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Beclin1 is critical for Rab5 endosomal-mediated trafficking of plasma membrane proteins

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by

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The Dissertation of Mark Lampert is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

I dedicate this dissertation to the many mentors I have had in my life, without whom I would not be here today. I thank my family and friends for their unyielding support throughout.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ad-	Adenovirus
αMHC	α-myosin heavy chain
ANOVA	Analysis of variance
Atg	Autophagy
Atg7	Autophagy Related 7
Atg7 cKO	Tamoxifen-inducible $Atg7^{f/f}$ mice
Atg14L	Autophagy Related 14
ATP	Adenosine triphosphate
Baf A1	Bafilomycin A1
BAX/BAK	BCL2-associated X protein (BAX)/BCL2-antagonistic/killer (BAK)
BCL2	B-cell CLL/lymphoma 2
BCL2L13	BCL2 like 13
Beclin1 cKO	Tamoxifen-inducible $Beclin1^{f/f}$ mice
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BNIP3L/Nix	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 Like
cKO	Conditional knockout
CVD	Cardiovascular disease
CPCs	Cardiac progenitor cells
DM	Differentiation media
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNM1L	Dynamin related protein 1

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
EEA1	Early endosome antigen 1
EF	Ejection fraction
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EHD3	EH domain containing 3
EM	Electron microscopy
EPCs	Endothelial progenitor cells
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FS	Fractional shortening
FUNDC1	FUN14 domain containing 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
h	Hour(s)
H&E	Hematoxylin and Eosin

HSCs	Hematopoietic stem cells
HW	Heart weight
I/R	Ischemia/reperfusion
ICLAC	International cell line authentication committee
IGFRβ	Insulin-like growth factor receptor 1 beta
IMM	Inner mitochondrial membrane
LC3	Microtubule-associated protein 1 light chain 3
Mdivi-1	Mitochondrial division inhibitor 1
MEF	Mouse embryonic fibroblast
Mfn1/2	Mitofusin 1/2
MI	Myocardial infarction
MOI	Multiplicity of infection
MSCs	Mesenchymal stem cells
mtDNA	Mitochondrial DNA
MVB	Multivesicular body
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P62/Sqstm1	Sequestosome 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PHB2	Prohibitin 2

PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
POLG	DNA polymerase gamma, catalytic subunit
PPARGC1A	PPARG coactivator 1 alpha
PTM	Post translational modification
qPCR	Quantitative polymerase chain reaction
Rab5	Ras-Related Protein Rab-5A
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
siRNA	Small interfering ribonucleic acid
TEM	Transmission electron microscopy
TL	Tibia length
TOM	Translocase of the outer mitochondrial membrane
TRPM4	Transient Receptor Potential Cation Channel Subfamily M4
UVRAG	UV radiation resistance associated gene
Vps34	Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3
WT	Wild type

LIST OF FIGURES

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ABSTRACTS

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ABSTRACT OF THE DISSERTATION

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Proper cellular quality control is essential in all cell types but is especially important in a post-mitotic cell such as the cardiomyocyte to prevent cell death. Deficiencies in cellular quality control pathways such as autophagy or the endosomal degradation pathway are known to lead to a variety of neurodegenerative and cardiovascular

diseases. Here, I focus on the regulation of plasma membrane receptor trafficking in the heart through the endosomal pathway by the scaffolding protein Beclin1. The endosomal pathway is mediated by Rab proteins, a family of GTPases. While Beclin1 is well-known as a positive regulator of autophagy, binding to the PI3-K Vps34 and autophagy protein Atg14L, its mechanistic role in the endosomal pathway is less clear. I report that Beclin1 mediates endosomal trafficking through early endosome marker Rab5. Loss of Beclin1 leads to accumulation of the EGF receptor in Rab5 positive vesicles in the cytosol. Additionally, I demonstrate that Beclin1 is required for UVRAG protein stability, a protein involved in a variety of cellular trafficking pathways, and that Beclin1, UVRAG, and Rab5 all colocalize at the early endosome. *In vivo,* I found that loss of Beclin1 in the heart leads to rapid heart failure and reduced survival. Furthermore, loss of Beclin1 in the heart led to increased protein levels of early endosome markers Rab5 and EEA1. Finally, restoration of UVRAG alleviated the cardiac phenotype observed after loss of Beclin1, indicating the importance of the endosomal pathway in maintaining heart function.

I also report a novel mechanism for selective removal of mitochondria by autophagy, or mitophagy, through mitochondrial outer membrane receptors FUNDC1 and BNIP3L in cardiac progenitor cells (CPCs). This pathway is distinct from the traditional mitophagy pathway mediated by PINK1/PARKIN and relies on direct binding to the autophagosome by FUNDC1 and BNIP3L. I show that FUNDC1 and BNIP3L are required for the formation of a functional, interconnected mitochondrial network in CPCs during differentiation. Also, I found that knockdown of FUNDC1 and BNIP3L decreased maximal respiration and increased CPC susceptibility to H_2O_2 mediated cell stress. Notably, impaired FUNDC1 and BNIP3L function reduced cell viability after injection into the

myocardium post-myocardial infarction, indicating functional mitophagy is crucial for CPCs to persist in the unfavorable conditions of the injured heart. Together, these data uncover new mechanism(s) of cellular quality control in the heart and could have significant impact on the design of therapeutics targeting either the endosomal pathway or mitophagy receptors in cardiovascular disease.

CHAPTER 1: INTRODUCTION

1.1 Heart Disease and Cellular Quality Control by Beclin1

Cardiovascular disease (CVD) remains the number one cause of death in the United States, with an age-adjusted death rate of 219.4 per 100,000 people (Virani et al., 2020). Between 2013 and 2016, 121.5 million Americans had some form of CVD. This number is only expected to increase, as age is a significant risk factor for CVD and modern advances in medicine have increased lifespan. CVD encompasses diseases from diabetic cardiomyopathy to ischemic heart disease. The indirect and direct cost of treating CVD alongside losses in productivity is estimated to be $351.3 billion annually, with this number expected to rise to nearly $1 trillion by 2030. CVD places an immense health and financial problem that must be addressed. Thus, there is a great need for new therapeutics to help alleviate such diseases.

Upstream regulation of intracellular quality control pathways in the heart is a tightly regulated process. Different stressors require different responses from the cell to maintain cardiomyocyte viability and respond to the damage in an appropriate manner. Beclin1 is a scaffolding protein that forms unique protein complexes in order to regulate different intracellular quality control pathways. For example, it is well-known that Beclin1 is a positive regulator of autophagy and is essential for nucleation of the autophagosome by binding to the class III PI3-K Vps34 and autophagy protein Atg14L (Itakura et al., 2008). However, despite its well-known role in autophagy, evidence suggests roles for Beclin1 in regulating multiple pathways. Beclin1 global knockout mice are known to be embryonic lethal, while autophagy deficient mice survive until birth but die soon after(Itakura et al., 2008). This suggests that Beclin1 plays an important role beyond that of autophagy.

1.2 The Endosomal Degradation Pathway and Receptor Trafficking

The endosomal degradation pathway is regulated by the Rab GTPase family of proteins and mediates plasma membrane receptor degradation and recycling in the cell (Jovic et al., 2010). Early endosomes are characterized by the presence of Rab5, which in its active GTP bound state will bind to the membrane of the early endosome to mediate its maturation. At the early endosomes, cargo is sorted into one of two respective pathways: recycling back to the plasma membrane or sorted for degradation. Different proteins play key roles in the recycling pathway, including Rab4 and Rab11, while maturation of the early endosome into a late endosome is characterized by the replacement of Rab5 by Rab7 on the endosome (Figure 1.1). These late Rab7-positive endosomes subsequently fuse with the lysosome for degradation of cargo (Babst, 2011). Recent evidence has demonstrated that loss of Beclin1 leads to impaired plasma membrane receptor degradation (McKnight et al., 2014). Mechanistically, it remains unclear how Beclin1 is regulating the endosomal pathway, especially in the heart. There is some evidence indicating that the binding of Beclin1 to UV Radiation Resistance Associated Gene (UVRAG) is important for regulating endosomal activity (Liang et al., 2008; Nagy et al., 2016). UVRAG was initially reported to function as a tumor suppressor in colon cancer (Liang et al., 2006), while additional studies demonstrated that UVRAG regulates autophagy alongside Beclin2 at the level of initiation (He et al., 2013) and maturation (Liang et al., 2008). Despite this, recent work has challenged the role of UVRAG as an essential regulator of autophagy. First, truncating mutations in UVRAG in colon cancer cells has no effect on autophagy (Knaevelsrud et al., 2010). Second, UVRAG depletion in HeLa cells disrupts plasma membrane receptor EGFR degradation

but does not change autophagosome formation or autophagic flux (Jiang et al., 2014). Third, UVRAG-mediated activation of the endosomal-lysosomal degradation pathway, but not autophagy, is required for axon pruning in *Drosophila* (Issman-Zecharya and Schuldiner, 2014). Lastly, Beclin1$^{-/-}$ mouse embryonic fibroblasts (MEFs) have extremely low levels of UVRAG which is correlated with impaired endosomal activity (McKnight et al., 2014). In summary, these studies raise the possibility that Beclin1 and UVRAG form a unique protein complex in the cell to regulate endosomal activity instead of autophagy (Figure 1.2). This is an important question that will be addressed in chapters 3 and 4 of this dissertation.

1.3 Autophagic Degradation in the Heart

Autophagy is a well-conserved intracellular quality control pathway that is responsible for degrading protein aggregates and dysfunctional organelles such as mitochondria (Tanida, 2011). Autophagy was first discovered in yeast (Ohsumi, 2014), and its mammalian orthologs have been shown to target intracellular components for encapsulation inside of autophagosomes. Once the target cargo is inside of autophagosomes, they are subsequently able to fuse with lysosomes for degradation by acid hydrolases. Deregulation of autophagy have been observed in a variety of CVDs where too little or too much autophagy can lead to cell death and disease development. For example, excessive activation of autophagy can be detrimental in type 2 diabetes, whereas too little autophagy leads to cardiac dysfunction in anthracycline-induced cardiotoxicity (Lampert and Gustafsson, 2018). It is clear that proper regulation of

autophagy is necessary to prevent development of CVD, and whether autophagy is beneficial or detrimental is context dependent.

One of the main functions of autophagy is the removal of aberrant mitochondria in a process termed mitophagy. Mitochondria are crucial for heart function, as they provide ATP to the beating myocytes by oxidative phosphorylation that is necessary for the heart to contract (Kubli and Gustafsson, 2012). Mitochondria are densely packed in cardiomyocytes, and the heart has among the highest concentration of mitochondria among all tissue types. In cardiomyocytes, mitochondria make up ~30% of each myocyte's volume (Schaper et al., 1985). Mitochondria can be damaged in the unfavorable conditions of the stressed or diseased heart, where they can then activate cell death by releasing pro-death factors and/or generating reactive oxygen species (ROS) (Tait and Green, 2013). Mechanistically, mitochondria are able to regulate cell death through the Bax/Bak pore or opening of the mitochondrial permeability transition pore (mPTP). Cardiomyocyte death is a hallmark of a variety of CVDs, including that of heart failure, myocardial infarction, and ischemia/reperfusion (I/R) (Chiong et al., 2011). As cardiomyocytes are post-mitotic, these cells are not easily replaced if lost and presents a significant issue for cardiac health. Thus, maintaining the proper levels of functional mitophagy in cardiomyocytes is especially important to help combat a variety of CVDs. Thus, the development of therapeutic options for lost cardiomyocytes is an important area of research. In the next section, I will discuss my research on stem cell therapies, specifically those in the heart, and the potential they represent to treat a variety of CVDs.

1.4 Stem Cell Therapy in the Heart

An area of significant research to help combat the effects of lost cardiomyocytes is through stem cell therapy. Stem cells in the heart, cardiac progenitor cells (CPCs), have multiple mechanisms in which they are able to help an injured heart. First, they are able to differentiate into various cardiac cells to replace lost cells and to help generate new blood vessels (Cho et al., 2017; Kulandavelu et al., 2016). Second, they release protective paracrine factors such as Abi3bp, which help to improve ventricular systolic function and reduce infarct size in patients with heart failure following myocardial infarction (Hodgkinson et al., 2016). However, despite these positive effects, the injection of cardiac progenitor cells as a viable therapy for CVD remains incomplete. CPCs are often unable to persist in the unfavorable conditions of the failing heart for a significant amount of time, making their impact limited. Thus, there is great need for a better understanding of the biology of these cells in order to better design them to be able to survive in the environment of the injured heart. Here, we will dive into the role of mitophagy as a CPC differentiates from a stem cell into a cardiomyocyte. We will focus on the two most commonly studied mitophagy pathways: the PINK1/PARKIN pathway, and the mitophagy receptor pathway.

1.4.1 Mitophagy during CPC differentiation

CPCs in their quiescent, stem-cell state rely primarily on glycolysis for energy generation (Orogo et al., 2015). They reside in stem cell niches, or areas of the tissue that favor a quiescent state such as those under hypoxic conditions, as well as glycolytic metabolism to maintain their ability for self-renewal to keep a significant number of available progenitor cells in the tissue at all times (Ito and Suda, 2014). Upon induction of differentiation, there is evidence mitophagy is involved through the removal of immature

mitochondria to allow the stem cell to make a metabolic switch to that of oxidative phosphorylation with the establishment of a new, interconnected mitochondrial network (Sin et al., 2016). Additionally, in other stem cell contexts, mitophagy has been shown to be important for metabolic reprogramming. The conversion of fibroblasts into induced pluripotent stem (iPS) cells involves the switch from oxidative phosphorylation to that of glycolysis requires the removal of mature mitochondria by mitophagy (Ma et al., 2015). Additionally, loss of mitophagy through PINK1 deletion decreased the rate and efficiency of iPS cell reprogramming, increasing spontaneous differentiation and the formation of cell populations with different, suboptimal cell types (Vazquez-Martin et al., 2016). These data indicate that mitophagy is crucial both for determining stem cell fate but also for the conversion of differentiated cells back into progenitor cells. Given the importance of mitophagy on both directions of a stem cell's life cycle, understanding the mechanisms behind how mitophagy is recognizing and degrading these mitochondria is critical.

1.4.2 PINK1/PARKIN Mediated Mitophagy

The most well-known and studied mechanism of mitophagy involves the PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin. Parkin was discovered as frequently mutated in young onset autosomal recessive Parkinson's disease (Mizuno, 2007), while binding partner PINK1 accumulates on the outer mitochondrial membrane upon loss of mitochondrial membrane potential (Jin and Youle, 2012). Once there, Parkin ubiquitinates a variety of proteins on the outer mitochondrial membrane, whose ubiquitination recruits downstream effectors that recognize the mitochondria for engulfment by an autophagosome. As an E3 ubiquitin ligase, Parkin has a variety of substrates, and such proteins have been demonstrated to include

MitoNEET/CISD1, Mitofusin (MFN), Miro, Translocase of the outer membrane20 (Tom20), and voltage dependent anion selective channel (VDAC) (Koyano et al., 2019). Adaptor proteins such as p62 which are directly bound to autophagosomes are then able to recognize the ubiquitinated proteins by binding to ubiquitin, mediating autophagosome engulfment of depolarized mitochondria. After maturation, the autophagosome is then able to fuse with the lysosome for degradation (Figure 1.3).

Failure of the PINK1/Parkin pathway to remove aberrant mitochondria has been demonstrated to play an important role in a variety of CVD contexts. Most of the emerging evidence on the importance of Parkin in the heart comes from *in vivo* mouse knockout models. For instance, germ-line deletion of Parkin demonstrated changes in the mRNA levels of hundreds of cardiac-related genes, indicating a potential compensatory mechanism for the loss of Parkin (Bhandari et al., 2014), while there is little change in the transcriptome when Parkin is deleted in the adult mouse heart (Song et al., 2015a). These findings suggest that adaptations are able to be made in Parkin-deficient hearts if lost from early embryogenesis, while the adult heart is less able to do so. Cardiomyocyte-specific deletion of Parkin in mice does not lead to any change in heart function at baseline, but instead have reduced survival and increased infarct size after myocardial infarction (Kubli et al., 2013b). Additionally, as these mice aged, they had smaller and less organized mitochondria that developed electron dense inclusions (Kubli et al., 2013a). Notably, Parkin transcript levels and protein levels are relatively scarce in the adult mouse heart compared to other mitophagy proteins such as Mfn2 or PINK1 (Dorn, 2016). However, there is a variety of evidence that Parkin is upregulated in the heart in response to stress. In PINK1 knockout hearts, Parkin protein is significantly increased

(Kubli et al., 2015), similar to in the mouse hearts with deletion of mitochondrial fission regulator Drp1. Thus, there is significant evidence that Parkin functions as a stress response protein in the heart compared to being required to maintain cardiac homeostasis. Due to its importance in CVD, it is clear that PINK1/Parkin-mediated mitophagy is essential to maintain proper cardiac function and cardiomyocyte viability, especially under pathological conditions.

1.4.3 Mitophagy Receptor Mediated Mitophagy

1.4.3.1 Mitophagy Receptors

Although PINK1/Parkin mediated mitophagy is the most well studied mechanism of mitophagy, recent work has discovered alternative mechanisms of mitochondrial removal through mitophagy receptors. Mitophagy receptors are a group of proteins and lipids that are mostly present on the outer mitochondrial membrane (OMM) (although there is evidence of inner mitochondrial membrane receptors as well) which are able to bind directly to the autophagosome either through LC3 or gamma-aminobutyric acid receptor-associated protein (GABARAP) (Hanna et al., 2012; Schwarten et al., 2009) (Figure 1.3). Some examples of mitophagy receptors in the OMM include BNIP3, BNIP3L, BCL2L13 and FUNDC1 and inner mitochondrial membrane PHB2 (Lampert et al., 2019; Wei et al., 2017). While a majority of these mitophagy receptors are known to have pro-apoptotic functions due to their BH3 domain (BNIP3, BNIP3L, BCL2L13), it is clear that they also serve roles in clearing mitochondria directly. Loss of mitophagy receptors FUNDC1 and BNIP3L in cardiac progenitor cells was shown to affect mitochondrial function and network expansion upon differentiation (Lampert et al., 2019). Additionally, mice deficient in both casein kinase 2α and FUNDC1 had increased sensitivity to

myocardial ischemia/reperfusion injury, with reduced mitophagy, electron transport chain inhibition, and increased oxidative stress (Zhou et al., 2018). In other CVD contexts, the amount of mitophagy through mitophagy receptors such as BNIP3L will help determine whether it is beneficial or pathological to cardiomyocytes. For example, in stroke, excessive mitochondrial clearance by BNIP3L have deleterious effects, while some mitophagy is beneficial (Shi et al., 2014; Yuan et al., 2017). It is clear that the cell has multiple mechanisms of mitophagy, and its regulation is essential to maintaining cardiomyocyte function and cell viability.

1.4.3.2 Fission/Fusion

The regulation of mitochondrial dynamics, especially in the context of stem cells, is a unique process that requires a balance of mitochondrial fission and fusion. In mitophagy, cells can undergo asymmetric fission in order to sequester damaged parts of the mitochondria for selective removal by autophagosomes. Here, the mitochondria can traffic its damaged proteins to a daughter mitochondrion, which it can then separate from the second, healthy daughter mitochondrion. The healthy daughter mitochondrion remains and is then able to fuse back into the mitochondrial network, while the damaged daughter mitochondrion is removed by degradation. There is ample evidence that the protein Drp1 (DNM1L) regulates mitochondrial fission by enclosing around a mitochondrion to activate fission and create two separate daughter mitochondrion (Yamano and Youle, 2011). Similar to levels of mitophagy, Drp1 mediated mitochondrial fission is an important balancing act in the cardiomyocyte. Loss of Drp1 in mouse hearts results in cardiomyocyte cell death and lethal dilated cardiomyopathy (Song et al., 2015b),

while overexpression of Drp1 in the heart led to increased mitochondrial fragmentation without an effect on cardiac function (Song et al., 2017).

Mitochondrial fission is clearly important for regulating mitochondrial function and removal, but reintegration into the mitochondrial network and prevention of further mitophagy is coordinated by mitochondrial fusion. It is has been reported that fusion also functions to dilute the amount of damaged proteins in one particular mitochondria by fusing two mitochondria together (Twig and Shirihai, 2011). Fusion of the outer mitochondrial membrane involves two main proteins: Mfn1 and Mfn2. Knockdown of either Mfn1 or Mfn2 leads to increased mitochondrial fission, while deletion of Mfn2 has a strong disruption in the placental trophoblast giant cell layer (Chen et al., 2003). Additionally, Mfn2 KO mice have decreased autophagy, while Mfn1 overexpression leads to a 40% decrease in mitochondrial volume (Twig and Shirihai, 2011). Cardiac specific deletion of either Mfn1/2 is embryonic lethal despite a lack of cardiomyocyte cell death (Song et al., 2015b). Meanwhile, fusion of the inner mitochondrial membrane is mediated by Opa1, which also maintains the cristae structure of the newly fused mitochondrion (Lee and Yoon, 2018). Similar to Mfn1/2, knockout of Opa1 is embryonic lethal, with increased mitochondrial fragmentation observed. Additionally, Opa1 is regulated by proteolytic cleavage, creating an IM-anchored long and short soluble forms of Opa1 to mediate mitochondrial membrane fusion. Finally, Opa1 KO was shown to increase sensitivity to apoptosis and decrease mtDNA levels (Lee and Yoon, 2018). These data demonstrate the essential role for a balance of both mitochondrial fusion and fission in mitophagy to maintain a healthy mitochondrial network and prevent CVD disease.

1.5 Rationale and Specific Aims of the Thesis

CVD places a large health and economic burden on our society, and there is great need for new therapeutics to combat it. While autophagic degradation has been well-studied in the heart, other intracellular quality control pathways have been overlooked. The endosomal pathway is well-known for its trafficking of plasma membrane receptors through receptor recycling or degradation and is known to be important for neuron viability (McKnight et al., 2014). However, studies of this pathway in the heart are still limited. Similarly, Beclin1 is known to be an important regulator of autophagy and implicated as a regulator of the endosomal pathway. It is surprising that despite its importance, the endosomal role of Beclin1 in the heart has yet to be investigated.

Additionally, stem cell therapies have great promise for restoration of heart function in a variety of different CVDs, but cell retention after injection is a major issue. Functional mitochondria are an integral component of cardiac health, including in cardiac progenitor cells, and understanding how they expand to provide energy for a differentiating cell is important to keep them functioning optimally. Damaged mitochondria, whether through accumulation of mtDNA mutations with age or through environmental stressors, can lead to cell death. Thus, the replacement of these damaged mitochondria through mitophagy is critical to avoid cardiomyocyte loss and help maintain physiological cardiac function. Given this, we sought to better understand the role of mitophagy in cardiac stem cells while also investigating the role of Beclin1 in mediating the endosomal pathway in the heart. Proper cellular quality control is an essential process in cardiomyocytes, and therefore I hypothesize that **1.) Beclin1 regulates the endosomal pathway through its**

interaction with UVRAG and is necessary for cardiac function and 2.) Mitophagy is essential for mitochondrial expansion and cardiac progenitor cell viability.

Aim 1: To examine the role of Beclin1 in regulating the endosomal pathway through its interaction with UVRAG and its importance in heart function *in vivo*. *Hypothesis: Beclin1 is necessary for proper heart function and interacts with UVRAG to mediate endosomal activity.*

Aim 2: To characterize the role and mechanism of mitophagy in cardiac progenitor cells (CPCs). *Hypothesis: Mitophagy is mediated by PINK1/Parkin upon differentiation in CPCs and is important for mitochondrial network expansion.*

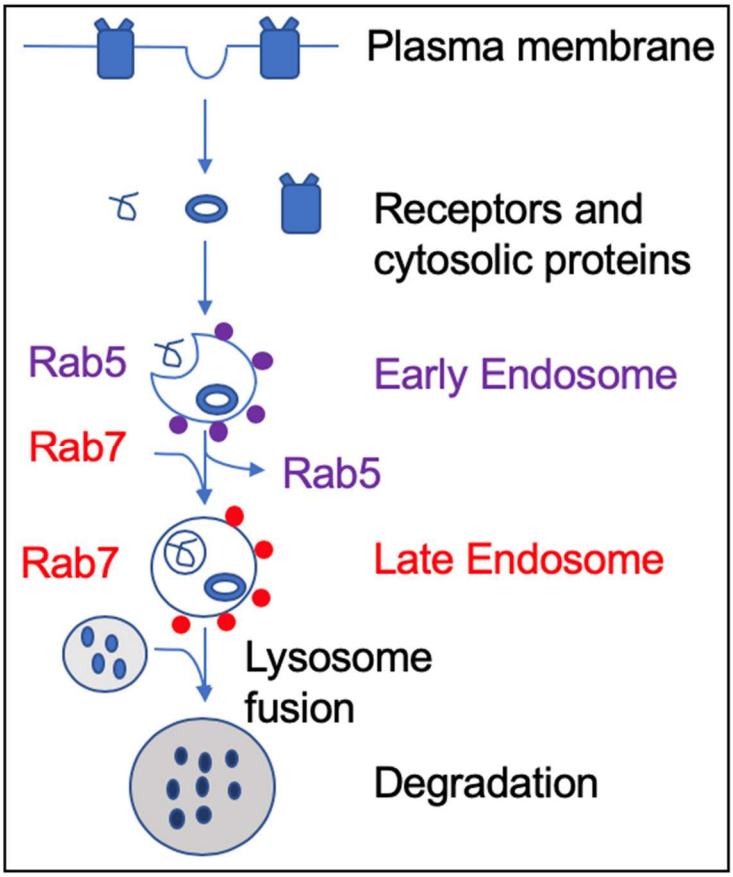

Figure 1.1 Regulation of plasma membrane receptor degradation by the endosomal pathway.
Plasma membrane receptors are internalized and trafficked to Rab5-positive early endosomes. Maturation of the endosomes leads to replacement of Rab5 with Rab7 to form the late endosome (also known as a multivesicular body). Rab7-positive late endosomes then fuse with the lysosome for target degradation.

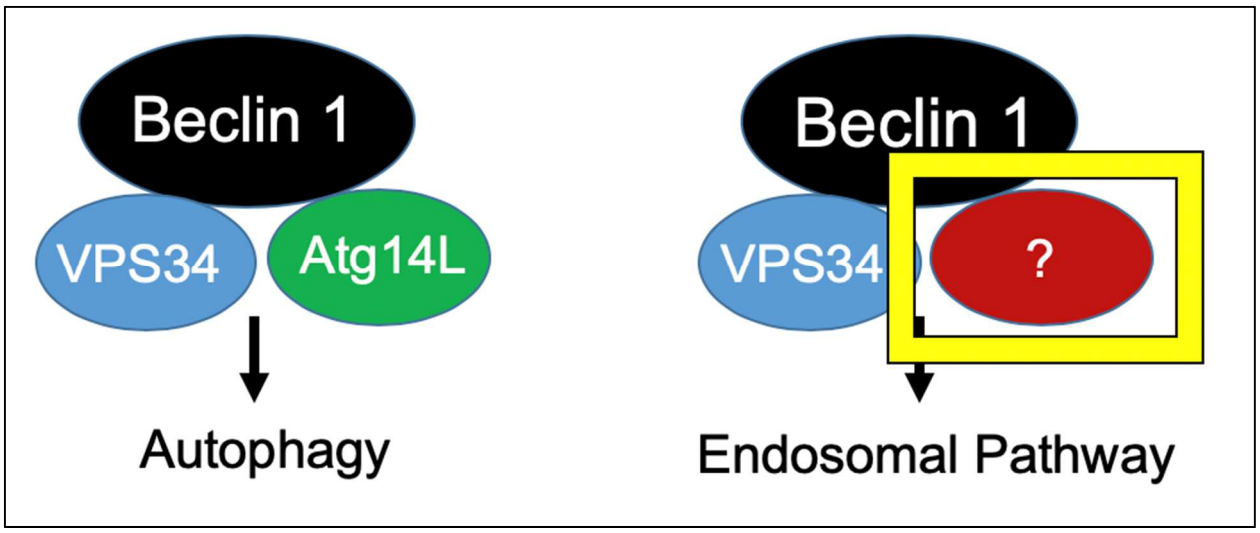

Figure 1.2. Beclin1 forms unique protein complexes to regulate different intracellular quality control pathways.
As a scaffolding protein, Beclin1 is known to form a protein complex with the class III PI3-K Vps34 and autophagy related protein Atg14L in order to regulate autophagy and autophagosome nucleation. However, despite being implicated as a regulator of the endosomal pathway, the Beclin1 protein complex involved in regulating this pathway remains unclear.

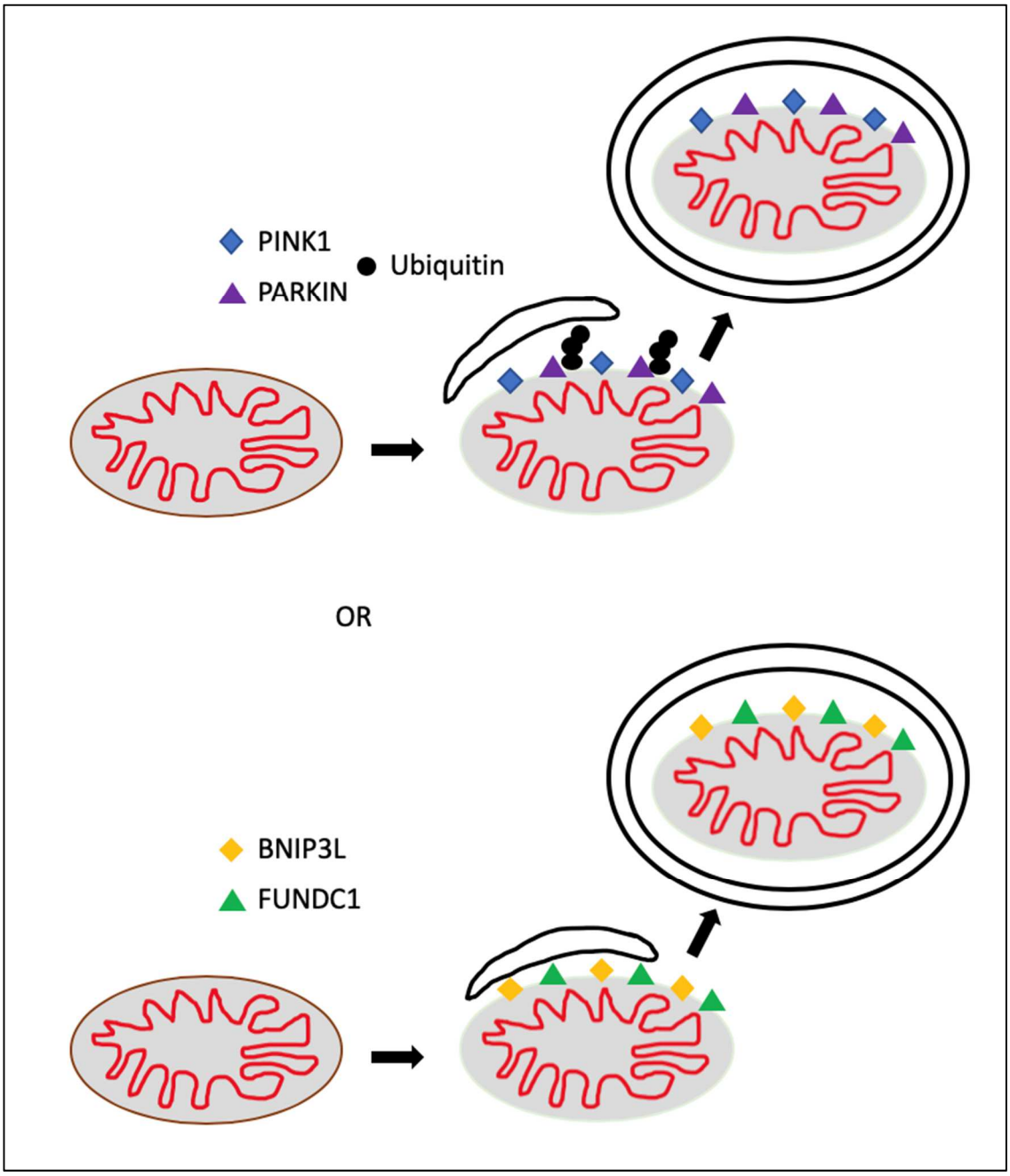

Figure 1.3. Loss of mitochondrial membrane potential leads to degradation by PINK1 and PARKIN or Mitophagy Receptors.
Upon loss of mitochondrial membrane potential, kinase PINK1 accumulates on the outer mitochondrial membrane (OMM) and recruits E3 ubiquitin ligase PARKIN. PARKIN proceeds to ubiquitinate substrates on the OMM, leading to downstream signaling and recognition of the mitochondria by a double membrane autophagosome for engulfment. Alternatively, mitophagy can proceed directly by binding of the autophagosome to OMM mitophagy receptors such as FUNDC1 or BNIP3L.

CHAPTER 2: EXPERIMENTAL METHODS AND MATERIALS

Antibodies

The following antibodies were used for immunofluorescence (IF) and Western blotting (WB) experiments: Actin (WB 1:1000; Genetex; no. 110003), Beclin1 (WB 1:1000; Santa Cruz; no. sc-11427), Bnip3l (WB 1:500; ProSci; no. 2289), DNM1L (WB 1:1000; BD transduction laboratory; no. 611113), p-DNM1L-S616 (WB 1:1000; Cell Signaling Technology; no. 3455), EGFR (WB 1:1000; Millipore Sigma; no. 06-847), FUNDC1 (WB 1:1000; Aviva Biosystems; no. ARP53280_P050), Gapdh (WB 1:2000, Genetex; GTX627408), IGFRβ (WB 1:1000; Cell Signaling Technology; no. 3027), LC3 (WB 1:1000; Cell Signaling; no. 4108), MFN1 (WB 1:1000; Santa Cruz Biotechnology; no. 50330), MFN2 (WB 1:1000; MilliporeSigma; no. M6319), MYC (WB 1:1000; MilliporeSigma; no. M4439), p62 (WB 1:1000; Abcam; no. ab56416), Parkin (WB 1:1000; Cell Signaling Technology; no. 4211), OXPHOS (WB 1:1000; Abcam; ab110413), Rab5 (IF 1:100; WB 1:1000; Cell Signaling; no. 3547), Tim23 (WB 1:1000; BD Biosciences; no. 611222), Tom20 (IF 1:200; Santa Cruz; sc-11415), Tubulin (WB 1:2000; MilliporeSigma; no. T6074), and UVRAG (WB 1:1000; MBL Life Science; no. M160-3). Secondary antibodies used were goat anti-mouse or goat anti-rabbit HRP, Alexa Fluor 488 or 594 (Life Technologies). Membranes were imaged using a ChemiDoc XRS+ System (Bio-Rad).

***In vivo* mouse generation, echocardiography, and myocardial infarction**

All experimental procedures were performed in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of the University of California San Diego. αMHC-MerCreMer mice were obtained from Jackson Labs (Stock no. 005657). Beclin1$^{flox/flox}$ mice were obtained from Dr. Zhenyu Yue, Icahn School of Medicine at Mount Sinai, New York, New York, USA (McKnight et al., 2014). Atg7$^{flox/flox}$ mice were obtained from Dr. Tomoki Chiba, Metropolitan Institute of Medical Science, Japan (Komatsu et al., 2005).

All animal protocols and studies were approved by the review board of the Institutional Animal Care and Use Committee at San Diego State University. Myocardial infarctions were carried out on 8-week old C57/B6 male mice under 2% isoflurane anesthesia as previously described (Gao and Koch, 2013). Briefly, the heart was popped out through the fourth intercostal space, and the left anterior descending artery was ligated at the distal diagonal branch with a 7–0 suture. Infarction was confirmed by blanching of anterior left myocardium wall. Following ligation, either control CPCs or *Bnip3l* and *Fundc1* siRNA CPCs stably expressing GFP together with DiI stain as an injection tracer (Thermo Fisher Scientific, D3911; 25 μg/mL) were delivered intramyocardially at three sites at the border of the blanching area with a total of 100,000 cells/10 μL per heart. The heart was immediately placed back into the intrathoracic space followed by muscle and skin closure.

Echocardiography was executed as previously described (Kubli et al., 2013b) using a Vevo770 In Vivo Micro-Imaging System with an RMV707B 15-45 MHz imaging transducer (VisualSonics Inc.). Mice were anesthetized through a nose cone (1.5%

isoflurane, 98.5% O_2). Mice were placed in a supine position on a warming pad kept at 42°C. Data analysis was performed using the VisualSonics software.

Mitochondrial Function

Mitochondrial membrane potential was measured by incubating CPCs with tetramethylrhodamine methyl ester (Invitrogen, T668; 25 nM) and Hoechst 33342 (Life Technologies, H3570; 10 µg/ml) for 20 min at 37°C. Live cells were imaged using a Carl Zeiss Axio Observer Z1 at 40x magnification and fluorescence intensity was quantified using ImageJ software. At least 30 cells were quantified per condition in replicate. Mitochondrial oxygen consumption of monolayers of CPCs was measured using the Seahorse XF96 analyzer (Agilent Technologies, Santa Clara, CA), adapted from a previously described protocol (Rikka et al., 2011). 1×10^4 cells/well were equilibrated in XF Base medium (Agilent Technologies, 102353-100) supplemented with 1 mM sodium pyruvate (MilliporeSigma, S8636), 3 mM glutamine (ThermoFisher Scientific, 25030149), and 10 mM glucose (MilliporeSigma, G8769) for 1 h before addition of 2 µM oligomycin (MilliporeSigma, 75351) to measure ATP-linked respiration. Three successive additions of 1.5 µM FCCP (MilliporeSigma, C2920) were added to measure maximal respiration, with the full respiration profile obtained after addition of antimycin A (MilliporeSigma, A8674). Oxygen consumption rates were normalized against cell number.

Endothelial Tube Formation Assay

Adapted from a protocol as described previously(Castaldi et al., 2017), CPCs were incubated for 3 d in DM to generate conditioned media. Bovine aortic endothelial cells

(75,000 cells/well) were plated on Geltrex (Invitrogen, A14132-02) coated 24-well plates with conditioned media from CPCs (Castaldi et al., 2017). Tube formation was assessed using a Carl Zeiss Axio Observer Z1 at 10x magnification after 14 h and tube formation was quantitated as previously described(Moc et al., 2015). Each condition was quantitated from at least 15 images per well in replicate.

Cells and Culture Conditions

CPCs were isolated from ~2 month old homozygous *polg*$^{D257A/D257A}$, *prkn*$^{-/-}$ (Goldberg et al., 2003), and wild-type mice as described previously (Orogo et al., 2015). CPCs were cultured in DMEM/F12 (GIBCO, 11330-032; 1:1) media containing embryonic stem cell grade FBS (GIBCO, 10439-016; 10%), leukemia inhibitory factor (MilliporeSigma, LIF2010; 1:1000), insulin transferrin selenium (Lonza, 17-838Z; 1:500), EGF/epidermal growth factor (MilliporeSigma, E9644; 1:5000), FGF2/basic fibroblast growth factor (Peprotech Inc., 100-18B; 1:5000), and antibiotic-antimycotic (GIBCO, 15240-062; 1X). To induce differentiation, CPCs were incubated in α-Minimal Essential Medium containing 10 nM dexamethasone (Alfa Aesar Chemicals, A17590) for up to 7 d (Orogo et al., 2015).

GFP-stable CPCs were generated by lentiviral infection. In short, WT CPCs were infected with 10 MOI of Lenti-PGK-EGFP (puromycin) in the presence of 8 μg/mL polybrene (MilliporeSigma, TR-1003). Cells were incubated for 48 h, after which GFP-positive cells were selected for using 10 μg/mL puromycin (MilliporeSigma, P9620). GFP-CPCs were subsequently cultured in CPC media with puromycin until a stable line was established.

Human CPCs for single cell RNA seq experiments were isolated as previously described (Monsanto et al., 2017). The National Institutes of Health guidelines for human subject research are consistent with Institutional Review Board exemption based on the use of tissues that are waste discards from normal and routine clinical procedures of LVAD surgery (45 CFR 46.101). Briefly, cardiac biopsies were obtained from patients undergoing LVAD implantation. The cardiac tissue was minced and digested in collagenase solution (Worthington Biochemical Corporation, LS004174; type II 225 U/mg dry weight) for 1.5–2 h at 37°C with continuous shaking. After digestion, the suspension was centrifuged at 350 g for 5 min and resuspended in human CPC media. The cells were filtered through a 100 μm filter (Corning, 352360) followed by a 40-μm filter (Corning, 352340) and centrifuged at 150 g for 2 min to eliminate cardiomyocytes. The supernatant was further centrifuged at 350 g for 5 min and resuspended in human CPC media and incubated overnight at 37°C in a CO_2 incubator.

Mouse embryonic fibroblasts (MEFs) were maintained in MEF culture media containing DMEM+Glutamax, (GIBCO, 10569-044) supplemented with 10% FBS (GIBCO, 16000-044) and antibiotic-antimycotic (GIBCO, 15240-062) in a 5% CO_2 atmosphere at 37 °C. WT and Beclin1$^{-/-}$ MEFs were generously provided by Dr. Zhenyu Yue (Icahn School of Medicine at Mount Sinai, USA); Neither of these cell lines are listed in the database of commonly misidentified cell lines maintained by ICLAC. Cell lines have been tested for mycoplasma contamination and were negative.

Infections, Transfections, Plasmids, and siRNA Knockdown

Cells were transiently transfected with DNA using Fugene 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Transfection plasmids: pcDNA4-Beclin1-HA, a gift from Qing Zhong (Addgene plasmid 24399) (Sun et al., 2008), mCherry-UVRAG, a gift from Do-Hyung Kim (Addgene plasmid 86743) (Kim et al., 2015), GFP-Rab5Q79L, a gift from Sergio Grinstein (Addgene plasmid 35140) (Bohdanowicz et al., 2012), EGFR-GFP, a gift from Alexander Sorkin (Addgene plasmid 32751) (Carter and Sorkin, 1998), and/or pCLBW-*Cox8*-EGFP-mCherry, a gift from David Chan (Addgene plasmid 78520) (Rojansky et al., 2016). For siRNA knockdowns, CPCs were transfected with 5 nM of *Bnip3l, Fundc1*, and/or *Bnip3* siRNA (Sigma Mm01_00038852, Mm01_00049694, and Mm01_00115985, respectively) using the HiPerfect transfection reagent according to the manufacturer's instructions (Qiagen, 301705). CPCs were re-transfected mid-way through differentiation for 7 d experiments. For knockdown of Beclin1 and UVRAG in MEFs, cells were incubated with UVRAG or Beclin1 siRNA (50 nM, 72 hours) using RNAiMax transfection reagent (ThermoFisher, 13778030). All experiments were performed 24 hours after the infection.

Cells were infected with adenoviruses in DMEM + 2% heat inactivated serum for 4 h and rescued with growth media. Experiments were performed 20 h later, for a total of 24 hours post initial infection. CPCs were infected with adenoviruses (MOI: 100) encoding *β-Gal, mCherry-PRKN*, or *GFP-LC3* as previously described (Hanna et al., 2012). All experiments were performed 24 h after the infection.

MEFs were infected with an adenovirus encoding HA-Beclin1 (MOI: 200) or β-gal (MOI: 75) to assess rescue of UVRAG protein. For *in vivo* infections, mice were infected

by intravenous tail injections at $5*10^{11}$ pfu/ml of AAV9-FLAG-UVRAG or AAV9-GFP for 3 weeks prior to loss of Beclin1 (Vigene Biosciences).

Cell Death Assays

CPCs in DM were incubated with vehicle or 10 μM Mdivi-1 (MilliporeSigma, M0199) for 16 h prior to addition of 200 μM H_2O_2 (MilliporeSigma, H1009). After 8 h, CPCs were stained with YOPRO1 (ThermoFisher Scientific, Y3603; 1:1000) and Hoechst 33342 (Life Technologies, H3570; 10 μg/ml) for 5 min at 37°C. Cells were imaged using a Carl Zeiss Axio Observer Z1 at 10x magnification and cell death was quantified with ImageJ software. At least 175 cells were quantitated per condition in replicate.

Single Cell RNA Sequencing

Freshly isolated (P0) or cultured (P5) CPCs were loaded on a Chromium™ Controller (10x Genomics) to generate single-cell Gel Bead-In-EMulsions (GEMs). Single-cell RNA-Seq libraries were prepared using Chromium™ Single Cell 3' Library & Gel Bead Kit v2 (10x Genomics, 120267). GEM-reverse transcription (RT) was performed in a C1000 Touch Thermal cycler. After RT, GEMs were broken and the single-strand cDNA was cleaned up with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific, 37002D) and SPRIselect Reagent Kit (Beckman Coulter, B23317; 0.6X SPRI). cDNA was amplified and then cleaned up with the SPRIselect Reagent Kit. Indexed sequencing libraries were constructed using the reagents in the Chromium™ Single Cell 3' Library & Gel Bead Kit v2, following these steps: (1) end repair and A-tailing; (2) adapter ligation; (3) post-ligation cleanup with SPRIselect; (4) sample index PCR and cleanup. For quality

control of libraries, each library was tested with Bioanalyzer (average library size: 450–490 bp). The sequencing libraries were quantified by quantitative PCR using KAPA Biosystems Library Quantification Kit (Illumina, KK4824) and Qubit 3.0 with dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851). Sequencing libraries were loaded at 2 pM on an Illumina HiSeq2500 with 2 × 75 paired-end kits using the following read length: 98 bp Read1, 8 bp i7 Index, and 26 bp Read2. For the single-cell RNA sequencing of mouse CPCs, 1608 and 850 cells were recovered from freshly isolated and cultured CPC samples, respectively. For the single-cell RNA sequencing of human cardiac stem cells, 814, 1693, and 1765 cells were recovered from cultured CPCs, EPCs, and MSCs, respectively. Samples were down-sampled until all samples have an equal number of confidently mapped reads per cell. The gene-cell barcode matrix was filtered based on number of genes detected per cell (any cells with less than 1000 genes per cell were filtered) and percentage of mitochondrial UMI counts (any cells with more than 10% of mitochondrial UMI counts were filtered). Altogether, 2383 cells and 15786 genes were kept for analysis by Seurat (Macosko et al., 2015). 2221 variable genes were selected based on their expression and dispersion (expression cutoff = 0.0125, and dispersion cutoff = 0.5), and the first 10 principal components were used for clustering analysis.

Immunofluorescence

Similar to as described previously (Hammerling et al., 2017), cells were fixed with 4% paraformaldehyde (Ted Pella Inc.) in Dulbecco's Phosphate Buffered Saline without calcium chloride and without magnesium chloride (PBS) (Gibco, no. 14200-075), permeabilized with 0.2% Triton X-100 in PBS, and blocked in 5% normal goat serum.

Cells were incubated with primary antibodies (4ºC, overnight) in PBS, washed with PBS, incubated with secondary antibodies (37ºC, 1 h), and counter-stained for nuclei with Hoechst 33342 (Invitrogen). Fluorescence images were captured using a Carl Zeiss Axio Observer Z1 fitted with a motorized Z-stage with a 63X Plan-Apochromat (oil immersion) objective. Z stacks were separated by 0.6 µm along the *z*-axis were acquired with an ApoTome using a high-resolution AxioCam MRm digital camera and Zeiss AxioVision 4.8 software (Carl Zeiss). Mitophagy was scored by count of red-only puncta of Cox8-EGFP-mCherry transfected cells on maximal image projections and averaged per cell or by colocalization of GFP-LC3 autophagosomes and Tom20 labeled mitochondria. Enlarged vesicle formation of GFP-Rab5Q79L was assessed using ImageJ and scored as positive if vesicles were a minimum of 3 µm in diameter, and then averaged per condition (minimum of 30 cells per condition per replicate). For split GFP analysis, images were quantified in ImageJ for fluorescence units per cell and averaged per condition (minimum of 20 cells per condition per replicate).

Western Blot Analysis

Similar to as previously described(Hammerling et al., 2017), cells were homogenized in lysis buffer on ice consisting of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science, 11873580001). Cells were homogenized using a 27-gauge needle (BD sciences). After boiling at 95ºC for 5 minutes, the proteins were run on NuPAGE Bis-Tris gels (Invitrogen, NP0302BOX) and then transferred to nitrocellulose membranes (GE Healthcare Life Sciences, 10600001). Membranes were incubated with the indicated

antibodies and imaged with a Bio-Rad ChemiDoc XRS+ imager. Band densitometry quantification was performed using the software Image Lab 4.1 (Bio-Rad).

Split GFP Constructs

Cells were transfected with sequences from the split GFP system(Kamiyama et al., 2016) to assess Rab5A activity. In short, we designed sequences containing the GFP 1-10 barrel bound to Rab5AWT and a 3x-FLAG-GFP 11-Rab5 binding domain (R5BD) from Rabenosyn-5. GFP fluorescence will only be observed when the constructs interact to reform the complete GFP barrel, and thus Rab5 activity can be monitored. Cells were treated with 25 μM FCCP for 4 hours to stimulate Rab5 activity.

Plasma Membrane Fractionation and Proteinase K Receptor Degradation

Cells were subjected to sub-cellular fractionation by centrifugation as adapted from this protocol to assess receptor localization (Yamamoto et al., 2016). In short, cells were subjected to a series of spins to isolate the whole cell lysate, cytosolic, and plasma membrane fractions. Cytosolic and plasma membrane fractions were run on a NuPAGE Bis-Tris gel and analyzed by western blot.

Cells were harvested, spun down at 400 x g for 5 minutes and treated with either PBS, heat-inactivated proteinase K, or proteinase K (50 ng/ul) for 15 minutes on ice. Cells were spun down again, the supernatant was removed, and treated with protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 5 mM) for 5 minutes on ice. After a final spin, cells were lysed in 1% Triton X buffer as described above and run on a NuPAGE Bis-Tris gel.

EGFR Internalization Assay

Similar to as described previously (McKnight et al., 2014), cells were plated and serum-starved for 2 hours before stimulation with EGF (200 ng/ml) in serum-free media. Cells were then harvested on ice and lysed in 1% triton-X lysis buffer and homogenized with a 27-gauge needle (BD sciences) before subsequent processing and Western blot analysis.

Electron Microscopy

Mouse hearts were fixed, mounted, and sectioned as previously described (Kubli et al., 2013b). Sections were imaged using a TECNAI G2 Spirit BioTWIN Transmission Electron Microscope equipped with an Eagle 4k HS digital camera (FEI).

Real Time quantitative PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. qPCR was performed with standard TaqMan primers and TaqMan Universal Mastermix II (Applied Biosystems) or SYBR Green (Applied Biosystems, 4309155) on a CFX96 real-time PCR detection system (Bio-Rad Laboratories). Fold difference was calculated by the comparative C_T($2^{-\Delta\Delta C}$T) method against 18s (Schmittgen and Livak, 2008).

Primers for 18S *Rn18s* (18S Mm03928990), *Prkn* (Mm00450187), *Bnip3* (Mm01275600), *Bnip3l* (Mm00786306), *Fundc1* (Mm00511132), *Gata4* (Mm00484689), *Gata6* (Mm00802636), *Mef2c* (Mm01340842), *Il6* (Mm00446190), *Myh7* (Mm01319006), *Nppa* (Mm01255747),

Nppb (Mm01255770), *TGF-β1* (Mm01178820), *UVRAG* (Mm00724370), *Il6* (Mm00446190) and *Ppargc1a* (PGC-1α Mm01208835) were obtained from ThermoFisher Scientific. *Bcl2l13* (Mm.PT.58.41776912), *Phb2* (Mm.PT.58.7430511), *Ccl2* (Mm.PT.58.42151692), and *Wnt5a* (Mm.PT.58.16402801) were obtained from Integrated DNA Technologies. *Beclin1* primers were designed to recognize exon2 (FWD: 5'- GCATGGAGGGGTCTAAGGCGTC-3' and REV 5'- GTTCCTGGATGGTGACCCGGTC-3') and were obtained from Eton Bioscience Inc.

Immunohistochemistry

Mice were sacrificed at 7 days post tamoxifen injection. Hearts were fixed in 10% neutral buffered formalin for 24 hours and subsequently processed (Thermo Scientific STP 120). Hearts were then embedded in paraffin and sectioned at 5-10 μm thickness (Leica Biosystems) for histological analysis. Deparaffinization and rehydration were performed before staining with Hematoxylin and Eosin (H&E) or Masson's Trichrome (MilliporeSigma) according to the manufacturer's instructions. Staining for Wheatgerm Agglutinin was also done according to manufacturer's protocol (ThermoFisher, W11261). Similarly, apoptosis was measured using a TUNEL staining kit (Roche Applied Science) according to the manufacturer's instructions. Imaging was performed using a Nikon Eclipse microscope (10x and 20x magnification).

For myocardial infarction sectioning, mice were sacrificed at 6 d post injection by chloral hydrate sedation. Hearts were infused with heparin and arrested in diastole under $CdCl_2$ and KCl solution and perfused with 1% paraformaldehyde (ThermoFisher, 28908)

in PBS for 5 min at 80–100mmHg via retrograde cannulation of the abdominal aorta. Retroperfused hearts were removed from the thoracic cavity and fixed overnight in 1% paraformaldehyde at 4ºC. Fixed hearts were gradually dehydrated by 30% sucrose (Fisher Chemical, S3-212) and 1:1 mix of 30% sucrose:Neg50 frozen section medium (ThermoFisher Scientific, 6502) before mounting in Neg50 on dry ice, followed by cryosectioning at 20-µm thickness at −18-20ºC. Heart sections were rehydrated with 1X PBS (GIBCO, 14200-075), permeabilized and blocked with 0.1% Triton X-100 (Sigma, T9284-500ML), 0.1 M glycine (Fisher, BP381-500), 1% BSA (Sigma, A9418), and 10% donkey serum (Millipore, S30-100mL) in PBS for 45 min at room temperature. Sections were incubated in primary antibodies anti-GFP (Rockland Immunochemicals Inc., 600-101-215) and anti-DES/desmin (Abcam, 15200) overnight at 4ºC, followed by incubation in secondary antibodies (ThermoFisher Scientific, A-11055 and A-31573) for 90 min at room temperature. Representative images were scanned using Leica SP8 confocal microscopy.

Statistical Analysis

All experiments were independently repeated in the laboratory. Data were collected from experiments performed in at least triplicate and expressed as mean ± S.E.M. Sample size was not predetermined before start of experiments. Differences between groups were assayed using repeated-measure ANOVA tests with Tukey or Dunnett's post hoc test or by Student's t-test through Prism 6 software. Each test was used based on number of samples being analyzed according to the test's assumptions and requirements. Differences were considered to be significant when $p < 0.05$. Analyses

were done un-blinded with respect to sample identity. Scientific reasons to exclude data included very low transfection efficiency (<25%) or if cell viability was affected in control conditions.

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CHAPTER 3: LOSS OF BECLIN1 IN THE HEART LEADS TO RAPID CARDIAC DYSFUNCTION AND DECREASED SURVIVAL

3.1 Introduction

The heart is an essential organ that requires constant upkeep in order to function properly. Cardiomyocytes are densely packed with mitochondria that utilize oxidative phosphorylation to generate ATP for the beating myocytes (Piquereau et al., 2013). Many studies have demonstrated a potential contribution of dysfunctional mitochondria in different CVDs, such as in dilated cardiomyopathy, cardiac hypertrophy, and perinatal cardiomyopathy, among others (Bravo-San Pedro et al., 2017). However, both organelle and protein quality control pathways are important to prevent development of cardiovascular disease. For example, the recycling and degradation of various membrane proteins through the endosomal pathway is critical to maintain heart function and properly balance Ca^{2+} influx and efflux (Curran et al., 2015). Additionally, overexpression of β1-adrenergic receptor in mice led to dilated cardiomyopathy and heart failure (Madamanchi, 2007), suggesting that accumulation of receptors can lead to negative effects on the heart. Despite this work, the functions of the endosomal pathway in the heart have not been extensively studied. Some work has implicated its importance in preventing neurodegeneration (McKnight et al., 2014), but its role in the cardiovascular system remains unclear.

The endosomal system can play a variety of roles in cells, from receptor degradation/recycling to that of mitochondrial quality control. Cellular excitability of myocytes is regulated by both the number and type of ion channels at the plasma

membrane of the cell. Notably, it has been reported that recycling endosomes in the cytosol are able to store cardiac pacemaker channels HCN2 and HCN4 for rapid delivery to the plasma membrane if necessary (Hardel et al., 2008). Additionally, recent work by our lab has demonstrated the ability of endosomes to recognize and degrade aberrant mitochondria through the endosomal-lysosomal pathway (Hammerling et al., 2017). This alternative form of mitochondrial clearance is thought to be a quicker response than that of autophagy, with a response of hours rather than 1 to 2 days (Hollville et al., 2014). Finally, the endosomal pathway is also essential for proper trafficking of membrane proteins in the cell. It plays an important role in degrading, recycling, or transporting membrane proteins across the cell (Elkin et al., 2016). Despite the importance of the endosomal pathway in ensuring many functions in cardiac myocytes involving proteins in the plasma membrane, its mechanisms of regulations have not been well elucidated. In this chapter, I will investigate the *in vivo* role of Beclin1 in the heart with a specific focus on its role in regulating the endosomal pathway and its subsequent effects on cardiac function.

3.2 Results

3.2.1 Beclin1 is essential for cardiac health and survival

Global knockout of Beclin1 is known to be embryonic lethal at E7.5, demonstrating its essential role during development (Yue et al., 2003). Beclin1 has been studied in a variety of diseases in the heart, with a particular focus on its role in regulating autophagy. For example, Beclin1 has been shown to play a cardioprotective role in sepsis by enhancing autophagy in the heart (Sun et al., 2018). Despite this, additional physiological

functions of Beclin1 in myocytes have yet to be identified. To examine the function(s) of Beclin1 in the heart, we generated cardiac-specific, inducible Beclin1 knockout mice by crossing *Beclin1*$^{flox/flox}$ mice with transgenic mice expressing *MerCreMer* under the control of the *α-myosin heavy chain* promoter to permit tamoxifen-inducible deletion of the *Beclin1* allele in the adult heart. Interestingly, we found that loss of Beclin1 (Beclin1 cKO) in myocytes led to a significant decrease in cardiac function by echocardiography in both ejection fraction and fractional shortening (Figure 3.1). The contractile defects and reduced cardiac output were evident as early as one week after the deletion of *Beclin1* in myocytes. The Beclin1 cKO mice also have reduced survival compared to WT mice or control MerCreMer mice (Figure 3.1).

Histological analysis revealed thinner left ventricular (LV) walls and dilated LV chambers 1 week after deletion of Beclin1, alongside increases in heart weight to body weight and heart weight to tibia length ratios (Figure 3.2). Pathological hypertrophy is often also accompanied by activation of genes that are similarly upregulated during fetal/embryonic development (Dirkx et al., 2013) (Zhu et al., 2019). We found that fetal genes *Myh7, Nppa, and Nppb* were significantly upregulated after loss of Beclin1 (Figure 3.3).

Next, we explored the effect of Beclin1 loss in myocytes at the ultrastructural level. Transmission electron microscopy allows for visualization of a variety of cardiac structures, including sarcomeres, tissue junctions, and organelles. We found that Beclin1 cKO mice displayed substantial myocardial disarray and loss of tissue structure at 1-week post tamoxifen injection (Figure 3.4). Progression towards heart failure from myocardial disarray can quickly lead to cell death and significantly reduced survival (Orogo and

Gustafsson Å, 2013). We found that Beclin1 cKO mice had increased cell death by TUNEL-staining for DNA damage (Figure 3.4). Loss of cardiomyocytes is accompanied by a fibrotic response to repair the damaged heart tissue (Piek et al., 2016). However, excessive fibrosis can lead to organ dysfunction and eventual failure (Piek et al., 2016). We found that Beclin1 cKO mice had significantly increased cardiac fibrosis compared to WT as assessed by Masson's Trichrome staining of heart sections (Figure 3.5). Additionally, cardiac inflammation is often associated with fibrosis and plays a significant role in cardiac dysfunction in a variety of cardiac pathologies (Suthahar et al., 2017). We discovered that mRNA levels of pro-inflammatory marker *Il-6* and fibrotic marker *Tgf-β1* were simultaneously increased in Beclin1-deficient mouse hearts (Figure 3.5). Together, these data illustrate the importance of Beclin1 in maintaining proper cardiac function and homeostasis.

3.2.2 Loss of autophagy alone does not affect cardiac function

Beclin1 is a well-known positive regulator of autophagy. It interacts with the PI3-Kinase Vps34 and Atg14L to form a unique protein complex that is important in the nucleation of the autophagosome (McKnight and Zhenyu, 2013). Global heterozygous knockout of Beclin1 mice have been shown to have reduced autophagy in tissues (Qu et al., 2003) and many studies have investigated the role of Beclin1-mediated autophagy in a variety of disease states (Zhu and He, 2015). Here, we assessed whether autophagy was altered in Beclin1-deficient hearts. LC3-I is a cytosolic protein that is conjugated to the autophagosome during the activation of autophagy and converts to LC3-II, making it a biomarker of autophagosome formation (Tanida et al., 2008). Additionally, autophagic activity can be monitored by the accumulation of adaptor protein p62, which binds

autophagic cargo and is also degraded with cargo during autophagy (Mathew et al., 2009). We found that loss of Beclin1 in the heart led to increased LC3I and p62 levels, suggesting that autophagic flux is reduced in these hearts (Figure 3.6). To investigate whether this impairment of autophagy was responsible for the cardiac phenotype observed in the Beclin1 cKO mice, we generated cardiac specific, inducible Atg7 knockout mice by crossing *Atg7*$^{flox/flox}$ mice to αMHC-MerCreMer transgenic mice. Atg7 is critical for autophagosome formation and deletion of *Atg7* in cells leads to a disruption in autophagy (Komatsu et al., 2005). We confirmed that deletion of *Atg7* in the heart by tamoxifen administration disrupted autophagosome formation as assessed by lack of LC3II and accumulation of p62 protein (Figure 3.7). Interestingly, we found that disruption autophagy in Atg7 cKO mice did not have a significant impact on cardiac function or mortality (Figure 3.8). These data demonstrate that the severe cardiac phenotype observed in the Beclin1 cKO mice is not caused by a defect in autophagic activity and suggests that Beclin1 regulates additional pathways that are essential for cardiac function and homeostasis.

3.2.3 Endosomal activity is impaired in Beclin1 cKO mice

Beclin1 is a component of a PI3K complex that can regulate both autophagy and the endosomal pathways (Itakura et al., 2008). Therefore, we investigated whether loss of Beclin1 had an effect on proteins involved in regulating the endosomal pathway *in vivo*. The endosomal pathway has traditionally been characterized for its role in trafficking, recycling, and degradation of plasma membrane proteins. Receptors are first synthesized in the Endoplasmic Reticulum and subsequently trafficked to the Golgi apparatus, where they are sorted to early endosomes for either degradation or shuttling to the plasma

membrane (Pavlos and Friedman, 2017). As Beclin1 has been implicated as a regulator of the endosomal pathway, I first investigated whether the protein levels of plasma membrane receptors were changed in hearts of Beclin1 cKO mice. I found that Insulin Growth Factor Receptor Beta (IGFR-β) was significantly increased in Beclin1 cKO mouse hearts, suggesting impaired endosomal activity (Figure 3.9). Next, I investigated the effect of the loss of Beclin1 on key endosomal regulators. We found that early endosome markers Rab5 and EEA1 were both significantly increased in the Beclin1 cKO hearts (Figure 3.9). Additionally, Rab6 is localized to the Golgi apparatus and helps facilitate receptor trafficking through the Golgi compartment (Martinez et al., 1994). Due to the trafficking of receptors between the early endosome and the Golgi, we examined Rab6 levels in the Beclin1 cKO mice and found it to be similarly increased (Figure 3.9). In contrast, the late endosome marker Rab7, which delivers cargo to lysosomes for degradation, was significantly decreased in Beclin1 cKO mice (Figure 3.9). Interestingly, although Rab5 mRNA levels were decreased after loss of Beclin1, Rab5 protein levels were increased (Figure 3.9). This suggests that the turnover of Rab5-positive endosomes might be reduced in the absence of Beclin1. Additionally, we saw no change in EEA1 mRNA levels in Beclin1 cKO mice compared to WT (Figure 3.9), which further indicates that loss of Beclin1 leads to changes only at the protein level. Overall, our data suggest that Beclin1 plays key roles in regulating multiple processes in myocytes, including the autophagic-lysosomal degradation pathway and endosomal trafficking.

3.2.4 Overexpression of UVRAG increases Beclin1 and restores heart function in mice

UVRAG is also a Beclin1 binding protein that has previously been reported to be impacted by loss of Beclin1 in MEFs (McKnight et al., 2014). Here, I wanted to examine if deletion of Beclin1 in myocytes had an effect on UVRAG levels *in vivo*. First, I examined UVRAG protein levels in the Beclin1 cKO mice and I discovered that UVRAG protein levels were significantly decreased in the Beclin1-deficient hearts while transcript levels were unchanged (Figure 3.10). This suggests that UVRAG stability is reduced in the absence of Beclin1. Next, I wanted to assess whether restoring UVRAG in hearts of Beclin1 cKO mice would delay the rapid cardiac dysfunction by stabilizing existing Beclin1 protein. GFP or Flag-UVRAG were overexpressed in hearts via adeno-associated virus 9 (AAV9)-mediated gene delivery for three weeks prior to tamoxifen administration to delete *Beclin1*. Interestingly, we observed that overexpression of UVRAG in WT mice led to increased Beclin1 protein levels at baseline (Figure 3.10). Increased Beclin1 protein levels were observed despite decreased levels of Beclin1 mRNA levels in the mice that overexpressed UVRAG (Figure 3.10). This could indicate a potential negative feedback mechanism to prevent excessive levels of Beclin1, as excessive cellular quality control can also contribute to the pathology of a variety of diseases (Sridhar et al., 2012). As we have shown that the loss of Beclin1 is important for cardiac function, we wanted to investigate whether overexpression of UVRAG would rescue the rapid decline in cardiac function in the Beclin1 cKO mice. Notably, we found that overexpression of UVRAG was able to prevent the rapid decline in fractional shortening and ejection fraction in Beclin1

cKO mice (Figure 3.10). These data suggest that the presence of UVRAG is important for Beclin1 stability and vice versa.

3.3 Discussion

The heart is a dynamic organ that requires constant maintenance in order to function properly. In chapter 3, I have shown data that provide new insights into the importance of Beclin1 in cardiac homeostasis likely through its dual role as a regulator of autophagy and the endosomal pathway. I propose that Beclin1 interacts with UVRAG to mediate endosomal trafficking of plasma membrane receptors through Rab5-positive early endosomes. Early endosomes are known to be the sorting center of the cell (Jovic et al., 2010) and either traffic cargo to the plasma membrane or into late endosomes for eventual degradation by the lysosome. Upregulation of Rab5 has previously been reported to have negative effects on neurodegenerative diseases such as Alzheimers, where increased Rab5 in the hippocampus led to mild cognitive impairment (Ginsberg et al., 2010), but we are the first to report a relationship between Beclin1, increased Rab5 and negative cardiac phenotypes. Our data reveals a severe loss in cardiac function only 1-week after deletion of Beclin1 in mice. Previous work had shown that Beclin1 global knockout mice were embryonic lethal (Yue et al., 2003), while autophagy deficient mice were viable but died soon after birth (Kuma et al., 2017). However, the role of Beclin1 in the heart had not been well characterized. The rapid pathological cardiac hypertrophy, upregulation of cardiac stress markers, and decreased survival seen in Beclin1 cKO mice upon loss of Beclin1 confirms its importance in myocytes. Future studies will need to uncover the precise mechanisms that Beclin1 utilizes to regulate endosomal activity. For example, although it is thought that Beclin1 binds to Atg14L to mediate autophagy and

UVRAG to mediate the endosomal pathway, it may bind to other effector proteins to mediate specific endosomal processes. The EH domain (EHD) family of proteins are thought to play a role in endocytic transport and are also present at the early endosome (Naslavsky et al., 2009). Guanine exchange factors (GEFs) also help to regulate Rab5 activity and facilitate recruitment of Retromer to coordinate membrane protein recycling (Bean et al., 2015). Our data suggest that Beclin1 might play a role in the trafficking of Rab5 positive vesicles, but other downstream regulators of endosomal trafficking have yet to be elucidated.

Additionally, I have demonstrated that loss of autophagy alone does not result in a loss of cardiac function at baseline. Disrupting autophagy in myocytes had little effect on cardiac function and there was no significant effect on survival within the timeframe of the experiment. Recently published reports have shown that loss of autophagy after high fat diet does lead to impaired cardiac function (Tong et al., 2019) or after ischemia-reperfusion injury (Li et al., 2016a) demonstrating its importance under conditions of stress. Given the crosstalk between both the autophagic and endosomal degradation pathways, it is possible that there is some compensation when one pathway is inhibited at baseline but is insufficient under conditions of stress. More research is needed to uncover whether the endosomal pathway is able to compensate under some but not other conditions of stress such as I/R injury or high fat diet.

Lastly, I have shown that UVRAG protein is lost upon deletion of Beclin1 *in vivo* while its transcript levels are unaffected. Overexpression of UVRAG increased Beclin1 levels in the heart and ablated the rapid decline in cardiac function observed in the Beclin1 cKO mice. Considering the reported roles of UVRAG and Beclin1 in mediating both

autophagy and the endosomal pathways, it is perhaps unsurprising that a severe cardiac phenotype is observed when both are lost. These data suggest that the interaction between Beclin1 and UVRAG is important in maintaining cardiac homeostasis and act in a protein complex *in vivo*. Previous studies have demonstrated the importance of the Beclin1-UVRAG interaction in the brain (McKnight et al., 2014). Additionally, unveiling of the crystal structure demonstrated a strengthened interface due to hydrophobic groups and electrostatically favorable interactions in the Beclin1-UVRAG complex. A failure of this complex to form led to reduced endolyosomal degradation of EGFR, suggesting that the Beclin1-UVRAG complex has an important role in regulating endocytic pathways (Wu et al., 2018). Additional studies are necessary to further understand the mechanisms and effects of Beclin1-UVRAG binding in various tissue and stress contexts.

Interestingly, it appears that either autophagy or endosomal activity is required at baseline to maintain proper cardiac function. Since endosomal activity has not been well-studied in the context of cardiac function, it is currently unclear in which contexts that endosomal activity is able to compensate for autophagy. I propose that diseases that have been attributed to mutations in plasma membrane receptors, including a variety of cancers, will be more susceptible to treatment by restoration of endosomal activity than through autophagy. More studies are required to pick apart the role of each of these pathways, in the heart and in other tissues, and the conditions that drive them.

Parts of Chapter 3 are in preparation for a manuscript for submission to a journal. Lampert, M. A., Najor, R. H., Cortez, M.Q., Leon, L. J., Gustafsson, A. B. The primary author is the lead investigator and author of this manuscript.

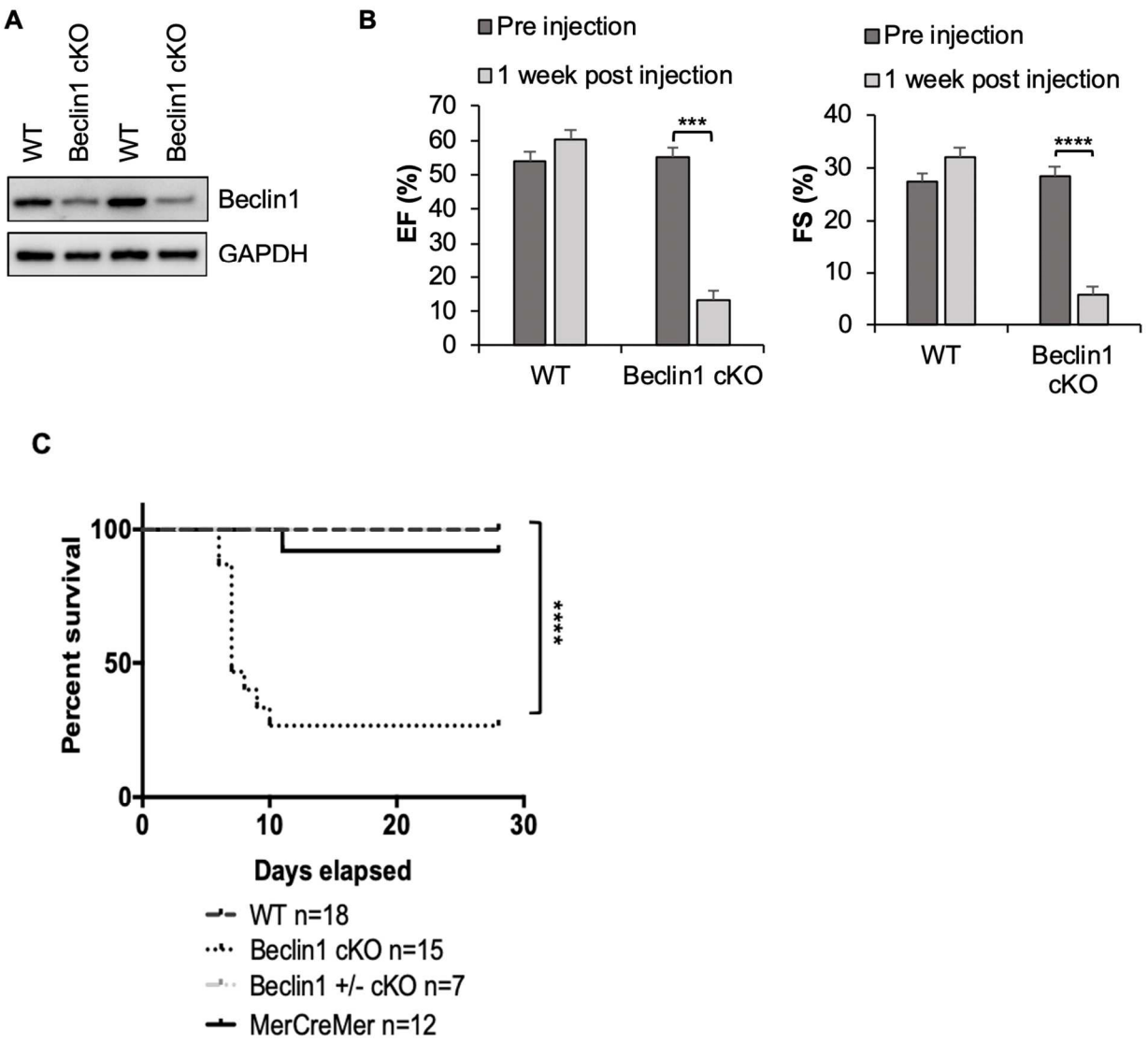

Figure 3.1. Deletion of Beclin1 in the heart results in cardiac dysfunction and reduced survival.

(**A**) Representative Western blot of Beclin1 in WT and Beclin1 cKO mice 1-week post tamoxifen injection. (**B**) Echocardiographic analysis revealed reduced ejection fraction (EF) and fractional shortening (FS) in Beclin1 cKO mice (n=7-8). (**C**) Kaplan-Meier survival plot for WT and Beclin1 cKO mice alongside heterozygous Beclin1 cKO mice (Beclin1 +/- cKO) and MerCreMer controls up to 4-weeks post tamoxifen injection (n=7-18). Data are mean ± SEM. ***$p < 0.001$; ****$p < 0.0001$.

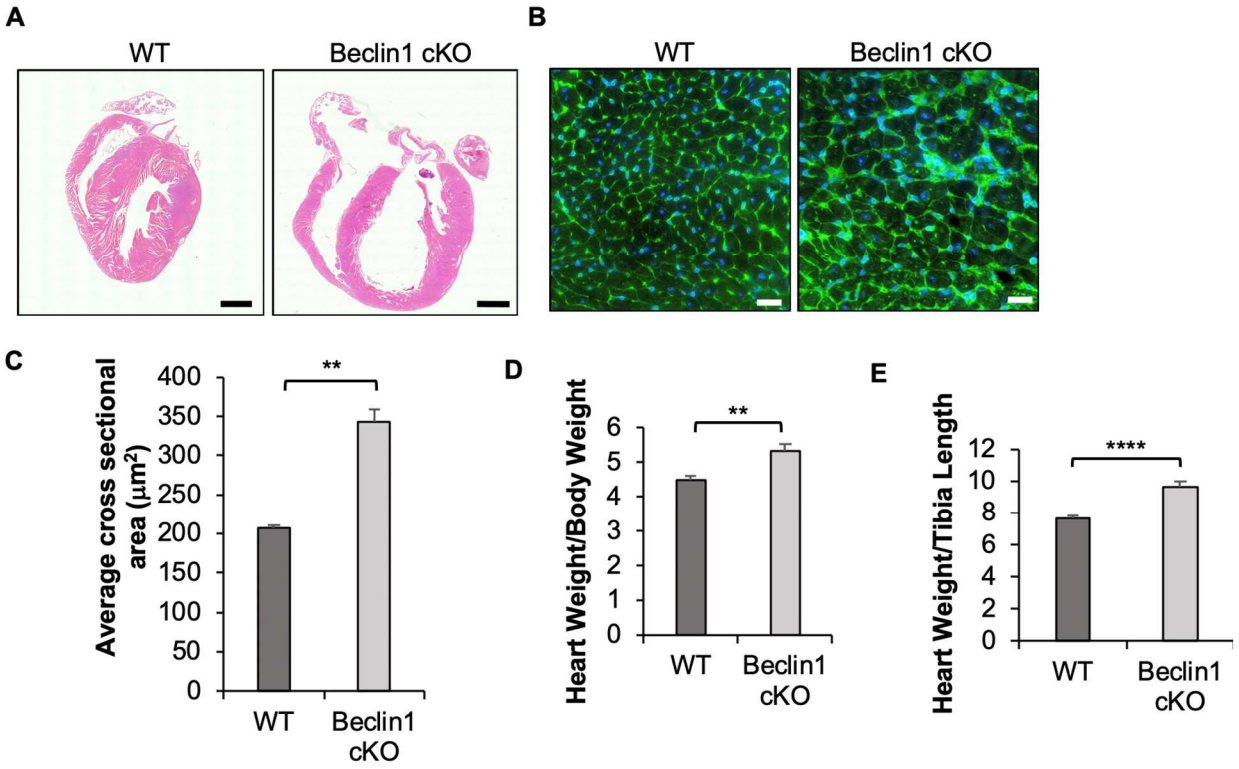

Figure 3.2. Deletion of Beclin1 in myocytes leads to cardiac dilation and hypertrophy.

(**A**) Representative images of H&E staining show thinning of ventricular walls in Beclin1 cKO mice 1-week post tamoxifen injection. Scale bar=1 mm. (**B**) Representative images of Wheatgerm Agglutinin staining of hearts sections from WT and Beclin1 cKO mice 1-week post tamoxifen injection. Scale bar=20 μm. (**C**) Quantitation of myocyte cross-sectional area (n=3). (**D**) Heart weight/body weight in WT and Beclin1 cKO mice 1-week post tamoxifen injection (n=7-8). (**E**) Heart weight/tibia length in WT and Beclin1 cKO mice 1-week post tamoxifen injection (n=7-8). Data are mean ± SEM. **p < 0.01; ****p < 0.0001.

A

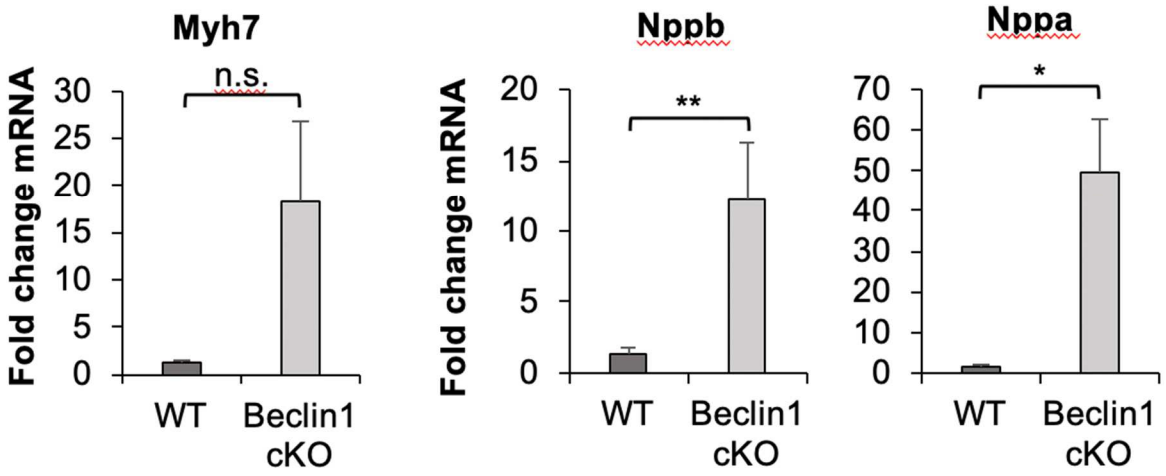

Figure 3.3. Beclin1 cKO mice have increased cardiac stress.
(**A**) Real-time PCR analysis of transcript levels for cardiac stress markers *Myh7, Nppa,* and *Nppb* in WT and Beclin1 cKO heart tissue 1-week post tamoxifen injection (n=7-9). Data are mean ± SEM. *p < 0.05; **p < 0.01.

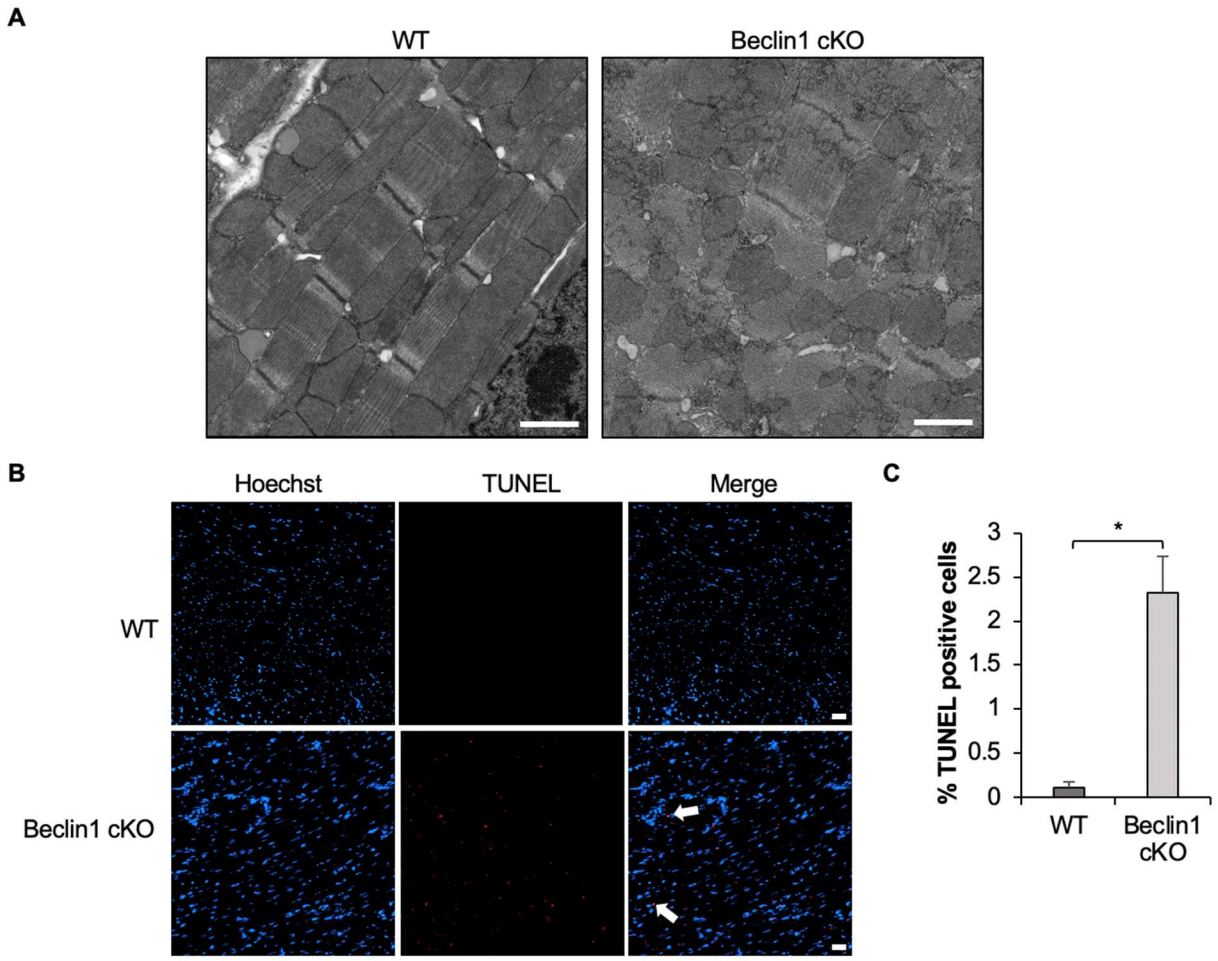

Figure 3.4. Beclin1 deficiency leads to myocardial disarray and cell death.
(**A**) Representative transmission electron microscopy images of WT and Beclin1 cKO mice 1-week post tamoxifen injection. Scale bar=1 μm. (**B**) Representative images of TUNEL staining of heart sections prepared from WT and Beclin1 cKO mice 1-week post tamoxifen injection. Nuclei are stained with Hoechst. Scale bar=20 μm. (**C**) Quantitation of TUNEL positive nuclei in WT and Beclin1 cKO heart tissue (n=3). Data are mean ± SEM. *$p < 0.05$.

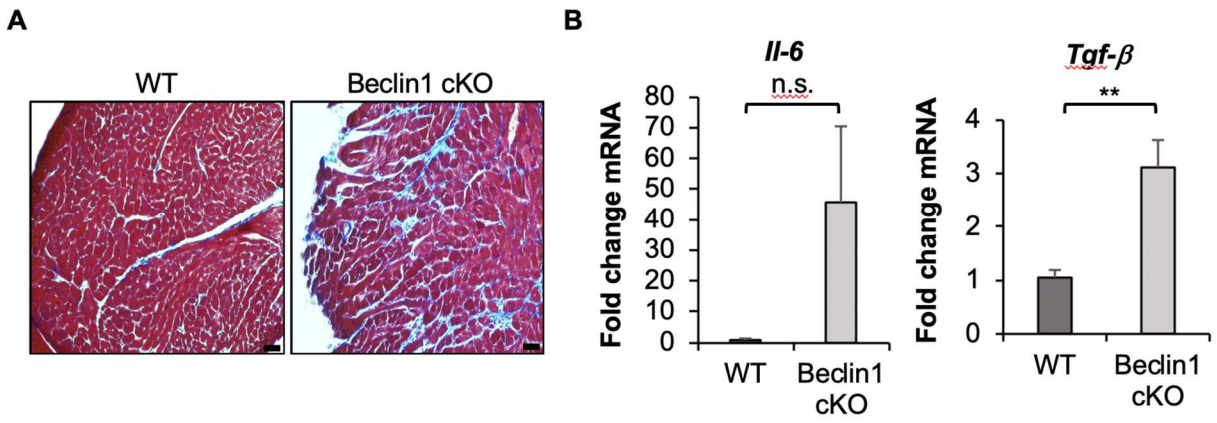

Figure 3.5. Increased cardiac fibrosis after loss of Beclin1 *in vivo*.
(**A**) Representative images of WT and Beclin1 cKO heart sections stained with Masson's Trichrome. Hearts were harvested for analysis 1-week post tamoxifen injection. Scale bar=40 μm. (**B**) Real-time PCR analysis of transcript levels for *TGF-β1* and *Il-6* in WT and Beclin1 cKO hearts 1-week post tamoxifen injection (n=7-8). Data are mean ± SEM. *p < 0.05; **p < 0.01.

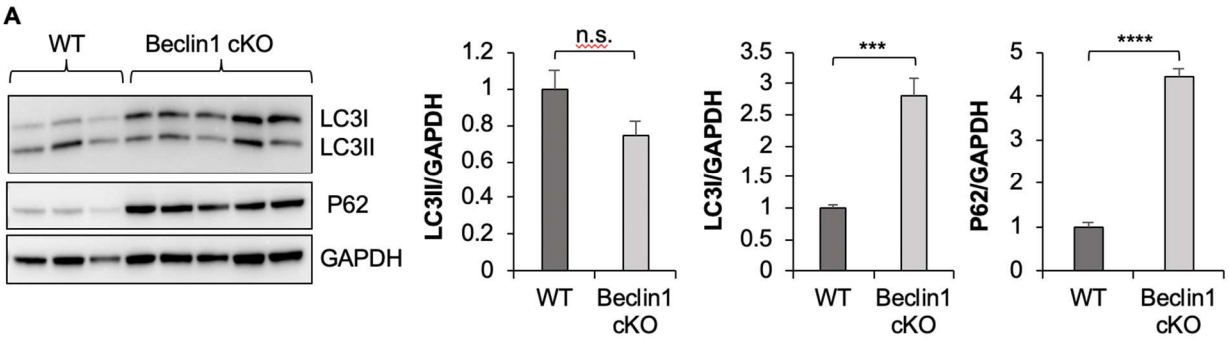

Figure 3.6. Beclin1 cKO mice have reduced autophagic flux.
(**A**) Representative Western blots of autophagy markers LC3I, LC3II and p62 in hearts of WT and Beclin1 cKO mice 1-week post injection. Quantitation of LC3II/GAPDH, LC3I/GAPDH, and p62/GAPDH (n=8). Data are mean ± SEM. ***p < 0.001; ****p< .0001; n.s., not significant.

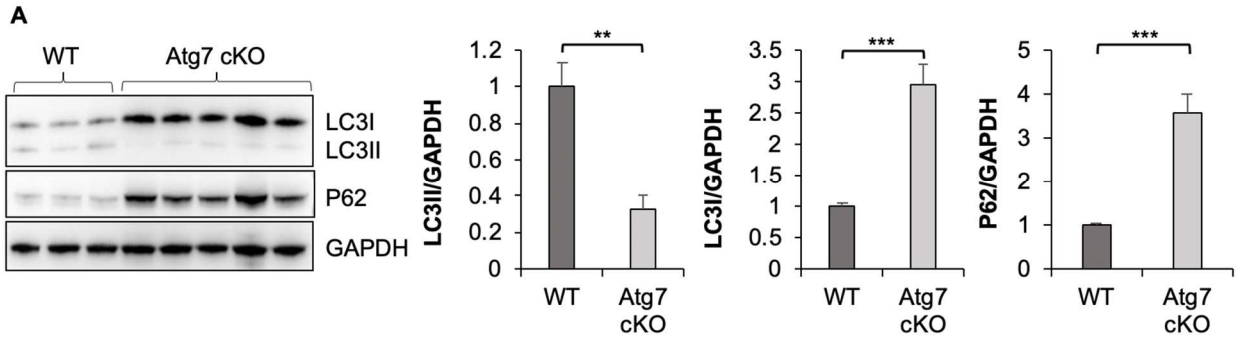

Figure 3.7. Deletion of Atg7 in heart leads to disruption of autophagy.
A) Representative Western blots of autophagy markers LC3I, LC3II, and p62 in WT and Atg7 cKO mice 1-week post injection. Quantitation of LC3II/GAPDH, LC3I/GAPDH, and p62/GAPDH (n=6-9). Data are mean ± SEM. **p < 0.01; ***p < 0.001.

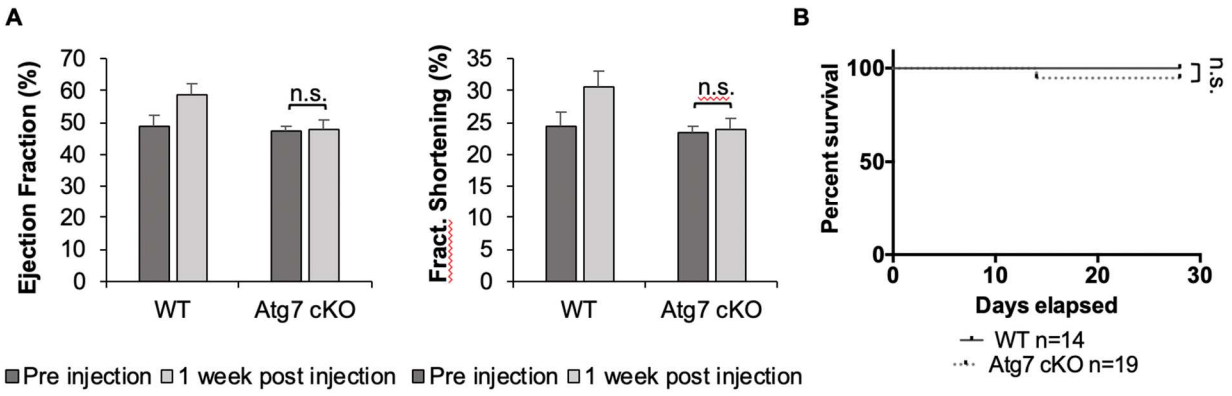

Figure 3.8. Deletion of Atg7 and disruption of autophagy has no effect on cardiac function or survival.

(**A**) Ejection fraction and fractional shortening are similar in WT and Atg7 cKO mice (n=6-8). (**B**) Kaplan-Meier survival plot for WT and Atg7 cKO mice up to 4-weeks post tamoxifen injection (n=14-19). Data are mean ± SEM. n.s., not significant.

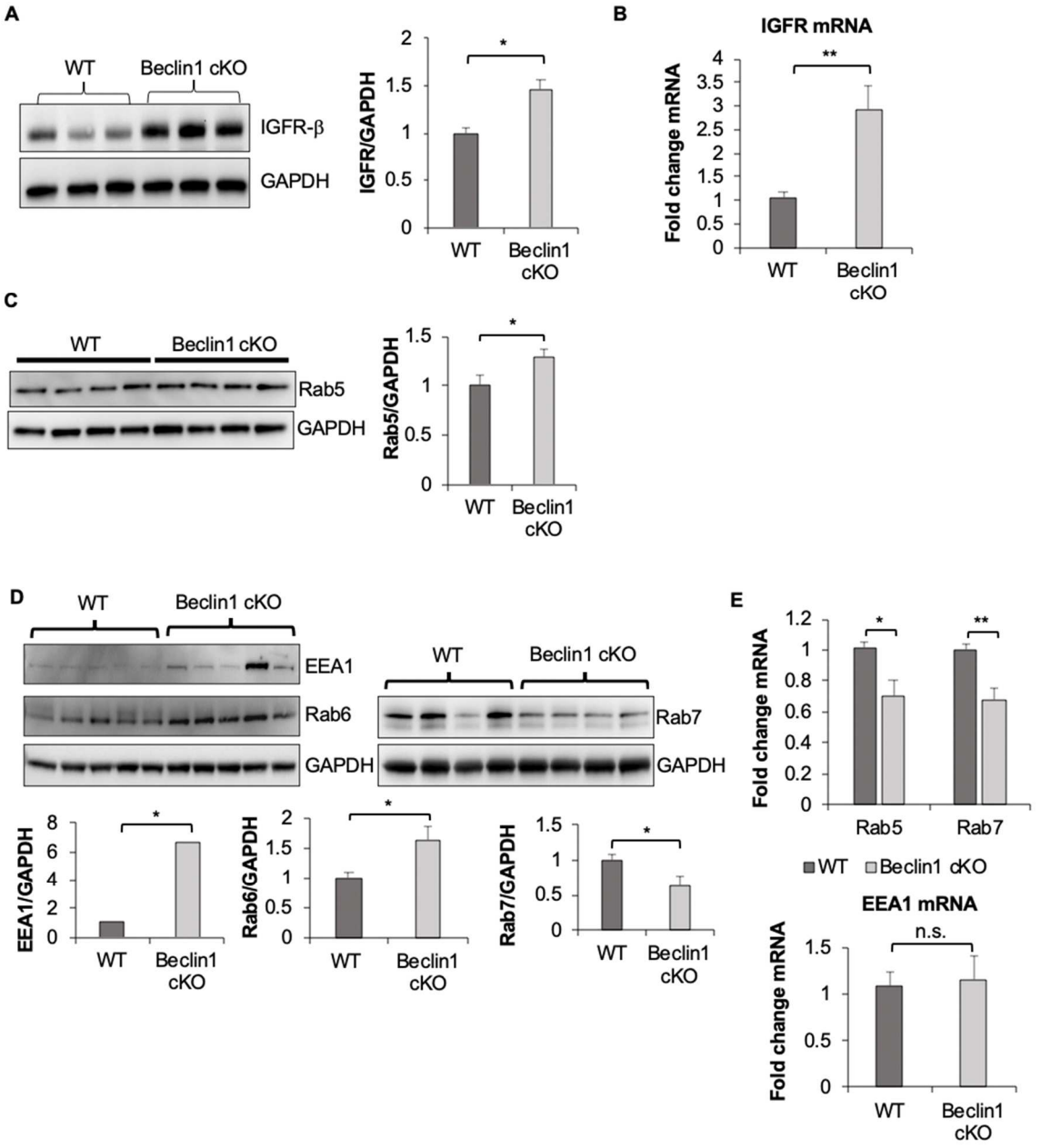

Figure 3.9. Endosomal activity is altered in Beclin1 cKO mouse hearts.
(**A**) Representative Western blot of IGFR-β protein levels in WT and Beclin1 cKO mice 1-week post tamoxifen injection. Quantitation of IGFR-β/GAPDH (n=3-5). (**B**) mRNA levels of IGFR-β from WT and Beclin1 cKO heart tissue (n=7). (**C**) Representative Western blot of Rab5 in WT and Beclin1 cKO mice 1-week post tamoxifen injection. Quantitation of Rab5/GAPDH (n=9). (**D**) Representative Western blots of EEA1, Rab6 and Rab7 in WT and Beclin1 cKO hearts 1-week post tamoxifen injection. Quantitation of EEA1/GAPDH, Rab6/GAPDH and Rab7/GAPDH (n=8). (**E**) mRNA levels of Rab5a, Rab7, and EEA1 from WT and Beclin1 cKO heart tissue (n=7-8). Data are mean ± SEM. *$p < 0.05$; n.s., not significant.

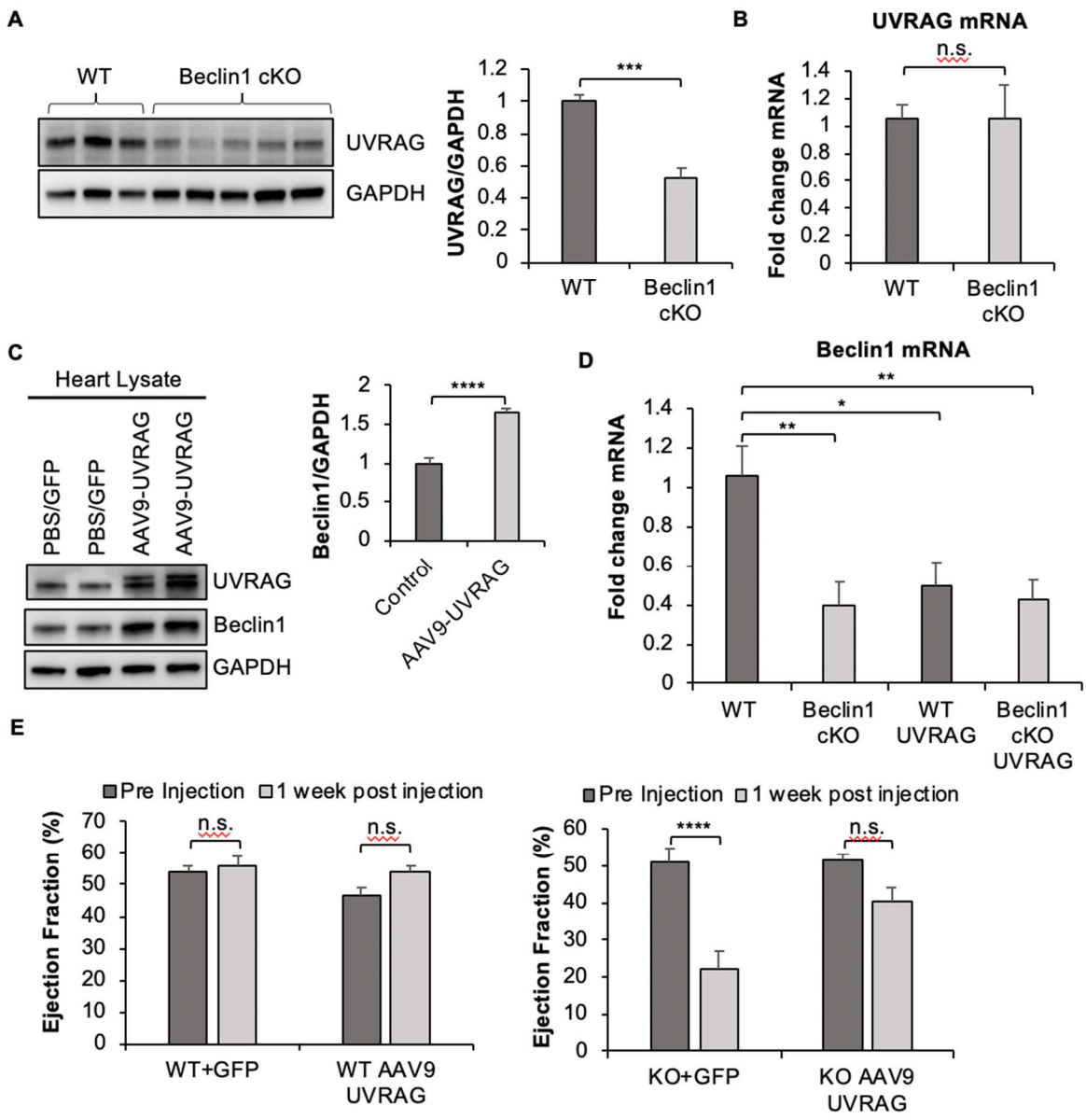

Figure 3.10. AAV9-UVRAG rescues cardiac function in Beclin1 cKO mice.
(**A**) Representative Western blot of UVRAG protein in WT and Beclin1 cKO mice 1-week post tamoxifen injection. Quantitation of UVRAG/GAPDH (n=8). (**B**) Real-time PCR analysis of *UVRAG* in WT and Beclin1 cKO heart tissue (n=5). (**C**) WT mice were infected with AAV9-FLAG-UVRAG for 3 weeks before being sacrificed. Representative Western blots of UVRAG and Beclin1 protein levels with quantitation of Beclin1/GAPDH protein levels (n=5). (**D**) Real-time PCR analysis of *Beclin1* in WT and Beclin1 cKO heart tissue after overexpression of AAV9-FLAG-UVRAG (n=6-8). (**E**) Quantitation of echocardiograms in WT and Beclin1 cKO mice for ejection fraction after infection with AAV9-UVRAG 1-week post tamoxifen injection (n=5-14). Data are mean ± SEM. ***p < 0.001; ****p < 0.0001; n.s. not significant.

CHAPTER 4: BECLIN1 REGULATES THE ENDOSOMAL PATHWAY THROUGH RAB5 AND UVRAG

4.1 Introduction

The endosomal pathway is primarily known for its role in receptor recycling and degradation and is regulated by the Rab family of proteins. Rab proteins are GTPases that cycle between active GTP and inactive GDP states through binding of guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs) (Müller and Goody, 2018). Once active, they are important regulators of endosome biogenesis and function (Zeigerer et al., 2012). Rab5 is present on early endosomes and helps mediate endocytosis of target cargo into the vesicle alongside the ESCRT complex (Elkin et al., 2016; Stenmark, 2009). There are two main pathways that diverge from sorting at the early endosome; the endosomal recycling pathway and the endosomal degradation pathway (Elkin et al., 2016). In the endosomal degradation pathway, endosomes mature and Rab5 is replaced by late endosome marker Rab7, which is then able to fuse with the lysosome for degradation of cargo. Alternatively, in the endosomal recycling pathway, other Rab proteins, such as Rab4 and Rab11, are involved to mediate recycling of internalized cargo to the plasma membrane in lieu of degradation. Rab4 has been shown to be involved in rapid recycling of plasma membrane receptor such as class A GPCRs, while Rab11 recycles at a slower rate and has been shown to recycle somatostatin receptors and other class B GPCRs (Li et al., 2008).

Beclin1 has also been implicated as a regulator of the endosomal pathway. Loss of Beclin1 in cells led to decreased EGF receptor degradation (McKnight et al., 2014),

while keratinocyte-specific deletion of Beclin1 led to a mislocalization of integrins and abnormal skin development due to a defect in Rab11 positive recycling endosomes (Noguchi et al., 2019). As a scaffolding protein, there is evidence that Beclin1 forms a unique protein complex with Vps34 and UVRAG to regulate endosomal activity. Notably, homotypic fusion of early endosomes has also been shown to be impaired in the absence of Beclin1 (McKnight et al., 2014). In the previous chapter, I demonstrated the importance of Beclin1 in maintaining cardiac homeostasis and function, and loss of Beclin1 led to impairment in both the endosomal and autophagy pathways and rapid cardiac dysfunction. While the role of Beclin1 in regulating autophagy is well established, the role of Beclin1 in regulating the endosomal pathway is not well understood. In this chapter, I investigate the role of Beclin1 in mediating receptor trafficking through Rab5-positive early endosomes and its effect on protein complex partner UVRAG.

4.2 Results

4.2.1 Loss of Beclin1 leads to altered Rab5 Activity

We sought to test the hypothesis that Beclin1, as a scaffolding protein, is important for endosomal activity and sorting at the early endosome. It is possible that Beclin1 binds to different protein effectors such as UVRAG or Rab proteins to mediate endosomal recycling and/or endosomal degradation. First, we wanted to investigate the role of Beclin1 in regulating early endosomal activity. We utilized a constitutively active mutant of Rab5, Rab5Q79L, that maintains Rab5 in its active GTP state. As Rab5 also regulates homotypic fusion of the early endosome, the Q79L mutation leads to the formation of enlarged vesicles through continuous fusion. We discovered that mouse embryonic

fibroblasts deficient in Beclin1 (Beclin1$^{-/-}$ MEFs) failed to form enlarged Rab5Q79L-positive vesicles compared to WT MEFs (Figure 4.1). Rab5 vesicle fusion is also an indication of Rab5 activity (Gautreau et al., 2014), and its inability to form enlarged vesicles suggests an impairment in the endosomal pathway. Thus, we sought to further assess Rab5 activity in cells lacking Beclin1. We designed a split GFP Rab5 activity reporter that utilizes the split beta barrel of GFP into two constituents, GFP 1-10 and GFP 11 (Kamiyama et al., 2016), which were used to tag Rab5 and a Rab5 binding domain, respectively. GFP will only fluoresce when these two components recombine, and thus we can use it monitor Rab5 activity. Interestingly, we found no significant difference in GFP fluorescence at baseline between WT and Beclin1$^{-/-}$ MEFs (Figure 4.2A). However, when treated with FCCP, a mitochondrial uncoupler and known stimulant of Rab5 activity (Hammerling et al., 2017), Beclin1$^{-/-}$ MEFs had no increase in GFP fluorescence, while it increased to a significant extent in WT MEFs (Figure 4.2A). Similar to the baseline data, we saw no difference in number of endogenous Rab5+ vesicles between WT and Beclin1$^{-/-}$ MEFs (Figure 4.2B). Together, these data suggest that the absence of Beclin1 does not affect formation of Rab5 positive early endosomes, but the homotypic fusion of the vesicles is impaired.

Given the importance of the endosomal pathway in mediating trafficking and degradation of plasma membrane receptors, we next sought to characterize the role of Beclin1 in regulating trafficking. We discovered that the absence of Beclin1 in MEFs led to increased levels of the plasma membrane receptor EGFR (Figure 4.3A). Transcript levels of EGFR were similar in WT and Beclin1$^{-/-}$ MEFs (Figure 4.3), indicating accumulation of the EGFR in the absence of Bcllin1. EGFR is internalized into the cell

upon binding of its ligand EGF and is then sorted for degradation. To investigate if the increased levels of EGFR in the Beclin1$^{-/-}$ MEFs were due to decreased endosomal-mediated degradation, we treated the WT and Beclin1$^{-/-}$ MEFs with EGF and monitored the levels of EGFR. Unexpectedly, we observed no difference between WT and Beclin1$^{-/-}$ MEFs in EGFR degradation at either time point of 1 or 2 hours (Figure 4.3B). We also confirmed that Texas Red labeled EGF co-localized with Rab7 in both WT and Beclin1$^{-/-}$ MEFs (Figure 4.3C), confirming delivery of the ligand bound receptor to the late endosomes. This data suggests that EGFR degradation by the endosomal pathway is still functional in Beclin1 deficient cells.

Another defining characteristic of the endosomal pathway is receptor trafficking to and from the plasma membrane, either from biosynthesis or recycling of internalized receptors (Fölsch et al., 2009). Notably, we found that overexpression of GFP-EGFR in WT MEFs led to primarily plasma membrane localization of the receptor, while overexpression in Beclin1$^{-/-}$ MEFs led to accumulation of receptor in vesicular structures in the cytosol (Figure 4.4). To identify the EGFR-positive vesicles, we stained cells with anti-Rab5 and we found that EGFR co-localized with Rab5 in Beclin1$^{-/-}$ MEFs, suggesting that the receptor was accumulating in early endosomes (Figure 4.4). Next, we confirmed our fluorescence microscopy data with a biochemical assay to assess cellular localization of EGFR. We subjected the WT and Beclin1$^{-/-}$ MEFs to subcellular fractionation by centrifugation to separate the crude cytosol containing vesicles from the heavy plasma membrane fraction. We found increased levels of EGFR in the crude cytosolic fraction in Beclin1$^{-/-}$ MEFs (Figure 4.4). To further confirm that EGFR is accumulating inside cells in Beclin1$^{-/-}$ MEFs, we treated intact cells with Proteinase K, which will digest exposed

proteins in the plasma membrane. We found that treatment with Proteinase K led to significant loss of EGFR in WT but not in Beclin1$^{-/-}$ cells (Figure 4.5). Together, these data suggest that Beclin1 is essential for endosomal trafficking of EGFR to the plasma membrane.

4.2.2 UVRAG protein stability is dependent on Beclin1

As a scaffolding protein, Beclin1 binds to a variety of proteins to regulate different intracellular quality control pathways. While its binding partners to regulate autophagy are known to include Vps34 and Atg14L, the components of the protein complex to regulate endosomal activity are less clear. UVRAG has been implicated as a regulator of the endosomal pathway and is a known binding partner of Beclin1 (Itakura et al., 2008). In the previous chapter, we found that Beclin1-deficiency led to reduced UVRAG levels in the heart. Here, we confirmed that UVRAG protein levels were also decreased in Beclin1$^{-/-}$ MEFs, while UVRAG mRNA levels were unaltered (Figure 4.6). Notably, although UVRAG mRNA levels were significantly increased upon FCCP treatment in Beclin1$^{-/-}$ MEFs compared to WT, UVRAG protein levels did not increase (Figure 4.6). This suggests that UVRAG protein is unstable in the absence of Beclin1. Therefore, we investigated whether UVRAG was subjected to proteasomal degradation in the absence of Beclin1. We treated Beclin1$^{-/-}$ MEFs with MG-132, a specific 26S proteasomal inhibitor, and found that proteasomal inhibition led to preserved UVRAG protein levels (Figure 4.7). Additionally, restoration of Beclin1 in Beclin1$^{-/-}$ MEFs using an adenovirus expressing Beclin1 rescued UVRAG protein levels (Figure 4.7). Finally, we utilized specific siRNA constructs that target either UVRAG or Beclin1 to knockdown each protein individually in WT MEFs and assess its impact on the protein levels of the other. Interestingly,

knockdown of Beclin1 alone led to a significant decrease in UVRAG protein levels, while knockdown of UVRAG alone had no effect on Beclin1 (Figure 4.8). This indicates that while Beclin1 is essential for UVRAG protein stability, UVRAG is not important for Beclin1 protein stability. Together, these data suggest that UVRAG is unstable in the absence of Beclin1 and subject to degradation and that restoring Beclin1 is sufficient to prevent UVRAG degradation.

4.2.3 UVRAG interacts with Beclin1 at the early endosome

Next, we sought to better understand the relationship between UVRAG and Beclin1 in regulating the endosomal pathway. First, we confirmed that UVRAG and Beclin1 interact in cells. We performed co-immunoprecipitation experiments in MEFs and confirmed that UVRAG co-immunoprecipitated with Beclin1 (Figure 4.9). Notably, we found that there was a significant increase in both UVRAG and Beclin1 protein levels upon overexpression of both proteins in Beclin1-/- MEFs, further indicating that they interact to enhance each other's stability. UVRAG has been implicated as a regulator of endocytic trafficking through its interaction with the HOPS complex, but its exact role in the endosomal pathway remains undefined (Liang et al., 2008). We further investigated whether overexpression Beclin1, UVRAG or Beclin1+UVRAG could restore homotypic fusion of Rab5+ endosomes in Beclin1$^{-/-}$ MEFs. We found that while overexpression of Beclin1 alone led a significant increase in homotypic fusion of Rab5Q79L positive vesicles, simultaneous overexpression of Beclin1 and UVRAG led to an even further increase in vesicle fusion (Figure 4.10). This restoration of homotypic fusion was not seen with overexpression of UVRAG alone, further underlining the necessity of Beclin1 for early

endosome fusion (Figure 4.10). The increase in homotypic fusion of Rab5-positive vesicles in cells overexpressing Beclin1 alone is likely due to preserved stability of endogenous UVARG in these cells (Figure 4.7). Taken together, this data supports the hypothesis that Beclin1 interacts with UVRAG to regulate endosomal activity.

4.3 Discussion

Here, I report that Beclin1 is essential for Rab5 activity and its intracellular trafficking of plasma membrane receptors. Additionally, the interaction between Beclin1 with its binding partner UVRAG further enhances endosomal activity and suggests that the formation of a protein complex between UVRAG, Beclin1, and Rab5 exists in the cell. Additional studies are necessary to determine the exact mechanisms of regulation of endosomal activity between Beclin1, UVRAG, and Rab5. For example, whether a post-translational modification, such as phosphorylation, regulates a potential interaction between Beclin1, UVRAG, and Rab5, or the presence of some other protein effector binding, is yet to be determined.

I also report that Beclin1 is essential for intracellular trafficking of plasma membrane receptors such as EGFR. Proper localization and amount of different receptors are crucial for proper cell signaling and function (Joseph et al., 2013). Without Beclin1, the majority of EGFR accumulates inside the cell in Rab5 positive vesicles, suggesting that their trafficking to the plasma membrane is impaired. How this affects a variety of different cellular pathways is of interest - are cellular growth and proliferation rates affected by the inability of a significant portion of EGFR to make it to the plasma membrane? How does this affect trafficking of other cellular components that are sorted

at the early endosome? Answering these questions would provide new insights into the implications and importance of Beclin1-mediated cellular trafficking and help potentially explain why Beclin1 global knockout mice are embryonic lethal (Yue et al., 2003).

Interestingly, I also report here that activation of the endosomal degradation pathway is unaffected by the loss of Beclin1. A previous study reported that EGFR degradation is reduced in Beclin1$^{-/-}$ MEFs, but only at the 30 minute time point (McKnight et al., 2014). Similar to our data, they observed no difference in EGFR degradation at later time points, including as early as 1 hour post EGF treatment (McKnight et al., 2014), suggesting that while endosomal degradation may be delayed without Beclin1, there is no appreciable difference in the long-term. Additionally, this is in spite of the increased levels of EGFR observed at baseline in the Beclin1$^{-/-}$ MEFs. Furthermore, we are the first to report an accumulation of EGFR in the cytosol as a result of Beclin1 deficiency. Recent reports have indicated that Beclin1 was necessary for the sorting of receptors into intraluminal vesicles (Matthew-Onabanjo et al., 2020), further indicating its importance in intracellular trafficking. It is possible that Beclin1 plays an important role in mediating delivery of newly synthesized receptors to the plasma membrane but is not essential for receptor internalization and subsequent degradation or recycling. For example, lack of Beclin1 and subsequent decreased Rab5-regulated trafficking could lead to a build-up of newly synthesized receptors in the cell. As these receptors are unable to be properly trafficked to the plasma membrane, they accumulate in the cytosol and could account for the increased levels of EGFR observed in the Beclin1$^{-/-}$ MEFs. Despite this defect, the receptors that are able to make it to the plasma membrane can be internalized and degraded as normal with or without Beclin1. More research is necessary to understand

fully these distinctions and what effector proteins beyond that of UVRAG may play a role in mediating receptor trafficking, especially in disease contexts such as heart disease, neurodegeneration, and cancer.

I also report that Beclin1 is required for UVRAG protein stability. UVRAG is a known Beclin1 binding partner (Liang et al., 2008), but the exact functions of their interactions remained unclear. Here, I show that UVRAG stability is dependent on Beclin1 and that lack of Beclin1 leads to its rapid degradation. Additionally, overexpression of both UVRAG and Beclin1 led to enhanced restoration of homotypic fusion of Rab5Q79L-positive vesicles. Recent reports have shown that enhancing the interaction between UVRAG and Beclin1 at the coiled-coil domain leads to increased endolysosomal degradation of EGFR (Wu et al., 2018), as a stable UVRAG-Beclin1 interaction was able to outcompete Beclin1-Atg14L binding. This further substantiates our findings that Beclin1 is important for UVRAG stability and that this complex is important in the endosomal pathway. Lastly, we have shown that UVRAG protein stability is reliant on Beclin1, but not vice versa. The finding that there is some restoration of Rab5 activity upon overexpression of Beclin1 alone is likely due to stabilization of endogenous UVRAG. Nonetheless, similar to Beclin1, UVRAG global knockout mice are known to be embryonic lethal (Afzal et al., 2015). It is clear that both Beclin1 and UVRAG are necessary for a variety of essential cellular functions, and here we report a novel function with their importance in regulating endosomal trafficking at the early endosome.

In conclusion, here I have provided evidence that Beclin1 is important for endosomal trafficking of plasma membrane receptors through its interaction with UVRAG at the Rab5 positive early endosome. This is a novel function for Beclin1 and an

expansion of previous efforts to characterize its role in the cell. The ability to dynamically traffic proteins to their proper compartments is especially important in a beating cardiomyocyte, which is constantly contracting to provide energy for the body (Curran et al., 2015). Thus, Beclin1 is likely to have an important role in maintaining proper cardiac function.

Parts of Chapter 4 are in preparation for a manuscript for submission to a journal. Lampert, M. A., Najor, R. H., Cortez, M.Q., Leon, L. J., Gustafsson, A. B. The primary author is the lead investigator and author of this manuscript.

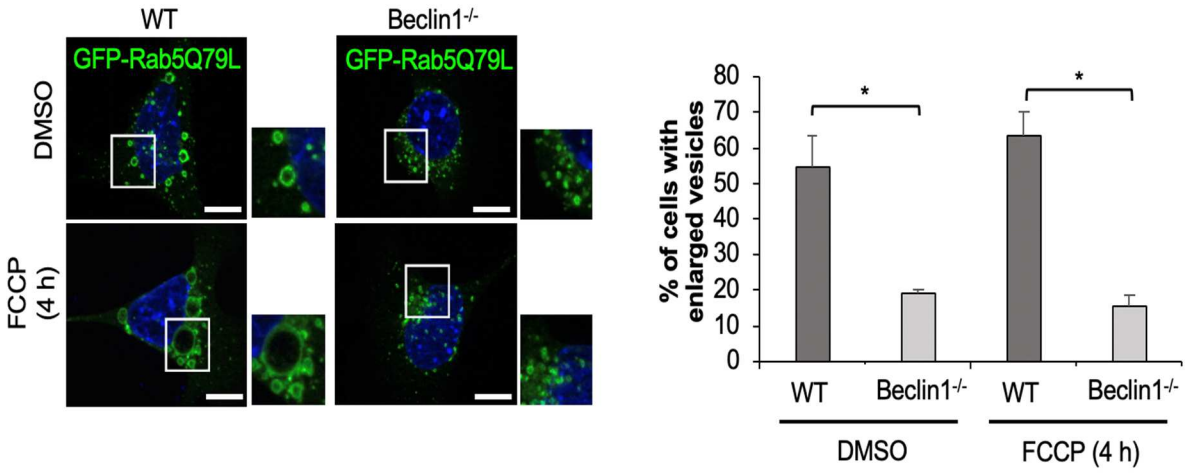

Figure 4.1. Loss of Beclin1 leads to impaired Rab5 homotypic vesicle fusion. Representative images of WT and Beclin1$^{-/-}$ MEFs overexpressing the constitutively GFP-Rab5Q79L before and after treatment with DMSO or FCCP (4 hours, 25 μM). Scale bar=10 μm. Quantitation of % of cells with enlarged vesicles (n=3). Data are mean ± SEM. *$p < 0.05$.

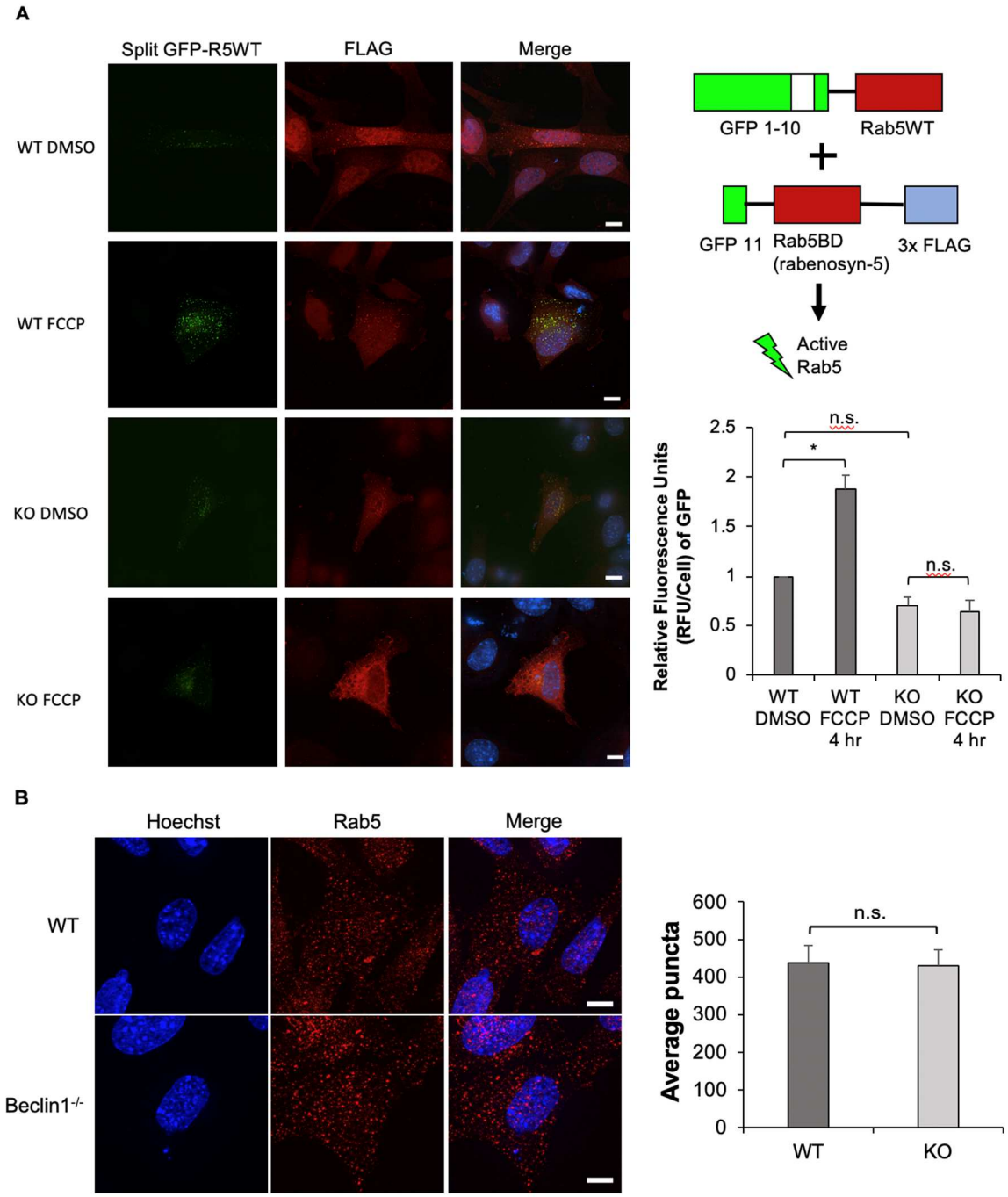

Figure 4.2. Baseline Rab5 activity and endosome formation are similar in WT and Beclin1$^{-/-}$ MEFs.

(**A**) Representative images of WT and Beclin1$^{-/-}$ MEFs overexpressing the split GFP reporter system of GFP1-10-R5WT and GFP11-FLAG-R5BD before and after treatment with DMSO or FCCP (4 hours, 25 μM). Scale bar=10 μm. Quantitation of relative fluorescent units per cell of GFP (n=4). (**B**) Representatives images of endogenous Rab5 in WT and Beclin1$^{-/-}$ MEFs. Scale bar=10 μm. Quantitation of average Rab5 puncta per cell (n=3). Data are mean ± SEM. *$p < 0.05$; n.s., not significant.

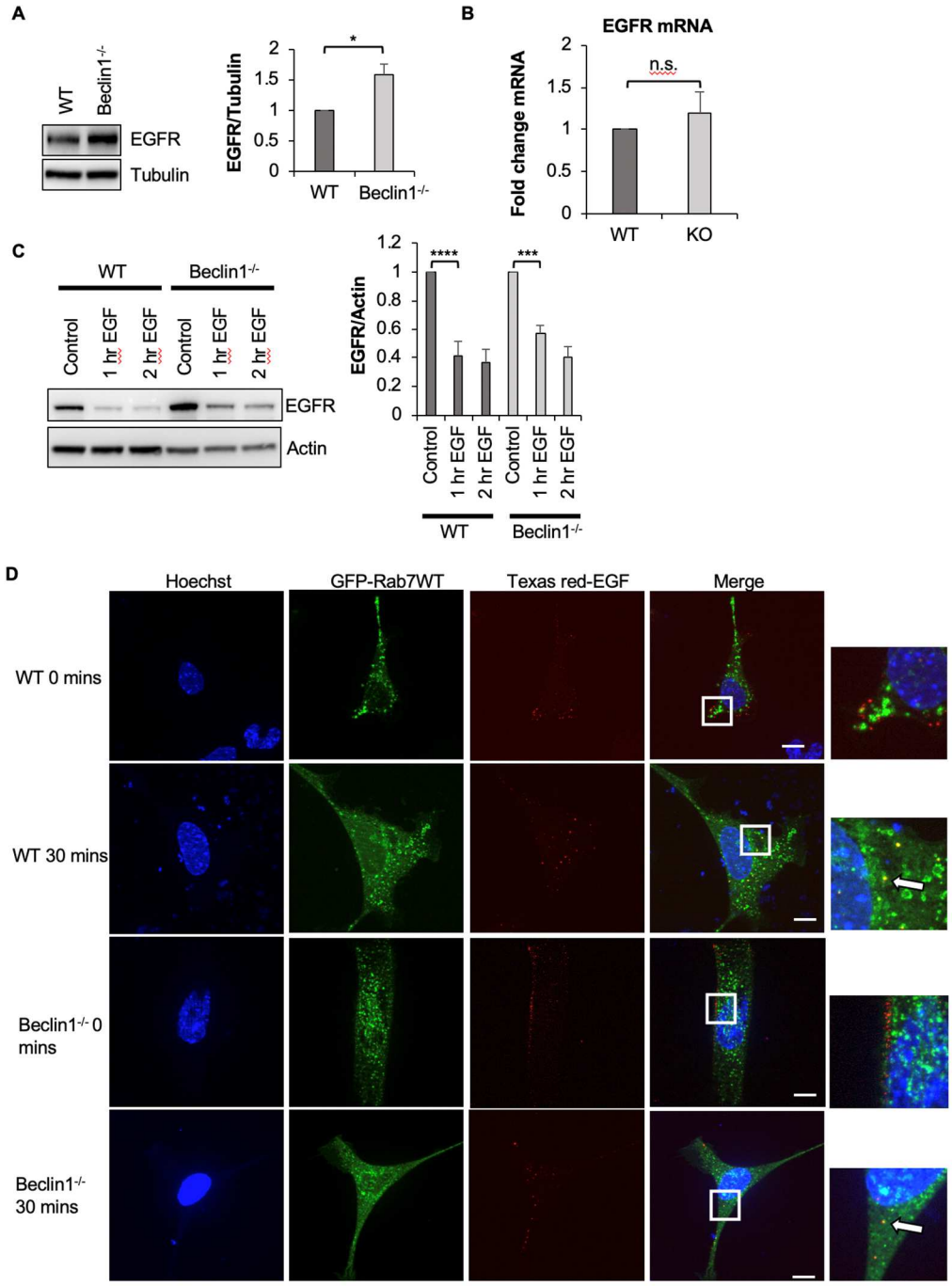

Figure 4.3. EGFR is increased in Beclin1$^{-/-}$ MEFs despite functional endosomal-mediated degradation.

(**A**) Representative Western blot of EGFR protein levels at baseline in WT and Beclin1$^{-/-}$ MEFs. Quantitation of EGFR/Tubulin (n=5). (**B**) mRNA levels of EGFR in WT and Beclin1$^{-/-}$ MEFs at baseline (n=4). (**C**) Representative Western blot of EGFR protein levels after treatment with EGF (200 ng/ul) for the indicated time points. Quantitation of EGFR/Actin normalized to each cell line's control (n=6). (**D**) Representative images of WT and Beclin1$^{-/-}$ MEFs assessing co-localization between GFP-Rab7 and Texas Red-labeled EGF. Data are mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

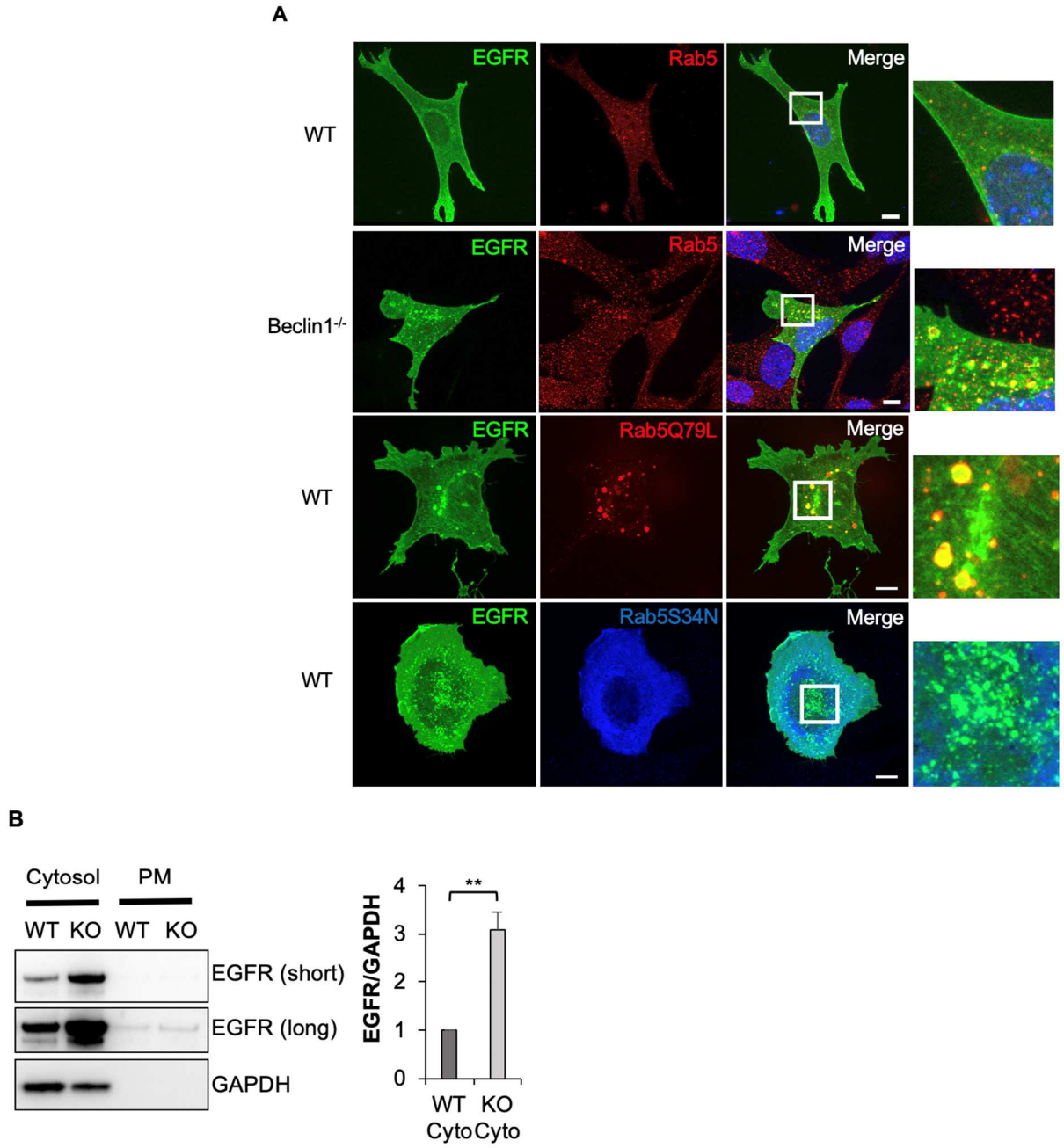

Figure 4.4. EGFR accumulates in Rab5 positive vesicles in the cytosol in Beclin1$^{-/-}$ cells.

(**A**) Representative images of WT and Beclin1$^{-/-}$ MEFs overexpressing GFP-EGFR and either stained for endogenous Rab5 or also overexpressing GFP-Rab5S34N or GFP-Rab5Q79L (n=3). Scale bar=10 μm. (**B**) Representative Western blot of endogenous EGFR protein levels in cytosolic and plasma membrane fractions prepared from WT and Beclin1$^{-/-}$ MEFs. Quantitation of cytosolic EGFR/GAPDH (n=4). Data are mean ± SEM; **$p < 0.01$.

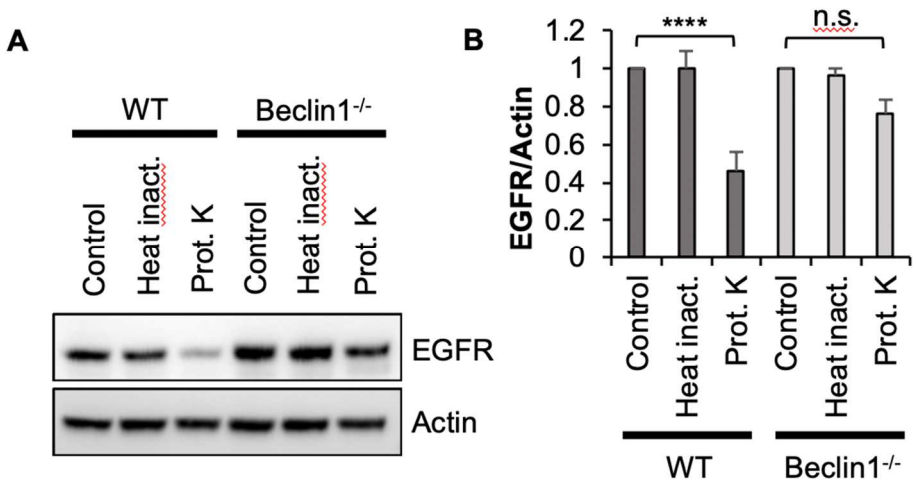

Figure 4.5. EGFR is resistant to proteinase K digestion in Beclin1$^{-/-}$ MEFs.
(**A**) Representative Western blot of EGFR protein levels in WT and Beclin1$^{-/-}$ MEFs after treatment with Proteinase K (50 ng/ul, 15 mins). (**B**) Quantitation of EGFR/Actin protein levels (n=4-5). Data are mean ± SEM; ****p < 0.0001; n.s., not significant.

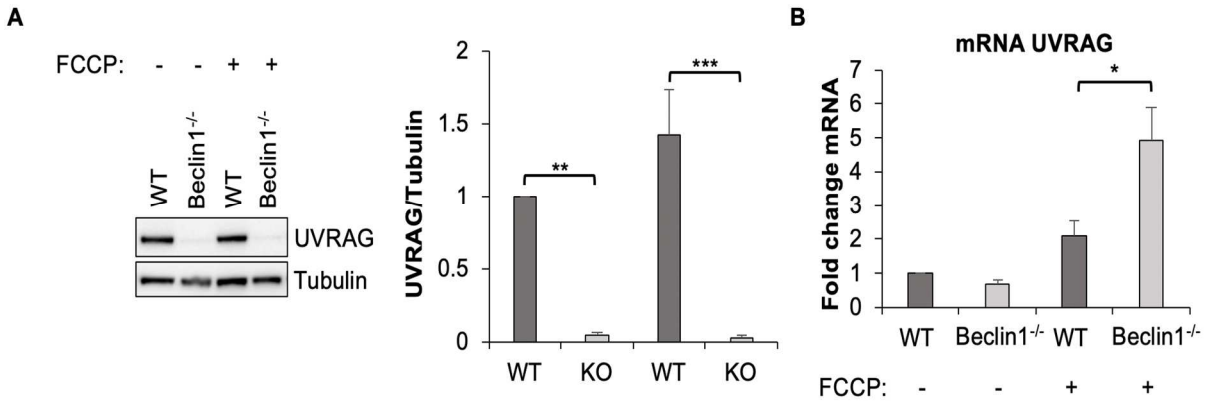

Figure 4.6. UVRAG protein is undetectable in Beclin1$^{-/-}$ MEFs.
(**A**) Representative Western blot of UVRAG protein levels before and after treatment with DMSO or FCCP (4 hours, 25 μM) in WT and Beclin1$^{-/-}$ MEFs. Quantitation of UVRAG/Tubulin (n=3). (**B**) Real-time PCR analysis of *UVRAG* transcript levels in WT and Beclin1$^{-/-}$ MEFs before and after treatment with DMSO or FCCP (4 hours, 25 μM) (n=3). Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

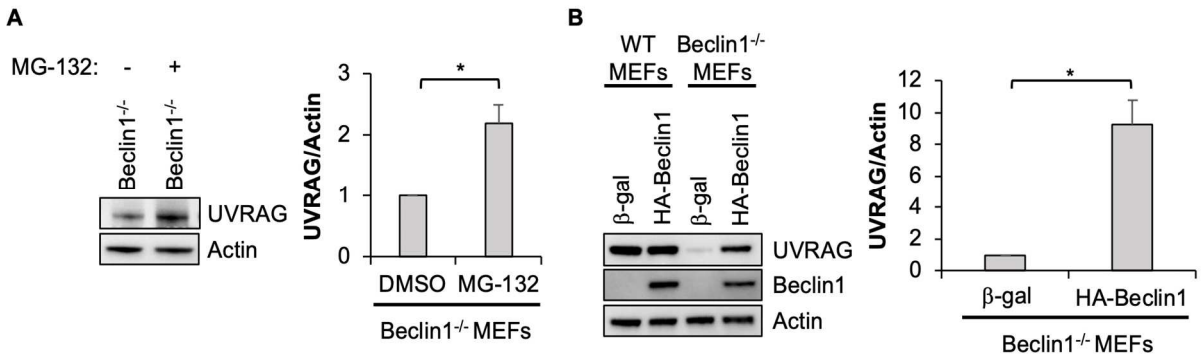

Figure 4.7. UVRAG protein is subjected to proteasomal degradation in the absence of Beclin1.

(**A**) Representative Western blot of UVRAG protein levels before and after treatment with DMSO or MG-132 (4 hours, 5 μM) in Beclin1$^{-/-}$ MEFs. Quantitation of UVRAG/Actin (n=4). (**B**) Cells were infected with *β-gal* or *HA-Beclin1* for 24 hours prior to harvesting. Representative Western blots of UVRAG and Beclin1 protein levels in WT and Beclin1$^{-/-}$ MEFs. Quantitation of UVRAG/Actin Beclin1$^{-/-}$ MEFs (n=3). Data are mean ± SEM. *$p < 0.05$.

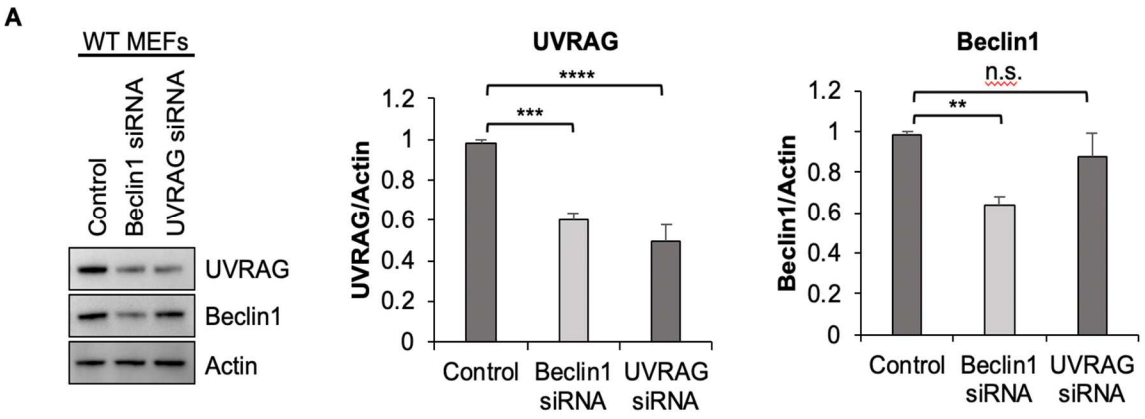

Figure 4.8. Beclin1 is essential for UVRAG protein stability.
(**A**) Representative Western blots of UVRAG and Beclin1 protein levels upon siRNA knockdown (50 nM, 72 hours) of Beclin1 or UVRAG in WT MEFs. Quantitation of UVRAG/Actin and Beclin1/Actin (n=5). Data are mean ± SEM. **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s. not significant.

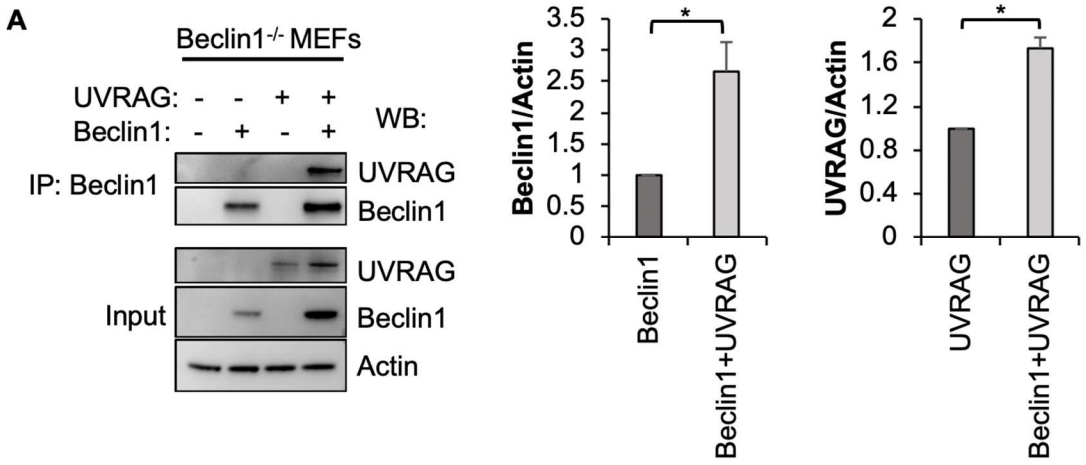

Figure 4.9. Beclin1 interacts with UVRAG in cells.
(**A**) Representative Western blot of UVRAG and Beclin1 protein levels after co-immunoprecipitation of Beclin1 from Beclin1$^{-/-}$ MEFs. Quantitation of Beclin1/Actin and UVRAG/Actin (n=3-4) for input blots. Data are mean ± SEM. *$p < 0.05$.

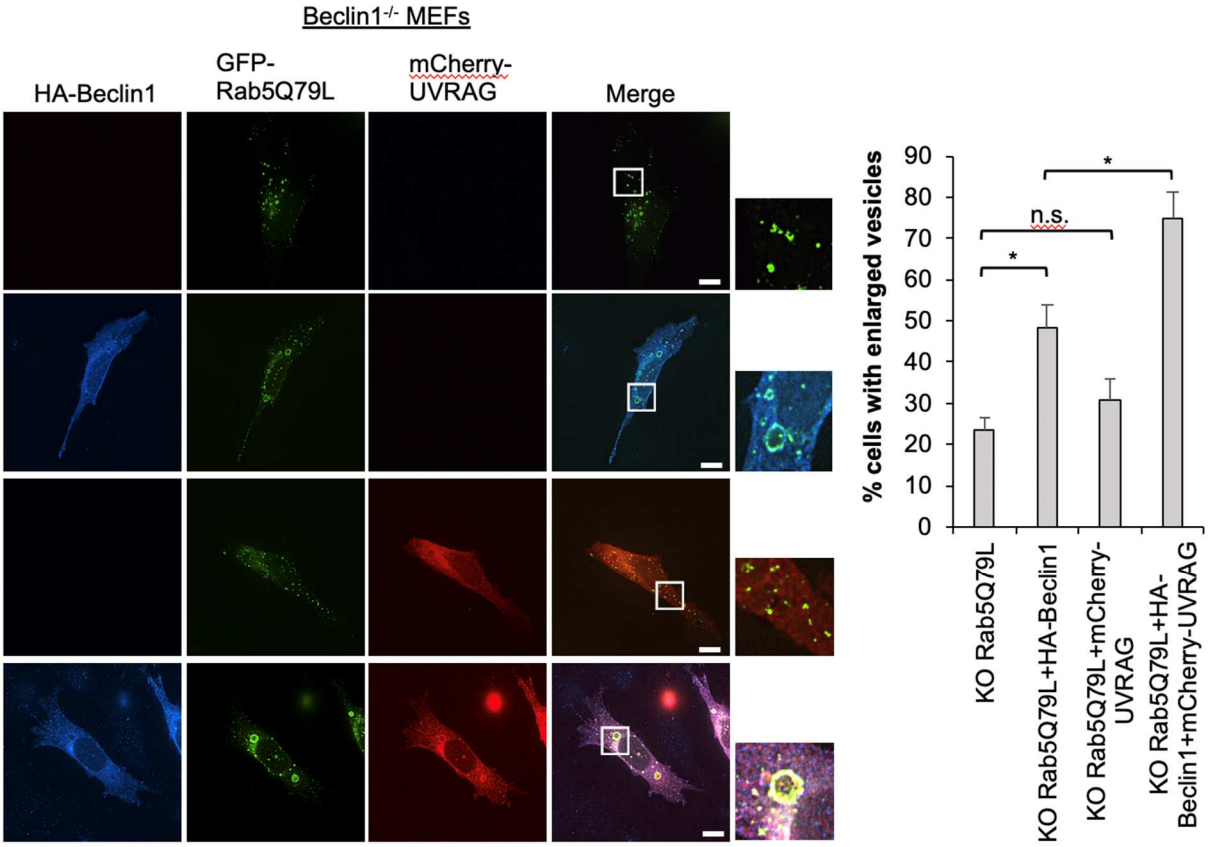

Figure 4.10. Simultaneous overexpression of Beclin1 and UVRAG restores homotypic fusion of Rab5Q79L-positive vesicles.
Representative images of Beclin1$^{-/-}$ MEFs overexpressing GFP-Rab5Q79L, mCherry-UVRAG, and/or HA-Beclin1. Scale bars=10 μm. Quantitation of % of cells with enlarged vesicles (n=4). Data are mean ± SEM. *$p < 0.05$; n.s., not significant.

CHAPTER 5: MITOPHAGY RECEPTORS DRIVE MITOPHAGY IN CPCs

5.1 Introduction

Stem cell therapy has held significant promise as a therapeutic option to treat a variety of heart diseases (Tehzeeb et al., 2019). Much of the success of current stem cell therapies have been due to the ability of the stem cells to differentiate into necessary cell types (Cho et al., 2017; Hatzistergos et al., 2015; Kulandavelu et al., 2016) or secretion of beneficial paracrine factors (Hodgkinson et al., 2016). However, despite its potential, many stem cell treatments fail due to issues in delivery to the myocardium or inability of the cells to survive once in the unforgiving conditions of the failing heart (Guo et al., 2017; Li et al., 2016b; Terrovitis et al., 2010). In fact, a great majority of infused stem cells do not survive longer than a few days in the myocardium. Thus, there is a great need to better understand the biology of these cells to enhance their retention and survivability in the stressful environment of the injured heart.

Endogenous adult progenitor cells are relatively few in number and reside in a variety of tissues, generating energy primarily through glycolysis. As such, endogenous adult progenitor cells have a relatively low density of mitochondria compared to a differentiated mature cell (Chung et al., 2007). Upon activation of the differentiation program, progenitor cells switch from a primarily glycolytic state to one of oxidative phosphorylation, which is accompanied by an increase in mitochondrial biogenesis and mitochondrial network expansion (Chen et al., 2008; Rodriguez-Colman et al., 2017). Mitochondrial function and expansion are crucial for differentiation, as defects in mitochondria has been shown to disrupt proper maturation of progenitor cells (Norddahl

et al., 2011; Wahlestedt et al., 2014). However, the molecular mechanisms behind mitochondrial biogenesis and network expansion in progenitor cells remain unclear.

Autophagy is a highly conserved intracellular degradation pathway that marks unwanted or damaged organelles for degradation by the lysosome (Goldman et al., 2010). Autophagosomes degrade mitochondria in a process known as mitochondrial autophagy, or mitophagy. Mitophagy is important in stem cell maintenance as stem cells are long-lasting cells that must recognize and degrade aberrant mitochondria to maintain homeostasis. There has been recent evidence that mitophagy is required to suppress oxidative metabolism in hematopoietic stem cells (HSCs) and maintain stemness (Ho et al., 2017). While it has become clear that autophagy is activated during differentiation (Vessoni et al., 2012; Zhang et al., 2009), it is still unclear what role it plays in stem cell differentiation and commitment. For instance, autophagy may remove damaged or immature cellular components to allow the immature stem cell to expand and differentiate. Recent work has shown that removal of immature mitochondria is necessary to establish a respiring mitochondrial network (Sin et al., 2016). Additionally, the reprogramming of fibroblasts into iPS cells requires the opposite, with the removal of mature mitochondria necessary during the switch from oxidative phosphorylation to glycolysis (Ma et al., 2015; Vazquez-Martin et al., 2016). In this chapter, I investigate the role of mitochondria and mitophagy during differentiation in cardiac progenitor cells (CPCs). We have performed experiments to determine the molecular mechanisms behind mitophagy activation during differentiation and how this can affect cardiac progenitor cell maturation and viability in the heart.

5.2 Results

5.2.1 Differentiation activates mitophagy and autophagy in CPCs

To investigate whether autophagy is activated in CPCs, we incubated WT and POLG CPCs in differentiation media (DM). POLG CPCs have a mutation in polymerase gamma, which leads to an increased mutation rate and increased mtDNA damage(Orogo et al., 2015). To assess autophagy, we looked at autophagosome formation, which is characterized by lipidation of LC3I into autophagosomal membranes as LC3II. We found that autophagy is activated upon differentiation in both WT and POLG CPCs (Figure 5.1). Treatment with Bafilomycin A1 (Baf), a vacuolar H+ ATPase inhibitor, prevents fusion of autophagosomes with the lysosome and thus can be used to monitor autophagic flux. We found that both at baseline and after 4 days of incubation in DM, WT and POLG CPCs had increased autophagosome formation after treatment with Baf, with no significant differences between the cell types at either time point (Figure 5.1). Next, we wanted to assess whether mitophagy was affected by accumulation of mtDNA mutations. We utilized an EGFP-mCherry-*Cox8* reporter, which fluoresces yellow at baseline. As GFP fluorescence is lost inside the acidic environment of the lysosome, red-only puncta can be counted as autophagosomes with mitochondria inside and indicative of mitophagy (Rojansky et al., 2016). We discovered that POLG CPCs have decreased mitophagy after 3 days of incubation in DM (Figure 5.2). These results indicate that autophagy is activated by differentiation in CPCs, and that although mitophagy is activated by differentiation in both WT and POLG CPCs, mtDNA mutations do significantly decrease mitophagy.

5.2.2 Mitophagy receptors, not Parkin, regulate mitophagy in CPCs

Mitochondria are important organelles in the cell that contain their own genome to generate ATP through oxidative phosphorylation. Excess or dysfunctional mitochondria are eliminated from the cells via mitochondrial autophagy or mitophagy. The most well-known mechanism of mitophagy is via Parkin-mediated mitophagy. Parkin is an E3 ubiquitin ligase that is activated by phosphorylation from PINK1, which then proceeds to ubiquitinate damaged mitochondria. Ubiquitination will then result in engulfment by autophagosomes and subsequent degradation in lysosomes. To study whether Parkin is involved in mitophagy in CPCs, we utilized Parkin KO ($Parkin^{-/-}$) CPCs. We found that after treatment with FCCP, a mitochondrial uncoupler, there was no significant difference in mitochondrial clearance as assessed by western blotting for the inner mitochondrial membrane protein TIM23 between WT and Parkin KO CPCs (Figures 5.3C and 5.3D). This indicates that Parkin mediated mitophagy is not required for CPCs to clear their damaged mitochondria. Notably, Parkin-mediated mitophagy is not the only mechanism of mitochondrial clearance the cell has access to. Recently, a lot of research has focused on a distinct mitophagy pathway that is regulated by mitophagy receptors in the outer mitochondrial membrane that bind directly to the autophagosome to mediate mitophagy. These receptors include FUNDC1, BNIP3L, and BNIP3 (Liu et al., 2014). We found that transcript levels of mitophagy receptors through RNA sequencing both immediately after isolation and upon incubation in culture media were significantly increased compared to Parkin (Figures 5.3E and 5.3F). This held true both in mouse CPCs and in human CPCs for FUNDC1, BNIP3L, and BNIP3 (Figures 5.3E and 5.3F). Recently discovered mitophagy receptors BCL2L13 and PHB2 (Otsu et al., 2015; Wei et al., 2017) had similarly

increased transcript levels (Figures 5.3E and 5.3F), further indicating that CPCs undergo mitophagy receptor-mediated mitophagy. Together, this data indicates that Parkin is not required for mitophagy in CPCs and that mitophagy receptors likely play an important role in mitochondrial clearance.

5.2.3 Mitophagy receptors increase upon differentiation in WT CPCs

Differentiation leads to an increase in mitochondrial biogenesis and mitochondrial network expansion to facilitate the bioenergetic switch from relying primarily on glycolysis for energy to that of oxidative phosphorylation (Chen et al., 2008). Thus, we wanted to investigate whether mitophagy receptors were similarly upregulated in response to differentiation in WT and POLG CPCs. We found that transcript levels of FUNDC1 and BNIP3L, but not BNIP3, were significantly increased upon incubation of WT CPCs in DM (Figure 5.4A-C). In contrast, transcript levels of FUNDC1 and BNIP3L were significantly decreased upon 4 days of incubation in DM in POLG CPCs, indicating a defect in mitophagy receptor transcription (Figure 5.4A-C). Western blot analysis showed that protein levels of FUNDC1 and BNIP3L were also significantly increased upon 7 days of incubation in DM for WT CPCs, while there was no significant difference in POLG CPCs (Figure 5.4D and 5.4E). Thus, our data demonstrate that CPCs do upregulate mitophagy receptors FUNDC1 and BNIP3L in response to differentiation.

Next, we wanted to assess whether FUNDC1 and BNIP3L were required for functional mitophagy in CPCs after differentiation. We utilized specific siRNAs to knock down both FUNDC1 and BNIP3L mRNA levels. Using a similar mitophagy reporter as described before, we found that after 3 days of incubation in DM, WT CPCs that had both

FUNDC1 and BNIP3L siRNA had significantly less mitophagy in the form of red-only puncta than in the scrambled siRNA control cells (Figures 5.5D and 5.5E). Notably, we only saw this effect when both FUNDC1 and BNIP3L were knocked down, indicating the presence of a compensatory mechanism when only a single mitophagy receptor is depleted. Because we discovered that FCCP-mediated mitophagy occurs independent of Parkin, we next wanted to investigate whether FUNDC1- and BNIP3L-mediated mitophagy could be responsible. We treated WT CPCs +/- FUNDC1 and BNIP3L knockdown with FCCP but found no difference in TIM23 protein levels (Figures 5.5F and 5.5G). These data indicate that FUNDC1 and BNIP3L do not mediate FCCP related mitophagy in CPCs but instead regulate mitophagy upon activation of differentiation.

5.2.4 FUNDC1 and BNIP3L are not required for lineage commitment or mitochondrial biogenesis

Increased production of mitochondria is a hallmark of stem cell differentiation, alongside the upregulation of various lineage markers. We investigated whether FUNDC1 and BNIP3L were involved in regulating lineage commitment of WT CPCs. We found that transcript levels of different lineage commitment markers Gata-4, Gata-6, and Mef2c were all unaffected by knockdown of FUNDC1 and BNIP3L (Figure 5.6A). Ppargc1a, or PPARG coactivator 1 alpha, is a master regulator of mitochondrial biogenesis (Fernandez-Marcos and Auwerx, 2011). Thus, we wanted to investigate whether FUNDC1 and BNIP3L were involved in affecting pPARGC1a levels after differentiation. We found that knockdown of FUNDC1 and BNIP3L had no effect on Ppargc1a transcript levels after 7 days of incubation in DM (Figure 5.6B). Finally, we wanted to assess

whether BNIP3L and FUNDC1 were involved in regulating mitochondrial OXPHOS protein levels. Similarly, we found no difference between siRNA control and FUNDC1 and BNIP3L knockdown in OXPHOS protein levels after 7 days of incubation in DM in WT CPCs (Figures 5.6C and 5.6D). Taken together, these data demonstrate that BNIP3L- and FUNDC1-mediated mitophagy is not required for lineage commitment and mitochondrial biogenesis upon induction of differentiation in CPCs.

5.2.5 BNIP3L and FUNDC1 are required for the formation of an interconnected, functional mitochondrial network

Formation of an interconnected mitochondrial network is another notable hallmark of stem cell differentiation (Sin et al., 2016). We observed significant expansion of the mitochondrial network through outer membrane mitochondrial protein TOMM20 upon 7 days of differentiation in CPCs by immunofluorescence (Figure 5.7A). Interestingly, knockdown of FUNDC1 and BNIP3L led to the formation of fragmented mitochondria and change in mitochondrial network morphology (Figures 5.7A and 5.7B). Next, we used transmission electron microscopy (TEM) to assess the ultrastructural changes in mitochondrial morphology upon knockdown of FUNDC1 and BNIP3L. Notably, we discovered that knockdown of FUNDC1 and BNIP3L led to the formation of donut-shaped, circular mitochondria after differentiation in WT CPCs (Figure 5.7C). These mitochondria were characterized by electron-dense material in the lumen and only seen after differentiation. Based on these results, it is clear that FUNDC1 and BNIP3L are critical for maintaining mitochondrial morphology after differentiation in CPCs.

To uncover the mechanism behind the FUNDC1- and BNIP3L-mediated mitochondrial fragmentation, we next analyzed the role of mitochondrial fission regulator DNM1L. DNM1L is known to regulate mitochondrial fission by assembling on the cytoplasm of mitochondrial tubules (Bleazard et al., 1999). It is regulated by phosphorylation of various sites, with phosphorylation on serine 616 increasing DNM1L activity (Kashatus et al., 2015). While we found that total DNM1L protein levels were unchanged after differentiation in CPCs, we found that levels of p-DNM1L S616 were significantly increased upon knockdown of FUNDC1 and BNIP3L compared to control (Figure 5.8D-G). While this indicates an increase in mitochondrial fission when mitophagy is impaired, there may also be a defect in mitochondrial fusion. Thus, we investigated the role of mitochondrial fusion proteins Mitofusin1 and Mitofusin2 (Mfn1 and Mfn2). We found no significant difference in MFN1 and MFN2 protein levels after differentiation between control or FUNDC1 and BNIP3L knockdown CPCs (Figures 5.8D and 5.8E). These data indicate that BNIP3L- and FUNDC1-mediated mitophagy lead to sustained DN1ML activation after differentiation and subsequent increase in mitochondrial fragmentation.

Finally, we wanted to assess the importance of FUNDC1- and BNIP3L-mediated mitophagy on mitochondrial function. We found that maximal mitochondrial respiration, analyzed by a seahorse flux analyzer, was significantly decreased in FUNDC1 and BNIP3L knockdown CPCs after 7 days of differentiation (Figure 5.9C). Additionally, we found that CPCs with FUNDC1 and BNIP3L knockdown had increased susceptibility to H_2O_2 mediated cell death (Figure 5.9D). Retention of CPCs *in vivo* is critical for any stem cell therapy to be viable. Thus, we wanted to assess whether impaired mitophagy would

affect the ability of CPCs to survive in the heart. Notably, we found that after 7 days of differentiation, CPCs with FUNDC1 and BNIP3L knockdown were undetectable in the border zone of mouse hearts 5 days after myocardial infarction (Figure 5.9E). Taken together, our data indicate that mitophagy is important for the formation of an interconnected mitochondrial network upon differentiation in CPCs and provides protection against stress, increasing cell survival both *in vitro* and *in vivo*.

5.3 Discussion

In this chapter, I report that CPCs undergo mitophagy mediated by mitophagy receptors FUNDC1 and BNIP3L and not through the traditional Parkin pathway. Mitophagy was activated in CPCs after induction of differentiation, as has been reported in multiple stem cell types (Ma et al., 2015; Vazquez-Martin et al., 2016). Interestingly, we found that loss of Parkin did not affect mitophagy in CPCs. Parkin-mediated mitophagy is the most well-known and characterized mitophagy pathway and is activated by a variety of stressors in different cardiac diseases. However, emerging evidence demonstrates that Parkin-mediated mitophagy is not the only mechanism of removal of mitochondria by the cell. Mitophagy is an important cellular process and thus it is clear that it has redundancies in case of deficiency in one pathway to maintain cell viability.

Notably, in CPCs, I found that mitophagy receptors FUNDC1 and BNIP3L were upregulated both at the transcript and protein levels upon activation of differentiation in WT but not in POLG CPCs. This highlights the impact that an accumulation of mtDNA mutations can have on stem cell function. Knockdown of both FUNDC1 and BNIP3L, but not alone, led to a significant decrease in mitophagy, indicating a possible compensatory

mechanism in CPCs. Previously, it was already known that POLG CPCs have premature aging and susceptibility to cell death (Orogo et al., 2015). Our data is the first to uncover the specific mechanism of mitophagy that CPCs utilize upon activation of differentiation through mitophagy receptors FUNDC1 and BNIP3L. Notably, our data suggest that lack of upregulation of mitophagy receptors could contribute to the pathological phenotype present in POLG CPCs.

Additionally, we found that mitophagy is dispensable in CPCs for lineage commitment or for mitochondrial biogenesis. As differentiation occurs, stem cells must generate more mitochondria to fuel the development of the cell and thus increase mitochondrial biogenesis. We found that knockdown of mitophagy receptors FUNDC1 and BNIP3L had no effect on the induction of the master mitochondrial biogenesis regulator PPARGC1a. This indicates that deficiencies in mitophagy do not affect the ability of the cell to generate more mitochondria. We also found that lineage commitment markers were unaffected. CPCs also release protective paracrine factors upon differentiation which contribute to tissue repair after injury (Baraniak and McDevitt, 2010). Interestingly, we found that loss of mitophagy impaired the ability of CPCs to form endothelial tubes after differentiation. This indicates that mitophagy is important for cell secretion and thus could affect the ability of CPCs to aid in cardiac repair.

Mitochondria are believed to undergo asymmetric fission to separate damaged or dysfunctional proteins from the healthy part of the mitochondria (Dorn and Kitsis, 2015). This division allows the mitochondria to continue to function properly while the dysfunctional components can be degraded by the cell. The data here demonstrate that mitophagy in CPCs is required for the formation of an interconnected mitochondrial

network upon differentiation. Loss of mitophagy leads to increased mitochondrial fragmentation mediated by DNM1L, which remains in its active, phosphorylated state. As mitophagy is impaired, the cell is unable to remove dysfunctional mitochondrial components, leading to increased mitochondrial fragmentation and impaired network formation. Ultrastructural analysis confirmed the presence of fragmented, donut-like mitochondria after differentiation in mitophagy deficient CPCs. Other studies have noted the donut-like mitochondrial structure after different stressors such as CCCP (Ding et al., 2012). Finally, we found that loss of mitophagy led to downstream functional consequences in CPCs, including decreased maximal respiration, increased susceptibility to cell death, and decreased retention after injection *in vivo*. These functional consequences underlie the importance of mitophagy during differentiation in CPCs.

Based on these findings, it is clear that mitophagy is important in CPC differentiation and is mediated by mitophagy receptors FUNDC1 and BNIP3L rather than the PINK1-Parkin pathway. It is important to further investigate how an inability to remove select mitochondria contributes to a defect in mitochondrial network formation. The ability to better understand the biology of stem cells is critical for designing better therapeutics that can have a lasting positive effect on the injured heart.

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Autophagy. 15(7): 1182-1198, 2019; doi:10.1080/15548627 © 2019 Autophagy. PMID: 30741592. The dissertation author was the primary investigator and author of this paper.

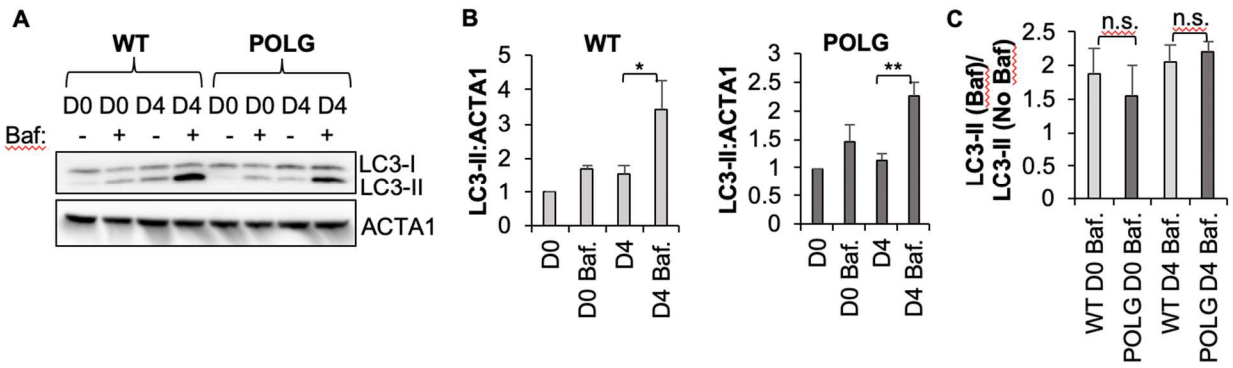


Figure 5.1. POLG CPCs have functional autophagic flux after differentiation.
(A) Representative Western blots of LC3II and ACTA1 in WT and POLG CPCs after incubation in differentiation medium (DM). To assess flux, cells were incubated with 50 nM Bafilomycin A1 (Baf) for 4 hours before harvesting cells. (**B**) Quantitation of LC3II:ACTA1 in WT CPCs and POLG CPCs (n=3). (**C**) Quantitation of LC3II:ACTA1 +/- Baf in WT and POLG CPCs (n=3). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant.

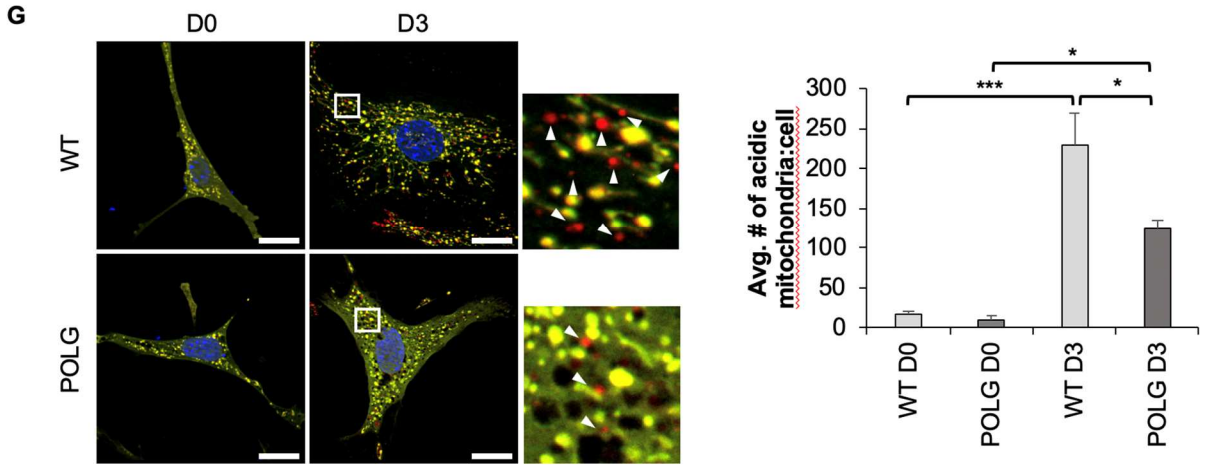
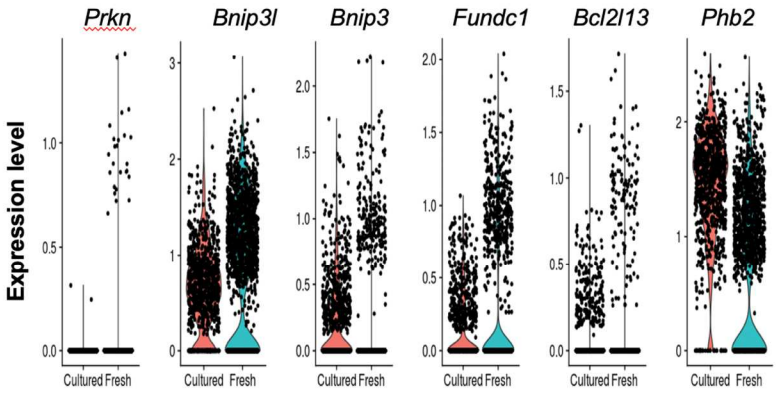


Figure 5.2. POLG CPCs have impaired mitophagy.
(**G**) Representative fluorescent images of WT and POLG CPCs overexpressing *Cox8*-EGFP-mCherry at D0 and D3. Quantitation of acidic (red-only) mitochondria in WT and POLG CPCs (n=3). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant.

E

	P5 (cultured)		P0 (fresh)	
	# of cells	%	# of cells	%
Prkn	2	0.2	25	1.6
Bnip3l	717	84.4	1075	66.9
Bnip3	404	47.5	226	14.1
Fundc1	331	38.9	424	26.4
Bcl2l13	203	23.9	128	8.0
Phb2	819	96.4	888	55.2
Total	850		1608	



F

	CPC		EPC		MSC	
	# of cells	%	# of cells	%	# of cells	%
PRKN	2	0.2	3	0.2	7	0.4
BNIP3L	686	84.3	1173	69.3	1285	72.8
BNIP3	781	95.9	1527	90.2	1459	82.7
FUNDC1	286	35.1	491	29.0	488	27.6
BCL2L13	364	44.7	685	40.5	692	39.2
PHB2	738	90.7	1495	88.3	1512	85.7
Total	814		1693		1765	

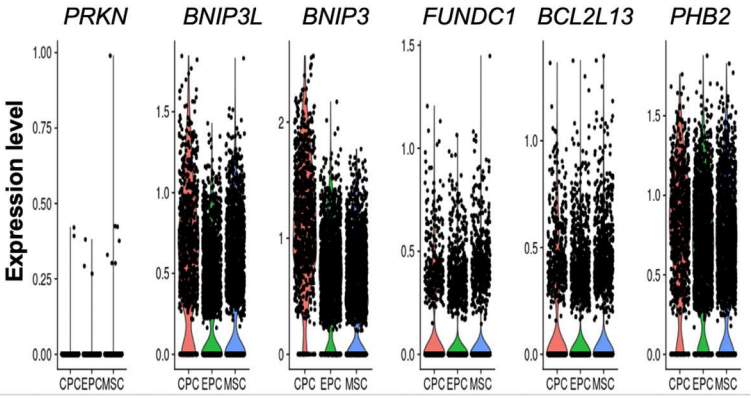


Figure 5.3. Mitophagy in CPCs does not require Parkin.
C) Representative Western blots of TIMM23 and ACTA1 in WT and *prkn*$^{-/-}$ CPCs after treatment with 25 μM FCCP for 24 hours. (**D**) Quantitation of TIMM23:ACTA1 in WT and *prkn*$^{-/-}$ CPCs (n=3). (**E**) The number and percentage of cells with mRNA detected by single cell RNA sequencing for *Prkn* and mitophagy genes in mouse CPCs at passage 0 (fresh) or passage 5 (cultured). Violin plots display gene expression of mitophagy genes in mouse CPCs. (**F**) The number and percentage of cells with mRNA detected by single cell RNA sequencing for *PRKN* and mitophagy receptors in human CPCs at passage 5 (cultured). Violin plots display gene expression of mitophagy genes in human CPCs. Data are mean ± SEM. ***p < 0.001; n.s., not significant.

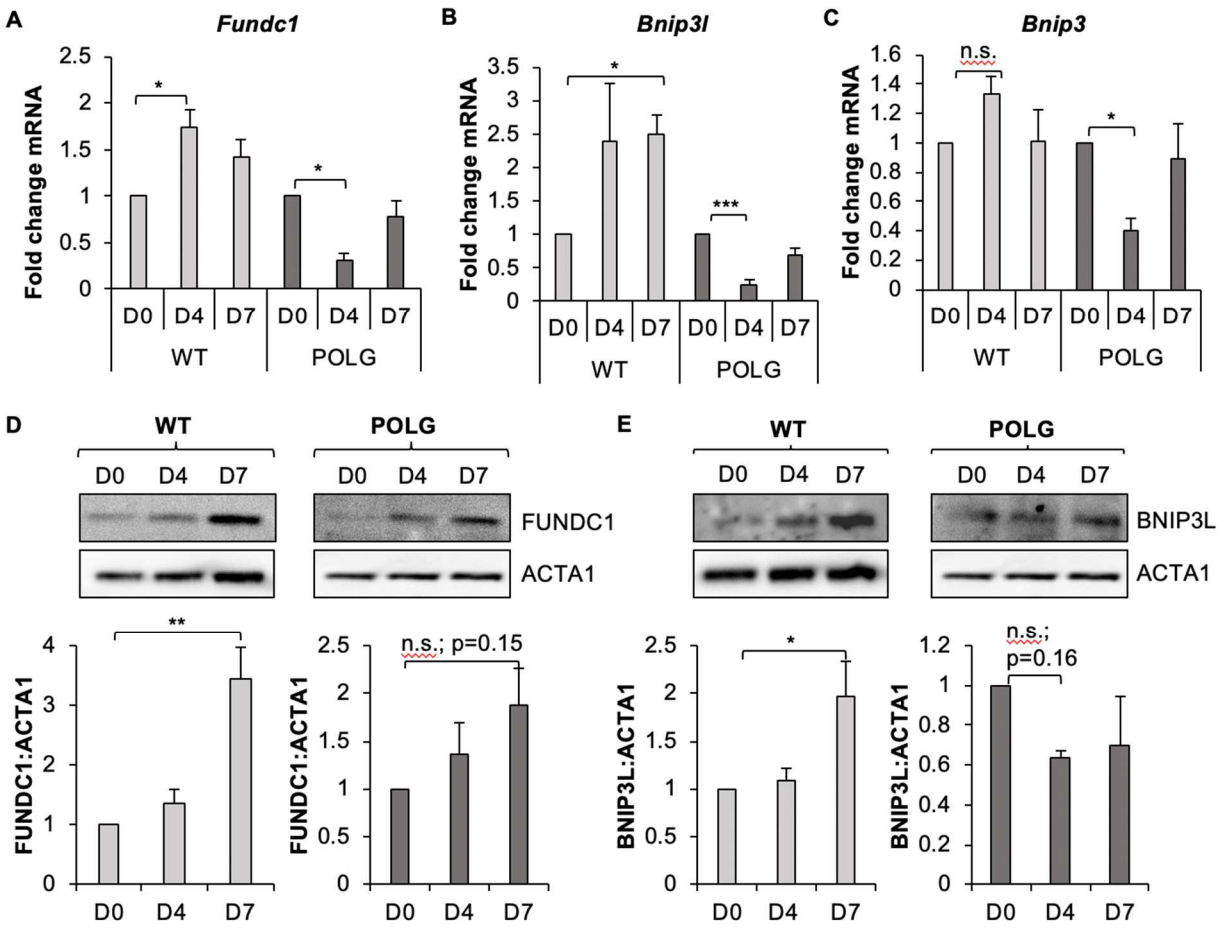


Figure 5.4. Increase in mitophagy receptors after differentiation in WT CPCs.
Real-time PCR analysis of mitophagy receptors (**A**) *Fundc1*, (**B**) *Bnip3l* and (**C**) *Bnip3* transcript levels in WT and POLG CPCs at baseline and after incubation in DM (n=4). (**D**) Representative Western blots and quantitation of FUNDC1:ACTA1 in WT and POLG CPCs (n=4). (**E**) Representative Western blots and quantitation of BNIP3L:ACTA1 in WT and POLG CPCs (n=4). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.

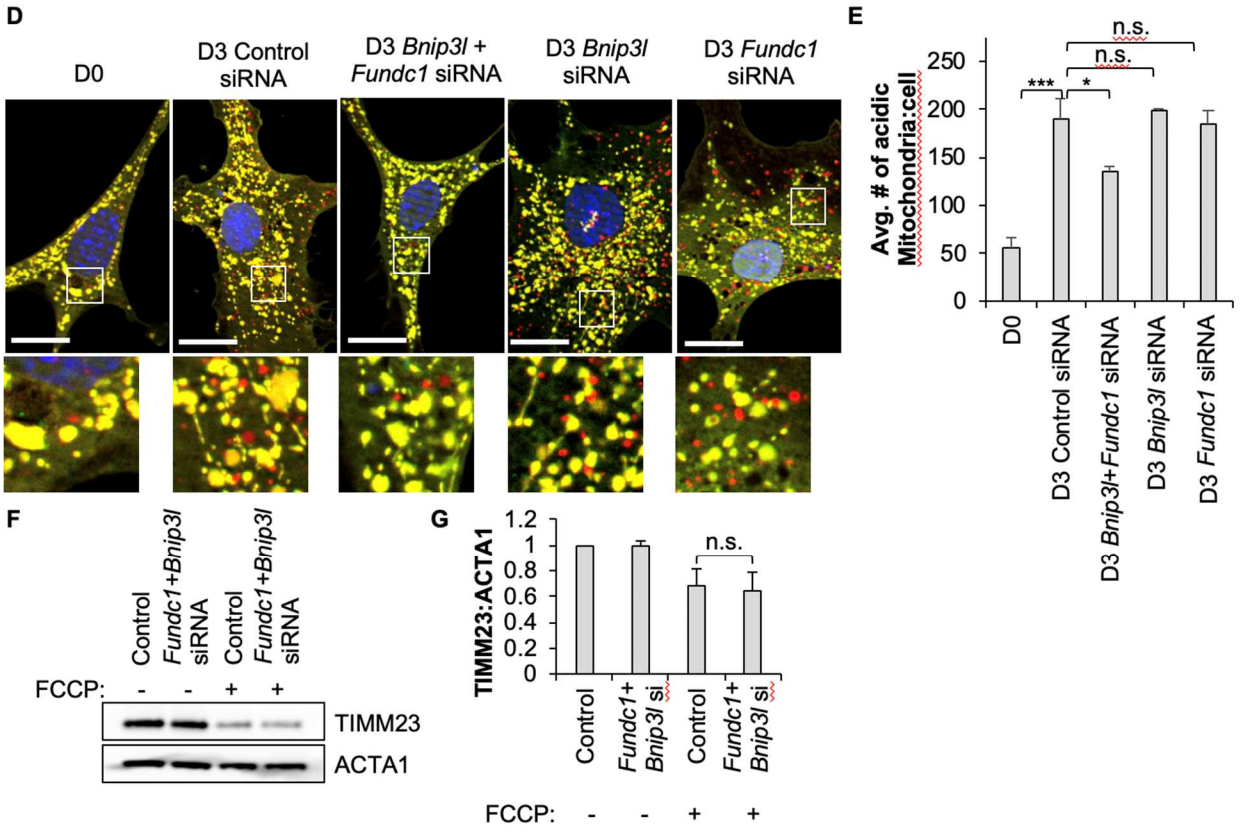


Figure 5.5. Knockdown of mitophagy receptors *Fundc1* and *Bnip3l* leads to decreased mitophagy in CPCs.

D) Representative fluorescent images of CPCs overexpressing *Cox8*-EGFP-mCherry. Cells were transfected with siRNA against *Bnip3l*, *Fundc1*, or *Bnip3l*+*Fundc1*, and incubated in DM for 3 days. Scale bar=20 μm. (**E**) Quantitation of acidic (red-only) mitochondria in CPCs (n=3). (**F**) Representative Western blots of TIMM23 after siRNA knockdown of *Bnip3l*+*Fundc1* and treatment with 25 μM FCCP for 24 hours. (**G**) Quantitation of TIMM23:ACTA1 in CPCs (n=3). Data are mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant.

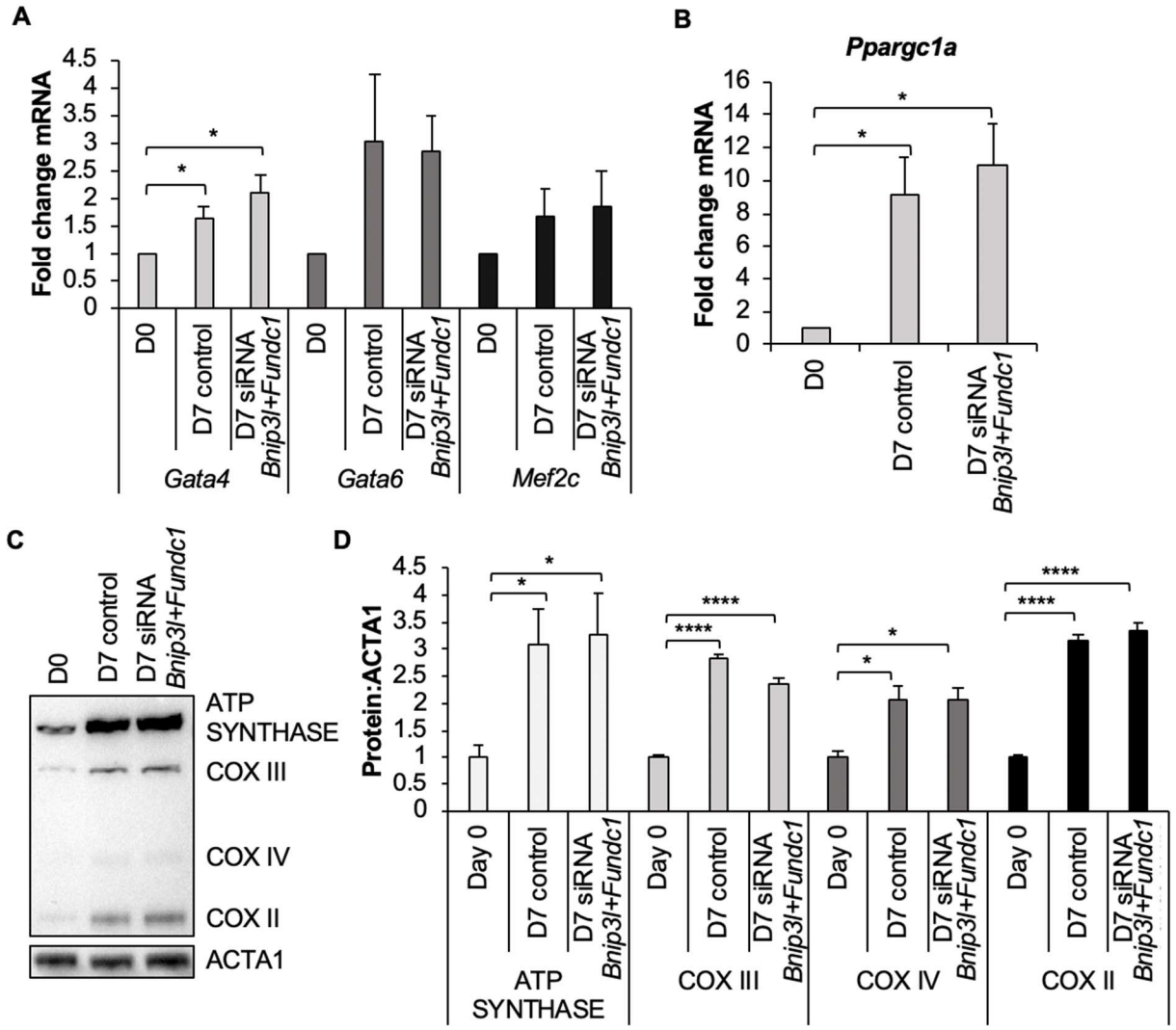


Figure 5.6. Mitophagy receptors are not required for lineage commitment or mitochondrial biogenesis.

(**A**) Real-time PCR analysis of *Gata4*, *Gata6*, and *Mef2c* transcripts in CPCs (n=4). (**B**) Real-time PCR analysis of *Ppargc1a* transcript levels in CPCs (n=4). (**C**) Representative Western blots of mitochondrial OXPHOS subunit proteins in CPCs. (**D**) Quantitation of mitochondrial OXPHOS subunit protein levels (n=4). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant.

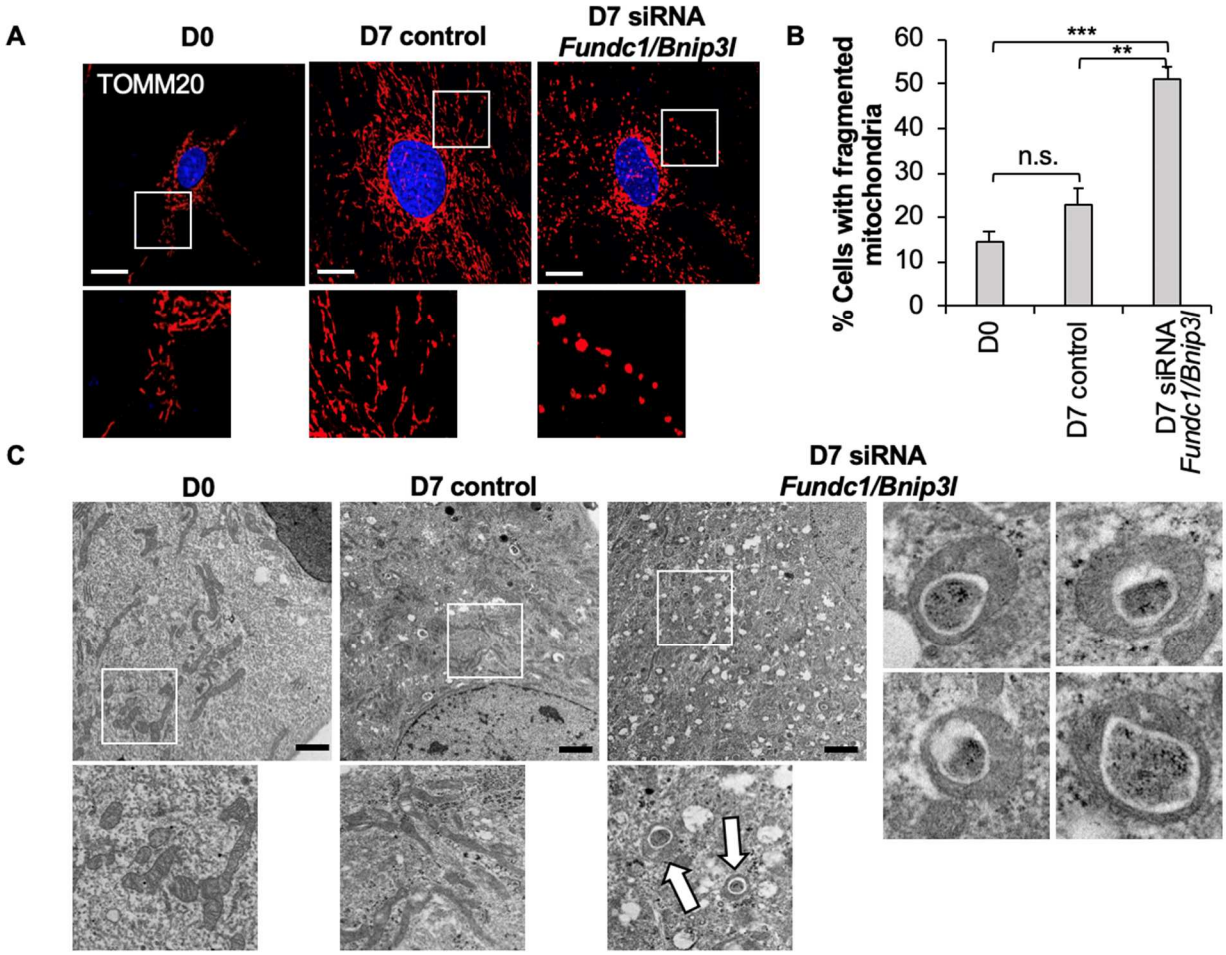


Figure 5.7. Loss of mitophagy receptors leads to mitochondrial fragmentation after differentiation in CPCs.

(**A**) Representative fluorescent images of mitochondrial network in CPCs before and after incubation in DM. Scale bar=20 μm. (**B**) Quantitation of CPCs with fragmented mitochondria (n=3). (**C**) Representative electron microscopy images of mitochondria in CPCs before and after incubation in DM. Arrows point to donut-shaped mitochondria. Scale bar=2 μm. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant.

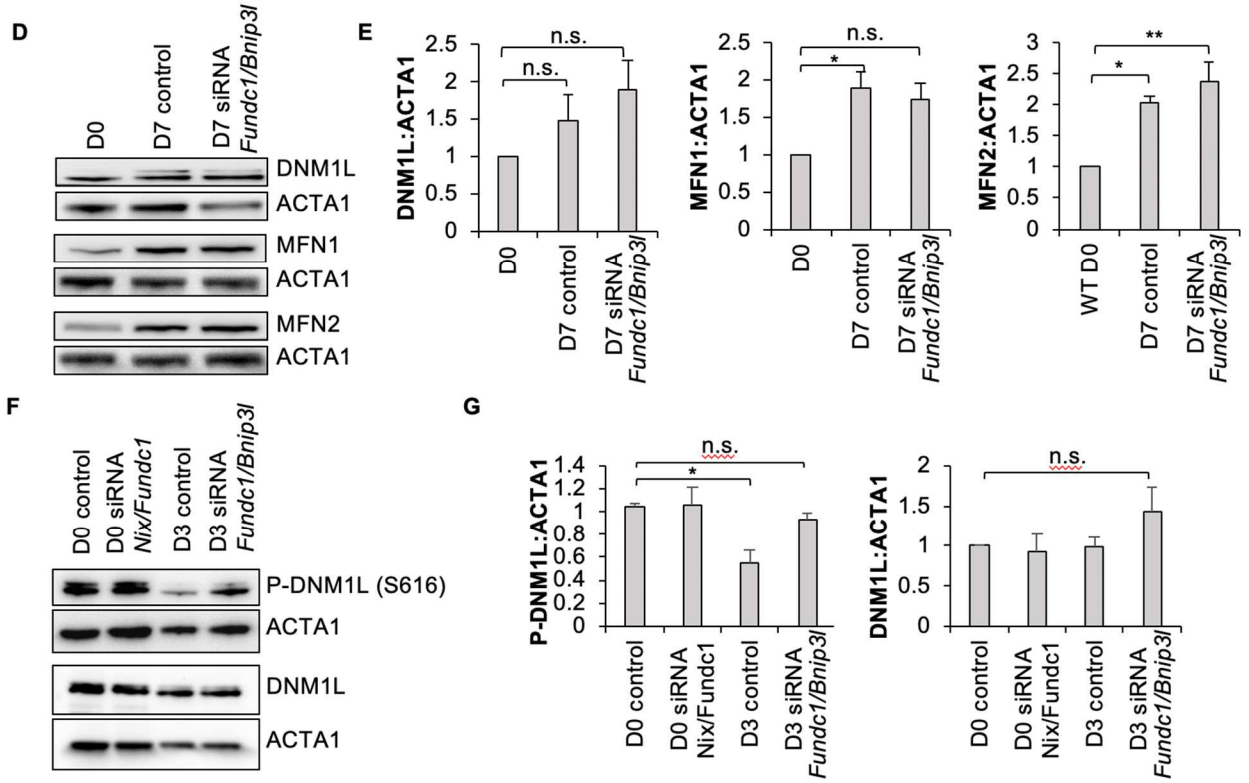


Figure 5.8. Mitochondrial fragmentation is mediated by DNM1L activity in CPCs. (**D**) Representative Western blots of DNM1L, MFN1 and MFN2. (**E**) Quantitation of DNM1L (n=5), MFN1 (n=3) and MFN2 (n=3) protein levels. (**F**) Representative Western blots of P-DNM1L (Ser 616) and DNM1L. (**G**) Quantitation of P-DNM1L (n=3) and DNM1L (n=3) protein levels. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; n.s. not significant.

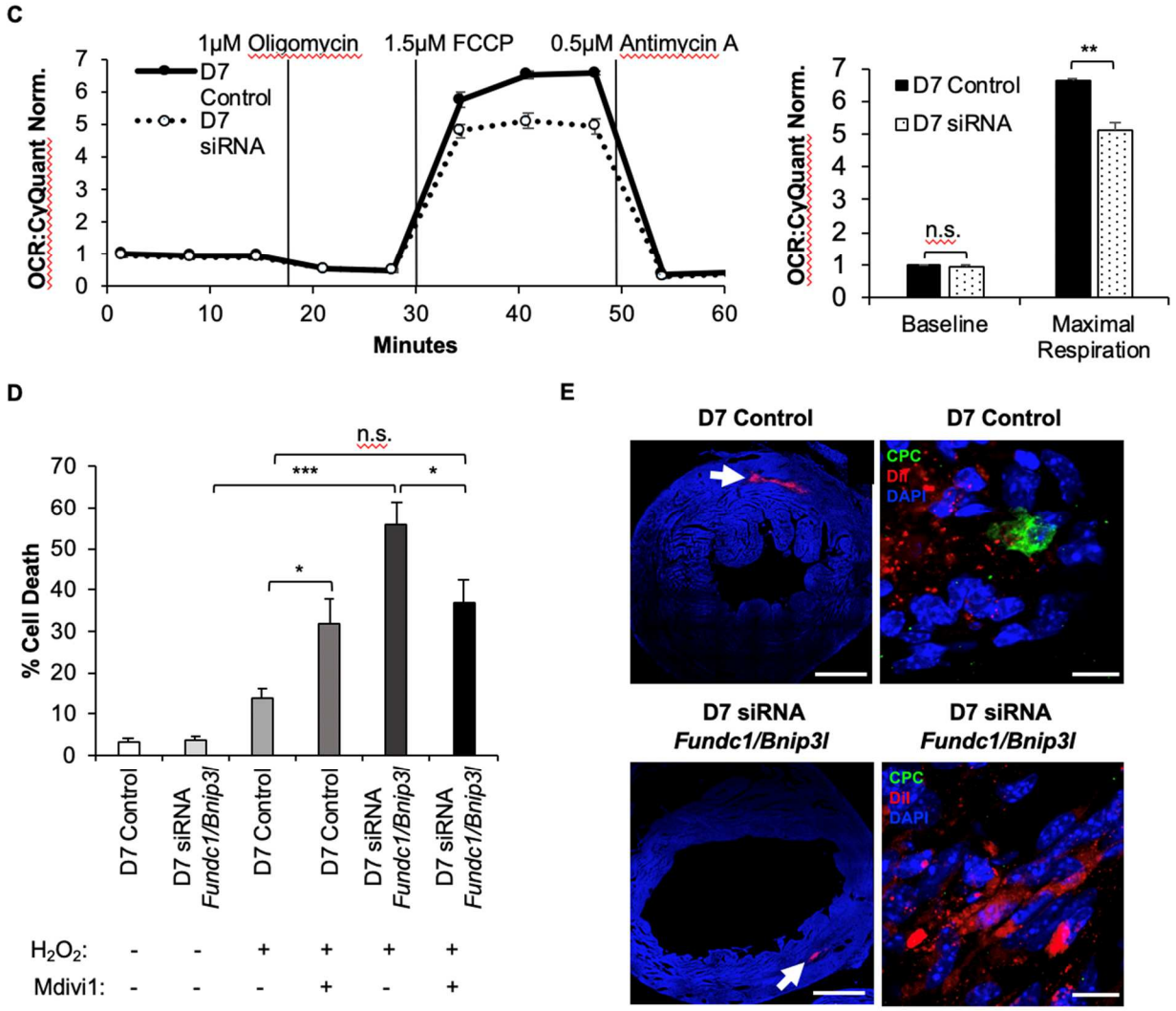


Figure 5.9. Knockdown of mitophagy receptors decreases mitochondrial function and susceptibility to cell death.

(**C**) Mitochondrial respiration in CPCs at D7. Oxygen consumption rate (OCR) was normalized to cell number (n=4). (**D**) Quantitation of cell death in differentiating CPCs (D7). Cells were treated with 200 μM H_2O_2 for 8 hours in serum-free DM in the presence or absence of 10 μM Mdivi-1 (n=3). (**E**) Representative scans of mouse hearts for retention of GFP-positive CPCs. Hearts from animals injected with either control CPCs or *Bnip3l/Fundc1* knockdown CPCs were immunostained with GFP to detect surviving CPCs at 5 days after infarction. DiI dye was utilized to track the injections through the heart. GFP-positive control CPCs were detected in the border zone of infarcted hearts (n=2), while GFP-positive *Bnip3l/Fundc1* knockdown CPCs were not present in the infarcted hearts (n=5). Scale bar=1 mm and 10 μm, respectively. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n.s., not significant.

CHAPTER 6: DISCUSSION

6.1 Introduction

Beclin1 is a cellular scaffolding protein that forms unique protein complexes in the cell to regulate a variety of different processes. Traditionally, Beclin1 has been studied in the context of autophagy where it initiates formation of autophagosomes through its interaction with Vps34 and Atg14L (Liang et al., 1999). However, its additional role as a regulator of the endosomal pathway was recently described in neurons (McKnight et al., 2014). Our data demonstrate for the first time that the presence of Beclin1 is essential for cardiac homeostasis and that it regulates both autophagic activity and endosomal trafficking. Specifically, loss of Beclin1 in cells led to accumulation of the plasma membrane receptor EGFR in Rab5 positive early endosomes. Additionally, Beclin1-deficiency was associated with reduced homotypic fusion of Rab5 positive endosomes and their trafficking of cargo to plasma membrane. In the heart, cardiac specific deletion of Beclin1 led to rapid loss of myocytes, severe contractile dysfunction and increased mortality *in vivo*. A variety of endosomal markers were affected by the loss of Beclin1, including early endosome markers Rab5 and EEA1 and Golgi marker Rab6. Overexpression of UVRAG delayed both Beclin1 degradation and the decline in cardiac function upon deletion of *Beclin1*. Together, these data uncover a novel role for Beclin1 in its regulation of endosomal trafficking and its importance in the heart.

6.2 Beclin1 as a regulator of endosomal trafficking and heart function

Our study provides novel insights into the roles of Beclin1 in maintaining cardiac homeostasis through its regulation of pathways beyond that of autophagy, with a focus on the endosomal pathway. First, we demonstrate that loss of Beclin1 is detrimental to the heart, with severe cardiac dysfunction seen in mice only 1 week after deletion of Beclin1. Second, the cardiac dysfunction is not a result of impaired autophagy, as the autophagy deficient mice lacked a cardiac phenotype within the same timeframe. Third, Beclin1 is essential for early endosome GTPase Rab5 activity as loss of Beclin1 impairs Rab5 activity *in vitro* and leads to a significant increase of Rab5 effector EEA1 *in vivo.* Fourth, we found that loss of Beclin1 disrupts endosomal biosynthetic receptor trafficking but not receptor internalization. Finally, we discovered that Beclin1 is required for UVRAG protein stability *in vitro* and *in vivo* and that overexpression of UVRAG *in vivo* can delay the negative cardiac phenotype by preserving Beclin1 protein levels. Thus, our findings demonstrate the importance of Beclin1 in regulating intracellular trafficking pathways in the heart to maintain cardiac homeostasis and function.

In the data presented in chapter 4, it is clear that Beclin1's role as a scaffolding protein is critical to the regulation of multiple intracellular degradation pathways. Proper cellular control of protein localization and recruitment allows for proper signaling for a variety of processes. It is interesting to note that in a variety of diseases, mislocalization of receptors leads to poor outcomes due to defective intracellular transport, including in choroideraemia, cystic fibrosis, and Wilson's disease (Gissen and Maher, 2007; Kuwahara and Marumo, 1996). We are the first to identify a role for Beclin1 in the trafficking of receptors as important for heart function. Additionally, our data suggest that while Beclin1 is important for biosynthetic receptor trafficking, endocytic receptor

degradation is unaffected. There are two main pathways that allow for receptors to be trafficked to the plasma membrane, which include after biosynthesis and recycling after endocytosis (Grant and Donaldson, 2009). During biosynthesis, receptors are translated in ribosomes in the ER, undergo folding, and are then transported to the Golgi apparatus. There, they are post-translationally modified to determine where in the cell they will reside. For instance, addition of mannose-6-phosphate to N-glycans traffics lysosomal hydrolases from endosomes to lysosomes (Hickman et al., 1974). Once their fate has been determined, they will be transported by endosomes to the plasma membrane. Given the role of early endosomes in biosynthetic trafficking, it appears likely that Beclin1 would be recruited at this late step. The other main form of receptor trafficking is after being endocytosed at the plasma membrane. Once internalized, receptors are sorted at the early endosome for either degradation (Rab7 into lysosomes) or recycling (Rab4, Rab11) back to the plasma membrane. Our data indicates that Beclin1 does not affect endocytosis and trafficking of the receptors through the degradation pathway. Future work should focus on determining whether Beclin1 is able to bind to recycling endosomes to facilitate their trafficking.

The data presented in chapter 3 illustrates that Beclin1 is essential for cardiac function, with its role in autophagy alone not being able to account for the rapid cardiac dysfunction observed upon Beclin1 deletion in myocytes. The loss of contractility and increased mortality in mice without cardiac Beclin1 underline for the first time its importance in myocardial homeostasis. Beclin1 has previously been thought to regulate the endosomal pathway with UVRAG, and here our data demonstrate that Beclin1 is required for UVRAG protein stability. Overexpression of UVRAG was sufficient to prevent

the rapid cardiac dysfunction observed upon deletion of *Beclin1*. Given our previous data, this raises an interesting mechanistic question: which pathways are most affected by loss of Beclin1/UVRAG that contribute to loss of myocytes and development of heart failure? Are there specific plasma membrane proteins that are dependent on Beclin1 for trafficking to the plasma membrane? Previous work has identified Ca2+ ion channel receptors as important for heart function (Curran et al., 2015). Future work should investigate the importance of various receptor pathways in the heart, including β_1 adrenergic receptors given their role in regulating cardiac contractility, as well as muscarinic receptors such as M_2 given their impact on overall cardiac output (Gordan et al., 2015). If such pathways could be identified, we would have a better understanding on the mechanistic role of Beclin1 in mediating endosomal trafficking.

Although the work I have presented here is novel and impactful, there are some limitations to these investigations. One concern is that while cell lines are a useful model to study signaling, they often do not translate into animal models, let alone human models for eventual development of therapeutics. To alleviate this concern, we have focused on the results from the Beclin1 cKO animal model, which shows a defect in endosomal activity in concordance with the cell data. Another concern is that the cardiac impairment observed in the Beclin1 cKO mice could be related to cre toxicity or tamoxifen toxicity (Lexow et al., 2013). To address this, we treated MerCreMer only mice with tamoxifen and observed little cardiotoxicity (Figure 5.3). Additionally, we used the same Cre recombinase (MerCreMer) for our conditional knockout of Atg7 and found little to no evidence of reduced survival or toxicity in those mice (Figure 5.8). Finally, another limitation of this study is due to the rapid loss of cells and decline in cardiac function in

Beclin1 cKO mice, we are unable to fully examine downstream signaling pathways as pro-death pathways are activated. In the future, utilizing Beclin1 heterozygous knockouts in the heart may be useful to better investigate Beclin1's role in the heart.

6.3 Identification of Mitophagy Receptors as the primary mechanism of mitochondrial clearance in CPCs

Cellular quality control pathways have long been studied for their role in a variety of diseases, with an increased research focus over the past 10 years (Ohsumi, 2014). Notably, the importance of autophagy was recognized with the nobel prize in medicine in 2016 to Dr. Yoshinori Ohsumi for his discovery of the mechanisms of autophagy. This is due in part to the multifaceted role that autophagy plays in a variety of diseases, from different forms of cardiovascular disease to neurodegenerative diseases such as Alzheimer's. Even in stem cell therapy, activation of autophagy has been shown to be important during differentiation of stem cells into mature cells (Ho et al., 2017). Additionally, the removal of immature mitochondria during differentiation by mitophagy has been shown to be required for stem cell differentiation and the establishment of a new mitochondrial network (Sin et al., 2016).

Previously, mitophagy was primarily thought to occur through the PINK1-Parkin pathway, which utilizes the E3 ubiquitin ligase Parkin to mark mitochondria for degradation (Narendra et al., 2008). In my dissertation research, I have discovered a different mechanism of mitophagy during cardiac progenitor cell (CPC) differentiation. My data demonstrate that PINK1-Parkin mitophagy is dispensable upon activation of differentiation in CPCs, which instead utilize mitophagy receptors BNIP3L and FUNDC1

to remove immature mitochondria. Mitophagy receptors are able to bind directly to the autophagosome to mediate mitochondrial clearance and are present both on the inner and outer mitochondrial membranes. Interestingly, knockdown of either BNIP3L or FUNDC1 led to a compensatory increase in the other, suggesting the presence of compensatory pathways in CPC differentiation. Surprisingly, deficiencies in mitophagy did not affect CPC differentiation or lineage commitment but did affect its ability to form a functioning, interconnected mitochondrial network. Additionally, mitophagy deficient CPCs were more susceptible to stress and had reduced retention after injection *in vivo*. Together, this data demonstrates a novel mechanism of mitophagy in the context of stem cells and creates new therapeutic targets for better design and retention in stem cell therapy.

In Chapter 5, the data presented show that mitophagy is primarily regulated by OMM mitophagy receptors BNIP3L and FUNDC1 in CPCs. Most studies have focused on the role of PINK1-Parkin mediated mitophagy, but we found that Parkin was dispensable and did not play a role in mitophagy during differentiation in CPCs. Very little is currently understood about the regulation of mitophagy receptors, especially on the transcriptional level. It may be that different signals in the cell activate transcription factors to increase mitophagy receptor levels at the OMM when needed, such as during differentiation. BNIP3L specifically has previously been shown to play a role in the differentiation of retinal ganglion cells (Esteban-Martinez et al., 2017), while FUNDC1 has not been shown to be important in any other type of stem cell. Mechanistically, I propose that BNIP3L/FUNDC1 mediated mitophagy is a programmed step during differentiation for the maturation of the stem cell.

Another important finding demonstrated in Chapter 5 showed that mitophagy was essential for the formation of an interconnected mitochondrial network. If mitophagy was impaired, mitochondrial fission was uninterrupted and the mitochondria took on a more circular, donut-like morphology. The exact components of these mitochondria require further investigation, but previous identification of donut-like mitochondria by another group found that they were electron dense with cytosolic material (Ding et al., 2012). Mitochondrial morphology is a tightly regulated process that requires a balance of mitochondrial fusion and fission. Too much fission will lead to a depleted mitochondrial network, while too little creates an abundance of inefficient damaged mitochondria (Serasinghe and Chipuk, 2017). On a similar note, mitochondrial fusion also requires balance, as deletion of key mitochondrial fusion proteins Mfn1/2 was shown to impair differentiation of embryonic stem cells into cardiomyocytes (Kasahara et al., 2013). Our study shows that CPCs with abrogated mitophagy have sustained mitochondrial fission through activation of DNM1L. While progenitor cells rely primarily on glycolysis for energy, it is possible that during differentiation, mitophagy must be activated to remove any immature or fetal mitochondria that remain in order to develop a mature network. Finally, we demonstrate the importance of mitophagy in maintaining CPC retention and viability both *in vitro* and *in vivo*. One of the major issues that stem cell therapy has had is cell retention; stem cells simply aren't surviving long enough to distribute their beneficial effects, whether that be through its release of paracrine factors or by differentiation into mature cardiomyocytes. Our data suggest that increasing mitophagy could help stem cells last longer in the hypoxic environment of an injured heart.

Lastly, we also showed in Chapter 5 how the accumulation of mtDNA mutations, which increase with age, can also affect mitophagy in CPCs. POLG CPCs were unable to effectively activate mitophagy after differentiation, which could help explain why they were so susceptible to cell death in previous reports (Orogo et al., 2015). A different study has reported that POLG mutant mice had a defect in mitophagy during erythrocyte maturation (Li-Harms et al., 2015). However, they reported that this defect was due to excessive mTOR activation (mechanistic target of rapamycin kinase) and reduced autophagy, but they did not examine BNIP3L or FUNDC1 levels. This difference could be due to the fact that erythrocytes have a different mechanism to regulate mitophagy compared to CPCs, or it could be due to the different time point used in the study (2 versus 4 months). Either way, it would be interesting to research in the future the prevalence of mitophagy-receptor mediated mitophagy in different cell types.

One limitation on the study presented here is the limited *in vivo* work involving CPC retention and viability. While we investigated the role of mitophagy after injection into the mouse myocardium, it would have been interesting to look at the long-term effects of CPCs on the recovery of mice under different stressors such as myocardial infarction after being supplemented with mitophagy functional CPCs. Despite this limitation, we did test the CPCs *in vitro* under a variety of different physiological stressors, including damage by reactive oxygen species, which are a problem in a variety of CVD contexts. Additionally, our work demonstrating the effect of mitophagy on the beneficial aspects of CPCs, including its protective paracrine effects through the endothelial tube formation assay, should translate well into designing any therapeutics for future *in vivo* studies.

6.4 Model

My findings demonstrate a new role for Beclin1 in the endosomal pathway, and I propose a model of intracellular trafficking of receptors and its importance in maintaining myocyte function (Figure 6.1). In this model, loss of Beclin1 leads to an accumulation of certain plasma membrane receptors in Rab5-positive early endosome after their biosynthesis due to impaired trafficking to the plasma membrane. Receptors are translated in ribosomes at the ER, where they are trafficked to the Golgi apparatus for further processing. From the Golgi, receptors are placed in vesicles which normally facilitate them to the plasma membrane for signaling. In the absence of Beclin1, Rab5 activity is altered, and receptors accumulate in Rab5-positive vesicles in the cytosol (Figure 6.2). This mislocalization of receptors has significant consequences for cell signaling and viability, and when coupled with simultaneous impairment of autophagy, leads to negative effects on the hearts ability to function properly. This work demonstrates that Beclin1 is essential for heart function and helps uncover a new mechanism in receptor trafficking in which Beclin1 contributes to maintaining cardiac homeostasis.

6.5 Relevance to Disease and Therapeutic Potential

While dysregulation of Beclin1 is known to play a role in a variety of diseases through its role in autophagy, only recently has its role as a regulator of the endosomal pathway been connected to different pathologies. Previously, Beclin1's role in the endosomal pathway was only connected to neurodegeneration. We are the first to link Beclin1 as a regulator of endosomal trafficking as essential for cardiac health and

survival. Now that we understand more about the importance of Beclin1 in receptor trafficking, this pathway can be studied in a variety of disease states, including heart failure, cancer and Alzheimer's disease.

It is possible that given Beclin1's formation of unique protein complexes, it will be possible to target its binding to specific effector proteins in order to combat different diseases. For example, targeting of the coiled-coil domain that enhances binding of Beclin1-UVRAG may be useful in disease contexts that are contingent on aberrant receptor signaling, such as the case in many non-small cell lung carcinomas with EGFR. In this disease state, it could be beneficial to enhance endolysosomal degradation of EGFR by stabilizing the Beclin1-UVRAG interaction and thus reduce its signaling. Additionally, this may have indirect therapeutic benefits in different disease states due to its activation of autophagy, which is generally pro-survival. Careful consideration of the amount of activation will need to be considered, as too much recycling or trafficking of components could have negative consequences in the form of uncontrolled growth (from an excessive amount of growth receptors) or excessive degradation of essential proteins and organelles. Previous work involving global heterozygous deletion of Beclin1 has demonstrated how affecting Beclin1 levels can be useful in helping treat disease. During ischemia-reperfusion injury, Beclin1 mediated autophagy has been shown to be cardioprotective (Sala-Mercado et al., 2010). Additionally, recent studies have shown that Beclin1-mediated autophagy is protective in the heart during sepsis (Sun et al., 2018). Future studies should further examine how modulation of Beclin1, given its unique role as a regulator of multiple cellular quality control pathways, can help treat different diseases, including cardiovascular disease.

Stem cell therapies also represent an exciting therapeutic option for a variety of diseases (Garbern and Lee, 2013), especially in cardiovascular disease, where they can replace post-mitotic cells in cardiomyocytes. Our research has highlighted the importance of mitophagy during cardiac progenitor cell differentiation in the formation of an interconnected mitochondrial network. Stem cells have been shown to have therapeutic benefit, but stem cell retention in the failing heart has proved elusive. Our data suggests that expression of mitophagy is essential for stem cell protection against cell death from reactive oxygen species in the failing heart. Thus, it will be interesting to investigate in the future whether overexpression of mitophagy (either through BNIP3L and FUNDC1) would help augment stem cell therapy. A better understanding of the biology of stem cells will help refine these therapies until precision medicine for each patient is a reality.

6.6 Future Studies

The data presented here will have a significant impact on the fields of mitophagy, stem cell therapies, endosomal activity, and cardiac function. It highlights the impact that intracellular quality control pathways can have on cell viability and on disease. With these advances, new questions have followed that should be further investigated in future work.

My work demonstrates the importance of Beclin1 in the heart, specifically through its regulation of endosomal trafficking of plasma membrane receptors. Given its importance as a scaffolding protein and upstream regulator of autophagy, Beclin1 likely plays a significant role in a variety of disease states, including those of the heart. Exciting new work has uncovered interactions between immune sensing receptor TLR9 and Beclin1 to mediate cellular quality control during exercise (Liu et al., 2020). However,

despite these new discoveries, questions remain on the exact role of Beclin1. Although our data demonstrates a relationship between Beclin1 and early endosome protein Rab5, it is likely that Beclin1 plays a role in other trafficking pathways. Recycling endosomes are regulated by different Rab GTPases such as Rab4 and Rab11 (Lock and Stow, 2005; Mohrmann et al., 2002), and it is possible that Beclin1 plays a role in regulating their activity. Additionally, post-translational modifications (PTMs) play a significant role in helping to determine the end location of a target receptor in the cell. While many PTM's that regulate Beclin1 are known, they have primarily been studied for their effects on autophagy, such as ULK1 phosphorylation of Beclin1 Serine 15 (Menon and Dhamija, 2018). With the new discovery of the role of Beclin1 as a regulator of endosomal trafficking, future studies should focus on potential phosphorylation sites that regulate Beclin1 endosomal activity. Of note, these studies could include Beclin1 phosphorylation sites by EGFR, such as tyrosine 229 or 233, which have already been shown to have a negative effect on autophagy, but whose role in the endosomal pathway is unknown (Wei et al., 2013).

Another question that merits further investigation is whether Beclin1 coordinates exocytosis in cells. As some recent work has indicated, recycling endosomal marker Rab11 has been identified as a dual regulator of exocytosis at the plasma membrane (Takahashi et al., 2012). This opens up the possibility that some targets of endocytosis could be marked for exit from the cell if necessary. Research into the field of exosomes is growing by the year, and a potential role for Beclin1, given its membrane-binding capabilities in its evolutionarily conserved domain (Huang et al., 2012), warrants further investigation. Finally, our data suggest that Beclin1 is important for maintaining systolic

function and thus contractility of the heart. Further investigation is warranted to better understand the downstream effects of this pathway and how cell death is induced. It is likely due to a combination of trafficking defects, such as in ion channels or structural proteins such as integrins and connexins. Given the role of Beclin1 in a variety of pathways, the possibilities for future research are wide-ranging, and with the extensive array of Beclin1 research into other diseases beyond the heart such as Alzheimer's disease and cancer, it is likely that breakthroughs in one field will have some transfer into the other.

Additionally, I have unveiled the importance of mitophagy receptors BNIP3L and FUNDC1 in regulating CPC function. Additionally, I have found that mitophagy is essential not for lineage commitment of CPCs but for the formation of a functioning, interconnected mitochondrial network during differentiation. Despite this, I have not yet identified how the initiation of the differentiation program signals to induce mitophagy and how it determines which mitochondria to remove during the expansion of the mitochondrial network. My data suggests that DNM1L-mediated fission is involved in sequestering inefficient mitochondria during differentiation, but this needs to be further investigated. Additionally, why does reduced mitophagy lead to failure to form an interconnected mitochondrial network during differentiation? Is this something that could be overcome by overexpression of mitochondrial fusion proteins, or does the formation of the mitochondrial network need to occur early on in differentiation? It is interesting to note that deficient mitophagy during differentiation led to the formation of spherical, donut-shaped mitochondria. The molecular components of these mitochondria, including their membrane potential and respiration capacity, would be interesting to further explore.

Altogether, my work dives into the molecular mechanisms behind mitophagy during CPC differentiation that had previously been unexplored, and future work should also focus on new pathways that have yet to be discovered to truly unlock the potential of stem cells in regenerative medicine.

6.7 Concluding Remarks

Intracellular quality control pathways are essential for cellular health, and dysregulation of these pathways contribute to a variety of diseases. In addition, stem cell therapies represent a promising avenue for cardiac regeneration (Broughton and Sussman, 2016; Zakrzewski et al., 2019), and enhancing mitophagy is one way to help augment these myocytes against cell death in the unfavorable environment of the diseased heart. Dysfunctional mitochondria are known to contribute to heart failure (Rosca and Hoppel, 2013). While mitophagy is well-known to remove dysfunctional mitochondria, recent work has shown the endosomal pathway is also able to do so (Hammerling et al., 2017). Our work highlights the role(s) of Beclin1 as an upstream regulator of both autophagy and the endosomal pathway and for its importance in preventing cardiac dysfunction. We uncover a novel mechanistic role for Beclin1 as a regulator of endosomal trafficking that will aid in the design of therapeutics to treat a wide range of diseases.

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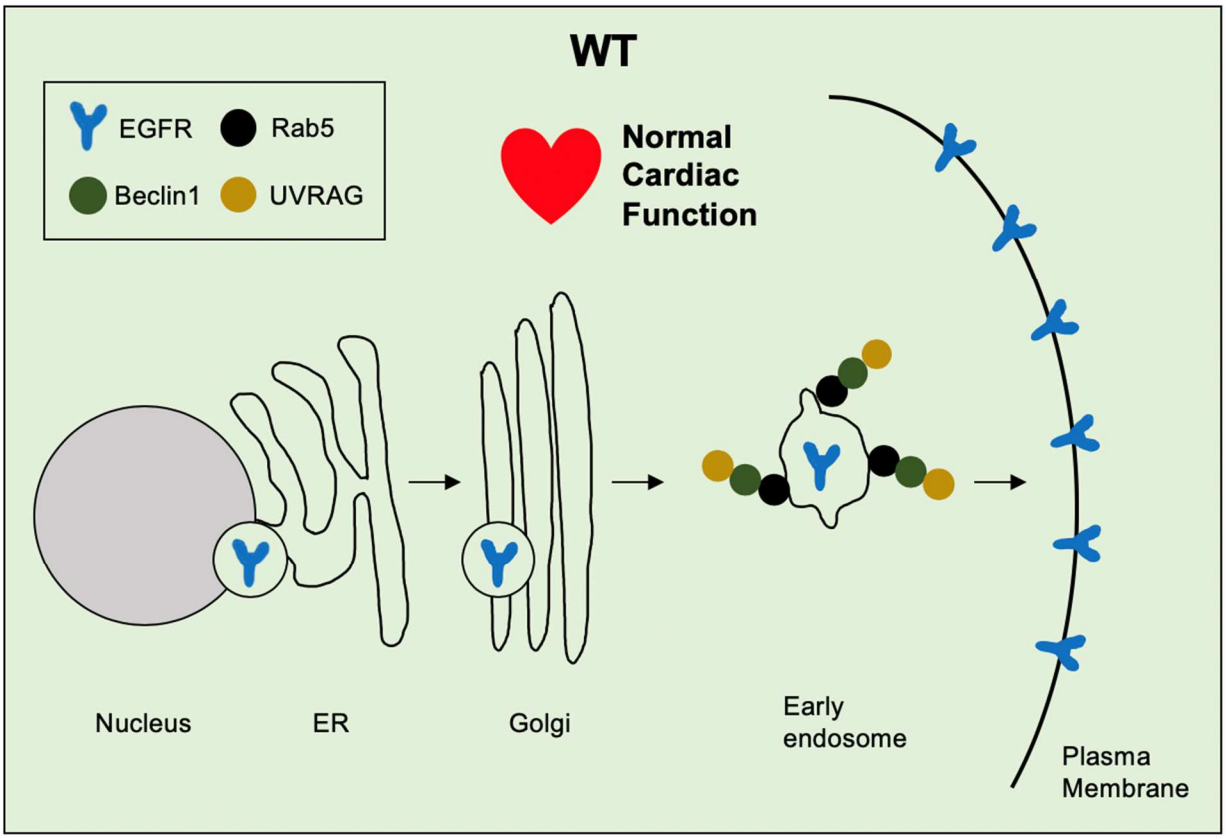

Figure 6.1. Model of Beclin1 mediated biosynthetic endosomal trafficking (WT). Biosynthesis of receptors is facilitated by translation at the ER, where proteins are folded and subsequently trafficked to the Golgi apparatus for further processing. After modification at the Golgi, receptors are sorted at early endosomes to be brought to the plasma membrane for downstream signaling.

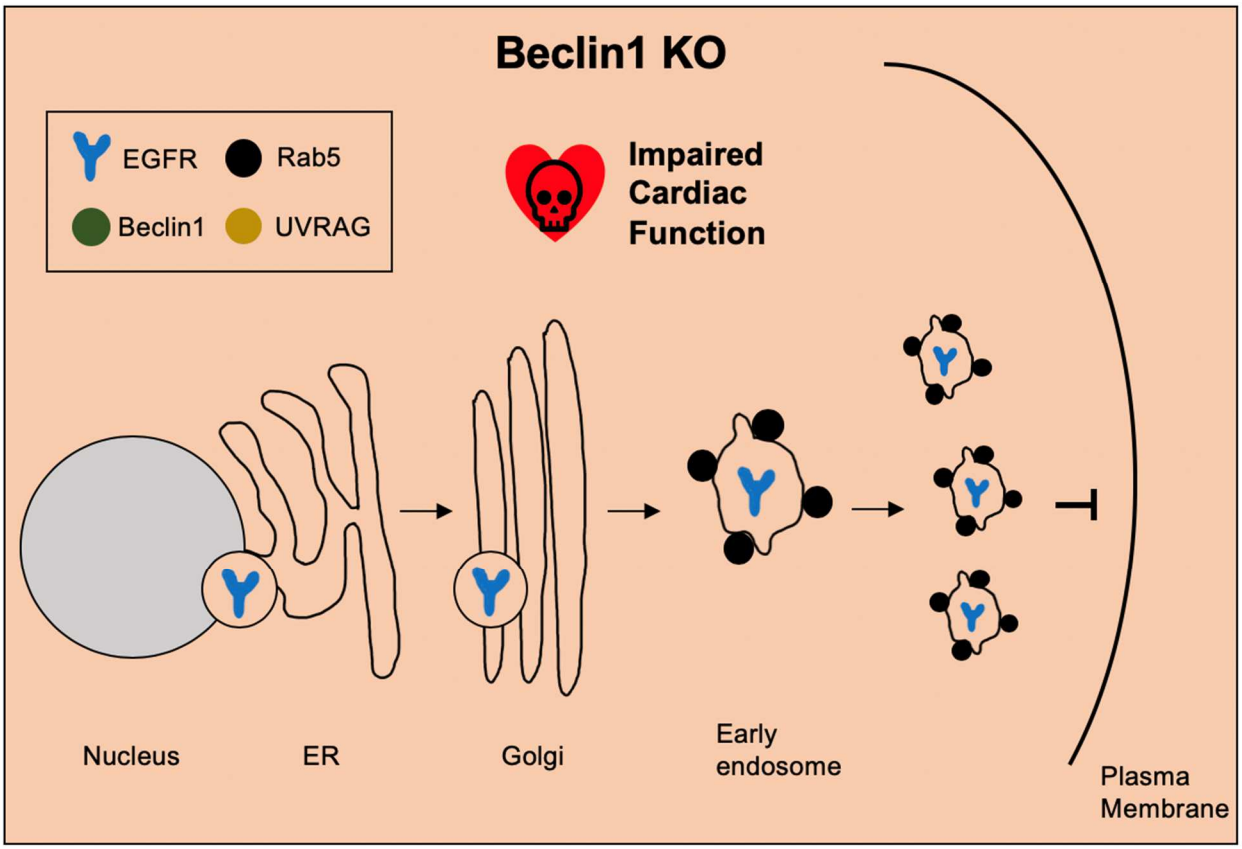

Figure 6.2. Model of Beclin1 mediated biosynthetic endosomal trafficking (KO). Biosynthesis of receptors is facilitated by translation at the ER, where proteins are folded and subsequently trafficked to the Golgi apparatus for further processing. After modification at the Golgi, receptors are sorted at early endosomes. Without Beclin1, receptors are unable to be trafficked to the plasma membrane for downstream signaling, resulting in cell death and reduced survival.

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