UNIVERSITY OF CALIFORNIA SAN DIEGO

Delineating Roles of Beclin1 and Beclin2 in Autophagy

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Monica C. Jeung

Committee in charge:

Professor Åsa B. Gustafsson, Chair
Professor Sonya Neal, Co-Chair
Professor Amy Kiger

2021

The thesis of Monica C. Jeung is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Åsa Gustafsson for allowing me to complete the research for my thesis in her lab. Without her support, my research and success would not have been possible

I would also like to acknowledge Eileen Gonzalez for her mentorship and guidance. Her instruction and support have been crucial to my success in this lab and in my research.

I would like to acknowledge the entire Gustafsson lab for their support and encouragement, without whom I could not have finished my research.

Chapters 1, 2, and 4 are currently being prepared for submission for publication of the material. Gonzalez, Eileen, Jeung, Monica, Gustafsson, Åsa B. The thesis author was a coauthor of this material.

ABSTRACT OF THE THESIS

Delineating Roles of Beclin1 and Beclin2 in Autophagy

by

Monica C. Jeung

Master of Science in Biology

University of California San Diego, 2021

Professor Åsa B. Gustafsson, Chair
Professor Sonya Neal, Co-Chair

Autophagy is a highly conserved degradation pathway which is regulated by the autophagy protein Beclin1. Beclin1 has been reported to be essential for initiation of autophagy. Here, we characterize the roles of Beclin1 and Beclin2 in autophagy and mitophagy. Lack of Beclin1 or Beclin2 did not prevent the initiation of autophagy in cells. However, Beclin1 was found to be important for Parkin-mediated mitophagy, while Beclin2 was dispensable. Our findings suggest that autophagy is functional without Beclin1 or Beclin2, but Beclin1 is required for functional mitophagy.

Introduction

Autophagy

Autophagy is an evolutionarily conserved degradation pathway in which cytosolic cargo is sequestered in vesicles called autophagosomes. Turnover of proteins and organelles in cells is necessary for maintaining homeostasis and adapting to stress. Autophagy also removes cytotoxic protein aggregates, damaged organelles, and regulates organelle numbers. It is activated in response to various forms of metabolic and oxidative stressors, and generally limits the effects of organelle damage or cell death. Importantly, autophagy is also one of the primary cellular responses to starvation. It facilitates the recycling of key materials, such as amino acids and fatty acids from proteins and lipids, which can be used in processes like gluconeogenesis when resources and energy are limited (Sciarretta et al., 2018). Early studies of autophagy demonstrated that autophagy-deficient mutants of *S. cerevisiae* were more susceptible to death than wild-type (WT) under nitrogen-starvation conditions (Tsukada & Ohsumi, 1993). Later studies showed that nutrient starvation induced autophagy in most tissues *in vivo* (Mizushima et al., *MBoC,* 2003) and that inhibition of autophagy led to lower amino acid levels and survival rates in mice during the neonatal starvation period after birth (Kuma at al., 2004).

Under baseline conditions, autophagy is the primary mechanism for removing cytotoxic protein aggregates, which is particularly important in terminally differentiated cells such as cardiomyocytes and neurons. Autophagic processes are also involved in immune responses, aiding in the degradation of intracellular bacteria and viruses, as well as antigen presentation and lymphocyte development (Choi et al., 2013). Autophagy has a wide variety of roles in the heart, including clearance of dysfunctional mitochondria and protecting against cardiomyocyte

apoptosis (Ikeda et al., 2014). It is also activated as a protective response in response to ischemia (Matsui et al., 2007).

During autophagy, vesicles called autophagosomes engulf damaged proteins or organelles and fuse with lysosomes for degradation and elimination (Gustafsson & Dorn, 2019) (Fig 1). Specifically, induction of autophagy starts with the formation of a phagophore membrane, which is usually derived from ER membranes. Phagophore formation is initiated by the unc-51-like-autophagy-activating kinase macromolecular complex (ULK complex), which is composed of Atg13, ULK1 or ULK2, FIP200 and Atg101 (Itakura et al., 2008). The ULK1/2 complex phosphorylates Beclin1 (Atg6) which leads to activation of the Beclin1-Vps34 complex (also referred to as PI3KC3) (Fig. 2). This complex is composed of Beclin1, Atg14L, Vps34, and Vps15 (Russell et al., 2013). The Vps34 complex is responsible for the formation of phosphatidylinositol-3-phosphate (PI3P) which promotes recruitment of various autophagy proteins to the phagophore, resulting in the growth of the phagophore. As the membrane grows, the phagophore closes around its cytoplasmic cargo (Fig. 1).

Elongation of the phagophore is mediated by the Atg5-Atg12 conjugation system. Atg12 is linked to Atg5 (Mizushima et al., 1998), and then the Atg12-Atg5 complex is conjugated with Atg16L (Mizushima et al., *J. Cell Sci.,* 2003). This complex is necessary for elongation but is removed when the autophagosome is mature. As elongation proceeds, microtubule-associated protein 1 light chain 3 (LC3) is conjugated to phospatidylethanolamine to form LC3II. Conjugated LC3 (LC3II) is incorporated into the phagophore, resulting in maturation of the autophagosome (Kabeya et al., 2000) (Fig. 2). The autophagosome fuses with a lysosome to form an autolysosome. Atg14 also plays a role in maturation, localizing to autophagosomes and

promoting interaction with lysosome-associated proteins (Diao et al., 2015). Acid hydrolases from the lysosomes then degrade the autophagosomal contents (Sciaretta et al., 2018).

Autophagy has been demonstrated to be dysregulated in neurodegenerative diseases such as Parkinson's (Webb et al., 2003), Huntington's (Ravikumar et al., 2004) and Alzheimer's (Pickford et al., 2008). Failure in autophagy has also been implicated in some forms of cardiomyopathy (Tannous et al., 2008) and in pancreatitis (Helin et al., 1980). Autophagy has also been reported to have both protective and damage-inducing roles in the pathologies of various cancers by clearing reactive oxygen species and damage in healthy cells or promoting these same protective behaviors in malignant cells (Ravikumar et al., 2010). Similarly, it has been reported to have protective and damaging effects in strokes, either by recycling injured cells or promoting increased cell death (Rami et al., 2008). Appropriate regulation of autophagy is vital to the overall health of an organism.

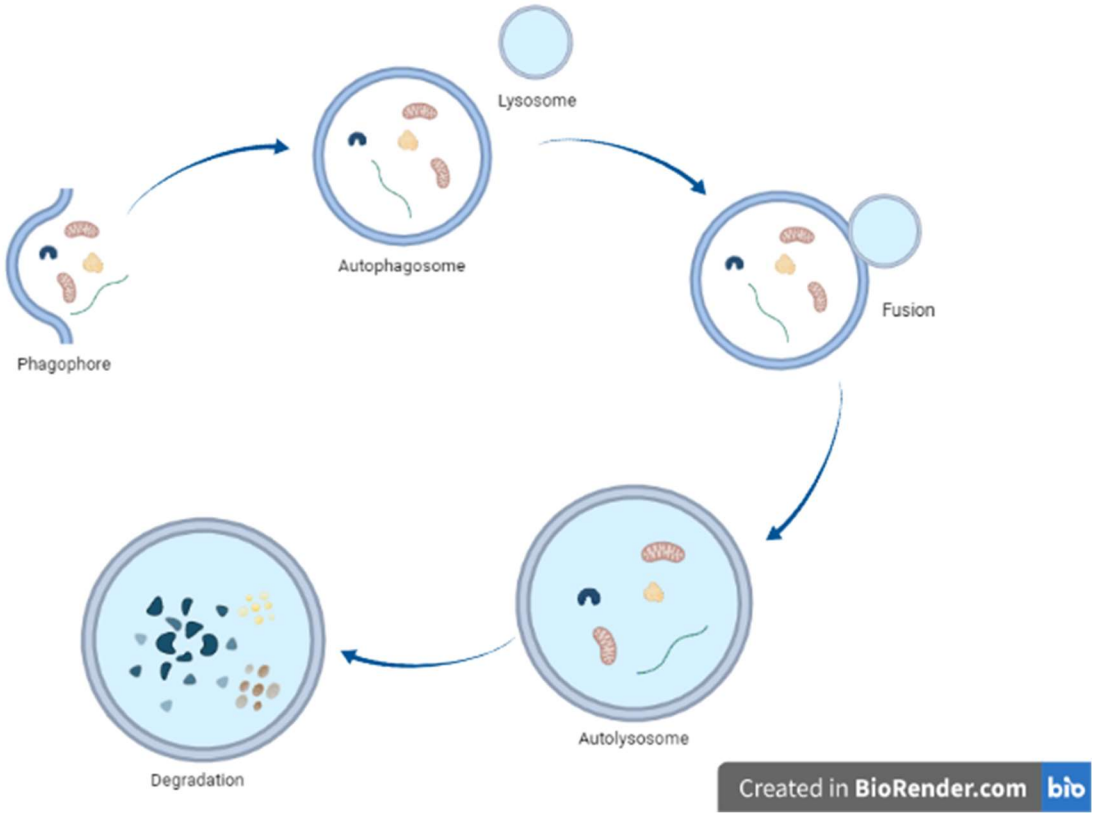

Figure 1: Overview of autophagy. Proteins and organelles are engulfed by a growing autophagosome. The autophagosome fuses with a lysosome to degrade the protein or organelle cargo, forming autolysosomes. Cargo is degraded and components are recycled.

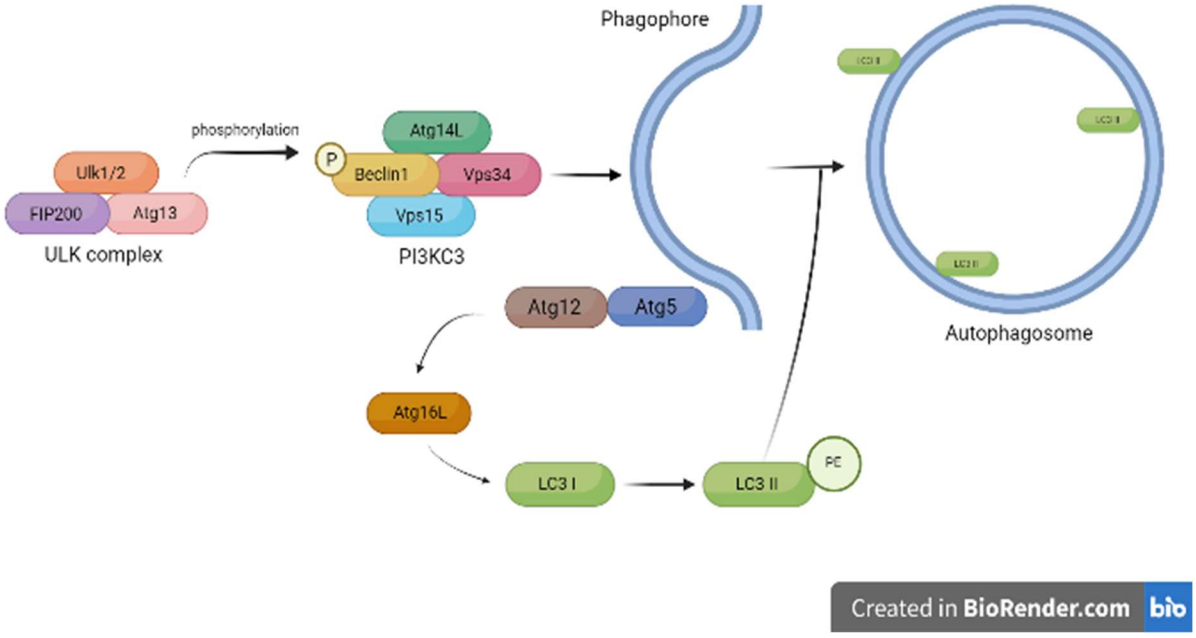

Figure 2: Mechanism of autophagy. ULK1/2 forms a complex with FIP200 and Atg13 to phosphorylate Beclin1. Beclin1 then forms the PI3KC3 complex with Vps34, Vps15, and Atg14L, initiating the formation of the phagophore. Atg12 is conjugated with Atg5, and the Atg12-Atg5 complex conjugates with Atg16L. Atg16L facilitates the conjugation of LC3I to phosphatidylethanolamine to form LC3II. LC3II attaches to the growing phagophore.

PINK1/Parkin-mediated mitophagy

Mitochondrial autophagy (mitophagy) is a form of organelle-specific autophagy that involves degradation of mitochondria. Initiation of mitophagy requires selective targeting of aged or damaged mitochondria. Mitophagy of dysfunctional mitochondria is regulated by the PINK1/Parkin pathway. PINK1 is a serine/threonine kinase which contains a mitochondrial targeting sequence (MTS) directing it to mitochondria in the cell. Upon contact with healthy mitochondria, it is rapidly imported and cleaved by mitochondrial proteases. The cleaved protein is subsequently degraded by the ubiquitin proteasome system (Eiyama & Okamoto, 2015). Damaged mitochondria that lack mitochondrial membrane potential no longer import PINK1. Instead it accumulates on the surface of the mitochondria, where it associates with the outer membrane protein TOM20. PINK1 is subsequently autophosphorylated, converting it to an active kinase which recruits and activates Parkin (Lazarou et al., 2012). Parkin is an E3 ubiquitin ligase that ubiquitinates proteins on the mitochondrial outer membrane. The ubiquitin is recognized and bound by various adaptor proteins such as p62, which subsequently bind to LC3 on the autophagosome surface to tether the mitochondria to the autophagosome (Lazarou et al., 2015) (Fig. 3).

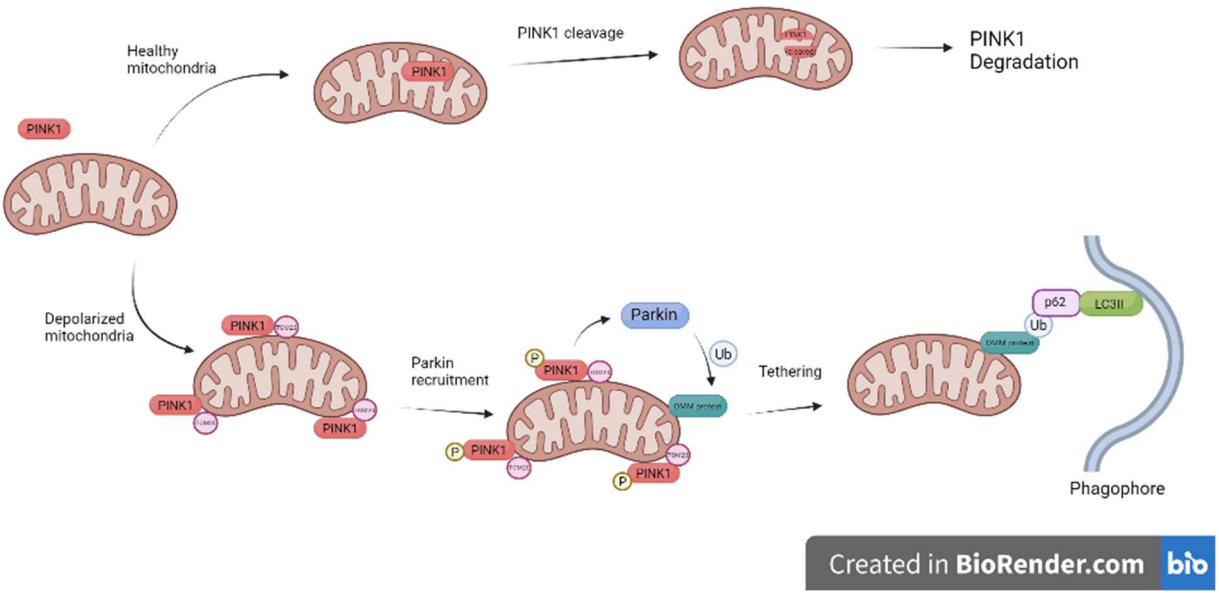

Figure 3: Regulation of PINK1/Parkin-mediated mitophagy. PINK1 is imported, then cleaved in healthy mitochondria, but accumulates on the surface of depolarized mitochondria. PINK1 interacts with TOM20, and recruits Parkin to the mitochondrial surface. Parkin, in turn, ubiquitinates different proteins on the outer mitochondrial membrane. The ubiquitinated protein interacts with adaptor proteins such as p62, which in turn interacts with LC3II, tethering the mitochondria to the growing phagophore.

Beclin1: role in autophagy and mitophagy

Beclin1 is an upstream regulator for the initiation of autophagy and a critical part of an organism's development and survival, as Beclin1 knockout mice demonstrate early embryonic lethality (Levine et al., 2015). Beclin1 heterozygous mice also have a higher occurrence of spontaneous tumors, suggesting that it functions as a tumor suppressor (Yue et al., 2003). In addition, Beclin1 has been demonstrated to play a role in mitophagy, where knockdown of Beclin1 leads to reduced mitochondrial clearance (Gelmetti et al., 2017). Beclin1 is a component of the class III phosphatidylinositol 3-kinase complex (PtdIns3K) consisting of Vps34, Atg14 (an autophagy-specific protein), and Vps15, which initiates the formation of the phagophore (Fig 2), the precursor membrane that will form the autophagosome (Gelmetti et al., 2017). It has been reported that the PI3KC3 complex is recruited to the site of phagophore formation by Atg-14 or UVRAG (Itakura & Mizushima, 2009).

Structural studies of Beclin1 suggest that the Beclin1 N-terminal is involved in regulating PI3KC3-complex lipid kinase activity (Levine et al., 2015). The ER is known to donate the membrane for the growing autophagosome. Thus, it is not surprising that Beclin1 localizes to mitochondria-ER contact sites (MAMs). MAMs have been shown to be the initial site of autophagosome formation during mitophagy, and there is increased localization of Beclin1 to MAMs upon initiation of autophagy (Gelmetti et al., 2017).

Beclin2

Beclin2 is a Beclin1 homolog, and little is known about its functions in cells. Its protein structure is similar to Beclin1, but while Beclin1 orthologs are present in multiple non-mammalian vertebrate species, Beclin2 appear to only be expressed in mammalian cells (He et

al., 2013). Beclin2 has been demonstrated to play a role in autophagy, where knockdown of Beclin2 in HeLa cells led to decreased autophagosome formation. Beclin2 has also been shown to interact with some of the binding partners of Beclin1. For instance, Beclin2 interacts with VPS34 and Atg14 in the PtdIns3K complex (He et al., 2013). Beclin2 also regulates endosomal trafficking of G-protein coupled receptors (GPCRs) to lysosomes for degradation (He et al., 2013). Further studies of Beclin2 have demonstrated that it is involved in a specific autophagy pathway that suppresses innate immune signaling through the degradation of kinases involved in pro-inflammatory signals. (Zhu et al., 2020).

Rationale

Autophagy and mitophagy are key processes involved in maintaining cellular homeostasis and preventing development of various diseases. Therefore, it is important to understand the proteins involved in regulation of these processes. Beclin1 is known to be an important regulator of autophagy, but little is currently known about its ortholog Beclin2. Beclin2 has been demonstrated to participate in autophagy, but the extent of its role has not yet been characterized. Whether or not it regulates mitophagy is also unknown. The objective of my research is to explore these questions.

In my research I have used in vitro models to examine the hypothesis that Beclin2 can compensate for the loss of Beclin1 in autophagy but not in Parkin-mediated mitophagy. To test this hypothesis, I have examined the following questions:

1) Are Beclin2 knockout HeLa cells deficient in autophagy?
2) Do Beclin2 knockout HeLa cells have a defect in mitophagy?

Additionally, to further mitophagy research both in this laboratory and outside it, I have participated in the development of a novel mitophagy reporter for use both in cells via adenoviral infections and in transgenic mice.

Materials and Methods

Cell Culture

WT and *Becn1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), and WT, BECN1$^{-/-}$ and BECN2$^{-/-}$ HeLa cells were cultured in media consisting of Dulbecco's Modified Eagle Medium (DMEM) with GlutaMax (Gibco), 10% fetal bovine serum (FBS, Gibco), 100U/mL penicillin (Gemini) and 100g/ml streptomycin (Gemini). Cells were maintained at 37°C in 5% CO_2.

Induction of Autophagy and Mitophagy

Cells were subjected to nutrient deprivation using DMEM lacking D-glucose, sodium pyruvate (Gibco), and FBS. Mitochondrial autophagy was induced with FCCP (Sigma-Aldrich) or oligomycin A (Sigma-Aldrich)/antimycin A (Sigma-Aldrich). Autophagy activity was evaluated using Bafilomycin A (Millipore).

cDNA Constructs, Transfections and Adenoviral Infections

The MTS-pH2-mCh-TM (mitophagy reporter) construct and the Beclin1S15A mutant were synthesized by GenScript. pJG/alpha-MHC was a gift from Jeffrey Robbins (Addgene plasmid #55594) (Gulick et al., 1991). MTS-pH2-mCh-TM forward primer was designed to include SalI and recognize the mitochondrial targeting sequence (MTS) (5' gcgctGTCGACATGGCCTCCACT CGTGTCCTCGC 3') and the reverse primer was designed to include HindIII and recognize the transmembrane region (TM) (5' gcgctAAGCTTTCATATTAGATATGCCAGACC AGC CCC CTA CTC CAG 3').

The myc-Parkin plasmid (Addgene plasmid #17613) and the GFP-LC3 plasmid (Addgene plasmid #11546) were generously gifted by Ted Dawson. The mCherry-Parkin and GFP-LC3 adenoviruses have been previously described (Kubli et al., 2013). For experiments involving transfections, HeLa cells were transfected with cDNA using FuGene 6 Transfection Reagent

(Promega) according to manufacturer instructions. For adenoviral infections, cells were infected with viruses containing the relevant DNA in media consisting of DMEM + GlutaMax supplemented with 2% heat-inactivated serum for 3 hours. All experiments were performed 24 hours after infection or transfection.

Assessment of Autophagy and Mitophagy by Fluorescence Microscopy

HeLa cells and MEFs were plated on Mattek dishes, then infected the next day. 24hrs after infection, cells were treated and then fixed with 4% paraformaldehyde for 25 minutes. Cells were infected with GFP-LC3 or transfected with myc-Parkin and MTS-ph2-mCh-TM. For myc staining, cells were permeabilized with 0.2% Triton X-100, then blocked with 5% normal goat serum (Vector) and incubated with anti-myc (MilliporeSigma) in 5% normal goat serum for 3hrs at 37°C. Cells were washed with PBS and then incubated with secondary antibodies at 37°C for 2hrs. Nuclei were counterstained with Hoescht 33342 (1mg/mL) (Invitrogen). Images were captured using a Nikon ECLIPSE Ti2 inverted microscope with a 60x objective. Autophagy was assessed in 25 individual cells per experiment by counting the number of GFP-LC3 puncta in each cell. Mitophagy was assessed in 25 individual cells per experiment using our mitophagy reporter. Mitochondria not undergoing mitophagy were indicated by yellow fluorescence. Mitophagy was indicated by the presence of mCherry (red) puncta, and the number of red puncta per cell was counted.

Western Blot Analysis

MEFs and HeLa cells were harvested and lysed in ice-cold lysis buffer consisting of 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 20,000xg for 20 minutes. A Bradford assay was performed on lysates to determine the protein concentration. 4X

NuPAGE LDS Sample Buffer (Novex) and 50uM dithiothreitol (DTT) were added to the samples. Proteins were separated on 12% NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes. Proteins were detected using antibodies against LC3A/B (Cell Signaling), p62 (Abcam), COX IV (subunit 1) (Invitrogen), Beclin1 (Cell Signaling), TOM20 (Santa Cruz), TIM23 (BD Biosciences), Actin (GeneTex), and GAPDH (GeneTex). Proteins were visualized using a BioRad ChemiDoc XRS+, and bands were quantified using ImageLab.

Statistical Analysis

Comparisons between experimental groups were performed using one-way ANOVA followed by Bartlett's test, or by two-way ANOVA followed by a Tukey test. A p-value of less than 0.05 was considered statistically significant. Data are mean ± SEM.

Chapter 1

Autophagy is intact in Beclin1-deficient cells

Beclin1 is a key protein involved in the initiation of canonical autophagy. It is part of the PI3KC3 complex which initiates the growth of the phagophore, the membrane which precedes the autophagosome. However, there are reports that autophagy can be induced independent of Beclin1. Here, we investigated the effects of Beclin1 deficiency on autophagosome formation in MEFs.

To investigate the effect of Beclin1 deficiency on baseline autophagic flux in cells, WT and *Becn1*$^{-/-}$ MEFs were treated with Bafilomycin A1 (BafA1). BafA1 disrupts lysosomal acidification, leading to accumulation of autophagosomes. During autophagosome maturation, LC3I is converted to LC3II via conjugation to phosphatidylethanolamine. LC3II accumulates on the autophagosome surface, and p62 acts as an adaptor protein, tethering ubiquitinated proteins to LC3II. LC3II and p62 accumulation are therefore a common method to assess autophagy. Significant increases in LC3II and p62 were observed in both WT and *Becn1*$^{-/-}$ MEFs treated with BafA1 (Fig. 4A & 4B). Accumulation of p62 was significant and comparable in WT and *Becn1*$^{-/-}$ MEFs. These results were confirmed in WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3. A similar percentage of WT and *Becn1*$^{-/-}$ cells exhibited punctate GFP-LC3 accumulation in response to BafA1 (Fig. 4C & 4D). Overall, these results indicate that autophagosome formation and baseline autophagic flux are maintained in *Becn1*$^{-/-}$ MEFs.

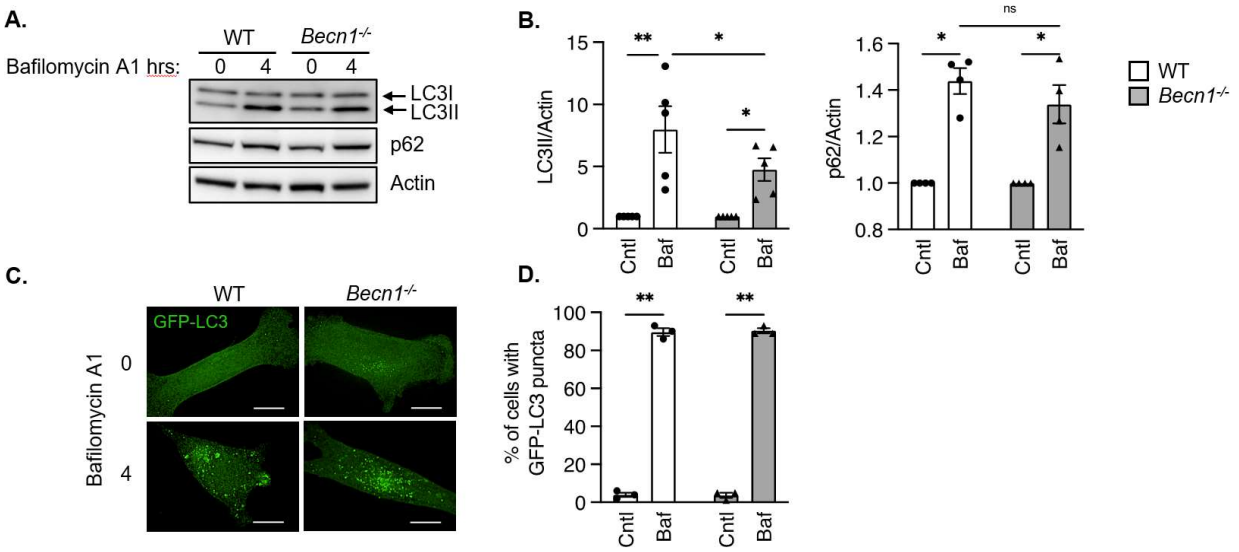

Figure 4: Baseline autophagic flux is preserved in *Becn1*$^{-/-}$ MEFs. A) WT and *Becn1-/-* MEFs were treated with 100nM BafA1 for 4hrs and then harvested for Western blot analysis. Representative blots of LC3 and p62 protein levels. Actin was used as a loading control. **B)** Quantifications of LC3II and p62 levels. (n=5 (LC3II), n=4 (p62) *p<0.05, **p<0.01, ns = not significant) **C)** WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3 were treated with 100nM BafA1 for 4hrs. Scale bar=10µm. **D)** Percentage of WT and *Becn1*$^{-/-}$ MEFs positive for GFP-LC3 puncta out of 100 cells per experiment. (n=3, **p<0.01).

We next examined whether induction of autophagy was altered in response to stress in *Becn1*$^{-/-}$ MEFs. WT and *Becn1*$^{-/-}$ MEFs were subjected to nutrient deprivation to induce autophagy. LC3II protein levels significantly increased in both WT and *Becn1*$^{-/-}$ MEFs in response to starvation (Fig. 5A & 5B), indicating that autophagosome formation was intact in *Becn1*$^{-/-}$ MEFs. Autophagosome formation was confirmed in WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3 in response to nutrient starvation. A significant increase in the percentage of cells containing GFP-LC3 puncta was observed following starvation in both the WT and *Becn1*$^{-/-}$ MEFs, and similar proportions of cells containing punctate GFP-LC3 were observed in WT and *Becn1*$^{-/-}$ MEFs both before and after starvation (Fig. 5C & 5D).

FCCP induces mitochondrial depolarization and is a known inducer of mitophagy. Treatment with FCCP led to a significant increase in LC3II in WT, but not *Becn1*$^{-/-}$ MEFs (Fig. 6A & 6B). However, during fluorescence imaging, similar levels of autophagosome formation was observed in WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3 in response to FCCP. (Fig. 6C & 6D). These results suggest that although stress-induced autophagy is intact in *Becn1*-deficient MEFs, it might be slightly reduced in response to mitochondrial depolarization.

Chapters 1, 2, and 4 are currently being prepared for submission for publication of the material. Gonzalez, Eileen, Jeung, Monica, Gustafsson, Åsa B. The thesis author was a coauthor of this material.

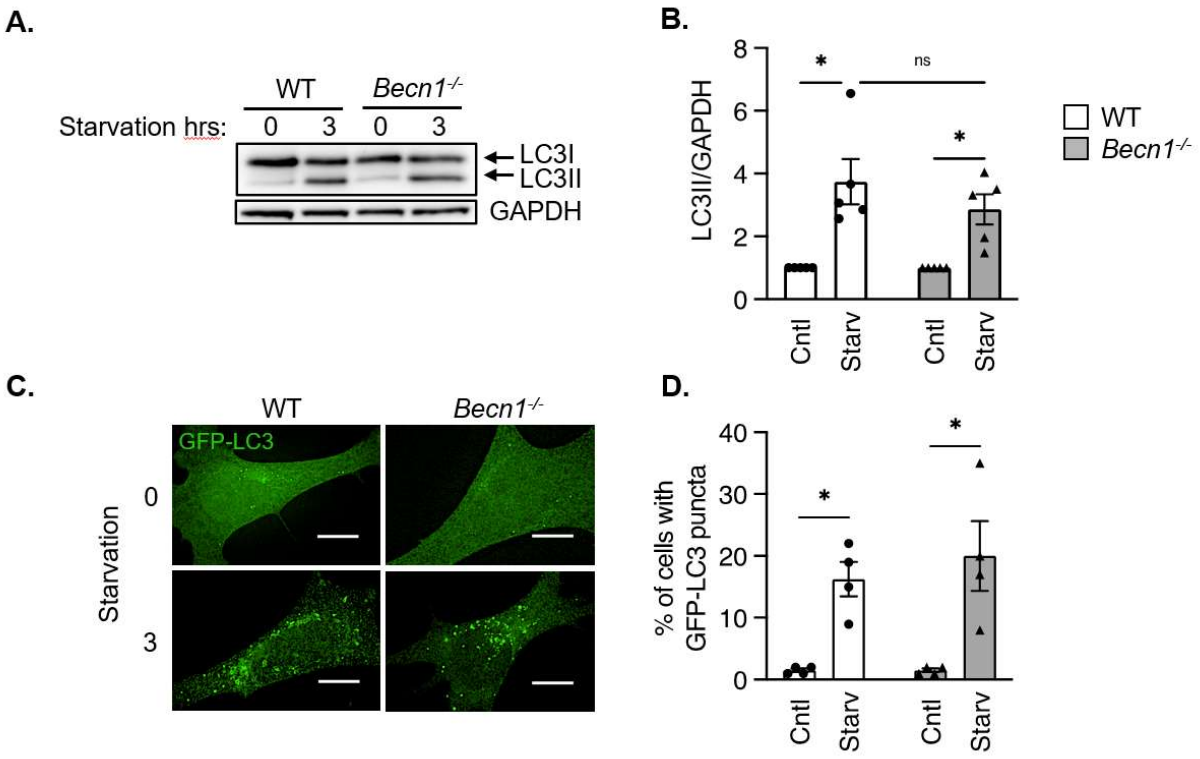

Figure 5: *Becn1*$^{-/-}$ MEFs form autophagosomes in response to starvation. A WT and *Becn1*$^{-/-}$ MEFs were exposed to nutrient deprivation for 3hrs, then harvested for Western blot analysis. Representative blot of LC3I/II protein levels. Actin was used as a loading control. **B)** Quantification of LC3II protein levels (n=5, *p<0.05, ns=not significant). **C)** WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3 were exposed to nutrient starvation conditions for 3hrs. Scale bar=10μm. **D)** Percentage of WT and *Becn1*$^{-/-}$ cells positive for GFP-LC3 puncta out of 100 cells per experiment (n=4, *p<0.05).

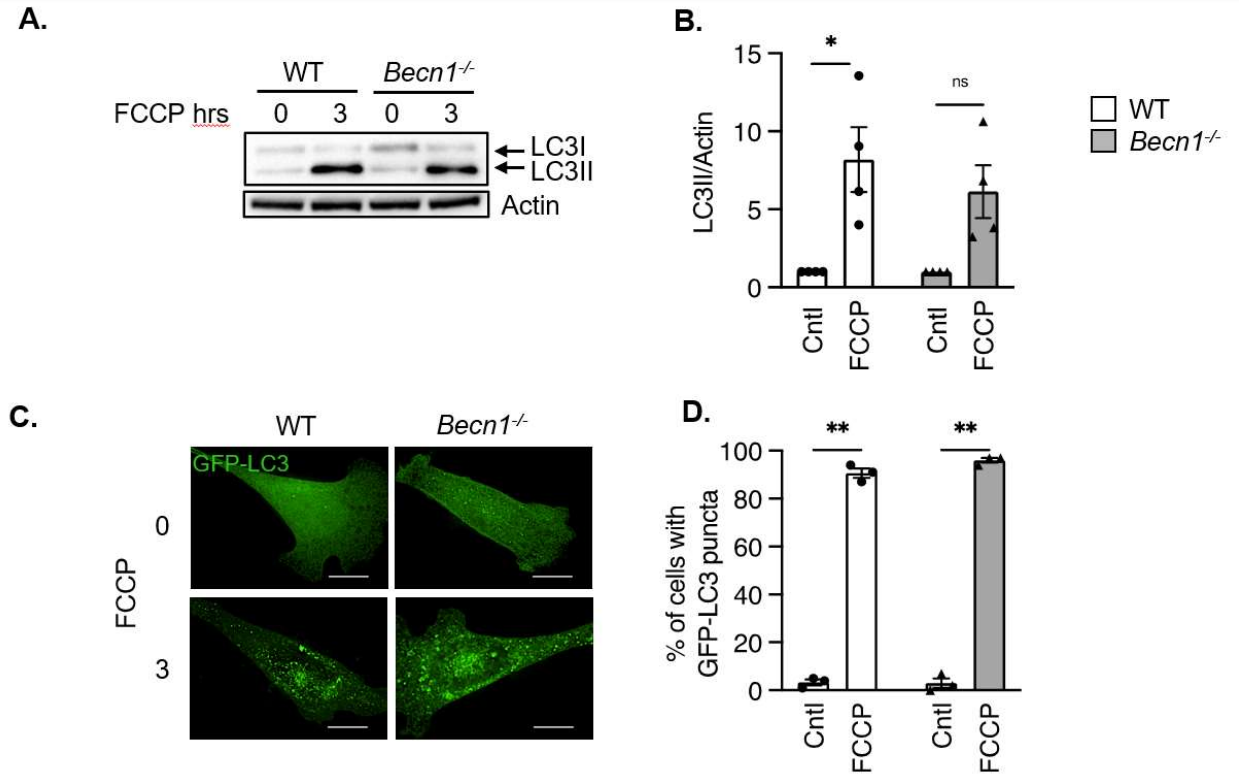

Figure 6: *Becn1*$^{-/-}$ MEFs form autophagosomes in response to mitochondrial depolarization. A) WT and *Becn1*$^{-/-}$ MEFs were treated with 10μM FCCP for 3hrs and harvested for Western blot analysis. Representative blot of LC3 protein levels. Actin was used as a loading control. **B)** Quantification of LC3II protein levels (n=4, *p<0.05, ns=not significant). **C)** WT and *Becn1*$^{-/-}$ MEFs overexpressing GFP-LC3 were treated with 10μM FCCP for 3hrs. **D)** Percentage of WT and *Becn1*$^{-/-}$ MEFs positive for GFP-LC3 puncta out of 100 cells per experiment (n=3, **p<0.01).

Chapter 2

Autophagy is intact in Beclin2-deficient HeLa cells

Beclin2 is a homolog of Beclin1, with 57% sequence identity between human *BECN1* and *BECN2*, and 47% sequence identity between the mouse variants (He et al., 2013). Beclin2 has been demonstrated to play a role in autophagy, although its specific mechanism of action is unknown (He et al., 2013). We observed comparable baseline autophagy and intact autophagosome formation in WT and *Becn1*$^{-/-}$ MEFs, suggesting that a compensation mechanism exists to accommodate for the lack of Beclin1. Given the protein sequence similarity between Beclin1 and Beclin2, we investigated whether Beclin2 was responsible for maintaining autophagy in *Becn1*$^{-/-}$ cells. We successfully used CRISPR/Cas9 to generate both *BECN1* and *BECN2* knockout HeLa cell lines. Knockout of *BECN1* and *BECN2* in HeLa cells was confirmed by Western blot and PCR respectively (data not shown). Our lab also attempted to create a *BECN1/2* double-knockout cell line in HeLa cells, but we were unsuccessful.

To investigate the effect of Beclin1 or Beclin2 deficiency on autophagic flux, WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells were treated with BafA1. Similar to previous experiments, autophagy was assessed by monitoring the conversion of LC3I to LC3II and the accumulation of p62. Exposure to BafA1 in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells resulted in a significant increase in LC3II and p62 levels (Fig. 7A & 7B). Overall, there was significantly less LC3II accumulation in *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ cells compared to WT cells (Fig. 7A & 7B). To further investigate autophagosome formation, WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells overexpressing GFP-LC3 were treated with BafA1. WT and *BECN2*$^{-/-}$ HeLa cells demonstrated a significant increase in the number of GFP-LC3 puncta per cell. While an increase in GFP-LC3 puncta was observed in the *BECN1*$^{-/-}$ cells, it was not significant (Fig. 7C & 7D). Unexpectedly, *BECN1*$^{-/-}$

HeLa cells had higher levels of autophagy at baseline compared to WT, while *BECN2*$^{-/-}$ cells had similar levels to the WT (Fig. 7E). Overall, these findings suggest that baseline autophagy is intact in Beclin2-deficient HeLa cells and reduced in Beclin1-deficient HeLa cells.

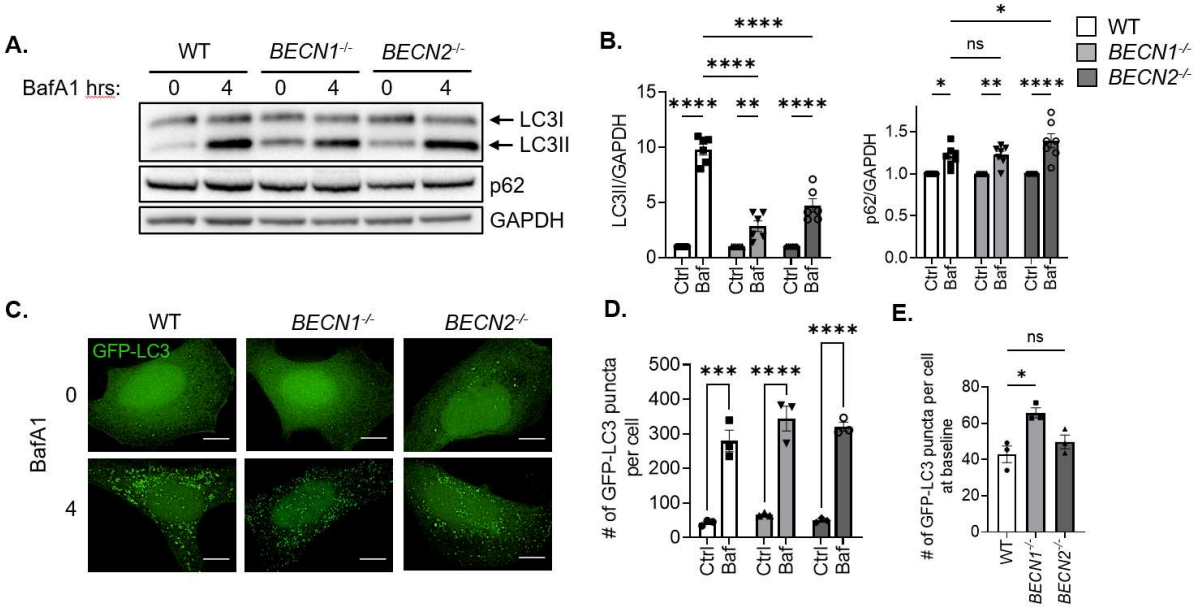

Figure 7: Baseline autophagic flux is preserved in *BECN2*$^{-/-}$ HeLa cells. A) WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ cells were exposed to 100nM BafA1 for 4hrs and harvested for Western blot analysis. Representative blot of LC3 and p62 protein levels. GAPDH was used as a loading control. **B)** Quantifications of LC3II and p62 protein levels. Analysis was performed using two-way ANOVA with Tukey post-test. (n=6 (LC3II), n=7 (p62), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant). **C)** WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells overexpressing GFP-LC3 were treated with 100nM BafA1 for 4hrs. Scale bar=10µm. **D)** Number of GFP-LC3 puncta per cell in 25 individual cells per experiment in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells. Analysis was performed using two-way ANOVA with Tukey post-test. (n=3, ***p<0.001, ****p<0.0001). **E)** Comparison of number of GFP-LC3 puncta found in controls in 25 individual cells per experiment. Analysis was performed using one-way ANOVA with Bartlett's post test (n=3, ns=not significant).

Next, we investigated the induction of autophagy in response to starvation and mitochondrial depolarization. WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells were exposed to nutrient deprivation, and we observed a significant increase in LC3II protein levels in WT and *BECN2*$^{-/-}$ HeLa cells (Fig 8A & 8B). *BECN1*$^{-/-}$ HeLa cells had a modest increase in LC3II levels after starvation that did not reach significance. (Fig. 8A & 8B). To further assess autophagosome formation, GFP-LC3 was overexpressed in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells, which were then exposed to starvation. All three cell lines displayed a significant increase in the number of GFP-LC3 puncta per cell in response to starvation (Fig. 8C & 8D).

We also observed that mitochondrial depolarization with FCCP resulted in a significant increase in LC3II accumulation in all three cell lines (Fig. 9A & 9B). WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells overexpressing GFP-LC3 exhibited a significant increase in GFP-LC3 puncta in response to FCCP (Fig. 9C & 9D). Collectively, these results indicate that stress-induced autophagy is maintained in *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells.

Chapters 1, 2, and 4 are currently being prepared for submission for publication of the material. Gonzalez, Eileen, Jeung, Monica, Gustafsson, Åsa B. The thesis author was a coauthor of this material.

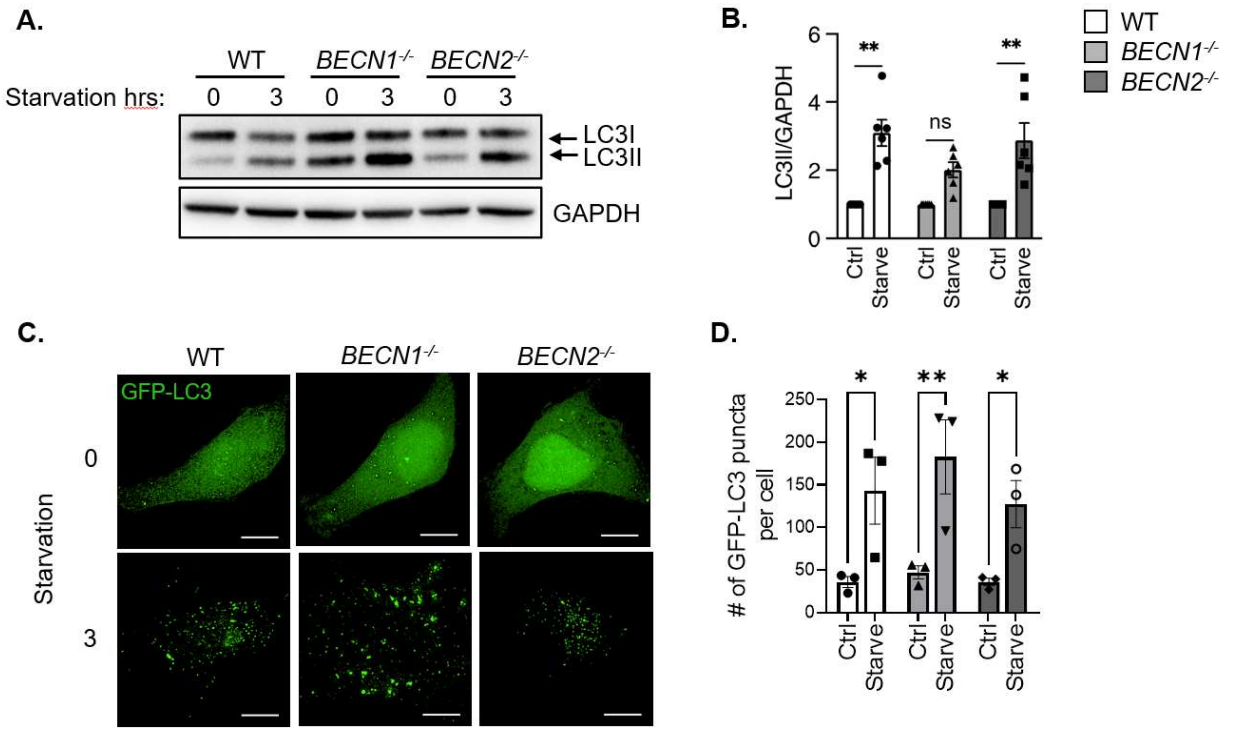

Figure 8: *BECN2*$^{-/-}$ HeLa cells form autophagosomes normally in response to starvation. A) WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ cells were subjected to nutrient starvation for 3hrs, then harvested for Western blot analysis. Representative blot of LC3 protein levels. GAPDH was used as a loading control. **B)** Quantification of LC3II protein levels. Analysis was performed using two-way ANOVA with Tukey post-test (n=5, **p<0.01, ns=not significant). **C)** Representative images of WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells overexpressing GFP-LC3 after exposure to nutrient starvation conditions for 3hrs. Scale bar=10µm. **D)** Number of GFP-LC3 puncta per cell in 25 individual cells per experiment in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells. Analysis was performed using two-way ANOVA with Tukey post-test (n=3, ***p<0.001).

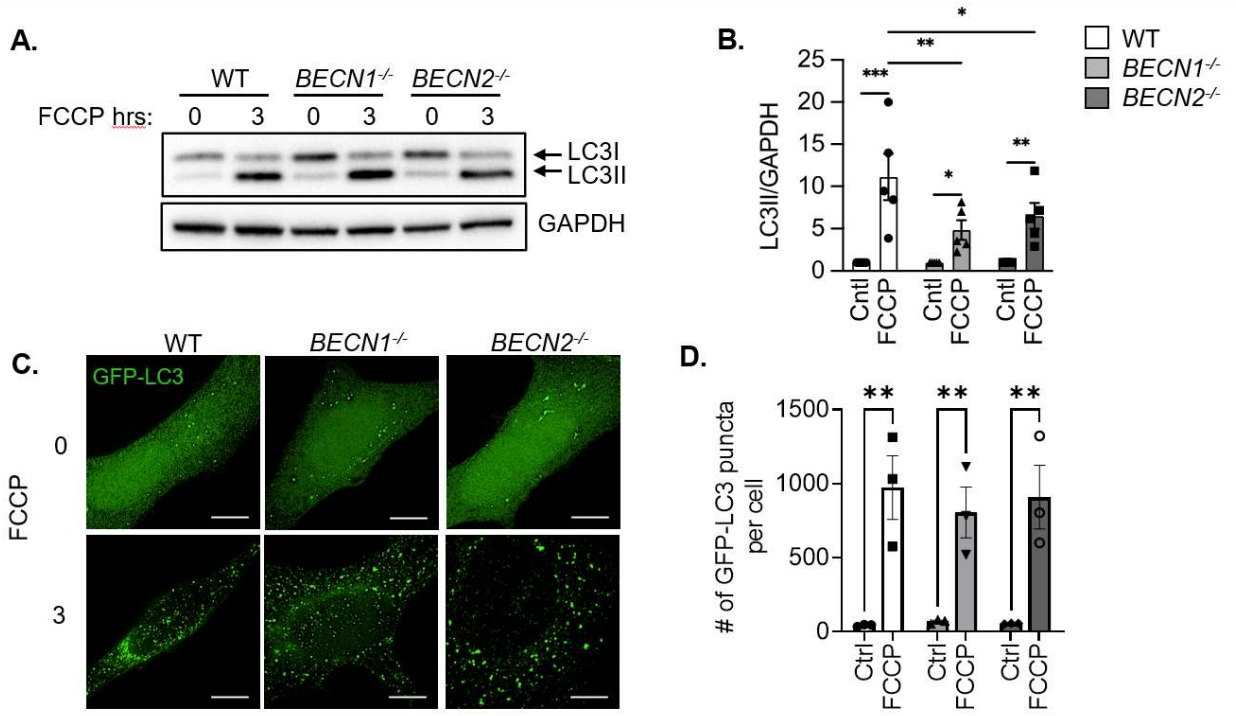

Figure 9: *BECN2*$^{-/-}$ HeLa cells form autophagosomes normally in response to mitochondrial depolarization. A) WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ cells were treated with 10μM FCCP for 3hrs, then collected for Western blot analysis. Representative blot of LC3 protein levels. GAPDH was used as a loading control. **B)** Quantification of LC3II protein levels (n=5, *p<0.05, **p<0.01, ***p=0.001). **C)** Representative images of WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells overexpressing GFP-LC3 after treatment with 10μM FCCP for 3hrs. Scale bar=10μm. **D)** Number of GFP-LC3 puncta per cell in 25 individual cells per experiment in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells. Analysis was performed using two-way ANOVA with Tukey post-test (n=3, **p<0.01, ***p<0.001).

Chapter 3

Preparation of a novel mitophagy reporter for use *in vivo*

Genetically encoded fluorescent tags are a common method for imaging proteins and organelles. These are protein sequences which are fused to the protein of interest and may be naturally fluorescent, or may bind to endogenous or exogenous fluorophores made by or provided to the cells respectively. (Thorn, 2017). The brightness of the fluorescent tag depends on a variety of factors, including the folding and maturation rate of the protein it is fused to, the cell type it is expressed in, and environmental factors such as pH and salt concentration (Thorn, 2017).

pH-sensitive variants of green fluorescent protein (GFP) are commonly used in the study of proteins involved in vesicular trafficking (Mahon, 2011) and can be particularly useful in the study of autophagy and mitophagy due to the decrease in pH that occurs during autophagosome to lysosome fusion. pHluorin2 is a variant of GFP derived from radiometric pHluorin, a version of GFP which has increased sensitivity to pH changes, and GFP2, a GFP variant containing mammalianized codons and with higher fluorescence than standard GFP. Much like traditional GFP, pHluorin2 fluorescence is quenched at low pH. (Mahon, 2011). In contrast, mCherry has high pH resistance and remains fluorescent in low pH environments (Shaner et al., 2004). The objective was to develop a mitophagy reporter utilizing pHluorin2 and mCherry. The tandem mCherry-pH2 fluorophores were linked to a mitochondrial targeting sequence (MTS) to target the construct to the mitochondrial matrix (MTS-mCh-pH2-TM). During mitophagy, mitochondria are engulfed in autophagosomes, which fuse with lysosomes, forming a low-pH environment inside the vesicle. Before lysosome fusion with the autophagosome, both mCherry (red) and pHluorin2 (green) will fluoresce, making the mitochondria appear yellow. Following

lysosome fusion, pHluorin2 is quenched, leaving only mCherry fluorescence (red) visible. This process allowed us to visualize mitochondria undergoing mitophagy (Fig.10A). The reporter was validated in MEFs and HeLa cells by comparing images produced using the new reporter against images produced with existing mitophagy reporters (data not shown).

After validation of the reporter in cells, the lab wanted to generate a mouse line overexpressing the reporter in the heart. I assisted in this project by cloning the reporter cDNA into the α-MHC plasmid. α-MHC is a cardiac-specific promoter, which will ensure expression of the gene only in cardiac myocytes. The construct was cloned into the α-MHC plasmid in between SalI and HindIII, with the mitochondrial targeting sequence ligated to SalI and the transmembrane region ligated to HindIII (Fig. 10B). The vector was then linearized using NotI for injection into mice. The construct was sent to the transgenic mouse core for injection. Successful transgene expression was confirmed via PCR genotyping using primers generated to recognize the pHluorin2 gene. We have 2 positive mouse pups that will be used to expand the line.

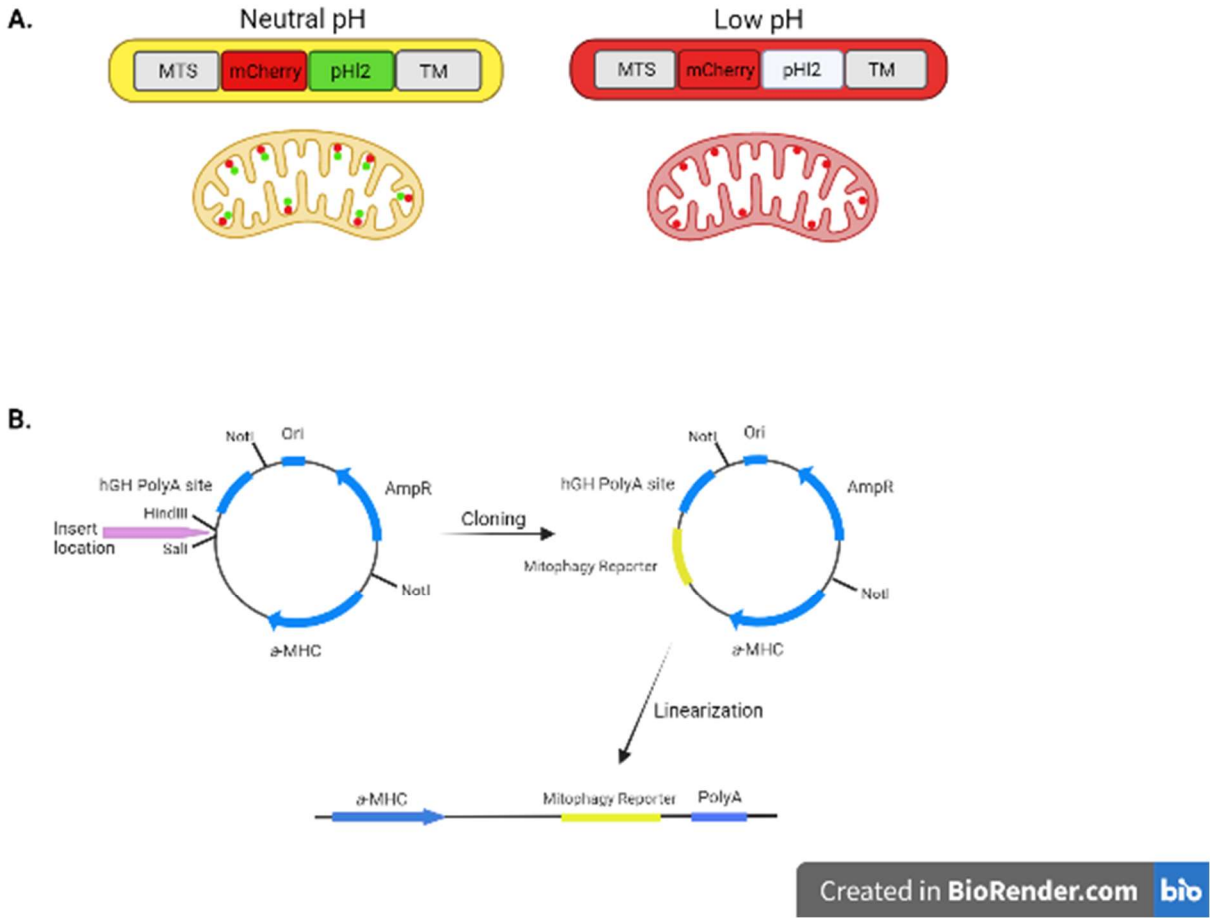

Figure 10: Preparation of mitophagy reporter for generation of transgenic mice. A) Description of mitophagy reporter. At neutral pH, both mCherry and pHluorin2 fluoresce, producing yellow mitochondria. At low pH inside autolysosomes, pHluorin2 is quenched, resulting in red-only mitochondria. **B)** The mitophagy reporter was cloned into the α-MHC plasmid backbone between HindIII and SalI, and the plasmid was linearized using NotI for injection into mice.

Chapter 4

Characterization of the role of Beclin2 in Parkin-mediated mitophagy

Previous studies have reported that the loss of Beclin1 results in a reduction of mitophagy (Sun et al., 2018). Prior research conducted by the lab has confirmed that *Becn1*$^{-/-}$ MEFs have reduced Parkin-mediated mitochondrial clearance, and that *BECN1*$^{-/-}$ HeLa cells also have reduced Parkin-mediated mitophagy (data not shown). While it has been demonstrated that Beclin2 deficiency results in reduction of autophagy, no previous studies have been conducted on the effect of Beclin2 deficiency in Parkin-mediated mitophagy. Here I compared whether Beclin2 deficiency leads to a similar defect in Parkin-mediated mitophagy as observed in the absence of Beclin1.

To confirm previous reports of impaired Parkin-mediated mitophagy in Beclin1-deficient cells, MEFs overexpressing mCh-Parkin were exposed to FCCP to induce mitochondrial depolarization. *Becn1*$^{-/-}$ MEFs displayed impaired mitochondrial clearance in comparison to WT MEFs after FCCP exposure. Unexpectedly, *Becn1*$^{-/-}$ MEFs had increased levels of COX IV following FCCP treatment, suggesting potential activation of mitochondrial biogenesis (Fig. 11A & 11B). This experiment was also conducted in our HeLa cell lines. WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells overexpressing mCh-Parkin were exposed to FCCP. WT and *BECN2*$^{-/-}$ HeLa cells demonstrated a significant level of mitochondrial clearance, while *BECN1*$^{-/-}$ had impaired clearance (Fig. 11C & 11D). We then sought to characterize the effects of oligomycin/antimycin A treatment in the HeLa cell lines. Antimycin A inhibits the mitochondrial respiratory complex III, leading to accumulation of reactive oxygen species (ROS) and minor depolarization. Oligomycin inhibits the compensatory reverse hydrolysis activity of the F1Fo-ATP-synthase for stronger depolarization (Georgakopoulos et al., 2017). WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells

expressing mCh-Parkin were treated with oligomycin/antimycin A, and levels of COX IV clearance were used to assess mitophagy. Following oligomycin/antimycin A exposure, WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells demonstrated a significant level of mitochondrial clearance. Similar levels of mitochondrial clearance were observed in *BECN2*$^{-/-}$ and WT cells, while *BECN1*$^{-/-}$ HeLa cells demonstrated reduced mitochondrial clearance compared to WT (Fig. 11E & 11F). In agreement with previous literature, Beclin1 deficiency impairs Parkin-mediated mitophagy. Overall, the data suggests that Beclin2 is not essential in Parkin-mediated mitophagy.

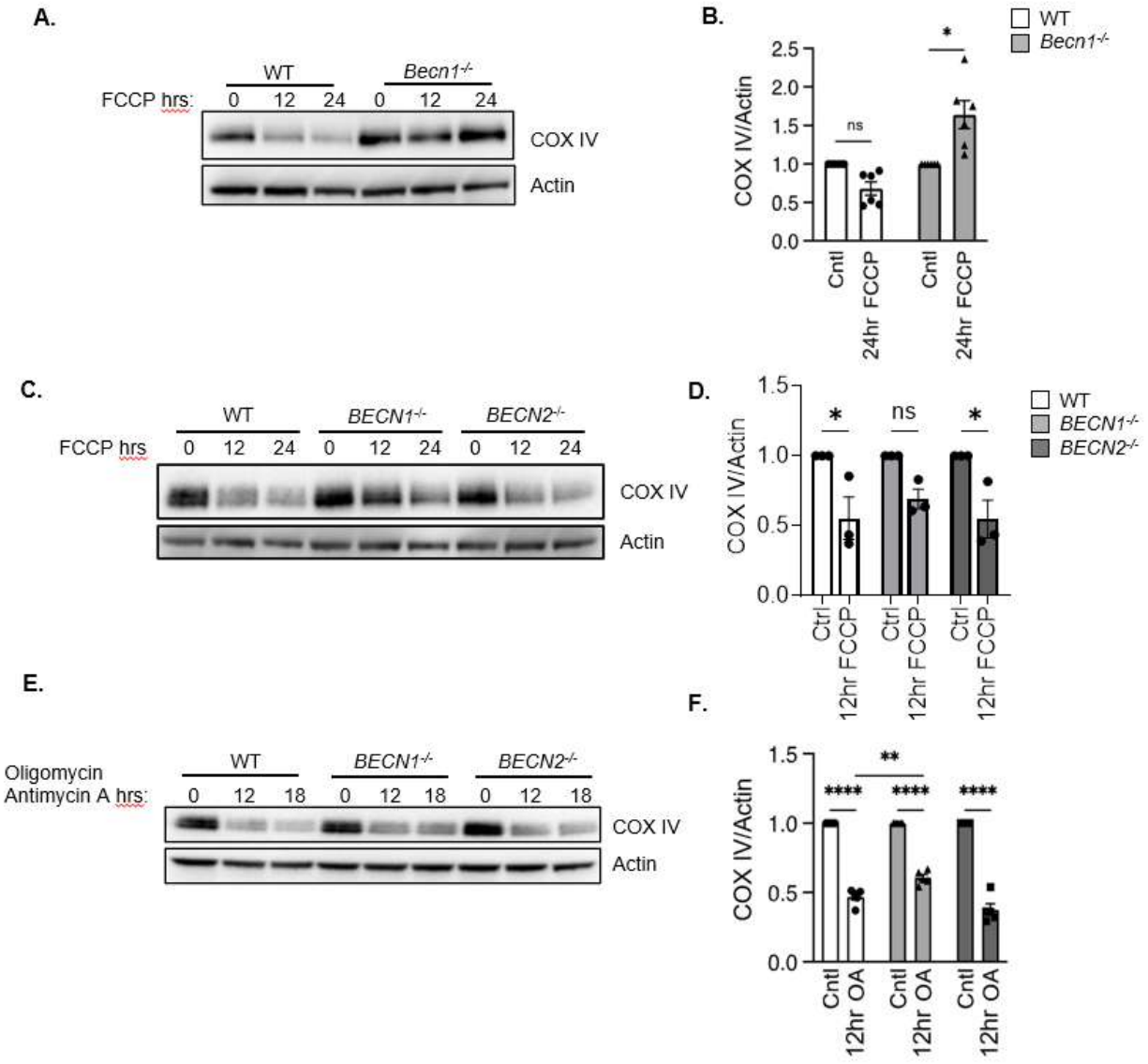

Figure 11: Evaluation of mitochondrial clearance in *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ cells. A) WT and *Becn1*$^{-/-}$ MEFs expressing mCherry-Parkin were treated with 25μM FCCP for 12 and 24hrs, and then collected for Western blot analysis. Representative blot showing COX IV protein levels. Actin was used as a loading control. **B)** Quantification of COX IV protein levels at 24hrs (n=5, $p<0.05$, ns=not significant). **C)** WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells expressing mCh-Parkin were treated with 25μM FCCP for 12 and 24hrs, and then collected for Western blot analysis. Representative blot of COX IV protein levels. Actin was used as a loading control. **D)** Quantification of COX IV protein levels at 12hrs (n=3, *$p<0.05$, ns=not significant). **E)** WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells expressing mCh-Parkin were treated with 0.2mM oligomycin and 4μM antimycin A for 12 and 18hrs, and then collected for Western blot analysis. Representative blot of COX IV protein levels. Actin was used as a loading control. **F)** Quantification of COX IV protein levels at 12hrs (n=5, **$p<0.01$, ****$p<0.0001$).

We confirmed these results using the MTS-mCh-pH2-TM mitophagy reporter. WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells expressing myc-Parkin plus the mitophagy reporter were treated with oligomycin/antimycin A to induce mitophagy. After treatment, cells were fixed and stained with anti-myc to identify cells transfected with both myc-Parkin and MTS-mCh-pH2-TM. *BECN1*$^{-/-}$ HeLa cells did not demonstrate a significant increase in mitophagy following oligomycin/antimycin A exposure (Fig. 12A & 12B). WT and *BECN2*$^{-/-}$ HeLa cells both demonstrated significant increases in mitophagy following treatment. These results are consistent with the earlier HeLa cell data, which also suggest that Beclin1-deficient HeLa cells have impaired mitophagy and Beclin2-deficient HeLa cells have intact mitophagy. Unexpectedly, *BECN2*$^{-/-}$ HeLa cells displayed higher levels of mitophagy compared to the WT after oligomycin/antimycin A treatment (Fig 12A & 12B). These results further confirm that Beclin1 plays a role in Parkin-mediated mitophagy, while Beclin2 is dispensable.

Chapters 1, 2, and 4 are currently being prepared for submission for publication of the material. Gonzalez, Eileen, Jeung, Monica, Gustafsson, Åsa B. The thesis author was a coauthor of this material.

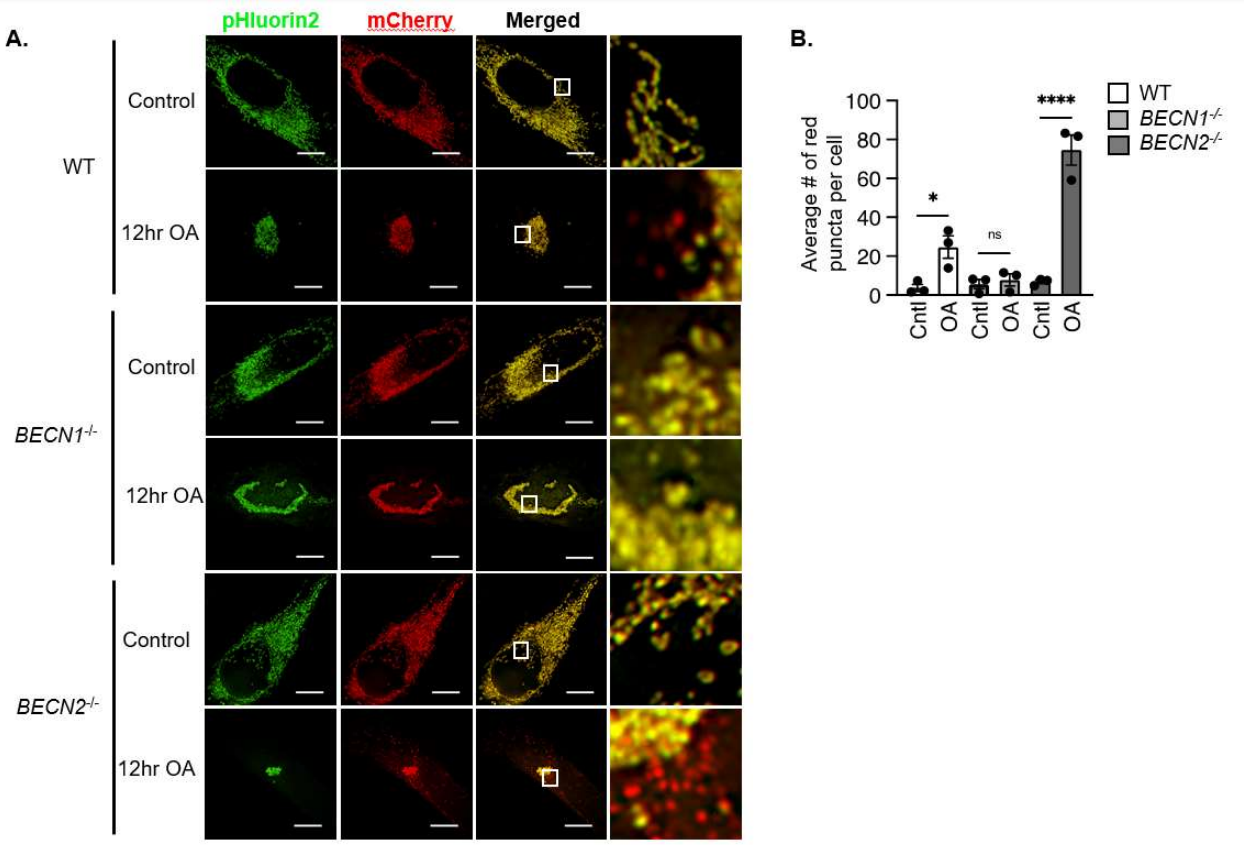

Figure 12: Fluorescence imaging analysis of mitophagy. A. WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells expressing myc-Parkin and the mitophagy reporter were treated with 0.2mM oligomycin and 4µM antimycin A (OA) for 12hrs. Scale bar=10µm. **B.** Quantification of the number of red puncta per cell. 25 cells per experiment were scored for mitophagy events. Subtraction of yellow puncta was performed via ImageJ, then remaining red puncta were manually counted. Statistical analysis was performed using one-way ANOVA with post-test (n=3, *p<0.05, ****p<0.0001, ns=not significant).

Summary

The data shown displays a comparison of the roles of Beclin1 and Beclin2 in autophagy and in Parkin-mediated mitophagy. Both Beclin1- and Beclin2- deficient cells demonstrate intact baseline autophagic flux and stress-induced autophagosome formation. However, Beclin1-deficient cells have reduced Parkin-mediated mitophagy, while BECN2-deficient cells retain intact mitophagic activity. Generation of a mouse line containing a novel pH-based mitophagy reporter localized to the heart which can be used in simple imaging experiments both *in vivo* and *in vitro* will be useful for future studies of cardiac mitophagic activity in live animals.

Discussion and Conclusions

These results provide deeper insight into the roles of the Beclin proteins in autophagy. We first demonstrated that autophagic flux and stress-induced autophagy can still proceed in the absence of Beclin1 in MEFs and HeLa cells. We also observed that loss of Beclin2 has no significant effect on autophagy, likely because our Beclin2 deficient cells retained Beclin1. We then determined that Beclin1 plays a role in Parkin-mediated mitophagy, while Beclin2 does not seem to play a role in this process. Finally, we generated a novel reporter to assess mitophagy *in vitro* and *in vivo*. Therefore, our research suggests possible compensatory functions of Beclin1 and Beclin2 in autophagy, but differences in their role in mitophagy.

Traditional autophagy is induced by Beclin1 as a part of the PtdIns3K complex which initiates the formation of autophagosomes. However, several studies have reported the existence of non-canonical, Beclin1-independent autophagy pathways. Examples of Beclin1-independent autophagy that have been described to date occur during very specific circumstances, such as the treatment of breast cancer cells with the diterpene carnasol (Al Dhaheri et al., 2014) or colorectal cancer cells with *Rhus coraria* extract (Athamneh et al., 2017). Thus, it is assumed that the

induction of general autophagy during starvation is regulated by Beclin1. It has been assumed that baseline autophagy involved in the turnover of organelles and long-lived proteins is also Beclin1 dependent. It is interesting to note that because viral infections may be cleared in cells by autophagy, Beclin1 is a common target for viruses to inhibit autophagy (Ahmad et al., 2018). This suggests that it is evolutionarily advantageous to both have Beclin1-independent autophagy pathways as well as redundancy in the functions of Beclin2. In our research, we observed a smaller decrease in autophagy in Beclin1-deficient cells than expected during starvation or mitochondrial stress, as autophagy in these cases should be Beclin1-dependent. It is possible that Beclin2 compensates for the loss of Beclin1 and participates in maintaining functional autophagy in its absence. Beclin2 is a homolog of Beclin1 with similar amino acid sequences but different genomic organization, and it has been demonstrated that siRNA directed against Beclin2 does not decrease expression of Beclin1 (He et al., 2013), as well as that Beclin2 is involved in immune-signaling based autophagy pathways (Zhu et al., 2020). This suggests the possibility that Beclin2 would not be vulnerable to the same viral interference as Beclin1, and that Beclin2 may partially compensate for Beclin1 during basal autophagy to maintain a minimal level of autophagy even in cases where Beclin1 might be inhibited. Regardless, our data demonstrate that Beclin1 deficiency does not lead to a total loss of autophagy.

Another interesting finding was that our untreated Beclin1-deficient cells seemed to have elevated levels of basal autophagy relative to untreated cells from our other cell lines (WT MEFs and HeLa cells, and Beclin2-deficient HeLa cells). This effect was clearly observed during the various fluorescence experiments, as *BECN1*$^{-/-}$ HeLa cells had significantly higher numbers of puncta compared to the WT at baseline (Fig. 7E & 9E). The reason for the enhanced autophagy is unclear. It is possible that this effect is due to reduced elimination of the autophagosome.

Beclin1 may play a role in autophagosome and lysosome fusion. It is also possible that the upregulation of autophagosome formation intended as a compensatory mechanism for the loss of Beclin1 for immune-responsive reasons, as mentioned above, since Beclin1 is a common target of viral interference.

We also examined the effects of both Beclin1 and Beclin2 deficiency on Parkin-mediated mitophagy. These experiments were restricted to HeLa cells because we were unsuccessful in generating a *Becn2*$^{-/-}$ MEF line. The behavior of Beclin2 deficient cells during mitophagy has not previously been characterized. In agreement with previous research, we observed that Beclin1 deficiency resulted in decreased mitochondrial clearance (Gelmetti et al., 2017). In contrast, we observed that Beclin2 deficiency had no impact on mitochondrial clearance. Unexpectedly, *BECN2*$^{-/-}$ HeLa cells had increased mitophagy response compared to the WT during use of the mitophagy reporter (Fig. 11A & 11B). However, this was merely an observed trend, and no further research has been done to examine this apparent increase in mitophagy within this paper. We have demonstrated here that Beclin2 deficiency did not decrease Parkin-mediated mitophagy in HeLa cells, and that *BECN2*$^{-/-}$ cells are capable of functional mitophagy. Overall, this suggests that unlike Beclin1, Beclin2 does not have a role in for Parkin-mediated mitophagy.

Additional research is necessary to clarify Beclin2's role in autophagy. Previous work on Beclin2 has demonstrated that it participates in autophagy (He et al., 2013), and we have more closely characterized its role in autophagy and mitophagy. Additionally, Beclin2 has been shown to have specific roles in immune-related autophagy (Zhu et al., 2020), but the mechanisms by which Beclin2 regulates other forms of autophagy have not been investigated. As Beclin2 remains a mostly uncharacterized protein, there are also no studies on the effects of Beclin2 overexpression. This could potentially provide further insights on its function in autophagy.

Other unexplored areas of study involve the interaction of Beclin1 and Beclin2 and the effects of simultaneous overexpression of both proteins, or of the effects of a double-knockout cell line on autophagy. A manuscript in preparation from our lab will be discussing the effects of simultaneous Beclin1 and Beclin2 deficiency to begin to examine whether cells still form autophagosomes following a double-knockout of the two Beclin proteins. Our observed increase in autophagy at baseline in our Beclin1 HeLa cells also leaves new areas of study to be explored. This shows that there is additional research to be done on Beclin1's role in the regulation of autophagy and mitophagy.

The generation of the mitophagy reporter mouse line also has potential to be invaluable for future investigation of mitophagy *in vivo*. The ability to assess mitophagy *in vivo* allows our lab to examine the roles of a wide variety of mitophagy-related proteins that we have examined in cells *in vivo*. Our lab is in possession of *Becn1*$^{+/-}$ heterozygous mice, and in combination with the new homozygous mitophagy reporter mice, a future direction for the lab could involve *in vivo* exploration into the role of Beclin1 during mitophagy. Clearly, there is much more work to be done to explore both the individual roles and the possible interacting roles of Beclin1 and Beclin2 in general autophagy and mitophagy. With the avenue of *in vivo* cardiac research into the Beclin proteins now open, it is important to attempt to fully understand how both Beclin1 and Beclin2 regulate autophagy, as this could lead to the discovery of new therapeutic interventions.

References

Sciarretta, S., Maejima, Y., Zablocki, D., & Sadoshima, J. (2018). The Role of Autophagy in the Heart. *Annual Review of Physiology, 80*, 1-26. https://doi.org/10.1146/annurev-physiol-021317-121427

Tsukada, M., & Ohsumi, Y. (1993). Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS letters*, *333*(1-2), 169–174. https://doi.org/10.1016/0014-5793(93)80398-e

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., & Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Molecular biology of the cell*, *15*(3), 1101–1111. https://doi.org/10.1091/mbc.e03-09-0704

Choi, A., Ryter, S., & Levine, B. (2013). Autophagy in Human Healh and Disease. *N Engl J Med*, 368:651-662. https://doi.org/10.1056/NEJMra1205406

Ikeda, Y., Shirakabe, A., Maejima, Y., Zhai, P., Sciarretta, S., Toli, J., Nomura, M., Mihara, K., Egashira, K., Ohishi, M., Abdellatif, M., & Sadoshima, J. (2014). Endogenous Drp1 Mediates Mitochondrial Autophagy and Protects the Heart Against Energy Stress. *Circulation Research, 116*(2), 264-278. https://doi.org/10.1161/CIRCRESAHA.116.303356

Matsui, Y., Takagi, H., Qu, X., Abdellatif, M., Sakoda, H., Åsano, T., Levine, B., & Sadoshima, J. (2007). Distinct Roles of Autophagy in the Heart During Ischemia and Reperfusion. *Circulation Research, 100*(6), 914-922. https://doi.org/10.1161/01.RES.0000261924.76669.36

Gustafsson, Å. B., & Dorn, G. W., 2nd (2019). Evolving and Expanding the Roles of Mitophagy as a Homeostatic and Pathogenic Process. *Physiological reviews*, *99*(1), 853–892. https://doi.org/10.1152/physrev.00005.2018

Itakura, E., Kishi, C., Inoue, K., & Mizushima, N. (2008). Beclin 1 Forms Two Distinct Phosphatidylinositol 3-Kinase Complexes with Mammalian Atg14 and UVRAG. *Molecular Biology of the Cell, 19*(12). https://doi.org/10.1091/mbc.e08-01-0080

Russell, R., Tian, Y., Yuan, H., Park, H., Chang, Y., Kim, J., Kim, H., Neufeld, T.P., Dillin, A., & Guan, K. (2013). ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol 15*, 741–750. https://doi.org/10.1038/ncb2757

Mizushima, N., Sugita, H., Yoshimori, T., & Ohsumi, Y. (1998). A New Protein Conjugation System in Human. *Journal of Biological Chemistry, 273*(51), 33889-33892. https://doi.org/10.1074/jbc.273.51.33889

Mizushima N., Kuma A., Kobayashi Y., Yamamoto A., Matsubae M., Takao T., Natsume T., Ohsumi Y., & Yoshimori T. (2003). Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci 116*: 1679–1688. https://doi.org/10.1242/jcs.00381

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., & Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J 19*, 5720-5728. https://doi.org/10.1093/emboj/19.21.5720

Diao, J., Liu, R., Rong, Y., Zhao, M., Zhang, J., Lai, Y., Zhou, Q., Wilz, L.M., Li, J., Vivona, S., Pfuetzner, R.A., Brunger, A.T., & Zhong, Q. (2015). ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature* 520, 563–566. https://doi.org/10.1038/nature14147

Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N., Rubinsztein, D.C. (2003). α-Synuclein Is Degraded by Both Autophagy and the Proteasome. *Journal of Biological Chemistry* 278(27), 25009-25013. https://doi.org/10.1074/jbc.M300227200

Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J., & Rubinsztein, D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* 36, 585–595 . https://doi.org/10.1038/ng1362

Pickford, F., Masliah, E., Britschgi,M., Lucin,K., Narasimhan,R., Jaeger, P.A., Small, S., Spencer, B., Rockenstein, E., Levine, B., & Wyss-Coray, T. (2008). The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. *J Clin Invest.* 118(6), 2190-2199. https://doi.org/10.1172/JCI33585.

Tannous, P., Zhu, H., Johnstone, J.L., Shelton, J.M., Rajasekaran, N.S., Benjamin, I.J., Nguyen, L., Gerard, R.D., Levine, B., Rothermel, B.A., & Hill, J.A. (2008). Autophagy is an adaptive response in desmin-related cardiomyopathy. *PNAS* 105(28), 9745-9750. https://doi.org/10.1073/pnas.0706802105

Helin, H., Mero, M., Markkula, H. & Helin, M. (1980), Pancreatic acinar ultrastructure in human acute pancreatitis. *Virchows Arch. A Path. Anat. and Histol.* 387, 259–270. https://doi.org/10.1007/BF00454829

Ravikumar, B., Sarkar, S., Davies, J.E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z.W., Jimenez-Sanchez, M., Korolchuk, V.I., Lichtenberg, M., Luo, S., Massey, D.C.O., Menzies, F.M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F.H., Underwood, B.R., Winslow, A.R., & Rubinsztein D.C. (2010). Regulation of Mammalian Autophagy in Physiology and Pathophysiology. *Phys Rev 90*(4). https://doi.org/10.1152/physrev.00030.2009

Rami, A., Langhagen, A., & Steiger, S. (2008). Focal cerebral ischemia induces upregulation of Beclin 1 and autophagy-like cell death. *Neurobiology of Disease,* 29(1) 132-141. https://doi.org/10.1016/j.nbd.2007.08.005.

Eiyama, A., & Okamoto, K. (2015). PINK1/Parkin-mediated mitophagy in mammalian cells. *Current opinion in cell biology*, *33*, 95–101. https://doi.org/10.1016/j.ceb.2015.01.002

Lazarou, M., Jin, S., Kane, L.A., & Youle, R.J. (2012). Role of PINK1 Binding to the TOM Complex and Alternate Intracellular Membranes in Recruitment and Activation of the E3 Ligase Parkin. *Developmental Cell*, 22(2) 320-333. https://doi.org/10.1016/j.devcel.2011.12.014.

Lazarou, M., Sliter, D., Kane, L., Sarraf, S., Wang, C., Burman, J.L., Sideris, D.P., Fogel, A.I., & Youle, R.J. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 524, 309–314. https://doi.org/10.1038/nature14893

Levine, B., Liu, R., Dong, X., & Zhong, Q. (2015). Beclin orthologs: integrative hubs of cell signaling, membrane trafficking, and physiology. *Trends in cell biology*, *25*(9), 533–544. https://doi.org/10.1016/j.tcb.2015.05.004

Yue, Z., Jin, S., Yang, C., Levine, A. J., & Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(25), 15077–15082. https://doi.org/10.1073/pnas.2436255100

Gelmetti, V., De Rosa, P., Torosantucci, L., Marini, E. S., Romagnoli, A., Di Rienzo, M., Arena, G., Vignone, D., Fimia, G. M., & Valente, E. M. (2017). PINK1 and BECN1 relocalize at mitochondria-associated membranes during mitophagy and promote ER-mitochondria tethering and autophagosome formation. *Autophagy*, *13*(4), 654–669. https://doi.org/10.1080/15548627.2016.1277309

Itakura, E. & Mizushima, N. (2009). Atg14 and UVRAG: Mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. *Autophagy 5*(4), 534-536. https://doi.org/10.4161/auto.5.4.8062

He, C., Wei, Y.,Sun, K., Li, B., Dong, X., Zou, Z., Liu, Y., Kinch, L.N., Khan, S., Sinha, S., Xavier, R.J., Grishin, N.V., Xiao, G., Eskelinen EL., Scherer, P.E., Whistler, J.L., & Levine, B. (2013). Beclin 2 Functions in Autophagy, Degradation of G Protein-Coupled Receptors, and Metabolism. *Cell 154*(5), 1085-1099. https://doi.org/10.1016/j.cell.2013.07.035

Zhu, M., Deng, G., Tan, P., Xing, C., Guan, C., Jiang, C., Zhang, Y., Ning, B., Li, C., Yin, B., Chen, K., Zhao, Y., Wang, H. Y., Levine, B., Nie, G., & Wang, R. F. (2020). Beclin 2 negatively regulates innate immune signaling and tumor development. *The Journal of clinical investigation*, *130*(10), 5349–5369. https://doi.org/10.1172/JCI133283

Gulick J, Subramaniam A, Neumann J, & Robbins J. (1991). Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem. 266(14):9180-5.*

Kubli, D.A., Zhang, X., Lee, Y., Hanna, R.A., Quinsay, M.N., Nguyen, C.K., Jimenez, R., Petrosyan, S., Murphy, A.N., & Gustafsson, A.B. (2013). Parkin Protein Deficiency Exacerbates Cardiac Injury and Reduces Survival following Myocardial Infarction. *Journal of Biological Chemistry 288*(2), 915-926. https://doi.org/10.1074/jbc.M112.411363

Thorn K. (2017). Genetically encoded fluorescent tags. *Molecular biology of the cell*, *28*(7), 848–857. https://doi.org/10.1091/mbc.E16-07-0504

Mahon M. J. (2011). pHluorin2: an enhanced, ratiometric, pH-sensitive green florescent protein. *Advances in bioscience and biotechnology (Print)*, *2*(3), 132–137. https://doi.org/10.4236/abb.2011.23021

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature biotechnology*, *22*(12), 1567–1572. https://doi.org/10.1038/nbt1037

Sun, Y., Yao, X., Zhang, Q. J., Zhu, M., Liu, Z. P., Ci, B., Xie, Y., Carlson, D., Rothermel, B. A., Sun, Y., Levine, B., Hill, J. A., Wolf, S. E., Minei, J. P., & Zang, Q. S. (2018). Beclin-1-Dependent Autophagy Protects the Heart During Sepsis. *Circulation*, *138*(20), 2247–2262. https://doi.org/10.1161/CIRCULATIONAHA.117.032821

Georgakopoulos, N., Wells, G. & Campanella, M. (2017). The pharmacological regulation of cellular mitophagy. *Nat Chem Biol* 13, 136–146. https://doi.org/10.1038/nchembio.2287

Al Dhaheri, Y., Attoub, S., Ramadan, G., Arafat, K., Bajbouj, K., Karuvantevida, N., AbuQamar, S., Eid, A., & Iratni, R. (2014). Carnosol Induces ROS-Mediated Beclin-1-Independent Autophagy and Apoptosis in Triple Negative Breast Cancer. *PLOS ONE*. https://doi.org/10.1371/journal.pone.0109630

Athamneh, K., Hasasna, H.E., Samri, H.A., Attoub, S., Arafat, K., Benhalilou, N., Al Rashedi, A., Al Dhaheri, Y., AbuQamar, S., Eid, A., & Iratni, R. (2017). *Rhus coriaria* increases protein ubiquitination, proteasomal degradation and triggers non-canonical Beclin-1-independent autophagy and apoptotic cell death in colon cancer cells. *Sci Rep* **7**, 11633. https://doi.org/10.1038/s41598-017-11202-3

Ahmad, L., Mostowy, S., Sancho-Shimizu, V. (2018). Autophagy-Virus Interplay: From Cell Biology to Human Disease. *Frontiers in Cell and Developmental Biology 6*, 155. https://doi.org/10.3389/fcell.2018.00155