curve. Npc1 -/-; Mapt +/- (green line) and Npc1 -/-; Mapt -/- (blue line) are significantly different (p<0.0001 by Log-rank test) than WT, Mapt null (black line) and Npc1 -/-; Mapt +/+ (red line) mice. (C) Age of death for Npc1 -/-; Mapt +/+ (red triangles), Npc1 -/-; Mapt +/- (blue squares) and *Npc1 -/-*; *Mapt -/-* (black triangles) mice.**



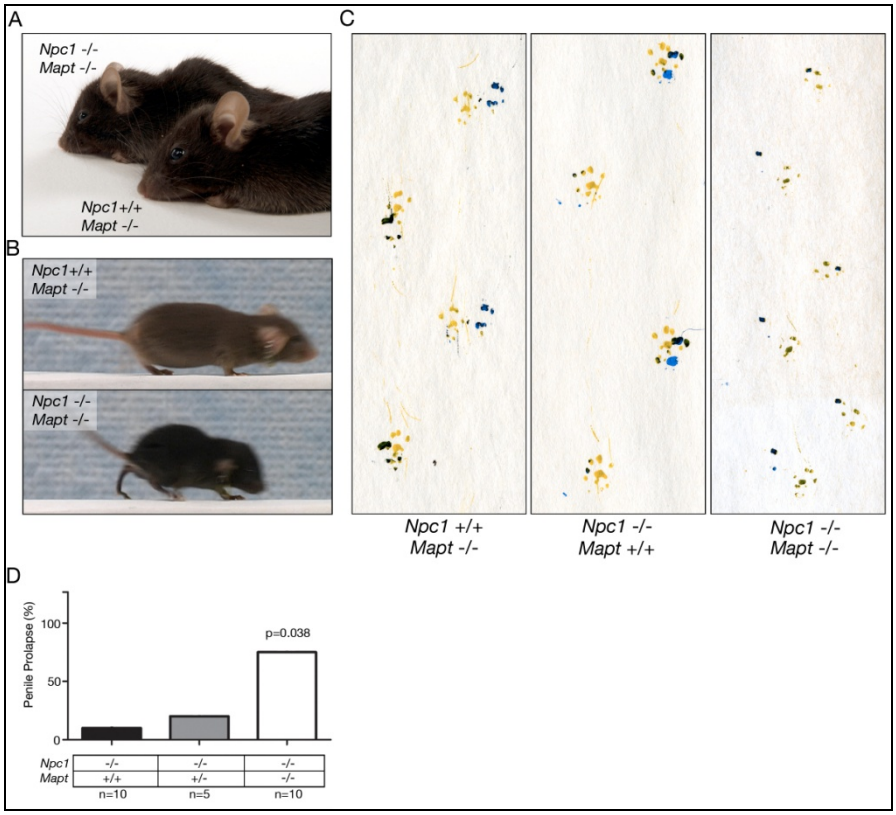
3.4.2 Tau knock-down impairs autophagy in NPC1 deficient cells

We next sought to establish the mechanism by which tau deletion exacerbates the NPC phenotype. Since tau is a microtubule binding protein implicated in the regulation of intracellular trafficking, we hypothesized that impairments of critical microtubule-dependent processes might underlie the worsened phenotype of double null mutants. Our prior work established that NPC1 deficiency increases basal autophagy (25), and others have shown that transport along microtubules is required for autophagic protein degradation (26, 27). We therefore examined whether tau deletion affects autophagy in NPC1 deficient mice and cells.

To monitor the autophagic pathway, we used the microtubule-associated protein 1 light chain 3 (LC3) (30, 31). This protein is modified from its LC3-I cytosolic form to a more rapidly migrating, lipidated LC3-II form associated with autophagosome formation and the induction of autophagy. Immunohistochemical analysis of liver from 10-week old mice demonstrated an increased accumulation of LC3-positive vacuoles in NPC1/tau double null mutants compared to NPC1 single null mutants (Fig.3.4, A). A similar accumulation of LC3-positive vacuoles has been associated with impaired autophagic flux in several *in vitro* model systems (32, 33) and in some lysosomal storage disorders (34, 35)

Figure 3.3 Systemic phenotypes associated with NPC1/tau double null mutant mice

(A) Double null mutant mice (*Npc1 -/-*, *Mapt -/-*) demonstrate shortened snout and kyphosis when compared to control mice (*Npc1 +/+*, *Mapt -/-*). (B) Still images of video reveal abnormal gait of double null mutant mice (*Npc1 -/-*, *Mapt -/-*, bottom panel). (C) Footprint analysis demonstrates tip toe walking gait of 4 week old double null mutant mice (*Npc1 -/-*, *Mapt -/-*) when compared to single null (*Npc1 -/-*, *Mapt +/+*) and control mice (*Npc1 +/+*, *Mapt -/-*) of the same age and sex. (D) Nearly all double null mutant males that survive past 5 weeks of age demonstrate penile prolapse, while NPC1 single null males and WT males rarely exhibit this phenotype (p=0.038 by ANOVA)

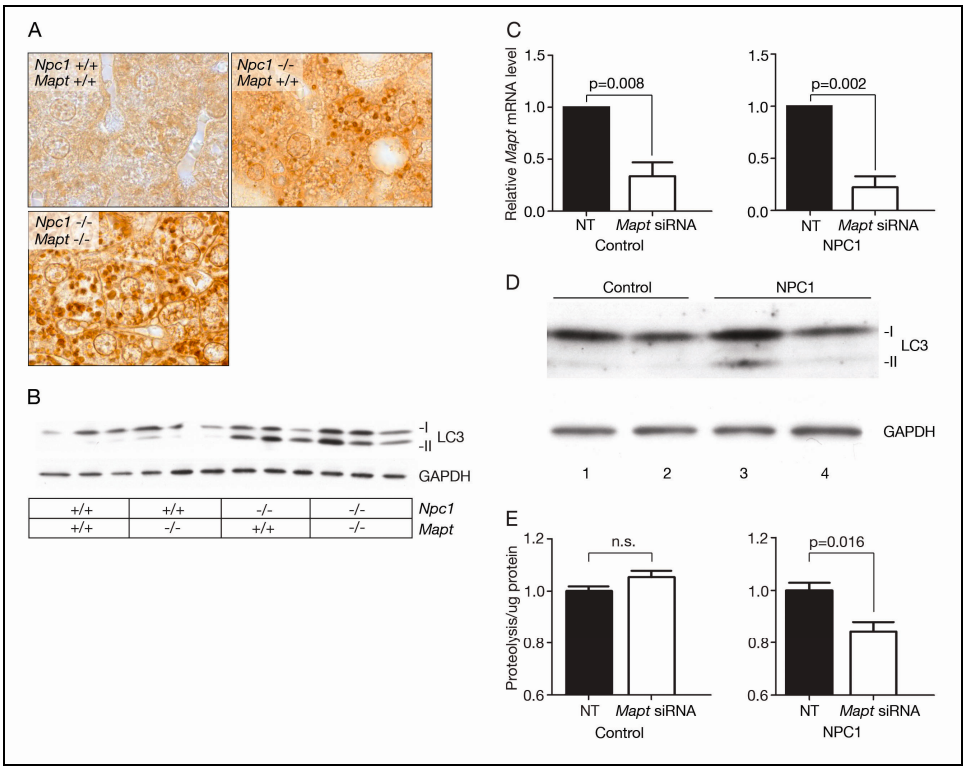


These results prompted us to examine levels of LC3-II and p62 in tissue lysates from NPC1/tau double null mutants. LC3-II is a robust marker of autophagy induction and p62 is a marker of autophagic flux. p62 links ubiquitinated proteins to LC3, and is itself incorporated into autophagosomes and degraded upon fusion with lysosomes (36). Therefore, impaired flux through the

autophagic pathway can lead to the accumulation of insoluble p62. Western blot analyses demonstrated increased soluble LC3-II in liver (Fig. 3.4, B) and brain (Fig. 3.5, A – C), and increased SDS-insoluble p62 in brain (Fig. 5A – C) of NPC1 deficient mice. These findings demonstrate that NPC1 deficiency *in vivo* leads to the robust induction of autophagy coupled with impaired autophagic flux, a situation that may lead to autophagic stress and predispose to autophagic cell death (37). Alterations in LC3-II and p62 expression were also observed in tissue lysates from NPC1 single null mutants as young as 4 weeks (data not shown), which is prior to phenotype onset, and similarly occurred in NPC1/tau double null mutants.

Figure 3.4 Reduction of tau impairs autophagy in NPC1 deficiency.

(A) Immunohistochemical staining for LC3 demonstrates frequent LC3-positive vacuoles in liver of 10 week old double null mutant (*Npc1 -/-*; *Mapt -/-*) when compared to single null mutant (*Npc1 -/-*; *Mapt* +/+) or wild type mice. (Original magnification, 1000 x.) (B) Liver lysates from 10 week old mice were probed by western blot for expression of LC3. Both double null (*Npc1 -/-*, *Mapt -/-*) and single null mutant mice (*Npc1 -/-*, *Mapt* +/+) demonstrate increased levels of LC3-II compared to controls. (C) Relative *MAPT* mRNA levels (mean +/- SEM) 72 hours post treatment with non-targeted (black bars) or *MAPT* siRNAs (white bars), as determined by TaqMan assay. Targeted siRNAs significantly reduced endogenous *MAPT* mRNA expression in control (p=0.008 by Student's unpaired *t*-test) and NPC1-deficient (p=0.002) cells. (D) Human fibroblast lysates from control (lanes 1 and 2) and NPC1-deficient (lanes 3 and 4) cells were analyzed by western blot for expression of LC3 (top) and GAPDH (bottom) 72 hours post treatment with non-targeted (lanes 1 and 3) or *MAPT* siRNAs (lanes 2 and 4). (E) Degradation of long-lived proteins in control (left panel) and NPC1-deficient human fibroblasts (right panel) following transfection with non-targeted (black bars) or *MAPT* siRNAs (white bars). Data (mean +/- SEM) are reported relative to non-targeted siRNA-transfected cells at 72 hours. Relative proteolysis is significantly decreased in NPC1-deficient cells (p=0.016 by unpaired Student's *t*-test), but not in controls (p>0.05).



While *in vivo* deletion of tau in NPC mice led to subtle changes in autophagic markers manifest by the accumulation of LC3-positive vacuoles in the liver, the acute down-regulation to tau expression *in vitro* markedly reduced both autophagic induction and flux in NPC1 deficient cells. To reduce *MAPT* expression, we used pooled, targeted siRNAs to specifically knockdown *MAPT* in control (Fig. 3.4, C, left panel) and NPC1-deficient primary human fibroblasts (Fig. 4C, right panel). These cells were selected for analysis since prior studies have established that they express all human tau isoforms (38). Treatment with *MAPT* siRNAs significantly decreased endogenous *MAPT* mRNA levels. Reduction of tau in NPC1-deficient but not control cells reduced the amount of LC3-II (Fig. 3.4, D), signifying an overall reduction in the amount of autophagosomes present. These results demonstrate that an acute reduction of *MAPT* expression is sufficient to decrease levels of basal autophagy in NPC1-deficient cells.

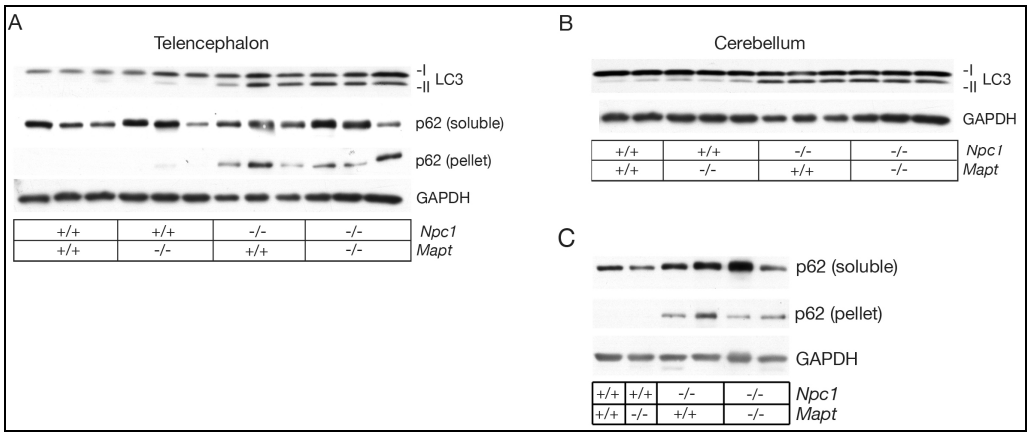


Figure 3.5 Increased LC3-II and p62 in brains of NPC1-deficient mice.

(A-C) Lysates from the telencephalon (A) and cerebellum (B, C) of NPC1 single null and NPC1/tau double null mutant mice demonstrate increased levels of LC3-II and pelleted p62 compared with controls.

To independently confirm these results, we sought to determine if *MAPT* knock-down decreased flux through the autophagic pathway by measuring the degradation of long-lived proteins (39). This assay provides a quantitative measure of flux through autophagy and provides a functional readout of this pathway (40, 41). Control and NPC1-deficient cells were treated with *MAPT* or non-targeted siRNAs, and then labeled for 48 hours with ^{3}H-leucine. The cells were washed, re-fed and trichloroacetic acid soluble radioactive counts released into the media were measured 24 hours after labeling. NPC1-deficient fibroblasts treated with *MAPT* siRNAs demonstrated significantly lower levels of long-lived protein degradation than cells treated with non-targeted siRNAs (Fig. 3.4, E, right panel). In contrast, long-lived protein degradation was unchanged in similarly treated control cells (Fig. 3.4, E, left panel). From these results, we

conclude that an acute reduction of *MAPT* expression in NPC1-deficient cells is sufficient to inhibit the autophagic pathway.

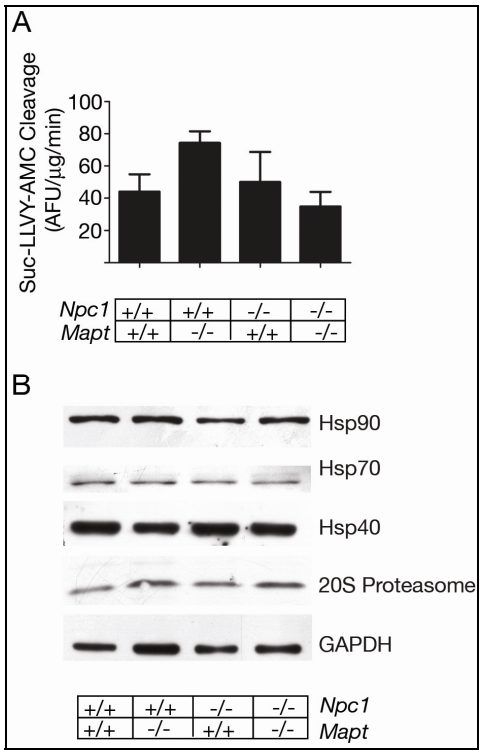


Figure 3.6 The ubiquitin-proteasome pathway remains intact in NPC1 deficient animals.

(A) Activity of 20S proteasome assayed in liver lysates of 10 week old mice (mean +/- SEM). p>0.05 by ANOVA. (B) Western blot analyses of liver lysates from 10 week old mice demonstrate equal levels of expression for hsp40, stress-inducible hsp70, hsp90 and 20S proteasome subunits.

Trafficking along microtubules is needed for efficient protein quality control, processes that in addition to autophagy depend on protein degradation by the ubiquitin-proteasome pathway (42). To determine whether tau deletion also affected this pathway, we evaluated proteasome function in NPC1 single null and NPC1/tau double null mutants. Liver lysates were isolated and used as

a source of active 20S proteasomes. Cleavage of Suc-LLVY-AMC, a substrate for the chymotrypsin-like activity of the proteasome, was unchanged by *Npc1* and *Mapt* genotype (Fig. 3.6, A). Similarly, expression of 20S proteasome subuints was unchanged in single and double null mutant mice compared with wild type controls (Fig. 3.6, B). Additionally, no induction of heat shock proteins indicative of a stress response was detected in single or double null mutants (Fig 3.6, B). These data indicate that the function of the ubiquitin-proteasome pathway is intact in NPC1/tau double null mutant mice, and indicate that tau deletion primarily impairs protein degradation through autophagy in NPC1 deficiency.

3.5 Discussion

The tauopathies are a large and diverse collection of neurodegenerative disorders that have in common the accumulation of hyperphosphorylated and aggregated tau species (45). This group of diseases includes forms of frontotemporal lobar dementia caused by *MAPT* mutations, establishing a role for tau pathology in human disease (46, 47). However, most tauopathies, including NPC, lack these mutations, and the contribution of tau to the pathogenesis of these disorders has remained poorly defined. Using NPC as an example of this latter group of disorders, here we show that tau deletion exacerbates the disease phenotype *in vivo*. Through the generation of NPC1/tau double null mutants, we establish that tau deficiency decreases litter size and survival, and worsens the systemic phenotype of NPC1 null mutants. Our findings indicate that diminishing functional tau has dire consequences in the setting of certain neurodegenerative disorders. The effects of *Mapt* deletion on the phenotype of NPC mice are

distinct from toxicity mediated by tau protein aggregation, a mechanism that has attracted widespread attention. In contrast to the results reported here, a recent study demonstrated that reduction of endogenous tau partially ameliorates the phenotype of Alzheimer disease mice (48). This suggests that both tau loss-of-function and toxic gain-of-function mechanisms modulate disease progression in tauopathies, implying the existence of two distinct classes within this large and diverse group of neurodegenerative disorders. Furthermore, we suggest that alterations in tau mediated by hyperphosphorylation may similarly act through a loss-of-function mechanism to exacerbate the phenotype of NPC patients.

We observed that tau deletion causes an accumulation of autophagic vacuoles in the liver of NPC mice, and that knockdown of *MAPT* expression decreases autophagic induction and flux in NPC1-deficient fibroblasts. These results indicate that tau contributes to the regulation of autophagy in NPC1 deficiency, and that loss of functional tau impairs this pathway. Our findings build on published data showing that NPC1 deficiency robustly increases basal autophagy (24-26) and stimulates flux through the autophagic pathway in a Beclin-1 dependent manner (26). As diagrammed in Figure 7, we propose that this induction of basal autophagy acts as a pro-survival response to facilitate recycling of critically needed cellular components in NPC1 deficient cells. Tau deletion diminishes this response, perhaps through affects on microtubule based trafficking. The resulting exacerbation of systemic pathology suggests that tau-dependent processes normally buffer the severity of the NPC phenotype. Our studies have identified a critical role for tau in the regulation of autophagy in

NPC1 deficient cells, and suggest that other disorders where autophagy contributes to pathogenesis may be similarly sensitive to affects of tau deletion.

Our findings suggest the intriguing conclusion that tau loss-of-function may be more deleterious to the progression of NPC pathology than the accumulation of hyperphosphorylated tau species. This novel mechanism for modulating the severity of the disease phenotype indicate that new treatment approaches aimed at restoring normal tau function could ameliorate or delay NPC disease progression. Other tauopathies may be similarly affected by tau loss-of-function, suggesting that this approach could prove beneficial in multiple disorders characterized by tau abnormalities. Additionally, our data raise the possibility that allelic differences in tau expression levels may modify the NPC phenotype. Indeed, it is well established that patients with the same mutation in NPC1 can have divergent clinical presentations, suggesting the existence of genetic modifiers (49, 50). The work presented here identifies *MAPT* as a candidate modifier, a possibility that will be explored in future studies.

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Chapter 4

Generation of a Conditional Mouse Model of Niemann Pick Type C Disease

4.1 Abstract

Niemann-Pick C Disease is a recessive sphingolipid storage disorder characterized by global progressive neurodegeneration for which there is no cure or effective treatment. About 95% of cases are caused by loss of function mutations in the *Npc1* gene. It is currently unknown which cell types within the central nervous system are causally involved in disease pathogenesis and whether these effects occur at a specific time in development. To address these questions, we generated a conditional null mutant of the mouse *Npc1* gene. A 9.3 kb fragment of *Npc1* genomic sequence was isolated from a C57BL/6 BAC library, and used to generate a 3.0 kb 5' arm and a 5.2 kb 3' arm of a targeting construct. These arms were cloned into pFlox-Flp-Neo, a vector containing the neomycin resistance gene and the PGK promoter flanked by two *FRT* sites. *Npc1* exon 9 and flanking intronic sequence were amplified by high fidelity PCR, and then inserted between *loxP* sites to allow for Cre mediated excision. To

functionally test the *loxP* and *FRT* sites, we utilized arabinose inducible Cre and Flp recombinase *E. coli* strains. Restriction enzyme digestion after arabinose

induction confirmed appropriate Cre- and Flp-mediated recombination. The construct was then electroporated into Bruce4 embryonic stem (ES) cells, a line derived from C57BL/6J mice. Approximately 900 neomycin resistant clones were selected and screened by Southern blot for appropriate integration. Correctly targeted clones that contained 40 chromosomes were injected into albino C57BL/6J blastocysts to generate ES cell-mouse chimeras. Two male chimeras, which were generated from two independent ES cell clones, were bred with albino C57BL/6 females (B6-tyr^{c-2J}). These breedings yielded multiple mice which harbor *loxP* flanked exon 9 of the *Npc1* allele. Studies utilizing these mice are expected to provide insights into the progression and mechanism of this devastating neurodegenerative disorder.

4.2 Introduction

The progressive and disabling neurodegeneration that occurs in patients with Niemann-Pick C has been studied in several animal models. The best characterized and most commonly used are mice in which an insertional mutation into exon 9 of the *Npc1* gene causes a frame shift and truncation of the encoded protein (*npc1* -/- mice) (1). In these mice, as in patients with the disease (2-5), neurons accumulate lipids including glucosylceramide, lactosylceramide, complex gangliosides and cholesterol (6-8), abnormally swollen axons are frequent, and decreased myelination is present in the cerebral white matter (9). These histological changes are associated with behavioral deficits, including impaired gait and rotarod performance, and early death. This progressive neurological impairment is due to a loss of functional NPC1

expression in the nervous system. In support of this conclusion are studies in mice demonstrating that prion-promoter driven transgenic over-expression of NPC1 primarily in the brain of *npc1 -/-* mice prevents neurodegeneration in the face of moderate continued visceral pathology (1). However, it remains poorly understood how loss of functional NPC1 results in such marked disease.

NPC1 is widely expressed in the central nervous system by neurons, oligodendrocytes and astrocytes, all which accumulate lipids in this disorder (10, 11) and traffic cholesterol abnormally in *npc1 -/-* mice (12-14). Recent studies have demonstrated that NPC1 deficiency leads to cell autonomous neuronal death in chimeric mice that express functional protein in only some cells (15). While these data provide important information on the mechanism of toxicity, it remains uncertain whether dysfunction or death of cells other than neurons also contributes to the Niemann-Pick C disease phenotype. For example, oligodendrocytes, which normally form the lipid rich myelin sheath, do not appropriately myelinate axons in *npc1 -/-* mice (9) and may thereby contribute to neuronal pathology. This effect may be mediated in part by disordered trafficking of glycosphingolipids, components of the myelin sheath that are critical for its interaction with axons (16). Similarly, dysfunction of astrocytes, which enable normal brain development and repair through the production and transport of cholesterol (17, 18), has been implicated in disease pathogenesis. Astrocyte-derived cholesterol may impact neuronal function by promoting synaptogenesis (19) and by stimulating axon growth (20) . Additionally, inflammatory mediators released by activated microglia have been implicated in the pathogenesis of

Niemann-Pick C disease, and are known to participate in neurodegeneration in Sandhoff disease (21, 22), another lysosomal storage disease. These data indicate that loss of functional NPC1 in cells other than neurons may significantly contribute to disease pathogenesis, although proof of such involvement is currently lacking. For these reasons and more, the generation of conditional *Npc1* null mice will be an important resource for understanding the mechanisms underlying neurodegeneration seen in this devastating disorder.

4.3 Materials and Methods

4.3.1 Materials

We acquired a pFlip-Flox-Neo vector from the Transgenic Animal Model Core at the University of Michigan. The vector was a kind gift from Dr. James Shayman (23). The *Npc1* genomic BAC clone, RPCI23-37J18, was acquired from BACPAC Resources at Children's Hospital Oakland Research Institute (CHORI). The pcDNA3 construct was purchased from Invitrogen. We obtained the Bruce4 embryonic stem cell line from the Transgenic Animal Model Core at the University of Michigan. The line was provided to the core by Dr. Colin Stewart and has been previously characterized (25).

4.3.2 Deletion of FRT and loxP flanked sequence *in vivo*

The arabinose inducible FLP and Cre recombinase *E. coli* strains, E250 and EL350 respectively, were a kind gift of Dr. Elizabeth Engle and were used as described previously (24). Briefly, both lines were electroporated with the

targeting construct, and transformed clones were selected for on LB-Amp plates. Individual clones were grown to log phase in LB-Amp media. Diluted cultures were induced to express either FLP or Cre recombinase in LB-Amp with 0.1% arabinose for 2 hours. The cells were plated and plasmids from individual clones were analyzed for the deletion of *FRT* or *loxP* flanked sequence by restriction enzyme digestion.

4.3.3 Identification of embryonic stem cells with the targeted *Npc1* allele

Genomic DNA from ES cell clones was digested with EcoRV and analyzed by Southern blot. 5' and 3' probes were generated by PCR amplification of the *Npc1* genomic BAC clone. These probes contained sequence for exon 5 (5'-AGTTGTTATCTTTCCTGCTTTGAGTC-3', 5'-ACATGTCTGACTTTTCTCTCTGAATTT-3') and exon 18 (5'-TCTTTAATCTTCTTTTCAGGATTCGTA-3', 5'-GAACTAGTCTAACCCTAACACTCAGGA-3'), respectively, both of which fall outside the targeting vector.

4.4 Results

4.4.1 Generation of Npc1 targeting construct

We have targeted the *Npc1* gene in Bruce 4 embryonic stem (ES) cells derived from C57BL/6 mice. This strategy will enable us to more rapidly generate mice carrying the floxed allele on a uniform genetic background without needing to backcross to C57BL/6J mice for ten generations. A recent study co-

authored by the Transgenic Animal Model Core at the University of Michigan supports the use of this strategy, and suggests that C57BL/6-derived ES cells offer additional advantages including an increased frequency of germ line transmission (26).

We chose to flank exon 9 of the *Npc1* gene with *loxP* sites. Deletion of this exon is expected to result in a frame shift mutation and truncation of the encoded protein, similar to that caused by an insertional mutation into exon 9 present in the constitutive *Npc1* null mutant (1). A BAC containing the C57BL/6J *Npc1* genomic clone was identified by a database search and digested with BamH1 to obtain a 9.3 kb fragment of the *Npc1* gene that includes exon 9. This fragment was subcloned into pcDNA3, and then digested with Ssp1 and EcoRV to generate a 3.0 kb 5' arm, and with Asp718 to generate a 5.2 kb 3' arm of a targeting construct. These arms were cloned into pFlox-Flp-Neo, a vector that contains the neomycin resistance gene and the PGK promoter flanked by *FRT* sites. Included within the neomycin resistance gene is an EcoRV site that was used during screening for recombinants by Southern blot. *Npc1* exon 9 and flanking intronic sequence were amplified from C57BL/6J genomic DNA by high fidelity PCR. During amplification, a two base pair substitution of intronic sequence was made that introduced an EcoRV site 3' to exon 9. This site will be used during Southern blot analyses to identify deletion of exon 9 by Cre recombinase *in vivo*. Both strands of the entire PCR product were sequenced, and then the fragment was inserted between *loxP* sites to allow for Cre mediated excision. Sequencing across the cloning sites, and *loxP* and *FRT* sites

confirmed the accuracy of the completed targeting construct. Schematic representations of the targeting construct, its relationship to genomic sequence,

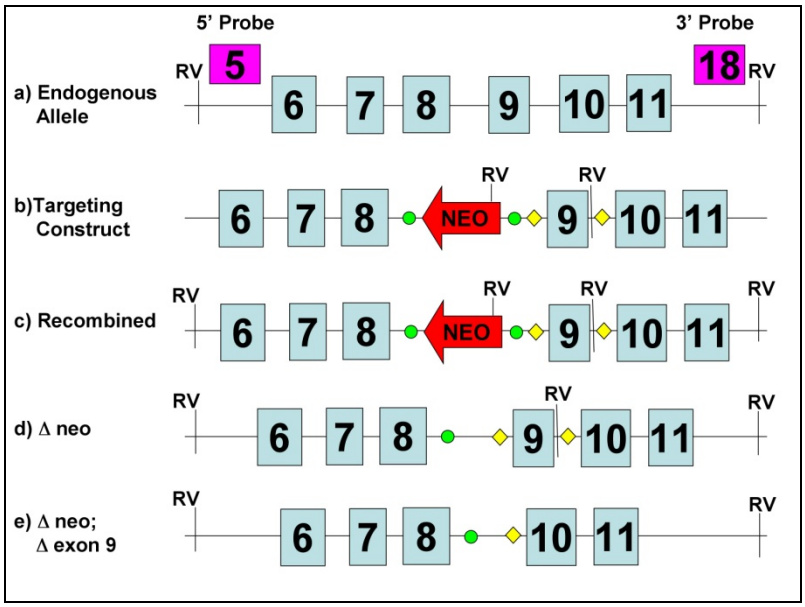

Figure 4.1 Strategy for generation of a conditional null mutation of the mouse *Npc1* gene

(a) Endogenous *Npc1* allele. Exons are represented by numbered boxes. Shown are 5' and 3' probes containing exons 5 and 18, respectively. (b) Schematic representation of targeting construct. *FRT* sites are shown as green circles and *loxP* sites as yellow diamonds. RV indicates EcoRV sites. (c) Predicted structure of allele after homologous recombination. (d) Representation of targeted allele following Flp– and (e) Cre–mediated excision events.

and the targeting construct, its relationship to genomic sequence, and the expected results of Cre and Flp mediated recombination are shown in Figure 4.1.

Upon completion of the targeting construct, we confirmed that the *loxP* and *FRT* sites flanking exon 9 and the neomycin resistance cassette were functional. The targeting construct was transformed into *E. coli* that express

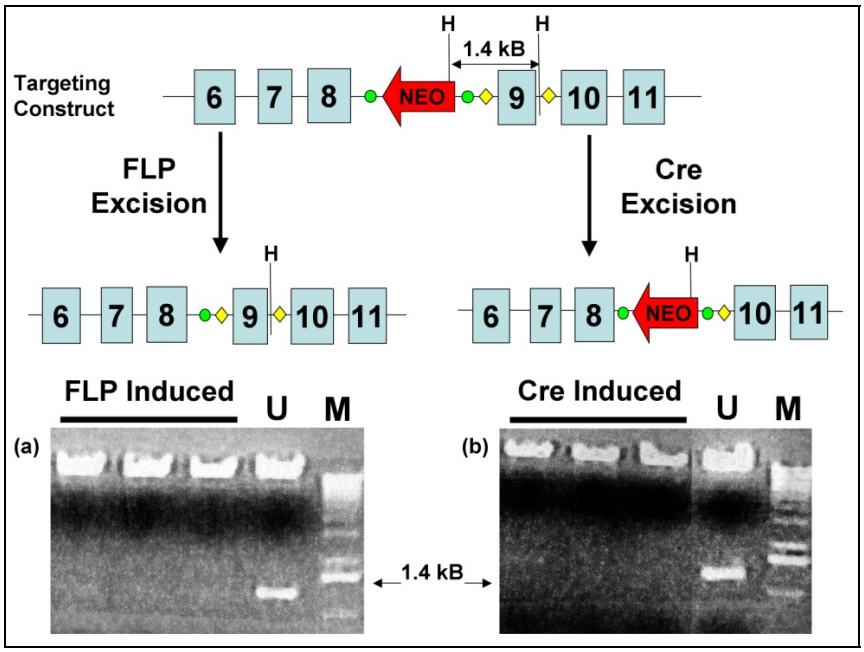

Figure 4.2. LoxP and FRT sites are functional

The completed targeting construct was transformed into E. coli that express arabinose inducible Flp (on left) or Cre (on right) recombinase. Following arabinose induction, individual colonies isolated from ampicillin-containing plates were used as a source of plasmid DNA for restriction enzyme digestion. Three individual clones from each induction were compared with plasmid isolated from uninduced bacteria (U). The non-recombined plasmid contained HindIII sites (H) between the loxP and FRT sites, and yielded a 1.4 kb fragment after digestion. Flp or Cre mediated recombination deleted one of these sites, resulting in the loss of the 1.4 kb band and the generation of a plasmid that was linearized by HindIII. M indicates 1 kb marker.

arabinose inducible Cre or Flp recombinase (24). *In vivo* recombination was confirmed in several independent bacterial colonies by digestion with HindIII (Fig. 4.2). These data and the sequence analysis established that we have generated the *Npc1* targeting construct diagrammed in Figure 4.1.

4.4.2 Embryonic stem cells with the targeted *Npc1* allele

The targeting construct was electroporated into Bruce4 embryonic stem (ES) cells, a line derived from C57BL/6 mice. Approximately 900 neomycin resistant clones were selected and screened by Southern blot for appropriate integration. Multiple correctly targeted clones were identified (Fig. 4.3), and three euploid clones were selected for further use. The identification of correctly targeted clones indicates that our vector is able to recombine with the mouse *Npc1* gene to generate a mutant allele.

4.4.3 Generation of ES cell–mouse chimeras

The Transgenic Animal Core at the University of Michigan injected these targeted clones into albino C57BL/6J blastocysts to generate ES cell-mouse chimeras. Two male chimeras generated from injection of independently targeted ES cell clones were mated with albino C57BL/6 females (B6-tyr$^{c\text{-}2J}$), and germ line transmission was monitored by the appearance of black fur, by PCR

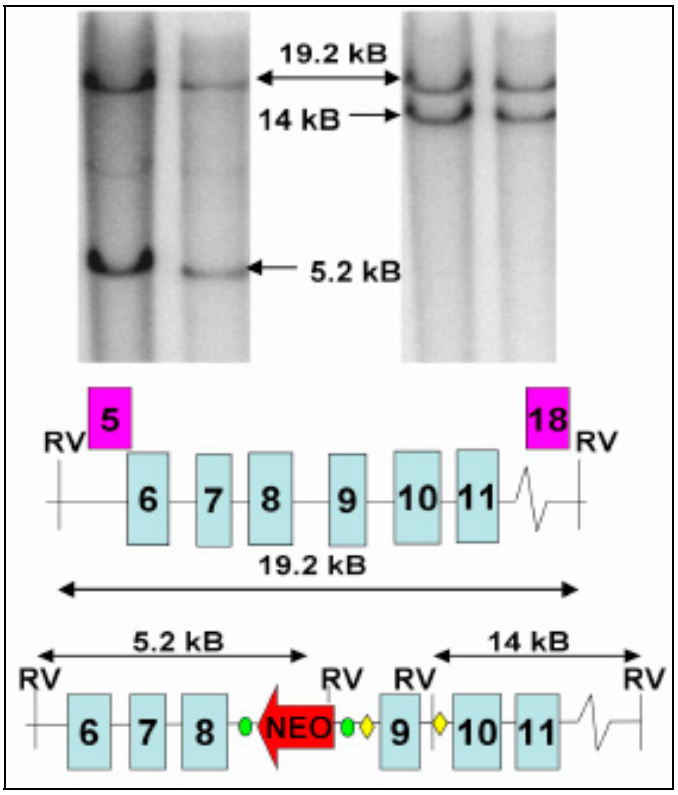

Figure 4.3 Southern blot confirms homologous recombination in ES cells

Genomic DNA isolated from ES cell clones was digested with EcoRV and analyzed by Southern blot using probes that fall outside the targeting construct. The 5' exon 5 *(on left)* and 3' exon 18 *(on right)* probes both generate a 19.2 kb band from the non-recombined allele. The recombined *Npc1* allele generates a 5.2 kb band with the 5' probe and a 14 kb band with the 3' probe. Shown is an example of a Southern blot from two recombined clones.

across the *loxP* site 3' to exon 9 and by Southern blot. Both male chimeras sired multiple offspring with black fur, approximately half of which carried the floxed *Npc1* allele (Figure 4.4). These data demonstrate that we have established two lines of mice, derived from independently targeted ES cell clones, which carry the targeted *Npc1* allele with exon 9 flanked by *loxP* sites.

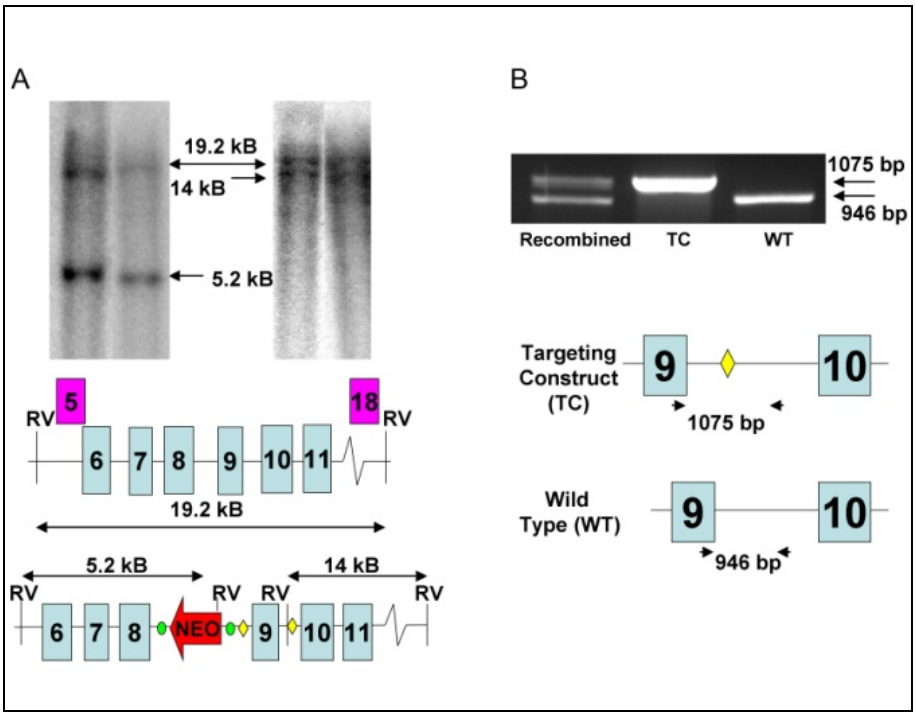

Figure 4.4. Germ line transmission of the targeted *Npc1* allele

(a) Genomic Southern blot of tail biopsy derived DNA of black pups from two independent mouse lines after digestion with EcoRV. 5' exon 5 *(on left)* and 3' exon 18 probes *(on right)* were used, as described in Figure 3. (b) PCR amplification across exon 9 and flanking intronic sequence that includes the 3' *loxP* site generates a 946 bp band from wild type (WT) alleles. Incorporation of a *loxP* site 3' to exon 9 generates a 10754bp product that can be distinguished on argarose gel. This larger product was detected when either the targeting construct (TC) or tail DNA from a mouse with the targeted allele (recombined) was used as template DNA. Arrows indicate primers used for PCR amplification.

4.5 Discussion

Our data establish that we have generated two independently derived lines of mice that carry the targeted *Npc1* allele in which exon 9 is flanked by *loxP* sites. We expect that this resource will significantly impact our understanding of disease mechanisms and approaches to therapy, particularly if

one cell type were shown to play a dominant role in the development of neuropathology. Such information could lead to therapeutic strategies targeted to a specific cell type. One example is the potential role of inflammatory mediators released by activated microglia, a target that may be responsive to existing anti-inflammatory drugs.

It is also unclear whether the neuropathology of Niemann-Pick C disease is predominantly developmental or degenerative in origin. Conditional *Npc1* null mice are an ideal system for resolving this question. Recent data suggest that loss of functional NPC1 exerts an effect in late development that significantly contributes to neuropathology. Evidence in support of this hypothesis comes from the observation that *npc1 -/-* mice treated with a single dose of allopregnanolone at postnatal day seven exhibit decreased storage of cholesterol and gangliosides in neurons, reduced Purkinje cell loss, and prolonged survival (27). This observation led to the notion that NPC1 deficient cells are simultaneously burdened by cholesterol and ganglioside storage in late endosomes, yet also exhibit functional evidence of cholesterol deficiency. This paucity of cholesterol in critical subcellular compartments causes an inability of the cell to generate cholesterol-derived mediators which influence neuronal survival and differentiation. Data also demonstrate that continued weekly treatment with allopregnanolone enhances this survival effect (28), indicating that cholesterol deficiency contributes to the Niemann-Pick C disease phenotype beyond the first postnatal week. To determine the extent to which NPC1 deficiency exerts its effects during development or in the mature nervous system,

floxed *Npc1* exon 9 mice could be crossed with animals that globally express tamoxifen-inducible Cre recombinase. Inactivation of the floxed *Npc1* allele at different ages may identify a critical window for disease development and therapeutic intervention. Furthermore, gene inactivation in the mature nervous system may lead to a slowly progressive disorder that is similar to the late-onset cases of Niemann-Pick C disease, for which there are no current animal models.

To resolve these critical and complicated issues in the study of Niemann-Pick C disease neuropathology, we have generated a conditional null mutant of the mouse *Npc1* gene. This mouse model will enable studies to define the role of individual cell types in disease pathogenesis and to determine the effects of NPC1 deficiency during development and in the mature nervous system. As such, this model will be an important resource to the neuroscience community and hopefully aid in the creation of an effective therapy for this devastating and untreatable disorder.

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Chapter 5

Conclusion

During the first part of my doctoral research, I elaborated on a previously reported relationship between loss of function mutations in the *Npc1* gene and the highly conserved pathway of autophagy. I showed that loss of NPC1 function increased activation of autophagy and increased the expression of Beclin 1, a genetic regulator of autophagy. My recent studies demonstrated a genetic interaction between *Mapt* and *Npc1*. Simultaneous deletion of both genes caused a disruption in the autophagic pathway *in vitro* and an exacerbation of the Niemann-Pick type C disease (NPC) phenotype *in vivo*. Finally, I generated an important resource – a conditional mouse model of NPC. In these animals, exon 9 of the *Npc1* allele is floxed, enabling the gene to be functionally deleted in a certain cells when animals are crossed with mice expressing Cre recombinase under the control of a cell type specific promoter.

5.1 NPC and autophagy

My initial studies further characterized the relationship between autophagy and NPC (1). We examined cerebellum and liver from *Npc1-/-* and wild type mice since these are two known sites of pathology in mutant animals (2). Western blots demonstrated elevated levels of lipidated LC3 (LC3-II) as occurs during induction of autophagy (3, 4). Similar analyses confirmed elevated levels of LC3-II in primary fibroblasts from patients with NPC as compared to age and sex matched controls. Increased levels of basal autophagy in NPC1 deficient fibroblasts were confirmed by enhanced staining with monodansylcadaverine, by demonstrating frequent autophagosomes and autolysosomes by transmission electron microscopy, and by measuring significantly increased degradation of long-lived proteins. That these differences were due to NPC1 deficiency was confirmed by stable expression of NPC1 protein in mutant fibroblasts, a manipulation that decreased basal LC3-II levels (1). These results confirmed earlier observations of increased autophagy in *Npc1 -/-* mice (5) and were the first demonstration of increased autophagy in human samples. By demonstrating the activation of this pathway in both humans and mice, we opened the door to additional studies that will define the role of autophagy in NPC pathogenesis and will establish the extent to which this pathway is a therapeutic target.

Autophagy is controlled by the Akt – mTOR – p70 S6K signaling pathway and by Beclin-1, a crucial regulator of autophagosome formation which forms a protein complex with the Class III PI3K (6, 7). To determine which of these was responsible for increased basal autophagy in NPC, we examined lysates from

NPC1 deficient fibroblasts and mice. Surprisingly, we found no evidence that enhanced basal autophagy was due to signaling through the mTOR pathway. In contrast, NPC1 deficient cells and mice both showed a small, but consistent increase in the expression of Beclin-1 protein (1). To determine the extent to which Beclin-1 contributed to increased basal autophagy, we used siRNA to decrease Beclin-1 expression. Knockdown of Beclin-1 in NPC1 deficient fibroblasts was sufficient to reduce the basal rate of long-lived protein degradation by ~20%, while knockdown of Beclin-1 in control fibroblasts had no significant effect. Taken together, these results indicated that enhanced levels of basal autophagy were mediated by increased Beclin-1 expression. Similar Beclin-1 dependent, mTOR independent activation of autophagy has been observed in models of another neurodegenerative disorder, Huntington disease (8). Subsequent to our report, it was demonstrated that Beclin-1 expression is decreased in Alzheimer disease brains (9), suggesting that the regulation of autophagy through a Beclin-1 dependent pathway is common to a diverse group of neurodegenerative disorders.

A critical, unanswered question is whether activation of this pathway in NPC is detrimental and promotes cell death, or is compensatory and promotes cell survival. To resolve this issue, autophagy will need to be modulated in NPC model systems and the effects on disease phenotype established. To determine if increasing autophagy is beneficial to the progression of NPC, our lab has begun studies to administer autophagy activating compounds like rapamycin to the existing mouse model of NPC. Intraperitoneal (IP) injection of rapamycin to

Npc1 deficient mice will enable us to determine the extent to which induction of autophagy *in vivo* will ameliorate or enhance the phenotype in these animals. If the induction of autophagy delays the onset of NPC, these studies could suggest a possible therapeutic option as rapamycin has already passed the initial stages of human clinical trials. Conversely, to determine whether autophagy is deleterious to NPC, inhibition of this pathway by genetic mutations or small molecules will prove useful. A search for compounds that specifically modulate the activity of the autophagic pathway in the CNS will likely yield important reagents for investigational studies of disease mechanisms, and may provide lead compounds for the development of new treatments.

There are a variety of different endosomal sorting pathways within cells, and there is a distinct possibility that many of these are perturbed in NPC. In addition to autophagy, another interesting candidate pathway has emerged as interacting with autophagy and the endosomal/lysosmal system (10, 11). Multivesicular bodies (MVBs) are critical for the maintenance of a variety of cell functions, such as receptor down regulation, viral budding and the generation of lysosome-related organelles (12). MVBs are compartments that enable the delivery of cytoplasmic and transmembrane domains of proteins to the lysosome for degradation, which is also the final stage of autophagy. The rationale of investigating these structures comes from recent studies identifying numerous MVBs in a fly model of NPC (13) and a paper describing how autophagic clearance of aggregated proteins is dependent upon functional MVBs (11). It is possible that dysfunction of MVBs and other endosomal sorting pathways that

intersect with autophagy contributes to the pathogenesis of NPC and related lysosomal storage disorders.

5.2 NPC and tau

Our laboratory has recently demonstrated a genetic interaction between the microtubule associated protein tau (encoded by the *MAPT* gene) and *NPC1*. In primary human fibroblasts from patients with NPC, siRNA knockdown of *MAPT* reduced the degradation of long-lived proteins, a functional assay of autophagic flux. In contrast, in control fibroblasts, *MAPT* knock-down caused no significant reduction in autophagic flux. *In vitro* down regulation of *MAPT* expression was also sufficient to reduce LC3-II levels in NPC1 null cells, further demonstrating that loss of tau function decreased induction and flux through the autophagic pathway. These results are the first demonstration of a genetic interaction between *NPC1* and *MAPT*. Before these studies, most believed that the tau pathology associated with NPC was an epiphenomenon and not involved in the disease pathogenesis. From our cell culture studies, we have established that loss of *MAPT* function modulates the critical pathway of autophagy in NPC1 deficient cells.

Tau is a microtubule binding protein, and as such is positioned to influence the efficiency of numerous intracellular trafficking events. In addition to autophagy, other protein quality control pathways such as the proteasome and MVBs require microtubule based trafficking for efficient protein degradation (14-18). Dysfunction of each of these pathways has been implicated in

neurodegeneration (8, 19-22), and it is possible that loss of tau function influences several of these pathways in NPC. In order to determine the extent to which dysfunction of the proteasome or MVBs occurs in NPC1 null cells and is modulated by tau, one could knockdown *MAPT* in NPC1 deficient fibroblasts and then monitor the degradation of specific proteins. Appropriate targets include a form of GFP protein targeted for degradation by the proteasome (GFPu) (23), or the receptor for PDGF which traffics to MVBs after ligand binding. These proposed studies would determine the extent to which impairments of protein quality control pathways are involved in NPC pathogenesis and if genetic deletion of *MAPT* modulates them.

Our *in vivo* studies conclusively showed that deletion of *Mapt* exacerbates the NPC phenotype. While attempting to generate double null mice (*Mapt -/-*; *Npc1 -/-*), we noticed a significant decrease in litter size. These small litters were not due to a selective loss of NPC1 null mice since the frequency distribution of the *Npc1*-null allele remained constant compared to litters generated by mice with normal levels of tau. Instead, these data provided the first demonstration that NPC1 and tau interact *in vivo* to affect fertility. Although the precise mechanism underlying this affect is currently unknown, it is interesting to note that functional autophagy is critical for early embryogenesis in mice (24).

Of the few double null animals that were generated, these mice demonstrated a much more severe phenotype than single null mutants (*Mapt* +/+; *Npc1* -/-). These animals showed decreased weight by 5 weeks of age, increased occurrence of penile prolapse and decreased survival. Many of the

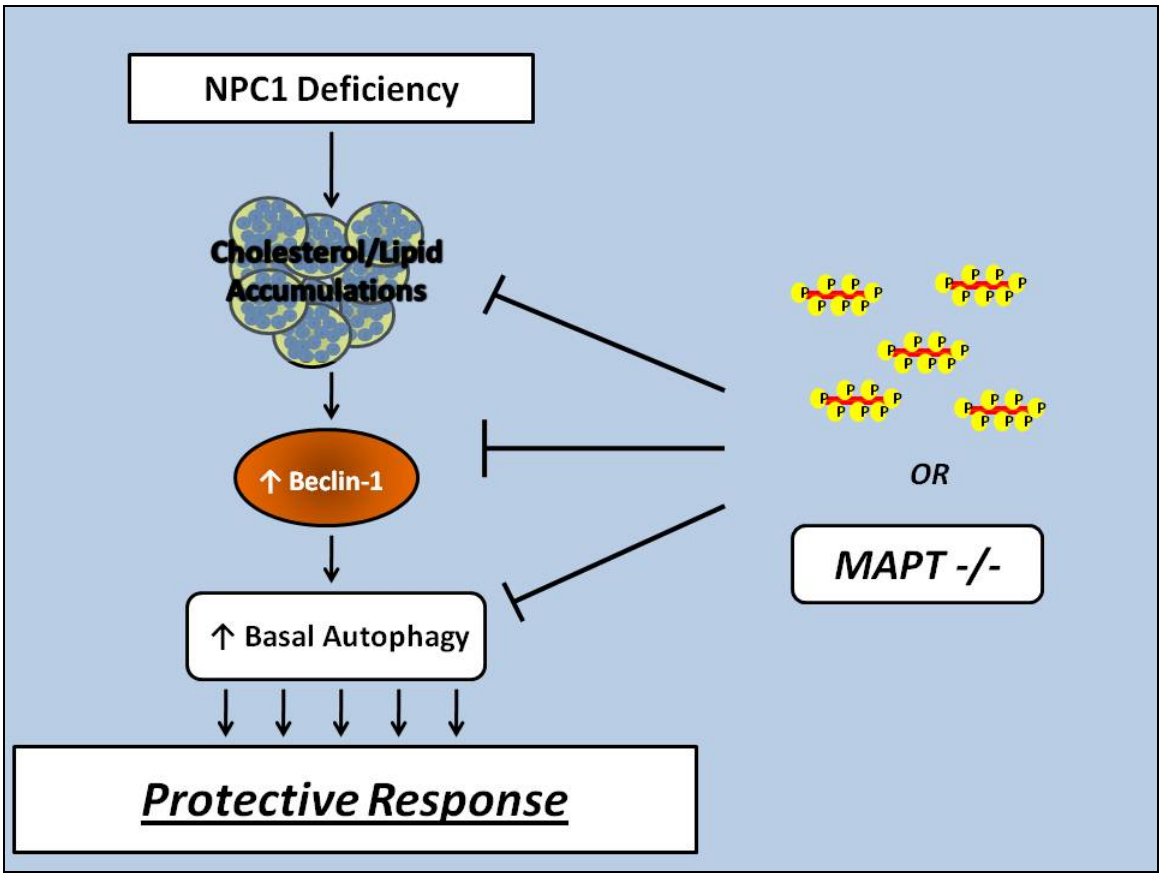
double null animals also showed mild facial dysmorphia with a shortened snout, kyphosis and an abnormal, “tip-toe” walking gait. These data provide the first *in vivo* evidence that *Mapt* loss of function exacerbates a disease phenotype. Previous reports in Alzheimer disease (AD) have demonstrated that loss of function mutations in *Mapt* actually ameliorate some of the disease phenotypes in a mouse model (25). Our data highlight the fact that tau pathology plays different roles in disease pathogenesis in various tauopathies.

Histochemical analyses of the double null mutants (*Mapt* -/-; *Npc1* -/-) demonstrated numerous large autophagic vacuoles in the liver, a common site of pathology in NPC. In contrast, autophagic vacuoles in the livers of single null mutants (*Mapt* +/+; *Npc1* -/-) were qualitatively smaller. Biochemical analyses showed a slight, but not statistically significant, increase in LC3-II levels in the livers of double null mutants compared to single nulls. No change in soluble or pelleted p62 was observed. These results suggest a possible blockage of the autophagy in double null mutants. However, perhaps because of more subtle effects *in vivo* than *in vitro,* because of compensatory processes, or because our assays to measure autophagy in living mammals are relatively insensitive, we were unable to definitively establish that *Mapt* deletion impairs autophagy in NPC mice.

Our studies have yielded compelling data demonstrating a previously unreported relationship between the microtubule-associated protein tau and *Npc1*. Our data demonstrate an important role for loss of normal tau function in the disease, and suggest that the restoration of this function could ameliorate

aspects of the NPC phenotype. Our data also highlight the fact that tau hyperphosphorylation, which occurs similarly in NPC, AD and other tauopathies, can contribute to disease by triggering a loss of function and acting through pathways that are distinct from the formation of toxic protein aggregates.

Our studies demonstrate that NPC1 deficiency leads to increased levels of basal autophagy and that this activation is dependent upon Beclin-1. We further demonstrate that reduction of functional tau causes a disruption of autophagy and an exacerbation of the NPC phenotype *in vivo*. Our model in Figure 5.1 demonstrates the relationship between loss of function mutations in NPC1 and activation of autophagy via increased expression of Beclin-1. We hypothesize that this increase in basal autophagy is a protective response, and that reduced tau function, by either hyperphosphorylation or genetic deletion, inhibits this protective response. We propose that the diminished autophagic capacity causes increased autophagic stress and exacerbates the NPC phenotype.



5.1 Proposed relationship between NPC1 deficiency, autophagy and tau

Functional deficiency of NPC1 impairs intracellular lipid trafficking and leads to the accumulation of unesterified cholesterol, sphingolipids and complex gangliosides in the late endosomal/lysosomal network. These defects stimulate a Beclin-1 dependent increase of basal autophagy, which we propose exerts a protective effect by facilitating the re-use of critical cellular components. Deletion of endogenous tau abrogates this response and exacerbates the NPC phenotype. We speculate that similar effects may be triggered by tau hyperphosphorylation as occurs in NPC patients.

5.3 Conditional *Npc1* mutant mouse

We have successfully generated a conditional mouse model of NPC with a floxed exon 9 of the *Npc1* allele. We have deleted this sequence *in vivo*, and confirmed that deletion of this floxed sequence is able to recapitulate a null allele

(unpublished data). These animals will aid in determining whether a single cell type is primarily responsible for the pathogenesis of NPC and therefore is a primary therapeutic target. There are a number of possible avenues to investigate with these animals, and our lab has begun to cross these mice with various Cre-expressing mice to elucidate the cell type specific contributions to NPC.

The Purkinje cells of the cerebellum are the most sensitive to NPC1 loss of function and die rapidly in the mouse model of the disease. Because of these attributes, Purkinje cell specific deletion of *Npc1* makes for an attractive first cross. We have obtained mice (Jackson Labs Tg(Pcp2-cre)1Amc/J) which express Cre recombinase specifically in Purkinje cells of the cerebellum by expressing the gene under a modified mouse Purkinje cell protein 2 (Pcp2) promoter. This cross will help determine the extent to which NPC1 deficiency causes cell autonomous neuronal toxicity.

Additional interesting crosses would include two glial cell type specific Cre recombinase lines enabling microglia (26) and astrocyte (27) specific gene deletion. Emerging evidence supports the role of these glial cells in the progression of NPC (28) and other neurodegenerative disorders such as AD (29). Microglia are the macrophages of the brain, and inflammation has been implicated in many neurodegenerative disorders (30) including NPC (31). Deletion of *Npc1* in microglia specifically will lead to a better understanding of the role of neuroinflammation in NPC pathogenesis. The NPC1 protein is also highly expressed in astrocytes, and a recent report suggested that over-expression of

Npc1 in astrocytes increased the lifespan of NPC mice (32). Astrocytes promote normal brain development and repair through the production and transport of cholesterol (33, 34), and their dysfunction has been implicated in disease pathogenesis. Astrocyte-derived cholesterol may impact neuronal function by promoting synaptogenesis (35) and by stimulating axonal growth (36). These data suggest deletion of NPC1 expression in astrocytes could yield interesting results, however, cell type specific deletion of *Npc1* (or any other gene) in astrocytes has been difficult to achieve. The most commonly used astrocyte specific promoter, from the glial fibrillary acidic protein (GFAP) gene, is highly expressed in post-mitotic astrocytes but is also weakly expressed in neuronal precursor cells (37). In order to keep *Npc1* expression intact for all cells of the CNS besides astrocytes, a tamoxifen-inducible system could be employed to express Cre recombinase in this glial cell population alone (Mutant Mouse Resource Center B6 (C3)-Tg (GFAP-cre/Esr1)/13Kdmc). These crosses could help determine the extent to which astrocytic involvement contributes to the neuropathology seen in NPC.

In addition to helping define the contribution of specific cell types to disease pathogenesis, the new conditional mouse model could be used to determine whether there is a temporal component to NPC disease. By crossing the conditional animal to tamoxifen inducible Cre mice (Jackson Labs B6.Cg-Tg (cre/Esr1)5Amc/J), the experimenter will globally delete the floxed sequence when tamoxifen is introduced. These studies will allow for *in vivo* ablation of *Npc1* expression at different time points. Although the assumption has been that

NPC is a degenerative disorder of childhood and not a disorder of fetal development (38), reagents to specifically test this notion have not been previously available. To establish the consequences of *Npc1* loss of function during development and in the adult nervous system, tamoxifen injections could be administered to animals of different ages and the consequences determined.

The studies proposed utilizing the conditional mouse model I generated will help define the roles of many different cell types in the pathogenesis of NPC, and will establish the extent to which a specific developmental window is critical for disease pathogenesis. The answers to these questions may help determine treatment targets and timing, and will likely yield important insights into the normal function of NPC1. The generation of this reagent has been a valuable experience for me as a scientist, and it is my hope that it will be a useful reagent for the entire NPC community.

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