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NOVEL INSIGHT INTO THE AUTOPHAGY- INDEPENDENT FUNCTIONS OF BECLIN 1 IN TUMOR GROWTH

A Dissertation Presented

By

ASIA NAOMI MATTHEW-ONABANJO

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

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DOCTOR OF PHILOSOPHY

June 27, 2019

CANCER BIOLOGY

DEDICATION

John 14: 2-3 My Father's house has many rooms; if that were not so, would I have told you that I am going there to prepare a place for you? And if I go and prepare a place for you, I will come back and take you to be with me that you also may be where I am.

This work is dedicated to my family that passed away during the course of my PhD. To my angels Trenaya L. Johnson and Kearns Louis-Jean, thank you for providing me a reason to keep working. Boo Boo, Nay Nay, watching your battle with cancer brought my work into a surreal context. It provided a new meaning to my work and more reasons to remain passionate about science. During those troubling hours, I often thought of your strength to keep me going. To my Kearns, I love you little brother. I will never forget your support of my educational goals. Thank you for giving me a reason to "let my hair down" and remember to keep the fun in science. Here's to continuing to reach new educational heights!

Until we meet again...

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Nelson Mandela once said “It always seems impossible until it’s done”. Thank you to my family, friends, and mentors who help to make my “impossible dream” a reality. I may not be able to list everyone I need to give thanks to but please charge it to my head and not my heart. It is with my humblest gratitude that I give you all thanks.

Jeremiah 29: 11 “For I know the plans I have for you”, declares the Lord, “plans to prosper you and not harm you, plans to give you hope and a future.”

ABSTRACT

Beclin 1 is a haploinsufficient tumor suppressor gene that is monoallelically deleted or epigenetically silenced in multiple human tumor types. In human breast cancer, 40% of tumors exhibit monoallelic deletion of Beclin 1. Additionally, low Beclin 1 mRNA expression is more commonly observed in ER negative (ER-) tumors (HER2 and basal-like subtypes) than ER+ luminal tumors and reduced expression is an independent predictor of overall patient survival. Previous studies have implicated a role for Beclin 1 in breast cancer progression. For example, heterozygous loss of Beclin 1 in mice results in mammary tumorigenesis following parity. Furthermore, overexpression of Beclin 1 in an orthotopic xenograft model reduces tumor growth. The role of Beclin 1 in cancer has almost exclusively been attributed to its function in autophagy. However, recent work from our lab demonstrated an alternative role for Beclin 1 in the regulation of growth factor receptor trafficking and signaling *in vitro* that could contribute to cancer. More knowledge of the role of Beclin 1 in breast cancer is necessary to understand its mechanism of action and to develop novel therapeutic approaches for patients with aggressive disease. Therefore, the major objective of my thesis project was to understand the molecular basis by which Beclin 1 contributes to breast cancer tumor growth and progression *in vivo*.

Using *in vivo* models, I discovered that Beclin 1 promotes endosomal recruitment of hepatocyte growth factor tyrosine kinase substrate (HRS), which is necessary

for sorting receptors to intraluminal vesicles for signal silencing and degradation. Beclin 1-dependent recruitment of HRS results in the autophagy-independent regulation of endocytic trafficking and degradation of the epidermal growth factor (EGFR) and transferrin (TFR1) receptors. When Beclin 1 expression is low, endosomal HRS recruitment is reduced and receptor function is sustained to drive tumor proliferation. An autophagy-independent role for Beclin 1 in regulating tumor metabolism was also observed. Collectively, my results demonstrate a novel role for Beclin 1 in impeding tumor growth by coordinating the regulation of growth promoting receptors. These data provide an explanation for how low levels of Beclin 1 facilitate tumor proliferation and contribute to poor cancer outcomes, independently of autophagy.

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Figure 1.2: Adapted from Hansen,M., Rubinsztein, DC and Walker,D, W., Nat Rev Mol Cell Biol. 2018 July 19 (579-593) The Macroautophagy Process. Copyright 2018, with permission from Springer Nature Publishing Group (License Number: 4593790804499).

Figure 1.3: Adapted from Williams R.L. and Urbé S., Nat Rev Mol Cell Biol. 2007 May; 8(5):355-68). The ESCRT Machinery involved in Sorting Cargos to the Lysosome via MVB Formation Copyright 2007, with permission from Springer Nature Publishing Group (License Number: 459379004)

LIST OF ABBREVIATION

BECN1	Beclin 1
BARKOR	Beclin-1 associated autophagy related key regulator
BRCA1	BReast CAncer gene 1
CavME	Caveolae-mediated endocytosis
CIE	Clathrin Independent Endocytosis
CME	Clathrin-mediated Endocytosis
CCD	Coiled-coil domain
FYVE	Conserved in Fab 1, YOTB, Vac 1, EEA1
EE	Early Endosome
ER	Endoplasmic reticulum
ESRCT	Endosomal-Sorting Complex Required for Transport
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ECD	Evolutionary Conserved Domain
GAPR-1	Golgi associated plant pathogenesis-related 1
	Hepatocyte Growth Factor-regulated Tyrosine Kinase
HRS	Substrate
HER2	Human Epidermal Growth Factor 2
iBMKs	Immortalized Baby Mouse Kidney Cells

IGF1	Insulin Like Growth Factor 1
IGF2	Insulin Like Growth Factor 2
IGF1-R	Insulin Like Growth Factor Receptor 1
IR	Insulin Receptor
ILVs	Intraluminal Vesicles
LE	Late Endosome
PI3KC3	Lipid Kinase, Class III Phosphatidylinositol-3 kinase
mTORC1	Mechanistic Target of Rapamycin Complex I
LC3	Microtubule Associated protein 1 light chain 3 (MAP1LC3)
MVB	Multivesicular Body
NGF	Nerve Growth Factor
NES	Nuclear Export Signal
PE	Phosphatidylethanolamine
PI3P	Phosphatidylinositol 3-phosphate
PR	Progesterone Receptor
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RE	Recycling Endosome
AKT	Serine/threonine kinase or Protein Kinase B
STAM	Signal Transducing Adaptor Molecule
TCGA	The Cancer Genome Atlas
TF	Transferrin

TFR1	Transferrin Receptor
TSG101	Tumor susceptibility gene 101
UEV	Ubiquitin E2 variant
UIM	Ubiquitin Interacting Motif
UBL	Ubiquitin Like
ULK1	Unc-51-like kinase 1
ULK2	Unc-51-like kinase 2
UVRAG	UV irradiation resistance-associated gene
VPS	Vacuolar Protein Sorting

CHAPTER I

Introduction

Beclin 1 and its Functional Complexes

Bcl-2-interacting myosin-like coiled-coil protein (Beclin 1) is a 60 KD protein that is the mammalian orthologue to yeast Atg6/Vps30 that was first identified through a yeast two-hybrid screen for its role in viral protection¹. The Beclin 1 protein consists of 450 amino acids that form multiple domains. These domains include a BCL-2 binding domain, coiled-coil domain (CCD), evolutionary conserved domain (ECD) and a nuclear export signal (NES) (Figure 1.1A). Each domain is important for mediating interactions between Beclin 1 and multiple interacting partners. The CCD and ECD are important for interacting with binding partners that mediate Beclin 1's biological functions. The NES motif of Beclin 1 promotes the export of Beclin 1 from the nucleus, thereby promoting its cytosolic localization².

Core Complex

Beclin 1 functions in two main complexes that are mutually exclusive but contain the same core complex (Figure 1.1B)³⁻⁵. This core complex consists of Beclin 1, the lipid kinase class III phosphatidylinositol-3 kinase PI3KC3 (mammalian homologue to yeast Vps34), and the regulatory element p150 (mammalian homologue to yeast Vps15)⁶. PI3KC3 interacts with and is activated by Beclin 1 through its evolutionary conserved domain (ECD)⁷. PI3KC3 is member of a kinase family that phosphorylates inositols to generate 3-phosphoinositides⁸.

Beclin 1 binds and activates the kinase activity of PI3KC3 to generate phosphatidylinositol 3-phosphate (PI3P), a lipid product that facilitates multiple membrane fusion events^{8,9}. ATG14 and UV irradiation resistance-associated gene (UVRAG) interact with this core complex by binding the coiled-coil domain (CCD) of Beclin 1. These two proteins bind the same domain of Beclin 1 but never at the same time; therefore they form mutually exclusive complexes and these complexes mediate the distinct functions of Beclin 1 (Figure 1.1B).

Complex I-ATG14

In Complex I, Beclin 1 interacts with ATG14L (mammalian homologue to yeast Atg14) by heterodimerizing with its CCD. ATG14L binding to Beclin 1 has been shown to initiate autophagy, one of Beclin 1's well known functions. Originally discovered in yeast, Atg14 was found to be indispensable for autophagy in yeast strains¹⁰. ATG14L also helps localize this core complex to the endoplasmic reticulum and phagophore¹¹. Additionally, ATG14L is important for targeting this core complex to membrane curvatures because it contains an intrinsic domain which allows it to sense PI3P rich membranes¹². Because of the known role of ATG14L in autophagy, ATG14L is also known as Beclin-1 associated autophagy related key regulator or BARKOR. Recent studies show that Dapper1, a protein that helps target Dishevelled to lysosomes to inhibit WNT signaling, is important for regulating the ATG14L interaction with Beclin 1 and PI3KC3 to promote autophagy¹³. Overexpression of Dapper 1 in HEK293T cells was shown to

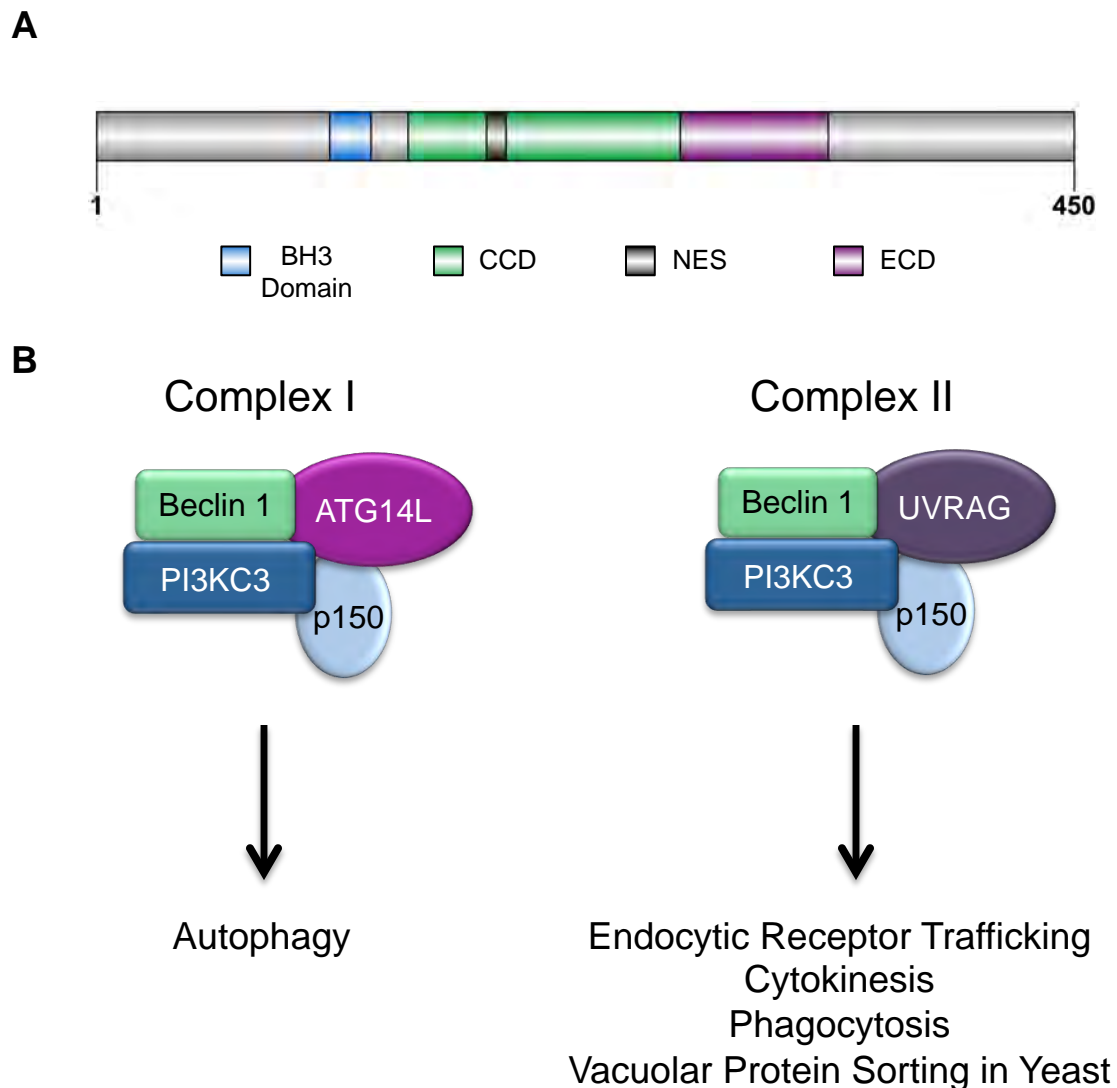


Figure 1.1. Schematic of Beclin 1 and its Complexes. (A) Beclin 1 is a 450 kd protein that consist of multiple domains which mediate its function. Beclin 1 contained a BH3 domain, a coiled-coil domain (CCD) which interacts with ATG14L or UVRAG, an evolutionary conserved domain (ECD) which interacts with PI3KC3 and a nuclear export sequence. Protein schematic was generated with DOG 1.0 software (*Cell Research* (2009) 19: 271-273) (B) Beclin 1 functions in two mutually exclusive complexes with the binding partners ATG14L and UVRAG. These two independent complexes mediate Beclin 1's functions. Figure adapted from Wirawan et al⁵⁵.

increase the interaction of ATG14L, PI3KC3 and Beclin 1 and this interaction was also confirmed through a yeast two-hybrid assay¹³. On the other hand, knockout of *Dpr1*, the mouse gene of Dapper 1, in mouse embryonic fibroblasts reduced the interaction of *Atg14*, *Vps34*, and *Beclin 1*¹³.

Complex II-UVRAG

In Complex II, Beclin 1 interacts with UVRAG (mammalian homology to yeast Vps38) via interaction with the CCD. This interaction is thought to mediate the autophagy-independent functions of Beclin 1. Initially it was thought that UVRAG was important for autophagosome formation, but other groups have shown that UVRAG's homologue did not function in the autophagic process in yeast. For example, loss of *Atg14* in yeast results in decreased autophagosome formation which does not occur with loss of *Vps38*¹⁴. In fact, loss of *Vps38* in yeast led to dysfunctional vacuolar protein sorting due to missorting of the Carboxypeptidase Y, a hydrolase that is known to be trafficked from the endosome to the vacuole in yeast¹⁵. This same finding was also confirmed in recent studies in Arabidopsis plant species as loss of *Vps38* resulted in impaired vacuolar protein sorting but did not interrupt the autophagic process¹⁶. In mammalian cells UVRAG mediates endosome-endosome and endosome-lysosome fusion via its interaction with Class C Vps complex, a major regulator of endosomal fusion¹⁷. These studies suggest that the Complex II interaction of UVRAG and Beclin 1 is important for mediating Beclin 1's function in endosomal trafficking.

Functions of Beclin 1: Autophagy Dependent vs Autophagy Independent

Autophagy-Dependent

Beclin 1 is well known for its role in the initiation of macroautophagy, hereafter referred to as autophagy. Autophagy, which translates to “self eating” in Greek, is a conserved homeostatic process that cells use to recycle or degrade different cargos into macromolecules (carbohydrates, lipids, amino acids) that can be used for energy supply. Degradation of these cargos occurs via the lysosome. Cells utilize this process during times of nutrient stress or when macromolecules become limited to promote survival. Additionally, cells use autophagy during the immune response to get rid of intracellular pathogens¹⁸. The autophagic process can be selective or nonselective; nonselective autophagy usually involves the degradation of bulk cargos whereas selective autophagy usually involves the degradation of damaged protein and organelles.

There are three well characterized types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy¹⁹⁻²¹. In microautophagy, the lysosomal membrane invaginates to capture cytosolic material which is then degraded¹⁹. In Chaperone-mediated autophagy, proteins are directly taken up into the lysosome via the LAMP-2A transmembrane protein that is located on the lysosome. A chaperone protein mediates this process (i.e. Heat Shock 70, Hsc70) as well as co-chaperones that recognize a specific sequence on substrates²². This targets cytosolic substrates to the lysosome for degradation

and they are translocated into the lysosome via LAMP2. During macroautophagy, an isolation membrane (or phagophore) captures some amount of cytoplasm. The isolation membrane elongates and eventually encloses to form an autophagosome, a double-layered membrane vesicle. This autophagosome can then fuse with a lysosome (i.e. autolysosome) to digest its contents including the inner membrane. Additionally, autophagosomes can fuse with other cellular components such as an endosome (known as an amphisome) before fusing with a lysosome. Macroautophagy is the most common type of autophagy and is regulated by Beclin 1. Therefore the machinery involved in this process will be described in greater detail in this thesis.

Phases of Autophagy

There are 3 main phases of autophagy that occur: Initiation/nucleation of the phagophore, expansion/elongation of the phagophore and autophagosome fusion/recycling/degradation of autophagosome contents (Figure 1.2).

- I. Initiation/nucleation of the phagophore:
 - a. Autophagy is under tonic inhibition through the mechanistic target of rapamycin complex I (mTORC1). During the fed state, mTORC1 is bound and phosphorylates a complex of Unc-51-like kinase family (ULK1 or ULK2), ATG13, and RB1-inducible coiled-coil 1 which are required for autophagy induction²³⁻²⁵. During starvation,

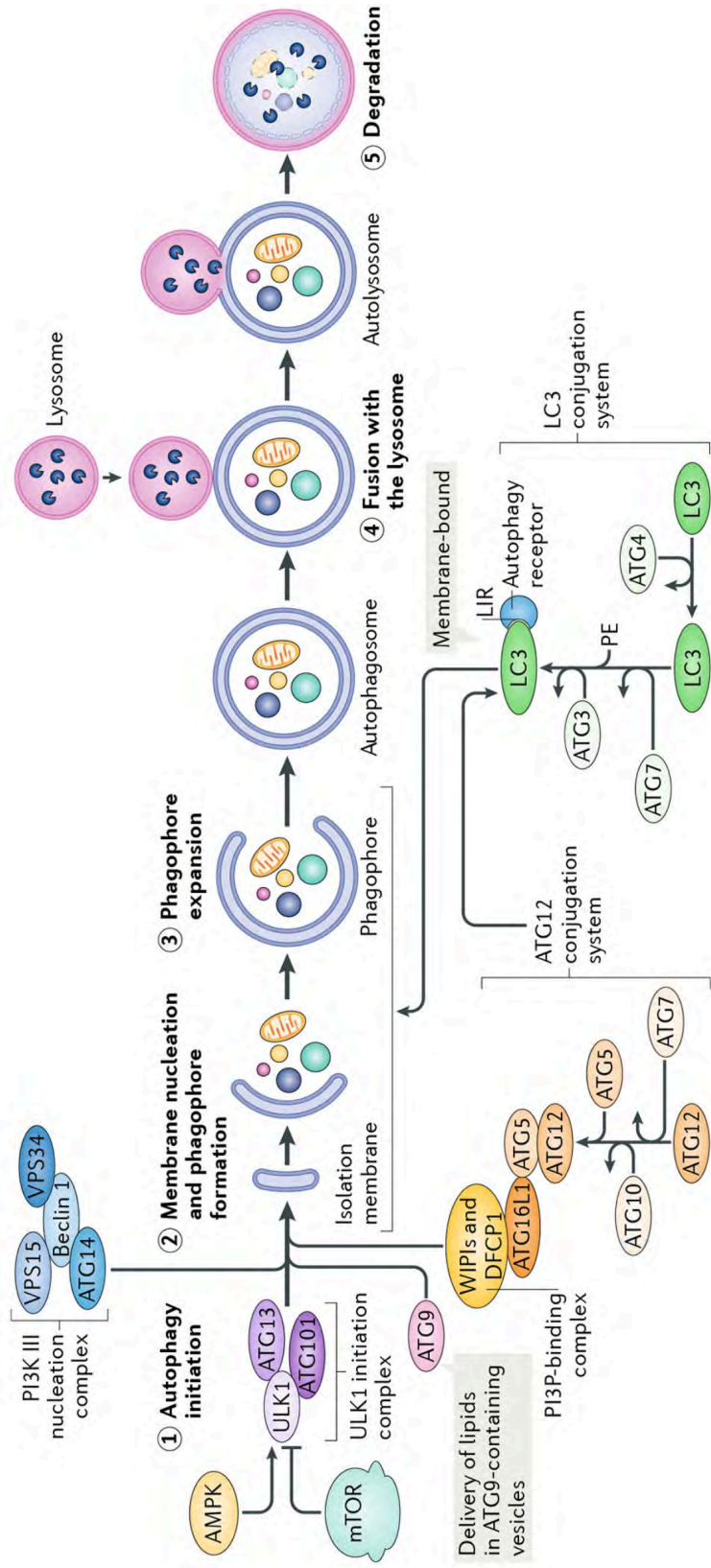


Figure 1.2. Schematic of the Macroautophagy pathway. Autophagy is induced by starvation or metabolic stress. Upon induction, the inhibitory stimulus of mTORC1 on ULK1 is released. The released inhibition of ULK-1 with the activation Beclin 1-VPS34-ATG14 axes allows for autophagy to be induced. An isolation membrane encloses around cytoplasmic cargo and elongates through the ATG8/LC3 and ATG12 E3-like ligase systems to generate an autophagosome. The autophagosome fuses with a lysosome to degrade the inner contents which can be used as building blocks for cellular processes. Adapted from Hansen et al³².

mTORC1 is no longer active and the ULK1/ATG13/RB1 complex is able to initiate the autophagic process²⁶.

- b. Following induction, the Beclin 1-VPS34-ATG14L complex is recruited and nucleation of the isolation membrane or phagophore occurs³. Removal of tonic inhibition stimulus of mTORC1 results in ULK1 phosphorylation of Beclin 1²⁷. This results in Beclin 1 activation of PI3KC3 to generate PI3P, which is necessary for phagophore formation. Many studies suggest that the mammalian isolation membranes arise from endoplasmic reticulum (ER) or other ER associated organelles²⁸.

II. Expansion/elongation of the phagophore:

- a. Elongation of the phagophore is mediated by two ubiquitin like (UBL) conjugation systems. The first system involves the multiprotein complex of ATG12, ATG5, and ATG16. In this conjugation system ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme) conjugates ATG12 to ATG5 in an irreversible manner²⁹. Following conjugation, the ATG12/ATG5 complex then binds to ATG16 to create a multiprotein complex³⁰. In the second conjugation system, ATG8/LC3 is processed by cysteine protease ATG4 to generate LC3I³¹. LC3-I is then lipidated with phosphatidylethanolamine (PE) in a series of steps involving ATG7 and ATG3 to generate LC3II³⁰. This series of lipidation steps help to

elongate the phagophore and LC3II is found on both the inner and outer layer of the mature autophagosome.

III. Autophagosome fusion and degradation/recycling of autophagosome contents:

- a. After expansion, the phagophore elongates and fuses to form an autophagosome (double membrane vesicle). The autophagosome can then be trafficked to a lysosome where it fuses and degrades its inner contents. The trafficking of the autophagosome to lysosomes is dependent on microtubules as microtubule disrupting agents have been shown to prevent fusion of autophagosome and lysosomes³². Additional studies suggest that SNARE proteins mediate fusion of autophagosomes with lysosomes³³. Following fusion with lysosomes, autolysosome contents are digested and materials can be used as building blocks for cellular processes.

Autophagy can be either tumor suppressive or tumor promoting. The process of autophagy can remove damaged organelles and protein aggregates that can cause elevated ROS, which can lead to genomic instability. Therefore, autophagy acts in a tumor suppressive manner by actively surveying for these damaged cargos. For example, immortalized mouse mammary epithelial cells that are heterozygous for *beclin 1*, showed impaired autophagy and increased genomic instability as evidenced by increase phosphorylation of DNA damage

protein γ -H2AX that indicates double stranded DNA breaks³⁴. This increase in phosphorylation of γ -H2AX during metabolic stress was also observed in immortalized baby mouse kidneys that were heterozygous for *beclin 1*³⁵. Damaged or misfolded proteins are directed to the autophagosome through several autophagy receptors such as p62, a substrate for autophagy that binds protein aggregates. Increased susceptibility to metabolic stress was observed in autophagy defective immortalized baby mouse kidney epithelial cells (*atg5* ^{-/-} or *beclin 1* ^{+/-}) as they exhibited accumulation of p62, damaged mitochondria, and increased endoplasmic reticulum chaperone proteins³⁶. This stress was also associated with elevated ROS and chromosomal instability³⁶. Additionally, p62 overexpression resulted in increased tumorigenesis in autophagy defective cells and tumors exhibited increased ROS and DNA damage³⁶. Taken together, these results indicate that autophagy can suppress ROS production to prevent DNA damage, genomic instability and tumorigenesis.

Another line of evidence that autophagy can act in a tumor suppressive manner is the tumorigenesis exhibited by deletion of autophagy specific genes in mice. For example, systemic mosaic knockout of *Atg5* in mice renders mice susceptible to liver adenomas, or benign liver growths³⁷. This same phenotype is also observed in mice with liver-specific knockout of *Atg7*³⁷. Additionally, frameshift mutations in other autophagy genes such as *ATG2B*, *ATG5*, *ATG9B* and *ATG12*

are observed in human gastric and colorectal tumors with microsatellite instability³⁸. These results indicate a tumor suppressive role for autophagy.

Interestingly, liver tumors observed in *Atg5* systemic mosaic knockout or *Atg7* liver-specific knockout mice never progress to hepatocellular carcinoma suggesting that autophagy may be necessary for tumor progression³⁷. Autophagy can also behave in a tumor-promoting manner. This is often observed in ischemic areas of tumors where there is increased nutrient stress. By activating autophagy, tumor cells can acquire macronutrients to promote survival in a stressful tumor microenvironment. For example, a study done by Eileen White showed that under ischemic conditions, a reduction of *Beclin 1* and *Atg5* expression resulted in reduced viability of immortalized baby mouse kidney cells (iBMKs)³⁹. Autophagy has also been shown to be tumor promoting in p53 dependent tumors. In an *in vivo* model for pancreatic tumors, knockout of *Atg7* or *Atg5* prevented tumor progression and resulted in premalignant pancreatic lesions that did not progress to pancreatic ductal adenocarcinoma⁴⁰. Other studies in pancreatic ductal adenocarcinoma show that pancreatic cancer cells are dependent on autophagy and are sensitive to autophagy inhibition by knockdown of autophagy genes or chemical modulation⁴¹. These studies suggest a role for autophagy that is tumor promoting as knockdown or knockout of autophagy genes prevent survival in ischemic conditions or prevents malignant tumor formation in genetic mouse models.

Autophagy-Independent Functions of Beclin 1

While the majority of studies investigating Beclin 1 have focused on its role in autophagy, there is considerable evidence to support that Beclin 1 has essential autophagy-independent functions and it is likely that these functions impact the role of Beclin 1 as a tumor suppressor. For example, homozygous deletion of *Becn1* in mice results in embryonic lethality due to failed cavitation of the blastocyst⁴². In contrast, deletion of other essential autophagy genes such as *Atg5* or *Atg7* in mice results in healthy-appearing pups that succumb to death in the perinatal period (1 day following delivery)^{43,44}. This difference in knockout phenotype supports that Beclin 1 has other essential autophagy-independent roles. An additional line of evidence that Beclin 1 has autophagy-independent functions is apparent when comparing tumor formation in heterozygous *Becn1* mice compared to mice deficient in *Atg5* or *Atg7*. *Becn1*^{+/-} mice develop spontaneous lung and liver adenocarcinomas, as well as lymphomas^{42,45}. As stated previously, mice deficient in *Atg5* or *Atg7* do not develop malignant tumors but form benign hepatic adenomas (non-malignant liver growth)³⁷. This difference in tumorigenesis between these essential autophagy genes provides support that the autophagy-independent functions of Beclin 1 contribute to the development and maintenance of malignant tumors.

Beclin 1 mediates its autophagy-independent functions through Complex II with the binding partner UVRAG. Reported functions include cytokinesis, endocytosis/endocytic receptor degradation, phagocytosis, and vacuolar protein sorting in yeast⁴⁶. Complex II was shown to play a role in cytokinesis by the Stenmark group. Initially they discovered that PI3KC3 and Beclin 1 contributed to cytokinesis as loss of PI3KC3 and Beclin 1 resulted in a defective cytokinesis and resulted in cells that were multinucleated⁴⁷. Complex II was implicated in cytokinesis in a follow up study that showed that knockdown of UVRAG but not ATG14L in HeLa cells resulted in impaired detachment of the midbody structure, which is present at the cleavage furrow⁴⁸. Failure of the midbody to detach prevents the separation of two daughter cells during mitosis. This study implicated Complex II in the regulation of chromosomal number during mitosis and the prevention of aneuploidy, which can lead to genomic stress resulting in pathogenic processes such as cancer development.

Multiple studies have implicated a role for Beclin 1 in endocytosis that is mediated through UVRAG/Complex II, as restoration of UVRAG but not ATG14L in *Becn1* deficient mouse embryonic fibroblasts was able to rescue a deficiency in endosome formation and support neuronal development⁴⁹. The role for Beclin 1 in endocytosis is well conserved from lower to higher organisms. Beclin 1 homologues in *Drosophila Melanogaster* as well as *C. elegans* have been shown to play a role in endocytosis. In *Drosophila*, Atg6 mutant animals show defects in

endocytosis as reduced uptake of Texas Red-avidin in larval fat bodies is observed⁵⁰. Additionally, Atg6 mutant larval fat bodies have reduced Rab5 perinuclear staining, a marker for early endosomes⁵⁰. This change is not observed in Atg1 (ULK1/2 in mammals) mutant flies. Another study in flies showed that loss of Atg6 results in an accumulation of early and late endosomes as well as endolysosomes⁵¹. This phenotype was phenocopied by UVRAG knockdown but not ATG14 knockdown, which again indicates that the role of Beclin 1 in endocytosis is mediated through Complex II and is independent of its functions in autophagy. In *C. elegans*, a defect in endocytosis was observed by performing fluid uptake assays with Texas-Red in animals mutant in BEC-1 (*C. elegans* homologue to mammalian Beclin 1)⁵². Additionally, BEC-1 mutants showed defects in recycling MIG/Wntless protein from endosome to the Golgi. Instead it was trafficked to the lysosome for degradation⁵². These studies in lower organisms indicate a role for Beclin 1 in endocytosis and this role is mediated through its interaction in Complex II with UVRAG.

A role for Beclin 1 in endocytosis has also been shown in mammalian systems as well. The Stenmark group showed that knockdown of Beclin 1, VPS34, and UVRAG in HeLa cells led to decreased degradation of the Epidermal Growth Factor Receptor (EGFR) through Rhodamine-EGF pulse chase and confocal microscopy assays⁴⁸. Additionally, they determined that this function was specific to Complex II as knockdown of ATG14L did not alter EGFR degradation⁴⁸.

However, other conflicting studies suggest that Beclin 1 does not regulate EGFR degradation⁵³. The Maltese group showed that suppression of Beclin 1 in glioblastoma cells impaired autophagy but did not change EGFR degradation⁵³. These studies suggest that the role of Beclin 1 in endocytosis could be cell type specific.

Deregulation of both endocytic receptor degradation and cytokinesis can serve as a nidus for cancer development. As mentioned previously, UVRAG has been implicated for having a role in both endocytic receptor degradation and cytokinesis. Therefore, studies indicate that UVRAG has tumor suppressive behavior. In support of this, UVRAG is located in a tumor susceptible locus that is commonly mutated in multiple cancers such as colon, gastric, and breast. Microsatellite unstable colon carcinomas cells with monoallelic UVRAG mutations exhibit increased tumorigenic potential⁵⁴. Frameshift mutations in UVRAG were identified in gastric tumors with microsatellite instability⁵⁵. Additionally, UVRAG negatively regulates proliferation as colon cancer cells with monoallelic UVRAG mutations exhibit enhanced proliferation⁵⁶. Loss of UVRAG promotes genomic instability independent of autophagy, as cells that lack UVRAG are prone to DNA damage in the form of double stranded DNA breaks⁵⁷. Loss of UVRAG also gives rise to cellular aneuploidy due to destabilization of centrosomes⁵⁷. Interestingly, UVRAG mutated cell lines do not exhibit impaired autophagy suggesting that the function of UVRAG in cancer is independent of

autophagy^{54,57}. These studies all highlight the tumor suppressive activity of UVRAG, which mediates the autophagy-independent functions of Beclin 1 through Complex II. Moreover, ATG14L, which is essential for the autophagic process through Complex, I, has not been shown to be mutated in cancer or to suppress tumorigenesis.

Beclin 1 and Cancer

Since its discovery, multiple functions for Beclin 1 have been identified including a role for Beclin 1 in cancer. Beclin is a haploinsufficient tumor suppressor as heterozygous loss of *Becn1* in mice leads to spontaneous tumorigenesis. *Becn1*^{+/-} mice develop lung and liver adenocarcinomas as well as multiple lymphomas when compared to control mice^{42,45}. In addition, mammary glands from these mice show evidence of pre-malignant hyperplastic changes. Moreover, *Becn1*^{+/-} mice exhibit enhanced mammary tumorigenesis following parity⁵⁸. In humans, monoallelic loss of *BECN1* is observed in 40% of breast, 50% of prostate, and 75% of ovarian cancers⁵⁹. Furthermore, overexpression of Beclin 1 in MCF7 cells in an orthotopic xenograft model prevents tumor formation and proliferation, further suggesting a tumor suppressive role for Beclin 1.

The role of Beclin 1 as a tumor suppresser has been questioned due to the chromosomal positioning of *BECN1*. *BECN1* is located on human chromosome 17q21. It is positioned next to *BRCA1*, a well know tumor suppressor gene that

regulates DNA repair and is commonly deleted in breast cancer. In one analysis of The Cancer Genome Atlas (TCGA) data it was determined that loss of *BECN1* occurred because of large deletions of the region in which *BRCA1* was located⁶⁰. These data suggested that *BECN1* loss occurred because it was passenger gene that is lost when *BRCA1* deletions occur. This co-deletion questions a role for Beclin 1 as a tumor suppressor. However other evidence to support Beclin 1 as a tumor suppressor in multiple cancers has emerged. In a study of human breast tumors, low Beclin 1 expression was more commonly observed in ER negative (ER) breast tumors (HER2 and basal-like) than luminal tumor subtypes and reduced expression is associated with poor prognosis⁶¹. This same study found that reduced *BRCA1* expression was not associated with basal like or HER2 enriched tumors and was not associated with patient prognosis⁶¹. This finding suggests that *BECN1* is not a passenger deletion but may have important independent functions in cancer. In human breast tumors, aberrant methylation of *BECN1* decreases expression⁶¹. Beclin 1 expression is reduced in multiple TNBC cells lines⁵⁹. In addition, low Beclin 1 expression is an independent predictor of patient prognosis in multiple cancers in addition to breast cancer, including oral tongue squamous cell carcinoma, gastric cancer and hypopharyngeal cancer⁶²⁻⁶⁴.

While Beclin 1 expression is reduced in multiple cancers, this thesis will highlight breast cancer because a large majority of research on Beclin 1 and cancer has

focused on breast. The role of Beclin 1 in breast cancer has been studied in multiple genetic models of mammary tumor development in mice. Tumorigenesis was enhanced in mice with overexpressed WNT activation and heterozygous loss of *BECN1* following parity. Additionally in an orthotopic xenograft model, overexpression of Beclin 1 in MCF7 breast cancer cells was shown to reduce tumor formation in mouse mammary glands of nude mice⁶⁵. However, in a polyoma middle T oncogene driven tumor model and a HER2/ErBB2 overexpressed tumor model, tumorigenesis was not enhanced in mice with heterozygous loss of *BECN1*. This suggests that the role of Beclin 1 in mammary tumorigenesis is context dependent and warrants further investigation to identify the molecular mechanism by Beclin 1 suppresses tumor formation^{58,66}.

Endocytic Pathway and Growth Factor Receptor Regulation

Beclin 1 plays a role in endocytosis and reduced expression of Beclin 1 results in reduced degradation of some receptors (i.e. EGFR) that undergo endocytosis. Deregulation of growth factor receptor signaling can promote tumorigenesis. Therefore, insight into the function of Beclin 1 as a tumor suppressor can be gleaned by understanding the role of endocytosis in the regulation of growth factor receptor expression and function. Endocytosis is a process in which cells internalize membrane surface proteins or macromolecules that are targeted to different cellular compartments. Once internalized, cargos are sorted into intracellular vesicles called endosomes. Cargos that are packaged into

endosomes can have multiple fates ranging from recycling back to the plasma membrane or targeting to the lysosome for degradation. Deregulation of this intricate movement of internalized cargo has been implicated in multiple disease pathologies including neurodegenerative disorders, metabolic syndromes and cancer.

There are multiple forms of endocytosis but the main two mechanisms by which endocytosis occur is through either Clathrin-mediated or caveolae-mediated endocytosis.

- I. Clathrin-mediated endocytosis (CME): Clathrin mediated endocytosis, also known as receptor-mediated endocytosis, is the most commonly studied form of endocytosis⁶⁷. This mechanism involves the formation of clathrin coated pits as well as clathrin coated vesicles. Two of the most well studied receptors that are trafficked by CME are the low-density lipoprotein (LDL) receptor and the Transferrin receptor (TFR1). The core machinery needed to facilitate CME includes clathrin, adaptor proteins and dynamin⁶⁸. CME initiation occurs when adaptor protein 2 (AP2) is recruited to lipid rich portions the plasma membrane⁶⁹. Recruitment of AP2 allows for the assembly and recruitment of clathrin heavy and light chains creating the “pit”. This then allows for the docking of different cargo to the clathrin-coated pit. Clathrin coated vesicle formation is then mediated by dynamin, a GTPase which

recognizes BAR domain proteins that interact through Dynamin's proline-rich domain⁷⁰. Dynamin encircles clathrin coated invaginations to cause scission⁷¹. This creates the separation between the plasma membrane and the new clathrin coated vesicle. Clathrin is then removed from the vesicle through heat shock cognate 70 (Hsc70) and newly formed vesicles are trafficked to different cellular compartments.

- II. Caveolae-mediated endocytosis (CavME): Caveolae, the location of CavME, are flask or omega shaped invaginations that are present in most eukaryotic plasma membranes^{72,73}. While not well understood, the main structure of caveolae consists of caveolin-1, caveolin-2 and caveolin-3, integral membrane proteins that insert into inner leaflets of the membrane bilayer and bind cholesterol to serve as a scaffold for different cargos^{72,74}. Budding of caveolae is mediated by different kinases (src-family of kinases) and phosphatases^{75,76}. Similar to CME, dynamin triggers fission of caveolae to separate them from the plasma membrane⁷⁷. Caveolae that are removed from the plasma membrane are then trafficked to different cellular localizations via the actin and microtubule cytoskeleton⁷⁸.

The endocytic pathway is made up of three main types of endosomes. The early endosome (EE) is responsible for receiving cargo from multiple endocytic entry points⁷⁹. The late endosome (LE) targets endosomal contents to the lysosome or trans-golgi network⁷⁹. The third component is the recycling endosome (RE),

which takes cargo from the EE population, and recycles this cargo back to the cell surface⁷⁹. A small family of GTPases, a superfamily of enzymes that hydrolyze Guanosine Triphosphates (GTPs), known as Rab GTPases, regulate the endocytic process. Additionally, these Rab GTPases localize to different endosomal compartments (i.e. EE, LE, and RE). Over 60 Rab GTPases have been identified in the human genome and different Rabs localize to and are markers of specific endosomal compartments⁷⁹. The early endosome is characterized as being Rab5 and Rab4 positive. Rab5 is one of the most commonly characterized Rab GTPases present on the early endosomal vesicle. Late endosome are characterized as Rab7 positive and recycling endosomes are Rab11 and Rab4 positive. Trafficking of cargos through these different endosomes (EE, LE, and RE) is crucial in the regulation of intracellular signaling⁷⁹.

Endocytic Regulation and Growth Factor Signaling

The endocytic pathway can regulate the trafficking of multiple membrane proteins to different intracellular compartments. Additionally, the endocytic pathway can also regulate growth factor receptor signaling by recycling or degrading receptors thereby maintaining or inhibiting their signals, respectively. For example, multiple receptor tyrosine kinases (RTKs) are regulated by endocytosis. RTKs are transmembrane receptors for ligands such as growth factors, cytokines, and hormones that regulate multiple cellular processes. Upon ligand binding, a series

of tyrosine phosphorylation events leads to signal transduction triggering activation of downstream pathways. In order for the receptor to be recycled or degraded, RTKs undergo endocytosis through various endocytic compartments to be removed from the cell surface. Signaling through RTKs is tightly regulated through both space and time by intracellular trafficking components, and the process of endocytic internalization, degradation, and recycling of RTKs is fundamental for this regulation. Deregulation of these signaling pathways has been implicated in multiple disease pathologies including cancer initiation and progression. I will describe the regulation of RTKs by using the Epidermal Growth Factor receptor (EGFR) as an example because the endocytic regulation of EGFR has been studied most extensively.

EGFR, also referred to as ErbB1/HER1, is one of the most commonly studied members of the EGFR family of RTKs that includes ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. Signaling through the EGFR leads to a multitude of functional downstream outcomes including cellular proliferation, differentiation, migration, growth and inhibition of apoptosis. The activation and downstream activity of the receptor is mediated by an extracellular ligand binding domain, transmembrane domain, kinase domain and the C-terminal tail domain. Following ligand binding, single EGFR monomers undergo dimerization and transphosphorylation of the C-terminal domain. Dimerization of the EGFR was first appreciated by Yarden and Schlessinger in the presence of the EGF

ligand^{80,81}. Transphosphorylation of the EGFR allows for the phosphorylation of multiple tyrosine residues in the C-terminal tail domain. Following transphosphorylation, several proteins are recruited, such as GRB2 and SOS, which activate downstream RAS/RAF/MEK/ERK to promote cellular proliferation as well as PI3K to activate downstream AKT/mTOR signaling which promotes cell survival⁸². In addition to the RAS/RAF/MEK/ERK and AKT/mTOR pathways, dimerization of EGFR monomers can also activate other downstream signaling pathway axes such as PLC γ 1-PKC, JNK, and JAK-STAT. After activation of downstream signaling, EGFR signaling must be terminated. Activated EGFR is internalized through CME or CavME to attenuate signals^{83,84}. Similar to other RTKs, once internalized, EGFR can be recycled back to the membrane or targeted to the lysosome for degradation.

RTK Degradation and Recycling

Activation of RTKs and subsequent degradation or recycling of receptors is regulated by the endocytic process. Internalized receptors can be ubiquitinated and this ubiquitination controls trafficking through the endosomal pathway. EGFR is internalized from the plasma membrane through CME and CIE pathways in an EGF ligand concentration dependent manner. Studies suggest that at low concentrations of EGF, CME was shown to be the route of internalization, however at high concentrations of EGF, CIE was determined to be the main mechanism of internalization⁸⁵. However, a controversial study indicated that

EGFR internalization is impaired at both high and low concentrations of EGF in CIE⁸⁶. EGFR is ubiquitinated by the Cbl family of ubiquitin ligases⁸⁷. c-Cbl binds activated EGFR, which leads to mono-ubiquitination of the receptor⁸⁸. Cbl along with the adaptor protein CIN85 were shown to interact with EGFR to mediate its endocytosis and degradation⁸⁹. Ubiquitination of receptors allows for binding of other proteins that will change the fate of the receptor for either degradation or recycling. Similar to the EGFR, other RTKs such as the insulin-like growth factor receptor 1 (IGF-1R) are targeted for degradation through ubiquitination but this occurs through different adaptor proteins. Nedd4, an E3 ligase, mediates ubiquitination of the IGF-1R, which is internalized by both CME and CIE mechanisms^{90,91}. Additional ubiquitin ligases have been shown to ubiquitinate IGF-1R to mediate endocytosis such as Mdm2 and c-Cbl^{92,93}.

Once internalized, key decision-making steps occur where RTKs are sorted into different endosomal compartments. Such compartments include the recycling endosome (prolongs receptor signaling) or lysosome (attenuates receptor signaling). Ubiquitination is the key step in the degradation of RTKs as it tags proteins that are destined for lysosomal degradation. Ubiquitinated cargos in endosomal membranes are detected by the endosomal-sorting complex required for transport (ESCRT) machinery (Figure 1.3)⁹⁴. The ESCRT machinery, first discovered in yeast, is highly conserved in mammalian systems⁹⁵. In yeast, depletion of the class E vacuolar sorting proteins (*Vps*) was found to cause

defects in endosomal sorting as well as enlarged prevacuolar compartments that lacked internal vesicles⁹⁶. These *Vps* proteins were found to assemble into multi-subunit machinery consisting of ESCRT-0, I, II, and III. The ESCRT subunits are recruited to endosomal membranes in a sequential manner (i.e. 0 then I, II, and III) and facilitate trafficking of proteins to intraluminal vesicles (ILV), which go on to form the multivesicular body (MVB). The contents of the MVB get degraded upon fusion with the lysosome. The sequential recruitment of the ESCRT complexes to the endosome is termed the conveyor belt method⁹⁷.

ESCRT-0 consist of Hepatocyte Growth Factor-regulated Tyrosine Kinase Substrate HRS (mammalian orthologue to yeast *Vps27*) and Signal Transducing Adaptor Molecule STAM (mammalian orthologue to yeast *Hse1*) and initiates MVB formation by recognizing ubiquitinated cargo^{98,99}. HRS is recruited to endosomes through binding of its Fab 1, YOTB, Vac 1, EEA1 (FYVE) zinc finger domain to PI3P and binds ubiquitinated proteins through its ubiquitin-binding motif (UIM)¹⁰⁰⁻¹⁰². Following recruitment of ESCRT-0, ESCRT-I is recruited to endosomal membranes through HRS of ESCRT-0. ESCRT-I member TSG101 binds HRS through its UEV domain¹⁰³. ESCRT-I contains TSG101 (mammalian orthologue to yeast *Vps23*), *Vps28*, *Vps37A-D*, and ubiquitin associated protein 1 (UBAP1). ESCRT-II is recruited to the endosomal membrane by *Vps28* of ESCRT-I, which binds the GLUE domain of EAP45 (mammalian orthologue to yeast *Vps36*)¹⁰⁴. ESCRT-II consists of EAP45 (mammalian orthologue to yeast

Vps36), EAP22 (mammalian orthologue to yeast Vps22) and two EAP20 (mammalian orthologue to yeast Vps25). ESCRT-III is made up of 4 core subunits CHMP6, CHMP4(A-D) CHMP3, and CHMP2(A-B), (mammalian orthologue to yeast Vps20, Snf7, Vps24, Vps2 respectively). Multiple ESCRT-III complexes are recruited to the endosome by CHMP6 and once activated forms a ring structure around ubiquitinated cargos¹⁰⁵. Vps4 is AAA+ ATPase is involved in the final steps of MVB biogenesis and helps to cycle ESCRT complexes off of endosomal membranes^{97,106}.

EGFR is targeted to the lysosome via the endosomal-sorting complex required for transport (ESCRT) machinery. The ESCRT-0 proteins HRS and STAM, which initiate the targeting of this receptor to the lysosome, recognize ubiquitinated EGFR. Ubiquitination increases trafficking of the EGFR to the multivesicular body to enhance lysosomal degradation¹⁰⁷. HRS and STAM are phosphorylated by EGFR at the endosomal membrane¹⁰⁸. Recruitment of ESCRT-0 allows for the initiation of the degradative process of EGFR. Although degradation of EGFR has been shown to occur through the lysosome, studies have also implicated a role for the proteasome in the degradation of EGFR. Cells treated with proteasome inhibitors exhibit delayed degradation of EGFR¹⁰⁹. Similar to EGFR, studies suggest that IGF-1R is degraded at the proteasome as proteasome inhibitors reduce the degradation of the receptor¹¹⁰. This inhibition could be due to a decrease in ubiquitination which is needed for EGFR or IGF-1R

to be trafficked to the lysosome or because a portion of EGFR or IGF-1R is degraded at the proteasome.

Although EGFR is degraded at lysosomes, another fate of the receptor is recycling to the cell surface. Studies show minimally ubiquitinated EGFR escapes degradation at the lysosome¹¹¹. EGFR is trafficked to the recycling endosome through Rab35 or Rab11 via the perinuclear recycling compartment. The clathrin adaptor protein Eps15 was shown to mediate recycling of EGFR via Rab11 to traffic back to the membrane¹¹².

Intracellular Signaling Through Endosomes

RTKs were originally thought to only transmit signals from the plasma membrane. However, this idea was challenged when subcellular fractionation identified downstream signaling proteins that are activated downstream of RTKs in endosomes. In rats injected with EGF prior to euthanization, fractionation experiments in liver tissues found that downstream adaptor proteins such as GRB2 and SHC localized with EGFR in large endosomes¹¹³. These same findings were identified following activation of the insulin receptor, another RTK, as activated MAPK accumulated in endosomes when detected by magnetic microbeads¹¹⁴. These experiments highlighted endosomes as potential platforms for signal propagation in the cytoplasm upon RTK activation beyond the plasma membrane.

The functional relevance of endosomal signaling was identified in studies examining signaling in neurons. Nerve Growth Factor (NGF), which activates the TrkA receptor, propagates signals through endosomes down long neuronal axons and disruption of this signaling allowed for sustained signaling downstream of the TrkA receptor^{115,116}. The actual transport of endosomes was visualized in axons by live imaging using quantum dot labeled NGF; transport of NGF was found to be unidirectional¹¹⁷. These results established a role for receptor trafficking of signaling downstream of the plasma membrane and identified a role for endosomes as signaling-competent compartments in the cytoplasm. These findings suggested that these signaling-competent endosomes regulate signaling in different subcellular locations upon ligand activation of receptors.

The ability of RTKs to propagate signals through endosomes has been shown in other studies. EGFR is associated with multiple of its downstream effectors in different endosomal compartments using endosomal isolation and immunofluorescence techniques¹¹⁸. In one study, the Wiley group used a reversible biotinylated EGFR antibody to isolate EGFR and associated proteins in different subcellular compartments and generated phospho-specific EGFR antibodies to examine activity of the receptor¹¹⁸. Using the biotinylated antibody and fluorescence techniques, they determined that EGFR remains active in

endosomes and associated with different adaptor proteins¹¹⁸. A more recent study using FRET microscopy determined that EGFR remains phosphorylated and active in endosomes and can recruit adaptor protein Shc1¹¹⁹. These studies indicate further that signaling can be propagated in endosomes.

Signaling from endosomes and silencing of signals is tightly linked. The ESCRT machinery, which binds ubiquitinated cargos destined for degradation, is required for the generation of intraluminal vesicles. The generation of intraluminal vesicles that merge to create MVBs removes active receptors from a signaling competent compartment to a signaling-incompetent compartment, thus attenuating signals. Deregulation of receptor trafficking can lead to increased signaling in signaling competent compartments and this aberrant signaling can promote disease pathogenesis. This delay may help to explain why many cancers, including breast, have elevated activity of RTKs.

Alternative subcellular mechanisms to regulate signaling:

While the endosomal pathway is one way to regulate receptor signaling, other mechanisms for controlling signaling exists. For example, the cellular cytoskeleton is an important regulator of growth factor receptor signaling downstream of multiple RTKs. The cytoskeleton has the ability to regulate receptors and aid in their activation. The cytoskeleton can impact receptor signaling, dimerization, and endosomal trafficking. The actin cytoskeleton can

also work with the microtubule axis to drive movement of different endosomal vesicles (EE, LE, and RE), which aid in the movement of receptors or different cargos to multiple subcellular sites¹²⁰. This can also influence receptor signaling. For example, the RTK and proto-oncogene c-Met was shown to have sustained signaling in perinuclear endosomes through activation of Rac-1, a Rho GTPase family member. Rac-1 is known to initiate actin cytoskeleton changes that promote cellular migration^{121,122}. In this study they were able to show that Rac-1 dependent changes in the cytoskeleton promoted enhanced signaling from active c-Met in perinuclear endosomes¹²³. Another example of RTK signal regulation by the cytoskeleton was shown through the neurofibromatosis type 2 (NF2) tumor suppressor Merlin. Merlin interacts with alpha-catenin, an actin binding protein, and can localize to the cortical cytoskeleton¹²⁴. Merlin regulates EGFR signaling by immobilizing EGFR to the plasma membrane and preventing its internalization¹²⁵. Additionally loss of Merlin was shown to enhance recycling of the EGFR¹²⁶. These studies indicate an important role for cytoskeletal regulation of growth factor receptors.

Another factor that can influence RTK signaling is plasma membrane lipid compositions. Lipid rafts, or area of the plasma membrane that are high in cholesterol and sphingolipids can either negatively or positively regulate RTK signaling. For example, EGF stimulation in methyl- β -cyclodextrin (cholesterol inhibitor) treated NIH 3T3 cell, leads to increase activation of MAPK downstream

of EGFR^{127,128}. Analysis of other RTK signaling pathways such as the Insulin receptor (IR) or Platelet derived growth factor receptor (PDGFR) reveal that depletion of lipid raft cholesterol impairs downstream signaling^{129,130}. The results of these studies indicate the importance of cholesterol and lipids to RTK function and signaling. It also highlights a receptor dependent role for lipids as loss of cholesterol can either promote or inhibit signaling.

Beclin 1 Regulation of Growth Factor Receptor Signaling

As previously mentioned, Beclin 1 has been implicated in having a role in the endocytic process that has been conserved from lower organisms to complex mammals. Beclin 1 regulates vacuolar protein sorting in yeast. Atg6 mutant *Drosophila* show defects in endocytosis as well as reduced Rab 5 expression in their larval fat bodies⁵⁰. Additionally, *C. elegans* with mutant BEC-1 have reduced endocytosis of Texas-Red marker uptake and defects in the recycling and trafficking of the MIG/Wntless protein⁵². In a mammalian system, knockdown of Beclin 1 leads to reduced degradation of the EGFR through confocal microscopy⁴⁸. Together, this literature suggests a role for Beclin 1 in regulating endocytosis.

Given that multiple receptors and their signaling are regulated at the level of endocytosis, it begs the questions of whether or not Beclin 1 can regulate growth factor receptor signaling through its regulation of endocytosis? Several studies

provide evidence that Beclin 1 can regulate growth factor signaling at the level of the endosome. In lower organisms such as *Drosophila*, depletion of Atg6 increases Notch in early endosomes as well as enhanced Notch signaling from endosomes⁵¹. This enhanced Notch signaling was detected by a EGFP reporter⁵¹. In a mammalian system using mouse embryonic fibroblast (MEFs), knockdown of Beclin 1 resulted in reduced degradation of EGFR following EGF stimulation⁴⁹.

Previous work from our lab identified a novel role for Beclin 1 in regulating growth factor receptor signaling in multiple breast cancer subtypes. Knockdown of Beclin 1 resulted in enhanced and sustained signaling activity following EGF or IGF1 stimulation. We identified that loss of Beclin 1 results in delayed endosomal maturation as evidenced by retained APPL⁺ endosomes¹³¹. APPL is an adaptor protein that is present on early endosomes in the absence of PI3P¹³². Following accumulation of PI3P at endosomal membranes, APPL is displaced by FYVE domain carrying proteins to continue the endosomal maturation process¹³². *shBECN1* cells stimulated with EGF or IGF1 exhibited increased APPL⁺ endosomes suggesting a delay in maturation¹³¹. Additionally, analysis of human tumors showed an inverse correlation of Beclin 1 expression with AKT or MAPK expression. Taken together, these data suggest a role of Beclin 1 in the regulation of growth factor receptor signaling. Given that this work was performed

in vitro, it is important to understand whether Beclin 1 can regulate growth factor receptor signaling *in vivo*.

Rationale for Thesis Project

Multiple studies have implicated Beclin 1 in having a role in cancer progression. This role for Beclin 1 in cancer has been attributed almost exclusively to its role in autophagy. However multiple studies provide evidence for autophagy-independent functions of Beclin 1 that can impact cancer progression. Although Beclin 1 regulates processes that are important for all types of cancer, the majority of studies have been performed in breast cancer. Low Beclin 1 expression is associated with poor prognosis in multiple aggressive breast cancer subtypes. However, the exact mechanism by which loss of Beclin 1 promotes breast cancer progression has yet to be elucidated. Previous work from our lab identified a role for Beclin 1 in the endocytic regulation of growth factor receptor trafficking and signaling *in vitro* in a breast cancer model. However, a deeper understanding of the role of Beclin 1 in cancer is necessary in order to identify and develop novel treatment approaches for patients with aggressive disease. Therefore, for my thesis project, I sought to understand the mechanism by which Beclin 1 contributes to breast cancer tumor growth and progression *in vivo*. Given that Beclin 1 is able to regulate growth factor receptor signaling by modulating endocytic trafficking *in vitro*, it was important to assess whether this function affects tumor growth and progression *in vivo*. It was also important to assess whether Beclin 1 has roles in cancer progression that are

outside of its known role in autophagy. By understanding Beclin 1 function *in vivo*, it could potentially shed light on alternative avenues for treatment of patients with more advance stage disease.

CHAPTER II

Beclin 1 promotes endosome recruitment of hepatocyte growth factor tyrosine kinase substrate (HRS) to suppress tumor proliferation

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AUTHOR CONTRIBUTIONS

A.N.M.-O. and L.M.S. were involved in the conception and design of the project and wrote the manuscript; A.N.M.-O., J.M.-M., J.J., A.E.C. and F.L. were involved with the development of methodology and the acquisition and analysis of data. D.K., P.C.-G., R.R. and M.L. were involved in the analysis of data. All authors reviewed and approved the final version of the manuscript.

ABSTRACT

Beclin 1 has non-autophagic functions that include its ability to regulate endocytic receptor trafficking. However, the contribution of this function to tumor suppression is poorly understood. Here, we provide *in vivo* evidence that Beclin 1 suppresses tumor proliferation in an autophagy-independent manner by regulating the endocytic trafficking and degradation of the epidermal growth factor (EGFR) and transferrin (TFR1) receptors. We discovered that Beclin 1 promotes endosomal recruitment of hepatocyte growth factor tyrosine kinase substrate (HRS), which is necessary for sorting surface receptors to intraluminal vesicles for signal silencing and lysosomal degradation. In tumors with low Beclin 1 expression, endosomal HRS recruitment is diminished and receptor function is sustained. Collectively, our results demonstrate a novel role for Beclin 1 in impeding tumor growth by coordinating the regulation of key growth factor and nutrient receptors. These data provide an explanation for how low levels of Beclin 1 facilitate tumor proliferation and contribute to poor cancer outcomes, independently of autophagy.

INTRODUCTION

Beclin 1 is a haploinsufficient tumor suppressor that is associated with poor prognosis in a number of cancer types^{42,133}. In breast cancer, reduced Beclin 1 expression is an independent predictor of poor overall patient survival¹³⁴. Heterozygous loss of Beclin 1 (*BECN1*^{+/-}) promotes mammary tumorigenesis in response to parity and enhances WNT1-driven mammary tumor progression¹³⁵. The majority of studies that have investigated Beclin 1 function in cancer have focused on its role in regulating macroautophagy (hereafter referred to as autophagy). Autophagy is a conserved homeostatic and stress response pathway by which damaged proteins and organelles are engulfed within a double membrane vesicle and degraded upon fusion with lysosomes to prevent cytotoxicity and recycle macromolecules for energy supply^{136,137}. While a role for autophagy in suppressing tumor initiation has been supported by experimental studies¹³⁸, a paradoxical requirement for autophagy function in tumor progression has also been revealed^{139,140}. For example, knockout of *Atg5*, an essential autophagy gene that is required for the elongation and closure of the autophagosome, enhances tumor initiation in a *Kras* mouse model of pancreatic cancer, but these tumors remain benign and do not progress to invasive cancer⁴¹. Moreover, *Kras/p53*-driven lung tumors revert to benign oncocytomas upon acute knockout of *Atg7*, another essential autophagy gene important for autophagosome elongation¹⁴¹. These outcomes contrast with the enhanced tumor growth and progression observed in mice when Beclin 1 expression is

reduced^{42,133,135}. The requirement of autophagy for the development and maintenance of malignant tumors conflicts with the role of Beclin 1 as a tumor suppressor, and this discrepancy underscores the likelihood that alternative functions of Beclin 1 are involved in its regulation of tumor progression.

Autophagy-independent functions of Beclin 1 have been less studied in the context of cancer, although growing evidence supports their involvement in tumor suppression. Beclin 1 (Atg6/Vps30) regulates membrane trafficking events through its interaction with p150 (Vps15) and the lipid kinase class III phosphatidylinositol-3 kinase (PI3KC3/Vps34)^{142,143}. This Beclin 1 core complex interacts in a mutually exclusive manner with either ATG14L/BARKOR (Atg14; Complex I) or UVRAG (Vps38; Complex II) to regulate distinct vesicular trafficking functions^{144,145}. Complex I regulates autophagy and Complex II regulates autophagy-independent functions including vacuolar protein sorting, cytokinesis, phagocytosis, fluid phase endocytosis and endolysosomal receptor trafficking^{144,146,147}. Beclin 1, UVRAG and another Complex II-specific binding partner BIF-1 each suppress xenograft tumor growth when overexpressed, a finding not reported for ATG14L^{56,148,149}. This selective regulation supports a unique role for Beclin 1 and Complex II in cancer.

One mechanism by which Beclin 1 may regulate tumor growth and progression is through the control of endolysosomal trafficking, which plays an important role in

controlling the outcomes of cell surface receptor function^{150,151}. For growth factor receptors, ligand binding initiates internalization and entry into the early endosome compartment, which is required for the activation of some signaling pathways¹⁵². Other receptors, such as the transferrin receptor (TFR1), are internalized constitutively in a ligand-independent manner¹⁵³. Once internalized into early endosomes, receptors are sorted to either late endosomes/multivesicular endosomes (MVEs) where they are sequestered within intraluminal vesicles (ILVs) for signal termination and subsequent degradation upon fusion with the lysosome^{154,155}, or to the recycling endosomes for return to the cell surface¹⁵⁶. Beclin 1, UVRAG and BIF-1 have been reported to regulate the rate at which the epidermal growth factor receptor (EGFR) is degraded after stimulation with its ligand EGF^{146,157}. In previous work, we showed that Beclin 1 regulates phosphatidylinositol-3 phosphate (PI3P) production in response to growth factor stimulation and promotes the transition of PI3P-negative (PI3P⁻) early endosomes to PI3P⁺ endosomes^{158,159}. By doing so, Beclin 1 controls the length of time that growth factor receptors remain in the PI3P⁻ signaling competent compartment and consequently determines the duration of growth regulatory signals¹⁵⁹. The fact that Beclin 1 expression inversely correlates with AKT and ERK phosphorylation in human breast tumors is indicative that this Beclin 1-dependent regulation of growth factor receptor signaling occurs in human cancer¹⁵⁹.

Despite knowledge that Beclin 1 has been implicated in growth factor receptor signaling and trafficking, much remains to be learned about the mechanism by which this occurs. PI3P is necessary for the recruitment of FYVE (Fab1p, YOTB, Vac1p, EEA1) or PX (Phox homology) domain containing effector proteins that control the trafficking fate of cargo within the endocytic pathway. However, specific PI3P-interacting proteins that are regulated by Beclin 1 have not been identified. Moreover, the existing data on Beclin 1 regulation of trafficking were derived from *in vitro* studies and the impact of Beclin 1 on receptor trafficking and signaling *in vivo*, and the effect on tumor behavior, has not been demonstrated. In the current study, we identify an autophagy-independent mechanism by which Beclin 1 regulates the trafficking and function of growth factor and nutrient receptors that drive tumor cell proliferation *in vivo*. These findings provide novel insight into the mechanism by which Beclin 1 regulates receptor function and how loss of Beclin 1 expression contributes to tumor progression.

MATERIALS AND METHODS

Cells, antibodies and reagents. MDA-MB-231 LM2 4175 human breast cancer cells were purchased from the laboratory of Joan Massague (Memorial Sloan Kettering Cancer Center, Cornell University) and grown in DMEM media containing 10% FBS¹⁶⁰. Authenticated SUM-159 cells were a kind gift from Art Mercurio (UMass Medical School, Worcester, MA) and grown in F12 Hams media supplemented with 5% FBS, 500mM HEPES, 1.5mg Insulin and 1mg/mL hydrocortisone. Expanded stocks were frozen down and fresh knockdown cells

were generated after two months in culture. Cells tested negative for mycoplasma using the Morwell MD Biosciences EZ PCR Mycoplasma Test Kit (cat# 409010). Stable knockdown cell lines were generated using lentiviral vectors containing shRNAs that target human *BECN1* (TRCN0000033550, TRCN0000033552), *ATG5* (TRCN0000151963, TRCN0000151474) and *TFRC* (TRCN0000057660) (Open Biosystems, Lafayette, CO, USA). pLKO.1 puromycin containing shRNA that targets green fluorescent protein (*GFP*) was purchased through Addgene (Cat# 30323). For dual expression, shRNAs were sub-cloned into a pLKO.1 neomycin vector (Addgene; cat # 13425) using *EcoRI* and *MfeI* sites. For restoration of Beclin 1 expression, FLAG-Beclin 1 with silent mutations that disrupt shRNA targeting was subcloned into the pCDH-puro lentiviral vector¹⁵⁹. Stable cell lines were selected with 2ug/ml of puromycin (Gold Bio), 0.5ug/ml G418 Neomycin (Gold Bio), or both.

Antibodies recognizing Beclin 1 (cat# 3738), *ATG5* (cat# 2630), p44/42 MAPK (ERK1/2; cat#9102), pT202/Y204-MAPK (pERK1/2; cat# 4370), EGFR (cat# 4267), pY1068-EGFR (cat# 3777), HRS (cat# 15087), AKT (cat# 9272), pT308-AKT(cat# 4056) and phospho-Histone H3 (cat# 9701), as well as mouse IgG1 (cat# 5415) and normal rabbit IgG (cat# 2729) were purchased from Cell Signaling Technologies (CST) (Danvers, MA). Transferrin receptor (cat# 13-6800) and actin (cat# MA5-11869) antibodies were purchased from Invitrogen (Carlsbad, CA). LC3B (cat# L7543), Tubulin (cat# T5168) and pY334-HRS (cat#

SAB4504231) were purchased from Sigma (St. Louis, MO). Ki67 antibodies were purchased from Abcam (Cambridge, UK; cat# 66155).

Autophagic flux assays. Cells were plated in 24 well tissue culture dishes overnight and then incubated with complete DMEM containing 100nM Rapamycin (Sigma; cat# R0395), 40nM Bafilomycin (Sigma; cat# B1793), or both for 8 hours. Cells were extracted in radioimmune precipitation assay (RIPA) lysis buffer (25 mm Tris (pH 8.0), 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mm sodium chloride, 10 mm sodium fluoride, and 1 mm sodium orthovanadate) containing complete mini protease inhibitors (Roche). Cell extracts containing equivalent amounts of total protein were analyzed for LC3I to LC3II conversion by immunoblotting.

Orthotopic *in vivo* assays. LM2 cells were trypsinized, washed five times with PBS and cells (1×10^6) were resuspended in 35 μ L Matrigel (10mg/ml; Trevigen, Gaithersburg, MD; cat#3432-005-01) immediately prior to injection into the 3rd mammary fat pad of NOD/SCID mice. Tumors were measured twice weekly with calipers for 5-8 weeks. Tumor volume was calculated using the following equation: $4/3\pi[(L \times H \times W)/2]$. Tumors were excised and portions were either snap frozen for immunoblotting and mRNA analysis, fixed in 10% buffered formalin for immunohistochemistry or placed in culture medium for *ex vivo* analysis.

Ex vivo tumor analysis. Following tumor dissection, equal size tumor slices were equilibrated in DMEM containing 10% fetal bovine serum and supplemented with penicillin/streptomycin for 24 hours in a 5% CO₂ incubator. To assess pathway involvement in proliferation, tumor slices were incubated with DMSO (Sigma; cat# D5879), 5uM Lapatinib (Selleckchem; cat# S1028) or 10uM PD98059 (Selleckchem; cat# S1177) for 48 hours. Tissues were either flash frozen for protein extraction and analysis by immunoblotting or fixed in 10% buffered formalin and paraffin-embedded for IHC analysis.

Reverse Phase Protein Array. Frozen pieces of three tumors of each genotype (*shGFP*, *shBECN1*, and *shBECN1:Beclin 1*) were sent to the MD Anderson Cancer Center Reverse Phase Protein Array (RPPA) Core Facility. RPPA was performed according to their previously published protocol using the standard antibody list updated 3.

Immunoprecipitation and Immunoblotting. Cells were serum starved for 1 hr in serum-free medium and then stimulated with human recombinant EGF (Sigma; cat#9944) for the time periods indicated in the Figure Legends prior to extraction. Cells were solubilized at 4°C in a 20 mM Tris buffer, pH 7.4 containing 1% Nonidet P-40, 0.137 M NaCl, 10% glycerol, 10 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors (Roche). Frozen tumors were extracted at 4°C in Tissue Protein Extraction Buffer (Thermo Scientific; cat#

78510) containing 1mM sodium orthovanadate, 10mM NaF and protease inhibitors (Complete Mini Tab; Roche, Indianapolis, IN, USA). For immunoprecipitations, aliquots of cell or tumor extracts containing equivalent amounts of protein were pre-cleared for 1 hr with non-specific IgG and protein-A or -G sepharose beads (GE Healthcare) and then incubated for 3 hrs with specific antibodies and protein-A or -G sepharose beads with constant agitation. The beads were washed three times in extraction buffer and laemmli sample buffer was added to the samples.

Whole cell or tumor extracts containing equivalent amounts of protein or immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously^{161,162}. Bands were detected by chemiluminescence using a ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA) and band intensities were quantified by densitometry using Image Lab (Beta 1; Bio-Rad Laboratories) or Image J. Only signals within a linear range were used for quantitation and signals were normalized to total protein and/or housekeeping genes.

Immunofluorescent staining. Subconfluent, adherent cells plated on glass coverslips were serum starved for 2 hrs and then treated with or without EGF-Alexafluor 555 for 10 minutes. Cells were washed three times with cold Dulbecco's PBS and fixed in 3.8% paraformaldehyde in Dulbecco's PBS with

0.5% Tween (PBST) for 1 hr. Permeabilized cells were blocked for 1 hr using 3% BSA in PBST. Primary antibodies diluted in blocking buffer were added to cells and incubated at room temperature for 1 hr. Secondary antibodies were diluted in the same buffer and cells were incubated at room temperature for an additional 30 minutes. Cells were washed three times with PBST after each antibody incubation. Coverslips were then mounted on glass slides using Prolong Gold containing DAPI (Cell signaling) and the slides were viewed by confocal microscopy (Zeiss LSM700; 63X oil immersion objective). All images were adjusted equally for brightness/contrast using Adobe Photoshop.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (5 μ M) were deparaffinized and rehydrated, and antigen retrieval was performed in 10mM sodium citrate buffer, pH 6.0 with heating in a steamer for 1hr. Tissues were incubated with 0.3% Hydrogen peroxide to quench endogenous peroxides and then blocked using a dual avidin/biotin blocking kit (Vector Laboratories; cat # SP-2001) followed by a 1-hour incubation in 1x casein milk (Vector Laboratories; cat# SP-2050). Tissue sections were incubated with primary antibodies overnight followed by secondary antibody incubation with the elite ABC-HRP kit (Vector Laboratories; cat# PK6101). Sections were developed with diaminobenzidine (DAB) (Dako; cat# K3468) and then counterstained with hematoxylin. Stained tumor sections were viewed on an Olympus BX41 light microscope (Olympus). Images were captured with an Evolution MPColor

camera (Media Cybernetics). All images were adjusted equally for brightness and contrast using Adobe Photoshop.

Cell death was analyzed by TUNEL staining according to manufacturer's instructions (Promega, cat# G3250). Stained tissue sections were viewed and images captured on a Zeiss LSM-700 microscope. All images were adjusted equally for brightness and contrast using Adobe Photoshop.

Quantitative Polymerase Chain Reaction (qPCR). RNA was extracted from tumors using the RNA-easy kit (Qiagen; cat# 74134). cDNA was synthesized using a one-step cDNA kit (Biotool; cat# B22403). RT-qPCR was performed in a 20 uL reaction containing 0.5 uM primers, 20 ng cDNA template, and 1x SYBR green supermix (biotool; cat# B2120). Primers were designed using the Harvard PrimerBank (Table 1). Human R18S primers were used as a housekeeping control. RT-qPCR was performed using the Applied Biosystems QuantStudio 6 Flex apparatus. The delta –delta Ct method was used to determine relative mRNA expression.

Statistical Analysis. Statistical analysis between two groups was performed using the two-tailed unpaired student's t-test. Statistical analysis was performed using Prism7, Graphpad. A two-sided p-value of <0.05 was considered to indicate statistical significance. K means clustering was performed in MATLAB

using the built-in function 'kmeans' using the distance metric squared Euclidean. Fischer's exact test was performed to determine MAPK enrichment in clusters.

RESULTS

Beclin 1 regulates endosomal HRS recruitment

Our previous *in vitro* studies demonstrated that Beclin 1 regulates Insulin-like growth factor-1 (IGF-1R) and EGFR receptor trafficking and signaling by controlling the activation of VPS34 and generation of PI3P¹⁵⁹. Ligand-dependent receptor activation stimulates the production of PI3P and this increase is inhibited when Beclin 1 expression is suppressed¹⁵⁹. Reduced PI3P levels result in delayed receptor degradation, but the mechanism of this regulation is not known. A primary signal for sorting receptors that are destined for lysosomal degradation is receptor ubiquitination^{163,164}. Ubiquitinated receptors are recognized by the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), which contains both an ubiquitin binding (UIM) domain and a FYVE domain¹⁶⁵⁻¹⁶⁷. The HRS FYVE domain recognizes PI3P in the early endosomal membrane and is required for its recruitment to these vesicles^{168,169}. In cells treated with wortmannin to reduce PI3P levels and inhibit HRS recruitment to the early endosome, activated receptors escape sorting into intraluminal vesicles (ILVs) of multivesicular endosomes (MVE), a step prior to lysosomal degradation, and their signaling and expression are prolonged^{170,171}.

We hypothesized that suppression of Beclin 1 sustains growth factor receptor expression and signaling because HRS recruitment to the early endosome is limited, allowing receptors to escape sorting to the ILVs and delay degradation. To investigate this potential mechanism of Beclin 1 function, we used a variant of MDA-MB-231 cells (hereafter referred to as LM2 cells) because Beclin 1 expression is elevated in these cells when compared across a panel of TNBC cells¹⁷². Cells were generated that stably express shRNA targeting either *GFP* (control) or *BECN1*. This approach was taken to mimic the reduction, but not complete loss, of Beclin 1 expression that is commonly observed in human tumors^{134,173}. Beclin 1 expression was restored in the *shBECN1* cells using a construct in which silent mutations were introduced into the region of *BECN1* targeted by the shRNA to control for specificity of the knockdown¹⁵⁹. To visualize the recruitment of HRS to endosomes, cells were treated with EGF-AlexaFluor 555 (EGF-555) to stimulate and monitor trafficking of the EGFR and co-stained with HRS-specific antibodies (Figure 2.1). HRS localization was primarily diffuse in the cytoplasm of serum-starved cells, with a few puncta evident. After stimulation for 10 min, a similar number of EGF-555 positive puncta were detected in *shGFP*, *shBECN1* and *shBECN1:Beclin 1* cells, supporting an equivalent level of EGFR activation. The number of cytoplasmic HRS puncta increased markedly in *shGFP* cells after stimulation, and these puncta co-localized with EGF-555. Significantly fewer HRS puncta were induced by EGF stimulation in *shBECN1* cells, but rescue of Beclin 1 expression restored HRS

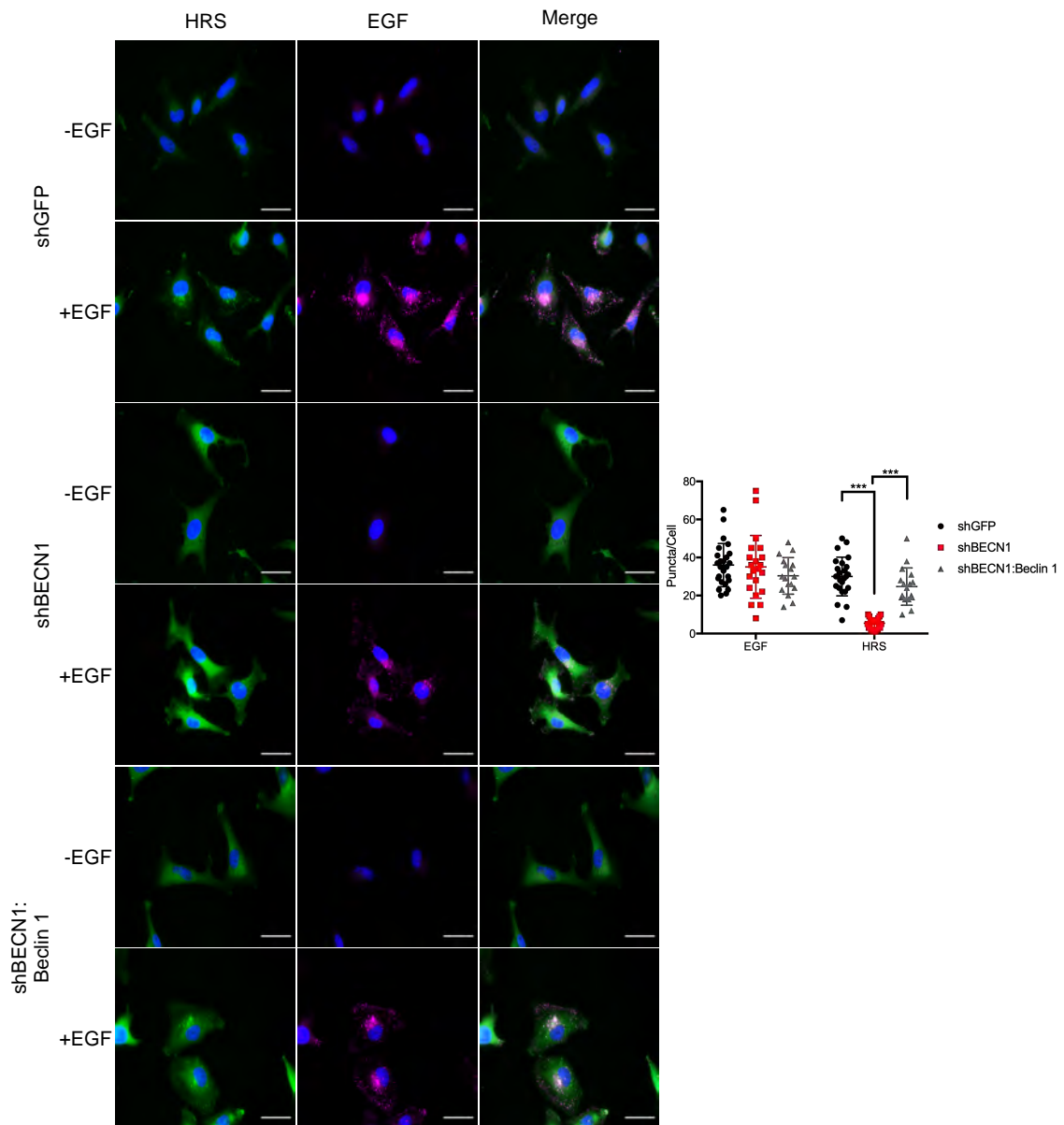


Figure 2.1. *shBECN1* cells exhibit reduced colocalization of EGF and HRS Puncta. MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1* or *shBECN1:Beclin 1* were serum starved and then stimulated with EGF-AlexaFluor 555 (200ng/ml) for 10 minutes. Cells were co-stained with HRS-specific Abs. Scale bar = 10uM. The data shown in the graph represent the number of HRS and EGF puncta per cell. *shGFP* n=25, *shBECN1* n=20 *shBECN1:Beclin1* n=17 *** , p<0.005

puncta formation. These results support that the EGF-stimulated recruitment of HRS to endosomes is regulated in a Beclin 1-dependent manner.

To investigate the Beclin 1-dependent regulation of HRS further, we evaluated the tyrosine phosphorylation of HRS in response to EGF stimulation. HRS is phosphorylated in response to EGFR activation and this phosphorylation event requires PI3P-mediated recruitment of HRS to endosomes, making it a surrogate marker for HRS endosome localization^{165,171,174}. Cell extracts from EGF-stimulated LM2 cells were immunoprecipitated with HRS-specific antibodies and immunoblotted with phosphotyrosine-specific antibodies to evaluate total HRS phosphorylation levels. EGF-stimulated HRS phosphorylation was decreased in cells expressing shRNA targeting two different sites within *BECN1* when compared with *shGFP* cells (Figure 2.2A). HRS phosphorylation was also reduced when Beclin 1 expression was suppressed in another TNBC cell line SUM-159PT (Figure 2.2B). A similar pattern of phosphorylation in *shGFP* and *shBECN1* cells was detected in immunoblots of whole cell extracts using a phospho-specific HRS antibody (pY334-HRS), and the reduced HRS phosphorylation in *shBECN1* cells was increased upon rescue of Beclin 1 expression (Figure 2.2C). In contrast, no difference in EGF-stimulated HRS phosphorylation was detected between *shGFP* cells and LM2 cells expressing two independent shRNA targeting *ATG5* (Figure 2.2D), supporting an autophagy-independent mechanism for this HRS regulation by Beclin 1.

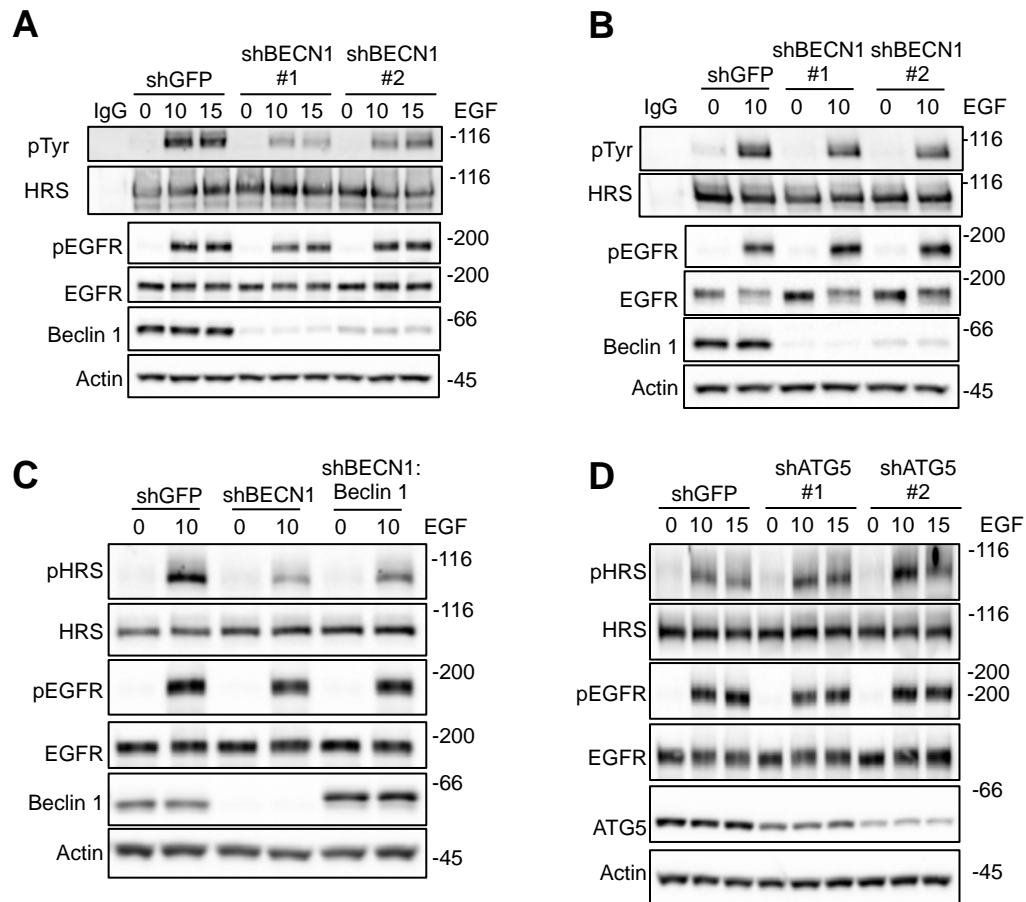


Figure 2.2. Beclin 1 regulates receptor trafficking through HRS. (A) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1* (#1) or *shBECN1* (#2) were stimulated with human EGF (50ng/ml) for the indicated time periods. Total cell extracts were immunoprecipitated with HRS-specific antibodies and immunoblotted with antibodies specific for phosphotyrosine (pTyr). The blot was stripped and re-probed with HRS-specific antibodies. Total cell extracts were also immunoblotted with the indicated antibodies. (B) SUM-159 cells expressing *shGFP*, *shBECN1* (#1) or *shBECN1* (#2) were stimulated with human EGF (50ng/ml) for the indicated time periods. Total cell extracts were immunoprecipitated with HRS-specific antibodies and immunoblotted with antibodies specific for phosphotyrosine (pTyr). The blot was stripped and re-probed with HRS-specific antibodies. Total cell extracts were also immunoblotted with the indicated antibodies. (C and D) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1* or *shBECN1:Beclin 1* (C) or *shGFP*, *shATG5* (#1) or *shATG5* (#2) (D) were stimulated with human EGF (50ng/ml) for the indicated time periods. Total cell extracts were immunoblotted with the indicated antibodies.

Beclin 1 regulates tumor proliferation in an autophagy-independent manner.

To investigate whether Beclin 1/HRS-dependent regulation of receptor trafficking impacts tumor growth, Beclin 1 knockdown and rescue cells were injected into the mammary fat pad (mfp) of NOD/SCID mice. *shBECN1* cells expressing reduced Beclin 1 grew at an increased rate, and the final tumor volume was significantly greater when compared with *shGFP* control tumors (Figure 2.3A). Rescue of Beclin 1 expression (*shBECN1:Beclin 1*) significantly diminished tumor growth rate and size (Figure 2.3A), confirming the specificity of the Beclin 1 knockdown. To explore the autophagy-dependent and independent functions of Beclin 1, LM2 cells expressing shRNA targeting *ATG5* were also evaluated for tumor growth. In contrast to the enhanced tumor growth observed upon suppression of Beclin 1 expression, the growth rate and final tumor volume of *shGFP* and *shATG5* tumors was similar (Figure 2.3C).

To determine the extent to which autophagy was inhibited by Beclin 1 or *ATG5* suppression, autophagic flux was examined by treating cells with either rapamycin to inhibit mTOR and stimulate autophagy, bafilomycin A1 to inhibit lysosomal degradation, or both together¹⁷⁵. A similar reduction in LC3-I to LC3-II conversion was evident in the *shBECN1* and *shATG5* cells (Figure 2.4A), demonstrating that inhibition of autophagy was similar in these cells. Moreover, autophagic flux was restored to control *shGFP* levels in the *shBECN1:Beclin 1*

rescue cells (Figure 2.4B). Importantly, the reduction in autophagy observed *in vitro* was maintained *in vivo* as the processing of LC3-I to LC3-II was decreased for both *shBECN1* and *shATG5* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Figure 2.3B and 2.3D).

Tumor sections were analyzed for either Phospho-Histone H3 (PH3) or TUNEL staining to determine if the enhanced growth observed for *shBECN1* tumors resulted from increased proliferation or decreased cell death, respectively. *shBECN1* tumors exhibited increased PH3 staining compared to *shGFP* and *shBECN1:Beclin 1* tumors (Figure 2.5A). In contrast, no differences in TUNEL staining were detected (Figure 2.5C). Both PH3 and TUNEL staining were equivalent in the *shGFP* and *shATG5* tumors, reflecting their similar growth rates (Figures 2.5B and 2.5D). Taken together, our results support the conclusion that the enhanced tumor growth observed for *shBECN1* tumors does not result from decreased autophagy alone and that alternative functions of Beclin 1 are involved in its regulation of tumor cell proliferation.

Regulation of EGFR and ERK1/2 signaling by Beclin 1 controls tumor proliferation

To explore further the hypothesis that Beclin 1 regulates tumor proliferation through the control of endocytic receptor trafficking, we performed an unbiased high-throughput, quantitative reverse phase protein array (RPPA) to assess the

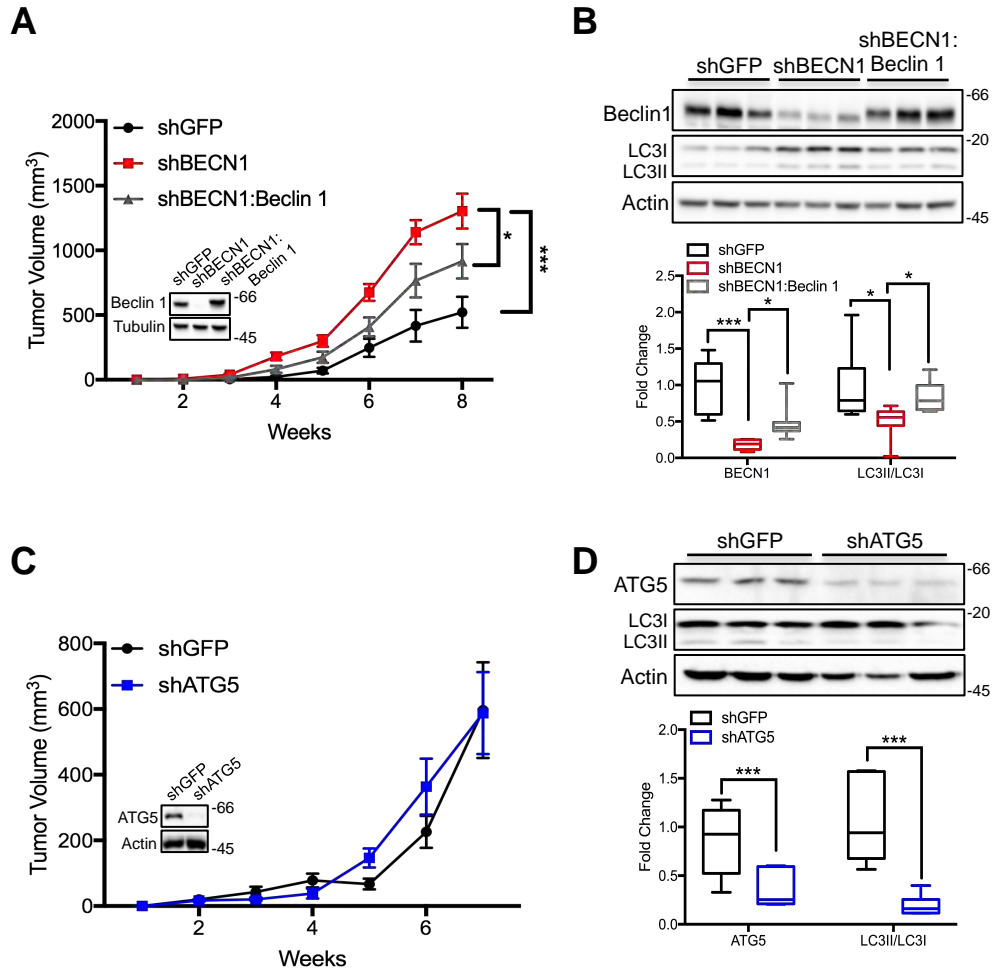


Figure 2.3. Comparison of tumor growth in autophagy deficient cell lines. (A) Tumor growth of orthotopic xenografts in NOD-SCID mice. Inset, Beclin 1 expression prior to injection. (B) Expression of Beclin 1 and LC3II/I in tumors. The data shown in the graph on the right represent the mean \pm SEM expression of seven tumors. (C) Tumor growth of orthotopic xenografts in NOD-SCID mice. Inset, ATG5 expression prior to injection. (D) Expression of ATG5 and LC3II/I in tumors. The data shown in the graph on the right represent the mean \pm SEM expression of six tumors. *, $p < 0.05$; ***, $p < 0.005$

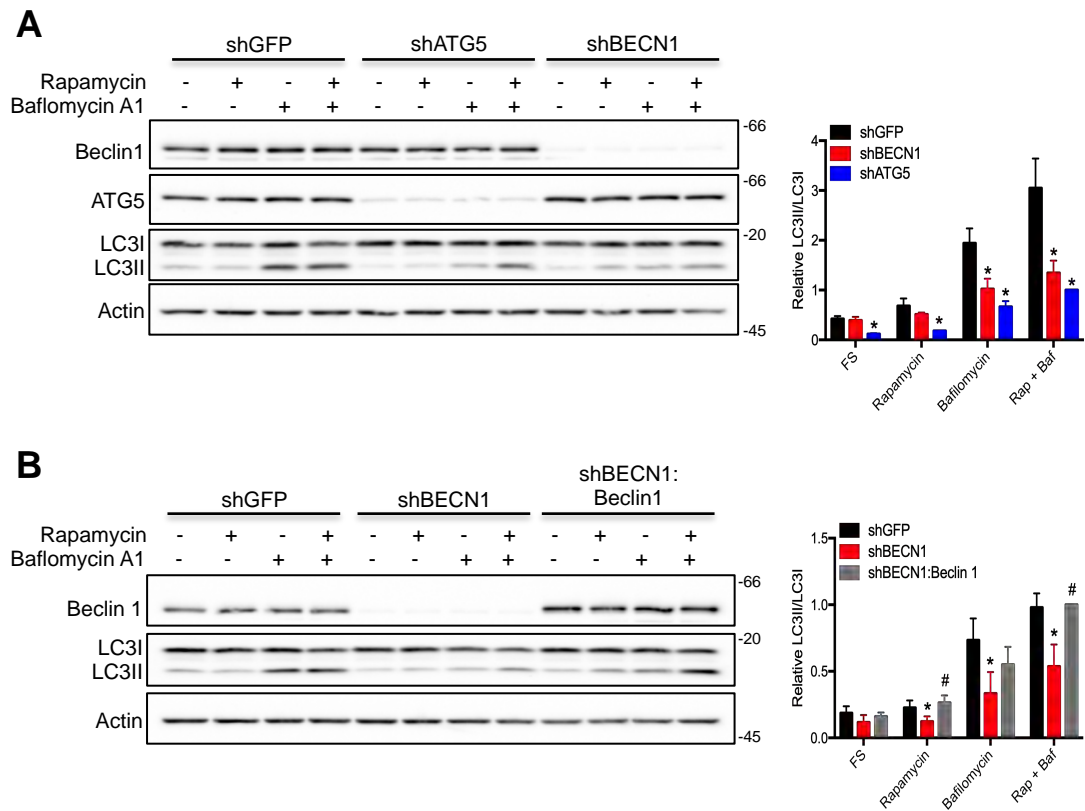


Figure 2.4. Comparison of autophagic flux in autophagy deficient or competent cell lines. (A-B) MDA-MB-231 LM2 cells expressing *shGFP*, *shATG5*, *shBECN1* or *shBECN1* with restored Beclin 1 expression (*shBECN1:Beclin 1*) were assayed for autophagic flux. Cells were incubated for 8 hours in complete serum alone or with 100nM Rapamycin (Rap), 40nM Bafilomycin A1 (Baf) or Rapamycin and Bafilomycin A1 combined (Rap + Baf). The data shown in the graphs on the right represent the mean \pm SEM LC3II/LC3I ratio of three independent experiments. *, $p < 0.05$ relative to *shGFP*; #, $p < 0.05$ relative to *shBECN1*

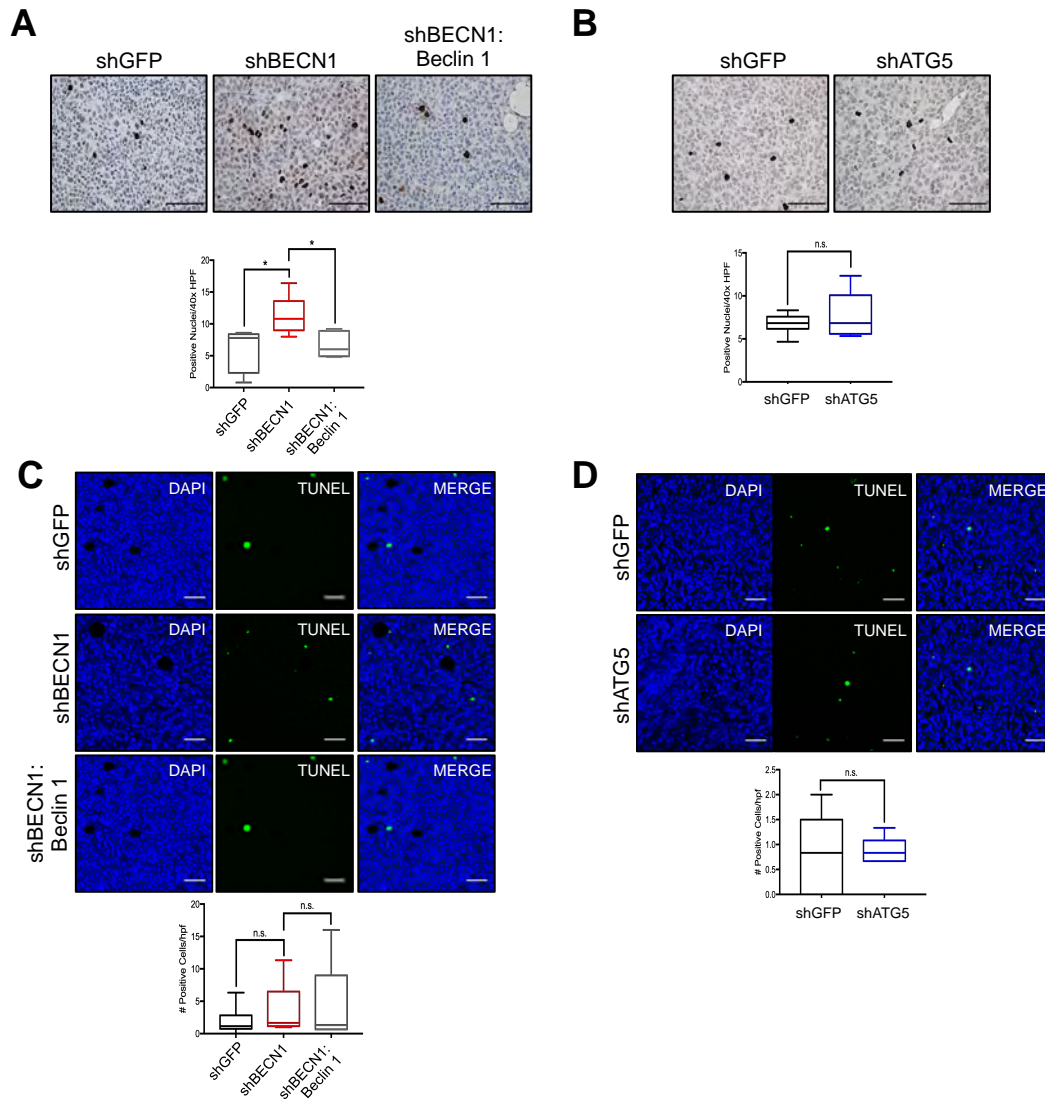


Figure 2.5. Comparison of proliferation and TUNEL staining in tumors. (A-B) Representative images of Phospho-histone H3 (PH3) staining in *shGFP*, *shBECN1*, *shBECN1:Beclin 1* and *shATG5* tumors. The data shown in the graphs represent the mean \pm -SEM positive nuclei/high powered field (hpf; five independent images/five tumors; $n = 25$). Scale bar = 50 μ M. (C-D) Representative images of TUNEL staining in *shGFP*, *shBECN1*, *shBECN1:Beclin 1* and *shATG5* tumors. The data shown in the graphs represent the mean \pm -SEM positive nuclei/hpf (three independent images/six tumors; $n = 18$). Scale bar = 50 μ M. *, $p < 0.05$

expression of 302 proteins and phosphoproteins that have important functions in cancer^{176,177}. This array included many growth factor receptors and downstream signaling effectors that have been implicated in tumor proliferation. Tumor lysates from three tumors of each genotype (*shGFP*, *shBECN1* and *shBECN1:Beclin 1*) were analyzed by RPPA. Unsupervised hierarchical clustering of the Z-scored data revealed segregation of the *shBECN1* tumors from the *shGFP* and *shBECN1:Beclin 1* tumors, with the exception of one *shGFP* tumor that co-segregated with the *shBECN1* tumors (Figure 2.6). K means clustering was used as an unbiased approach to identify changes in expression patterns that are unique to *shBECN1* tumors. Based on an analysis of the root-mean-square error (RMSE) we selected 18 clusters (K=18) as having the optimal balance between the similarity of the signaling profiles within each cluster while maintaining a small overall number of clusters (Figure 2.7A). Of the 18 distinct expression patterns that were identified, sub-clusters 1 and 11 contained proteins and phosphoproteins that exhibited increased expression in *shBECN1* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Figure 2.7B).

Analysis of sub-clusters 1 and 11 identified several growth factor (GF) and hormone receptors (EGFR, IR β , c-KIT, VEGFR2, phosphoHER3) and their downstream signaling intermediates (pY759-phospholipase C gamma2 (PLCg2), pS664-protein kinase C delta (PKCd), and pS116-PEA-15) that were increased in *shBECN1* tumors (Figures 2.8A and 2.8D). In addition, pT202/Y204-

extracellular regulated kinases 1/2 (pERK1/2), major regulators of cell cycle progression, as well as ERK1/2 substrates (pS383-ELK1 and pS318/S321-FOXO3A) were also increased in *shBECN1* tumors (Figures 2.8B and 2.8D). Analysis of all mitogen activated protein kinase (MAPK) pathway components (receptors, kinases and downstream substrates) that were included in the RPPA analysis revealed a significant enrichment for MAPK pathway activity in sub-cluster 1 and elevated pathway activity in sub-cluster 11 (Figure 2.8C). In contrast, increased PI3K/AKT pathway activity was not evident in the *shBECN1* tumors by RPPA analysis, indicating a selective activation of the MAPK signaling pathway in these tumors.

Immunoblot analysis of additional tumors (n=7) confirmed increased EGFR expression and activation of ERK1/2 in *shBECN1* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Figures 2.9A and 2.9B). This analysis also suggested that EGFR is preferentially localized within a signaling competent compartment in *shBECN1* tumors. Specifically, relative EGFR activation, as measured by phosphorylation of Y1068-EGFR, a GRB2 binding site, was similar across all tumors, but downstream ERK1/2 phosphorylation was significantly increased (Figures 2.9A and 2.9B). pS473-AKT levels were not elevated in the *shBECN1* tumors, confirming the RPPA findings that PI3K/AKT signaling is not enriched in the *shBECN1* tumors. Increased EGFR expression and pERK1/2 activity and equivalent AKT activity were also validated in a second cohort of

shGFP and *shBECN1* tumors (Figures 2.10A, 2.10B and 2.10D). In contrast, EGFR expression and ERK1/2 activity were not elevated in *shATG5* tumors (Figures 2.11A and 2.11B), providing further evidence that the regulation of this signaling pathway by Beclin 1 occurs independently of its regulation of autophagy.

EGFR mRNA levels were not significantly different across the three tumor genotypes indicating that Beclin 1 regulates EGFR at the level of protein expression (Figure 2.9C). To examine the hypothesis that this regulation occurs through EGFR endolysosomal trafficking, HRS tyrosine phosphorylation was assessed in the tumors. Overall HRS phosphorylation was lower in the tumors than detected after acute EGF stimulation *in vitro*. However, reduced HRS phosphorylation was detected in the *shBECN1* tumors when compared with *shBECN1:Beclin 1* tumors (Figure 2.9D). These results support our conclusion that Beclin 1 regulates HRS function *in vivo* to control receptor trafficking.

To assess the functional contribution of the EGFR/ERK signaling pathway to the enhanced proliferation observed in *shBECN1* tumors, *shGFP* and *shBECN1* tumor slices were incubated *ex vivo* for 48 hrs in the presence of either the EGFR/HER2 dual inhibitor Lapatinib or PD98059, an inhibitor of MEK, the upstream regulator of ERK1/2 activation¹⁷⁸⁻¹⁸⁰. Tumor morphology was maintained during the *ex vivo* culture period as evidenced by similar H&E

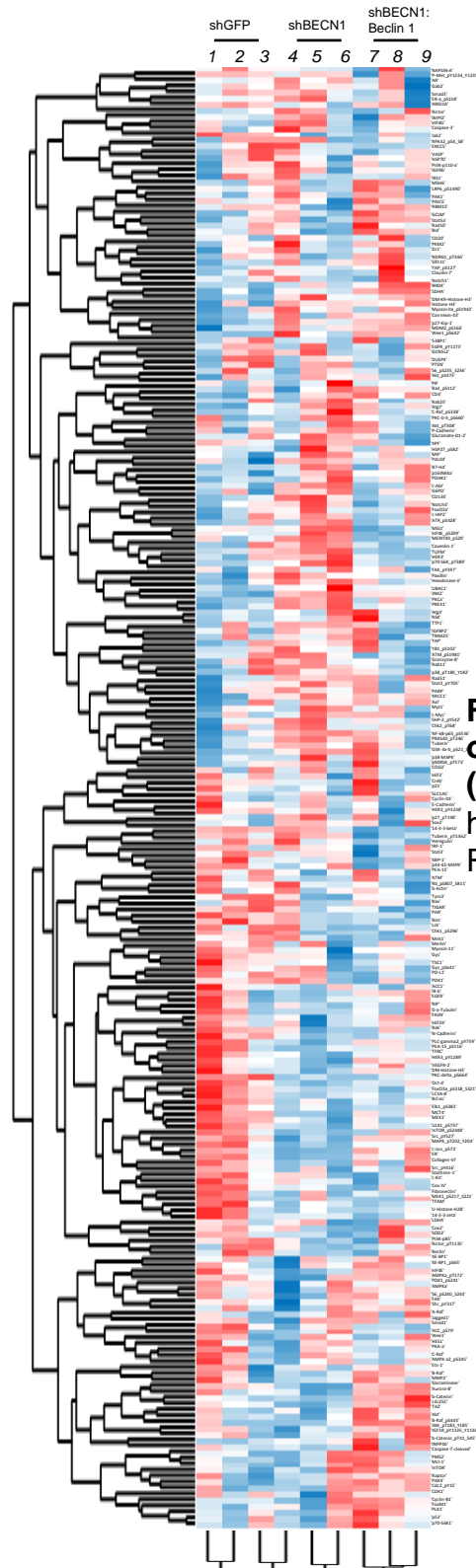


Figure 2.6 Hierarchical Clustering of Reverse Phase Protein Array (RPPA) data. Unsupervised hierarchical clustering of the Z-scored RPPA data.

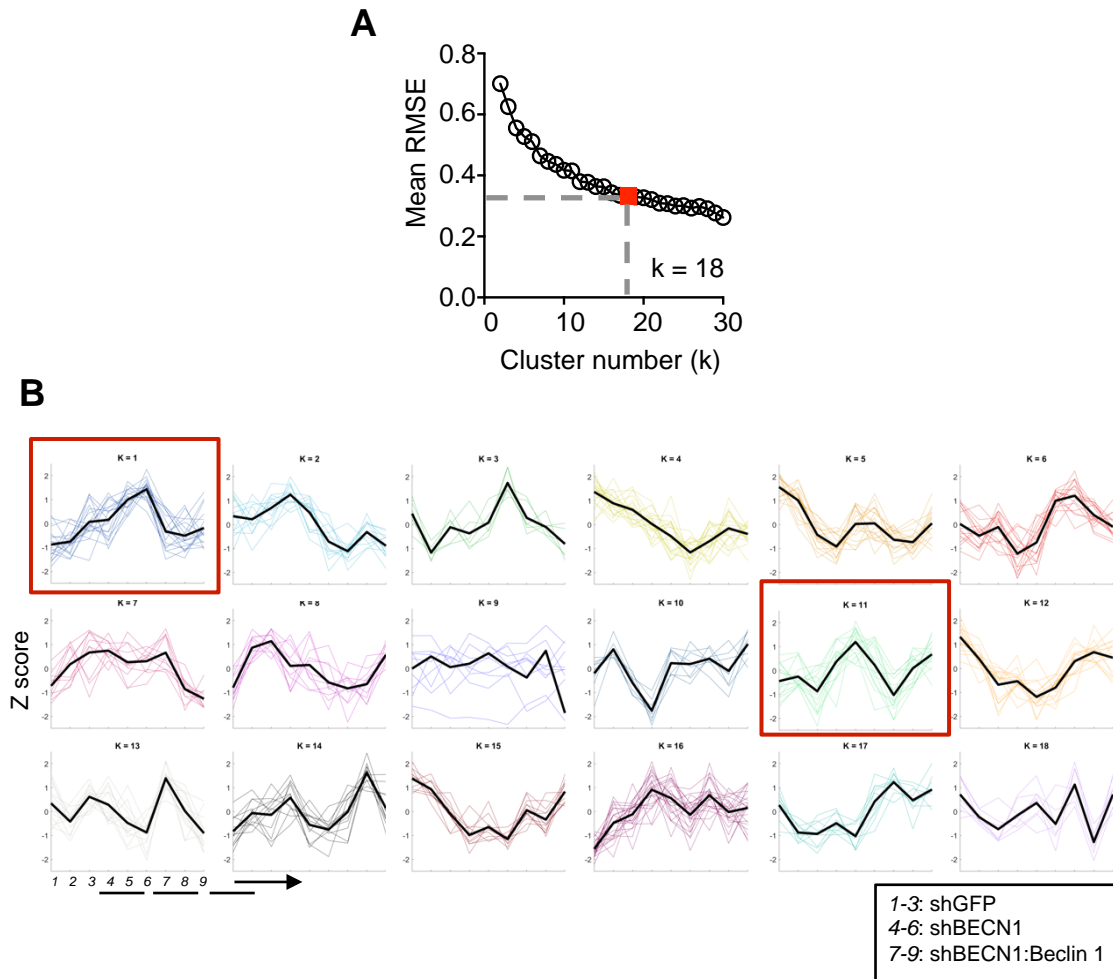


Figure 2.7. RPPA analysis clades of K=18. (A) Root-mean-square error (RMSE) graph from K means clustering analysis. (B) K means clustering analysis of RPPA data from three *shGFP* (1-3), *shBECN1* (4-6) and *shBECN1:Beclin 1* (7-9) tumors. Log^2 data was converted to Z-scores to perform K-means clustering analysis. Images represent consensus plots for K=18 (18 sub-clusters). Red boxes identify sub-clusters with elevated expression patterns in *shBECN1* tumors.

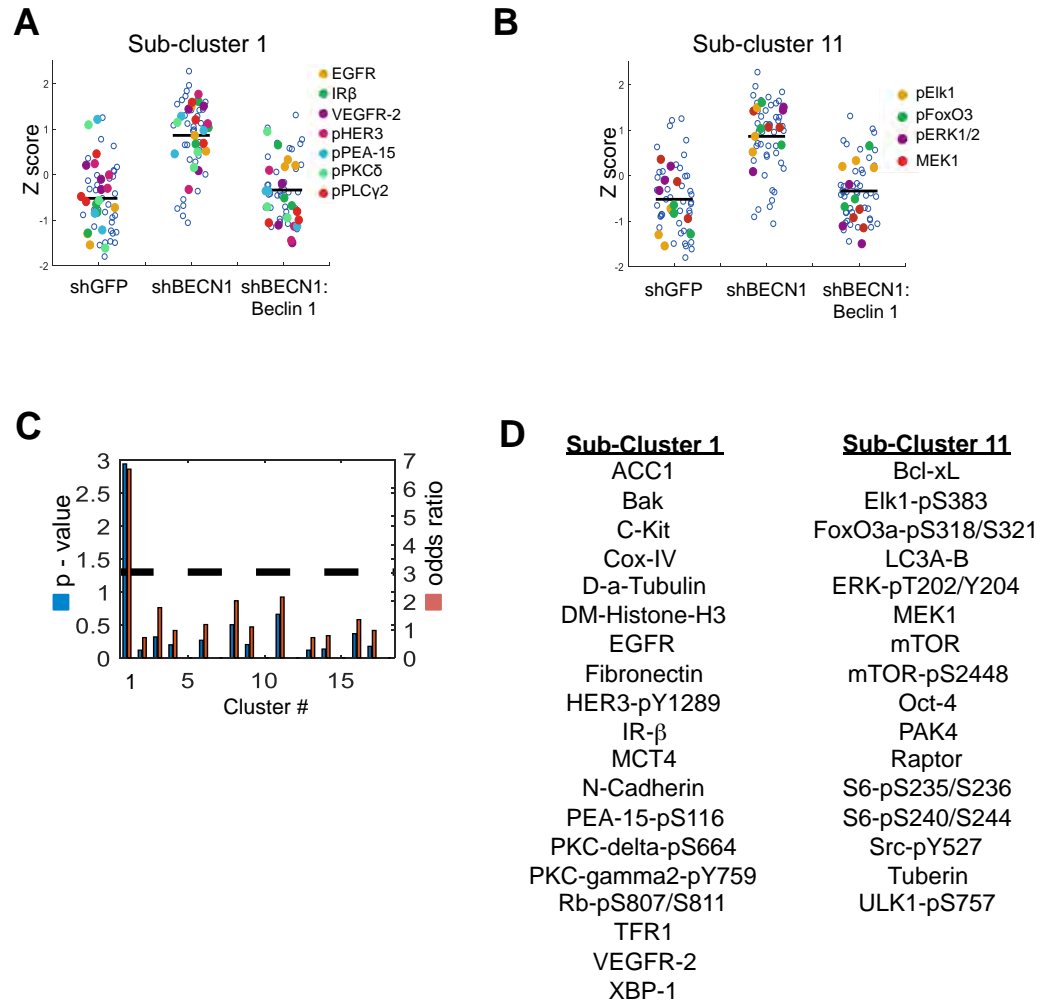


Figure 2.8. RPPA analysis identifies enhanced ERK1/2 signaling pathway activity in *shBECN1* tumors. (A-B) Scatter-plots of sub-cluster 1 (A) and sub-cluster 11 (B) highlighting growth factor/hormone receptors and ERK1/2 signaling pathway activity. (C) Enrichment analysis for a MAPK signaling signature. Orange, odds ratio; Blue, p-value. Dotted line represents $-\log_{10}(1.3)$ which indicates a p value of 0.05. (D) List of proteins/phosphoproteins identified in sub-clusters 1 and 11 from Cluster 18 of the K means clustering analysis.

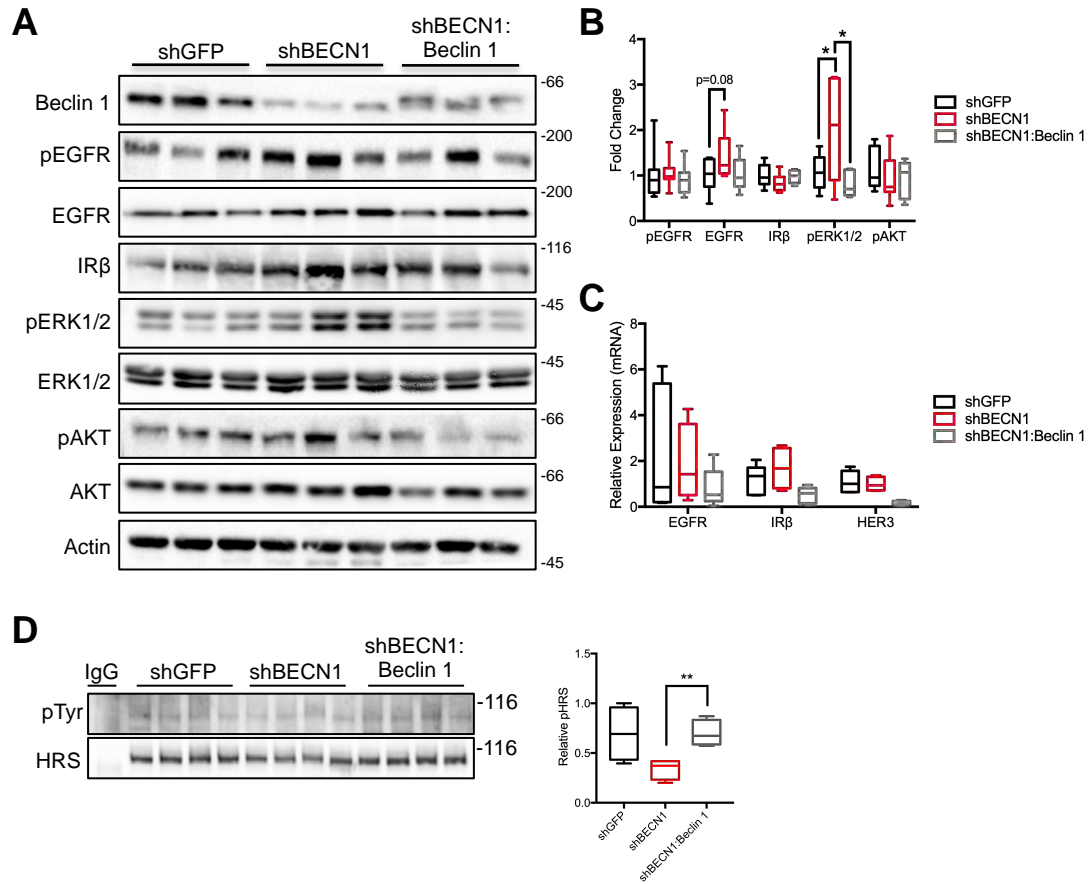


Figure 2.9. Validation of enhanced EGFR/ERK1/2 signaling pathway activity in shBECN1 tumors. (A) Immunoblot analysis of representative *shGFP*, *shBECN1* and *shBECN1:Beclin 1* tumors. (B) The data shown in the graphs represent the mean \pm SEM expression of seven tumors from each genotype and are shown as fold change in expression relative to *shGFP* tumors. (C) Relative mRNA expression was determined by real-time quantitative PCR (RQ-PCR). The data shown represent the mean \pm SEM mRNA expression of five (*shGFP* and *shBECN1:Beclin 1*) or four (*shBECN1*) tumors. $p < 0.05$. (D) Tumor extracts from representative *shGFP*, *shBECN1* and *shBECN1:Beclin 1* tumors were immunoprecipitated with HRS-specific antibodies and immunoblotted with antibodies specific for phosphotyrosine (pTyr). The blot was stripped and re-probed with HRS-specific antibodies. Lanes from the same immunoblot were merged as indicated by the black line. The data shown in the graph represent the mean \pm SEM HRS phosphorylation of four tumors of each genotype and are shown as relative phosphorylation. *, $p < 0.05$

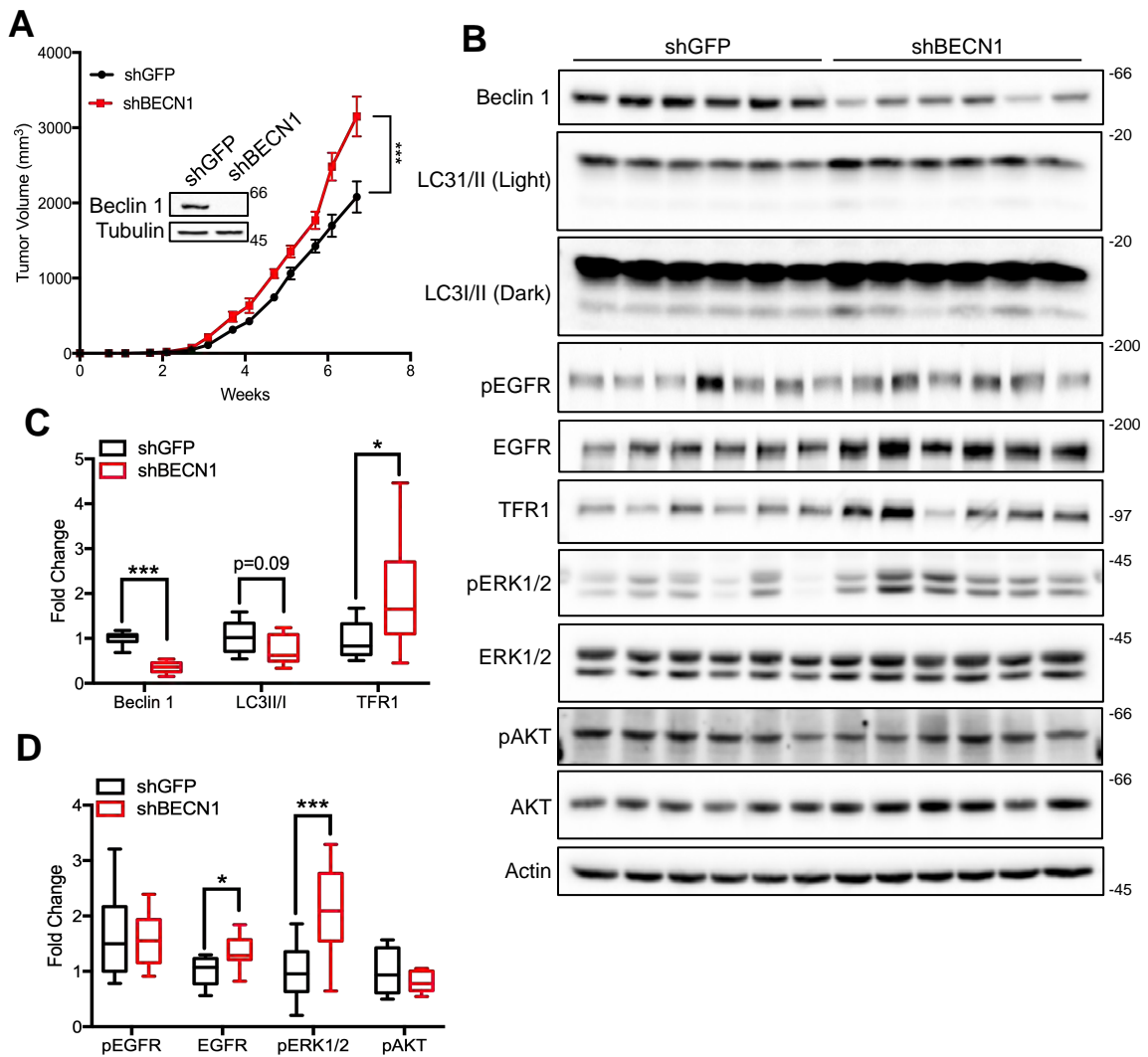


Figure 2.10. Validation of additional orthotopic xenograft tumor study.

(A) Tumor growth of *shGFP* and *shBECN1* LM2 orthotopic xenografts in NOD-SCID mice. Inset, Beclin 1 expression prior to injection. (B) Immunoblot analysis of *shGFP* and *shBECN1* tumors. (C-D) Graphs showing the mean \pm SEM expression of six tumors from each genotype. Data are shown as fold change in expression relative to *shGFP* tumors. $p < 0.05$; ***, $p < 0.005$

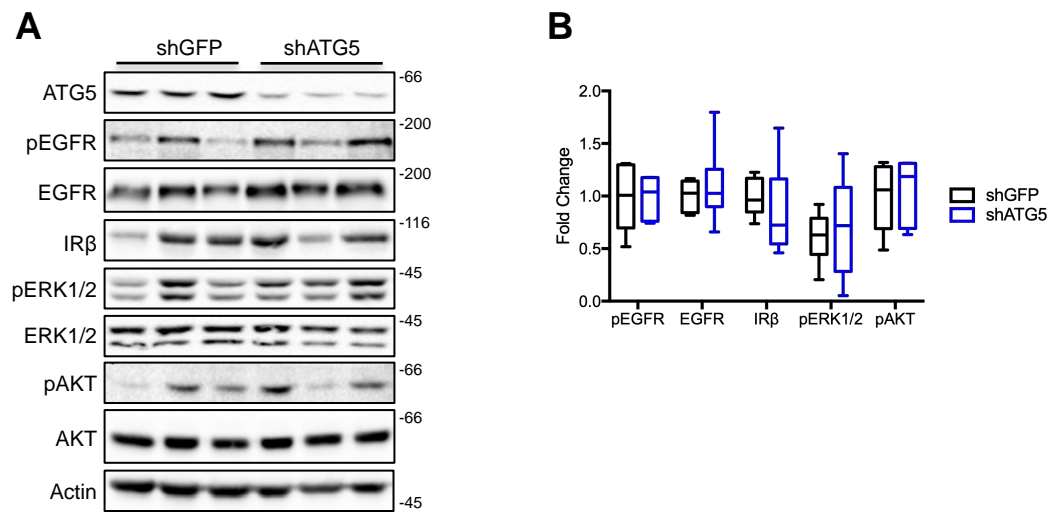


Figure 2.11. *shATG5* tumors do not exhibit enhanced EGFR/ERK1/2 signaling pathway activity. (A) Immunoblot analysis of representative *shGFP* and *shATG5* tumors. (B) The data shown in the graphs represent the mean \pm SEM expression of six tumors from each genotype and are shown as fold change in expression relative to *shGFP* tumors.

staining in tumors that were immediately fixed (untreated) or incubated *ex vivo* (DMSO) (Figure 2.13).

Pathway activity in the *ex vivo* tissue slices and inhibition of activity by the drugs were confirmed by immunoblotting tumor extracts (Fig 2.12A). EGFR activity (pY1068-EGFR) was inhibited significantly by Lapatinib in both *shGFP* and *shBECN1* tumors. MEK activity, as measured by pT202/Y204-ERK1/2 levels, was also inhibited significantly in both tumor genotypes by PD98059, but pEGFR levels remained the same in the presence of this drug. ERK1/2 phosphorylation was not inhibited in response to Lapatinib treatment, which may reflect the fact that these tumors express constitutively active mutant Ras that acts downstream of the EGFR and sustains ERK1/2 activation in the presence of this drug¹⁸¹. ERK1/2 function is regulated at both the level of activation (phosphorylation) and localization, with transition from the cytoplasm to the nucleus required for growth factor-dependent cell cycle entry¹⁸². Therefore, we assessed the localization of ERK1/2 in tumor sections treated with Lapatinib (Figure 2.12B). Homogeneous staining was evident in the DMSO treated tumors, indicating that ERK1/2 was present in both the cytoplasm and nucleus. In contrast, treatment with Lapatinib resulted in a decrease in nuclear staining (white arrows), indicating that ERK1/2 function was inhibited by this drug treatment.

The *ex vivo* tumor sections were analyzed for Ki67 expression by IHC staining to assess proliferation (Figure 2.13). *shBECN1* tumors exhibited increased Ki67 staining when compared with *shGFP* tumors, indicating that the enhanced proliferation observed *in vivo* was maintained during the *ex vivo* incubation period. Ki67 expression was reduced significantly in the *shBECN1* tumors in response to both Lapatinib and PD98059 treatment. Although a similar trend was observed for *shGFP* tumors, the decrease in Ki67 staining was not significant for either drug, suggesting that the enhanced EGFR/ERK signaling that occurs in *shBECN1* tumors renders their proliferation more dependent upon this signaling pathway and more sensitive to inhibition by these drugs.

Beclin 1 regulates Transferrin Receptor-1 (TFR1) expression to drive tumor proliferation

Additional analysis of our RPPA data revealed that TFR1 expression was significantly upregulated in *shBECN1* tumors (Figure 2.14A). The ability of cells to proliferate requires not only a growth factor stimulus but also the appropriate metabolic conditions to support the anabolic processes that must occur for a cell to divide¹⁸³. Iron is an essential nutrient cofactor for enzymes that are involved in DNA synthesis and cell cycle and it is required for proliferation^{184,185}. Extracellular iron is bound by transferrin and transported into cells by endocytic trafficking of TFR1¹⁵³. TFR1 expression correlates with proliferative capacity and receptor levels are elevated in tumor cells to satisfy the increased iron demand of

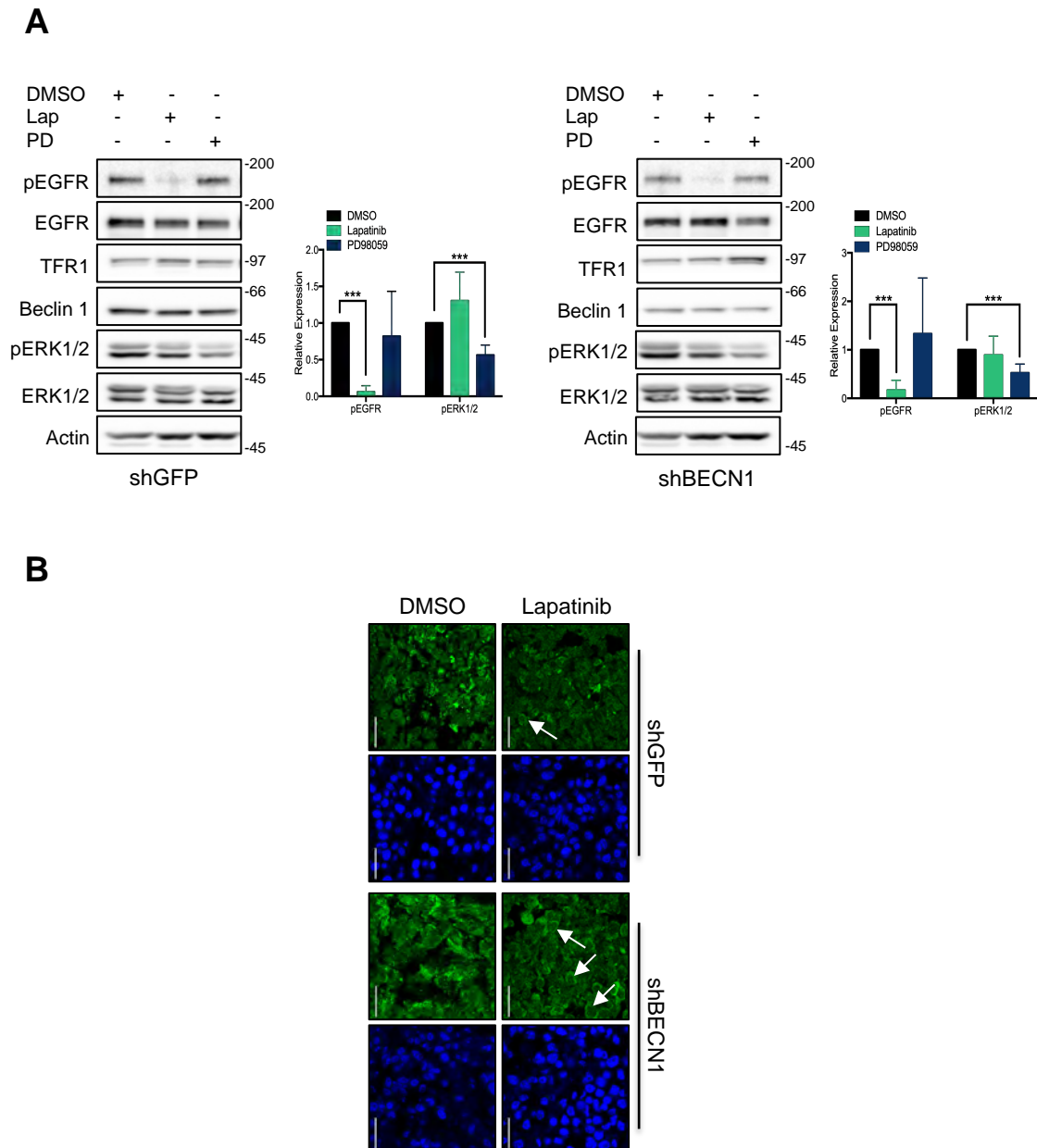


Figure 2.12. Inhibition of EGFR and ERK1/2 signaling in tumors. (A) Immunoblot analysis of representative *shGFP* and *shBECN1* tumors treated *ex vivo* for 48 hrs with DMSO, Lapatinib (Lap; 5uM), or PD98059 (PD; 10uM). The data shown in the graphs represent the mean \pm SEM expression of eight tumors of each genotype. (B) Immunofluorescent staining for ERK1/2 expression in representative *ex vivo* tumors treated with DMSO or Lapatinib. Arrows indicate representative cells with reduced nuclear localization of ERK1/2. Scale bar = 50uM. $p < 0.05$; ***, $p < 0.005$

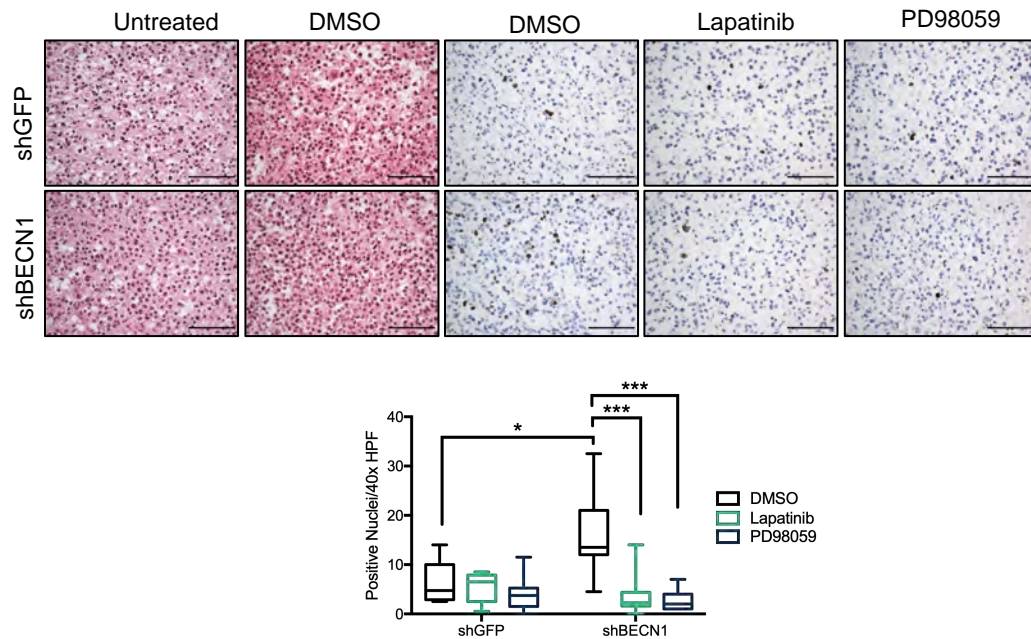


Figure 2.13. Proliferation in *shBECN1* tumors is sensitive to inhibition of EGFR and ERK1/2 signaling. Representative images of H&E or Ki67 staining of *shGFP* and *shBECN1* tumors treated *ex vivo* as indicated. The data shown in the graph below represent the mean \pm SEM positive nuclei/hpf (three independent images/five tumors; n = 15). Scale bar = 50uM. *, $p < 0.05$; ***, $p < 0.005$

these rapidly dividing cells^{184,186}. Increased expression of TFR1 in *shBECN1* tumors and restoration of expression to *shGFP* levels in *shBECN1:Beclin 1* tumors was confirmed by immunoblotting (n=13 tumors) (Figures 2.14B and 2.14C). Similar to EGFR mRNA expression, *TFRC* mRNA levels were equivalent across the tumor genotypes (Figure 2.14D), indicating that increased TFR1 expression in *shBECN1* tumors also occurs at the level of protein expression. TFR1 protein expression did not increase in *shATG5* tumors, supporting that the upregulation of TFR1 expression in *shBECN1* tumors occurs in an autophagy-independent manner (Figure 2.14E).

The link between Beclin 1 and TFR1 was unexpected because TFR1 is typically sorted in the early endosome for constitutive recycling back to the cell surface. As a result of this recycling, expression remains constant. However, TFR1 can be ubiquitinated by members of the membrane associated RING-CH (MARCH) family of ubiquitin ligases and this ubiquitination targets TFR1 for lysosomal degradation^{187,188}. We hypothesized that TFR1 is ubiquitinated in the tumor microenvironment and TFR1 levels increase in tumors with low Beclin 1 expression because these ubiquitinated receptors escape HRS-mediated sorting to the lysosome for degradation. In support of this mechanism of regulation by Beclin 1, elevated TFR1 expression was associated with increased ubiquitination in *shBECN1* tumors (Figure 2.14F).

To determine if increased TFR1 expression contributes to the enhanced proliferation of *shBECN1* tumors, LM2 cells were co-infected with shRNA targeting *BECN1* and *TFRC*. Cells with a modest suppression of TFR1 expression, resulting in expression levels equivalent to the levels observed in *shGFP* cells, were selected for further *in vivo* analysis. Restoration of TFR1 expression to control *shGFP* tumor levels inhibited the enhanced tumor growth observed in cells expressing *shBECN1* alone (Figure 2.15A and 2.15B). Tumor sections were analyzed for PH3 or TUNEL staining to determine if the reduced growth observed upon suppression of TFR1 expression in the *shBECN1* tumors was the result of decreased proliferation or increased cell death, respectively (Figures 2.15C and 2.15D). As we observed previously (Figure 2.5A and 2.5C), *shBECN1* tumors exhibited increased PH3 staining compared to *shGFP* tumors and no differences in TUNEL staining were detected. *shBECN1:shTFRC* tumors exhibited PH3 and TUNEL staining equivalent to *shGFP* tumors, indicating that Beclin 1-dependent control of TFR1 expression contributes to tumor cell proliferation.

We infer from our receptor trafficking and *in vivo* data that low HRS expression in human tumors should be associated with poor patient outcomes. To assess the significance of HRS expression in human breast cancer, the impact of HRS expression on patient outcomes was analyzed using Kaplan-Meier plotter¹⁸⁹. Low HRS expression significantly correlated with reduced relapse-free survival

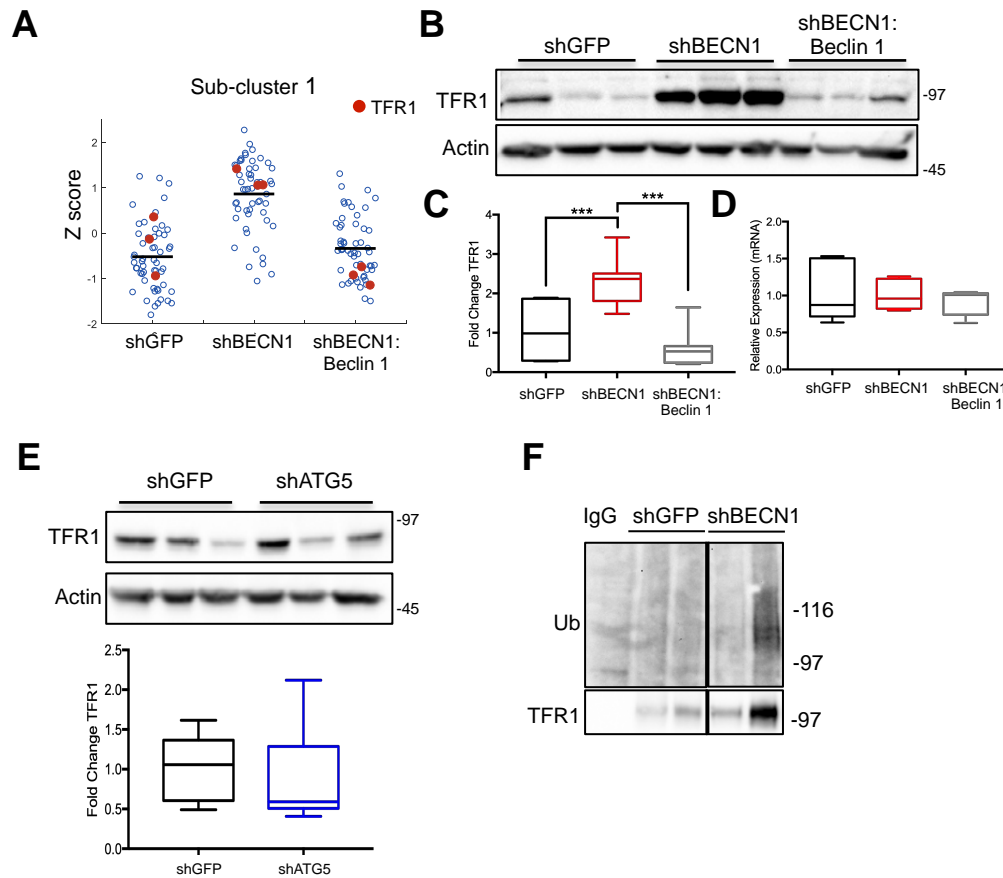


Figure 2.14. TFR1 expression is elevated in *shBECN1* tumors. (A) Scatter-plot of sub-cluster 1 from the K means clustering analysis of RPPA data highlighting TFR1 expression in the triplicate tumors of each genotype. (B) Expression of TFR1 in *shGFP*, *shBECN1* or *shBECN1:Beclin 1* tumors. (C) The data shown in the graph represent the mean \pm SEM TFR1 expression from thirteen tumors of each genotype and are shown as fold change in expression relative to *shGFP* tumors. (D) *TFRC* mRNA expression in *shGFP*, *shBECN1*, or *shBECN1:Beclin 1* tumors. The data shown represent the mean \pm SEM *TFRC* expression from five tumors of each genotype. (E) Expression of TFR1 in *shGFP* and *shATG5* tumors. The data shown in the graph represent the mean \pm SEM TFR1 expression from six tumors of each genotype and are shown as fold change in expression relative to *shGFP* tumor. (F) Tumor extracts from representative *shGFP* and *shBECN1* tumors were immunoprecipitated with TFR1-specific antibodies and immunoblotted with antibodies specific for Ubiquitin (Ub). The blot was stripped and re-probed with TFR1-specific antibodies. Lanes from the same immunoblot were merged as indicated by the black line. $p < 0.05$; $***$, $p < 0.005$

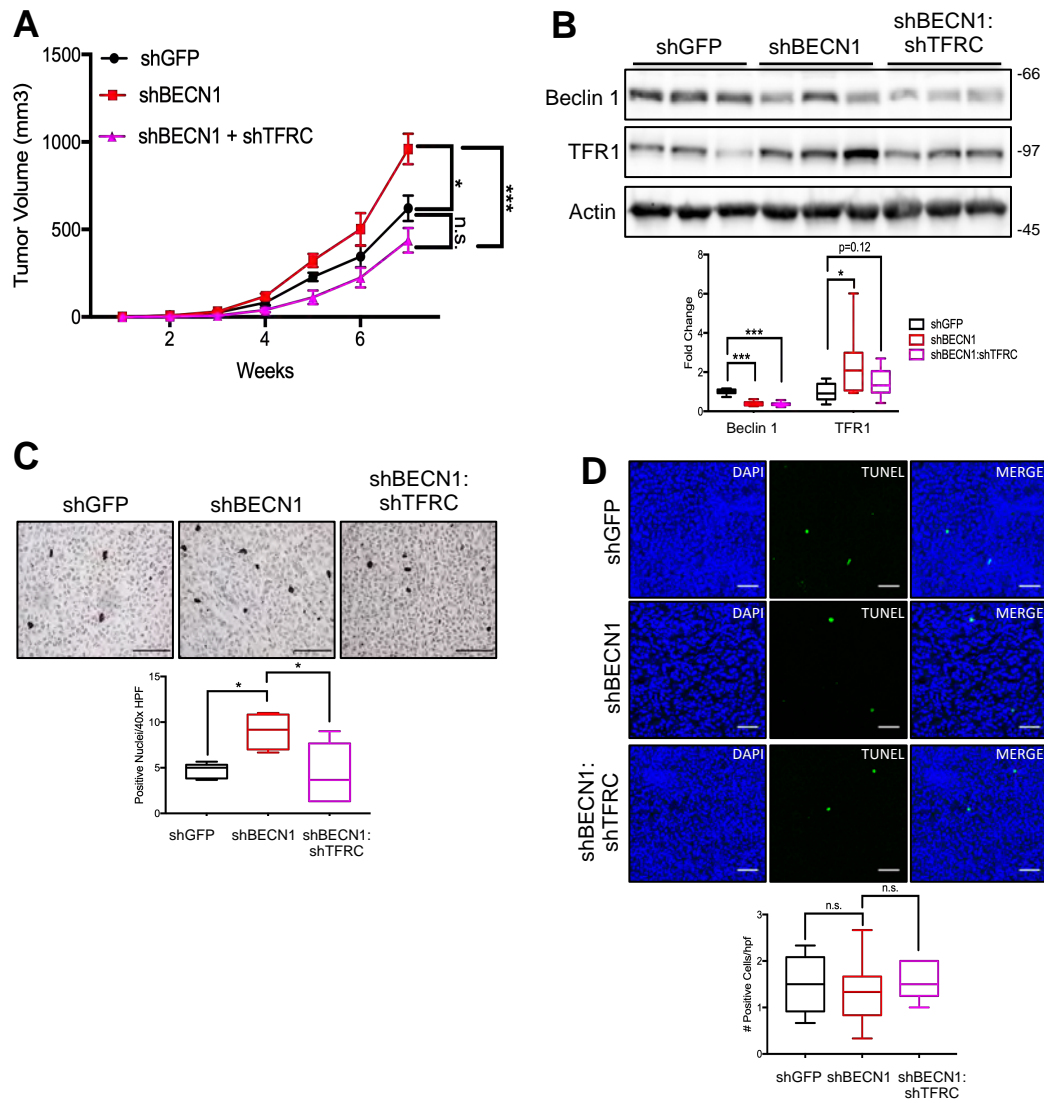


Figure 2.15. Reduction of TFR1 expression in *shBECN1* tumors suppresses tumor proliferation. (A) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1* or *shBECN1:shTFRC* were assayed for tumor growth as orthotopic xenografts in NOD-SCID mice. (B) Expression of Beclin 1 and TFR1 in tumors. The data shown in the graph below represent the mean \pm SEM expression from six tumors of each genotype. (C) Representative images of PH3 staining in *shGFP*, *shBECN1* and *shBECN1:shTFRC* tumors. The data shown in the graph represent the mean \pm SEM positive nuclei/hpf (five independent images/five tumors; $n = 25$). Scale bar = 50 μ M. (D) Representative images of TUNEL staining in *shGFP*, *shBECN1* and *shBECN1:shTFRC* tumors. The data shown in the graph represent the mean \pm SEM positive nuclei/hpf (three independent images/six tumors; $n = 18$). Scale bar = 50 μ M. *, $p < 0.05$; ***, $p < 0.005$

(RFS) when all breast cancer subtypes were analyzed together, and this significance was maintained upon analysis of only Basal subtype tumors (Figure 2.16A). By contrast, HRS expression did not correlate with RFS in HER2 positive tumors.

This lack of significant correlation likely reflects the fact that HER2 is not downregulated by HRS-dependent sorting to the lysosome and therefore the expression and activity of these receptors would not be enhanced if HRS expression was reduced^{190,191}. The inverse association of HRS with RFS supports that the control of receptor trafficking is important for the suppression of tumor progression.

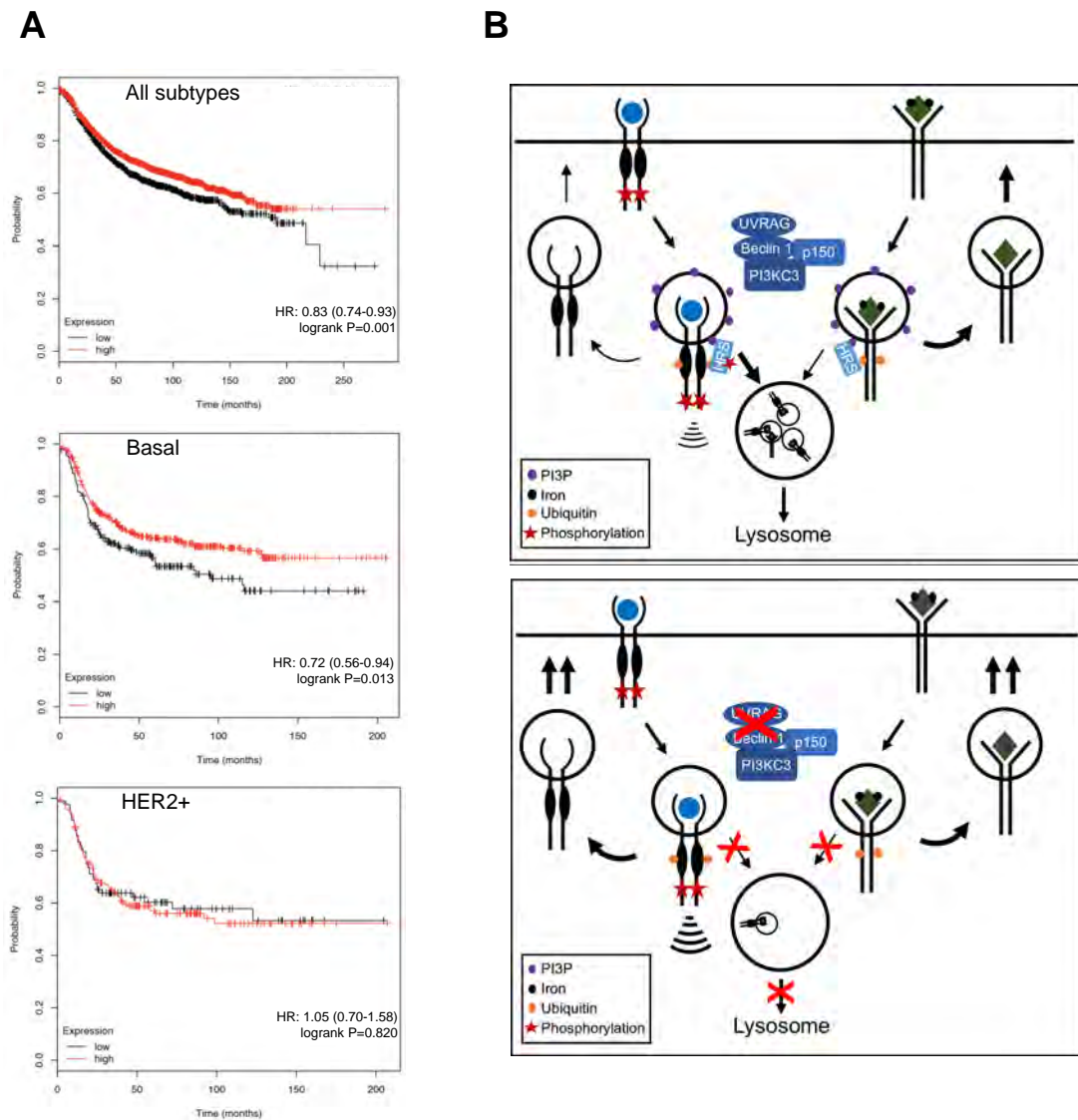


Figure 2.16. Mechanism for Beclin 1 regulation of receptor trafficking through HRS. (A) Kaplan Meier plots showing the impact of HRS expression on the relapse free survival (RFS) of human breast tumors. (B) Model of Beclin 1-dependent regulation of receptor trafficking. In cells expressing Beclin 1, ubiquitinated EGFR and TFR1 are targeted for degradation by HRS-dependent sorting to ILVs and fusion with the lysosome. In cells with reduced Beclin 1 expression, ubiquitinated receptors escape sorting to the ILVs and lysosome because PI3P levels are reduced and HRS recruitment recruited to the early endosomes is inhibited. As a result, EGFR expression and signaling and TFR1 expression are increased.

DISCUSSION

We demonstrate that Beclin 1 regulates endocytic receptor trafficking by an autophagy-independent mechanism and conclude that this function of Beclin 1 contributes to its role as a tumor suppressor. Specifically, we show that Beclin 1 regulates the endosomal recruitment of HRS, which is essential in the sorting of receptors for signal silencing and degradation. When Beclin 1 expression is reduced in tumors, early endosome recruitment of HRS is diminished and expression and activation of receptors that would normally be sorted for degradation persists (Figure 2.16B). A consequence of this prolonged expression and function is increased tumor proliferation. By RPPA analysis, we identified two independent growth regulatory receptors that contribute to enhanced proliferation when Beclin 1 expression levels are decreased. EGFR expression and function are elevated and downstream ERK1/2 activation is increased, and this enhanced activity renders tumor proliferation more sensitive to drugs that target this signaling pathway. Expression of the iron transporter TFR1 is also increased in tumors when Beclin 1 expression is low and this nutrient receptor supports enhanced tumor cell proliferation. Taken together, our data reveal an autophagy-independent mechanism by which Beclin 1 regulates receptor trafficking and provide insight into how reduced Beclin 1 expression in tumors contributes to progression.

Our demonstration that Beclin 1 controls the early endosome recruitment of HRS to impact receptor sorting identifies a novel mechanism by which the expression and functional outcomes of cell surface receptors can be regulated. This regulation can be mediated through either changes in Beclin 1 expression, which occurs in tumors and is modeled in our current studies, or function, such as through post-translational modifications of Beclin 1 that disrupt its interactions with PI3KC3. For example, phosphorylation of Beclin 1 by EGFR or AKT inhibits its interaction with PI3KC3, resulting in decreased lipid kinase activity^{192,193}. Ubiquitination of Beclin 1 also reduces PI3KC3 activation by targeting Beclin 1 for proteasomal degradation¹⁹⁴. These modifications of Beclin 1 inhibit PI3P production, which prevents HRS recruitment and delays receptor sorting to the lysosome. Beclin 1 post-translational modifications likely regulate the duration of receptor signaling and expression in normal cells in response to physiological stimuli, and may further alter receptor trafficking when these pathways are activated in tumors. Our data demonstrate an important role for Beclin 1/HRS regulation of EGFR trafficking in TNBC. However, many additional growth regulatory receptors are regulated by endolysosomal trafficking and would be impacted by Beclin 1 expression¹⁹⁵⁻¹⁹⁹. As one example, in *Drosophila*, Atg6 (the *Drosophila* homolog of Beclin 1) regulates Notch and Wingless signaling pathways through the control of lysosomal receptor degradation²⁰⁰. In *Atg6* mutant flies, receptor signaling is sustained which results in cell polarity and developmental defects. Future studies are warranted to determine if the

expression and activity of other receptors that are downregulated by endolysosomal trafficking are enhanced in tumors upon reduction of Beclin 1 expression and if this mechanism of regulation contributes to their oncogenic properties.

Although Beclin 1 has been implicated as a tumor suppressor, the mechanisms involved have not been well characterized^{42,133}. Our conclusion that Beclin 1 controls the endocytic trafficking of growth factor and nutrient receptors that drive tumor proliferation provides novel insight into this problem. Importantly, this mechanism of action may explain conflicting reports on the role of Beclin 1 as a tumor suppressor. We discovered that the expression and function of the EGFR and downstream activation of ERK1/2 increased in *shBECN1* tumors and that this enhanced signaling promoted tumor proliferation. This result is consistent with the fact that TNBC is frequently associated with elevated EGFR expression and activity²⁰¹. However, we posit that the functional impact of Beclin 1 loss in an individual tumor will likely reflect the level of addiction to a specific receptor signaling pathway and whether it is controlled by HRS and endocytic trafficking. For example, heterozygous Beclin 1 loss enhances tumor development and growth in a mouse mammary tumor model driven by WNT-1, which acts through Frizzled receptors¹³⁵. EGFR, TFR1 and the Frizzled receptors are cell surface receptors whose expression and function are regulated by endolysosomal trafficking^{153,202,203}. In contrast, mammary tumorigenesis and growth are not

enhanced by heterozygous loss of Beclin 1 in mouse models driven by either the polyoma-midline T oncogene (PyMT) or HER2²⁰⁴. PYMT is a cytoplasmic protein that regulates activation of PI3K, MAPK and Src signaling pathways independently of upstream receptor regulation²⁰⁵. Therefore, disruption of HRS-mediated endocytic sorting would not be anticipated to enhance signaling and promote tumor growth in this model. Although HER2 is a surface receptor that is internalized into the endocytic pathway, it is not targeted for degradation but instead is preferentially sorted to the recycling endosome¹⁹⁰. In fact, heterodimerization of HER2 with EGFR inhibits EGFR degradation and promotes recycling to the cell surface¹⁹¹. Therefore, disruption of the signals that promote receptor sorting to the endolysosomal pathway would not be expected to enhance HER2 expression or function, and tumor growth would not be promoted by loss of Beclin 1 expression. In this regard, HRS expression is not predictive of outcomes in HER2 positive tumors.

Our implication of Beclin 1 in the regulation of TFR1 expression is novel and significant for understanding how Beclin 1 affects tumor proliferation. Iron is an essential nutrient for cell growth and proliferation and enhanced iron metabolism is commonly observed in tumors to support their rapid proliferation^{184,206}. In breast cancer, iron levels are increased when compared with normal breast tissue and an iron-regulatory gene signature is prognostic for patient outcome²⁰⁷. As TFR1 is the major source of iron uptake into cells, regulating its expression is

key to maintaining iron homeostasis. TFR1 expression can be regulated in an iron-dependent manner at the level of mRNA stability through the binding of iron-responsive proteins-1 (IRP-1) and IRP-2) to elements in the 3' untranslated region^{208,209}. However, TFR1 protein expression can also be regulated through ubiquitination and sorting to the lysosome for degradation, a mechanism that allows for the acute regulation of metabolically available iron, or the labile iron pool^{188,210,211}. Our finding that Beclin 1 regulates TFR1 expression at the level of protein expression and that increased TFR1 ubiquitination is observed in *shBECN1* tumors can be explained by decreased HRS endosomal recruitment that allows ubiquitinated TFR1 to escape sorting to the lysosome. Collectively our results provide a novel mechanism by which Beclin 1 coordinates the regulation of both growth factor (EGFR) and nutrient receptors (TFR1) that are important for cell proliferation, and demonstrate how coordinated dysregulation of these pathways upon loss of Beclin 1 expression drives tumor proliferation.

Our study provides insight into opportunities for the clinical management of tumors with low Beclin 1 expression. We observed that *shBECN1* tumors were more sensitive to inhibition of proliferation by EGFR and MEK inhibitors than control tumors, indicating a greater dependence of these tumors on the enhanced EGFR/ERK signaling that occurs when Beclin 1 expression is reduced. Although EGFR expression is frequently upregulated in TNBC, clinical trials of EGFR inhibitors in these patients have not shown overall efficacy²¹². Screening

of patients with low Beclin 1 expression could identify subgroups of patients that would be more sensitive to these drugs, as well as inhibitors of other receptors that are regulated by trafficking, to improve outcomes. TFR1 is also of clinical interest both as a drug target and because of its potential for drug delivery¹⁸⁴. Tumors expressing elevated levels of TFR1, such as we observed in *shBECN1* tumors, would be more sensitive to the inhibition of iron-uptake by antibodies that block TFR1 function or iron chelators^{213,214}. In addition, transferrin-chemotherapeutic drug conjugates that are transported intracellularly by endocytosis of the TFR1 would be more effective in tumors that express low levels of Beclin 1 and elevated TFR1²¹⁵. Tumors with reduced Beclin 1 expression are also anticipated to be more sensitive to drugs that stimulate ferroptosis, an iron-dependent mechanism of cell death, due to their increased iron uptake²¹⁶. Given that Beclin 1 expression is frequently decreased across many human tumors, Beclin 1 could be a clinically relevant biomarker for many cancer patients.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

CHAPTER III

Beclin 1 loss alters systemic and local tumor metabolism

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AUTHOR CONTRIBUTIONS

A.N.M.-O. and L.M.S. were involved in the conception and design of the project and wrote the manuscript. H.L.N designed and performed experiments for Figures 3.1-3.7. J.K.K was involved with the development of methodology and the acquisition and analysis of data.

ABSTRACT

Beclin 1 is a haploinsufficient tumor suppressor that regulates growth factor receptor signaling through endocytic receptor trafficking in an autophagy-independent manner. Growth factor receptor signaling is important for regulating metabolic processes. Beclin 1 is also essential to the autophagic pathway. Reports indicate that autophagy is required for the maintenance of glycolytic capacity in *KRAS* mutant cells *in vitro*. However, a role for Beclin 1 in glycolysis has not been established. Here, we provide *in vivo* evidence for a role for Beclin 1 in tumor metabolism that is autophagy-independent. We discover that Beclin 1 alters glucose metabolism in mice on both the systemic and local tumor levels. Additionally, our results indicate that Beclin 1 may be a negative regulator of lipid metabolism. These data provide insight into the role of Beclin 1 as a tumor suppressor and identify novel clinical targets for future exploration.

INTRODUCTION

Beclin 1 is a haploinsufficient tumor suppressor as loss of one allele leads to the spontaneous development of tumors in mice^{42,45}. Reduced mRNA expression of Beclin 1 is associated with poor prognosis in many cancers, further supporting its role as a tumor suppressor. While Beclin 1 is essential for the initiation of autophagy and loss of this function is thought to be important for tumor initiation, Beclin 1 has additional autophagy-independent functions that contribute to its suppression of tumor progression^{8,46}. Previous studies from our lab demonstrated a role for Beclin 1 in regulating growth factor receptor signaling by controlling the duration of time that active receptors remain within a signaling competent endosome compartment. As discussed in Chapter II, I have demonstrated that this regulation of endocytic receptor trafficking controls tumor proliferation in a TNBC orthotopic mouse model. Specifically, when Beclin 1 expression is suppressed, epidermal growth factor (EGFR) expression is elevated and downstream MAPK signaling is increased. Furthermore, expression of the iron transporter transferrin receptor (TFR1) is also elevated in these tumors, and both pathways contribute to proliferation. Importantly, suppression of another essential autophagy gene, ATG5, does not increase proliferation or enhance EGFR or TFR1 expression, supporting an autophagy-independent role for Beclin 1 in this regulation.

The original hallmarks of cancer included the ability of tumor cells to evade apoptosis, enhance angiogenesis, sustain replicative potential, trigger invasion and metastasis, respond to growth signals and sustain proliferation²¹⁷. More recently, new emerging hallmarks of cancer have been proposed that include the capacity for tumor cells to reprogram their metabolism²¹⁸. This metabolic reprogramming refers to the tumor's ability to alter sugar, fat, and amino acid metabolism to meet increasing energy and biosynthetic intermediate demands for rapid proliferation. It has been known for many years that tumor cells perform glycolysis at higher rates than normal cells to keep up with their energy expenditure and anabolic needs, known as the Warburg effect²¹⁹. However, studies have emerged to show that not only is glucose metabolism reprogrammed, but glutamine and fatty acid metabolism can also be altered. For example, *KRAS* mutant and Myc overexpressing tumors have been shown to rely on glutamine metabolism to support tumor cell proliferation^{220,221}. Additionally, tumor cells can increase lipid content to meet metabolic demands either by endogenous means through de novo lipogenesis (DNL) or by increased uptake from exogenous sources^{222,223}.

There are many mechanisms by which tumors cells alter cellular metabolism. Of relevance to Beclin 1, autophagy has recently been shown to regulate glucose metabolism. Jay Debnath's group demonstrated that inhibition of autophagy in *KRAS* mutant mouse embryonic fibroblasts (MEFs) reduces glucose uptake and

glycolysis²²⁴. This occurs through an autophagy-dependent regulation of GLUT1 recycling to the cell surface that is mediated by sequestration of the RabGAP TBC1D5 by LC3+ autophagosomes. While this regulation was shown in MEFs deficient in *Atg7* and *Atg5*, both of which are essential for the elongation of the autophagophore, the involvement of other autophagy genes that have additional autophagy-independent functions, such as Beclin 1, has not been explored. In our *in vivo* tumor analysis, suppression of Beclin 1 and ATG5 expression led to an equivalent reduction of autophagy, but increased tumor growth was only observed when Beclin 1 expression was reduced. This finding supports the possibility that autophagy-independent functions of Beclin 1 may regulate tumor metabolism by alternative mechanisms to drive proliferation.

In this study, I investigated the contribution of Beclin 1 to tumor metabolism *in vivo*. Local and systemic glucose metabolism was assessed in mice bearing either *shBECN1* or *shATG5* RAS mutant TNBC tumors. My results support an autophagy-independent role for Beclin 1 in the regulation of both tumor metabolism and systemic metabolic homeostasis and reveal a potential role for Beclin 1 in the regulation of fatty acid metabolism.

MATERIALS AND METHODS

Cell Lines and shRNAs. MDA-MB-231 LM2 4175 cells (LM2 cells) were purchased from the laboratory of Juan Massagué (Sloan Kettering). Lentiviral vectors containing short hairpin RNA (shRNA) targeting *GFP*, *BECN1*, and *ATG5*

were obtained from Open Biosystems (Huntsville, AL). MDA-MB-231 LM2 4175 cells were infected with virus for each shRNA and stably expressing cells were selected with 2 μ g/mL puromycin (GoldBio).

Animal care. 6 week old NOD/SCID mice were purchased from Jackson laboratories (Bar Harbor, ME). Mice were housed and cared for in the animal facility of the University of Massachusetts Medical School. The facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and is up to date on regulations of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the National Institutes of Health. Mice were cared for according to university animal care guidelines.

Orthotopic xenograft tumor growth study. 7 week old NOD/SCID mice were injected with 1×10^6 LM2 cells expressing either *shGFP*, *shBECN1* or *shATG5* into the 3rd mammary fat pad. Mice were monitored twice weekly for body weight (g) and tumor dimensions. Tumor volume was calculated as follows: $V = 4/3\pi[(L \times W \times H)/2]$. *In vivo* metabolic studies were initiated at 3 weeks post injection to ensure similar tumor sizes between groups. Mice were euthanized with pentobarbital at the end of the hyperinsulinemic-euglycemic clamp study.

Hyperinsulinemic-Euglycemic clamp study. Survival surgery was performed 5 to 6 days prior to clamp experiments to place an indwelling catheter in the jugular

vein. Mice were fasted overnight the day before the clamp experiment (~12 hours). Conscious mice were continuously infused with human insulin (primed at 150 mU/kg body weight, followed by 2.5 mU/kg/min [Humulin; Eli Lilly, IN])²²⁵ for 2 hrs. To maintain euglycemia, 20% glucose was infused at variable rates during the clamps. Whole-body glucose turnover was assessed with a continuous infusion of [3-³H]glucose (PerkinElmer, Waltham, MA). A bolus of 2-deoxy-d-[1-¹⁴C]glucose (2-[¹⁴C]DG) (PerkinElmer, Waltham, MA) was administered at 75 min after the start of the clamp study to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamp study, mice were anesthetized and tissues were extracted for further analysis.

Biochemical analysis and calculation. Glucose concentrations during the clamp study were measured using 10 μ l of plasma by a glucose oxidase method. Plasma was analyzed on an Analox GM9 Analyser (Analox Instruments, Ltd., London, United Kingdom). Plasma [3-³H]glucose, 2-[¹⁴C]DG, and ³H₂O concentrations were measured after deproteinization of plasma samples²²⁵. Glucose uptake in tissues was analyzed by examining 2-[¹⁴C]DG-6-phosphate (2-[¹⁴C]DG-6-P) content in tissue homogenates. Ion exchange columns were used to separate 2-[¹⁴C]DG-6-P from 2-[¹⁴C]DG in supernatant from tissue homogenates. Plasma insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics, Salem, NH).

Basal hepatic glucose production (HGP) was determined as the ratio of basal [^3H] glucose infusion rate to specific activity of glucose at the end of the basal period²²⁵. Insulin stimulated whole body glucose turnover was determined as the ratio of [^3H] glucose infusion rate to specific activity of glucose at the end of the clamp period²²⁵. The insulin-stimulated rate of HGP was determined by subtracting the glucose infusion rate from whole-body glucose turnover. Insulin-stimulated glucose uptake in tissues was measured by analyzing tissue concentration of 2-[^{14}C]DG-6-phosphate and the plasma 2-[^{14}C]DG.

Labeled palmitate and glucose uptake study. Mice were starved for 5 hours prior to study initiation. Following starvation, mice were injected with [^{14}C]deoxy-D-glucose (10 uCi; NET-328, PerkinElmer) intravenously in awake mice. After 25 minutes, mice were given 2-[^3H] Palmitate (30 uCi) intravenously and blood samples were collected every minute for 5 minutes. At the 30-minute time point, mice were euthanized with sodium pentobarbital (150 mg/kg body weight). Tumor and lower limb muscles were removed and analyzed to measure tissue specific glucose and fatty acid uptake.

Body composition and energy balance. Magnetic resonance spectroscopy (^1H -MRS) (Echo Medical Systems, Houston, TX) was used as a noninvasive measurement for whole-body fat and lean masses. Metabolic cage studies were used to measure indirect calorimetry and energy balance parameters such as food/water intake, energy expenditure, respiratory exchange ratio, and physical

activity (TSE-Systems, Inc., Bad Homburg, Germany). TSE-Systems LabMaster platform was used with fully automated monitoring for food and water intake and activity. LabMaster cages are similar to UMMS facility cages; therefore UMMS bedding was used in cages to minimize animal anxiety during the experiment. TSE systems provide intuitive software that allows for flexible experimental design and data analysis.

RPPA Analysis. Frozen pieces of three tumors of each genotype (*shGFP*, *shBECN1*, and *shBECN1:Beclin 1*) were sent to the MD Anderson Cancer Center Reverse Phase Protein Array (RPPA) Core Facility. RPPA was performed according to their previously published protocol using the standard antibody list updated 3.

Immunoblotting. Frozen tumors were extracted on ice in Tissue Protein Extraction Buffer containing 1mM sodium orthovanadate, 10mM NaF and protease inhibitors (Complete Mini Tab). Tumor extracts containing equivalent amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously^{161,162}. Bands were detected by chemiluminescence using a ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA) and band intensities were quantified by densitometry using Image Lab (Beta 1) or Image J. Only signals within a linear range were used for quantitation and signals were normalized to total protein and/or housekeeping genes.

RESULTS

Beclin 1 and ATG5 are differentially required for glucose uptake *in vivo*. My *in vivo* tumor studies revealed an autophagy-independent role for Beclin 1 in the regulation of tumor proliferation that involves endocytic receptor trafficking (Chapter II). Proliferation requires both growth factor stimuli and appropriate metabolic conditions to support the biosynthetic processes necessary for cell division to occur. To investigate if Beclin 1 regulates tumor cell metabolism to support enhanced proliferation, I performed a hyperinsulinemic-euglycemic clamp study to evaluate tumor glucose uptake *in vivo*. Mice bearing either *shGFP*, *shBECN1* or *shATG5* tumors were analyzed at 3 weeks of tumor growth because tumor size and volume was equivalent across all three tumor groups at this time (Figure 3.1A and 3.1B).

Awake mice were kept euglycemic (100 mM basal glucose) while maintaining a hyperinsulinemic state to assay insulin stimulated glucose uptake in peripheral tissues and tumors (Figure 3.2A, clamp). Hepatic glucose production was suppressed following glucose infusion to ensure that all glucose measurements were from the exogenous glucose source (Figure 3.2B, clamp). Following the clamp study, tumors and peripheral insulin-sensitive tissues (i.e., muscle, adipose) were removed and glucose uptake was assessed. Glucose uptake was reduced significantly in *shBECN1* tumors when compared with uptake in *shGFP* tumors (Figure 3.3A). However, in contrast to published *in vitro* observations,

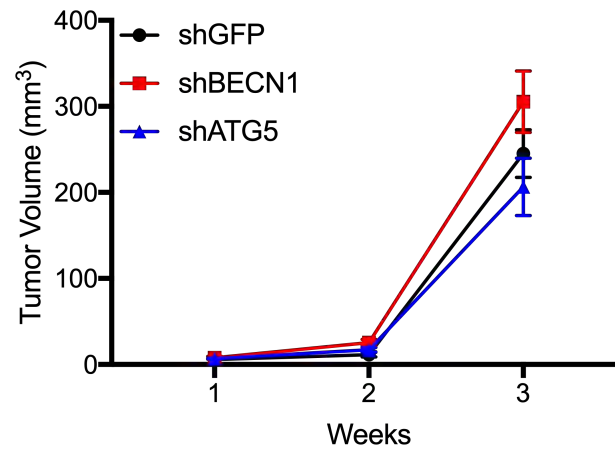
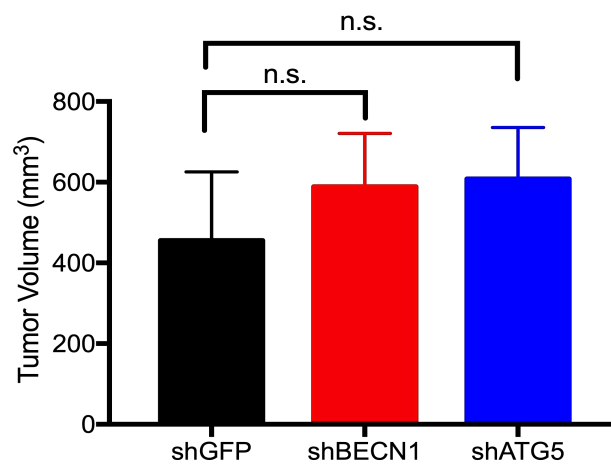
A**B**

Figure 3.1. Tumor growth is equivalent prior to Hyperinsulinemic-Euglycemic Clamp study. (A) Tumor growth of orthotopic xenografts in NOD-SCID mice. (B) Tumor volume of tumors during the hyperinsulinemic-euglycemic clamp study.

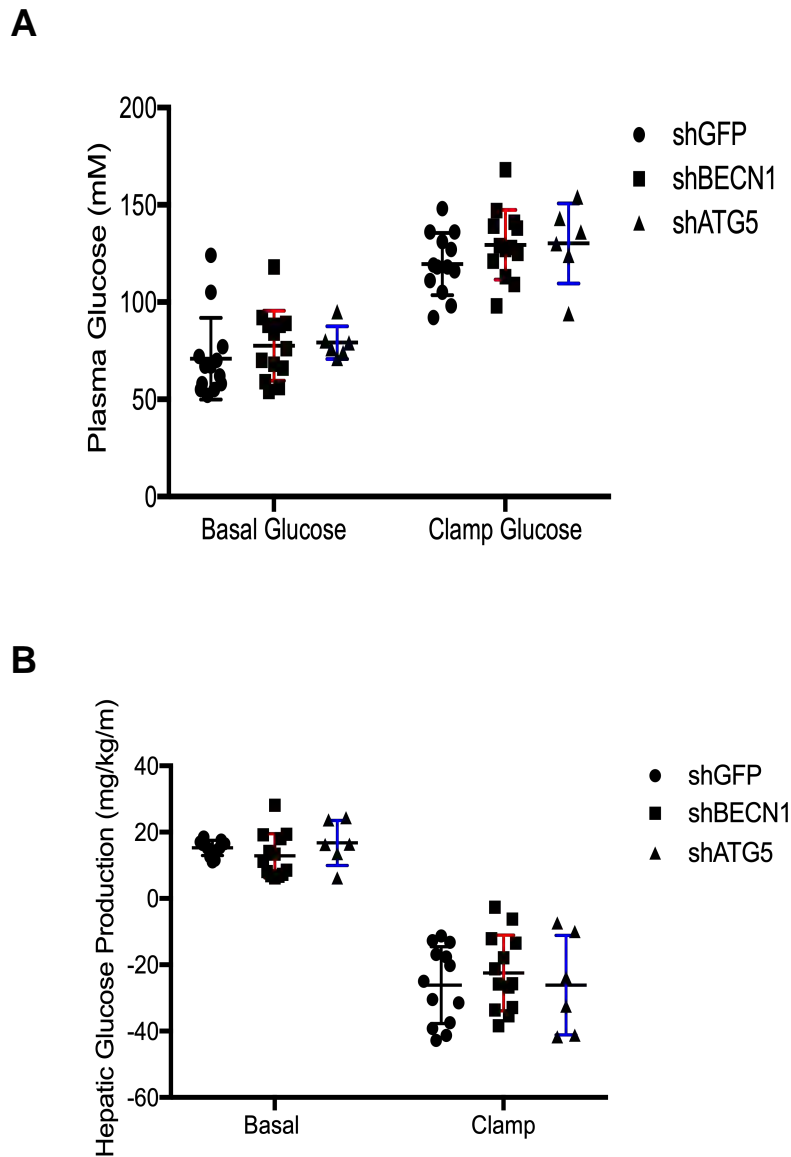
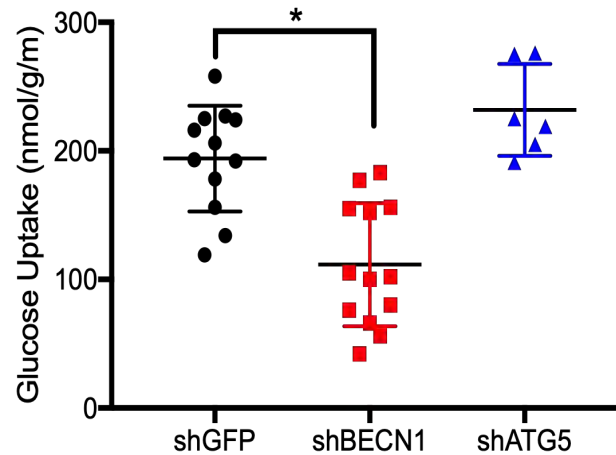


Figure 3.2. Hepatic glucose production is suppressed. (A-B) Mice with tumors expressing *shGFP*, *shBECN1* or *shATG5* were assayed for suppression of hepatic glucose production with (A) plasma glucose and (B) hepatic glucose production. Plasma glucose was measured with a clinical glucose analyzer in 10uL of blood. Hepatic glucose production was measured as a ratio of the glucose infusion rate compared to specific activity of glucose.

A



B

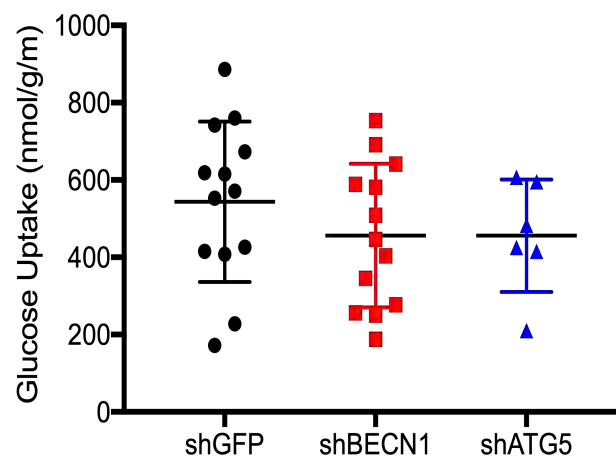


Figure 3.3. Beclin 1 depleted tumors exhibit decreased insulin stimulated glucose uptake. (A) Mouse tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for glucose uptake following insulin stimulation during a hyperinsulinemic-euglycemic clamp study. Glucose was measured by assessing labeled 2- ^{14}C DG-6-phosphate (2- ^{14}C DG-6-P) in tissues. (B) Lower limb skeletal muscle from mice with *shGFP*, *shBECN1*, or *shATG5* tumors was assessed for glucose uptake by measuring labeled 2- ^{14}C DG-6-phosphate (2- ^{14}C DG-6-P). $n=13$ for *shGFP* and *shBECN1* tumor bearing mice, and $n=6$ for *shATG5* tumor bearing mice. * $p<0.05$

glucose uptake in *shATG5* tumors was equivalent to uptake in *shGFP* tumors (Figure 3.3A)²²⁴. This difference in outcomes may reflect the fact that the *in vitro* studies were performed with *Atg5*^{-/-} MEFs and the current *in vivo* studies were performed with cells with reduced, but not complete loss, of *ATG5* expression. Glucose uptake was equivalent in other peripheral tissues suggesting that changes in glucose uptake were specific to the *shBECN1* tumors (Figure 3.3B). These findings support that Beclin 1 regulates insulin-stimulated glucose uptake and does so by autophagy-independent mechanisms.

The hyperinsulinemic-euglycemic clamp study is performed under insulin-stimulated conditions, which may not reflect basal glucose uptake potential. To determine basal rates of uptake in tumors, we assessed glucose uptake in non-stimulated mice. For this study, basal insulin levels were measured and determined to be similar in *shGFP*, *shBECN1* and *shATG5* tumor-bearing mice (Figure 3.4A). Awake mice were injected with [¹⁴C]deoxy-D-glucose and tumors and peripheral muscle tissue were removed after 30 minutes. Basal glucose uptake was variable within the *shGFP* and *shATG5* groups and, therefore, no significant differences in the rate of glucose uptake were observed (Figure 3.4B). However, a trend toward increased glucose uptake was evident in the *shBECN1* tumors. Additional analysis of a larger cohort of mice will be necessary to determine rigorously if Beclin 1 loss enhances tumor glucose metabolism to support proliferation.

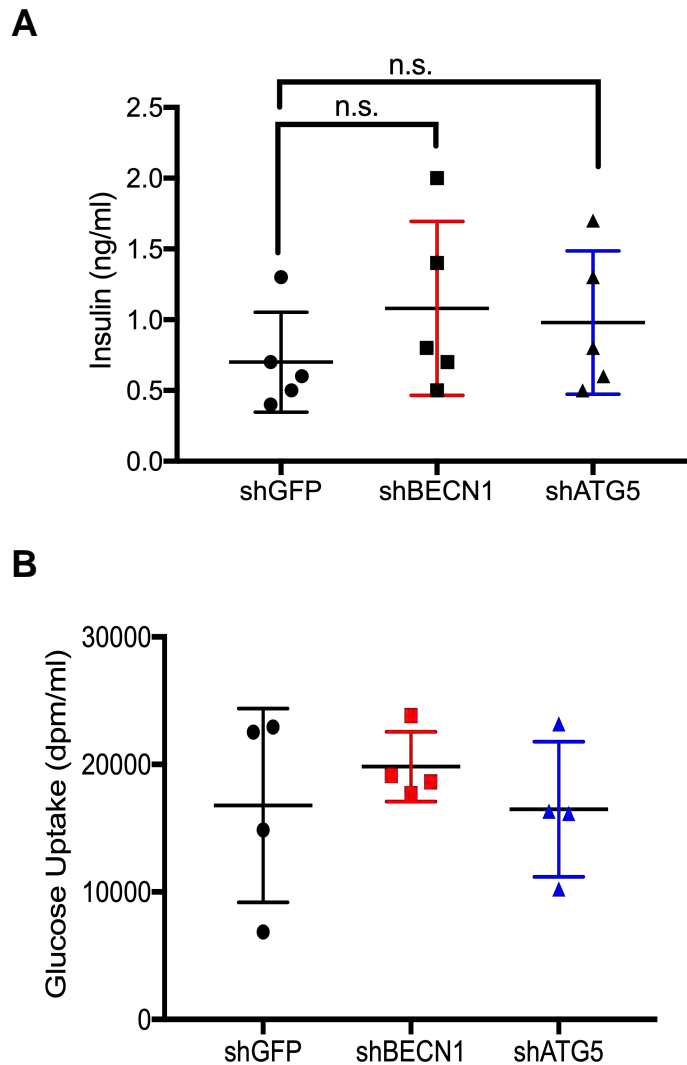


Figure 3.4. Basal glucose uptake is unchanged between tumors. (A) Mice with tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for serum insulin levels with an ELISA assay. (B) Following 5 hour starvation, mice with tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for basal glucose uptake following injection of carbon labeled glucose, ^{14}C deoxy-D-glucose. Glucose uptake in tumors was measured by assessing labeled 2- ^{14}C DG-6-phosphate (2- ^{14}C DG-6-P) in tissues. $n=4$ for each group, $*p<0.05$

Mice with *shBECN1* tumors exhibit early signs of cancer cachexia syndrome

Reduced tumor glucose uptake under insulin-stimulated, but not basal, conditions could represent an insulin-resistant phenotype in the tumors. Insulin resistance is an adaptive state that can occur in many physiologic conditions such as exercise and fasting²²⁶. Insulin resistance also occurs in pathological states such as diabetes and cancer cachexia, a wasting syndrome²²⁷. During the clamp study, two measures of systemic insulin resistance are glucose infusion rate and glucose turnover. Slower glucose infusion rates are suggestive of insulin resistance as it takes less glucose to maintain a euglycemic state. Glucose turnover is a systemic measure of glucose uptake. Less glucose uptake systemically is indicative of an insulin resistant state. Mice with *shBECN1* tumors showed decreased glucose infusion rates, as well as decreased glucose turnover, when compared with mice with *shGFP* and *shATG5* tumors (Figures 3.5A and 3.5B), indicating that mice with *shBECN1* tumors exhibit an insulin resistant state.

Systemic insulin resistance can be driven by multiple inflammatory cytokines. For example, interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor-alpha (TNF- α) are often increased in pathologic inflammatory states, mainly obesity and diabetes mellitus²²⁸. In order to understand whether the insulin resistance profile in mice with *shBECN1* tumors was driven by changes in

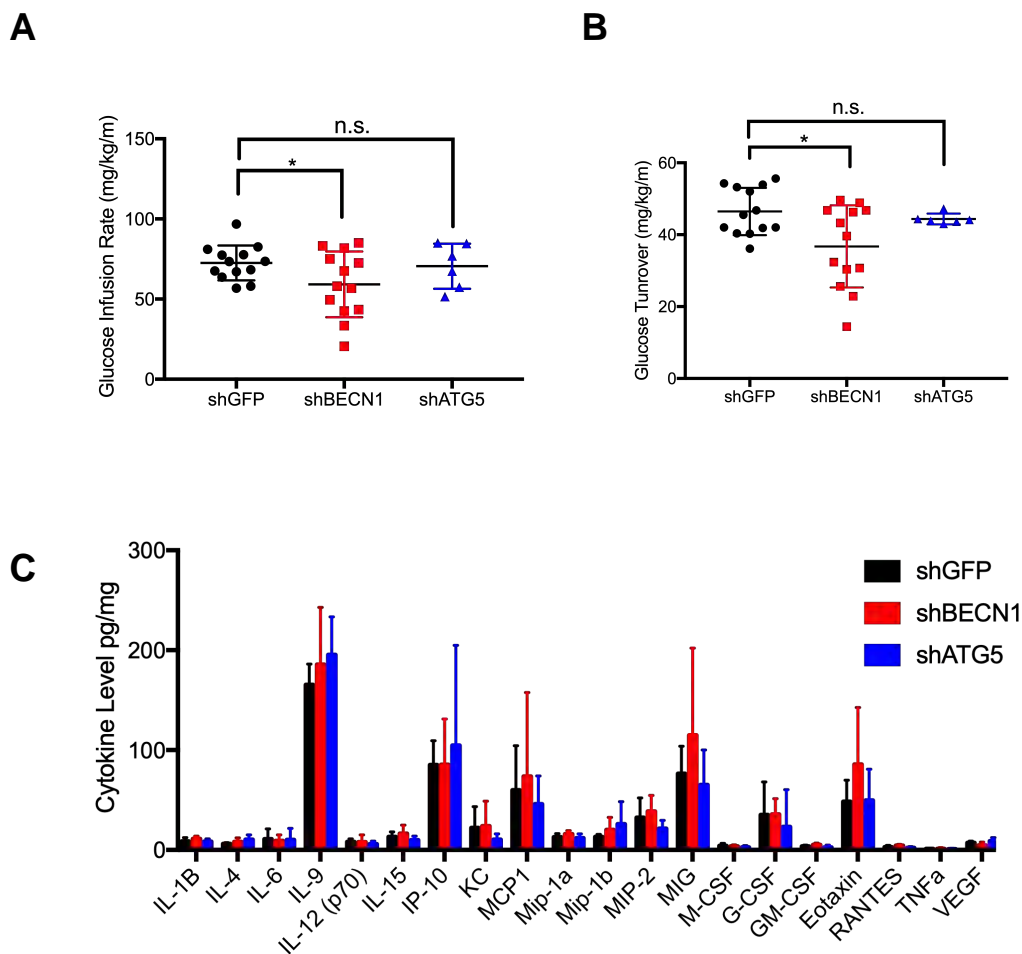


Figure 3.5. Mice with *shBECN1* tumors exhibit an insulin resistant profile. (A-B) Mice with tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for (A) glucose infusion rate and (B) whole body glucose turnover during the hyperinsulinemic-euglycemic clamp study. Whole body glucose turnover is calculated as the ratio between the clamp hydrogen labeled glucose infusion rate compared to the specific activity of plasma glucose in the final 30 min of the clamp study. (C) Luminex assay assessing cytokine levels in mouse serum of mice with *shGFP*, *shBECN1*, or *shATG5* tumors. n=13 for *shGFP* and *shBECN1* tumor bearing mice, and n=6 for *shATG5* tumor bearing mice. *p<0.05

inflammatory cytokines, we performed a Luminex assay to measure levels of circulating cytokines in the tumor-bearing mice. No significant changes in systemic inflammatory cytokines were observed in mice with *shGFP*, *shBECN1*, or *shATG5* tumors, although a trend toward increased MIG and Eotaxin in *shBECN1* tumors was observed (Figure 3.5C). Analysis of a larger cohort of mice will be necessary to evaluate if these factors are upregulated in *shBECN1* tumors.

Cancer cachexia syndrome is associated with insulin resistance and often seen in patients with end stage disease. The main sign of cancer cachexia is weight loss, generally due to loss of skeletal muscle and adipose tissue. This weight loss is usually caused by altered metabolic processing, increased energy expenditure and decreased oral intake. To explore further if Beclin 1 expression in tumors influences the development of cachexia, energy expenditure was measured in mice bearing *shGFP*, *shBECN1*, and *shATG5* tumors. To do so, activity, food intake and water intake were assessed in a metabolic cage study. While no changes in food intake were observed between the different tumor groups, mice with *shBECN1* tumors exhibited increased energy expenditure (VO_2 consumption) in a 24-hour period when compared with mice with *shGFP* and *shATG5* tumors (Figure 3.6A). This difference in VO_2 consumption was primarily observed during daytime hours, when mice typically are less active (Figure 3.6B).

Body composition was measured to evaluate changes in specific peripheral tissues that are commonly altered in cachexia. No significant changes were identified in total body weight or lean muscle mass. However, a trend toward decreased inguinal white adipose tissue (WAT) was observed in mice with *shBECN1* tumors (Figure 3.7A and 3.7B). Although muscle wasting is commonly observed in cancer cachexia, depletion of WAT usually precedes loss of muscle mass, indicating that mice bearing *shBECN1* tumors may be in early stages of cachexia.

***shBECN1* tumors show evidence of elevated de novo lipogenesis**

Tumors can utilize fat to support rapid proliferation either through uptake of fatty acids from the environment or through *de novo* lipogenesis. Our finding that mice with *shBECN1* tumors tend to have reduced WAT (Figure 3.7B) suggested that *shBECN1* tumors may utilize lipids as a metabolic source to support proliferation and in doing so, deplete the adipose tissue stores. To assess lipid uptake in the tumors, mice were injected with 2-[3-³H] Palmitate, a saturated long chain fatty acid. Unfortunately, this analysis was inconclusive due to the variability of the data within each tumor group (Figure 3.7C). Additional studies with a larger cohort of mice will be necessary to directly measure lipid uptake by the tumors.

As an alternative approach to assess lipid metabolism in the *shBECN1* tumors, we examined our RPPA dataset for alterations in the expression of proteins that may drive this metabolic phenotype. CD36, the cell surface glycoprotein that determines the rate of fatty acid uptake into cells, was not included in the RPPA analysis. However, enzymes important for *de novo* lipogenesis showed elevated expression in *shBECN1* tumors (Figure 3.7D). Specifically, the enzyme acetyl-CoA carboxylase 1 (ACC1) was significantly upregulated in *shBECN1* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Figure 3.7D). ACC1 is a key regulator of *de novo* lipogenesis as it catalyzes the conversion of acetyl CoA to malonyl CoA, shifting fat catabolism to fat generation^{229,230}. Expression of fatty acid synthase (FASN), which synthesizes palmitate from acetyl-CoA and malonyl CoA, was also elevated in *shBECN1* tumors (Figure 3.7D). In contrast, phosphorylation of AMP-activated protein kinase (AMPK), an inhibitor of ACC1 and *de novo* lipogenesis, was reduced in *shBECN1* tumors (Figure 3.7D). The upregulation of ACC in the *shBECN1* tumors (Figure 3.7E) was confirmed by immunoblot of additional tumors (n=7). The upregulation of ACC1 and FASN and downregulation of pAMPK provides evidence that *shBECN1* tumors may depend on lipid metabolism, in particular *de novo* lipogenesis, to support their enhanced proliferation.

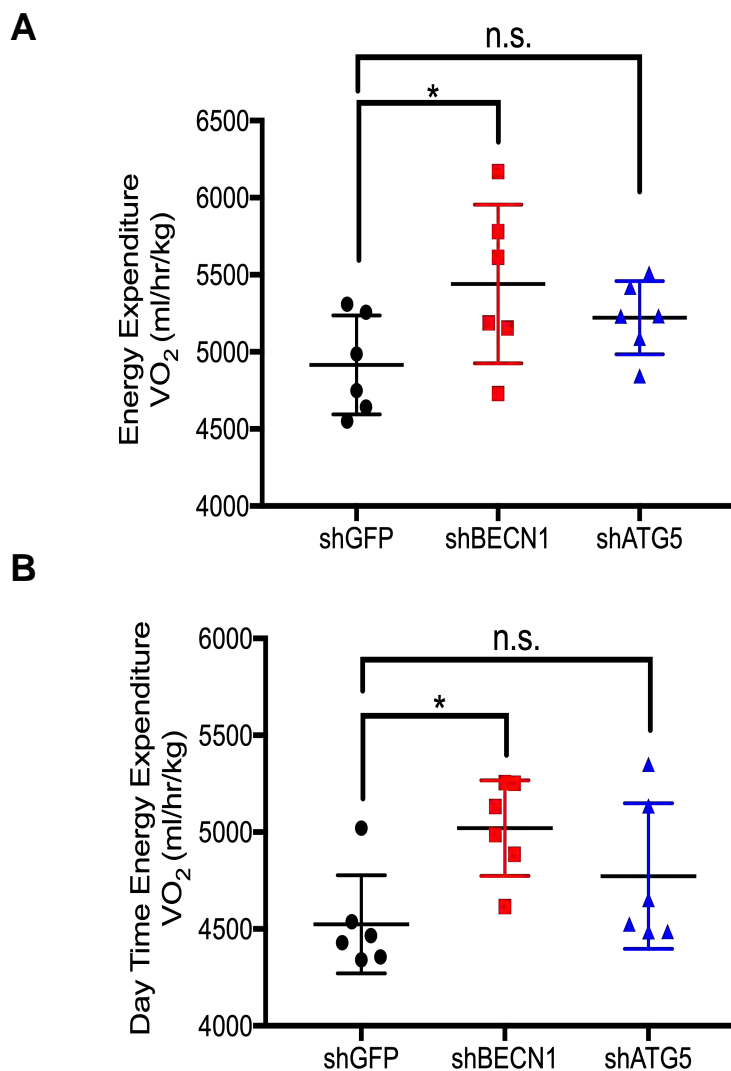


Figure 3.6. *shBECN1* tumor bearing mice exhibit increased energy expenditure. (A-B) Mice with tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for metabolic activity during a metabolic cage study. A metabolic cage from TSE-systems was used to automatically measure mouse movement/activity in a 24 hours period. Energy expenditure was measured as (A) total and (B) day time expenditure. * $p < 0.05$, $n = 13$ for *shGFP* and *shBECN1* tumor bearing mice, and $n = 6$ for *shATG5* tumor bearing mice.

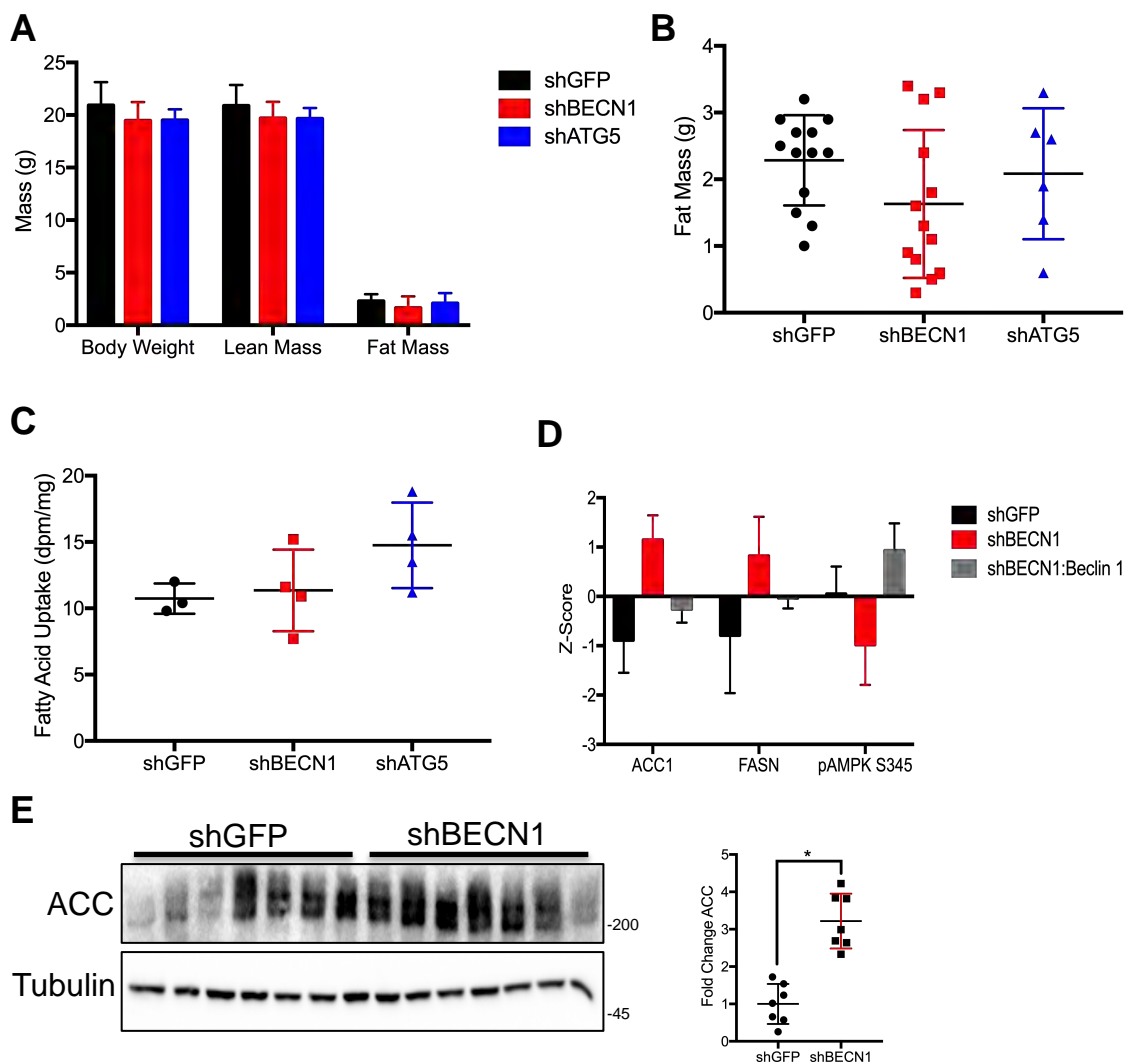


Figure 3.7. Mice with *shBECN1* tumors have decreased inguinal fat mass. (A-B) Mice with tumors expressing *shGFP*, *shBECN1*, or *shATG5* were assayed for body composition. Inguinal fat was measured following hyperinsulinemic-euglycemic clamp study. (C) Graph highlighting proteins involved in *de novo* lipogenesis that were assayed through reverse phase protein array (RPPA). (D) Tumor fatty acid uptake was measured under non-stimulated conditions by measuring hydrogen labeled palmitate (2-[3-³H] Palmitate) after a 5 minute injection. (E) Immunoblot validation of total ACC expression in *shGFP* and *shBECN1* tumors. n=7 tumors per group. *p<0.05

DISCUSSION

Our study demonstrates that Beclin 1 regulates both local and systemic glucose metabolism in an autophagy-independent manner and reveals a potential role for Beclin 1 in the regulation of lipid metabolism. Specifically, tumors with reduced Beclin 1 expression have decreased insulin-stimulated glucose uptake and mice bearing these tumors display systemic insulin resistance. *shBECN1* tumor bearing mice also have increased daytime energy expenditure and reduced white adipose fat depots. Together these phenotypes are evidence of early cancer cachexia, a condition that occurs often at the end stage of cancer and can lead to patient death. We note that *shBECN1* tumors have elevated expression of ACC1 and FASN, enzymes involved in *de novo* lipogenesis, which may signify a reliance of these tumors on lipid metabolism for proliferation. Taken together, our data support a novel mechanism by which Beclin 1 regulates tumor progression and impacts cancer outcomes.

Our data suggesting that Beclin 1 may play a role in regulating *de novo* lipogenesis contributes to our understanding of how Beclin 1 controls tumor proliferation. The ability of tumor cells to reprogram their metabolism is a hallmark of cancer and is essential to support the enhanced biosynthetic activity necessary for rapidly dividing cells. I observed a trend in increased basal glucose uptake, however this increase was modest and needs to be further validated. In contrast, I identified a significant increase in the expression of

enzymes that control fatty acid synthesis, ACC1 and FASN, and decrease in the expression of phosphorylated AMPK, an inhibitor of ACC1 activity. This “de novo lipogenesis signature” supports that loss of Beclin 1 expression results in the reprogramming of lipid metabolism. Lipid metabolism is highly active in breast tumors and metastatic tumors are reported to have high levels of fatty acid utilization. Triple negative breast carcinoma cells generate high levels of ATP through fatty acid oxidation²³¹. Silencing of ACC1 in breast cancer cells reduces *de novo* lipogenesis and induces apoptosis, suggesting that ACC is also important for breast tumor survival²³². Future studies to examine *de novo* lipogenesis and its role in the proliferation of tumors with low Beclin 1 expression are necessary to establish the contribution of this pathway to the aggressive behavior of these tumors. Of clinical relevance, an allosteric inhibitor of ACC1 and ACC2 has been developed that suppresses fatty acid synthesis and inhibits non-small cell lung cancer growth *in vivo*²³³. Tumors with low Beclin 1 expression may be more sensitive to this targeted therapy.

Our study highlights a role for Beclin 1 in the energy wasting syndrome of cancer cachexia²³⁴. Mice with *shBECN1* tumors have increased daytime energy expenditure, reduced inguinal fat mass and systemic insulin resistance, a finding not observed in mice with *shATG5* tumors. These symptoms are often the first signs of cancer cachexia, which affects cancer patients in the later stages of disease and contributes to their morbidity and mortality. Little is known about

mediators of cancer cachexia, although some cytokines including TNF- α and IL-6 are associated with cancer cachexia syndrome in different human cancers^{235,236}. However these factors cannot explain all cases of cachexia and the reasons why some patients develop this wasting syndrome and others do not are unknown. Identifying factors that drive cachexia is necessary for developing approaches to treat these patients. Given that Beclin 1 expression is reduced across multiple human tumors, it could serve as a biomarker for patients that may be prone to the development of cancer cachexia. Future studies using mouse models of cachexia may help to understand how Beclin 1 influences tumor function to induce cancer cachexia. Moreover, additional metabolic analyses of *shBECN1* tumors may reveal factors that control this devastating condition.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

CHAPTER IV

Discussion

Summary of Findings

BECN1 is a haploinsufficient tumor suppressor gene that is often reduced in multiple cancers such as breast, ovarian, and prostate. Additionally, low Beclin 1 expression is an independent predictor of prognosis in different cancers and is associated with poor outcomes. Despite this, many studies have been unable to elucidate why a reduction in Beclin 1 expression is associated with aggressive tumor behavior. My work was designed to understand the implications of reduced Beclin 1 expression in a TNBC model *in vivo*. In this thesis, I present an explanation as to why reduced Beclin 1 expression is associated with poor prognosis as well as a novel therapeutic approach for TNBC. Additionally, my studies provide evidence of autophagy-independent roles that add to our understanding of Beclin 1 as a tumor suppressor, where previously this role was attributed solely to autophagy. My work reveals a novel mechanism for Beclin 1 in the regulation of endocytic trafficking by regulating the recruitment of HRS to endosomes. HRS sorts ubiquitinated cargo to be degraded at the lysosome. I observed that loss of this recruitment leads to enhanced and sustained signaling of receptors, which enhances proliferation *in vivo*. This phenomenon occurs with both growth factor (EGFR) and nutrient (TFR1) receptors that are degraded through the endolysosomal machinery. As a result, tumors that express low levels of Beclin 1 are sensitive to inhibition of EGFR and downstream signaling effectors *ex vivo*, and respond with a reduction in overall proliferation, representing a possible therapeutic strategy to target these tumors in the clinic.

To further understand the impact of reduced Beclin 1 expression *in vivo*, I was able to show that Beclin 1 alters glucose metabolism in a hyperinsulinemic-euglycemic clamp study. *shBECN1* tumors have reduced local glucose uptake and have reduced systemic white adipose tissue suggesting that they use fat as an energy source. Additionally, I show a potential novel role for Beclin 1 in the negative regulation of *de novo* lipogenesis as *shBECN1* tumors upregulate proteins involved in this process. Interestingly, Beclin 1 may also be a biomarker for cancer cachexia, in which there are few known mediators, as mice with *shBECN1* tumors exhibit early signs of this wasting disease.

My study also adds to our knowledge of the autophagy-independent functions of Beclin 1. Knockdown of ATG5, another essential autophagy gene that is important for the elongation and closure of the autophagosome, does not result in the same outcomes as knockdown of Beclin 1. ATG5 does not alter HRS recruitment to affect degradation of ubiquitinated cargos nor does it alter glucose metabolism at either the local or systemic levels in mice with *shATG5* tumors. My work highlights the importance of these functions in breast cancer but these roles can impact other cancer types.

Beclin 1 as a tumor suppressor

Several studies support a tumor suppressive role for Beclin 1, but the mechanisms governing this role are still poorly understood. Heterozygous loss of

Beclin 1 *in vivo* leads to the development of lung adenocarcinomas, liver adenocarcinoma and lymphomas in mice. Additionally, these mice develop pre-malignant changes in the mammary gland compared to WT matched controls^{42,45}. In another model, overexpression of Beclin 1 in MCF7 cells prevents tumor formation in mice and suppresses proliferation *in vitro*, further supporting this tumor suppressive role⁶⁵. Beclin 1 as a tumor suppressor has been examined in the context of autophagy. Autophagy can clear damaged protein and organelles that can cause cellular stress. For example, immortalized baby mouse kidney (iBMK) cells with reduced *Beclin 1* expression show reduced autophagy and accumulate reactive oxygen species, p62 aggregates, and damaged mitochondria^{34,36}. The authors concluded that these aggregates promote tumor progression when Beclin 1 expression is reduced because these factors can cause genomic instability. My data indicating that Beclin 1 regulates growth factor and nutrient receptor signaling to regulate proliferation provides mechanistic insight into the role of Beclin 1 as a tumor suppressor that is independent of its function in autophagy. Regulating the recruitment of HRS to endosomes, governing the degradation of receptors that are often highly active in cancer and controlling signaling provide a molecular mechanism by which Beclin 1 functions as tumor suppressor.

Studies using genetic mouse models carrying heterozygous loss of Beclin 1 reached conflicting conclusions about the role of Beclin 1 as a tumor suppressor.

For example, heterozygous loss of Beclin 1 promotes mammary tumorigenesis in mice following parity and promotes WNT-1 driven tumorigenesis. On the other hand, in genetic models driven with either the polyoma middle T antigen (PyMT) or a HER2, heterozygous loss of Beclin 1 does not promote mammary tumorigenesis. My data showing that Beclin 1 regulates endocytic trafficking of certain receptors explains these different findings in Beclin 1 dependent tumorigenesis. Certain receptors are regulated through endolysosomal trafficking through HRS such as some RTKs and the Frizzled receptor family. Once activated, the Frizzled receptors then activate Wnt signaling downstream. It is possible that the enhanced mammary tumorigenesis following parity observed in mice was due to loss of Beclin 1 expression resulting in the reduction of endolysosomal degradation of the Frizzled receptors, resulting in enhanced WNT signaling and promotion of tumorigenesis. On the other hand, PyMT and HER2 are not regulated in the same manner. While HER2 is endocytosed, it is resistant to degradation and is instead recycled to the cell surface²³⁷. Interestingly, heterodimerization of EGFR and HER2 leads to delayed receptor degradation and increased recycling of this heterodimer to the cell surface^{238,239}. Given how HER2 receptor trafficking is regulated, loss of Beclin 1 expression is not likely to alter HER2 expression and function and is unlikely to change tumor growth in a HER2 model. These differences in receptor trafficking likely explain the discrepancies in previous studies regarding the role of Beclin 1 in the regulation of mammary tumorigenesis. These studies suggest that the role of Beclin 1 in

mammary tumor growth is context specific. However, my work reveals that the role of Beclin 1 in tumorigenesis is directly linked to the regulation of receptor trafficking. By understanding the context of how receptors are trafficked, we can identify signaling pathways that may be altered when Beclin 1 expression is reduced. To further study the regulation of receptor trafficking *in vivo*, I would use cell lines with sustained activity of different RTKs that undergo endolysosomal receptor degradation through HRS and examine tumor growth *in vivo* using a xenograft model. For example, I could generate *shBECN1* expressing non-small cell lung cancer cell lines that have an activating EGFR mutation and examine tumor growth in NOD/SCID mice. I hypothesize that *shBECN1* EGFR mutant cells would exhibit enhanced tumor growth due to reduced receptor degradation through the endolysosomal pathway compared to *shGFP* expressing EGFR mutant cells. This would further suggest that Beclin 1 regulates RTK expression and function of RTKs that are regulated through HRS and would also show that this regulation happens in other cancers in addition to breast cancer.

Beclin 1 and growth factor signaling in cancer

Growth factor receptor signaling is often aberrantly regulated in cancer pathogenesis. Enhanced signaling downstream of receptors such as RTKs leads to the activation of multiple downstream signaling pathways that promote cancer growth and survival. For example, both EGFR and IGF-1R activity are enhanced in multiple cancer types including breast cancer. Overexpression of EGFR and

IGF-1R are observed in over 50% and up to 46% of all TNBC respectively^{240,241}. This overexpression translates to elevated receptor activity and abnormal signaling. While there are multiple mechanisms for this aberrant signaling, one mechanism includes the inability of endocytosed receptors to be degraded. For example, disruption of endocytic processing of EGFR by interrupting endoplasmic reticulum contact sites has been shown to cause delayed degradation and enhanced signaling downstream of the EGFR²⁴². Additionally, overexpression of EGFR at the plasma membrane causes the increase of either homodimerization or heterodimerization, leading to enhanced kinase activity²⁴³. Overexpression of IGF-1R leads to enhanced signaling and can be observed when IGF-1R heterodimerizes with the Insulin Receptor (IR)²⁴⁴. The overall result of this enhanced receptor expression is the upregulation of downstream signaling, which is often observed in aggressive breast cancer subtypes. My work provides a novel mechanism into how the activity of certain signaling pathways can be enhanced in cancer and is the first to show that Beclin 1 regulates growth factor signaling *in vivo*. Taken together, my work emphasizes the importance of regulating growth factor signaling in cancer.

In my orthotopic xenograft model, I have shown that loss of Beclin 1 results in enhanced signaling downstream of the EGFR receptor because of the ability of Beclin 1 to regulate HRS recruitment to the endosome. Although the endolysosomal degradation of other RTKs such as IGF-1R, PDGFR and the IR is

regulated in a similar manner to EGFR, the ubiquitin ligases responsible for ubiquitinating these receptors are different. As mentioned previously, c-Cbl, a member of the Cbl family of ubiquitin ligases, ubiquitinates EGFR, while Nedd4, an E3 ubiquitin ligase, ubiquitinates IGF-1R^{87,90,91}. However, both of these receptors (EGFR and IGF-1R), when ubiquitinated, are recognized by HRS and sorted for degradation. Therefore, I hypothesize that in other breast cancer models with reduced Beclin 1 expression, enhanced signaling of these receptors (IGF-1R, PDGF, or Insulin) may also be due to diminished HRS recruitment to endosomes. I can test this *in vitro* by stimulating cells with the respective ligands of these receptors and immunoblotting for changes in phospho-HRS as a surrogate marker for HRS recruitment to endosome. I can also use immunofluorescence staining to look for reduced HRS puncta. I can further test whether Beclin 1 can regulate the signaling of these receptors *in vivo* by performing IPs for HRS and immunoblotting for phospho-tyrosine expression in *shBECN1* expressing tumors, specifically in tumor models that show enhanced signaling downstream of these receptors. Furthermore, I can explore the ability of Beclin 1 to regulate HRS recruitment to endosomes to modulate RTK signaling by examining other cancers. Beclin 1 expression is reduced in multiple aggressive tumor types including oral tongue squamous cell carcinoma, prostate, ovarian, gastric cancer and hypopharyngeal cancer^{59,62-64}. Therefore, it is important to explore whether the regulation of signaling by Beclin 1 in breast cancer is a conserved mechanism across multiple cancers.

My study models the reduction of Beclin 1 expression observed in many cancers that results in disruption of the PI3KC3 interaction. However, Beclin 1 can undergo post-translational modifications that interrupt its ability to interact with PI3KC3. For example, AKT phosphorylates Beclin 1 at the S295 site and EGFR phosphorylates Beclin 1 at Y229, Y233 and Y352 sites to disrupt the interaction of Beclin 1 and PI3KC3^{245,246}. In normal physiology, this short-term regulation may serve as a way to regulate the duration of signaling downstream of active receptors. However, this regulation may provide an advantage to cancer cells to promote sustained activation of receptor signaling. Beth Levine's group generated Beclin 1 mutant phosphorylation constructs that either mimic Beclin 1 phosphorylation (Beclin 1 EEE) or are unable to be phosphorylated (Beclin 1 FFF). The Beclin 1 EEE phosphomimetic was shown to increase proliferation and tumor growth in non-small cell lung cancer model while the Beclin 1 FFF mutant suppressed tumor growth²⁴⁶. This group concluded that the functional outcome of the phosphomimetic Beclin 1 mutant was due to a deregulation of autophagy. However, I hypothesize that the Beclin 1 phosphomimetic (Beclin 1-EEE) reduces HRS recruitment to endosomes and results in sustained signaling downstream of growth factor receptors which promotes tumor growth. One way to test this hypothesis is to generate *shBECN1* rescue cells that express the Beclin 1 EEE mutant and examine whether these cells, when stimulated with EGF, exhibit reduced pHRS via immunoblot or reduced HRS puncta via IF. I can

also stimulate cells that express this mutant to explore whether EGFR signaling is sustained. Since these Beclin 1 mutants were only explored in the context of autophagy, it would be important to elucidate whether or not these mutants affects endocytic receptor trafficking thereby affecting receptor signaling. If the Beclin 1 EEE or FFF mutant alter receptor signaling *in vitro*, it would be important to explore these mutants *in vivo* to further examine the role of Beclin 1 in the regulation of growth factor signaling and tumor growth.

Beth Levine's group also generated a TAT-Beclin 1 peptide that was shown to increase autophagy and reduce tumor growth in a xenograft model²⁴⁷. The TAT-Beclin 1 peptide contains 11 amino acids from the evolutionary conserved domain of Beclin 1 and an 11 amino acid sequence from HIV that allows it to be cell permeable²⁴⁸. This peptide increases autophagy in a dose dependent manner in a breast cancer cell line and is thought to activate autophagy in a canonical manner as treatment of *shBECN1* or *shATG7* cells with this peptide results in a reduction of LC3 puncta, indicating reduced autophagy²⁴⁸. Beth Levine's group hypothesized that TAT-Beclin 1 may exert its effects on autophagy by inhibiting the interaction of Beclin 1 and Golgi associated protein pathogenesis-related 1 (GAPR-1) which associates with the golgi complex, thereby releasing Beclin 1 from the golgi to function in autophagy²⁴⁸. However, they did not explore whether TAT-Beclin 1 could alter endocytic receptor degradation. First it would be important to determine whether the TAT-Beclin 1

peptide induces endocytic receptor degradation. If so, then it would be of interest to use this peptide to explore the novel role of Beclin 1 in the regulation of HRS recruitment *in vitro* and *in vivo*.

In my studies I use an *ex vivo* approach to treat tumor fragments with different RTK and downstream signaling inhibitors and monitor proliferation through IHC. Using this approach I was able to determine that low Beclin 1 expressing mammary tumors are sensitive to both Lapatinib and PD98059, and exhibit reduced proliferation in the presence of these drugs. Using this technique, I can use other inhibitors of RTKs to screen low Beclin 1 tumors for reduced proliferation following treatment. This is a great platform to screen different inhibitors to develop novel treatment approaches for multiple cancer types that have reduced Beclin 1 expression. Additionally, drug treatments that result in reduced proliferation *ex vivo* can be used to screen for efficacy in *shBECN1* tumors *in vivo*. Orthotopic xenograft studies can be used to examine tumor growth regression upon drug treatment as well as determine an adequate drug dosing schedule that is reasonable for human use²⁴⁹.

Beclin 1 and TFR1 in Cancer

Iron is an essential element that is important for multiple cellular enzymes that function in cellular metabolism and cellular proliferation. Given this critical requirement, cancer cells have generated mechanisms to increase intracellular

iron stores. The transferrin receptor (TFR1) is the major regulator of iron uptake into the cell. Many cancers including breast, bladder and lung cancer have increased TFR1 expression²⁵⁰. Conversely, iron transport out of the cell is mediated by the transporter ferroportin and consequently, ferroportin expression is reduced in many cancers including breast^{251,252}. This mechanism employed by cancer cells to regulate both iron influx and efflux allows for iron levels to remain elevated, which facilitates rapid proliferation. For example, breast tumors have elevated levels of TFR1 expression and reduced expression of ferroportin, supporting a tumor microenvironment that has elevated iron levels compared to normal tissue²⁵¹. Additionally, it was observed that breast tumors have an iron gene regulatory signature that can predict patient outcomes²⁵³. Our data shows, for the first time, that Beclin 1 can regulate TFR1 expression *in vivo*. Given that TFR1 regulates iron uptake into cells, one logical question would be to determine whether the proliferation phenotype findings observed in *shBECN1* tumors are sensitive to iron chelation. Studies suggest that iron chelation can have anti-tumor effects *in vivo*, therefore it would be of interest to use iron chelators in the orthotopic xenograft model to see if we can reduce proliferation in *shBECN1* tumors, thereby inhibiting tumor growth²⁵⁴. This could serve as a new mechanism to treat low Beclin 1 expressing tumors that have elevated TFR1 expression.

Our studies from Chapter II provide a novel mechanism by which TFR1 expression is regulated by endolysosomal degradation. Previously it was shown

that the iron-responsive proteins 1 & 2 regulate TFR1 mRNA expression. However, previous studies suggest that the endolysosomal pathway can also regulate TFR1 protein expression. TFR1 is ubiquitinated by membrane-associated RING-CH (MARCH) 8, a member of the MARCH family of ubiquitin ligases²⁵⁵. In Chapter II, I observed that TFR1 remains ubiquitinated in *shBECN1* tumors. I hypothesize that decreased HRS recruitment in *shBECN1* tumors allows for the escape of ubiquitinated TFR1 that leads to the increase in TFR1 expression. However, it would be important to look at this regulation more closely. Using immunofluorescence to examine colocalization of TFR1 and HRS would help to support a role for HRS in the regulation of TFR1 expression through the endolysosomal pathway. I hypothesize that *shBECN1* expressing cells would exhibit a reduction in HRS colocalization with TFR1 compared to *shGFP* control cells.

TFR1 is a very interesting candidate for anti-tumor therapy because this protein can be targeted to prevent iron uptake into cells or its physiological function can be utilized for drug delivery. As mentioned previously, iron chelators are being explored as a potential anti-tumor treatment because of the importance of iron in cellular proliferation. However, targeting TFR1 itself is being considered as a potential anti-tumor therapy²⁵⁶. For example, mice treated with single-chain antibodies targeting TFR1 exhibited reduced tumor growth in a leukemic mouse model by reducing intracellular iron²⁵⁷. Transferrin (TF) is the ligand for TFR1 that

binds iron and is constitutively taken up by cells. Another line of therapy is to use transferrin-chemotherapeutic conjugates. For example, TF-cisplatin or TF-doxirubicin have been used both *in vitro* and *in vivo* and are cytotoxic to cancer cells²⁵⁸. Our study suggests that Beclin 1 may be a biomarker to identify tumors with high TFR1 expression, which can be candidates for TF-chemotherapeutic conjugates. Additionally, using TF conjugated to inhibitors of downstream RTK signaling pathway members would be worth exploring in our tumor model. I can also use my *ex vivo* drug treatment system to determine which TF-conjugates are effective in multiple cancers and then use these same conjugates *in vivo* to explore their efficacy in reducing tumor growth in low Beclin 1 expressing tumors.

Beclin 1, HRS and the Endolysosomal Pathway

Several studies in lower organisms have implicated a role for Beclin 1 in endocytosis and have hinted at a potential role for Beclin 1 in the regulation of subcellular signaling^{50,52}. In the mammalian system, the Stenmark group showed that the Beclin-1-PI3KC3-UVRAG interaction (Complex II) was important for endosomal maturation and regulation of EGFR degradation⁴⁸. Our lab's previously published work supports this role for Complex II as we showed that Beclin 1 regulates endosomal maturation and reduced Beclin 1 expression allows receptors to signal from immature but signaling competent endosomes. Both of these studies were done in an *in vitro* model system. My work in this dissertation expands on these studies and now provides a novel explanation for the

regulation of endocytic trafficking by Beclin 1 *in vivo* using a breast cancer model. However, as mentioned, this regulation may also be present in other cancer types. Additionally it provides more support for the role of Beclin 1 in the regulation of growth factor receptor signaling which can contribute to its role as a tumor suppressor.

My work shows that Beclin 1 promotes the recruitment of HRS to endosomes, which sorts cargos for degradation in the endolysosomal pathway. By immunofluorescence staining, I show a reduction in HRS puncta in *shBECN1* cells as compared to control cells. Given that HRS is recruited to early endosomes and that our previous work shows that Beclin 1 regulates early endosomal maturation, using co-staining techniques with other endosomal markers would be necessary to confirm that the HRS puncta I visualized represent early endosomes. I can use Rab5, which is a marker of early endosomes, to confirm that the HRS is recruited to the early endosome. I can also stimulate breast carcinoma cells expressing shGFP or shBECN1 with EGF and perform an IP with HRS. I can then blot for early endosomal markers such as Rab 5 or early endosomal antigen 1 (EEA1) to show that there is decreased interaction between HRS and early endosomal markers in shBECN1 expressing breast carcinoma cells. This would help to further support a role for Beclin 1 in the endosomal recruitment of HRS. Signal transduction through active receptors and degradation of these receptors through HRS recruitment is a dynamic

process. One caveat to my study is that I examine recruitment of HRS in a short time course of either 10 to 15 minutes following EGF stimulation. Therefore additional studies are required to further look at the recruitment of HRS and longer time courses may be necessary to examine this dynamic process.

As mentioned previously, HRS is a member of the endosomal-sorting complex required for transport (ESCRT) machinery. This machinery sorts cargos into intraluminal vesicles (ILVs) that go on to form multivesicular bodies (MVB) which fuse with lysosomes to degrade their content. HRS is a member of ESCRT-0. I could examine TSG101 recruitment to HRS puncta since TSG101 is member of the ESCRT-I complex and is recruited to endosomes after ESCRT-0. I hypothesize that *shBECN1* expressing cells would exhibit a reduction in TSG101 positive staining as TSG101 is recruited to endosomes by HRS. To confirm that this is happening *in vivo*, I could use Amnis Flow Cytometry Flowsight to examine freshly dissociated tumor cells for colocalization of different markers such HRS, TSG101 and Rab5. Amnis Flow cytometry Flowsight is an imaging flow cytometer that takes images of individual cells and allows for the localization and quantification of colocalized proteins in single cells.

HRS is recruited to early endosomes in a PI3P-dependent manner^{100,102}. Previously our lab showed that loss of Beclin 1 leads to a reduction in growth factor stimulated PI3P production that results in delayed maturation of

endosomes¹³¹. We also observed a reduction in the recruitment of early endosome antigen 1 (EEA1) to early endosomes in *shBECN1* cells. EEA1 is also recruited in a PI3P-dependent manner¹³¹. In my work, I hypothesize that HRS recruitment to the endosome is reduced in *shBECN1* expressing LM2 cells because of a reduction in PI3P. However, it would be important to show that this regulation is occurring in the LM2 *shBECN1* cells and *shBECN1* tumors. To determine if reduced recruitment of HRS is due to reduced PI3P, I could examine the recruitment of other PI3P-dependent proteins to the endosome as our lab previously showed in another breast cancer cell line¹³¹. EEA1 is an early endosomal protein that is recruited to the endosome by the binding of its FYVE domain to PI3P^{259,260}. Since Beclin 1 regulates PI3P production, I would expect reduced EEA1 endosomal recruitment in *shBECN1* cells compared to *shGFP* control cells. Although we previously showed this finding in another cell line, it would still be of interest to show that this happens in the LM2 *shBECN1* cells. These results would support a hypothesis that the reduction in HRS recruitment to the endosome is due to reduced PI3P production.

As I mentioned previously, HRS initiates the MVB pathway by recognizing and binding ubiquitinated cargos destined for sorting into ILVs^{111,261}. This signal allows for the sequential recruitment of other ESCRT complexes. This key step of ubiquitination has been shown to be essential for degradation of multiple RTKs including the EGFR²⁶². In my work, I also show that TFR1 remains ubiquitinated

in *shBECN1* tumors. I hypothesize that ubiquitinated receptors escape degradation, which causes elevated receptor expression levels. It is important to investigate the ubiquitination of other receptors both *in vivo* and *in vitro* to further support a role for Beclin 1 in regulation of HRS and the degradation of different growth factor and nutrient receptors. In order to examine whether proteins escape degradation by remaining ubiquitinated, I could stimulate cells expressing *shBECN1* with EGF ligand (or other receptor ligands), treat with a deubiquitinase inhibitor such as *N*-ethylmaleimide (NEM), perform an IP for EGFR (or other RTKs), and blot for ubiquitin. Compared to *shGFP* expressing cells, *shBECN1* expressing cells may exhibit a prolonged ubiquitination of the EGFR receptor. This data would support that reduced Beclin 1 expression allows for enhanced signaling because receptors escape degradation and remain ubiquitinated. I can also express a mutant EGFR construct in the LM2 cells that is unable to be ubiquitinated. The Sorkin group generated a EGFR construct that has either 15 or 16 lysine-arginine (KR) mutations in the kinase domain of EGFR that prevent EGFR from being ubiquitinated²⁶³. I can express this mutant EGFR in EGFR knockout LM2 breast carcinoma cells that have reduced Beclin 1 expression and stimulate with EGF to examine downstream signaling. In *shGFP* cells, this mutant EGFR construct should exhibit reduced degradation and sustained signaling to a greater extent compared to *shBECN1* expressing cells. *shBECN1* cells may still exhibit an increase in signaling but the fold change will not be as significant as the *shGFP* control cells. I can also use these EGFR knockout cells

that express the EGFR ubiquitin mutant *in vivo* to examine tumor growth. I expect that *shGFP* LM2 cells that express the EGFR ubiquitin mutant will exhibit a more significant enhancement in EGFR signaling and increased tumor growth. LM2 *shBECN1* cells that express the EGFR ubiquitin mutant *in vivo* may exhibit increased growth however as stated previously, the fold change may not be a significant as observed for the *shGFP* tumors.

Autophagy-Independent/alternative Roles for Beclin 1 in Cancer

Autophagy can suppress tumor initiation and progression through multiple mechanisms. Autophagy can help to clear damaged proteins and organelles to suppress tumorigenesis by reducing reactive oxygen species, which promotes genomic instability. As stated previously, reduction of *Beclin 1* and *Atg5* expression in iBMK cells was shown to lead to the accumulation of both p62 and damaged mitochondria, resulting in elevated ROS and oxidative stress, which in turn promotes DNA damage^{34,36}. Autophagy can also prevent malignant tumor formation; mice with systemic mosaic deletion of *Atg5* or liver specific knockout of *Atg7* develop liver adenomas, which are non-malignant tumors, and accumulate ROS³⁷. However, these benign liver tumors fail to progress to hepatocellular carcinoma despite having increased ROS levels and genomic instability³⁷. On the other hand, mice heterozygous for *Becn1* develop multiple spontaneous malignancies including lung adenocarcinoma, liver adenocarcinomas and lymphomas. This difference in tumor development

between essential autophagy genes suggests that Beclin 1 has autophagy-independent or autophagy alternative functions that are required for cancer development.

My orthotopic xenograft studies indicate that *shBECN1* expressing tumors exhibit increased tumor growth compared to *shATG5* expressing tumors. I show that autophagy is equivalently reduced in these tumors, however there is a difference in tumor growth. *shBECN1* expressing tumors also exhibit an increase in EGFR/MAPK signaling due to diminished recruitment of HRS which prevents endolysosomal degradation of EGFR thereby promoting sustained signaling. This data suggest that it is the endolysosomal regulation by Beclin 1 that promotes enhanced tumor growth. I hypothesize that *shATG5* tumors do not exhibit increased tumor growth because ATG5 does not regulate endolysosomal degradation. It would be interesting to explore this further by manipulating endolysosomal degradation in *shATG5* expressing cells. I can express an HRS FYVE domain deletion mutant to perturb endolysosomal degradation. As stated previously, HRS is recruited to endosomes through its FYVE domain that binds PI3P. Using this mutant, I could compare tumor growth of *shATG5* (autophagy deficiency) and *shATG5:HRS-FYVE* domain deletion (autophagy and endolysosomal degradation deficient) cells. I hypothesize that *shATG5:HRS-FYVE* domain tumors would exhibit increased tumor growth compared to *shATG5* and *shGFP* control tumors because receptor degradation would be

disturbed. Expressing the HRS mutant in *shATG5* cells may allow the *shATG5* tumors to behavior more similarly to *shBECN1* tumor because they will now exhibit defective endolysosomal degradation.

My work highlights a novel role of Beclin 1 in the regulation of growth factor and nutrient receptor signaling *in vivo* that is autophagy-independent. As shown in Chapter II, knockdown of Beclin 1 or ATG5 results in equivalent reduction in autophagy. However, when I examined the functionality of this loss through tumor growth and proliferation, I note that reduction of ATG5 does not result in enhanced tumor growth or changes in proliferation compared to control tumors. Additionally, ATG5 does not regulate HRS recruitment to endosomes and therefore does not alter growth factor or nutrient receptor signaling. This finding helps to justify a role for Beclin 1 in cancer that is autophagy-independent.

In my study I knockdown Beclin 1 expression which affects both Complex I (autophagy) and Complex II (autophagy-independent). In order to determine if the autophagy-independent phenomenon I observed is regulated by Complex II, it would be important to investigate these complexes separately. I could knock down either ATG14 or UVRAG independently to examine their role in HRS recruitment. Based on my work, I hypothesize that Complex II, with UVRAG, mediates the recruitment of HRS to endosomes by Beclin 1. However, one caveat to this approach is that ATG14 and UVRAG are needed for complex

stability and knockdown of either of these proteins results in a reduction of Beclin 1²⁶⁴. It may be possible to prevent changes in Beclin 1 expression by targeting regulators of ATG14 or UVRAG. One group showed that Dapper 1 (Dpr1), a Dishevelled (Dvl)-interacting protein that can prevent both canonical and non-canonical WNT signaling, can regulate autophagy by enhancing the interaction of Complex I with Beclin 1, PI3KC3, and ATG14¹³. By targeting Dapper 1, I could reduce the interaction of Beclin 1 with ATG14 and increase the interaction of Beclin 1 with UVRAG. UVRAG and ATG14 bind Beclin 1 through its coiled-coil domain (CCD), however studies show that UVRAG has a stronger affinity for Beclin 1 and can out compete ATG14. Recent biochemical work has aimed to weaken the Beclin 1/UVRAG interaction by mutating the CCD of UVRAG²⁶⁵. These mutations help to enhance the binding of Beclin 1 to ATG14. As a result, this mutant causes an increase in Complex I activity, which mediates autophagy, but has reduced endocytic receptor trafficking. This UVRAG CCD mutant would be interesting to explore in future experiments. I hypothesize that expression of the UVRAG CCD mutant would cause a reduction in endocytic receptor degradation, while maintaining autophagy. In contrast, silencing Dapper 1 would increase Complex II activity, which would enhance recruitment of HRS to early endosomes and promote receptor degradation, resulting in reduced receptor signaling. Both of these methods would allow me to analyze HRS endosomal recruitment in a complex dependent manner.

Beclin 1 interacts with UVRAG and ATG14 through the coiled coil domain. This interaction with UVRAG or ATG14 regulates the functional outcomes of Beclin 1. As stated previously, Beclin 1 is a haploinsufficient tumor suppressor and expression of Beclin 1 is reduced in multiple cancer types. Given the reduction of Beclin 1, this begs the question, what regulates Beclin 1 complex formation with UVRAG or ATG14 in a setting of reduced Beclin 1 expression? It is important to explore this regulation closer to understand the role of Beclin 1 in cancer. I have shown that Beclin 1 regulates endocytic receptor trafficking by regulating HRS recruitment. I could stimulate cells with EGF and perform an IP with Beclin 1 and blot for UVRAG and ATG14. I hypothesize Beclin 1 regulates endocytic trafficking through Complex II. Therefore cell stimulated with EGF ligand may show an increased interaction between UVRAG and Beclin 1. Additionally, I could initiate autophagy in the same cells and examine whether this now increases the interaction of ATG14 and Beclin 1. I hypothesize that in the setting of low Beclin 1 expression, all Beclin 1 interacts with either Complex I or Complex II. However, both autophagy and endocytic receptor signaling can occur in a disease state. For example, RAS transformed cancer cells have increased autophagic activity and endocytic receptor signaling²²⁴.

Recent work has generated inhibitors of PI3KC3 (VPS34) to treat cancer by inhibiting autophagy²⁶⁶⁻²⁶⁸. PI3KC3 inhibitors were shown to have antitumor effects on cancer cell lines and synergize with mTOR inhibitors to block

proliferation²⁶⁸. Although VPS34 inhibitors may be useful for inhibiting autophagy in some cancers, they may not work well for others. In the setting of low Beclin 1 tumors, it is possible that adding a VPS34 inhibitor may worsen prognosis as this would lead to less HRS recruitment because of a reduction in PI3P. This would further suppress the degradation of both growth factor and nutrient receptors resulting in sustained signaling. However, if the downstream signaling is known, using a VPS34 inhibitor to reduce autophagy in combination with the inhibitors of downstream effectors is worth exploring in future experiments. My pilot studies suggest that knockdown of VPS34 in breast tumor cells causes sustained and enhanced signaling following IGF-1 ligand treatment. It would be interesting to explore combination therapy in these cells. I hypothesize that dual inhibition of VPS34 and downstream signaling effectors such as MEK, ERK, PI3K, or AKT would be cytotoxic to cancer cells because both the autophagy pathway and growth factor signaling advantage would be inhibited. However, it would be important to first identify which signaling pathways are elevated in different cancer cell lines to determine which inhibitor to use in combination with a VPS34 inhibitor.

Metastasis and Tumor Microenvironment

Our study supports previous work that shows that Beclin 1 suppresses tumorigenesis and proliferation^{58,59}. In this study we explore tumor progression in an *in vivo* model of breast tumor growth. However, it is important to explore a

role for Beclin 1 in tumor metastasis, because metastatic burden often leads to patient demise. In my study I use LM2 cells, which are a variant of MDA-MB-231 cells that were selected for their ability to metastasize to lung²⁶⁹. However, to observe metastatic lung lesions, survival surgery is necessary. Our previous work in breast carcinoma cells *in vitro* showed that low Beclin 1 expressing cells have both sustained AKT activation following IGF-1 ligand stimulation and enhanced invasion in a 2D Transwell Matrigel assay¹³¹. AKT signaling promotes tumor cell invasion which is an important initial step in the metastatic cascade^{270,271}. I hypothesize that mice with low Beclin 1 expressing tumors will have increased metastatic burden. Using different cancer cell lines, I could test metastatic burden following survival surgery. In this model I could look at cancer progression (xenograft tumor growth) and metastasis (survival surgery) in multiple cancer cell lines with reduced Beclin 1 expression. It is also important to assess low Beclin 1 expressing tumors in a syngeneic model. One model I could use would be the 4T1/67nr mammary cell model in which 4T1 is the metastatic counterpart of the 67nr non-metastatic cell line^{272,273}. Using the 4T1 and 67nr mammary cells derived from BALB/c mice, I could test a role for Beclin 1 in the promotion of metastasis by examining metastatic burden in BALB/c mice with 4T1 *shBECN1* tumors. I can also assess the ability of Beclin 1 to regulate the transformation of a non-metastatic cell line to a metastatic cell line by exploring the 67nr cells. In addition to survival surgery, another way to examine metastatic burden would be to use a luciferase reporter cell line. I could then use a non-

invasive approach to observe metastasis through imaging. It will also serve as a way to detect early metastatic lesions.

Our study shows that low Beclin 1 expressing tumors have elevated TFR1 expression. However, in *in vitro* studies performed with *shBECN1* cells, no increase in TFR1 expression was observed when compared to control *shGFP* cells. These data suggest that the tumor microenvironment may play a role in the modulation of TFR1 expression exhibited in *shBECN1* tumors. Given the importance of the supporting cells in the tumor microenvironment, it is important to investigate their role in promoting changes in TFR1 expression. For example, macrophages can uptake, store, and release iron into the microenvironment to modulate iron metabolism²⁷⁴. My model uses NOD/SCID mice, which are immunocompromised but still have detectable macrophages²⁷⁵. It would be important to assess the contribution macrophages provide to TFR1 expression regulation. It is possible that both the tumor microenvironment and effects of low Beclin 1 expression synergistically impact TFR1 expression and function.

Iron regulatory proteins 1 and 2 (IRP1/IRP2) regulate TFR1 mRNA expression. Specifically, in low iron conditions IRPs bind iron response elements (IREs) in *TFR1* mRNA to stabilize the mRNA and in high iron concentrations these proteins dissociate from *TFR1* mRNA to destabilize the mRNA promoting degradation²⁷⁶. While iron regulates IRP activity, other stimuli can also influence

IRPs such as hypoxia. IRP-2 RNA binding activity is induced in hypoxic conditions in HEK293 cells due to post-translational regulation²⁷⁷. As stated previously, *shBECN1* expressing LM2 cells do not exhibit increased TFR1 expression *in vitro*, whereas *shBECN1* expressing tumors have increased TFR1 expression *in vivo*. Given that IRPs bind to IREs in TFR1 mRNA, hypoxia may be able to induce TRF1 expression. The oxygen tension varies in different regions of tumors and certain areas are more hypoxic than others. Therefore, it is important to assess the affect of hypoxic induction of TFR1 expression in cells. *shBECN1* expressing LM2 cells can be cultured in hypoxic conditions to examine changes in TFR1 protein expression. It is possible that *shBECN1* cells are more sensitive to hypoxic induction of TFR1 expression compared to *shGFP* control cells. Low oxygen tension in rapidly proliferating *shBECN1* expressing tumors could drive increased TFR1 expression. The difference in oxygen content *in vivo* compared to *in vitro* may help to explain the discrepancy in TFR1 expression in low Beclin 1 expressing cells *in vitro* and tumors *in vivo*.

Beclin 1 in Tumor Metabolism

Cancer cells are efficient at metabolic reprogramming to create an excess of building blocks to support rapid proliferation and enhanced growth. This requirement to alter cellular metabolism has now been considered an additional “Hallmark of Cancer”²¹⁸. In addition to glucose utilization, cancer cells have been shown to rely on glutamine or fatty acids to support cellular processes. For

example, T cells that upregulate the transcription factor *c-myc* have elevated glutamine utilization and concordantly, deprivation of glutamine in these cells reduces their capacity to proliferate²⁷⁸. Additionally, cancer cells upregulate multiple genes that are important for lipid biogenesis and exhibit high levels of fat utilization²⁷⁹. In my work, I show that tumors with low Beclin 1 expression have reduced insulin-stimulated glucose uptake and that mice with these tumors exhibit systemic insulin-resistance. Additionally, mice with *shBECN1* tumors exhibit reduced inguinal white fat and increased energy expenditure, symptoms that are early signs of cancer cachexia. These results suggest that Beclin 1 can alter metabolism and upon further exploration may provide a novel mechanism by which Beclin 1 regulates tumor biology and promotes aggressive tumor phenotypes.

Recent work showed that autophagy can regulate glucose metabolism. Inhibition of autophagy in *KRAS* mutant mouse embryonic fibroblasts (MEF) reduces glucose uptake and glycolysis²²⁴. However, in my work I observe that *RAS* mutant tumors with low Beclin 1 expression have reduced glucose uptake while low ATG5 expressing tumors maintain glucose uptake. One explanation for this difference is that Jay Debnath's group used a complete knockout of autophagy genes *Atg7 and Atg5*. However, my study uses shRNA to reduce Beclin1 and ATG5 expression. Maintenance of some level of protein expression may allow

for some Beclin 1 and ATG5 specific functions to occur which may explain the difference in the glucose uptake phenotype observed in my study.

While preliminary, my data suggest a novel role for Beclin 1 in *de novo* lipogenesis, a lipid pathway that is often upregulated in multiple cancer types. *shBECN1* tumors appear to have elevated ACC1 and FASN expression, enzymes that are essential for *de novo* lipogenesis, as well as low pAMPK S345 expression. AMPK phosphorylates ACC1 to inhibit lipid metabolism²⁸⁰. To further understand whether Beclin 1 has a novel function in *de novo* lipogenesis, an investigation of lipid metabolism in Beclin 1 deficient cells and tumors is warranted as it could provide novel insight into Beclin 1 function in the aggressive behavior of tumors. Acetate incorporation into different lipid products such as triglycerides or phospholipids can be used to assess *de novo* lipogenesis. Additionally, I can probe for the upregulation of other proteins that are important for *de novo* lipogenesis such as ATP-citrate lyase (ACYL) which converts citrate to Acetyl-CoA, the substrate for fatty acid synthesis²⁷⁹. *De novo* lipogenesis is of clinical interest as inhibitors of ACC1 and FASN have been used to inhibit proliferation in cancer cells^{281,282}. Exploration of ACC1 inhibitors in my *ex vivo* drug analysis may reduce proliferation in *shBECN1* tumors and can potentially be used as a novel therapeutic treatment for low Beclin 1 expressing tumors. It is possible that Beclin 1 exerts its effects on tumor metabolism by modulating multiple metabolic pathways. In order to examine multiple metabolic pathways,

metabolomics profiling through liquid chromatography mass spectrometry can be performed. This technique can also provide more evidence for a role of Beclin 1 in *de novo* lipogenesis as well as other metabolic pathways that can be exploited for cancer therapy.

Deregulation of metabolism is present in multiple pathogenic diseases including cancer. My studies in Chapter III suggest that mice with *shBECN1* tumors exhibit early signs of cancer cachexia, a wasting disease present in end stage cancer that is a major cause of mortality and morbidity. Patients with cancer cachexia experience metabolic disturbances such as insulin resistance, muscle wasting, fat loss, and increase energy expenditure²⁸³. While my results are preliminary, it is important to address the role of Beclin 1 in the cancer cachexia phenotype as Beclin 1 may serve as biomarker for this disease in which few biomarkers exist. There are several rodent models of cancer cachexia such as the Yoshida ascites hepatoma rat model, a lung cancer model and murine colon cancer model²⁸⁴⁻²⁸⁶. I could use these models to examine the progression of cancer cachexia in mice with *shBECN1* tumors compared to control mice. Moreover, studying the role of Beclin 1 in cancer cachexia may provide novel therapeutic strategies for this devastating cancer related disease.

Significance of Findings

Beclin 1 is a predictor of prognosis in multiple cancer types but the role of Beclin 1 in these aggressive cancers has yet to be identified. Additionally, studies that focus on Beclin 1 attribute its function in cancer to autophagy without acknowledging its autophagy-independent functions. In my study, I have found that Beclin 1 is able to control the expression and function of both growth factor and nutrient receptors by regulating HRS recruitment to endosomes in an autophagy-independent manner. In regard to a TNBC model, I have shown that EGFR signaling is elevated in this breast cancer subtype and tumors with this aberrant signaling are sensitive to inhibitors of this pathway. My work also identifies a potential role for Beclin 1 in lipid metabolism that may provide an approach for treatment. Overall my study provides future strategies for the treatment of low Beclin 1 expressing breast tumors with the goal of improving patient outcomes.

APPENDIX A

Beclin 1 regulates IGFBP1 expression in an autophagy-independent manner

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AUTHOR CONTRIBUTIONS

A.N.M.-O. and L.M.S. were involved in the conception and design of the project and wrote the manuscript; D.R.-R. performed experiments for A.3. F.L. generated lentiviral constructs used to knockdown Beclin 1 and restore Beclin 1 or IGFBP1. M.L. was involved with the acquisition and analysis of data.

In Chapter II, I showed that loss of Beclin 1 expression in a triple negative breast cancer (TNBC) cell line resulted in enhanced tumor growth and proliferation. I demonstrated that Beclin 1 regulates HRS recruitment to endosomes to modulate growth factor and nutrient receptor degradation. To understand further the mechanism by which low Beclin 1 expression promotes tumor growth, I performed RNA-sequencing on 4 *shGFP* and 4 *shBECN1* tumors to identify differentially expressed genes. RNA was extracted from each tumor using the RNeasy kit (Qiagen). RNA-sequencing was performed by Applied Biological Material, Inc. (ABM, Canada) using single-end sequencing with a reading length of 75 base pairs and sequencing depth of 20 million reads/sample. Only 53 differentially expressed genes were identified (Appendix A, Figure 1A). Importantly, reduced *BECN1* expression was observed in *shBECN1* tumors (Appendix A, Figure 1A, red arrow).

One of the downregulated genes in *shBECN1* tumors encodes for Insulin-like growth factor binding protein-1 (IGFBP1), a 30 kd secreted protein that acts as a decoy for the IGF-1 and -2 ligands to prevent their binding to the IGF-1, IGF-2 and Insulin (IR) receptors (Appendix A, Figure 1A, gray arrow)^{287,288}. This target was of interest to us because we previously showed that Beclin 1 regulates growth factor receptor signaling downstream of the IGF-1R and IR and that reduced Beclin 1 expression in TNBC cells results in sustained and enhanced activation of AKT, a protein that promotes cancer cell invasion and survival¹³¹.

We hypothesized that a reduction in IGFBP1 expression could promote enhanced IGF-1R or IR signaling because IGFBP1 antagonizes their action. I used RQ-PCR to validate several of the up- and down-regulated genes identified by the RNA-Seq analysis in a larger panel of tumors and I confirmed that *IGFBP1* is a significantly down-regulated gene in *shBECN1* tumors (Appendix A, Figure 1B). IGFBP1 protein expression was also reduced in *shBECN1* tumors (Appendix A, Figure 1C). Furthermore, restoration of Beclin 1 expression increased *IGFBP1* mRNA expression (Appendix A, Figure 2A and 2B). These findings support that Beclin 1 regulates IGFBP1 expression at the level of gene expression. Moreover, regulation of IGFBP1 expression may serve as another novel mechanism by which Beclin 1 can regulate growth factor receptor signaling, in addition to its function in endocytic receptor trafficking.

As stated previously, the role of Beclin 1 in cancer has been attributed primarily to its function in autophagy. In Chapter II, we showed that Beclin 1 regulates growth factor receptor degradation through HRS and that this regulation is independent of autophagy. To determine if the regulation of IGFBP1 by Beclin 1 is also autophagy-independent, I examined IGFBP1 expression in breast carcinoma cells with low ATG5 expression. My data indicate that loss of ATG5 expression does not alter IGFBP1 mRNA expression (Appendix A, Figure 3A and 3B). The regulation of IGFBP1 expression by Beclin 1 but not ATG5 was also

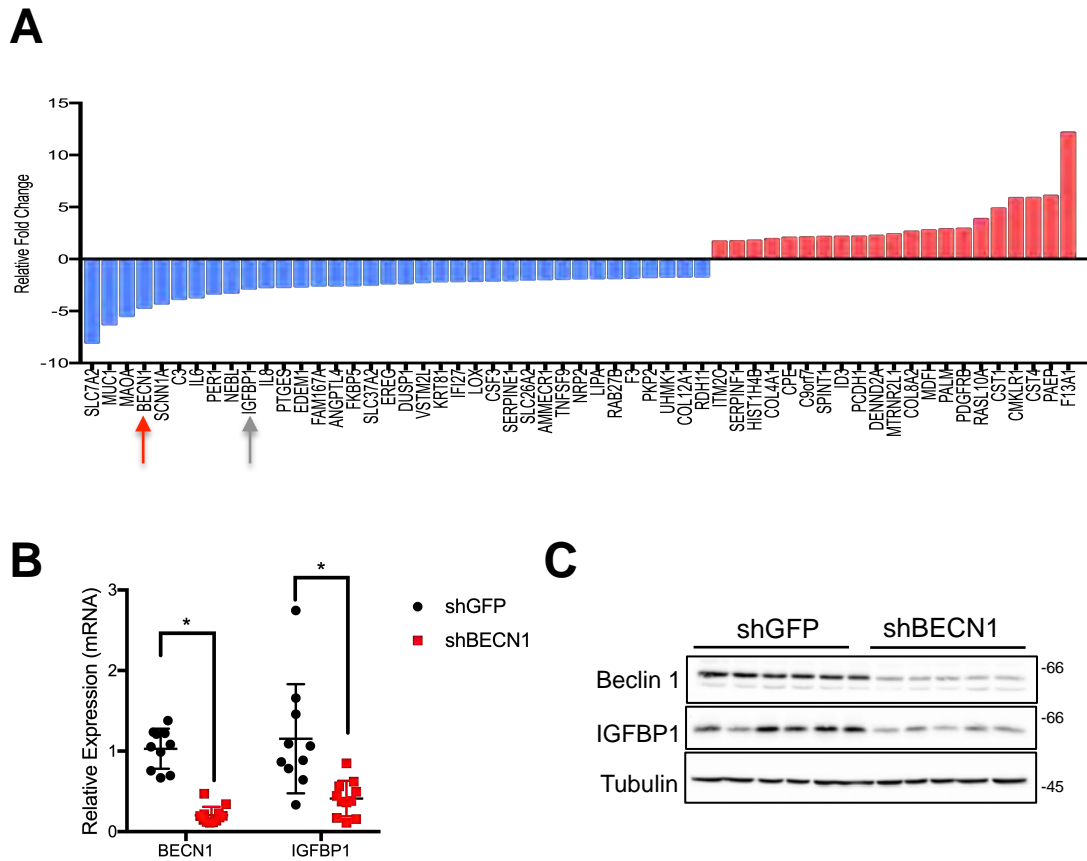


Figure A.1. IGFBP1 expression is reduced in *shBECN1* tumors. (A) RNA sequencing was performed on 4 *shGFP* and 4 *shBECN1* tumors. 53 differentially expressed genes were identified. Red arrow indicate *BECN1*. Gray arrow represents *IGFBP1*. (B) Relative mRNA expression of *IGFBP1* and *BECN1* were determined by real-time quantitative PCR (RQ-PCR) in tumor expressing *shGFP* and *shBECN1*. The data shown represent the mean \pm SEM mRNA expression from nine tumors. (C) Immunoblot analysis of Beclin 1 and IGFBP1 expression in tumors expressing *shGFP*, *shBECN1* and *shBECN1:Beclin 1*. * $p < 0.05$

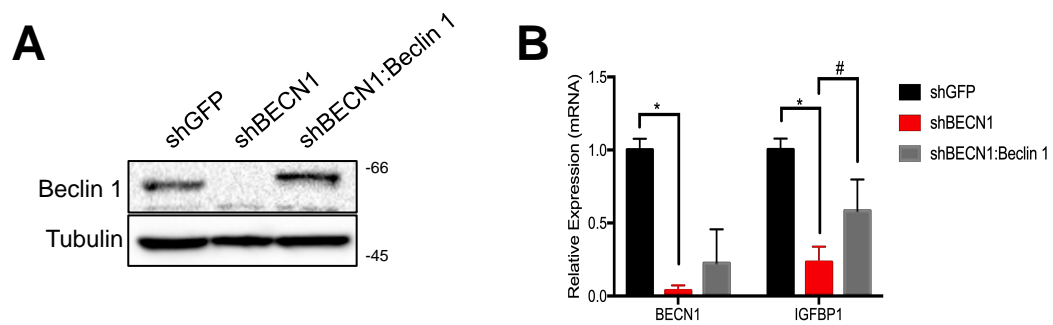


Figure A.2. Beclin 1 regulates IGFBP1 expression. (A) Immunoblot analysis of Beclin 1 expression in LM2 cells expressing *shGFP*, *shBECN1* and *shBECN1:Beclin 1*. (B) Relative mRNA expression of *IGFBP1* was determined by real-time quantitative PCR (RQ-PCR) in LM2 cells expressing *shGFP*, *shBECN1* and *shBECN1:Beclin 1*. The data shown represent the mean \pm SEM mRNA expression from three independent experiments. * $p < 0.05$

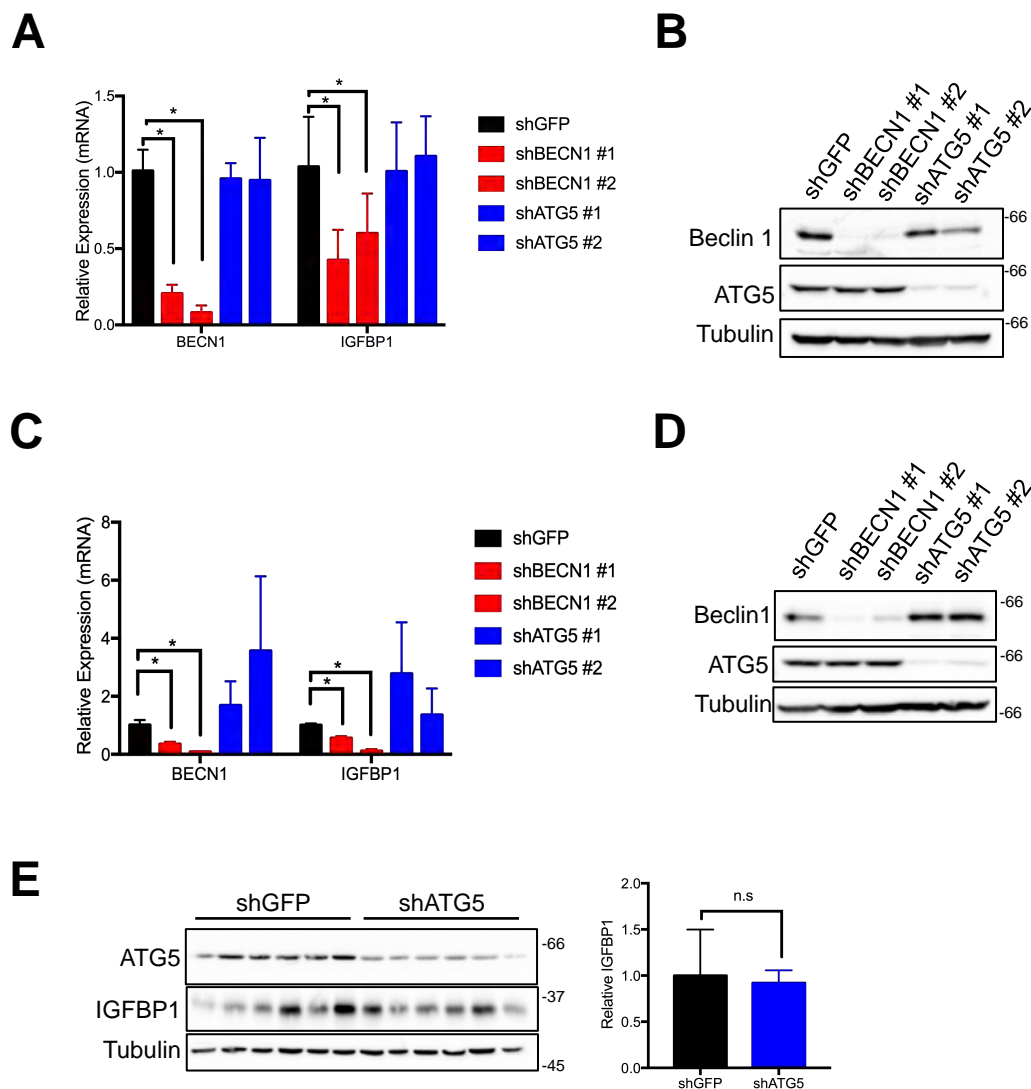


Figure A.3. ATG5 does not regulate IGFBP1 expression. (A) Relative mRNA expression was determined by real-time quantitative PCR (RQ-PCR) in LM2 cells expressing *shGFP*, *shBECN1* #1, *shBECN1* #2, *shATG5* #1 and *shATG5* #2. The data shown represent the mean \pm SEM mRNA expression from three independent experiments. (B) Immunoblot analysis of IGFBP1 expression in *shGFP*, *shBECN1* #1, *shBECN1* #2, *shATG5* #1 and *shATG5* #2 LM2 cells. (C) Immunoblot analysis of *shGFP* and *shATG5* tumors. The data shown in the graph represent the mean \pm SEM expression of six tumors from each genotype and are shown as fold change in expression relative to *shGFP* tumors. * $p < 0.05$

observed in a lung carcinoma cell line (Appendix A, Figure 3C and 3D). Furthermore, analysis of ATG5 deficient tumors showed no change in IGFBP1 protein expression when compared to control *shGFP* tumors (Appendix A, Figure 3E). These results suggest that Beclin 1 regulates IGFBP1 expression in an autophagy-independent manner.

Given that IGFBP1 can negatively regulate IGF-1R signaling it was important to determine whether restoration of IGFBP1 expression could suppress the growth of *shBECN1* tumors. To do so, an IGFBP1-myc tag cDNA was cloned into the pCDH-puro lentiviral plasmid to infect *shBECN1* cells. Beclin 1 knockdown cells were generated using a neomycin-selected shRNA for this study. Cells co-expressing *shBECN1* and either empty *pCDH* or *pCDH-IGFBP1* were injected into the mammary fat pad of NOD-SCID mice (Appendix A, Figure 4A). Overexpression of IGFBP1 in *shBECN1* tumors inhibited tumor growth to *shGFP* tumor levels (Appendix A, Figure 4B). Examination of PH3 staining in *shBECN1:IGFBP1* tumors revealed that restoration of IGFBP1 expression in *shBECN1* tumors reduced proliferation to the level of proliferation observed in *shGFP* tumors (Appendix A, Figure 4C). Together these results indicate that the reduction of IGFBP1 expression in tumors with low Beclin 1 expression may contribute to enhanced tumor growth.

Previous work has shown that autophagy competent *RAS* transformed cells secrete factors that promote invasion²⁸⁹. Knockdown of *Atg7* in *RAS* transformed mouse embryonic fibroblasts prevents the secretion of IL-6, a cytokine that promotes migration²⁸⁹. Treatment of *Atg7*^{-/-} mouse embryonic fibroblasts with media from autophagy-competent cells restored their capacity to invade²⁸⁹. Given that autophagy genes can govern secretion, I examined if Beclin 1 regulates IGFBP1 by a mechanism involving secretion of a regulatory factor. Conditioned media from *shBECN1* cells was used to treat *shGFP* cells. Interestingly, *shGFP* cells incubated in *shBECN1* conditioned media for 24 hours exhibited a reduction in *IGFBP1* mRNA expression (Appendix A, Figure 5). This preliminary finding suggests that *shBECN1* cells secrete a negative regulator of IGFBP1 expression. Additional experiments are necessary to confirm this mechanism of regulation and to identify this secreted factor to determine how Beclin 1 regulates IGFBP1 expression.

Our work presented in this Appendix indicates that Beclin 1 regulates IGFBP1 expression to suppress tumor growth. Ongoing studies are needed to understand the role of IGFBP1 in human tumors with low Beclin 1 expression. IGFBP1 antagonizes IGF1 and IGF2 action and prevents activation of the IGF1R and IR to promote cellular survival and invasion; therefore it would be an interesting clinical target. One hypothesis is that reduced IGFBP1 expression may increase levels of free IGF ligand resulting in increased activation of downstream

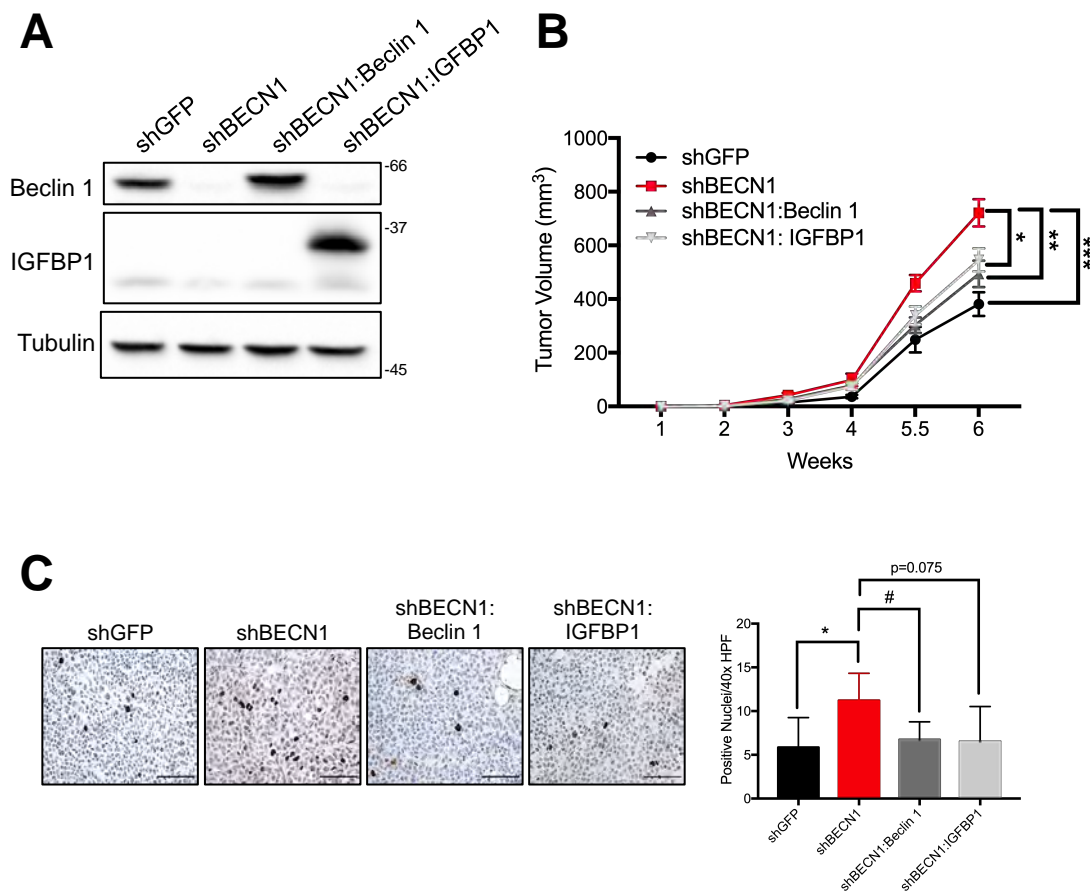


Figure A.4. Rescue of IGFBP1 suppresses growth of *shBECN1* tumors. (A) Expression of Beclin 1 and IGFBP1 in cell lines prior to mouse injection. (B) Tumor growth of orthotopic xenografts in NOD-SCID mice. (C) Representative images of Phospho-histone H3 (PH3) staining in *shGFP*, *shBECN1*, *shBECN1:Beclin 1* and *shBECN1:IGFBP1* tumors. The data shown in the graphs represent the mean \pm SEM positive nuclei/high powered field (hpf; five independent images/five tumors; n = 25). Scale bar = 50 μ m.

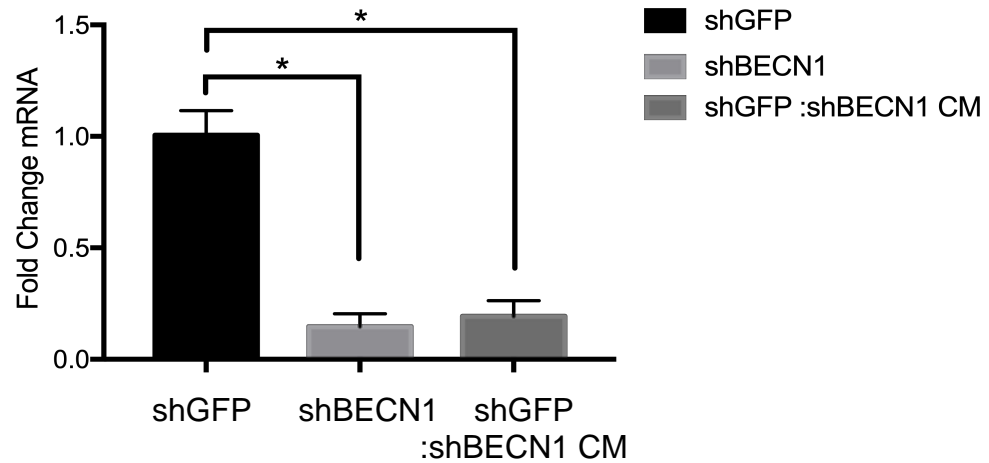


Figure A.5. Beclin 1 regulates IGFBP1 expression through a secreted factor. Conditioned media (CM) from *shBECN1* cells was collected and transferred to shGFP cells for 24 hours. Relative mRNA expression was determined by real-time quantitative PCR (RQ-PCR). The data shown represent the mean \pm SEM mRNA expression from two independent experiments. * $p < 0.05$

receptors in a feed forward loop. However, in my studies, *shBECN1* tumors do not exhibit increased IGF-1R or IR activity as evidenced by no changes in receptor phosphorylation or activation of their key downstream signaling effector PI3K in our RPPA dataset. This was confirmed through immunoblot analysis of *shBECN1* tumors. These findings suggest that IGFBP1 is likely acting independently of these receptors. IGFBP1 does have reported functions that are independent of its regulation of IGF1 and IGF2 action through their receptors. IGFBP1 contains an Arg-Gly-Asp (RDG) motif that promotes interactions with integrins²⁸⁷. In fact, IGFBP1 interacts with the $\alpha_5\beta_1$ integrin to promote migration in Chinese hamster ovary cells and these same results were seen in human trophoblastic cells^{290,291}. Integrins are important for cancer cell interactions and expression of integrins, either increased or decreased, can promote tumor progression²⁹². These alternative functions of IGFBP1 may play a role in the *shBECN1* tumor growth phenotype and warrant deeper exploration.

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