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Fbxo2 mediates clearance of damaged lysosomes and modifies neurodegeneration in the Niemann-Pick C brain

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Abstract

A critical response to lysosomal membrane permeabilization (LMP) is the clearance of damaged lysosomes through a selective form of macroautophagy known as lysophagy. Although regulators of this process are emerging, whether organ and cell specific components contribute to the control of lysophagy remains incompletely understood. Here, we examine LMP and lysophagy in Niemann-Pick type C disease (NPC), an autosomal recessive disorder characterized by the accumulation of unesterified cholesterol within late endosomes and lysosomes, leading to neurodegeneration and early death. We demonstrate that NPC patient fibroblasts show enhanced sensitivity to lysosomal damage as a consequence of lipid storage. Moreover, we describe a role for the glycan binding F-box protein Fbxo2 in CNS lysophagy. Fbxo2 functions as a component of the SCF ubiquitin ligase complex. Loss of Fbxo2 in mouse primary cortical cultures delays clearance of damaged lysosomes and decreases viability following lysosomal damage. Moreover, Fbxo2 deficiency in a mouse model of NPC exacerbates deficits in motor function, enhances neurodegeneration, and reduces survival. Collectively, our data identify a role for Fbxo2 in CNS lysophagy and establish its functional importance in NPC.
Introduction

Lysosomes are critical organelles that function in degrading and recycling cellular waste and play broader roles in signaling, membrane repair and metabolism (1). As lysosomes contain diverse hydrolytic enzymes, lysosomal membrane integrity is essential for organelle function and for containing enzymes within the lysosomal compartment. A variety of factors, including lysosomotropic drugs and oxidative stress, lead to lysosomal membrane permeabilization (LMP), releasing lysosomal enzymes and triggering cellular processes from inflammasome activation to apoptosis (2). LMP-induced cell death can occur under physiologic conditions as a homeostatic response, such as during mammary gland involution or to maintain neutrophil numbers during inflammation (3). However, LMP has also been observed in several neurodegenerative diseases, including Alzheimer and Parkinson diseases and lysosomal storage disorders (LSDs) (3).

One protective measure against LMP is the clearance of damaged lysosomes through a selective form of macroautophagy known as lysophagy. In this process, damaged lysosomes are sensed, and subsequent ubiquitination of lysosomal proteins leads to recruitment of autophagic machinery, engulfment by autophagic membranes, and clearance of the damaged organelles (2). Cytosolic galectins, including galectin-1, -3, -8 and -9, serve as sensors of lysosomal damage (4-7). In addition to their sensing function, galectins also play more active roles in lysophagy by recruiting autophagy adapters; galectin-3 interacts with TRIM16 (5), and galectin-8 recruits NDP52 (7). A key intermediate step for lysophagy progression is ubiquitination of lysosomal proteins. Polyubiquitination of organelle membrane proteins is a feature of many forms of selective autophagy, which mediates recruitment of autophagic
machinery, allowing for efficient organelle turnover (2,8). LRSAM1 (9), TRIM16 (5) and the SCF\textsuperscript{FBXO27} ubiquitin ligase complex (10) are ubiquitin ligases that have been implicated in lysophagy. Although components of lysophagy have been identified in recent studies, aspects of the machinery that function in organ and cell type specific regulation remain incompletely understood. Notably, LMP has been observed in an increasing number of neurodegenerative diseases, yet brain specific lysophagy machinery remains unknown.

Here, we probe LMP and lysophagy in Niemann-Pick C, an autosomal recessive LSD characterized by accumulation of unesterified cholesterol in lysosomes and late endosomes (11). LSDs are a heterogeneous group of more than 70 inherited disorders characterized by the accumulation of lysosomal substrates due to organelar dysfunction and frequently cause neurodegeneration (12). LMP has been implicated in an increasing number of lysosomal diseases, including Gaucher disease (13), late-infantile neuronal ceroid lipofuscinosis (14), Niemann-Pick A (15-17) and Niemann-Pick C (18,19).

Niemann-Pick C is a devastating illness that often begins with liver disease, followed by a gradually worsening neurological course, with loss of motor skills, cognitive decline, seizures and most often death by early adolescence (20). Most cases of Niemann-Pick C (~95%) are due to mutations in the \textit{NPC1} gene (21), although a small subset (~5%) is due to mutations in \textit{NPC2} (22). NPC1 is a multi-pass transmembrane protein in the limiting membrane of lysosomes, while NPC2 is a soluble protein in the lysosomal lumen. NPC1 and NPC2 function in concert to export cholesterol from lysosomes (23); thus, mutations in either of these proteins lead to cholesterol accumulation. Although lipid accumulation is a hallmark of disease, the pathogenesis of neurodegeneration in Niemann-Pick C remains incompletely understood. Prior studies have
demonstrated mislocalization of lysosomal cathepsins outside of the lysosomal compartment in neurons in the Niemann-Pick C mouse brain, suggestive of LMP (18,19). In addition, NPC1-deficient cells experience increased toxicity with oxidative stress, a known inducer of LMP, and Niemann-Pick C mice deficient in cystatin B, an inhibitor of cathepsins, exhibit exacerbated cerebellar degeneration (19).

Here, we sought to further establish the role of LMP in Niemann-Pick C pathogenesis and define mechanisms of lysophagy, a critical response to lysosomal damage. We show that Niemann-Pick C patient fibroblasts exhibit increased lysosomal damage after exposure to lysosome damaging agents, and that this sensitivity is dependent upon the presence of stored lipids. Furthermore, we describe a novel role for Fbxo2 in CNS lysophagy. Fbxo2 is a glycan binding F-box protein that functions in the S phase kinase-associated protein 1 (SKP1)-cullin 1 (CUL1)-F-box protein (SCF) ubiquitin ligase complex, one of the largest classes of E3 ubiquitin protein ligases. We demonstrate that Fbxo2 localizes to damaged lysosomes, and that Fbxo2 deficiency impairs clearance of damaged lysosomes and exacerbates the Niemann-Pick C disease phenotype.
Results

I1061T NPC1 patient fibroblasts are more sensitive to lysosomal damage

Prior work has described LMP in Purkinje neurons and cerebellar lysates of Niemann-Pick C mice, as evidenced by cytosolic mislocalization of cathepsins outside of the lysosomal compartment (18,19). To further investigate the role of LMP in Niemann-Pick C disease pathogenesis, we utilized control (Ctrl) fibroblasts homozygous for WT NPC1 and Niemann-Pick C patient fibroblasts homozygous for I1061T NPC1 (I1061T), the most common disease-causing allele in patients of Western European ancestry (24). Cells were treated with increasing doses of the lysosomotropic compound L-leucyl-L-leucine methyl ester (LLOMe), a widely used lysosomal damaging agent that accumulates in lysosomes and is converted to a membranolytic form, (Leu-Leu)n-OME (n>3), by a lysosomal thiol protease, dipeptidyl peptidase I (DPPI) (4,25,26). To detect damaged lysosomes, we quantified the number of galectin-3 (Gal3) puncta, which accumulate on damaged lysosomes and are a sensitive indicator of LMP (4,6). Even at the lowest doses of LLOMe, I1061T patient fibroblasts exhibited significantly higher levels of Gal3 puncta per cell (Figure 1A). As accumulated storage of lysosomal substrates leads to an increase in both size and number of lysosomes (27), we wondered if the greater number of damaged lysosomes also reflected a greater proportion of damaged lysosomes in Niemann-Pick C cells. To address this, we treated Ctrl and I1061T patient fibroblasts with LLOMe, stained for both Gal3 and LAMP-1, and quantified their co-localization (Figure 1B). A significantly higher portion of LAMP-1 signal co-localized with Gal3 in I1061T patient fibroblasts (~40%) compared to Ctrl (~10%), indicating a higher percentage of damaged lysosomes.
To limit cytotoxic consequences of LMP, damaged lysosomes are eliminated by a form of selective macroautophagy known as lysophagy (5,7,8,10,25,28). As impairments in autophagic flux have been characterized in Niemann-Pick C (29-35), we wondered whether the increased lysosomal damage was due to deficient autophagic clearance. Thus, we performed a time course, treating Ctrl and I1061T patient fibroblasts with LLOMe for 1 hr followed by washout, and Gal3 puncta were quantified up to 24 hrs. Cells that are deficient in autophagy exhibit impaired clearance of Gal3 puncta (25,28). Our time course confirmed significantly increased Gal3 puncta per cell in I1061T patient fibroblasts but showed that these puncta were cleared through time, decreasing markedly by 16 and 24 hrs (Figure 2A) and indicating functioning lysophagy. To assess if markers of autophagy also correlated with the transient accumulation and subsequent clearance of Gal3 puncta, we quantified LC3 puncta after LLOMe treatment. Similar to the pattern seen with Gal3 (Figure 2A), I1061T patient fibroblasts exhibited significantly higher numbers of LC3 puncta per cell compared to Ctrl, and these puncta were cleared out by 24 hrs (Figure 2B). Plotting the percentage of Gal3 and LC3 puncta clearance through time also showed that both Ctrl and I1061T patient fibroblasts clear Gal3 and LC3 puncta at similar rates (Supplementary Figure 1). These data support the induction of lysophagy and clearance of damaged lysosomes following treatment with LLOMe.

**Increased lysosomal damage is dependent upon lipid storage**

In addition to the marked accumulation of Gal3 puncta in NPC patient fibroblasts after inducing lysosomal damage with LLOMe, we also observed lysosomal damage in Npc1-I1061T knock-in mice (36). These mice develop age-dependent phenotypes including cholesterol
accumulation, neuron loss, motor impairment, and early death. Wild type (WT) and mutant mice were treated with vinblastine for 2 hrs to slow autophagosome maturation (37). This facilitated the identification of Gal3+, Lamp-2+ vesicles in liver macrophages of Npc1-I1061T mutants (Mander’s coefficient = 0.76 for I1061T), suggesting the occurrence of lysosomal damage in vivo (Figure 3A).

As accumulated lipids contribute to LMP (15-17,38-40), we reasoned that lipid storage in I1061T patient fibroblasts could increase sensitivity to lysosomal damage. To test this notion, we treated I1061T patient fibroblasts with hydroxypropyl beta cyclodextrin to remove stored lipids including unesterified cholesterol. Treatment with cyclodextrin for 48 hrs did not significantly change levels of LAMP-1 (Supplementary Figure 2) indicating that lysosome number was unaltered during the experiment. However, cyclodextrin treatment significantly reduced Gal3 puncta per cell in I1061T patient fibroblasts following treatment with LLOMe, demonstrating that sensitivity to lysosomal damage was dependent upon lipid storage (Figure 3B). The mutant I1061T NPC1 protein is known to misfold in the endoplasmic reticulum and be degraded, preventing its trafficking to the lysosome (37,41). To examine whether loss of NPC1 at the lysosomal membrane plays a role in sensitivity to lysosomal damage, we treated NPC2-mutant patient fibroblasts with LLOMe. These cells express WT NPC1 yet accumulate lipids due to functional deficiency of NPC2 (42-44). Similar to I1061T patient fibroblasts, two independent lines of NPC2-mutant fibroblasts (g.IVS1+2T>C/g.IVS1+2T>C and c.58G>T/c.140G>T) also exhibited significantly increased lysosomal damage (Figure 3C). These data corroborate our findings from NPC1-I1061T patient fibroblasts that lipid accumulation is linked to lysosomal
damage. Further, they support the notion that lipid accumulation, independent of loss of NPC1 at the lysosomal membrane, contributes to increased lysosomal damage.

**Increased lysosomal damage is not due to impaired clearance**

Our prior time course experiments demonstrated clearance of Gal3 puncta in I1061T patient fibroblasts (Figure 2A, Supplementary Figure 1) suggesting functioning lysophagy. To examine the rate at which damaged lysosomes were cleared from cells, we utilized a cycloheximide (CHX) chase assay (10) (Figure 4A). Cells were treated with LLOMe and CHX for 1 hr. Following LLOMe washout, CHX treatment was continued for various times to assess degradation rates of LAMP-1 or Gal3 as indicators of lysosomal clearance. We first corroborated the use of this assay as a readout of lysophagy. Without lysosomal damage, LAMP-1 and Gal3 levels remained constant for the duration of the CHX chase (Supplementary Figure 3). After lysosomal damage, LAMP-1 levels diminished with time in WT MEFs, but this effect was prevented in Atg5-/- MEFs, which are autophagy deficient (45) (Figure 4B). We next performed this analysis on Ctrl and I1061T patient fibroblasts, and consistent with prior time course experiments (Figure 2), found that Gal3 was cleared in both Ctrl and I1061T patient fibroblasts at equivalent rates (Figure 4C); similarly, LAMP-1 was efficiently cleared from I1061T patient fibroblasts (Supplementary Figure 4). We conclude that Niemann-Pick C cells exhibit enhanced sensitivity to lysosomal damage but clear damaged lysosomes at a similar rate to controls.

**Fbxo2 is the most highly expressed glycan binding F-box protein in the brain**
With evidence that Niemann-Pick C fibroblasts are more susceptible to lysosomal damage, we sought to further elucidate mechanisms of lysophagy, a critical cellular response to lysosomal damage. LMP exposes N-glycan-modified proteins of the limiting membrane of the lysosome to the cytosol, making them accessible to lectin binding. A recent study showed that following lysosomal damage, Fbxo27, a glycan binding F-box protein, ubiquitinates lysosomal proteins and targets damaged lysosomes for degradation by autophagy (10). F-box proteins function within the S phase kinase-associated protein 1 (SKP1)-cullin 1 (CUL1)-F-box protein (SCF) ubiquitin ligase complex, one of the largest classes of E3 ubiquitin protein ligases. There are ~70 different F-box proteins in humans, and the variable F-box protein determines substrate specificity (46). Confirming the importance of ubiquitination in lysophagy, inhibiting E1 ubiquitin-activating enzymes with MLN7423 (47) delayed clearance of damaged lysosomes (Figure 5A).

Prior studies have provided evidence of LMP in the Niemann-Pick C brain (19) and established neurons as a critical cell type for disease pathogenesis (48). Therefore, we were curious as to the function of Fbxo27 in the brain. Utilizing the Allen Brain Atlas, we found, however, that Fbxo27 exhibits very low expression in the brain (Figure 5B). As the importance of ubiquitination in lysophagy has been described (2), we wondered whether other glycan binding F-box proteins might play a more significant role in the brain. Fbxo2, Fbxo6 and Fbxo27 are in the FBA family of F-box proteins, which is the only family of ubiquitin ligase subunits thought to target glycoproteins (49). Utilizing the Allen Brain Atlas, we found that both Fbxo27 and Fbxo6 exhibit low brain expression; in contrast, Fbxo2 is highly expressed throughout the brain (Figure 5B). Indeed, Fbxo2 was originally identified as a brain enriched F-box protein (50),
with expression annotated in neurons, astrocytes, oligodendrocytes and microglia (51). We confirmed by qPCR that Fbxo2 is the most highly expressed glycan binding F-box protein in multiple brain regions, with no significant change in expression in WT compared to Npc1-I1061T knock-in mice (36), except for a slight increase in the cerebellum (Figure 5C). Similarly, protein levels of Fbxo2 were not significantly different between WT and Npc1-I1061T mice (Supplementary Figure 5).

**Fbxo2 localizes to damaged lysosomes**

Fbxo2 has been shown to play roles in glycoprotein quality control through ER-associated degradation (52). To our knowledge, it has not been shown to play a role in lysophagy, though prior work has demonstrated interaction with LAMP-1 and LAMP-2 after lysosomal damage (10). To begin to investigate whether Fbxo2 contributes to lysophagy, we overexpressed HA-FBXO2 in Ctrl and I1061T patient fibroblasts. As anticipated, this manipulation did not affect cholesterol accumulation in I1061T fibroblasts (Supplementary Figure 6). Notably, we found a strikingly altered distribution of HA-FBXO2 following lysosomal damage. Prior to LLOMe treatment, HA-FBXO2 exhibited diffuse, cytoplasmic staining, similar to Gal3 (Figure 6A). After lysosomal damage, HA-FBXO2 became punctate and partially co-localized with Gal3, indicating recruitment to damaged lysosomes (Mander’s coefficient = 0.80 (Ctrl), 0.81 (I1061T)) (Figure 6A). This recruitment of HA-FBXO2 to damaged lysosomes suggested that it may function in their clearance. Overexpressed FBXO6 and FBXO27 showed similar recruitment to Gal3 puncta after lysosomal damage (Supplementary Figure 7), raising the possibility that functional differences among the FBA family of F-box proteins is
determined, in part, by expression patterns, with Fbxo2 expression in the CNS being most prominent. Co-immunoprecipitation experiments after LLOMe treatment confirmed that HA-FBXO2 interacts with LAMP-2 and Skp1, demonstrating its interaction with damaged lysosomes in the SCF complex (Figure 6B). We also observed interaction of HA-FBXO2 with LAMP-2 and Skp1 following vehicle treatment (Supplementary Figure 8), consistent with prior co-immunoprecipitation experiments with FBXO2, FBXO6 and FBXO27 before and after lysosomal damage (10) and suggesting their recruitment to damaged lysosomes even in the absence of treatment with a lysosomal damaging agent. With evidence that HA-FBXO2 is recruited to damaged lysosomes in patient fibroblasts, we asked whether Fbxo2 plays a role in lysophagy in the CNS. In primary cortical cultures transfected with HA-FBXO2, we saw a similar transition from diffuse cytoplasmic to punctate staining in neurons after lysosomal damage (Figure 6C) and partial co-localization with LAMP-1 (Supplementary Figure 9), indicating recruitment to damaged lysosomes.

**Fbxo2 plays a role in CNS lysophagy**

To investigate a role for Fbxo2 in lysophagy in the brain, we established primary cortical cultures from WT and Fbxo2 deficient (Fbxo2-/-) mice (53). qPCR demonstrated that Fbxo2 was the most highly expressed glycan binding F-box protein in WT primary cortical cultures and that there was no compensatory upregulation of Fbxo6 or Fbxo27 in response to Fbxo2 deficiency (Figure 7A). Notably, clearance of damaged lysosomes was significantly delayed in Fbox2-/- cultures. When we overexpressed Gal3 in WT and Fbxo2-/- primary cortical cultures, we found significantly slower clearance of Gal3 puncta in Fbxo2-/- cultures (Figure 7B). Further, by CHX
chase assay (Figure 4A), the half-life of Gal3 was extended from ~30 min in WT cultures to ~105 min in Fbxo2-/- primary cortical cultures (Figure 7C). These data demonstrate delayed lysophagy progression and support a role for Fbxo2 in the clearance of damaged lysosomes. As lysosomal membrane damage releases luminal enzymes and can lead to cell death (2), we next compared cell viability in WT and Fbxo2-/- primary cortical cultures after lysosomal damage. When assessing toxicity 4 hrs after LLOMe treatment, we observed significantly decreased viability in Fbxo2-/- cultures compared to WT (Figure 7D), indicating increased cell death following lysosomal damage. Supporting on-target effects of LLOMe, this toxicity was rescued by preventing lysosomal damage with the cathepsin inhibitor E64D (54) (Figure 7E, Supplementary Figure 10).

**Loss of Fbxo2 exacerbates the Niemann-Pick C disease phenotype**

As our analyses indicated the occurrence of increased susceptibility to lysosomal damage in NPC1 or NPC2 deficient cells, we sought to determine whether loss of Fbxo2 modified the disease phenotype in a mouse model of NPC disease. To accomplish this, we generated Npc1-I1061T mutant mice deficient in Fbxo2. Fbxo2 deficiency is well tolerated in mice, except for the occurrence of hearing loss (53). Similarly, Fbxo2-/- mice were indistinguishable from WT littermates in our analyses (Figure 8). However, loss of Fbxo2 in Npc1-I1061T mice exacerbated deficits in motor function as quantified by performance on the balance beam (Figure 8A) and rotarod (Figure 8B) and significantly decreased survival (Figure 8C). Consistent with expression analysis in primary cortical cultures, loss of Fbxo2 did not lead to compensatory upregulation of Fbxo6 or Fbxo27 in the mouse brain (Figure 8D). Fbxo2
deficient mice also had similar expression levels of the SCF component Cul1 and a slight increase in expression of Rbx1 (Supplementary Figure 11).

Exacerbation of motor phenotypes prompted us to examine changes in neuropathology. We focused our analysis on Purkinje neurons, the major output neurons of the cerebellar folia, which degenerate in Niemann-Pick C brain (19,55) and whose loss is sufficient to trigger motor impairment (56). Npc1-I1061T mice deficient in Fbxo2 exhibited significantly increased Purkinje cell loss, correlating with their exacerbated motor phenotypes (Figure 8E). In contrast, Fbxo2 deficiency alone did not alter Purkinje cell density. Furthermore, brain tissue from Npc1-I1061T, Fbxo2-/- mice showed enhanced accumulation of p62 protein (Figure 8F, Supplementary Figure 12A) without upregulation of p62 mRNA (Supplementary Figure 12B), similar to findings in cell culture after lysosomal damage (14). Taken together, these data indicate that loss of Fbxo2 in Niemann-Pick C mice exacerbates behavioral phenotypes and neurodegeneration while altering markers of autophagy.
**Discussion**

In this study, we describe a novel role for Fbxo2 in CNS lysophagy and demonstrate the importance of lysophagy as a key compensatory pathway in Niemann-Pick C knock-in mice. We show that Niemann-Pick C patient fibroblasts are more sensitive to lysosomal damage by LLOMe and that this occurs in the context of functioning lysophagy (Figures 1-4). Our data suggest that the primary driver of increased sensitivity to lysosomal damage is a factor intrinsic to the lysosome that affects membrane stability. Increased oxidative stress has been posited as a contributor to LMP in Niemann-Pick C (19), but it is likely that additional factors also function in this context. We now show that lipid storage, independent of loss of the NPC1 protein, contributes to increased sensitivity to lysosomal damage (Figure 3). Consistent with this finding, studies have demonstrated that lipids including accumulated sphingomyelin (16,17,41) and cholesterol (39,40) induce lysosomal damage. As diverse storage materials in Gaucher disease (13) and late-infantile neuronal ceroid lipofuscinosis (14) also contribute to lysosomal damage, LMP is likely the consequence of aberrant accumulations within lysosomes. In contrast, studies have also described the importance of lipids in membrane rigidity and lysosomal membrane stability (57-59). Notably, the absence of functional NPC1 protein is expected to impair the movement of luminal cholesterol into the limiting membrane of the lysosome. Defining the role of altered luminal versus lysosomal membrane lipid composition and their effects on lysosomal membrane stability will be important in furthering our understanding of lysosomal dysfunction in Niemann-Pick C. Additionally, as some of our studies were conducted in patient fibroblasts, we acknowledge that these cells may not fully mirror events in neurons in a complex disorder like Niemann-Pick C disease, and further work on LMP in NPC neurons will be of importance.
Lysophagy is a critical response to LMP, and we show that of the FBA family of glycoprotein binding F-box proteins, Fbxo2 is most highly expressed in the brain (Figure 5) and is recruited to damaged lysosomes (Figure 6). Supporting its function in lysophagy, loss of Fbxo2 in primary cortical cultures delays clearance of damaged lysosomes and leads to decreased viability after lysosomal damage (Figure 7). This finding is consistent with work on the glycan binding F-box protein Fbxo27, where Fbxo27 deficiency slows but does not abolish lysophagy (10). Likely, additional pathways function to mediate lysophagy in the brain. TRIM16, a RING-type ubiquitin ligase, has also been shown to mediate lysophagy progression (5) and may function redundantly with Fbxo2.

Supporting the function of Fbxo2 in lysophagy, Niemann-Pick C mice deficient in Fbxo2 exhibit exacerbated disease phenotypes, with significantly worse impairments in motor function and decreased survival (Figure 8). This correlates with increased loss of Purkinje cells and an increase in the autophagic adapter protein p62 (Figure 8). These findings support a model wherein impairing lysophagy in a background of increased lysosomal damage exacerbates the disease phenotype. The characterization of Fbxo2 as a component of the machinery that regulates efficient lysophagy in the CNS is intriguing, as it demonstrates specificity of the mediators of this process. Among CNS cell types, RNA-seq datasets demonstrate broad expression of Fbxo2 in neurons and glia (51). Loss of functional Npc1 in mouse neurons and oligodendrocytes, but not astrocytes, has been shown to contribute to Niemann-Pick C neuropathology (30,48,56,60). As such, it is possible the Fbxo2 functions in multiple cell types to maintain efficient lysophagy and promote CNS homeostasis.
Collectively, our data describe a novel function for Fbxo2 in lysophagy and establish its proof-of-concept disease relevance in compensating for Niemann-Pick C pathophysiology. Our findings suggest that strategies aimed at targeting the lysosomal cell death pathway and enhancing lysophagy function may be protective against neurodegeneration in Niemann-Pick C. Furthermore, additional studies to probe the function of Fbxo2 will continue to advance our understanding of mechanisms involved in protein and organellar quality control pathways that likely contribute to the neuropathology in a diverse array of lysosomal diseases.
Methods

Antibodies:

Primary antibodies (antigen, dilution, vendor): galectin-3, 1:100 (IF), 1:500 (WB), Santa Cruz Biotechnology Inc. sc-20157 (discontinued); galectin-3, 1:100, Santa Cruz Biotechnology Inc. sc-23938; LAMP-1, 1:100 (IF), DSHB at the University of Iowa H4A3; LAMP-2, 1:10 (IF), DSHB at the University of Iowa ABL-93; LC3B, 1:500 (IF), 1:1000 (WB) Novus Biologicals NB600-1384; LAMP-1, 1:1000, abcam ab24170; Calbindin-D-28K, 1:500, Sigma-Aldrich C2724; HA.11, 1:500, BioLegend 901501; NeuN, 1:500, Sigma-Aldrich ABN90; β-Actin, 1:2000, Sigma-Aldrich A5441; Vinculin, 1:2000, Sigma-Aldrich V9131, p62, 1:1000, Sigma-Aldrich P0067; Skp1, 1:1000, BD 610530; LAMP2, 1:100 (WB), abcam ab25631; FBXO2, 1:100, Santa Cruz Biotechnology Inc. sc-393873; FLAG, 1:500, Sigma-Aldrich F1804

Secondary antibodies (antibody, dilution, vendor): Alexa Fluor™ 488 goat anti-rabbit IgG (H+L), 1:500, Invitrogen A11008; Alexa Fluor™ 495 Fab’2 fragment of goat anti-mouse IgG (H+L), 1:500, Invitrogen A11020, Alexa Fluor™ 488 goat anti-mouse IgG (H+L), Invitrogen A11029; Alexa Fluor™ 594 goat anti guinea pig IgG (H+L), Invitrogen A1076; Goat anti-mouse IgG (H+L)-HRP conjugate, Alexa Fluor™ 488 goat anti-rat IgG (H+L), Invitrogen A11006; 1:2000, Bio-Rad 170-6516; Goat anti-rabbit IgG (H+L)-HRP conjugate, 1:2000, Bio-Rad 170-6515

Reagents:
The following drugs and small molecules were used: MLN7243 (CT-M7243, Chemietek); Leu-Leu methyl ester hydrobromide (L7393, Sigma-Aldrich); Cycloheximide (C7698, Sigma-Aldrich); E-64d (13533, Cayman); 2-hydroxypropyl-beta-cyclodextrin (H-107, Sigma-Aldrich); vinblastine sulfate salt (cat. V1377, Sigma); filipin (F9765, Sigma). Plasmid encoding HA-FBXO2 was from Dr. Henry Paulson (University of Michigan, Ann Arbor). Plasmids encoding FLAG-FBXO2, FLAG-FBXO6 and FLAG-FBXO27 were from Dr. Yukiko Yoshida (Tokyo Metropolitan Institute of Medical Science, Japan). Plasmid encoding EGFP-hGal3 was from addgene (#73080).

**Cells**

The following cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: GM08399 (Ctrl), GM18453 (I1061T/I1061T), and GM18429 (NPC2-1), GM18455 (NPC2-2). Fibroblasts were cultured in MEM (Gibco 10370), PSG (Gibco), and 20% premium FBS (Atlanta Biologicals). WT and Atg5-/- MEF cell lines RCB2710 and RCB2711 were obtained from the RIKEN BRC Cell Bank and were cultured in MEM (Gibco 10370), PSG (Gibco), and 20% premium FBS (Atlanta Biologicals).

**Mice**

Npc1-I1061T mice (36) were a gift from Daniel Ory (Washington University in St Louis) and backcrossed to C57BL/6 (≥10 generations). Fbxo2-/- mice (53) were a gift from Henry Paulson (University of Michigan, Ann Arbor) and on the C57BL/6 background.
Western blot

Cell culture media was aspirated, cells were washed 1x with ice cold PBS, then cells were removed with a cell scraper and centrifuged at 1000xg for 5 min at 4°C. The cell pellet was resuspended in RIPA (Teknova) with complete protease inhibitor (Thermo Scientific 11836153001) and 0.625mg/ml N-ethylmaleimide (Sigma E3876) and sonicated. For primary cortical cultures, lysates were centrifuged at 12,000xg for 10min at 4°C and the supernatant was collected. For tissue preparation, mice were perfused with saline before tissue was collected and flash frozen in liquid nitrogen. Tissue was homogenized and sonicated in RIPA buffer. Protein concentrations were determined by DC™-protein assay (Bio-Rad) and normalized. Proteins were separated on NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo Scientific NP0336BOX) and transferred to Immobilon-P 0.45um PVDF (Merck Millipore). Immunoreactivity was detected with ECL (Thermo Scientific) or SuperSignal™ West Pico PLUS Chemiluminscent Substrate (Thermo Scientific) and an iBright (Thermo Fisher Scientific). Quantification was performed using Image Studio. Band intensity was normalized to the indicated loading control.

RT-qPCR

RNA was collected using TRIzol® (Thermo Fisher) according to manufacturer’s instructions and converted to cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems 4368814). Quantitative real-time PCR was performed using 10ng cDNA, FastStart Taqman Probe
Master Mix (Roche), and gene-specific FAM-labeled TaqMan probes (Thermo Scientific) for
mouse Fbxo2 (Mm00805188), Fbxo6 (Mm01257500), Fbxo27 (Mm01179110), Sqstm1
(Mm0044809), Rbx1 (Mm01705487), and Cul1 (Mm00516318). Gene expression was
normalized to mouse Cpsf2-Vic (Mm00489754) multiplexed within the same well. RT-qPCR was
performed using an ABI 7900HT Sequence Detection System and relative expression calculated
by the 2^(-ΔΔCt) method.

**Transfection**

Human patient fibroblasts: Cells were transfected with Lipofectamine® LTX with Plus™ Reagent
(Invitrogen). Briefly, 320ng of endotoxin-free plasmid was incubated in 43μL opti-MEM (Gibco)
and .425μL PLUS reagent for 5 min. Separately, 43μL opti-MEM was incubated with 1.28μL LTX
reagent for 5 min. Then, the LTX mixture was added to the plasmid mixture and incubated for
30 min before adding dropwise to cells.

Mouse primary cortical cultures: Cells were transfected on D2-4IV with Lipofectamine 2000
(Invitrogen). Briefly, cells were washed 2x with Neurobasal™-A Medium (Gibco). 200ng of
endotoxin-free plasmid was incubated with 50μL opti-MEM (Gibco) for 5min. Separately, 50μL
opti-MEM and 1μL Lipofectamine 2000 was incubated for 5min. After 5min, the Lipofectamine
mixture was added to the plasmid mixture and incubated 20 min before adding dropwise to
cells. Transfection mixture was kept on cells for 20 min before removing, and cells were washed
2x in Neurobasal™-A Medium (Gibco).
**Immunofluorescence staining**

Cells were washed 3x with HBSS and fixed with ice cold 100% methanol for 20min at -20°C. Cells were washed 3x with PBS and placed in 2.5mg/ml glycine for 10 min at room temperature. Cells were washed 3X with PBS, permeabilized with 0.1% Triton in PBS for 20min, then placed in blocking solution (10% goat serum, 1% BSA in PBS) for 1 hr. Cells were incubated with primary antibodies diluted in blocking solution overnight at 4°C. The next day, slides were washed 3x with PBS and incubated with secondary antibody diluted in blocking solution for 1 hr at room temperature. Slides were washed 3x with PBS and mounted with Vectashield + DAPI (Vector Laboratories). For LAMP-1 staining, after glycine incubation, cells were placed in saponin blocking solution (0.02% saponin, 5% NGS, 1% BSA) for 1 hr and then with primary antibody diluted in saponin blocking solution overnight at 4°C. Slides were washed with PBS + 0.02% saponin and incubated with secondary antibody diluted in saponin blocking solution, washed and mounted.

For tissue preparation, mice were perfused with saline and 4% PFA and tissue was removed and post-fixed in 4% PFA overnight at 4°C prior to paraffin embedding. Paraffin-embedded tissues were cut on a Reichert-Jung 2030 microtome into 5µm sections and placed on Fisher Scientific Superfrost Plus microscope slides. Sections were adhered onto slides in an oven at 55-60°C for 1 hr. Samples were deparaffinized and antigen retrieval was performed by boiling in 10mM sodium citrate (pH 6.0) for 10 min and incubating in hot citrate solution for an additional 20
min, then washed 3x in deionized water. For staining, slides were incubated in a solution with 0.1% Triton, 10% goat serum and 1% BSA in PBS for 20 min. Then, slides were placed in blocking solution (10% goat serum, 1% BSA in PBS) before incubating in primary antibody diluted in blocking solution overnight at 4°C. Slides were washed 3x in PBS and incubated for 1 hr with secondary antibody diluted in blocking solution. Slides were then washed 3x in PBS and mounted with Vectashield + DAPI (Vector Laboratories).

**Filipin staining**

Cells were stained following the immunofluorescence protocol described above, with the exception of being fixed in 4% PFA. Following the washes after secondary antibody incubation, cells were incubated with 1mL filipin staining solution (5% FBS + 40uL filipin solution (1mg filipin + 40uL DMSO) in PBS) for 2 hr at room temperature. Cells were washed 3x in PBS and slides were mounted with ProLong® Gold (Thermo Fisher).

**Primary cortical cultures**

Cortices were dissected using the Papain Dissociation System (Worthington) from P0-P1 WT or Fbxo2/- pups. Briefly, cortices were dissected free of meninges, placed in papain solution and incubated at 37°C for 20 min. Cortices were triturated 15x with a 10mL pipette tip and spun at 1200rpm for 5 min. Cells were resuspended and spun over a discontinuous density gradient at 1000rpm for 5 min. Cells were then resuspended in Neurobasal™-A Medium (Gibco) with B-27™
Supplement (Gibco), GlutaMAX™ (Gibco) and Pen Strep, counted and plated. Media was changed every 3 days.

**Cell Survival**

The XTT assay (ATCC) was used to assess cell survival according to manufacturer’s protocol. Briefly, 50µL of XTT solution was added to 100µL of cell culture media for 4 hrs in a CO₂ incubator at 37°C. Plates were read on a Synergy HTX multimode plate reader (BioTek) at 475 and 660nm.

**Neon™ Transfection System**

Cells were transfected by Neon™ Transfection System (Thermo Fisher) according to manufacturer’s protocol. Briefly, cells were counted and resuspended in DPBS (Gibco) along with plasmid DNA. Cell culture plates were pre-incubated with culture medium without antibiotics. Neon® Tube was set up with 3mL Electrolytic Buffer E2 and the 100µL Neon® Tip was used at 1200V, 40ms, 1 pulse.

**Co-Immunoprecipitation**

Cells were washed in PBS and cross-linked with DSP (Thermo 22585) for 30 min at RT. Tris-HCl pH 7.5 was added to a final concentration of 20mM and cells were incubated on a rotator for 15 min at 4°C. Cells were centrifuged for 5 min at 1000g and resuspended in lysis buffer (.025M
Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4) with complete protease inhibitor (Thermo Scientific 11836153001). Lysates were pre-cleared with protein A/G beads for 30 min at 4°C. Beads were pelleted at 1000g for 1 min and lysates were incubated with HA (BioLegend) antibody or control IgG on a rotator overnight at 4°C. Protein A/G beads were then added and lysates tumbled at 4°C for 1 hr. Beads were placed into spin columns (Pierce) and washed 6 times in lysis buffer. Finally, beads were boiled at 95°C for 5 min in 6X loading buffer and loaded on NuPAGE 4-12% 10 well gels (Invitrogen).

**Microscopy**

Confocal images were collected using a Nikon A-1 confocal with diode-based laser system. Co-localization and puncta quantification were determined using CellProfiler Analyst Software. For Gal3 puncta quantification, 100-200 cells per experiment were examined.

**Phenotype analysis**

Balance beam: The balance beam consists of a 5mm wide square beam suspended at 50cm. Mice were trained at 5 wks of age to cross the beam and then tested every other week starting at 6 wks. For testing, mice were run 3 times across the beam, and the average time was taken. Maximum time was set at 20 sec and falls were scored as 20 sec.
Rotarod: After acclimatizing to the testing room for 30 min, mice were gently placed on a moving (4 RPM) rotarod for 30 sec. Then, over a period of 235 sec, rotarod speed was increased to 40 RPM. The trial ended if mice stopped walking for two revolutions or dropped onto the paddle. Mice were trained three times per day with a 30 min interval between each training session over a period of 3 days. The following week, mice were tested on a single day using the training protocol.

Survival: All deaths were recorded. Mice that lost >20% maximal body weight were euthanized and recorded as deaths.

Purkinje Cell Quantification

Quantification of Purkinje cells was performed as described previously (19). Briefly, midline sagittal sections were stained with calbindin to identify Purkinje cells. The number of cells was normalized to the length of the Purkinje layer as measured by NIH ImageJ software.

Vinblastine treatment

Mice were injected with vinblastine (0.04mg/g) I.P. at 7 weeks of age as previously described (37). Liver was collected 2 hr post-injection and processed for immunofluorescence staining.
Statistics

Graphpad Prism 7.0 was used to determine significance (P<0.05), F (F-statistic) and t (T-statistic) values. Unpaired Student’s t-test (two-tailed) and one-way or two-way ANOVA were used as indicated in the figure legends. A P value less than 0.05 was considered significant. All error bars are s.e.m.

Study Approval

All procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals (PRO00008133) and conducted in accordance with institutional and federal guidelines.
Author Contributions

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Figure 1. I1061T NPC1 patient fibroblasts are more sensitive to lysosomal damage.
(A) Primary human fibroblasts homozygous for WT NPC1 (Ctrl) or I1061T NPC1 (I1061T) were treated with vehicle (Veh) or indicated doses of LLOMe for 1hr and stained for Gal3 to detect damaged lysosomes. Gal3 puncta per cell quantified below.

(B) Ctrl and I1061T patient fibroblasts were treated with Veh or 2mM LLOMe for 1 hr and stained for Gal3 and LAMP-1. Co-localization was performed on 4 fields each from 3 independent experiments, with 100-200 cells per experiment.

Data are shown as mean ± s.e.m. from 3 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by (A) one-way ANOVA with Tukey’s multiple comparisons (B) t-test ((A) F=24.04; (B) F=12.78). Scale bar: 25µm
Figure 2. Gal3 and LC3 puncta induced by lysosomal damage are cleared in I1061T NPC1 patient fibroblasts

(A) Ctrl and I1061T patient fibroblasts were treated with Veh or 2mM LLOMe for 1 hr and stained for Gal3 at indicated times after washout. Quantified at the right.

(B) Ctrl and I1061T patient fibroblasts were treated with Veh or 0.5mM LLOMe for 1 hr and stained for LC3 at indicated times. LC3 puncta per cell quantified at the right.

Data are shown as mean ± s.e.m. from (A) 3 or (B) 4 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by two-way ANOVA with Sidak’s test. Scale bar: 25µm
Figure 3. Increased lysosomal damage is dependent upon lipid storage.

(A) Seven-week-old WT and I1061T mice were treated with vinblastine for 2 hr. Liver was collected and stained for Gal3 and LAMP-2. Mander’s coefficient in I1061T liver: 0.76. Scale bar: 5µm

(B) Ctrl and I1061T patient fibroblasts were treated with Veh or 1mM cyclodextrin (Cyclo) for 48 hrs, then treated with 2mM LLOMe for 1 hr and stained for Gal3. Quantified at the right. Scale bar: 25µm

(C) Ctrl, I1061T and two independent lines of NPC2 patient fibroblasts were treated with Veh or 2mM LLOMe for 1 hr and stained for Gal3. Quantified at the right. Scale bar: 25µm
Data are shown as mean ± s.e.m. from (B-C) 4 independent experiments. n.s., not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by (B) t-test (C) one-way ANOVA with Tukey’s multiple comparisons ((B) F=2.845, (C) F=30.99).
Figure 4. Increased lysosomal damage is not due to impaired clearance.

(A) To examine lysophagy progression, cells were treated with 30μg/mL CHX and 2mM LLOMe for 1 hr and CHX treatment continued for indicated times before lysates were collected.

(B) WT and Atg5−/− MEFs were treated as in (A). LAMP-1 levels were analyzed and quantified at the right.

(C) Ctrl and I1061T patient fibroblasts were treated as in (A). Gal3 levels were analyzed and quantified at the right.

Data are shown as mean ± s.e.m. from (B) 4 or (C) 3 independent experiments. n.s., not significant, *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001 by one-way ANOVA with Tukey’s multiple comparisons ((B) F=2.862, (C) F=25.49)
Figure 5. Fbxo2 is the most highly expressed glycan binding F-box protein in the brain

(A) Ctrl patient fibroblasts were pre-treated with DMSO or 1µM MLN7243 for 4 hrs and then treated as indicated with 30µg/mL CHX and 2mM LLOMe. LAMP-1 levels quantified at the right.

(B) Allen Brain Atlas expression data of Fbxo2, Fbxo6 and Fbxo27 in mouse brain.

(C) Relative expression of Fbxo2, Fbxo6 and Fbxo27 was determined in the cerebellum, cortex and brainstem of WT and I1061T mice at 12 wks by qPCR. N=4-5 mice per genotype.

Data are shown as mean ± s.e.m from (A) 4 independent experiments. n.s., not significant, *P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0.0001 by (A, C) one-way ANOVA with Tukey’s multiple comparisons ((A) F=2.803, (C) F=78.88 (CB), F=95.66 (CX) F=128.7 (BS)).
Figure 6. Fbxo2 localizes to damaged lysosomes.

(A) Ctrl and I1061T patient fibroblasts were transfected with HA-FBXO2 and then treated with Veh or 1mM LLOMe for 1 hr and stained for HA and Gal3. Co-localization is indicated by yellow staining in merged image. Mander’s coefficients: 0.80 (Ctrl) and 0.81 (I1061T)

(B) I1061T patient fibroblasts were electroporated with HA-FBXO2, and after 48hr treated with 2mM LLOMe for 2hr. Lysates were immunoprecipitated with either HA antibody or control IgG. Arrowheads at ~50kD and ~25kD indicate immunoglobulin heavy and light chains, respectively. Asterisk denotes a non-specific band detected by the LAMP2 antibody.
(C) WT primary cortical cultures were transfected with HA-FBXO2 and treated with Veh or 2mM LLOMe for 1 hr on D9IV. Cell were stained for HA and NeuN.
Figure 7. Fbxo2 mediates CNS lysophagy.

(A) Relative expression of Fbxo2, Fbxo6 and Fbxo27 was determined in WT and Fbxo2-/- primary cortical cultures at D9IV by qPCR.

(B) WT and Fbxo2-/- primary cortical cultures were transfected with EGFP-hGal3 on D4IV, treated with Veh or 2mM LLOMe for 1hr, and stained at various times after washout. Percentage of cells with Gal3+ puncta was quantified.
(C) WT and Fbxo2-/- primary cortical cultures were treated on D9IV with 30µg/mL CHX and 2mM LLOMe for 1 hr and CHX treatment continued for indicated times before lysates were collected. Gal3 levels quantified at the right.

(D) WT and Fbxo2-/- primary cortical cultures were treated with Veh or LLOMe (0.5 or 2 mM) for 1 hr and cell viability was determined by XTT assay 4 hrs after LLOMe washout.

(E) Fbxo2-/- primary cortical cultures were pre-treated with Veh (-) or 100µM E64D for 30min, then treated with 2mM LLOMe for 1 hr and viability determined by XTT assay 4 hrs after LLOMe washout.

Data are shown as mean ± s.e.m. from (A, B, D) 3, (C) 5 or (E) 4 independent experiments. n.s., not significant, *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001 by (A, D-E) one-way ANOVA with Tukey’s multiple comparisons or (B-C) two-way ANOVA with Sidak’s test. ((A) F=81.08, (D) F=23.2, (E) F=14.55).
Figure 8. Loss of Fbxo2 exacerbates the Niemann-Pick C disease phenotype.

(A) Age-dependent performance on balance beam. Mice were trained at 5 wks and run every other week starting at 6 wks. The average of 3 trials was taken and max time was set at 20 s. N=5 males and 5 females per genotype.

(B) Performance on accelerating rotarod from 4-40 rpm at 9 wks. N=5 males and 5 females per genotype.
(C) Kaplan-Meyer survival curves. N=6-10 males and 6-10 females per genotype.

(D) Relative expression of Fbxo2, Fbxo6 and Fbxo27 was determined by qPCR in 8 wk brainstem. N=4 mice per genotype.

(E) Quantification of Purkinje cell density in lobules IV and V of midline cerebellar sections. N=3-4 mice per genotype.

(F) The relative abundance of p62 in brainstem from 8 wk mice was determined by western blot. N=5 mice per genotype.

Data are shown as mean ± s.e.m. n.s., not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by (A) two-way ANOVA, (B,E,F) one-way ANOVA or (C) Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test with (A,E) Bonferroni or (B, F) Tukey’s multiple comparisons (A) F=26.88, (B) F=63.59, (E) F=19.49, (F) F=11.64). Scale bar: 25µm