UNIVERSITY OF CALIFORNIA SAN DIEGO

Elucidating the Relationship Between *NPC1* Mutations and Autophagy Dysregulation Using CRISPR-engineered Human Neurons

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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DEDICATION

This thesis is dedicated to my family, friends, and mentors.

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ABSTRACT OF THE THESIS

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Emma Yating Wu

Master of Science in Biology

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Professor Lawrence Goldstein, Chair
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Niemann Pick Type C1 Disease (NPC1D) is a rare lysosomal storage disease that causes severe neurodegeneration. NPC1D has no treatment or cure, and leads to premature death. This disease is caused by mutations in *NPC1*, which affects the NPC1 lysosomal cholesterol transporter and results in sequestration of lysosomal cholesterol. While the effect

of *NPC1* mutations on cholesterol distribution is well-established, the mechanism underlying neuronal failure is not. Many groups have investigated the role of autophagy in NPC1D disease pathogenesis, as lysosomes, which are plagued in NPC1D, play an important role in autophagy. However, reported phenotypes of autophagy dysfunction have been inconsistent among models of NPC1D, potentially due to the differences among models and the dynamic nature of autophagy.

To further evaluate the role of autophagy, the CRISPR/Cas9 system was used to engineer a human *NPC1* knockout (KO) neuronal line. This new platform overcomes limitations associated with prior models, as it is a human neuronal line free of genetic background biases. This project aims to validate the KO line's ability to recapitulate NPC1D biology and further investigate the relationship between *NPC1* mutations and autophagy dysregulation. KO neurons recapitulate established phenotypes of NPC1D, abnormal distribution of cholesterol and decreased neuronal viability, proving it is a suitable model. Additionally, KO neurons exhibit increased induction of autophagy via Beclin-1 and impaired autophagy flow. These studies suggest that the *NPC1* KO neuronal line is a valuable model to study NPC1D and dysregulation of autophagy is directly related to the loss of function of *NPC1*.

INTRODUCTION

Niemann Pick Type C1 Disease (NPC1D) is a lysosomal storage disorder that results in neurodegeneration, cognitive decline, and ultimately causes premature death (J. Imrie et al., 2007; Jackie Imrie, Heptinstall, Knight, & Strong, 2015; Vanier, 2010). It is a rare disease, affecting only about 1:100,000 live births (Vanier, 2010). However, the incidence rate is likely higher because NPC1D is thought to be underdiagnosed due to multiple reasons, such as diverse clinical presentation and an extensive diagnostic process (Wraith et al., 2009). NPC1D is caused by loss of function mutations in *NPC1* (J. Imrie et al., 2007; Walkley & Suzuki, 2004) which impairs the function of the NPC1 cholesterol transporter, causing cholesterol to be trapped in lysosomes (Vanier, 2010; Walkley & Suzuki, 2004). Additionally, NPC1D is known to cause selective neuronal loss, specifically affecting cells in the cerebellum and brainstem (Sturley, Patterson, Balch, & Liscum, 2004).

Autophagy dysfunction has been linked to NPC1D as well as several other neurodegenerative diseases, such as Alzheimer's Disease (Boland et al., 2008; Komatsu et al., 2006; Nixon, 2013). Autophagy is the main cellular pathway to degrade and dispose of unwanted intracellular protein and damaged organelles (Eskelinen & Saftig, 2009; Mizushima, 2007). Autophagy begins when a phagophore forms around targeted cytosolic material, and when the target cargo becomes fully enclosed, the phagophore is then considered to be an autophagosome (Eskelinen & Saftig, 2009; Mizushima, 2007). The final stage of autophagy involves lysosomes fusing with an autophagosome to create an autolysosome, which is where the previously targeted materials are ultimately turned over (Eskelinen & Saftig, 2009; Mizushima, 2007). Due to the important role lysosomes play in autophagy, this degradation pathway has been studied in relation to NPC1D to determine if

autophagy plays a role in the disease pathogenesis. Some groups have found evidence of only impaired autophagy flux in NPC1D models (Maetzel et al., 2014; Sarkar et al., 2013), while another group observed both impaired autophagy flow and increased induction of autophagy (Ordonez et al., 2012). Interestingly, similar human-derived neuronal models have demonstrated differing phenotypes of autophagy dysfunction (Maetzel et al., 2014; Ordonez et al., 2012), which eludes to a complex relationship between NPC1D and autophagy.

The heterogeneity of NPC1D may play a role in why autophagy dysregulation is still a contested phenotype. Currently, there are over 300 described *NPC1* mutations (Bounford & Gissen, 2014), and NPC1D is known to affect multiple organ systems in patients (Vanier, 2010). Additionally, natural history studies have documented occurrences where patients with similar mutations have different clinical presentations and age of onset of symptoms (J. Imrie et al., 2007; Jackie Imrie et al., 2015). Furthermore, autophagy is difficult to measure, especially in vivo (Klionsky et al., 2016). Assays to properly examine autophagy in living organisms are not well-developed, and several factors can affect autophagy measurements (Klionsky et al., 2016). These limitations may contribute to why there are discrepancies in the autophagy literature for NPC1D. The complexity of both NPC1D and autophagy suggests the need to study this disease, as it relates to autophagy, in an isogenic model, specifically one that is human and neuronal based. Furthermore, elucidating autophagy dysfunction may aid drug development efforts, as autophagy is highly druggable and may be an effective target for potential therapeutics (Nixon, 2013).

To contribute to the diversity of NPC1D models and to further evaluate the role of autophagy in NPC1D, I used the CRISPR/Cas9 genome editing system to engineer an *NPC1* knockout (KO) human induced pluripotent stem cell (hiPSC)-derived neuronal line. I studied this KO line in parallel with hiPSC-derived neuronal lines of an identical genetic background. This investigational method overcomes the genetic bias associated with patient-derived lines and provides a more-accurate representation of NPC1D biology than in previously described non-human and non-neuronal models. Additionally, the *NPC1* KO neuronal line would add to the limited isogenic models (Maetzel et al., 2014) used to investigate NPC1D**,** thus providing another avenue to determine the direct effects of *NPC1* mutations. My thesis focuses on determining the feasibility of using an isogenic human neuronal model to study NPC1D biology and elucidating disease-relevant autophagy phenotypes. I hypothesize that the *NPC1* KO line will 1) prove to be a functional model to study NPC1D by recapitulating established phenotypes of NPC1D, and 2) exhibit aberrant autophagy, and thus indicate that the autophagy dysregulation seen in NPC1D is a direct consequence of the loss of function of NPC1.

RESULTS

Characterization of *NPC1* KO hiPSC-derived line

My prior work in the lab used the CRISPR/Cas9 genome-editing system to engineer a premature stop codon on exon 4 of the *NPC1* gene in a hiPSC donor line. Karyotype analysis indicated a normal karyotype for the *NPC1* KO hiPSC line, however, a micro-amplification on chromosome 1p was visually observed. I continued with my studies because the micro-amplification did not reach the threshold to be considered an abnormal observation and the micro-amplification is present on the KO's control line, therefore the KO and control lines are still isogenic to each other. Through the lab's established neuronal differentiation protocol (Israel et al., 2012), the *NPC1* KO hiPSC line and its control were directed into neural stem cells (NSC) and differentiated into mixed neuronal cultures, which contains both neurons and glial cells (Yuan et al., 2011). During the differentiation process, immunofluorescence staining and confocal microscopy were used to determine if the mutant and control cultures expressed cell-specific markers indicative of hiPSCs, NSCs, astrocytes, and neurons. At the hiPSC stage, both the KO and control hiPSC cultures express nanog, a pluripotency marker (Figure 1A), while the NSC cultures express Nestin, a neural progenitor marker (Figure 1B). The KO and control lines also express the neuronal marker, MAP2, and the astrocyte marker, GFAP, at the mixed neuronal culture stage (Figure 1C). These studies suggest that genetic disruption of *NPC1* does not affect a pluripotent stem cell's ability to successfully transition through the neuronal differentiation phases, and thus the lines can successfully become neuronal lines for future studies.

After observing the KO line's ability to generate relevant cell types, I sought to determine if NPC1 protein was completely ablated due to the introduction of a premature

stop codon. Through quantitative western blot, the *NPC1* KO neuronal line was observed to have almost undetectable levels of NPC1 protein (Figure 1D-E). RT-qPCR analysis of *NPC1* revealed significantly reduced levels of *NPC1* expression (Figure 1F), suggesting nonsense-mediated RNA decay played a role in the ablation of NPC1 protein. By observing transcription and translation levels of NPC1, I determined that *NPC1* is ablated in the KO neuronal line.

NPC1 KO neuronal line exhibits the established phenotypes of NPC1D

Two main cellular phenotypes of NPC1D have been established in the field: accumulation of cholesterol in lysosomes and decreased neuronal viability (Vanier, 2010). These phenotypes were studied to determine if the *NPC1* KO neuronal line recapitulates the hallmark features of NPC1D, which would ultimately validate the KO line as a feasible model to study NPC1D biology. Through established protocols in our lab, neurons were isolated to create a purified neuronal culture (Yuan et al., 2011). Filipin staining of cholesterol and confocal microscopy were used to visualize distribution of cholesterol in purified neurons. Filipin cholesterol staining of the control neurons showed a diffuse staining pattern, which is consistent with normal cholesterol distribution within a healthy cell (Figure 2A). However, filipin cholesterol staining of the *NPC1* KO neurons showed several puncta concentrated in the cell body (Figure 2A). This suggests cholesterol in the KO neurons is predominantly localized to vesicular structures in the cell body. By understanding NPC1D pathology, it can be hypothesized that the cholesterol is most likely concentrated in late endosomes or lysosomes, however, co-localization experiments were not performed to confirm this hypothesis. Quantifying the filipin mean intensity of neuronal

cell bodies further suggested abnormal cholesterol distribution, as the mean intensity was significantly higher in the KO compared to the control (Figure 2B). Additionally, cholesterol distribution was investigated by determining expression levels of HMG-CoA reductase (HMGCR), a key enzyme in the cholesterol biosynthesis pathway (Burg & Espenshade, 2011). Measurement of HMGCR expression levels through RT-qPCR revealed upregulation of HMGCR in the KO neuronal line (Figure 2C), suggesting upregulation of the cholesterol biosynthesis pathway compared to the control neurons. This increase suggests *NPC1* KO neurons are in a state of relative cholesterol starvation. A defective NPC1 transporter impairs efflux of cholesterol from the lysosome, and thus cells may rely on their own biosynthesis pathway to maintain cholesterol homeostasis. These experiments observing cholesterol phenotypes indicate abnormal distribution of cholesterol in the *NPC1* KO compared to the control.

Neuronal viability was observed through flow cytometry analysis of Calcein AM, a cell viability probe. Calcein AM is non-fluorescent until it enters a live cell, where intracellular esterases cleave off the AM ester, allowing the probe to fluoresce (Fritzsche & Mandenius, 2010). Therefore, cell viability can be determined by measuring the amount of Calcein AM green fluorescence. Flow cytometry analysis of Calcein AM in mixed neuronal cultures showed decreased cell viability in the KO compared to the control (Figure 2D). This experiment demonstrates that the *NPC1* KO neuronal line exhibits the decreased cell viability phenotype that leads to neuronal loss in NPC1D. Because the *NPC1* KO neuronal line recapitulates two well-established phenotypes of NPC1D, this line is a viable platform to further study NPC1D biology.

NPC1 KO neuronal line demonstrates autophagy dysregulation

To investigate the hypothesis that autophagy dysregulation contributes to the neurodegeneration in NPC1D, I sought to elucidate the relationship between ablation of *NPC1* and autophagy dysregulation. Induction of autophagy through upregulation of Beclin-1, an autophagy regulator that affects autophagosome development (Kang, Zeh, Lotze, & Tang, 2011), has been observed by a few groups (Ordonez et al., 2012; Pacheco, Kunkel, & Lieberman, 2007), but not others (Maetzel et al., 2014; Sarkar et al., 2013). To determine if autophagy is induced via Beclin-1, RT-qPCR was used to measure expression of Beclin-1. The *NPC1* KO neuronal model showed upregulation of Beclin-1 (Figure 3A), suggesting autophagy is strongly induced through the Beclin-1 pathway.

Autophagy flow was also investigated, as impaired autophagy flow has been observed in various NPC1D models (Ordonez et al., 2012; Sarkar et al., 2013). To examine this phenotype, I probed for LC3-II, an autophagosome marker, as turnover of LC3-II is indicative of autophagy flow (Klionsky et al., 2016). Additionally, protein starvation, which induces autophagy, was used to facilitate characterization of autophagy flow by magnifying the phenomenon, while leupeptin, a lysosomal poison, was used to block the termination of autophagy (Klionsky et al., 2016). As expected, leupeptin successfully blocked autophagy flow in the control neurons (Figure 3B), as there was a significant increase in LC3-II due to treatment (Klionsky et al., 2016). However, this effect was not observed in the KO neurons (Figure 3C), suggesting autophagy flow was already blocked before treatment with leupeptin. Comparing the effects of leupeptin in the control and KO neurons revealed that autophagy flow is relatively impaired in the KO neurons.

The results and discussion section, in part is currently being prepared for submission for publication of the material. Steele, John W.; Williams, Daniel; Miranda, Erika; Gastelum, Grady; Kidwell, Chelsea U.; Plaisted, Warren C.; Peters, Da'Kandryia; Wu, Emma; Cabebe, Laura; Chu, Allen; Goldstein, Lawrence S.B.; Ordoñez, M. Paulina. The thesis author is an author of this material.

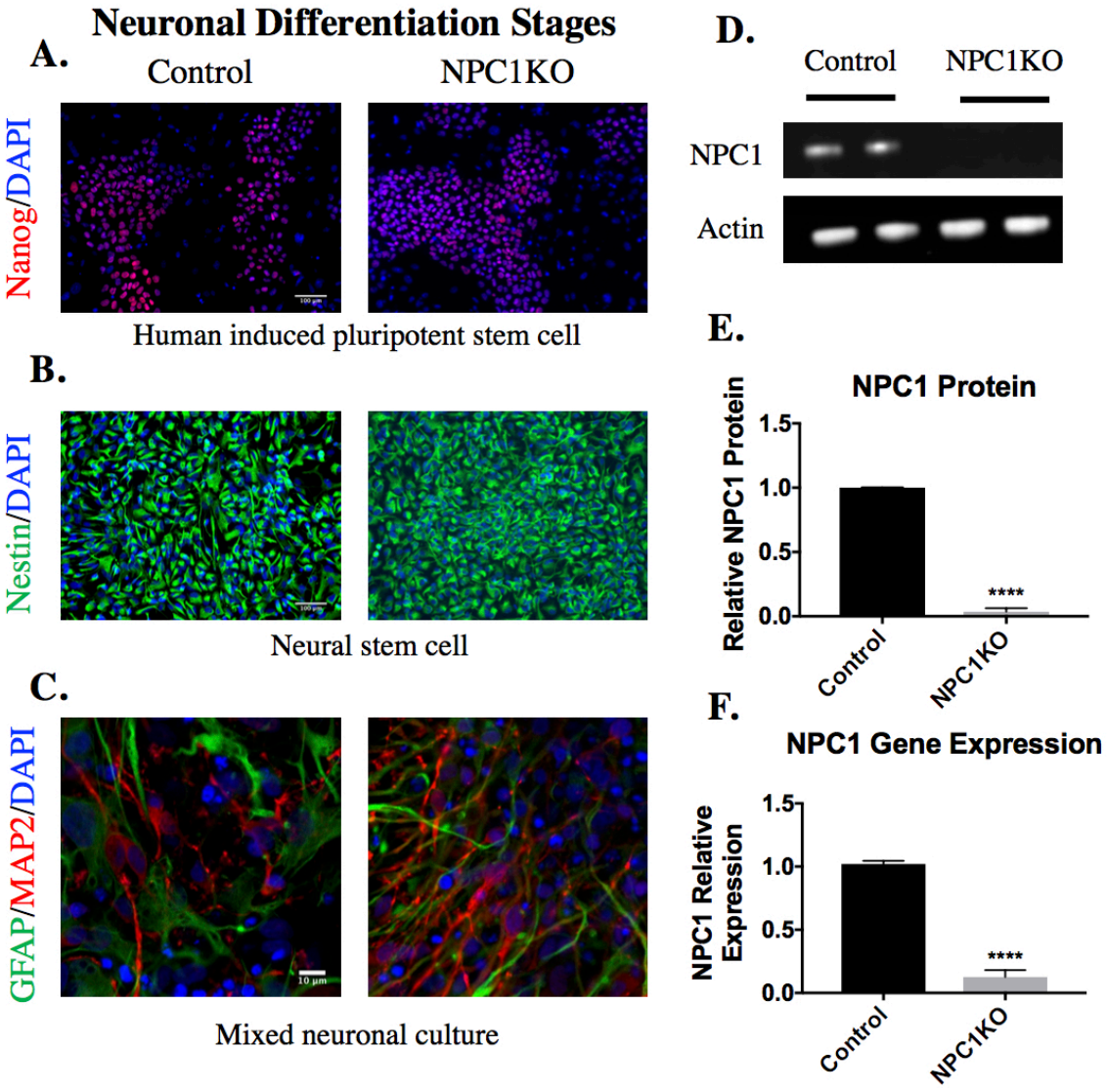

Figure 1. Characterization of *NPC1* KO hiPSC-derived neuronal line
A-C. Immunofluorescence staining and confocal microscopy indicates that the control and *NPC1* KO lines express cell-specific markers for **A.** hiPSCs, **B.** NSCs, **C.** neurons, and astrocytes. This suggests that ablation of *NPC1* does not affect a culture's differentiation capacity. **D.** Western blot of NPC1 protein indicates undetectable levels of NPC1 protein in the KO. **E.** Quantification confirmed ablation of NPC1 protein (n=3; $p<0.0001$). **F.** RT-qPCR measuring expression of *NPC1* showed decreased relative expression in the KO compared to the control (n=3; $p<0.0001$).

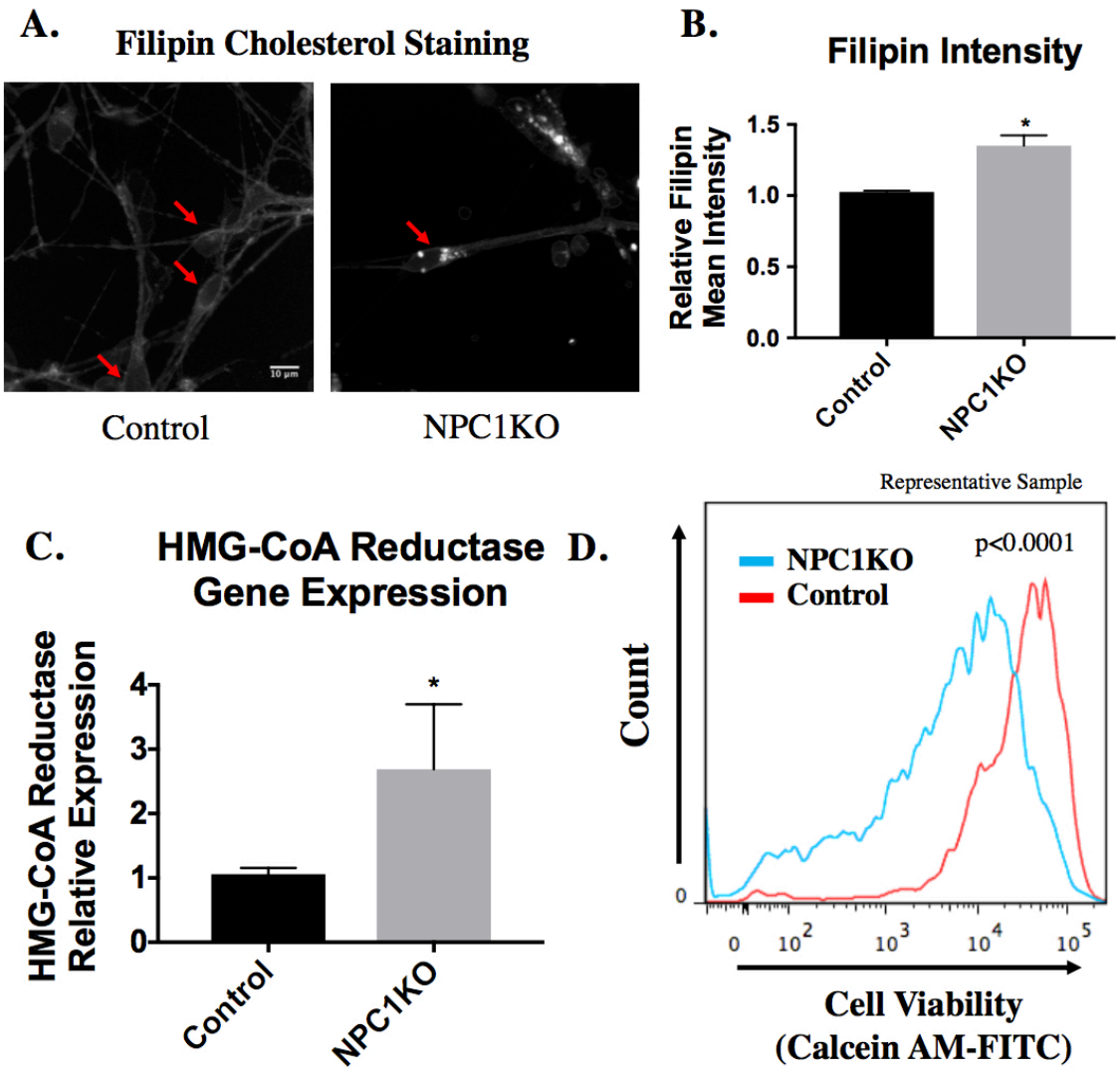

Figure 2. *NPC1* KO neurons exhibit established phenotypes of Niemann Pick Type C1 disease

A. Filipin cholesterol staining and immunofluorescence microscopy shows abnormal punctate distribution in NPC1 KO neuronal cultures. **B.** Quantification of filipin intensity in the cell body indicates abnormal distribution of cholesterol (n=2; p=0.0246). **C.** RT-qPCR measuring levels of HMG-CoA Reductase showed increased expression in the KO compared to the control (n=3; p=0.05). **D.** Analysis of cell viability by flow cytometry and Calcein AM fluorescent dye showed decreased cell viability in the NPC1 KO (p<0.0001).

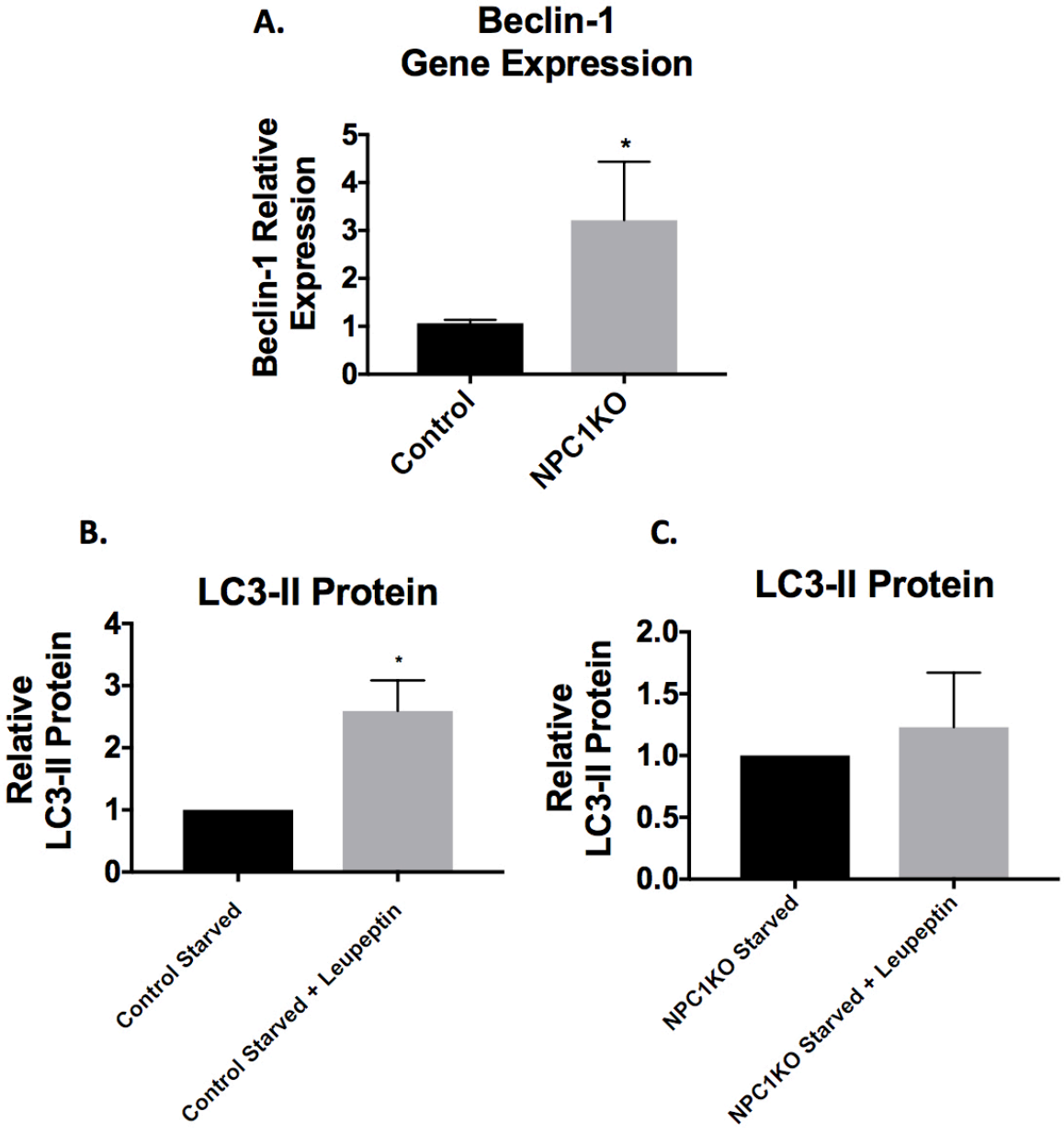

Figure 3. *NPC1* KO neurons exhibit increased induction of autophagy and impaired autophagy flow

A. RT-qPCR measuring levels of Beclin-1, an autophagy regulator, shows increased expression in the KO compared to the control (n=4; p=0.0126). **B-C.** Quantitative western blot examines autophagy dynamics by probing for LC3-II, an autophagosome marker. To observe autophagy flow, cultures are protein-starved and treated with the lysosomal poison, leupeptin. Leupeptin did not exert an effect on the KO neurons, suggesting that autophagy flow is relatively impaired in the KO cultures (Control: n=2, p=0.0443; *NPC1*KO: n=3, p=0.4202).

DISCUSSION

My thesis aims to characterize a newly developed isogenic neuronal line and elucidate the relationship between the loss of function of *NPC1* and autophagy dysfunction. My data show that the *NPC1* KO neuronal line recapitulates the established phenotypes of NPC1D, cholesterol sequestration in the lysosome and decreased neuronal viability (Vanier, 2010), thus validating the ability of this platform to be an informative NPC1D model. Additionally, using this innovative line, I have observed both impaired autophagy flow as well as increased induction of autophagy via Beclin-1. Because these studies were performed in an isogenic KO model, these results indicate that autophagy dysfunction in NPC1D is a direct cause of the loss of function of *NPC1*.

Interestingly, these studies shed light on the effect of cholesterol sequestration in NPC1D biology. The KO neurons exhibit complete knockout of NPC1, yet they do not have a diminished capacity to grow or differentiate effectively. If cholesterol sequestration solely and directly caused neurodegeneration, then the KO neurons should be severely impaired due to the inability to effectively distribute cholesterol, as cholesterol is critical to many neuronal functions (Zhang & Liu, 2015). Because severe impairment was not observed, it suggests that cholesterol sequestration is not the direct cause of the neuronal failure in NPC1D (Ko et al., 2005). Other mechanisms downstream of cholesterol sequestration may be responsible for the neuronal loss, which furthers the hypothesis that autophagy affects NPC1D pathogenesis.

Additionally, my thesis project adds to the current literature that describes how autophagy dysfunction contributes to the pathology of NPC1D. Initial characterization of autophagy dysregulation in KO neurons complements prior studies in patient-derived lines

by our group (Steele et al., 2017), as they both indicate increased induction of autophagy via Beclin-1 and impaired autophagy flow. Together, our studies provide insight in how autophagy is influenced in NPC1D, and the abnormal phenotypes observed can be used as a target for drug discovery. Successful therapies should not only address the induction of autophagy, but also help with autophagy flow. However, autophagy is a delicate pathway that is critical to neuronal survival (Hara et al., 2006; Komatsu et al., 2006). Therefore, therapies that target autophagy dysfunction have to be systematically investigated in human neurons to determine if they over-affect autophagy to a point that autophagy underperforms and can no longer participate in degradation or other homeostatic processes. Additionally, autophagy flow should be prioritized of the two dysfunction phenotypes (Ordonez et al., 2012), as it is the more downstream process and may be able to overcome increased induction of autophagy due to normal and efficient termination. This approach adds to other described therapeutic strategies that focus on autophagy dysfunction for NPC1D (Ordonez et al., 2012; Sarkar et al., 2013). Currently, there are no FDA-approved therapies for NPC1D, however many patients are treated with miglustat, a compound approved in Europe (Wraith et al., 2010), and 2-hydroxypropyl-β-cyclodextrin, which is moving to Phase 2b/3 clinical trials (Ory et al., 2017). While 2-hydroxypropyl-β-cyclodextrin is the most promising candidate, it still is not an ideal treatment option since it does not cross the blood-brain barrier, and thus must be administered intrathecally regularly (Ory et al., 2017). Therefore, any potential therapies that target autophagy dysfunction would add to the limited treatment options available for NPC1D.

Despite the promising findings reported in my thesis, there are also limitations of these studies. One limitation includes using only one *NPC1* KO neuronal line. Genetic

engineering through the CRISPR/Cas9 system may cause off-target effects (Fu et al., 2013; Pattanayak et al., 2013), in which multiple engineered lines could control for. However, due to the difficulties associated with genetic engineering, only one line was successfully created. In the future, more lines should be developed to follow up on my results. Additionally, the control used in these studies did not undergo the CRISPR process, which serves also as a limitation. Using controls that underwent the genetic engineering process also acts as a way to control for any unwanted effects of the genetic engineering process. Again, future experiments have been planned to generate such lines and to perform the necessary follow-up studies.

Furthermore, another group has also investigated autophagy in NPC1D using an isogenic hiPSC-derived neuronal line (Maetzel et al., 2014). However, instead of causing genetic disruption, they used TALEN genome-editing technology to correct a mutation in a patient-derived line (Maetzel et al., 2014). The mutant neuronal line exhibited impaired autophagy flow, but did not show upregulation of Beclin-1 (Maetzel et al., 2014). Genetic correction of the line's mutation rescued this autophagy dysfunction, which suggested that impaired autophagy flow is directly correlated to the loss of function of *NPC1* (Maetzel et al., 2014). Because this model does not identically mimic the dysregulation observed in the *NPC1* KO neurons, it suggests that there may be other underlying mechanisms contributing to NPC1D pathology besides loss of function of *NPC1*. Possible explanations include the influence of genetic background on NPC1D pathology or that dysfunction phenotypes are mutation-specific. If the type of mutation or genetic background plays a role in the disease pathogenesis, it would suggest that an isogenic model may be too simple to fully capture the complexity of NPC1D, and using a pool of patient-derived lines would be more effective for

future studies. Future studies should compare the severity of pathogenic phenotypes among isogenic and patient-derived lines in order to determine if isogenic NPC1D models truly capture the biology of NPC1D.

Importantly, my studies act as the first step in validating the mechanistic and drug discovery studies our group previously performed (Steele et al., 2017). Unpublished data from our group show similar autophagy dysfunction, but also indicate that disrupted autophagy contributes to mitochondrial dysfunction and the accumulation of depolarized mitochondria (Steele et al., 2017). Additionally, we identified a new class of potential NPC1D therapies that target mitochondrial dysfunction through a drug screen using FDA-approved compounds (Steele et al., 2017). However, these mechanistic and drug discovery studies were performed in patient-derived neuronal lines, and thus they may be biased from the patients' genetic backgrounds. My studies are following up on these results, specifically addressing and overcoming the limitations of patient-derived lines. Currently, we are moving from characterizing autophagy dysfunction to probing mitochondrial health in the KO neurons, specifically focusing on mitochondrial depolarization and mitochondrial fusion and fission dynamics. The NPC1KO neurons will be a valuable platform as we further validate the proposed mechanism underlying the neuronal failure in NPC1D and the recently discovered therapeutic candidates.

The results and discussion section, in part is currently being prepared for submission for publication of the material. Steele, John W.; Williams, Daniel; Miranda, Erika; Gastelum, Grady; Kidwell, Chelsea U.; Plaisted, Warren C.; Peters, Da'Kandryia; Wu, Emma; Cabebe, Laura; Chu, Allen; Goldstein, Lawrence S.B.; Ordoñez, M. Paulina. The thesis author is an author of this material.

MATERIALS AND METHODS

Cell Culture

hiPSCs were grown on mouse embryonic fibroblast feeder cells in iPSC media (knockout DMEM with knockout serum replacement, pen-strep, non-essential amino acids, glutamax, and 2-Mercaptoethanol) with FGF. NSCs were generated by co-culturing iPSCs with PA6 cells in PA6 differentiation media (Glasgow DMEM with knockout serum replacement, sodium pyruvate, nonessential amino acids, pen-strep, and 2-Mercaptoethanol) with 10 uM SB431542 and 0.5 ug/mL Noggin. NSCs were purified by fluorescence activated cell sorting (FACS) using established protocols (Yuan et al., 2011). NSCs were cultured in NPC base media (DMEM F12 + Glutamax with pen-strep, B27, and N2) with FGF. To initiate neuronal differentiation, FGF was withdrawn from NSC cultures. Mixed neuronal cultures were FACS-purified to isolate neurons using established protocols (Yuan et al., 2011). Purified neuronal cultures were grown in NPC base media without additional factors. NSCs, mixed neuronal cultures, and purified neuronal cultures were grown on plates coated with poly-L-ornithine and laminin. All cell types were dissociated using Accutase.

Western blot

Samples were harvested at $5x10^6$ cells/sample and syringed in NP40 lysis buffer with protease inhibitor. Thermo Scientific Pierce BCA protein assay kit was used to evaluate concentration of the protein samples. Samples were then loaded on a NuPAGE 4-12% Bis-Tris Protein Gel (Invitrogen) and then transferred to a PVDF membrane (Bio-rad). Membrane was then blocked for 30 minutes at room temperature with Li-cor Odyssey blocking buffer and then probed with a primary antibody overnight at 4°C with agitation.

Primary antibody was washed off using TBS-Tween and then incubated with secondary antibody for 1 hour at room temperature with agitation. Secondary antibody was washed off before being developed on the Li-cor Odyssey Imaging System.

Table 1. Primary antibodies used for western blot

Protein	Dilution	Manufacturer	Catalog Number
NPC1	1:1000	Abcam	ab36983
LC3	1:1000	Novus Biologicals	NB600-1384

RT-qPCR

Mixed neuronal cultures were harvested at $1x10^6$ cells/sample. mRNA was extracted using Qiagen RNeasy mini kit. Samples were DNase treated (Ambion) before being synthesized into cDNA via Invitrogen's SuperScript first strand synthesis system. RT-PCR was set up with Power SYBR Green PCR Master Mix and primers found in Table 2, and then ran in Applied Biosystems 7300 Real-Time PCR system. cDNA samples were ran in triplicates and analyzed using the ΔΔCT method. Data was normalized to a housekeeping gene.

Table 2. RT-PCR primer sequences

Gene	FWD Primer (5' to 3')	REV Primer (5' to 3')
NPC1	GTC CAG CGC AGG TGT TTT C	GCC GAA CAT CAC AAC AGA GAC
HMG-CoA Reductase	CGT GGA ATG GCA ATT TTA GGT CC	ATT TCA AGC TGA CGT ACC CCT
Beclin-1	GCC CAG ACA GGA CTC TCT TAG	TGA ACA CAC TTG CCA GTC TTC
RPL27	AAA CCG CAG TTT CTG GAA GA	TGG ATA TCC CCT TGG ACA AA
TBP	GAA CCA CGG CAC TGA TTT TC	CCC CAC CAT GTT CTG AAT G

Viability Assay

Flow cytometry was used to quantify the Calcein AM cell viability assay. Mixed neuronal cultures were incubated in Calcein AM fluorescent dye (Thermo Scientific; 1:3000) for 20 minutes at 37°C. After incubation, cells were washed once with PBS and then manually dissociated. Cells were resuspended and strained before being analyzed on the FACSAria II cytometer (BD Biosciences, San Jose, CA, USA). FlowJo was used for data processing.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 20-30 minutes, then permeabilized with 0.1% Triton X-100 for 10 minutes. After permeabilization, the samples were blocked with 10% fetal calf serum in PBS for 30 minutes and then incubated with a primary antibody overnight at 4 degrees. The primary antibody (Table 3) was then washed using 1% fetal calf serum in PBS before a secondary antibody (Table 3) was added. The secondary antibody was incubated for an hour at room temperature. The secondary antibody was washed with 1% fetal calf serum in PBS before being imaged on a Zeiss confocal microscope or Zeiss axio vert.A1 inverted microscope. DAPI was added before the final wash for 10 minutes.

Table 3. Primary and secondary antibodies used for immunofluorescence

Antibody	Dilution	Manufacturer	Catalog Number
Nanog	1:200	Santa Cruz	sc33759
Nestin	1:500	Millipore	ABD69
MAP2	1:500	Abcam	ab5392
GFAP	1:200	Agilent	Z033429-2
Anti-Chicken DyLight 650	1:500	Abcam	ab96954
Anti-rabbit Alexa 488	1:200	Invitrogen	A-21206
Anti-rabbit Alexa 568	1:200	Invitrogen	A10042

Filipin Staining

Purified neuronal cultures were fixed with 4% PFA, and then incubated with filipin (Sigma, 50 ug/ml) for an hour at room temperature. Samples were imaged using Zeiss confocal. ImageJ was used to quantify mean filipin intensity of the neuronal cell bodies.

Statistics

Data was analyzed through GraphPad Prism Version 7, and a two-tailed t-test with a 95% confidence level was used to determine significance.

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