

eScholarship@UMassChan

Autophagy-Independent Role for Beclin 1 in the Regulation of Growth Factor Receptor Signaling: A Dissertation

Item Type	Doctoral Dissertation
Authors	Rohatgi, Rasika
DOI	10.13028/M26S32
Publisher	University of Massachusetts Medical School
Rights	Copyright is held by the author, with all rights reserved.
Download date	2026-06-16 12:03:03
Link to Item	https://hdl.handle.net/20.500.14038/32249

**AUTOPHAGY-INDEPENDENT ROLE FOR BECLIN 1 IN
THE REGULATION OF GROWTH FACTOR RECEPTOR
SIGNALING**

A Dissertation presented

By

Rasika Rohatgi

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

January 15, 2015

Cancer Biology

**AUTOPHAGY-INDEPENDENT ROLE FOR BECLIN 1 IN
THE REGULATION OF GROWTH FACTOR RECEPTOR
SIGNALING**

A Dissertation Presented

By

Rasika Rohatgi

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

Leslie M. Shaw, Ph.D., Thesis Advisor

Silvia Corvera, MD, Member of Committee

Karl J Simin, Ph.D., Member of Committee

Brian Lewis, PhD., Member of Committee

Elizabeth P. Henske, MD, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Eric H. Baehrecke, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Cancer Biology Program
January 15, 2015

ACKNOWLEDGEMENTS

The last five and a half years have been an extremely exciting and adventurous period of my life. During this I made several friends and gained knowledge that will be treasured for the next several years to come.

I would like to start by expressing my gratitude towards my thesis advisor, Dr. Leslie Shaw. Her guidance and support have helped me think critically and become a better researcher. Her confidence in me, and constant encouragement helped me through both the good and bad phases of my journey towards attaining my PhD. I am grateful for the time, effort and patience that she has devoted in an attempt to extract the best out of me. She is not only a great scientist but also a very understanding human being who mentored me in both my scientific and non-scientific life.

I want to acknowledge the support of the members of my Thesis Research Advisory Committee Dr. Eric H. Baehrecke (Chair), Dr. Silvia Corvera, Dr. Karl J Simin, Dr. Jason Kim and Dr. Fumihiko Urano. Their guidance and suggestions have helped me grow as a scientist. I am also thankful to Dr. Brian Lewis and Dr. Elizabeth Henske (Harvard Medical School) for agreeing to serve in my Defense Examination Committee.

Next, I wish to thank Dr. Silvia Corvera for her time and patience in teaching me TIRF imaging and analysis. Her passion for science inspires me and

fills me with enthusiasm each time. A special mention goes to the Corvera lab members for making me feel comfortable and welcomed in their lab as I mastered the procedures they taught me. I especially want to thank Dr. Deborah Leonard for her assistance with TIRF microscopy as well as Dr. Karl Bellve and Kevin Fogarty from the Bioimaging group for their help with image acquisition and analysis.

I would also like to thank my lab members both past and present: Dr. Shannon Pankratz Dr. Katerina Mardilovich, Dr. Justine Landis, Dr. Jennifer Clark, Dr. Xiaoqing Yang, Dr. Anuradha Seshadri, Dr. Sha Zhu and Asia Mathew for making my PhD journey a wonderful learning experience. Special thanks goes to Jenny Janusis and Jose Mercado-Matos for all their support, advice and assistance with several experiments as well as for making the lab a fun place to work in. I am also appreciative of the help from Dr. Mihir Rajurkar, Dr. Sanjoy Samanta and Jennifer Cotton. I would like to acknowledge Dr. Usha Acharya for her advice and assistance with PI3P lipid analysis.

Finally, I would like to thank my loving family who has inspired, encouraged and supported me in every endeavor I have undertaken. I am thankful to my parents for the unconditional love and faith they have imparted throughout my life and my sisters and brother for their unwavering love and belief in me. I am also grateful to my loving husband Dr. Varun Kapoor for his certitude and confidence in me and for filling my life with happiness and bliss.

As I conclude this chapter in my life, I thank all the above-mentioned people as well as those I failed to mention. As without you I may not have been able to accomplish what I have now and what I will cherish forever. Thank you all!

ABSTRACT

Beclin 1 is a haplo-insufficient tumor suppressor that is decreased in many human tumors. The function of Beclin 1 in cancer has been attributed primarily to its role in the degradative process of autophagy. However, the role of autophagy itself in tumorigenesis is context-dependent and can be both preventive and promoting. Due to its dual function in cancer a better understanding of this process is necessary to develop potential novel cancer therapies. To gain insight into the role of autophagy in breast carcinoma, I analyzed the autophagy-dependency of different subtypes of breast cancer. My results implicate that triple-negative breast carcinoma cells are more dependent on autophagy than luminal breast carcinoma cells. Chemical inhibition of autophagy decreased the tumorigenicity of triple-negative breast carcinoma cells with regard to proliferation and anchorage-independent growth. However, RNAi-mediated suppression of two autophagy genes, ATG5 and Beclin 1, revealed different outcomes. While suppression of ATG5 decreased glycolysis, Beclin 1 depletion did not affect the glycolytic rates. These results suggest autophagy-independent pro-tumorigenic effects of loss of Beclin 1 in cancer.

Beclin 1 is a core component of the Vps34/Class III PI3K (PI3KC3) and Vps15/p150 complex that regulates multiple membrane trafficking events. I describe a novel mechanism of action for Beclin 1 in breast cancer involving its control of growth factor receptor signaling. I identify a specific stage of early

endosome maturation that is regulated by Beclin 1, the transition of APPL1-containing phosphatidylinositol 3-phosphate-negative (PI3P⁻) endosomes to PI3P⁺ endosomes. Beclin 1 regulates PI3P production in response to growth factor stimulation to control the residency time of growth factor receptors in the PI3P⁻/APPL⁺ signaling competent compartment. As a result, suppression of *BECN1* sustains growth factor stimulated AKT and ERK activation resulting in increased breast carcinoma cell invasion. In human breast tumors, Beclin 1 expression is inversely correlated with AKT and ERK phosphorylation. Taken together my data identify a novel role for Beclin 1 in regulating growth factor signaling and reveal a mechanism by which loss of Beclin 1 expression would enhance breast cancer progression independent of its impact on autophagy.

TABLE OF CONTENTS

TITLE PAGE	i
SIGNATURE PAGE	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	vi
TABLE OF CONTENTS	xii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
ABBREVIATIONS	xv
CHAPTER I : <i>INTRODUCTION</i>	1
Beclin 1	2
Beclin 1-Interacting Proteins	3
Functions of Beclin 1	7
Beclin 1 and Cancer	18
Rationale for the Thesis Project	24
CHAPTER II : <i>MATERIALS AND METHODS</i>	26
Cell lines	27
Plasmids	28
Immunoblotting and antibodies	29
Autophagic flux assay	30

Glucose uptake and lactate production assay	30
Proliferation assays	31
Soft agar colony formation assay	31
Growth factor stimulation assays	32
Quantitation of PI3P lipid	33
RNA extraction and real-time quantitative PCR (qRT-PCR)	33
Invasion and migration assays	34
Immunofluorescence staining and imaging	35
Total Internal Reflection Fluorescence (TIRF)/Epi-fluorescence Structure-illumination Microscopy (TESM) and image analysis	36
Statistical Analysis	38

CHAPTER III : CHARACTERIZATION OF AUTOPHAGY IN BREAST

CARCINOMA CELLS	39
Introduction	40
Results	43
Triple-negative breast cancer cell lines have higher levels of basal autophagy than luminal breast cancer cell lines	43
Triple-negative MDA-MB-231 breast carcinoma cells are more sensitive to autophagy inhibition than luminal T47D cells	48
Genetic disruption of autophagy inhibits the anchorage-independent growth of MDA-MB-231 cells	53
Suppression of ATG5 but not Beclin 1 inhibits aerobic glycolysis	55

Discussion.....	59
CHAPTER IV : <i>BECLIN 1 REGULATES EARLY ENDOSOME MATURATION AND GROWTH FACTOR RECEPTOR SIGNALING</i>	65
Introduction.....	66
Beclin 1 regulates growth factor receptor signaling	69
Beclin 1 in receptor degradation and signaling	78
Beclin 1 regulates growth factor receptor-dependent PI3KC3 activation and endosomal maturation.....	81
Beclin 1 negatively regulates invasion in breast carcinoma cell lines	98
Beclin 1 inversely correlates with phosphorylated AKT and ERK in human tumors	98
Discussion.....	104
Acknowledgements	107
CHAPTER V : <i>DISCUSSION</i>	108
Summary	109
Impact of Beclin 1 loss in cancer.....	112
Beclin 1 in human tumors.....	116
Regulation of Beclin 1 function by signaling pathway components.....	118
Role of Beclin 1 complexes in oncogenic signaling	119
The impact of Beclin 1 on endosomal maturation	121
Targeting autophagy for cancer treatment.....	123
Overall significance	124

APPENDIX : ANALYSIS OF EPIGENETIC REGULATION OF BECN1 IN BREAST CARCINOMA CELLS	126
BIBLIOGRAPHY	130

LIST OF TABLES

Table 3.1. Molecular classification of breast carcinoma cell lines.....	44
--	-----------

LIST OF FIGURES

Figure 1.1. Beclin 1 protein and its complexes	4
Figure 1.2. Regulators of autophagy	13
Figure 3.1. Comparison of basal autophagy in breast carcinoma cell lines	45
Figure 3.2. Comparison of autophagic flux between MDA-MB-231 and T47D cells	47
Figure 3.3. Comparison of aerobic glycolysis in breast carcinoma cell lines.....	49
Figure 3.4. The anchorage-dependent growth of MDA-MB-231 cells is more sensitive to autophagy inhibition when compared with T47D cells	51
Figure 3.5. The anchorage-independent growth of MDA-MB-231 cells is more sensitive to autophagy inhibition when compared with T47D cells	52
Figure 3.6. Autophagic flux in cells with suppression of ATG5 or Beclin 1 expression.....	54
Figure 3.7. Autophagy facilitates anchorage-independent growth in MDA-MB-231 cells	56
Figure 3.8. Suppression of ATG5, but not Beclin 1, expression inhibits glycolysis in MDA-MB-231 cells.....	58
Figure 4.1. Beclin 1 depletion increases IGF-1R protein levels.....	70
Figure 4.2. Beclin 1 regulates IGF-1-dependent signaling in MDA-MB-231 cells	72
Figure 4.3. Beclin 1 regulates IGF-1-dependent signaling in MCF7 cells	73
Figure 4.4. Beclin 1 regulates IGF-1-dependent signaling in MCF10A cells	74
Figure 4.5. Beclin 1 regulates EGF-dependent signaling	75
Figure 4.6. Ectopic expression of Beclin 1 decreases growth factor-dependent signaling	76

Figure 4.7. Rescue of Beclin 1 expression restores growth factor-dependent signaling to control levels	77
Figure 4.8. Regulation of EGFR degradation and signaling by Beclin 1	79
Figure 4.9. Regulation of IGF-1R degradation and signaling by Beclin 1.....	80
Figure 4.10. Regulation of receptor degradation and signaling by Beclin 1 is autophagy-independent.....	82
Figure 4.11. Chemical inhibition of autophagy does not enhance AKT activation	83
Figure 4.12. Beclin1 regulates growth factor signaling in early endosomes.....	85
Figure 4.13. Beclin1 regulates APPL1 ⁺ endosomes.....	87
Figure 4.14. Beclin1 regulates growth factor-dependent early endosome maturation	89
Figure 4.15. Beclin1 regulates growth factor-dependent transition to PI3P ⁺ early endosomes.....	90
Figure 4.16. Rescue of Beclin 1 expression restores PI3P ⁺ endosomes.....	93
Figure 4.17. Beclin 1 controls the maturation of early endosomes.....	94
Figure 4.18. Beclin 1 controls PI3P levels	97
Figure 4.19. Beclin 1 regulates breast carcinoma cell invasion.....	99
Figure 4.20. Rescue of Beclin 1 expression decreases enhanced invasion in Beclin 1-depleted cells	100
Figure 4.21. Inhibition of AKT signaling suppressed increased invasion in Beclin 1-deficient cells.....	101
Figure 4.22. Inverse correlation of Beclin 1 expression and AKT and ERK phosphorylation in human breast tumors	103
Figure 5.1. Model of Beclin 1's regulation of growth factor receptor signaling .	111
Appendix Figure 1. Epigenetic regulation of Beclin 1.....	129

ABBREVIATIONS

AMBRA1	Activating molecule in Beclin 1-regulated autophagy protein 1 or Autophagy/Beclin 1 regulator 1
APPL	Adaptor protein with phosphotyrosine binding, pleckstrin homology domains and leucine zipper motif
ATG	Autophagy related
Bcl-2	B-cell lymphoma 2
BECN1	Beclin 1
BH3	Bcl-2 homology-3
Bif-1	Bax-interacting factor-1
CCD	Coiled-coil domain
CHX	Cycloheximide
CQ	Chloroquine
ECD	Evolutionarily conserved domain
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

FYVE	Fab1p, YOTB, Vac1p, EEA1
GPCR	G-protein coupled receptor
HOPS	Homotypic fusion and vacuole protein sorting
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
LC3	Microtubule-associated protein 1 light chain 3 (MAP1LC3)
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
mTORC1/2	Mammalian/mechanistic target of rapamycin complex 1/2
PI	Phosphoinositides
PI3K	Class I phosphatidylinositol 3-kinase
PI3KC3	Class III phosphatidylinositol 3-kinase or Phosphatidylinositol 3-kinase, catalytic subunit type 3
PIKfyve	FYVE-containing phosphatidylinositol 3-phosphate 5-kinase
PI3P	Phosphatidylinositol 3-phosphate
p62/SQSTM1	Sequestosome 1
ROS	Reactive oxygen species

Rubicon	RUN domain, cysteine-rich domain containing, Beclin 1 interacting protein
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
TESM	TIRF/epi-fluorescence structured illumination microscopy
TIRF	Total internal reflection fluorescence
TNBC	Triple negative breast cancer
UVRAG	UV radiation resistance-associated gene

CHAPTER I

INTRODUCTION

Beclin 1

Beclin 1 is a 60kDa highly conserved mammalian orthologue of the yeast Atg6/vacuolar protein sorting-30 (Vps30) protein. It was initially identified as a gene regulating vacuolar protein sorting in yeast (1). This discovery was followed by its recognition as an essential protein involved in the yeast autophagic process (2). The same year it was also identified in the mammalian system through a yeast two-hybrid screen. In mammals it was originally identified as a Bcl-2-interacting myosin-like coiled-coil protein (Beclin) with a potential role in the host defensive system as it provided protection to mice against Sindbis virus induced encephalitis (3). Subsequently, Beclin 1 was shown to promote autophagy and tumorigenesis in mammals (4). Since then Beclin 1 protein has been shown to play a role in numerous biological functions including autophagy stress adaptation, vesicular trafficking, cytokinesis, and cell death and it is essential for normal development (5).

The Beclin 1 protein has three identifiable structural domains – the N-terminal Bcl-2 homology-3 (BH3) domain, a coiled-coil domain (CCD) and a C-terminal evolutionarily conserved domain (ECD) (5-7). It also has a short leucine-rich nuclear export signal that is required for its cytoplasmic localization (8). Through these domains it interacts with various proteins and carries out its diverse functions (Figure 1.1A).

Beclin 1-Interacting Proteins

One of the most important proteins in the Beclin 1 interactome is the lipid kinase Class III phosphatidylinositol 3-kinase (PI3KC3; homologue of yeast Vps34). PI3KC3 phosphorylates phosphoinositides (PI) at the 3' position of the inositol ring to generate phosphatidylinositol-3-phosphate (PI3P) (9). Beclin 1 binds to PI3KC3 via its ECD and it is responsible for activation of this enzyme (10). Together with p150 (a homolog of yeast Vps15), Beclin 1 and PI3KC3 form a core complex. The myristoylated p150 subunit targets and anchors the core complex to the membrane (11). The core complex forms the central platform that recruits additional regulatory proteins to form multiple, distinct complexes that may explain the various cellular functions of Beclin 1. Two major complexes have been described in both yeast and mammals (Figure 1.1B) (12, 13).

Complex I

The macromolecular Complex I is comprised of the core complex bound to autophagy related 14-like (ATG14L; a homolog of yeast Atg14). It is also known as Beclin 1-associated autophagy-related key regulator (Barkor). ATG14L heterodimerizes with the CCD region of Beclin 1 and directs the complex to the site of autophagosome initiation - the endoplasmic reticulum (5, 14). Complex I is required for initiation of the autophagic process. Activating molecule in Beclin 1-regulated autophagy protein (AMBRA1) is a Beclin 1-binding protein, which promotes interaction between Beclin 1 and PI3KC3 and thereby promotes auto-

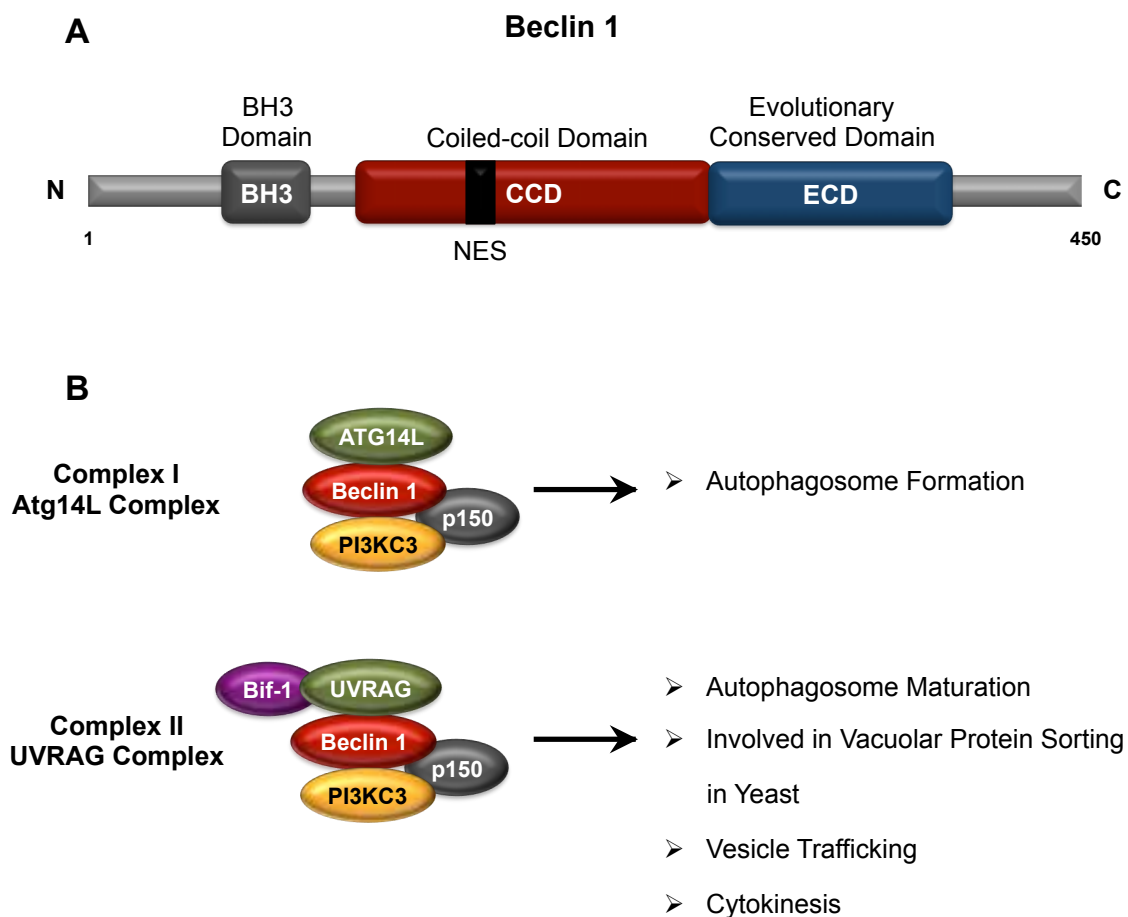


Figure 1.1. Beclin 1 protein and its complexes.

(A) Schematic of Beclin 1 and its structural domains. NES, Nuclear export signal. (B) The two complexes formed by Beclin 1 and their known functions. Beclin 1, PI3KC3 and p150 form the core complex to which ATG14L or UVRAG binds to form complex I or II respectively. Figure adapted from Wirawan *et. al.* (5).

phagosome nucleation (15).

Complex II

The macromolecular Complex II is formed by recruitment of UV radiation resistance-associated gene (UVRAG; a homologue of yeast Vps38) to the core complex. Binding of UVRAG to the CCD region of Beclin 1 further enhances PI3KC3 activity (16). Of note, the interaction of ATG14L and UVRAG with Beclin 1 is mutually exclusive and distinguishes Complex I from Complex II (13, 17). The UVRAG protein regulates endosome-endosome and endosome-autophagosome fusion, and hence vesicle maturation, by interacting with the homotypic fusion and vacuole protein sorting (HOPS)/class C-VPS complex and stimulating the endosomal fusion machinery (described ahead) (18). In yeast, Complex II is involved in vacuolar protein sorting but not autophagy. Deletion of the complex inhibits proper sorting of carboxypeptidase Y (CPY) from the pre-vacuolar compartment (endosome) to the vacuole (12). In mammals, Complex II was initially reported to play a role in the initiation and maturation of autophagosomes (16). However, recent studies have shown additional autophagy-independent functions for this complex including membrane trafficking events that regulate receptor degradation and cytokinesis (18, 19). Bax-interacting factor-1 (Bif-1; also known as Endophilin B1 or SH3GLB1) is recruited to Complex II via UVRAG and it further enhances PI3KC3 activity (20).

Additional Beclin 1 regulatory interactions

Beclin 1, and hence the two complexes, can also be regulated by additional interacting proteins. The anti-apoptotic Bcl-2 and Bcl-X_L proteins bind to the BH3 domain of Beclin 1 and inhibit its interaction with PI3KC3. This binding disrupts the core complex, thereby decreasing the lipid kinase activity and inhibiting autophagy (21, 22). Phosphorylation of Bcl-2 or Beclin 1 by stress-activated c-Jun N-terminal protein kinase 1 (JNK) and death-associated protein kinase (DAPK), respectively, inhibits interaction of Bcl-2/Bcl-X_L with Beclin 1 resulting in activation of Beclin 1-mediated autophagy (23, 24). Similarly, High mobility group box 1 (HMGB1), in response to enhanced reactive oxygen species (ROS), binds to Beclin 1 leading to dissociation of the Beclin 1-Bcl-2 complex (25). In addition, the positive regulator of autophagy Ambra 1 can bind to both Beclin 1 and Bcl-2, as well as compete with Bcl-2 for binding to Beclin 1 thereby inducing autophagy (26). The Beclin 1-Bcl-2 interaction can also be disrupted by ubiquitination of the two proteins. TNF receptor associated factor 6 (TRAF6), in response to Toll-like receptor (TLR) signaling, ubiquitinates Beclin 1 to promote oligomerization of the protein, hence promoting autophagy (27).

Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein), a negative regulator of autophagy, also reduces the kinase activity of the core complex and down regulates autophagy (28). Inhibition of Rubicon enhances autophagosome maturation and epidermal growth factor

receptor (EGFR) degradation, while over expression impairs these functions (29). Moreover, tagged Rubicon localizes with late endosome/lysosome markers, and partially with early endosomes, suggesting that this protein may mostly regulate Complex II (28, 30).

AKT and EGFR have recently been reported to inhibit autophagy by negatively regulating Beclin 1. AKT phosphorylates Beclin 1, enhancing its interaction with 14-3-3 and vimentin intermediate filament proteins and resulting in inhibition of autophagy (31). Activation of EGFR also leads to inhibitory phosphorylation of Beclin 1. Active EGFR increases binding of Bcl-2 and Rubicon, and decreases binding of PI3KC3, to Beclin 1 resulting in down-regulation of autophagy (32).

Functions of Beclin 1

Beclin 1 is a key regulator of the lysosomal-dependent catabolic process called macroautophagy and it is one of the most studied mammalian autophagy proteins. In contrast, studies reporting autophagy-independent functions for Beclin 1 in mammals have only recently begun to emerge. The role of Beclin 1 (Atg6/Vps30) in vacuolar protein sorting in yeast has been documented since the 1990s (1). Early studies showed that although human *BECN1* can restore autophagy in *atg6/vps30*-disrupted yeast, it was unable to restore vacuolar protein sorting function (4). One of the reasons for this lack of rescue could be the difference in the protein sequences of the two orthologues that share only

24.4% amino acid identity with 39.1% conservation (4). Nonetheless, identification of the two Beclin 1 complexes in mammalian systems (Complex I and II; described earlier), similar to yeast suggests autophagy-dependent and independent functions for Beclin 1 in mammals. Moreover, in contrast to other Atg gene-deficient mice (such as *Atg5*^{-/-} and *Atg7*^{-/-}) which survive birth but succumb to neonatal starvation, *Becn1* null mice die early during embryogenesis (33-35). This finding further highlights that both autophagic and non-autophagic functions of Beclin 1 are likely important for the biological processes that it regulates.

Autophagy

Complex I and Complex II play a role in the initiation and maturation steps of autophagy, respectively (5). Autophagy, which means “self-eating” in Greek, is an evolutionarily conserved, lysosome-mediated catabolic process utilized by cells to maintain homeostasis (36). A basal level of autophagy maintains intracellular protein and organelle quality by eliminating damaged, excess and abnormal organelles and protein aggregates. It is also a survival mechanism of the cell against various kinds of stress such as starvation, during which unwanted cytoplasmic material is recycled and adenosine triphosphate (ATP) molecules are synthesized (33, 37). Autophagy can also act as a defense system of the cell by degrading intracellular bacteria, viruses and parasites and thereby protecting it from pathogen invasion (38).

Autophagy has been categorized into three different types based on the mode of delivery of the cargo to the lysosomes (36):

- a. Chaperone-mediated autophagy (CMA):** During CMA target substrates are recognized and translocated by chaperone protein Hsc70 (heat shock cognate 70) to the lysosomes where lysosomal-associated membrane protein type 2A (LAMP-2A) mediates their internalization into the lysosomal lumen (39-41).
- b. Microautophagy:** In microautophagy cytoplasmic substrates are directly internalized into the lysosomes by invagination of the lysosomal membrane (42).
- c. Macroautophagy:** This process involves sequestration of cytoplasmic material into double-membrane vesicles called autophagosomes which ultimately fuse with lysosomes to degrade the enclosed contents. This thesis will focus on this form of autophagy.

Macroautophagy (hereafter referred to as autophagy) can be induced by a number of stress signals such as starvation, oxidative stress and infection (43). Upon induction of autophagy a membrane structure known as a phagophore, or isolation membrane, starts forming around the cytosolic substrate. The isolation membrane elongates and eventually engulfs a portion of the cytosol in a double-membrane sequestering vesicle called the autophagosome. The major membrane source for the nucleation and elongation of the phagophore are the

endoplasmic reticulum (ER) – Golgi intermediate compartment membranes (44, 45). However, plasma membranes and cytoplasmic organelles, including the Golgi, endosomes and mitochondria have also been reported to contribute (46-50). Once the autophagosome is formed, the outer membrane fuses with the late endosome to form the amphisome, or with the lysosome to form the autolysosome. Within the autolysosome the autophagosomal inner membrane and its contents are degraded by lysosomal proteases.

Autophagy can be non-selective with no cargo-specificity or selective for its cargo. The type of cargo sequestered can further classify selective autophagy. For example, in mitophagy the autophagosome encapsulates and selectively eliminates damaged or excess mitochondria thereby maintaining mitochondrial quality and quantity (51, 52). Selective autophagy utilizes receptors and adaptors to recognize substrates and target them for degradation. For example the autophagic adaptor proteins p62/SQSTM1 (sequestosome 1), NDP52 and NBR1 (neighbor of Brca1 gene) recognize and bind polyubiquitylated substrates and form aggregates (53-55). These adaptors also have an LC3-interacting region (LIR) via which they associate with the autophagy protein LC3 (also known as microtubule-associated protein 1 light chain 3, MAP1LC3; described ahead) anchored to the growing autophagosomal membrane. The autophagosome encapsulates and degrades the substrates.

Regulators of autophagy

The initiation of the autophagic process is primarily controlled by mTOR complex 1 (mTORC1). This complex consists of a serine/threonine protein kinase mTOR (mammalian/mechanistic target of rapamycin), regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (DEPTOR) and Proline-rich AKT/PKB substrate of 40 kDa (PRAS40) subunits (56). mTORC1, which plays a central role in sensing nutrient and growth factor availability as well as oxygen and energy status of the cell, is a negative regulator of autophagy (57, 58). Activation of upstream signaling pathways such as the class I phosphatidylinositol 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAPK) pathway by growth factors activates mTORC1 resulting in repression of autophagy (59-61).

Low nutrient conditions lead to an increase in the intracellular adenosine mono-phosphate/adenosine tri-phosphate (AMP/ATP) ratio, which in turn activates the cellular energy sensor AMP-activated protein kinase (AMPK) (62, 63). Activated AMPK suppresses mTORC1 indirectly by phosphorylating and enhancing the activity of the negative regulator of mTORC1, tuberous sclerosis 2 (TSC2) (64). Active AMPK also directly suppresses mTORC1 by phosphorylating and inactivating Raptor (65). AMPK can also directly promote autophagy by

phosphorylating and activating the ULK1 and Beclin 1-PI3KC3 complexes (see below) (66, 67).

Since autophagy plays an important role in maintaining homeostasis and promoting survival it is highly regulated to control the level, timing and cargo specificity. Autophagy-related (Atg) proteins regulate this process. There are more than 30 autophagy-related proteins that have been identified in yeast, of which orthologues of more than half have been identified in higher eukaryotes. In the mammalian system the core molecular machinery regulating the initiation and elongation stages of the autophagic process include the following hetero-oligomeric protein complexes.

a. ATG1/ULK kinase complex: This autophagy initiator complex consists of the serine/threonine kinase ULK1/2 (Uncoordinated-51-like kinase 1 or 2; also known as Atg1 in yeast), FIP200 (FAK family kinase-interacting protein of 200 kDa; also known as Atg17 in yeast), ATG13, and ATG101 subunits (68-70). This complex is negatively regulated by mTORC1. In the presence of nutrients, activated mTORC1 binds to the complex and phosphorylates ULK1 and ATG13 to inhibit autophagosome formation. Upon nutrient depletion, the inhibitory binding of mTORC1 is released resulting in dephosphorylation of the complex. ULK1/2 then autophosphorylates itself, as well as ATG13 and FIP200 leading to activation of the complex (71-73).

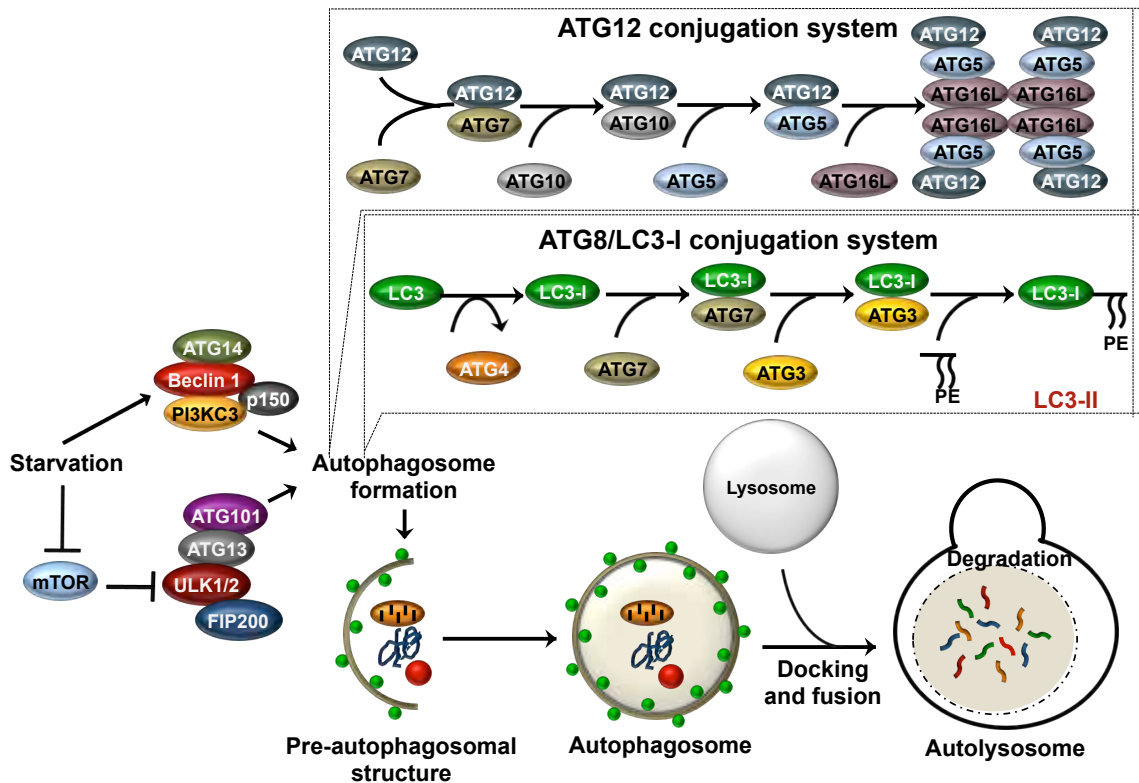


Figure 1.2. Regulators of autophagy.

Upon induction of autophagy by starvation, mTOR releases its inhibitory effect on the ULK1 complex, which together with the activated Beclin 1-PI3KC3 complex initiates autophagosome formation. The ATG12 and ATG8/LC3 conjugation systems are involved in the elongation and completion of the autophagosome membrane. The double-membraned autophagosome then fuses with the lysosome, where autophagosomal contents are degraded and recycled back to the cell. Figure adapted from Baehrecke, Nature reviews (74).

- b. Beclin 1-Class III PI 3-kinase complex:** This macromolecular complex consisting of Beclin1, PI3KC3, p150 and ATG14L subunits (Complex I), as mentioned earlier, is required for the nucleation of the isolation membrane (30). Beclin 1 activates PI3KC3 to generate phosphatidylinositol-3-phosphate (PI3P), which is essential for autophagosome formation. The ATG14L subunit mediates localization of the autophagy-specific PI3KC3 complex to the endoplasmic reticulum (ER) for phagophore nucleation (14). Recently, Russel *et. al.* reported that the PI3KC3 complex is regulated by ULK1. Upon inhibition of mTORC1 by amino acid withdrawal, Beclin 1 is phosphorylated by activated ULK1, resulting in increased lipid kinase activity of the complex and hence autophagy (75). As mentioned earlier, under low energy conditions due to glucose starvation, AMPK can directly phosphorylate Beclin 1 and activate Complex I (67).
- c. ATG12 and ATG8/LC3 conjugation systems:** These two ATG7-dependent ubiquitin-like protein conjugation systems are required for the elongation and completion of the autophagosome. They mediate the lipidation of three subfamilies of proteins in mammals – MAP1LC3 (microtubule-associated protein 1 light chain 3; also known as LC3), GATE16 (Golgi-associated ATPase enhancer of 16kDa) and GABARAP (γ -aminobutyric acid receptor-associated protein) (76). In the ATG12 conjugation system, the E1-like enzyme ATG7, together with the E2-like enzyme ATG10, catalyze the conjugation of ATG12 to ATG5 (77, 78). The ATG12-ATG5 complex then

non-covalently binds to Atg16L1 forming a large multimeric complex (79). In the ATG8/LC3 conjugation system the cysteine protease ATG4 cleaves LC3 at its carboxyl terminus forming cytosolic soluble LC3-I. Finally through a series of steps involving ATG7, ATG3 (E2-like enzyme) and the ATG12-ATG5-ATG16 complex (E3-like ligase) LC3-I is conjugated to phosphatidylethanolamine (PE) to form LC3-II (80). This LC3-II form is anchored to the growing phagophore, as well as to the outer and inner membranes of autophagosomes. After autophagosome-lysosome fusion, LC3-II present on the inner membrane is degraded while that on the outer membrane can be recycled back by ATG4, which cleaves off the PE group (81). Since LC3-II remains associated with the phagophore, mature autophagosomes and autolysosomes, it is frequently used to monitor autophagy.

Endocytosis

Beclin 1 performs autophagy-independent functions as a part of Complex II. In addition to yeast, Beclin 1 has been reported to play a role in endocytic vesicle trafficking in other organisms including *C. elegans* and *Drosophila*. Bec-1, the orthologue of Beclin 1 in *C. elegans* mediates endocytic retrograde transport from endosomes to Golgi. In *bec-1* mutants, MIG-14/Wntless cargo protein is misrouted from the endosome to the lysosome instead of the Golgi and degraded (82). Recently, Shrivage *et. al.* also demonstrated that Atg6 is essential for fluid

phase endocytosis in *Drosophila*. *Atg6*^{-/-} mutant drosophila cells showed negligible uptake of the endocytic tracer Texas Red-avidin (83). In mammalian cells, a study performed by Thoresen *et. al.* reported that RNA-mediated silencing of subunits of Complex II (Beclin 1, PI3KC3, p150, UVRAG and Bif-1) in HeLa cells inhibited epidermal growth factor receptor (EGFR) degradation (19). In contrast, depletion of Atg14L had no effect, implicating a role for Complex II in endocytic receptor degradation. However, these results were contrary to results from an earlier study which reported that inhibiting *BECN1* suppressed autophagy, but not EGFR degradation (84).

The Complex II subunit UVRAG, which is recruited to Complex II by Beclin 1, also regulates endocytic trafficking by mediating endosome-endosome fusion to promote maturation (18). Key players of the endocytic pathway are a subfamily of small GTPases, the Rab GTPases. Rabs can recruit specific cytoplasmic effector proteins and tethering factors to the endosomal membranes and thereby regulate trafficking (85). Rab5 associates with early endosomes and recruits the effector protein PI3KC3 to generate PI(3)P from phosphoinositides (86). PI(3)P mediates the recruitment of effector proteins containing PI(3)P-binding FYVE (Fab1p, YOTB, Vac1p, EEA1) and PX (Phox homology) domains to endosomal membranes (87, 88). This recruitment facilitates vesicular fusion and sorting events and is required for progression of endocytosis. Subsequently, Rab5 undergoes a process called Rab conversion during which Rab7 accumulates at the endosomal membrane with simultaneous removal of Rab5 (89). This

conversion drives the maturation of early endosomes to late endosomes. The PI3KC3 complex present on the endosomal membrane, in turn recruits UVRAG which activates the class C-VPS/HOPS (homotypic fusion and vacuole protein sorting) complex (18). HOPS is a tethering factor that also acts as a guanine nucleotide exchange factor (GEF) for Rab7. It catalyzes the exchange of GDP for GTP and activates Rab7. Stimulation of Rab7 activity promotes endosomal fusion and subsequent cargo degradation in the lysosome (18). However, Rab7 activation can be inhibited by a negative regulator of autophagy - Rubicon. Rubicon sequesters UVRAG making it unavailable for binding to C-VPS/HOPS, thereby preventing progression of endocytosis (29).

Cytokinesis

Complex II has also been reported to be involved in regulating cytokinesis(19). Suppression of components of the UVRAG-containing Complex II was reported to arrest the process. Moreover, both UVRAG and Bif-1, but not ATG14L, colocalize to the midbody (a sub-cellular structure present between dividing daughter cells implicated in controlling cytokinesis) (19). This study implicates that Complex II may contribute to maintaining chromosome number and stability.

Beclin 1 and Cancer

Autophagy is a self-eating process involved in degradation of excess and damaged macromolecules and it can be both cytoprotective and cytopathic. Uncontrolled autophagy can lead to cannibalistic, non-apoptotic cell death termed Type II programmed cell death, or autophagic cell death, while inefficient autophagy leads to the inability of cells to clear damaged cellular debris (90, 91). Dysfunctional autophagy has been implicated in various human diseases including cancer (92). *Beclin 1* (*BECN1*) was the first autophagy gene reported to link autophagy to cancer (4). The *BECN1* gene is located on human chromosome 17q21 and it is monoallelically deleted in 40% of prostate, 50% of breast and 75% of ovarian cancers (93). Its heterozygous disruption causes spontaneous tumorigenesis in mice (35, 94). These mice are prone to mammary hyperplasia and develop increased number of lymphomas, hepatocellular carcinomas and lung adenocarcinomas as compared to age-matched littermates. On the other hand, overexpression of Beclin 1 inhibits *in vitro* cancer cell proliferation and clonogenicity as well as tumor formation in nude mice (4). Comparison of *BECN1* expression in matched normal breast and breast carcinoma tissue from patients with invasive sporadic breast carcinoma showed lower expression levels in breast carcinoma samples compared to their normal counterparts (4).

Despite the above mentioned connection to cancer, a recent study by Laddha *et. al.* questioned the tumor-suppressive function of Beclin 1 (95). In their

analysis of human tumor sequencing data from The Cancer Genome Atlas (TCGA), they found no evidence for mutation or focal loss of *BECN1* gene. Mutational analysis revealed that *BECN1* deletion did not occur alone but in conjunction with the proximally located tumor suppressor gene *BRCA1* (breast cancer 1, early onset) (95). This new study therefore suggests that changes in Beclin 1 protein expression in tumors likely arise through epigenetic or transcriptional modifications. In humans, aberrant DNA methylation of the promoter and intron 2 of the *BECN1* gene have been reported in tumors with decreased Beclin 1 expression (96). Moreover, a reduction in Beclin 1 protein expression has been reported to be associated with poor prognosis in a number of cancers (97, 98).

Since Beclin 1 is a well-established regulator of autophagy, the tumor suppressive function of Beclin 1 has mostly been attributed to its regulation of the autophagic pathway. However, the role of autophagy in cancer is quite complex and may be stage and context dependent. It is considered as a “double-edged sword”, with both tumor promoting and tumor suppressing abilities. The current consensus is that autophagy suppresses early oncogenic transformation but can promote progression of established tumors.

Autophagy as a tumor suppressor

Autophagy plays a tumor suppressive role by eliminating protein aggregates and damaged organelles and thereby oxidative stress (99). Under

normal metabolic conditions mitochondria produce reactive oxygen species (ROS). The presence of damaged mitochondria elevates ROS levels, which triggers mitophagy (100). Dysfunction of this process leads to accumulation of damaged mitochondria resulting in excess ROS production (101). The increased intracellular ROS levels further increases metabolic stress in autophagy-defective cells causing DNA damage and genomic instability which can drive tumor formation (99). Quenching ROS with its scavenger N-acetyl cysteine (NAC) delays promotion of aneuploidy in autophagy-impaired *Becn1*^{+/-} cells. Thus, the inability of autophagy-deficient cells to suppress ROS production leads to chromosomal instability (99).

In cells with intact autophagy, damaged proteins and organelles are targeted for degradation in the autophagosome by the autophagy cargo-receptor p62/SQSTM1 (53). This receptor is also degraded during the process. Allelic loss of *Becn1* increases susceptibility to metabolic stress resulting in accumulation of p62, damaged mitochondria and endoplasmic reticulum chaperones *in vitro* and *in vivo* (99). Over-expression of p62 in *Becn1*^{+/-} cells results in p62 aggregate accumulation, elevated ROS production and DNA damage which promotes tumorigenesis while suppression of p62 reduced DNA damage induction. Autophagy-deficient *Atg5*^{-/-} cells also show similar defects (99). These results indicate that impaired p62 elimination due to defective autophagy leads to genomic instability and tumorigenesis (99). Persistent p62 can also activate the

transcription factor Nrf2 (nuclear factor, erythroid 2-like 2) resulting in transcription of genes that promote angiogenesis and cell survival, both of which are known to facilitate tumor progression (102).

In established tumors, regions of the tumor microenvironment are nutrient depleted and hypoxic due to increased proliferation rate and insufficient vascularization. Autophagy helps apoptosis-resistant cells in these ischemic regions survive metabolic stress (103). Suppression of either *Beclin 1* or *atg5* in Bcl-2-expressing apoptosis-resistant cells reduces autophagy induction and impairs survival in ischemia. Moreover, *Becn1*^{+/-} apoptosis-resistant tumors also show a prevalence of necrosis (103). Tumor necrosis is associated with inflammatory cell infiltration, which produces cytokines and chemokines and promotes angiogenesis, tumor progression and metastasis (103-105). Thus, autophagy can also suppress tumor growth by restricting necrotic death of metabolically stressed, apoptosis-resistant tumor cells, thereby preventing macrophage infiltration and chronic inflammation that can promote tumor growth and metastasis.

The role of autophagy as a tumor suppressive process is further supported by genetic inhibition of some ATG proteins other than Beclin 1. Frameshift mutations, which would lead to synthesis of truncated proteins and therefore impaired autophagy, have been reported in *ATG2B*, *ATG5* and *ATG9B* genes in gastric and colorectal cancers with microsatellite instability (106).

Knockout of another autophagy gene *Atg4C* increased the susceptibility of mice to develop fibrosarcomas induced by chemical carcinogens as compared with their wild-type littermates (107). Mice with systemic mosaic deletion of *Atg5* and liver-specific knockout of *Atg7* develop benign liver adenomas. However, these hepatomas do not progress to hepatocellular carcinoma suggesting that autophagy may be required for tumor progression (108).

Autophagy as a tumor promoter

In established tumors, autophagy can promote tumor growth by helping tumor cells adapt to the poorly vascularized, hypoxic tumor microenvironment (103, 109). It can help tumor cells survive in this metabolically stressful environment by supplying nutrients and maintaining energy homeostasis. In a study done by Yang *et. al.* autophagy has been reported to be required for tumorigenic growth of pancreatic cancer (110). They also show that pancreatic ductal adenocarcinoma (PDAC) cell lines have elevated basal autophagy and their growth can be suppressed by inhibiting this process. Both chemical and genetic inhibition of autophagy led to robust tumor regression and prolonged survival in pancreatic cancer xenografts and genetic mouse models (110).

Work done by Fung *et. al.* showed that inhibition of autophagy by depletion of Beclin 1, ATG5 or ATG7 enhanced apoptosis induced by detachment from extracellular matrix (111). Defective autophagy also decreased clonogenic survival of cells that were re-plated after detachment-induced

apoptosis, also known as anoikis (111). Since detachment of cells from the extracellular matrix is a critical step for dissemination and metastasis of tumor cells, autophagy may also promote metastasis by helping epithelial cells survive anoikis. Moreover, activation of autophagy can help cancer cells adapt to therapeutic insults making them resistant to anti-cancer treatment. Use of autophagy inhibitors or knockdown of Atg5 or Atg7 increases therapeutic cell killing, indicating autophagy contributes to acquired chemo-resistance of tumor cells (112).

Autophagy-independent role for Beclin 1 in cancer

Non-autophagic functions of Beclin 1 have been relatively understudied in the context of cancer. As stated earlier, mice with systemic mosaic deletion of *Atg5* and liver-specific knockout of *Atg7* develop only benign liver adenomas with no other neoplasms (108). Moreover, inhibition of p62 in these mice reduced these hepatomas indicating the possible cause to be the accumulation of p62 in the stressed liver. On the other hand, *Becn1*^{+/-} mice develop tumors in multiple organs including the liver suggesting a potential autophagy-independent role for Beclin 1 in cancer (35, 94).

In contrast with Atg14L (Complex I), which has not been shown to have tumor suppressor activity, UVRAG and Bif-1 (Complex II) have both been implicated as tumor suppressors. UVRAG, a positive regulator of PI3KC3 complex, is monoallelically mutated at a high frequency in human colon

carcinomas with microsatellite instability (113). Interestingly, colon cancer cell lines with UVRAG mutations did not exhibit a decrease in autophagy. Moreover, expression of wild-type UVRAG in a human colon cancer cell line, HCT116 (with reduced levels of endogenous UVRAG), suppressed tumorigenicity in nude mice (16). Frameshift mutations in the *UVRAG* gene have also been reported in gastric carcinoma with microsatellite instability (114). Bif-1, which interacts with Beclin 1 through UVRAG, is another protein that enhances the Beclin 1-PI3KC3 complex activity (20). Bif-1 is frequently deleted or down-regulated in a number of cancers including gastric, prostate, colorectal and pancreatic cancers (115-118). Allelic loss of *Bif-1* promotes chromosomal instability and accelerates the development of Myc-induced lymphoma (119). *Bif-1*^{-/-} mice have a higher frequency of developing spontaneous tumors, specially lymphoma, when compared to wild-type mice (20). Taken together these results support that autophagy-independent functions of Beclin 1 are likely to play a role in cancer.

Rationale for the Thesis Project

In recent years, emerging studies have revealed the existence of autophagy-independent functions for mammalian Beclin 1. To date, the tumor suppressor function of Beclin 1 has been ascribed primarily to its role in autophagy and the impact of alternate functions of Beclin 1 on cancer and its mode of action have yet to be demonstrated. Although Beclin 1 and its binding partners in Complex II have been shown to be involved in endocytic receptor

trafficking, the contribution of Beclin 1 to this pathway is still unclear (19). Additionally, the report refuting the involvement of Beclin 1 in EGFR degradation increases the ambiguity about the contribution of Beclin 1 to this regulatory pathway (84). For my thesis research work I sought to determine the role of Beclin 1 in breast cancer and focused on understanding its regulation of growth factor receptor endocytosis. The endocytic pathway modulates the strength and duration of growth factor signaling by regulating the downregulation and recycling of growth factor receptors. Deregulation of receptor endocytosis could lead to aberrant receptor signaling and contribute to the development and progression of cancer. Hence understanding the mode of action of Beclin 1 in regulating receptor endocytosis would help us better appreciate the potential impact of non-autophagic functions of Beclin 1 in cancer.

CHAPTER II

MATERIALS AND METHODS

Cell lines

MDA-MB-231, T47D and MCF7 human breast carcinoma cell lines were obtained from ATCC. SUM159, SUM149 and MCF10A cell lines were a gift from Dr. A. Mercurio (University of Massachusetts Medical School). MDA-MB-231 and T47D cells were maintained in RPMI 1640 and MCF7 cells were cultured in DMEM (all from GIBCO®). Media was supplemented with 10% fetal calf serum. SUM159 and SUM149 cells were cultured in F12 Nutrient mixture (Ham) containing 5% FBS, 25mM HEPES, 5 µg/ml Insulin and 1 µg/ml hydrocortisone. MCF10A cells were grown in DMEM/F12 (1:1) containing 5% horse serum, 20 ng/ml EGF, 10mM HEPES, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone and 100 ng/ml Cholera toxin.

Stable knockdowns were generated using lentiviral vectors from Open Biosystems, containing small hairpin RNAs (shRNA) targeting human *BECN1* (Clone IDs: TRCN0000033550, TRCN0000033552) human *ATG5* (Clone IDs: TRCN0000151963, TRCN0000150645) and GFP, or a scrambled shRNA. After infection, cells were selected for stable expression with puromycin (2 µg/ml). For over-expression and Beclin 1 rescue, Beclin 1 was expressed transiently using a lentiviral vector containing Flag-Beclin 1. 48 hours post-infection the cells were stimulated with IGF-1 (100 ng/ml) in media containing 0.1% BSA (no prior starvation) for the time periods indicated in the figures.

Plasmids

Lentiviral Flag-Beclin 1 was constructed by cloning Flag-Beclin 1 (Dr. Stephen Doxsey, University of Massachusetts Medical School) into the EcoRI cloning site of the lentiviral expression vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences). In order to rescue Beclin 1 expression, four silent mutations were introduced into the shRNA-targeting region of BECN1 using the QuikChange® site-directed mutagenesis kit (Stratagene). The primers used for mutagenesis were:

Forward 5'-CTGAAATTTTCAGAGATACCGACTgGTgCCTTACGGAAACCATTC-3'

Reverse 5'-CAGATATGAATGGTTTCCGTAAGGcACcAGTCGGTATCTCTGGAG-3'

Forward 5'-CAGAGATACCGACTgGTgCCaTAtGGAAACCATTCATATC-3'

Reverse 5'-GATATGAATGGTTTCCaTAtGGcACcAGTCGGTATCTCTG-3'

For imaging experiments cells were transfected with EGFP-FYVE, TagRFP-T-APPL1 and/or TagRFP-T-Beclin 1 using Cell Line Nucleofector® Kit V (Lonza Cologne AG, Germany) as per the manufacturer's protocol. EGFP-FYVE containing the FYVE domain of human SARA (589-656) and TagRFP-T-APPL1 were a gift from Dr. Silvia Corvera (University of Massachusetts Medical School) and have been described previously (120). TagRFP-T-BECN1 plasmid was generated by cloning Flag-Beclin 1 in frame with TagRFP-T at the N-terminus of the Flag-Beclin 1 protein using the EcoRI cloning site (121).

Immunoblotting and antibodies

Cells were extracted at 4°C for 10 minutes in a 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl, 1% NP-40, 10% Glycerol, 1 mM sodium orthovanadate, 10 mM NaF and protease inhibitors (Complete Mini Tab; Roche). Cell extracts containing equivalent amounts of total protein were resolved by SDS-PAGE, transferred to nitrocellulose filters. Nitrocellulose filters were blocked for 1 h in 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl, 0.05% Tween 20, and 5% (weight/volume) dry milk/Bovine serum albumin and then incubated overnight at 4°C in the same buffer containing the respective primary antibodies. This was followed by three 15 minute washes with 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20. The filters were then incubated for 1 h in blocking buffer containing peroxidase-conjugated secondary antibodies. After three more 15 minute washes with 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20, bands were detected by chemiluminescence using a ChemiDoc XRS+ system (BioRad Laboratories). Band intensities were quantified by densitometry using Image Lab (Beta 1) (Bio-Rad Laboratories).

Antibodies used for immunoblotting included: LAMP-1 (Catalog # H5G11; Santa Cruz Biotechnology), GAPDH (Catalog # A300-642A; Bethyl Laboratories), actin (Catalog # A2066; Sigma) and tubulin (Catalog # T5168; Sigma). All other antibodies were purchased from Cell Signaling Technology, Inc: pIGF-1R β (Y1135/1136) (Catalog # 3024), IGF-1R β (Catalog # 3027), pEGFR (Y1086)

(Catalog # 2220), EGFR (Catalog # 4267), pAKT(S473) (Catalog # 9271), pAKT(T308) (Catalog # 2965), AKT (Catalog # 9272), pERK1/2 (T202/Y204) (Catalog # 9106), ERK1/2 (Catalog # 9102), Beclin 1 (Catalog # 3738), ATG5 (Catalog # 2630) and LC3B (Catalog # 3868).

Autophagic flux assay

MDA-MB-231 cells expressing shGFP, shATG5 or shBECN1 after two washes with 1X PBS, were incubated in either RPMI 1640 supplemented with 10% fetal bovine serum or Hank's Balanced Salt Solution (GIBCO®), with and without 25 μ M Chloroquine diphosphate (Catalog # C6628; Sigma) for 4 and 8 hours. Cells were extracted as described above.

Glucose uptake and lactate production assay

Cells grown in triplicates in 12-well plates were serum-starved in 1ml media containing 0.1% BSA for 24 hours. After the 24-hour incubation conditioned media was collected and glucose and lactate present was assayed using the Glucose (GO) assay kit (Catalog # GAGO20; Sigma) and lactate kit (Trinity Biotech). The amount of cellular protein present in each well was also quantified by Bradford assay. Glucose uptake and lactate production were calculated and expressed as a rate measurement (mM/mg of total protein/hour) (122).

Proliferation assays

Proliferation was monitored using the MTT colorimetric assay. In this assay cleavage of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple colored formazan is measured. 5000 cells were plated in each well of a 48-well culture plate. To assess proliferation, cells were seeded in multiple plates and one plate was used per day. On the day of the assay, media from one plate was replaced with 400 μ l fresh media containing 0.5mg/ml MTT reagent and incubated at 37°C for 4 hours. Following incubation, the MTT containing medium was discarded and cells were solubilized in DMSO for 10 minutes at 37°C. Absorbance was measured at 595 nm. Proliferation was determined by the absorbance of the dye taken up by the cells. To examine the impact of autophagy inhibition on proliferation rates, cells were treated with 0 μ M, 6.25 μ M or 25 μ M of Chloroquine diphosphate starting at Day 0.

Soft agar colony formation assay

Wells of a 6-well plate was coated with 0.75% agar to prepare a base. After the bottom agar layer solidified, 2500-3000 cells resuspended in pre-warmed 0.3% agar were layered on top. 0.5 ml of media was added after the top agar layer containing the cells also solidified. Cells were plated in duplicates. Media was changed every 2 days. Images were taken of 5 separate fields per well after 20 days of incubation. The number and size of colonies were quantified

using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2014). To examine the impact of autophagy inhibition on anchorage-independent growth, MDA-MB-231 and T47D cells plated in soft agar were treated with 0 μ M, 12.5 μ M or 25 μ M of Chloroquine diphosphate.

Growth factor stimulation assays

Cells were serum-starved for either 2 hours or overnight in media containing 0.1% BSA and then stimulated with IGF-1 (100 ng/ml) or hEGF (50ng/ml) for the time periods indicated in the figures. To inhibit internalization, cells were incubated with 10 μ g/ml of Chlorpromazine HCl (Catalog # C8138; Sigma) for 1 hour prior to and during stimulation. For receptor degradation assays, 50 μ g/ml of cycloheximide (Catalog # C4859; Sigma) was added to serum-starved cells 1 hour prior to the addition of growth factors. To examine the impact of AKT inhibition on receptor degradation, cells were treated with 0.5 μ M MK2206 (Catalog # S1078; Selleck Chemicals) or DMSO together with cycloheximide 1 hour prior to growth factor stimulation. Cells were extracted as described above. To examine the effect of chemical inhibition of autophagy, cells were treated with 30 μ M Chloroquine diphosphate for 4 hours and then extracted.

Quantitation of PI3P lipid

1x10⁶ cells were plated in 10cm plates and serum starved overnight and then stimulated with IGF-1 (100ng/ml) or EGF (50ng/ml) for the indicated time periods. PI3P lipid levels were measured using the PI3P Mass ELISA Kit (Catalog # K-3300) according to manufacturer's instructions.

RNA extraction and real-time quantitative PCR (qRT-PCR)

Total mRNA was extracted and purified from cells using the RNeasy kit (Qiagen) and converted into cDNA using Superscript® II Reverse Transcriptase (Invitrogen). SYBR® Green RT-PCR reagents (Applied Biosystems) were used to quantify gene expression. IGF1R and BECN1 expression were normalized to human actin.

Primers used:

IGF1R forward 5'-CAAGTTGAGGATCAGCGAGA-3';

IGF1R reverse 5'-AACACAGGATCTGTCCACGA-3';

BECN1 forward 5'-CTCTGGCCAATAAGATGGGT-3';

BECN1 reverse 5'-CGGCAGCTCCTTAGATTTGT-3';

actin forward 5'-TGAGCGCGGCTACAGCTT-3';

actin reverse 5'-TCCTTAATGTCACGCACGATTT-3'.

Invasion and migration assays

For migration assays, 0.6ml of DMEM containing 15 $\mu\text{g/ml}$ collagen (PureCol® Bovine Collagen Solution, Type I, Catalog # 5005-B; Advanced BioMatrix) was added to the bottom of each 6.5 mm Transwell chamber (8 μm pore size; Costar), and the filters were coated for 1 hour at 37°C. Cells were re-suspended in DMEM containing 0.1% BSA and 10^4 cells were added to the top well of each Transwell chamber. After 4.5 hours, cells that had migrated to the lower surface of the filters were fixed in methanol for 10 mins. The fixed membranes were mounted onto glass slides using Vectashield mounting medium containing 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Matrigel invasion assays were performed as described previously (122). 10^4 cells were added to the top well of each Transwell chamber and allowed to invade through the Matrigel (Catalog # 356237; BD Biosciences) for 4.5 hours. Cells that had invaded to the lower surface were fixed and quantified as described above for migration assays. For Beclin 1 rescue experiments, cells were infected with shBECN1-resistant Flag-Beclin 1 48 hours prior to the assay. For inhibitor studies, cells were pre-incubated with 5 μM AG1024 (Catalog # S1234; Selleck Chemicals) for 16 hours or with 0.5 μM MK2206 for 1 hour prior to the assays. Inhibitors were also present in the upper and lower wells of the Transwell chamber during the assays. Migration and invasion were quantified by counting the number of stained nuclei in five separate fields of each Transwell

membrane using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2014).

Immunofluorescence staining and imaging

Cells were plated on glass coverslips, serum-starved overnight and stimulated with IGF-1 (100 ng/ml) for the time periods indicated in the figures. Cells were washed with 1X DPBS (GIBCO®) before fixing in 4% formaldehyde for 30 mins. Fixed cells were blocked for an hour in 1X DPBS containing 3% BSA and 0.5% Triton X-100 and then incubated in the same buffer containing primary antibody for 1 hour. Following three washes with 1X DPBS, cells were incubated in secondary antibody (Alexa Fluor 488 or Alexa Fluor 660 from Molecular Probes; Life Technologies Corporation) diluted in blocking buffer for 1 hour. After washing, coverslips were mounted on glass slides using Vectashield mounting medium containing DAPI. Stained cells were imaged using a Zeiss LSM700 confocal microscope with a 63X, 1.4 NA Plan Apochromat oil objective equipped with an AxioVision Imager.Z2 camera. Zen 2012 software was used to acquire the images. Number of APPL1 puncta in each cell was quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2014).

Antibodies used for immunofluorescence staining included APPL1 (Catalog # 3858) and EEA1 which was generated as described previously (121).

Total Internal Reflection Fluorescence (TIRF)/Epi-fluorescence Structure-illumination Microscopy (TESM) and image analysis

The TISM Optical System imaging hardware and software have been described previously (121). It is a custom-built microscope system that simultaneously combines Total Internal Reflection Fluorescence and wide-field epi-fluorescence modes. The latter incorporates structured illumination for fast optical sectioning and enhanced spatial resolution. Two diode-pumped solid-state 100mW lasers (Cobalt) produced 491 nm and 561 nm light while a 120mW diode laser (Blue Sky Laser) produced 660 nm light. The system uses a modified Olympus IX71 inverted microscope. The TIRF illumination is adjusted such that it passes through the edge of an Olympus TIRF 60X objective with an NA of 1.49 at an angle so as to visualize 100-200nm from the coverslip. Light from the fluorophores was collected and relayed onto a 1004x1002 Andor iXon 885 EMCCD camera, binned by 2. A Physik Instruments PIFOC was used for fine focus control. Focus stabilization was performed by pgFocus consisting of software and electronics developed by the Biomedical Imaging Group at UMass (<http://big.umassmed.edu/wiki/index.php/PgFocus>).

Cells were transfected with plasmids indicated in the figures using Cell Line Nucleofector® Kit V (Lonza Cologne AG, Germany) as per the manufacturer's protocol. Transfected cells were plated on glass coverslips and grown for an additional 48 hours. For imaging, cells were incubated at 35°C in

KRH buffer (125 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES pH 7.4, 2 mM sodium pyruvate and 0.5% BSA). Cells transfected with TagRFP-T-APPL1 alone were stimulated with Alexa-Fluor-488 EGF complex (Invitrogen) and cells co-transfected with EGFP-FYVE and TagRFP-T, TagRFP-T-BECN1 or TagRFP-T-APPL1 were stimulated with biotinylated EGF (Catalog # E3477; Life Technologies) complexed with streptavidin DyLight 650 conjugate (Catalog # 84547; Thermo Scientific). EGF was washed out after 8 minutes of stimulation and replaced with KRH buffer. Cells were imaged for another 12 minutes (approximately). For Figure 4.14 the time series protocol used lasers emitting 491 nm (EGF) and 561 nm (TagRFP-T-APPL1) while for Figure 4.15 – 4.17 the time series protocol used lasers emitting 491 nm (EGFP-FYVE/EGF), 561 nm (TagRFP-T-APPL1/ TagRFP-T-BECN1) and 660 nm (EGF/EEA1).

Quantitative TIRF image analysis was performed as described previously (121, 123). Time series raw images were first corrected by subtracting the background fluorescence followed by performing a temporal running average of three time points to reduce noise with negligible effect on the data. In order to quantitatively analyze structured fluorescence (i.e. vesicles) without interference from diffuse fluorescence, images were convolved with a small, 2D Gaussian spot ($\sigma = 150$ nm) that preserved the mean intensity and a larger, 2D Gaussian spot ($\sigma = 300$ nm) that estimated and subtracted the local background. A binary masking image was generated by visually thresholding the image so as to only select pixels belonging to the fluorescent structures (which were given a value of

one) and eliminate noise (pixel value was set to zero). Masked images were generated by multiplying the binary mask with the original image to display structures with intensity greater than the average local background. Co-localization was determined by two-way overlap of the masked images of each label and co-localized pixels are shown as white or as indicated by the accompanying color wheel. Co-localization values have been reported as percent of pixels of a label that co-localized with another label. Co-localization seen when pixel-rich regions were rotated 180 degrees relative to each other have been labeled spurious.

Epifluorescence images were generated from structured illumination data sets according to methods described previously (121). Raw images were processed as described above and binary masked images were used for quantifying the number of EGFP-FYVE puncta (between 10-10000 pixel size) in each cell (Figure 4.16) and for quantitative analysis of co-localization between labels (Figure 4.17).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Statistical analyses between two groups were performed using the two-tailed unpaired Student's t-test. Data are represented as mean \pm standard error unless mentioned otherwise. A p value of 0.05 was considered statistically significant.

CHAPTER III

***CHARACTERIZATION OF AUTOPHAGY IN BREAST
CARCINOMA CELLS***

Introduction

Autophagy is a lysosome-dependent degradation process that plays a major role in maintaining cellular homeostasis by degrading and recycling damaged and excess cytoplasmic components. It is also an adaptation process of the cell, activated under conditions of stress. Deregulation of autophagy has been implicated in the development of various diseases including cancer, neurodegeneration and inflammation (92).

The role of autophagy in cancer has been proposed to be both preventive and promoting (90). By eliminating aggregates of damaged proteins and organelles, autophagy prevents excess production of reactive oxygen species (ROS), oxidative stress and genome instability, thereby impeding tumor initiation. Autophagy can also prevent necrosis and a subsequent tumor promoting inflammatory response. Nonetheless, since it supports cell survival under various forms of stress, autophagy could also promote tumorigenesis by helping cancer cells survive in the hypoxic, nutrient-deprived tumor microenvironment. In addition, induction of autophagy can help protect cells from death due to extracellular matrix detachment or anoikis, which is required for dissemination of tumor cells to other parts of the body. Finally, autophagy can also provide resistance to chemotherapy. The function of autophagy in cancer is therefore quite complex and still under investigation, with the general consensus being that

this process prevents cancer initiation but can promote progression of established tumors.

Emerging investigations have revealed that the role of autophagy in cancer may also be context specific. The requirement of autophagy for various cancer types could be different depending on their metabolic requirements and activated oncogenic pathways. Aberrant activation of the PI3K pathway commonly found in many cancers activates the negative regulator of autophagy, mTORC1, thereby inhibiting autophagy (124). In contrast Ras transformation can upregulate basal autophagy (125). Interestingly, the tumor suppressor p53 that is frequently mutated in human cancers plays a dual role in autophagy. Nuclear p53 induces autophagy while cytoplasmic p53 represses the process (126).

Breast cancer is a heterogeneous disease, which has been classified into various categories based on different criteria such as histopathological type, grade and stage of tumors and expression of genes and proteins. Based on variations in gene expression patterns, breast tumors have been classified into five “Intrinsic” subtypes of breast cancer (claudin-low, basal-like, HER2-enriched, luminal A and luminal B) (127, 128). Claudin-low and basal-like breast cancers do not express significant levels of estrogen receptor (ER), progesterone receptor (PR) and HER2 and are hence called triple negative breast cancers (TNBCs). TNBCs have a worse prognosis than other breast cancer subtypes. The above mentioned molecular subtypes show crucial differences in incidence,

survival and response to treatment (128, 129). One of the major reasons for classifying breast tumors into these categories is to increase the efficacy of cancer treatment and to personalize therapy for each patient. Since autophagy is gaining importance as a potential therapeutic target, determining whether to therapeutically inhibit or induce autophagy for the treatment of a given tumor would be crucial and this could be dependent upon the subtype of breast cancer.

In this chapter I investigated the autophagy-dependency of triple-negative and luminal breast cancer subtypes. I first examined the levels of autophagy in a panel of cell lines consisting of both basal-B and luminal subtypes, and then evaluated the effect of inhibiting autophagy in representative cell lines of each subtype. My results suggest a greater dependency of TNBCs on autophagy than luminal breast cancers.

Results

Triple-negative breast cancer cell lines have higher levels of basal autophagy than luminal breast cancer cell lines

To determine basal autophagy levels in breast carcinoma cell lines, basal, triple-negative (MDA-MB-231, SUM149) and luminal (MCF7 and T47D) cells (Table 3.1) were compared. A common method of measuring autophagy is to assess the lipidation of the autophagy reporter protein LC3-I to LC3-II. Lipidation (conjugation of phosphatidylethanolamine to LC3-I) increases the electrophoretic mobility of the protein as shown in Figure 3.1. To measure basal autophagy, all cell lines were grown in nutrient-replete conditions for 24 hours before harvesting. Comparison of LC3-II levels indicate increased processing of LC3-I in the metastatic, TNBC subtypes as compared to the non-metastatic, luminal cells.

The increase in levels of LC3-II in the triple-negative breast cancer cell lines could be due to decreased fusion between the autophagosome and the lysosome resulting in reduced degradation of LC3-II (130). Depletion of lysosome associated membrane protein-1 (LAMP-1) and LAMP-2, the major components of the lysosomal membrane, leads to accumulation of autophagic vacuoles and hence LC3-II (131). Thus the levels of LAMP-1 protein in all the cell lines were assessed (Figure 3.1). However, no clear correlation between LC3-II and LAMP-1 protein levels was observed.

Table 3.1. Molecular classification of breast carcinoma cell lines.

Cell Lines	Subtype (128, 132)	Metastatic Potency	Hormone Receptor Status (129)	Mutations/oncogene expressed (129) (133)
MDA-MB-231	Basal-B/ Claudin- low	Metastatic	ER ⁻ , PR ⁻ , HER2 ⁻	BRAF G464V, CDKN2A, KRAS G13D, NF2, TP53 R280K, WNT7B, PDGFRA
SUM149	Basal-B/ Basal-like	Metastatic	ER ⁻ , PR ⁻ , HER2 ⁻	TP53 M237I, BRCA1, CDKN2A
MCF7	Luminal	Non- metastatic	ER ⁺ , PR ⁺ , HER2 ⁻	CDKN2A, PIK3CA E545K, WNT7B
T47D	Luminal	Non- metastatic	ER ⁺ , PR ⁺ , HER2 ⁻	PIK3CA H1047R, TP53 L194F, WNT7B

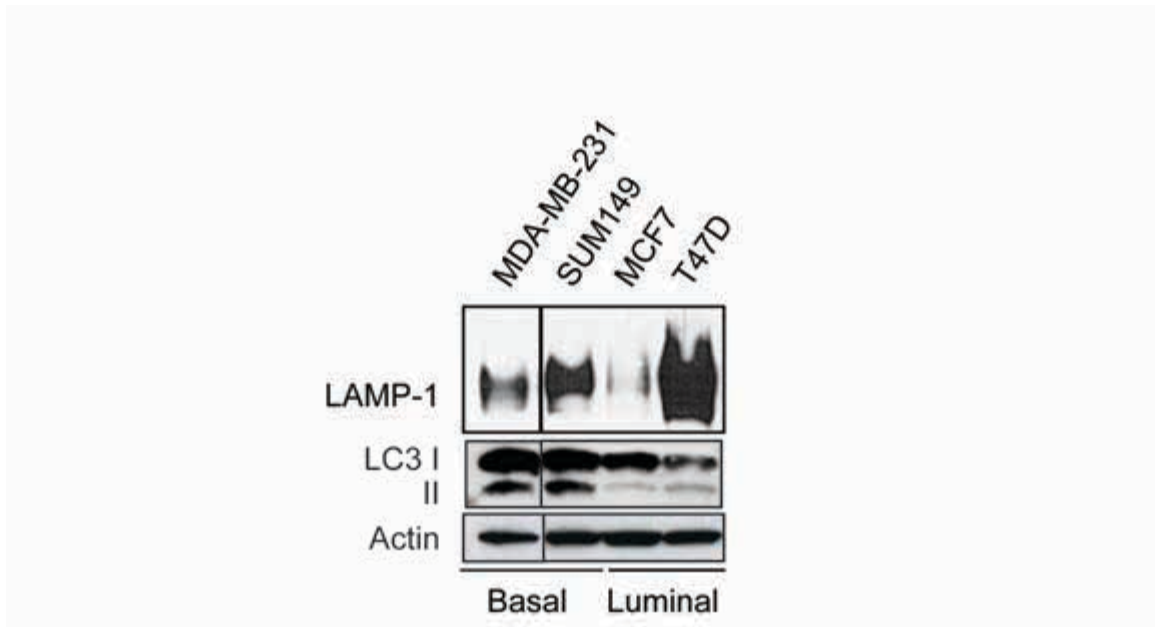


Figure 3.1. Comparison of basal autophagy in breast carcinoma cell lines.

Basal, triple-negative MDA-MB-231 and SUM149, and luminal MCF7 and T47D cells were lysed 24 hours after plating. Cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for LAMP-1, LC3 and actin. Images are from the same immunoblot that was cropped to remove lanes.

Accumulation of LC3-II can be due to induction of autophagy or inhibition of the downstream degradation pathway. Furthermore, low levels of LC3-II could be the result of high autophagic flux resulting in enhanced degradation of the protein (130). Hence to measure this dynamic process, LC3-II turnover was assessed in the presence of the lysosomal inhibitor chloroquine diphosphate (CQ) (130). Chloroquine diphosphate inhibits lysosomal acidification, thereby preventing degradation of autolysosomal contents including LC3-II. To compare autophagic flux between triple-negative and luminal cells, MDA-MB-231 and T47D cell lines were analyzed as representative cell lines. Cells were incubated for 4 and 8 hours in either nutrient replete media or in serum and amino acid-free Hank's Balanced Salt Solution (HBSS) with and without 25 μ M chloroquine diphosphate. MDA-MB-231 cells showed high levels of basal LC3-II lipidation, which was enhanced upon induction of autophagy by serum and amino acid deprivation (Figure 3.2A and B; Lanes 1 and 3). Addition of chloroquine diphosphate further elevated the LC3-II protein levels (compare Lane 1 with 2, and lane 3 with 4) indicating that increased processing of LC3-I to LC3-II was due to elevated autophagy and not impairment in LC3-II clearance.

In T47D cells, LC3-II protein accumulated upon addition of chloroquine diphosphate to cells in nutrient replete media indicating the presence of intact low basal autophagy levels in the cells (Figure 3.2A and B; Lanes 5 and 6). However, in contrast to the MDA-MB-231 cells, upon induction of autophagy by serum and amino acid deprivation, the protein levels did not increase (Figure 3.2A and B;

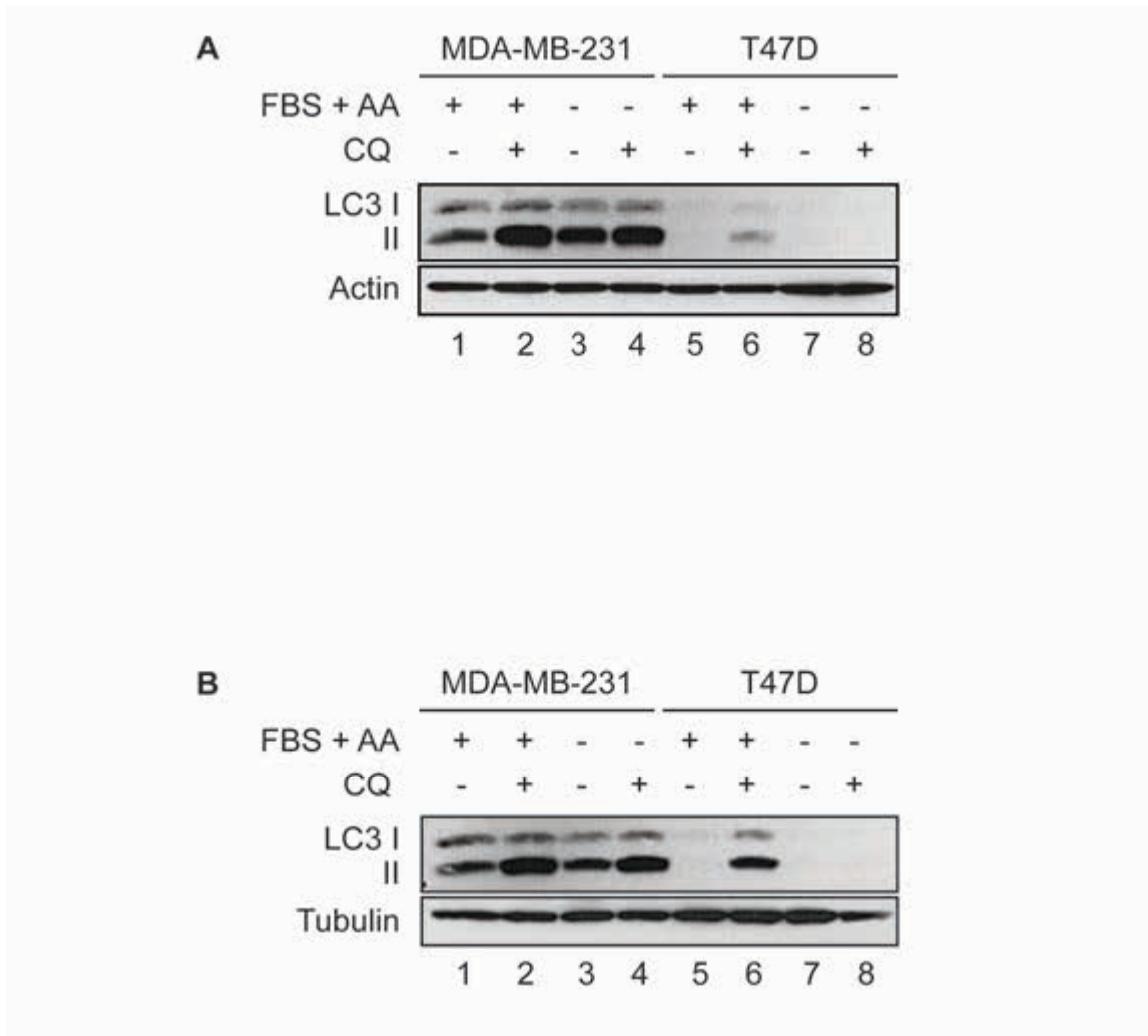


Figure 3.2. Comparison of autophagic flux between MDA-MB-231 and T47D cells.

MDA-MB-231 and T47D cells were incubated for 4 hours (A) or 8 hours (B) in either complete or serum and amino acid-free media with or without 25 μ M chloroquine diphosphate. Cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for LC3 and actin or tubulin. FBS, fetal bovine serum; AA, amino acids.

Lanes 7). Moreover, addition of chloroquine diphosphate did not enhance the LC3-II levels of starved cells, indicating that T47D cells do not upregulate autophagy to counteract starvation-induced stress (Figure 3.2A and B; Lanes 8). Thus, triple-negative MDA-MB-231 cells have higher autophagic flux than luminal T47D cells and further elevate the flux under starvation conditions.

Transformed cells undergo metabolic reprogramming known as the “Warburg effect” during which aerobic glycolysis is upregulated (134). This leads to increased glucose uptake and lactate production. Autophagy has been shown to facilitate increased glycolysis in Ras transformed cells and inhibition of autophagy in these cells reduces glucose uptake and metabolism (135). MDA-MB-231 cells harbor a K-Ras mutation (Table 3.1). Hence, in order to determine whether autophagic flux differences observed between MDA-MB-231 and T47D cells were due to differences in glycolysis, glucose uptake and lactate production were compared. Interestingly, MDA-MB-231 and T47D cells had similar levels of lactate production (Figure 3.3A) and glucose uptake (Figure 3.3B). These data implicate that the observed differences in autophagy were not due to differences in glycolytic metabolism.

Triple-negative MDA-MB-231 breast carcinoma cells are more sensitive to autophagy inhibition than luminal T47D cells

In order to determine the functional contribution of autophagy in the two cell lines, two key characteristics of tumor cells were measured – anchorage-

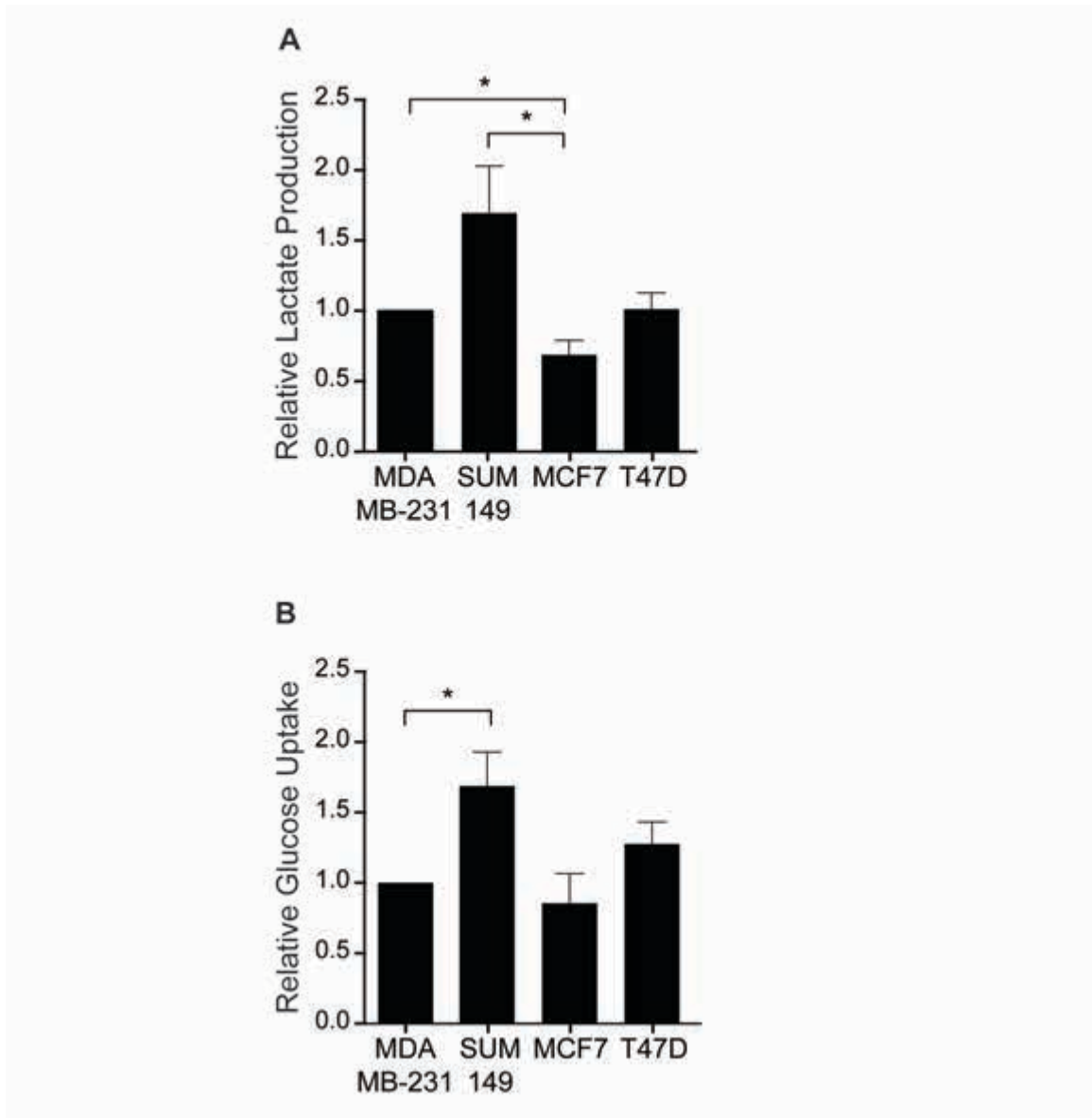


Figure 3.3. Comparison of aerobic glycolysis in breast carcinoma cell lines.

MDA-MB-231, SUM149, MCF7 and T47D cells were assayed for lactate production (A) and glucose uptake (B). The data are expressed as the rate of glucose depletion from or lactate production into the culture medium (mM/mg of total protein/hour) relative to MDA-MB-231 cells. The data shown represent the mean of three independent experiments performed in triplicate. *, $p < 0.05$.

dependent and anchorage-independent growth. To assess the effect of inhibiting autophagy on the growth the two cell lines, MDA-MB-231 and T47D cells were incubated with different concentrations of the lysosomotropic agent chloroquine diphosphate for three days. Both of the cell lines showed a decrease in cell number upon inhibition of autophagy with the highest concentration (25 μ M) of the inhibitor. A similar 30% - 40% decrease in growth of the MDA-MB-231 cells was observed with both 6.25 μ M and 25 μ M of chloroquine diphosphate (Figure 3.4A and B). However, no significant difference in cell number was observed when T47D cells were treated with the lowest concentration (6.25 μ M) of the inhibitor (Figure 3.4C and D). Thus, the growth of MDA-MB-231 cells with higher autophagy flux was more sensitive to inhibition of autophagy.

Anchorage-independent growth is one of the hallmarks of transformed cells. It gives tumor cells the potential to disseminate and metastasize (136). The dependence of the metastatic MDA-MB-231 and non-metastatic T47D cells on autophagy for anchorage-independent growth was evaluated. The cells were grown in soft agar in the presence of increasing concentrations of chloroquine diphosphate. Although T47D cells showed a 40% - 60% decrease in the number and size of soft agar colonies upon treatment with 25 μ M of chloroquine diphosphate, no significant decrease in colony formation was observed with 12.5 μ M of the inhibitor (Figure 3.5A and C). In contrast, MDA-MB-231 cells formed almost no colonies in the presence of both high (25 μ M) and low (12.5 μ M) concentrations of the inhibitor. Moreover, the few colonies that formed were

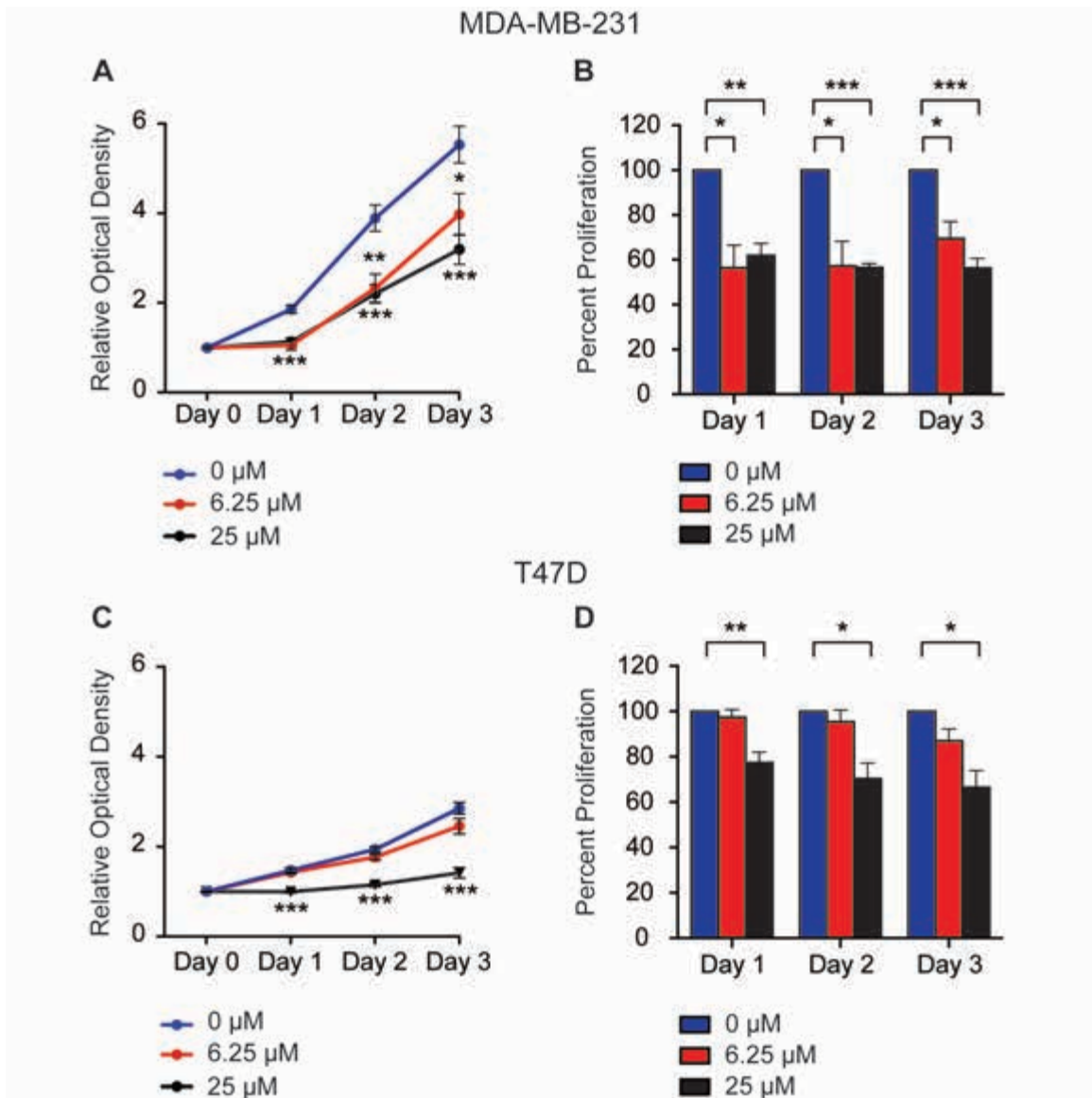


Figure 3.4. The anchorage-dependent growth of MDA-MB-231 cells is more sensitive to autophagy inhibition when compared with T47D cells.

MDA-MB-231 (A, B) and T47D (C, D) cells were treated with 6.25 μM or 25 μM chloroquine diphosphate and growth was determined on each day by MTT assay. Data are expressed as absorbance (optical density) relative to Day 0 (A, C) and as percent proliferation normalized to untreated cells. The data shown represent the mean of three independent experiments performed in quadruplicate. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$

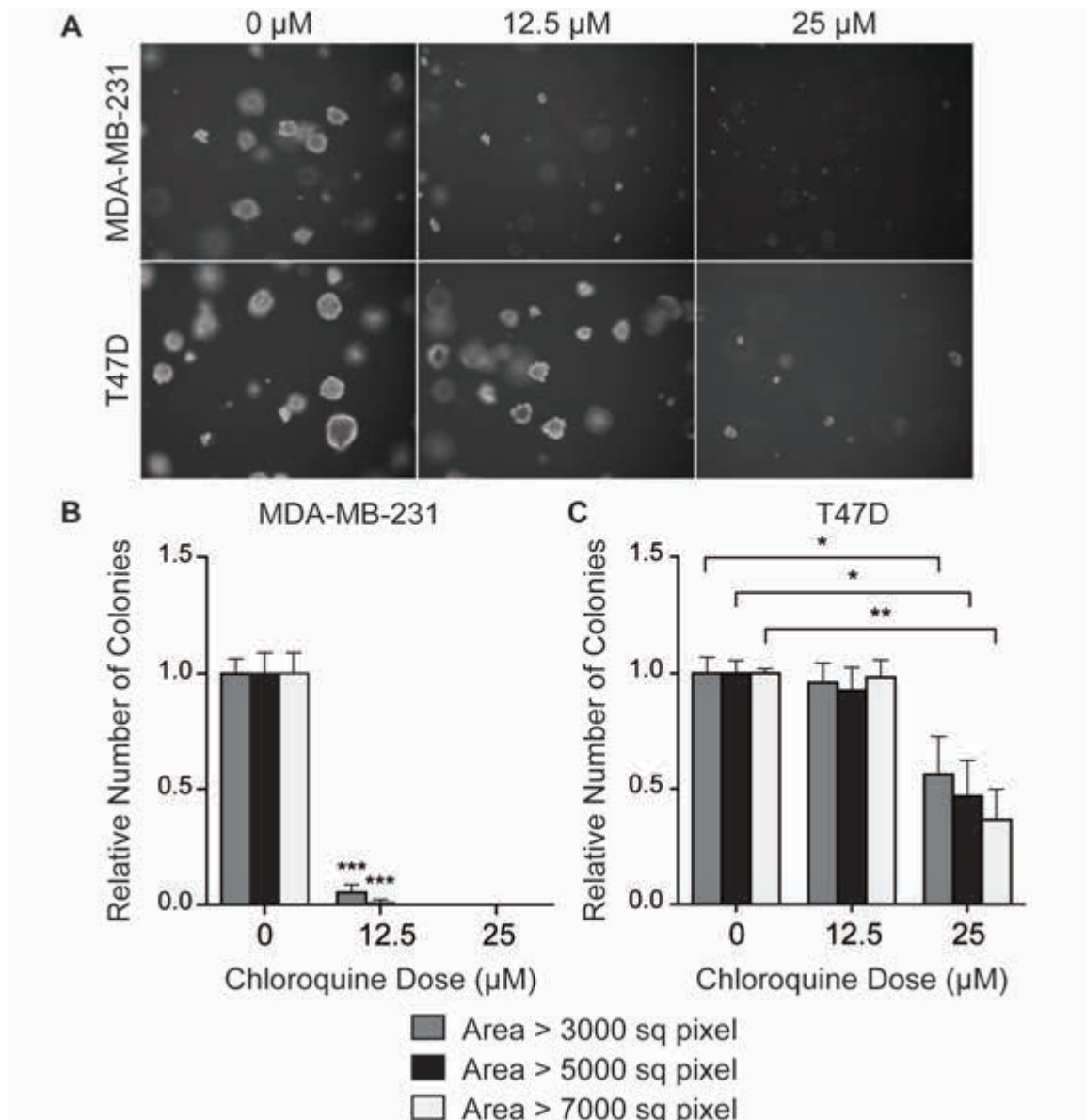


Figure 3.5. The anchorage-independent growth of MDA-MB-231 cells is more sensitive to autophagy inhibition when compared with T47D cells.

MDA-MB-231 and T47D cells were grown in soft agar and treated with or without 12.5 μM or 25 μM chloroquine diphosphate. (A) Representative images of the cells treated with the inhibitor. (B, C) The number of colonies formed was quantified and are represented relative to untreated cells. The data shown represent the mean of three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

reduced in size (less than 5000 square pixels in area) (Figure 3.5A and B). Thus, these data suggest that the two cell lines have differential sensitivities to autophagy inhibition, with the triple-negative, metastatic MDA-MB-231 cells being more dependent on autophagy for both anchorage-dependent and anchorage independent growth than the luminal, non-metastatic T47D cells.

Genetic disruption of autophagy inhibits the anchorage-independent growth of MDA-MB-231 cells

The contribution of autophagy in the previous experiments was determined by using the pharmacological inhibitor chloroquine diphosphate. However, since this anti-malarial agent inhibits lysosomal acidification, it can also have autophagy-independent effects (137). Hence, to directly inhibit autophagy in the MDA-MB-231 cells, a genetic approach was taken. The expression of two critical autophagy-regulators, ATG5 and BECN1, was suppressed with targeted lentiviral shRNA. Two stable cell lines expressing independent shRNA were generated for each of the autophagy proteins. From here on these cell lines will be referred to as shATG5 (#1), shATG5 (#2), shBECN1 (#1) and shBECN1 (#2) cells. Autophagic flux was monitored in these autophagy-deficient cells by incubating them for 4 hours in either nutrient replete media or in HBSS with and without 25 μ M chloroquine diphosphate. Depletion of ATG5 using shATG5 (#1) and to a lesser extent shATG5 (#2) decreased the autophagic flux. Induction of autophagy by serum starvation did not elevate LC3-II protein levels in these cell

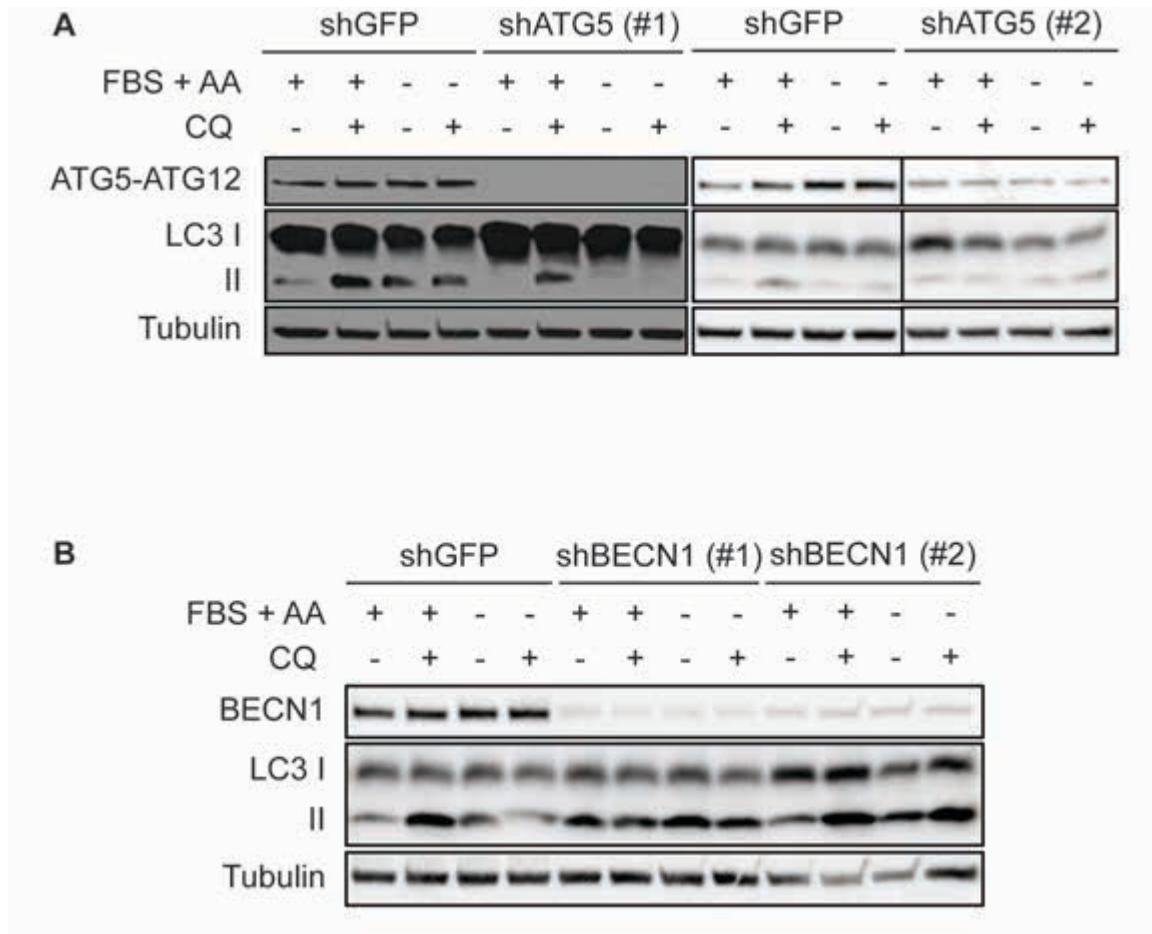


Figure 3.6. Autophagic flux in cells with suppression of ATG5 or Beclin 1 expression.

(A) MDA-MB-231 cells expressing shGFP and shATG5 (#1) and (#2) were incubated for 4 hours in either complete or serum and amino acid-free media with or without 25 μ M chloroquine diphosphate. Cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for LC3, ATG5 and tubulin. shGFP and shATG (#2) images are from the same immunoblot that was cropped to remove lanes. (B) MDA-MB-231 cells expressing shGFP, shBECN1 (#1) and (#2) were incubated for 4 hours in either complete or serum and amino acid-free media with or without 25 μ M chloroquine diphosphate. Cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for LC3, Beclin 1 and tubulin. FBS, fetal bovine serum; AA, amino acids.

lines (Figure 3.6A). In contrast to the results with shATG5, lipidation of LC3-I to LC3-II was not inhibited in the BECN1 depleted cells (Figure 3.6B).

To examine the contribution of autophagy to the anchorage-independent growth of MDA-MB-231 cells, shATG5 (#1) and (#2) and shBECN1 (#1) and (#2) cells were grown in soft agar to assess colony formation. Suppression of both ATG5 and Beclin 1 reduced the colony forming ability of MDA-MB-231 cells, similar to the results obtained with chemical inhibition of autophagy. A 40% - 50% decrease in the total number of colonies greater than 3000 square pixels was observed upon suppression of shATG5 (Figure 3.7). shBECN1 cells also showed a significant decrease (78% - 97%) in anchorage-independent growth. Overall the data indicate that autophagy facilitates anchorage-independent growth in MDA-MB-231 cells, which show sensitivity to both genetic and pharmacological inhibition of this degradative process. Of note, shBECN1 cells showed a more significant reduction in anchorage-independent growth when compared with shATG5 cells, suggesting that Beclin 1 may regulate additional pathways that are important for soft agar growth.

Suppression of ATG5 but not Beclin 1 inhibits aerobic glycolysis

Ras transformed cells have been shown to require autophagy to maintain their high glycolytic rates (135). Therefore, I next examined the glycolytic capacity of shATG5 and shBECN1 cells. Consistent with previous reports, ATG5 depletion reduced glucose metabolism in MDA-MB-231 cells. However,

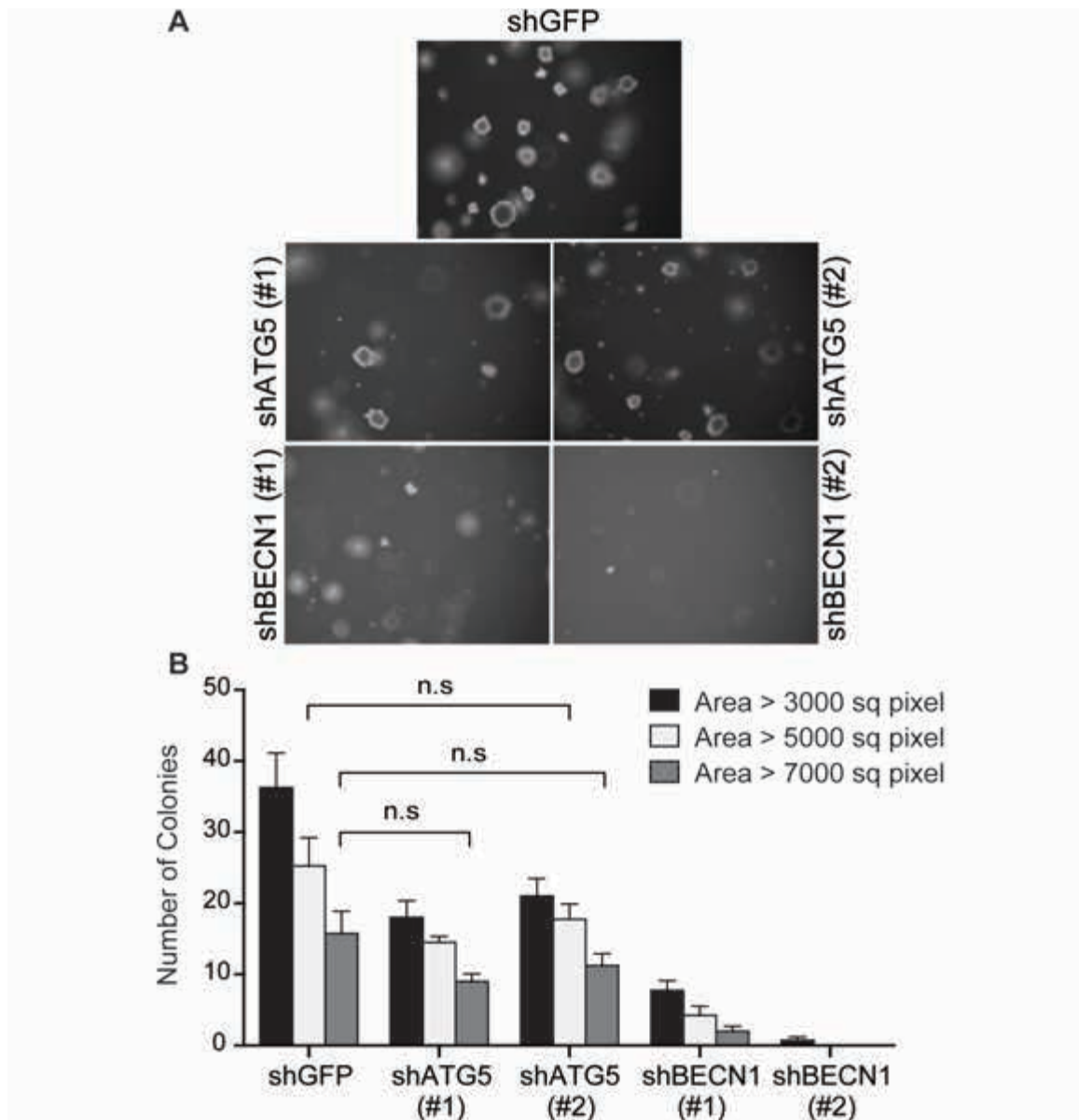


Figure 3.7. Autophagy facilitates anchorage-independent growth in MDA-MB-231 cells.

MDA-MB-231 expressing shGFP, shATG5 (#1 and #2) or shBECN1 (#1 and #2) were grown in soft agar. (A) Representative images of the cells. (B) The number of colonies formed was quantified and are represented relative to shGFP cells. The data shown represent the mean of two independent experiments performed in duplicate. All values are significant compared to shGFP except those marked as n.s. n.s, not significant.

suppression of Beclin 1 resulted in no difference in the aerobic glycolysis of the cells when compared to control shGFP cells. The differences between the shATG5 and shBECN1 cells implicate additional non-autophagic roles for Beclin 1, which enable Beclin 1-depleted cells to maintain their enhanced aerobic glycolytic rates.

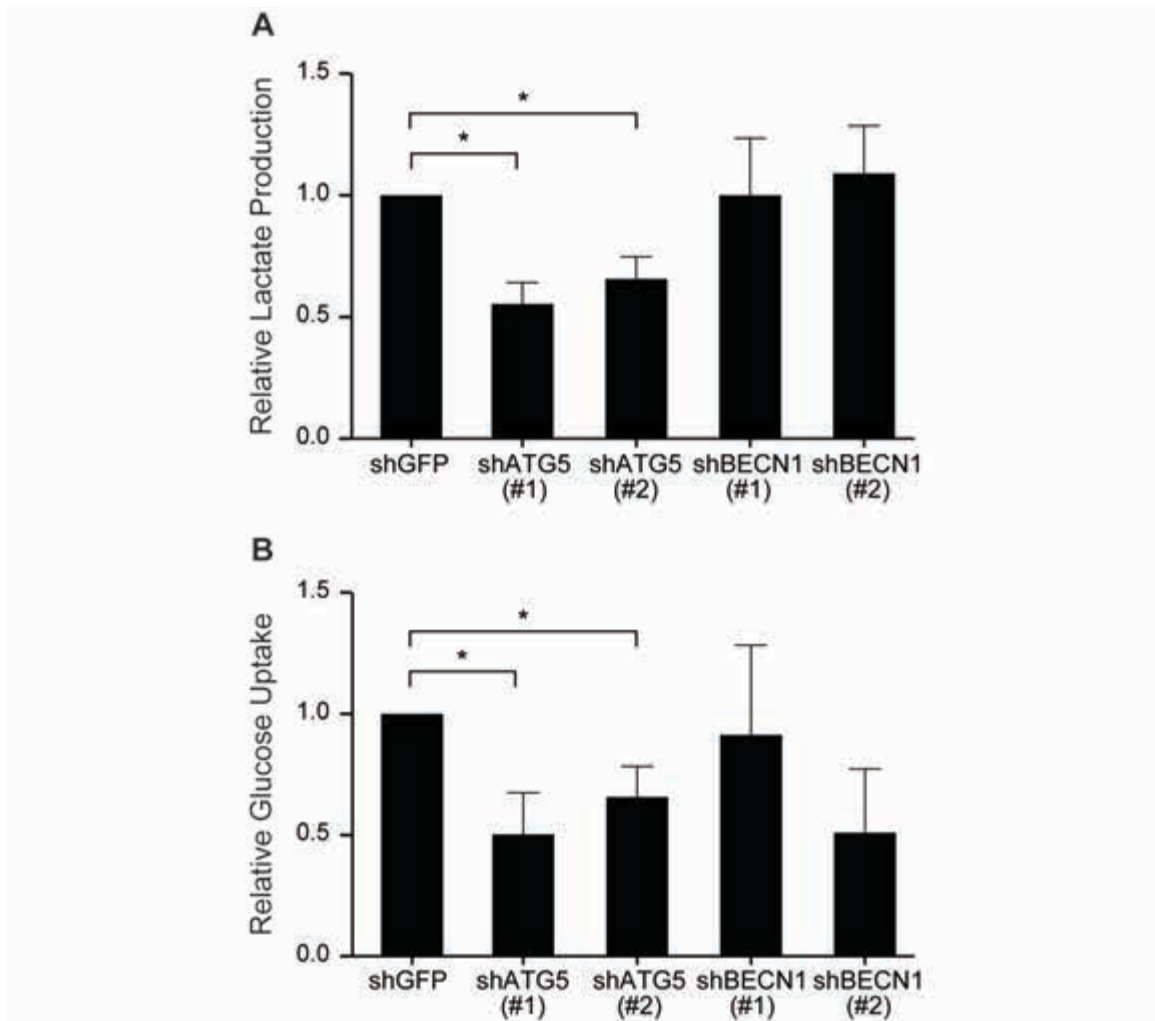


Figure 3.8. Suppression of ATG5, but not Beclin 1, expression inhibits glycolysis in MDA-MB-231 cells.

MDA-MB-231 cells expressing shGFP, shATG5 (#1 and #2) or shBECN1 (#1 and #2) were assayed for lactate production (A) and glucose uptake (B). The data are expressed as the rate of glucose depletion from or lactate production into the culture medium (mM/mg of total protein/hour) relative to MDA-MB-231 shGFP cells. The data shown represent the mean of three independent experiments performed in triplicate. *, $p < 0.05$.

Discussion

The study performed in this chapter indicates a tumor-promoting role for autophagy in a triple-negative, metastatic breast carcinoma cell line. I found that these cells had higher levels of basal and induced autophagy and were more sensitive to chemical inhibition of this degradative process when compared with the luminal breast cancer cells. Both anchorage-dependent and anchorage-independent growth of the triple-negative breast carcinoma cells were impeded to a greater extent upon inhibition of autophagy than observed for the luminal cells. Furthermore, I also demonstrate that RNAi-mediated suppression of the autophagy-regulating proteins ATG5 and Beclin 1 reduced anchorage-independent growth, with the decrease in soft agar colony formation being more significant upon Beclin 1 depletion. In addition, suppression of ATG5, but not Beclin 1, decreased glycolysis in the triple-negative MDA-MB-231 cells. My data suggest that Beclin 1 may have additional autophagy-independent functions that rescue glycolysis.

This study, as well as preliminary data (not shown), indicate that triple-negative breast carcinoma cell lines have higher levels of autophagy than luminal breast carcinoma cell lines. Moreover, starvation did not induce autophagy in luminal cells. MDA-MB-231 cells have a higher rate of proliferation than T47D cells and highly proliferating tumor cells are heavily dependent on glycolysis for supplying energy and biosynthetic intermediates (the Warburg effect). Activation

of several oncogenes including Ras has been shown to elevate aerobic glycolysis. The Ras oncogene impairs production of acetyl-CoA that feeds into the mitochondrial tricarboxylic acid (TCA) cycle, thereby decreasing mitochondrial respiration and increasing the production of lactic acid, a byproduct of glycolysis (90). Moreover, a study performed by Lock *et. al.* showed that autophagy facilitates increased glycolysis in Ras transformed cells and impairment of autophagy reduces their glycolytic capacity (135). Comparison of the amount of glucose taken up and lactate produced by MDA-MB-231 and T47D with high and low autophagic flux respectively, showed no difference in the level of glycolysis between the two cell lines. Since MDA-MB-231 cells have an activating K-Ras mutation, increased levels of autophagy may be required to support their glycolytic rates.

During the course of this work studies by Guo *et. al.*, Lock *et. al.*, Kim *et. al.* and Yang *et. al.* were published, which showed that Ras driven tumor cells upregulate autophagy and are “addicted” to this degradative process (110, 125, 135, 138). My studies suggest that differences in autophagy in the triple-negative and luminal cell lines were not due to differences in the glycolytic capacities of the MDA-MB-231 cells and the T47D cells. Increased glycolytic rates are accompanied by a decrease in mitochondrial oxidative phosphorylation. Nonetheless, functional mitochondrial metabolism and its generation of reactive oxygen species (ROS) to activate signaling pathways is required for K-Ras-mediated proliferation and tumorigenesis (139). Apart from maintaining the

quality of mitochondria by eliminating damaged organelles by mitophagy, autophagy can also support oxidative mitochondrial metabolism by providing biosynthetic intermediates to the TCA cycle. Degradation of protein and lipids by autophagy generates metabolites that can be used by the TCA cycle. In support of this, inhibition of autophagy in pancreatic ductal adenocarcinoma (PDAC) cells with activated Ras led to a decrease in TCA cycle metabolites and ATP (110, 125). These studies indicate that one of the reasons for elevated basal autophagy that we observed in the triple-negative MDA-MB-231 cells may be to maintain functional mitochondrial metabolism and ATP levels in rapidly dividing cells.

Luminal T47D breast cancer cells exhibited low levels of autophagy and low sensitivity to inhibition of anchorage-independent growth. T47D cells, as well as MCF7 cells, have gain-of-function mutations in the *PIK3CA* gene, which encodes for the p110 α catalytic subunit of class I PI3K (129, 140). These mutations lead to increased activation of PI3K and its downstream pathway. Elevated levels of AKT phosphorylation in T47D cells result in activation of the negative regulator of autophagy, mTORC1 (141). This inhibition could explain the lower autophagic flux observed in the luminal T47D cell line. These less aggressive cells could also resort to an alternate mode of maintaining metabolic fitness such as microautophagy and chaperone mediated autophagy (CMA). In microautophagy, proteins and organelles are directly engulfed into the lysosome (142). On the other hand during CMA, chaperone proteins recognize and bind

damaged and oxidized cytosolic proteins. These proteins are subsequently delivered into the lysosome after binding with the lysosomal protein LAMP-2A (36). Upregulation of CMA in various cancer cell lines including MCF7 cells has been reported (143). Additionally, elevated expression of LAMP-2A has also been observed in human breast tumor samples. Moreover, LAMP-2A over expression in T47D and MCF7 cells provided protection against oxidative damage (144). In my studies assessment of LAMP-1 protein levels indicated high lysosomal content in T47D but not MCF7 cells. The expression of LAMP-2A should be assessed in these cells to determine the involvement of CMA.

Luminal T47D cells were less sensitive to inhibition of lysosomal degradation with chloroquine diphosphate than triple-negative MDA-MB-231 cells. Under stress conditions such as contact-independent growth or uncontrolled proliferation, luminal cells could decrease protein synthesis as an alternative mechanism to overcome bioenergetic deficiencies. In support of this possibility, total LC3 levels were reduced in T47D cells upon serum starvation. These cells could also utilize the ubiquitin proteasome system to accelerate degradation of unwanted and damaged proteins. Further studies are required to determine how these cells maintain homeostasis when autophagy is inhibited.

As mentioned earlier, autophagy supports aerobic glycolysis in Ras mutant cells (135). Indeed, inhibition of ATG5 resulted in decreased glycolysis in MDA-MB-231 cells that have an activating Ras mutation. However, depletion of

Beclin 1 showed no effect on glucose metabolism. One explanation for this difference could be an increase in growth factor receptor signaling. Beclin 1 has been reported to regulate receptor degradation, with suppression of Beclin 1 protein levels decreasing EGFR degradation (19). Inhibition or delay in receptor degradation could result in increased growth factor receptor signaling that can regulate nutrient uptake by activating pathways such as the PI3K/AKT pathway. Increased signaling through this pathway in turn would enhance glycolysis in cancer cells by regulating translocation of the glucose transporter GLUT1 to the cell surface and activation of hexokinase and phosphofructokinase enzymes involved in glycolysis (145). Therefore, the decrease in glycolysis due to inhibition of autophagy could be compensated for by increased growth factor receptor signaling, thereby maintaining glycolytic rates in Beclin 1-suppressed MDA-MB-231 cells. Thus, my data implicates potential autophagy-independent functions of Beclin 1 that can regulate glycolysis in these cells.

My study and others demonstrate a dependency of triple-negative breast carcinoma cells on autophagy, suggesting that this subtype of breast cancer may be a good candidate for treatment with autophagy inhibitors (129). In addition to MDA-MB-231 cells, my preliminary experiments showed a similar increase in autophagic flux in additional triple-negative breast cancer cells lines. My findings are consistent with the recently published work by Maycotte *et.al*. Similar to my observations they found an increased sensitivity of triple-negative breast carcinoma cell lines to autophagy inhibition when compared with luminal and

non-tumorigenic cells (129). Thus, the efficacy of treatment of cancer by inhibiting autophagy is context-dependent, and for breast cancer it may depend upon the molecular subtype of the tumor.

CHAPTER IV

BECLIN 1 REGULATES EARLY ENDOSOME MATURATION AND GROWTH FACTOR RECEPTOR SIGNALING

Parts of this chapter represent work published as:

Beclin 1 regulates growth factor receptor signaling in breast cancer

Rasika A. Rohatgi^a, Jenny Janusis^a, Deborah Leonard^b, Karl D. Bellvé^c, Kevin E. Fogarty^c, Eric H. Baehrecke^a, Silvia Corvera^b and Leslie M. Shaw^a

^aDepartment of Cancer Biology, ^bProgram in Molecular Medicine and ^cBiomedical Imaging Group, University of Massachusetts Medical School, Worcester, MA 01605

Introduction

Beclin 1 is the mammalian homolog of the yeast Atg6/Vacuolar protein sorting 30 (Vps30) protein that plays an essential role in autophagy and vacuolar protein sorting (1, 2). Beclin 1 is required for normal mammalian development and it has been shown to play a critical role in pathogenesis, including cancer (35, 94). Mice heterozygous for Beclin 1 expression (*Becn1*^{+/-}) have an increased incidence of lymphoma and carcinomas of the lung and liver, and these mice also show preneoplastic changes in the mammary gland tissue as they age (35, 94). Additionally, heterozygous loss of Beclin 1 promotes mammary tumorigenesis in response to parity and WNT1 activation (146). These studies support a role for Beclin 1 as a haploinsufficient tumor suppressor gene in a context-dependent manner. In many human tumors Beclin 1 mRNA and protein expression are decreased, the result of both monoallelic loss of the *BECN1* gene and aberrant DNA methylation that suppresses gene expression (96). Mutation of *BECN1* has not been detected in tumors (95). Reduced Beclin 1 protein expression is associated with poor prognosis in several cancer types, including breast (97, 98).

The investigation of Beclin 1 function in cancer has focused primarily on its role in regulating autophagy. *Becn1*^{+/-} mice show evidence of decreased autophagy and immortalized *Becn1*^{+/-} mouse mammary epithelial cells exhibit a ROS-mediated DNA damage response that is attributed to the accumulation of

damaged mitochondria when autophagy is impaired. This increase in DNA damage has been implicated in transformation (109). However, additional membrane trafficking functions of Beclin 1 play significant roles in tissue homeostasis and their dysregulation could contribute to tumor cell biology. Beclin 1 regulates membrane trafficking through its interaction with Vps34/Class III Phosphatidylinositol-3 Kinase (PI3KC3), a lipid kinase that phosphorylates PI to generate the lipid second messenger PI-3 phosphate (PI3P) (147, 148). PI3P mediates the recruitment of proteins containing FYVE (Fab1p, YOTB, Vac1p, EEA1) and PX (Phox homology) domains to intracellular membranes to facilitate vesicular fusion and sorting events (149-151). Beclin 1 regulates distinct membrane trafficking pathways through mutually exclusive recruitment of additional binding partners. For example, Beclin 1 interacts with Atg14/ATG14L/Barkor (Complex I) to regulate autophagy initiation and Vps38/UVRAG (Complex II) to regulate vacuolar protein sorting, cytokinesis and receptor degradation (12, 13).

In the previous chapter, I demonstrated that Beclin 1 has autophagy-independent features that are likely to contribute to its role in cancer. These studies revealed a potential role for the regulation of growth factor receptor. Given the importance of growth factor receptor signaling in normal cell function and the consequences of its dysregulation in cancer, I assessed the role of Beclin 1 in regulating receptor endocytic trafficking and signaling, and sought to determine the mechanism involved. The data I report here reveal a novel role for

Beclin 1 in regulating growth factor receptor signaling that involves the control of early endosome maturation. My findings have important implications for understanding how Beclin 1 loss contributes to cancer.

Results

Beclin 1 regulates growth factor receptor signaling

Intracellular signaling by growth factor receptors is controlled by trafficking through compartments of the endocytic pathway. Factors that regulate receptor movement from the early endosome compartment to either the late endosome/lysosome compartment for signal termination and degradation or the recycling endosome compartment for restoration of surface expression control the strength and duration of signal transduction. To investigate a role for Beclin 1 in the regulation of growth factor receptor signaling, I examined the link between Beclin 1 and two growth factor receptors that have been implicated in breast cancer, the insulin-like growth factor-1 (IGF-1) receptor and the epidermal growth factor (EGF) receptor. The IGF-1R is frequently overexpressed in breast tumors and its activation correlates with poor prognosis (152, 153), whereas the EGFR is frequently elevated in expression and activation in the triple negative breast cancer (TNBC) subtype (154).

MDA-MB-231 cells (TNBC subtype), MCF7 cells (luminal breast cancer subtype) and MCF10A cells (immortalized mammary epithelial cells) were stably infected with shRNAs targeting BECN1 (shBECN1) or GFP (shGFP). IGF-1R protein levels were monitored in Beclin 1-depleted cells growing in nutrient-rich media. Suppression of Beclin 1 expression increased IGF-1R protein levels in all three cell lines (Figure 4.1A). Conversely, stable over-expression of flag-tagged

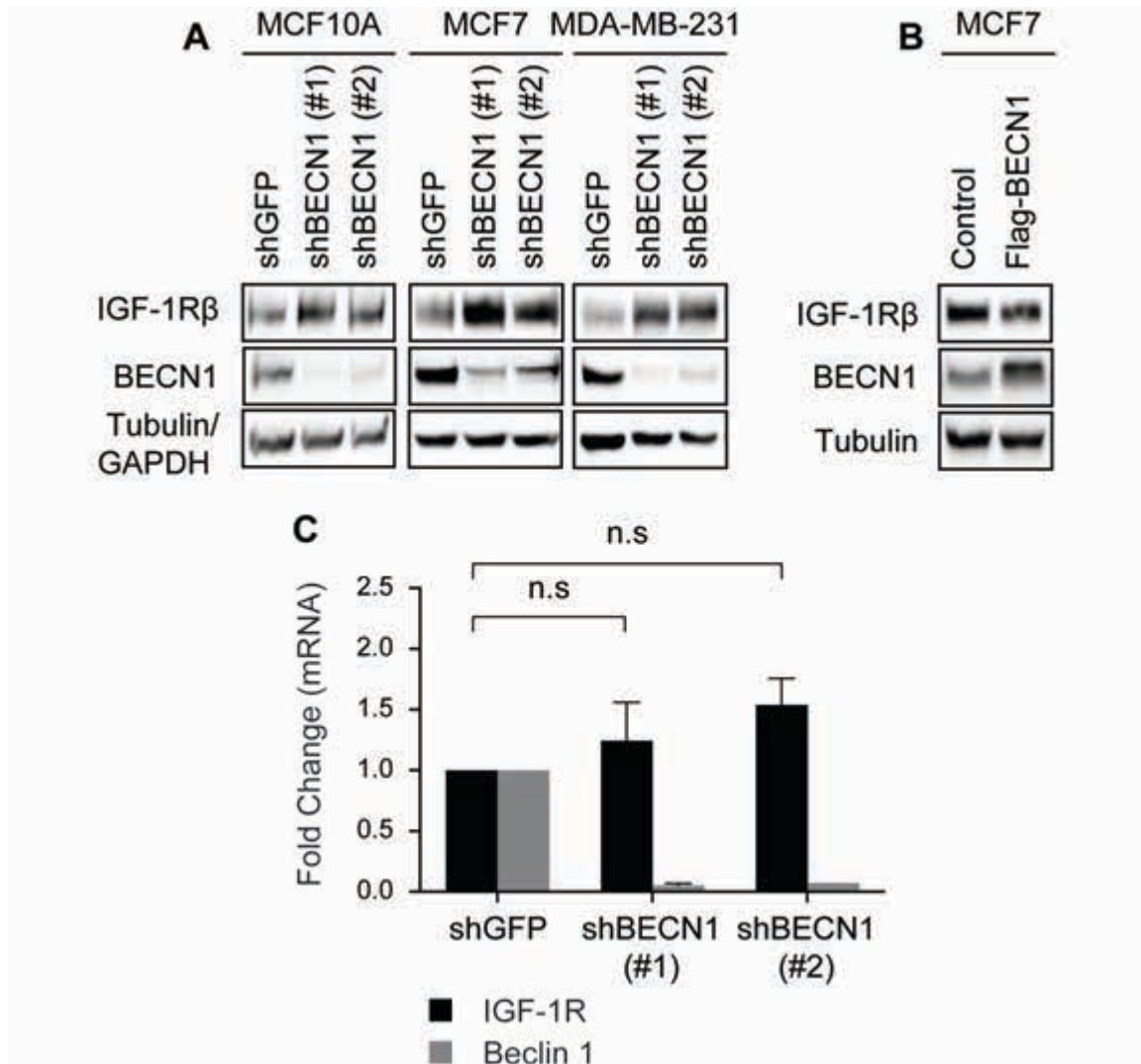


Figure 4.1. Beclin 1 depletion increases IGF-1R protein levels.

(A,B) Cell extracts from MCF10A, MCF7 and MDA-MB-231 cells expressing shGFP or shBECN1 (A) or MCF7 cells expressing Flag-Beclin 1 (B) were immunoblotted with the indicated antibodies. (C) *IGF-1R* and *BECLN1* mRNA levels were determined by qRT-PCR. The data shown represent the mean of three independent experiments. n.s., not significant.

Beclin 1 in MCF7 cells, which express the highest levels of IGF-1R, reduced IGF-1R expression (Figure 4.1B). Importantly, no significant change in mRNA expression was detected, indicating that Beclin 1 regulates the expression of IGF-1R at the level of protein expression and/or degradation (Figure 4.1C).

To investigate the impact of Beclin 1 on growth factor receptor signaling, phosphorylation of key downstream signaling effectors of the IGF-1R and EGFR, the serine/threonine kinases AKT and ERK1/2, was assessed in response to a time course stimulation with either IGF-1 (Figure 4.2-4.4) or EGF (Figure 4.5). AKT and ERK1/2 were phosphorylated in a transient manner in response to IGF-1 and EGF stimulation in control shGFP cells. In contrast, phosphorylation levels increased and were sustained at later time points in the shBECN1 cells (quantified in Figures 4.2-4.5). Although similar trends were observed for both AKT and ERK in response to both ligands, the impact on AKT phosphorylation was significant downstream of the IGF-1R and ERK1/2 phosphorylation downstream of the EGFR. These data reflect the dominant signaling pathways that are activated by each of these receptors. In addition, the IGF-1-dependent increase in AKT phosphorylation upon Beclin 1 loss was greater for T308 than for S473. Ectopic expression of Beclin 1 significantly decreased IGF-1-dependent AKT activation in the parental MDA-MB-231 cells (Figure 4.6), whereas the elevated signaling observed in the shBECN1 MDA-MB-231 cells decreased upon rescue of Beclin 1 expression (Figure 4.7). Taken together my data reveal that Beclin 1 negatively regulates the intensity and duration of growth factor receptor

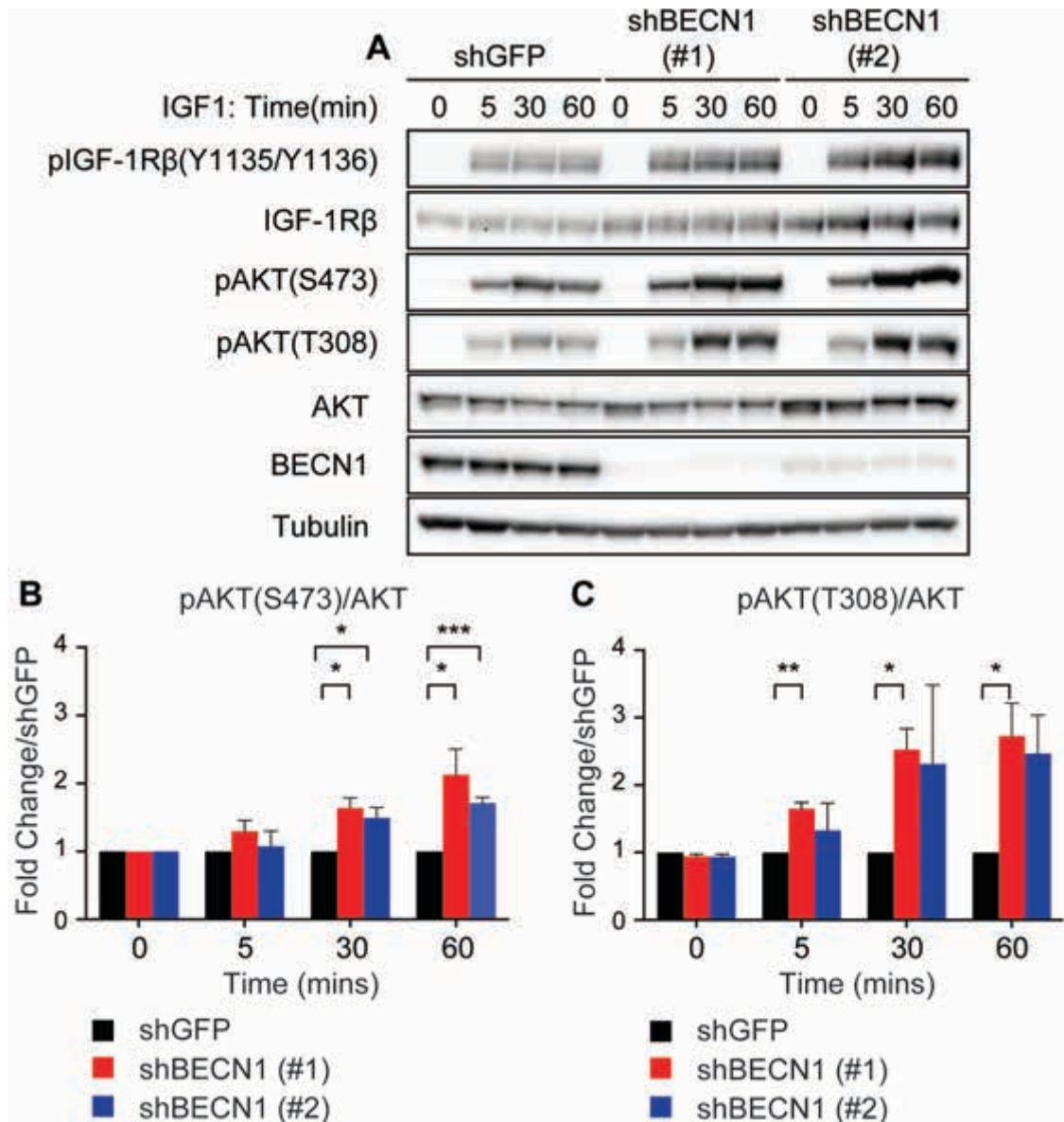


Figure 4.2. Beclin 1 regulates IGF-1-dependent signaling in MDA-MB-231 cells.

(A) MDA-MB-231 cells expressing shGFP, shBECN1 (#1) or shBECN1 (#2) were serum-starved overnight and then stimulated with IGF-1 (100ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(S473) (B) and phospho-AKT(T308) (C) levels were quantified by densitometry. The data shown represent the mean of three (C) or four (B) independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

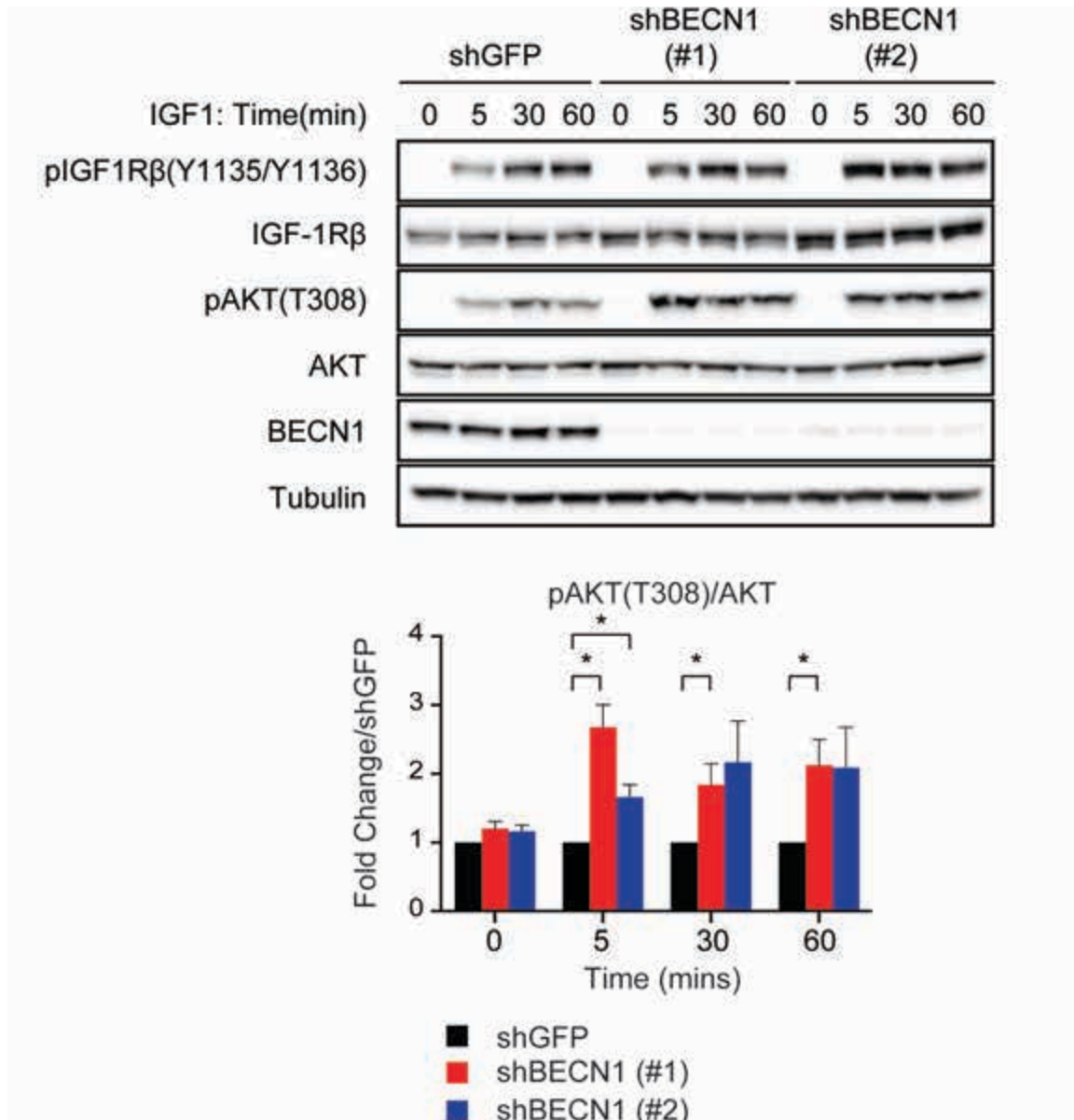


Figure 4.3. Beclin 1 regulates IGF-1-dependent signaling in MCF7 cells.

(A) MCF7 cells expressing shGFP, shBECN1 (#1) or shBECN1 (#2) were serum-starved overnight and then stimulated with IGF-1 (100ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(T308) levels were quantified by densitometry. The data shown represent the mean of three independent experiments. *, $p < 0.05$.

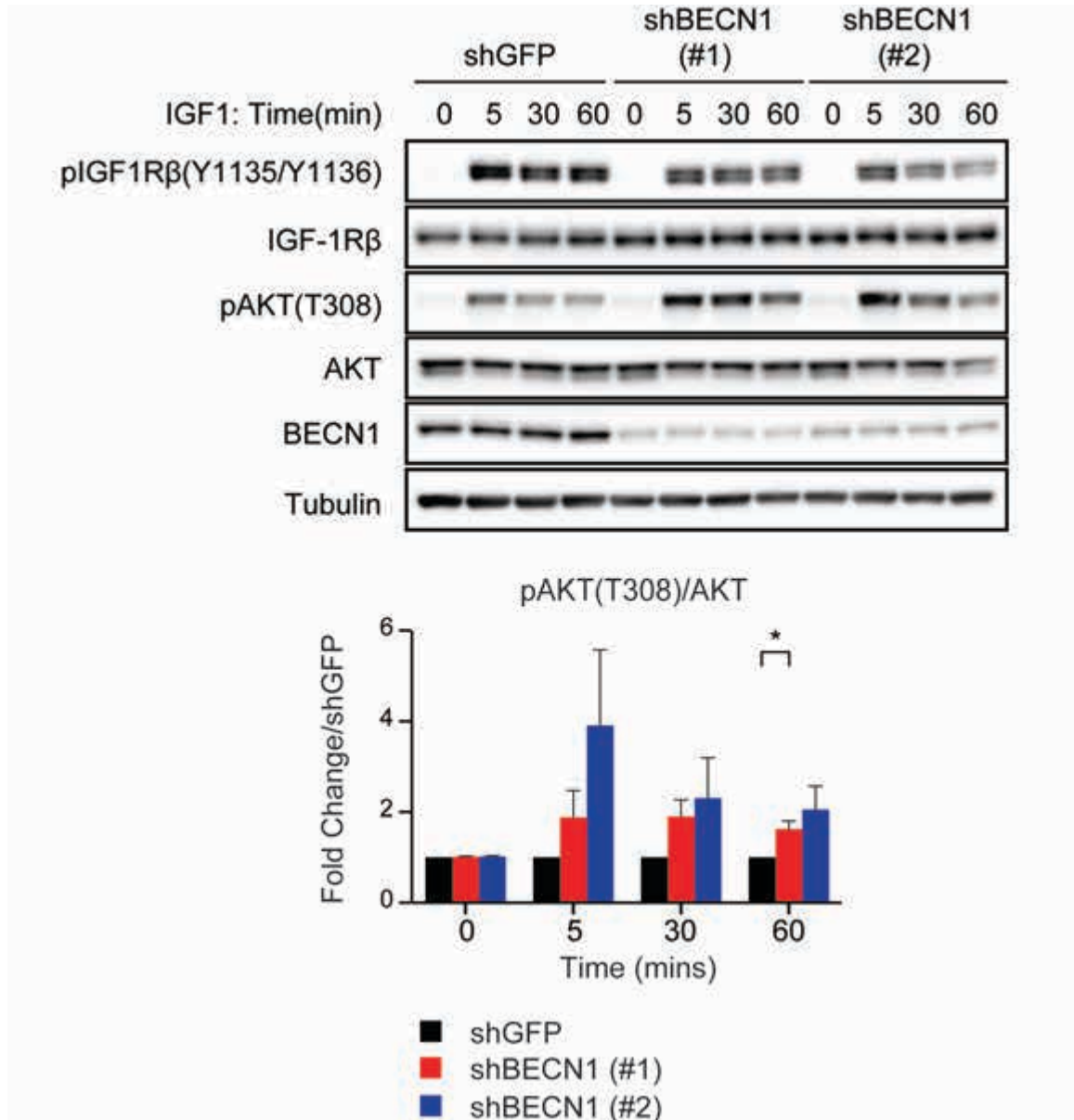


Figure 4.4. Beclin 1 regulates IGF-1-dependent signaling in MCF10A cells.

(A) MCF10A cells expressing shGFP, shBECN1 (#1) or shBECN1 (#2) were serum-starved overnight and then stimulated with IGF-1 (100ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(T308) levels were quantified by densitometry. The data shown represent the mean of three independent experiments. *, $p < 0.05$.

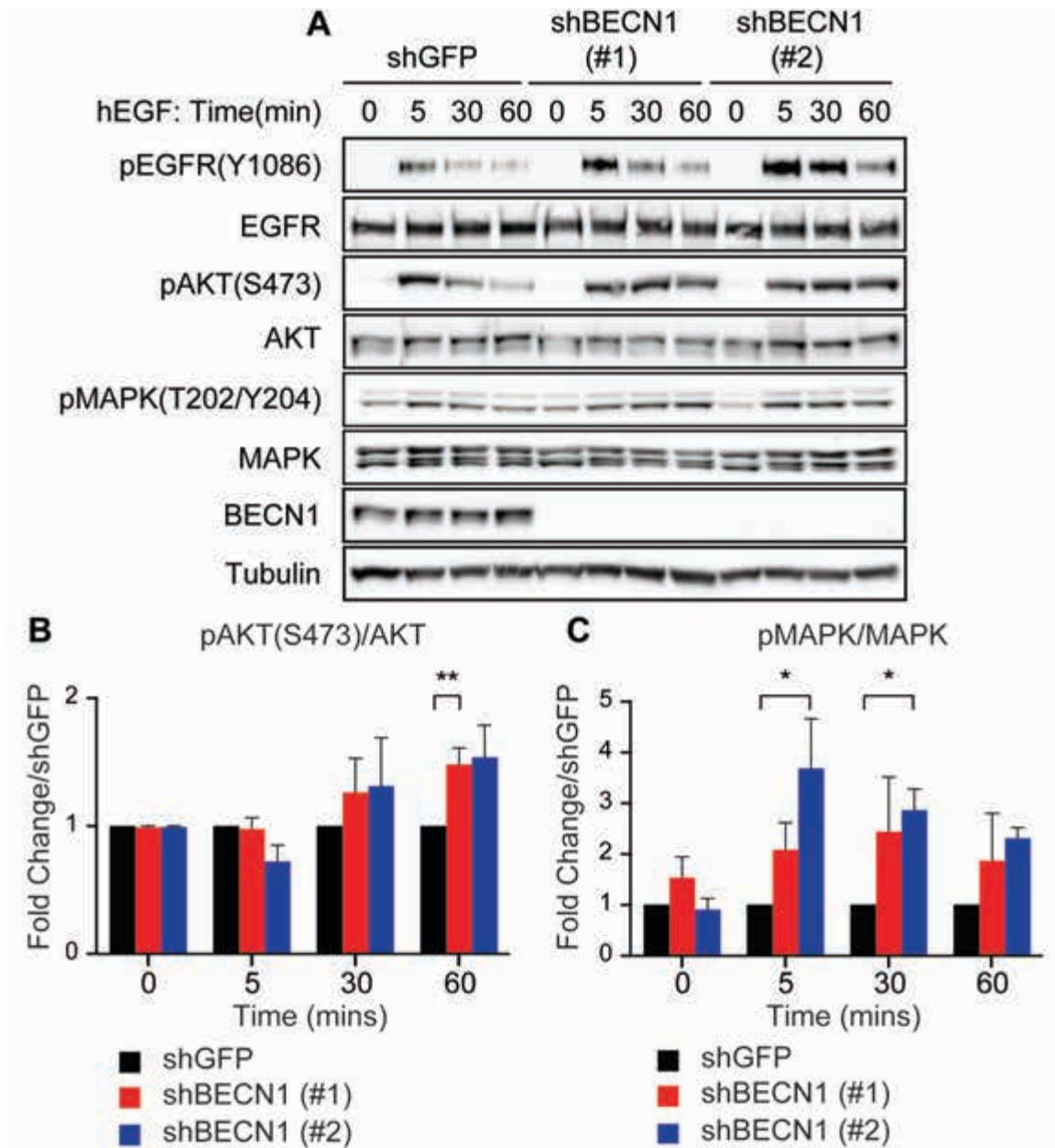


Figure 4.5. Beclin 1 regulates EGF-dependent signaling.

(A) MDA-MB-231 cells expressing shGFP, shBECN1 (#1) or shBECN1 (#2) were serum-starved overnight and then stimulated with EGF (50ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(S473) (B) and phospho-MAPK (T202/Y204) (C) levels were quantified by densitometry. The data shown represent the mean of three independent experiments. *, $p < 0.05$; **, $p < 0.005$.

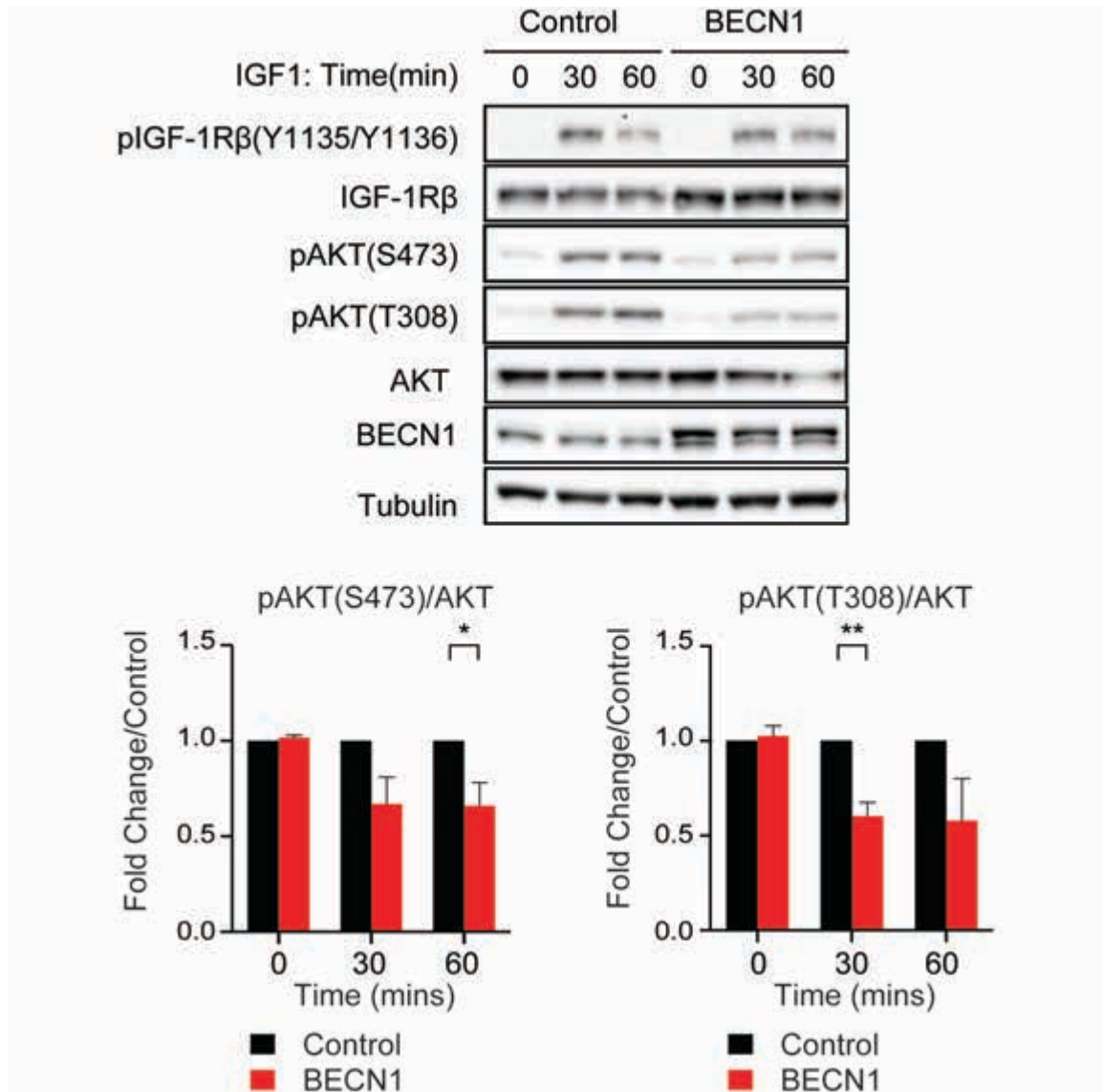


Figure 4.6. Ectopic expression of Beclin 1 decreases growth factor-dependent signaling.

MDA-MB-231 cells transiently expressing Flag-Beclin 1 were stimulated with IGF-1 (100ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(S473) and Phospho-AKT(T308) levels were quantified by densitometry. The data shown represent the mean of three independent experiments. *, $p < 0.05$; **, $p < 0.005$.

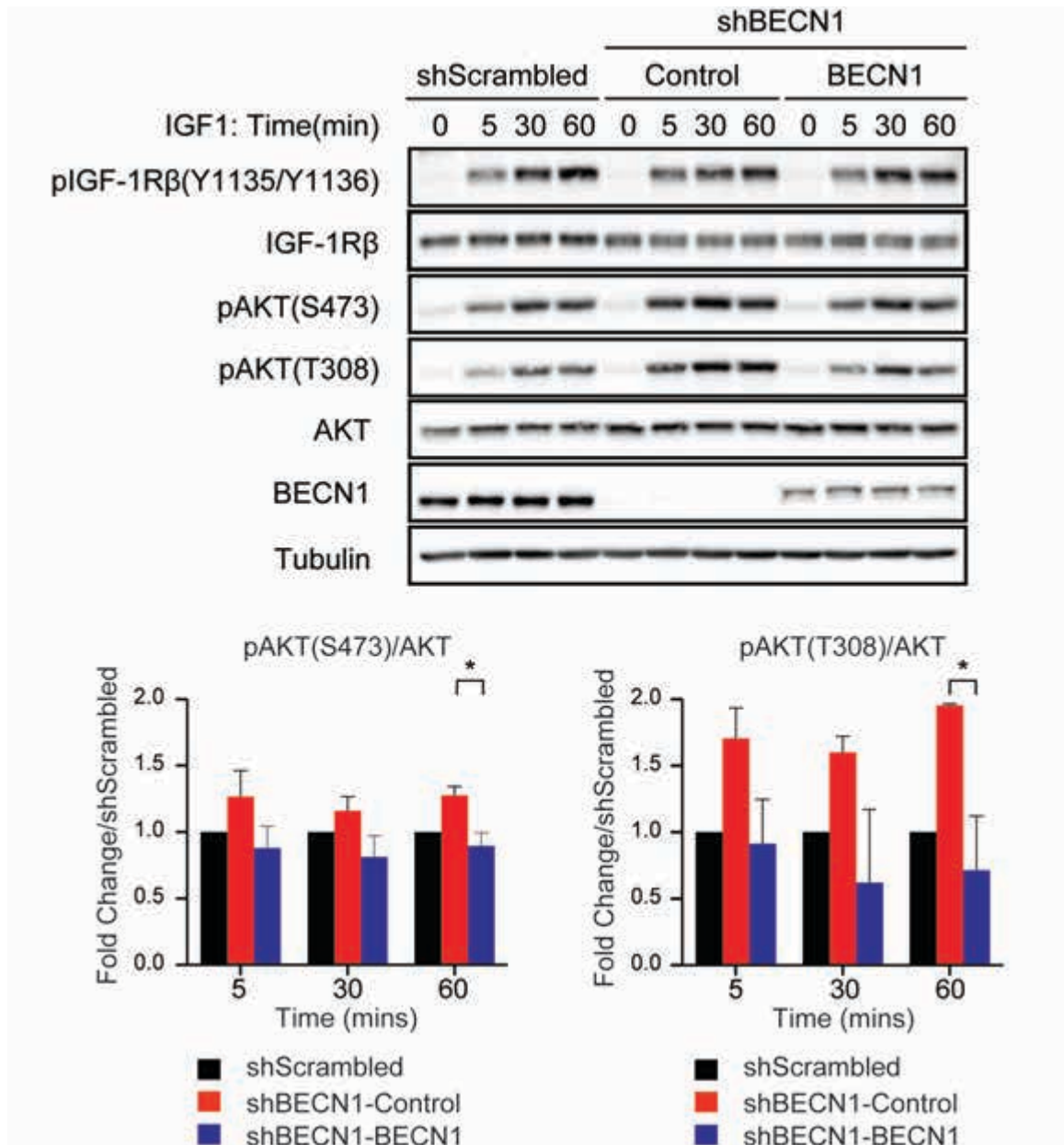


Figure 4.7. Rescue of Beclin 1 expression restores growth factor-dependent signaling to control levels.

MDA-MB-231 cells expressing shGFP, shBECN1 or shBECN1 and Flag-Beclin 1 were stimulated with IGF-1 (100ng/ml) for the indicated time periods. Cell extracts were immunoblotted with the indicated antibodies. Phospho-AKT(S473) and Phospho-AKT(T308) levels were quantified by densitometry. The data shown represent the mean of three independent experiments. *, $p < 0.05$.

signaling.

Beclin 1 in receptor degradation and signaling

My observation that suppression of Beclin 1 increased IGF-1R protein levels without a concomitant increase in mRNA levels (Figure 4.1) suggested a role for Beclin 1 in controlling receptor degradation. In mammalian systems, there have been conflicting reports regarding the role of Beclin 1 in the regulation of degradative endocytic trafficking. One study concluded that Beclin 1 does not participate in the regulation of EGFR endocytic trafficking and is only required for autophagic membrane trafficking (84), while a separate study identified a role for Beclin 1 in regulating the rate of EGFR degradation (19). To investigate this mechanism of action for Beclin 1, the rate of EGFR and IGF-1R degradation and signaling after stimulation with their cognate ligands was examined. Serum starved cells treated with cycloheximide to prevent *de novo* protein expression, were stimulated with either EGF or IGF-1 over a 6 hour period followed by protein analysis. Consistent with the latter study (19) a reduction in the rate of EGFR (Figure 4.8A and B) degradation was observed. However, IGF-1R was found to be more stable over the time course analyzed and only a slight decrease in its degradation during the early time points was detected (Figure 4.9A and B). Importantly, phosphorylation of AKT and MAPK remained elevated in the shBECN1 cells over this extended time course (Figure 4.8A, C, D and 4.9A, C).

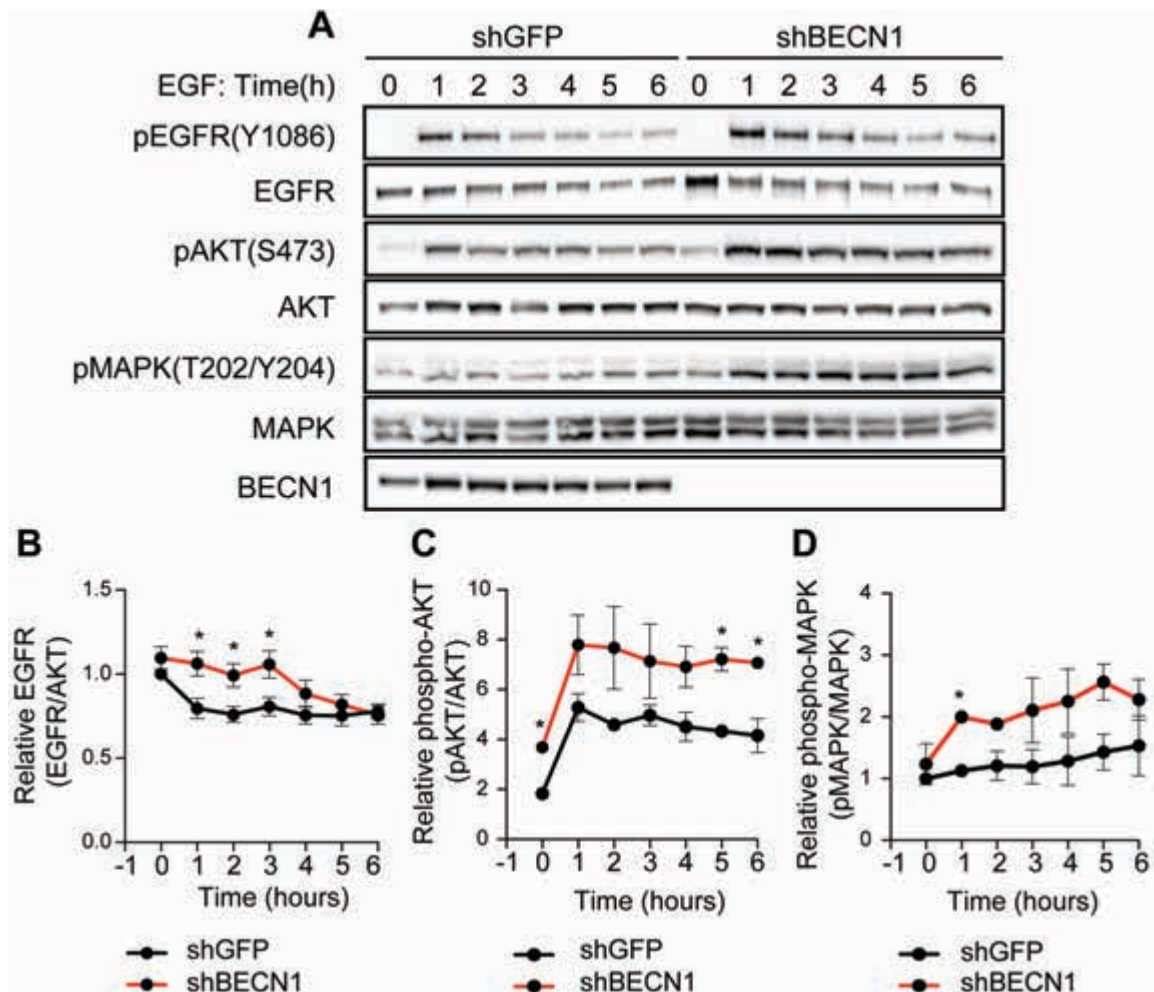


Figure 4.8. Regulation of EGFR degradation and signaling by Beclin 1.

MDA-MB-231 cells expressing shGFP or shBECN1 were pre-incubated with cycloheximide (50 μ g/ml) for 1hr and then stimulated with EGF (50ng/ml) in the presence of cycloheximide for 0-6 hours. Cell extracts were immunoblotted with the indicated antibodies. EGFR protein (B), phospho-AKT(S473) (C) and phospho-MAPK (T202/Y204) (D) levels were quantified by densitometry. The data shown represent the mean of two independent experiments. *, $p < 0.05$.

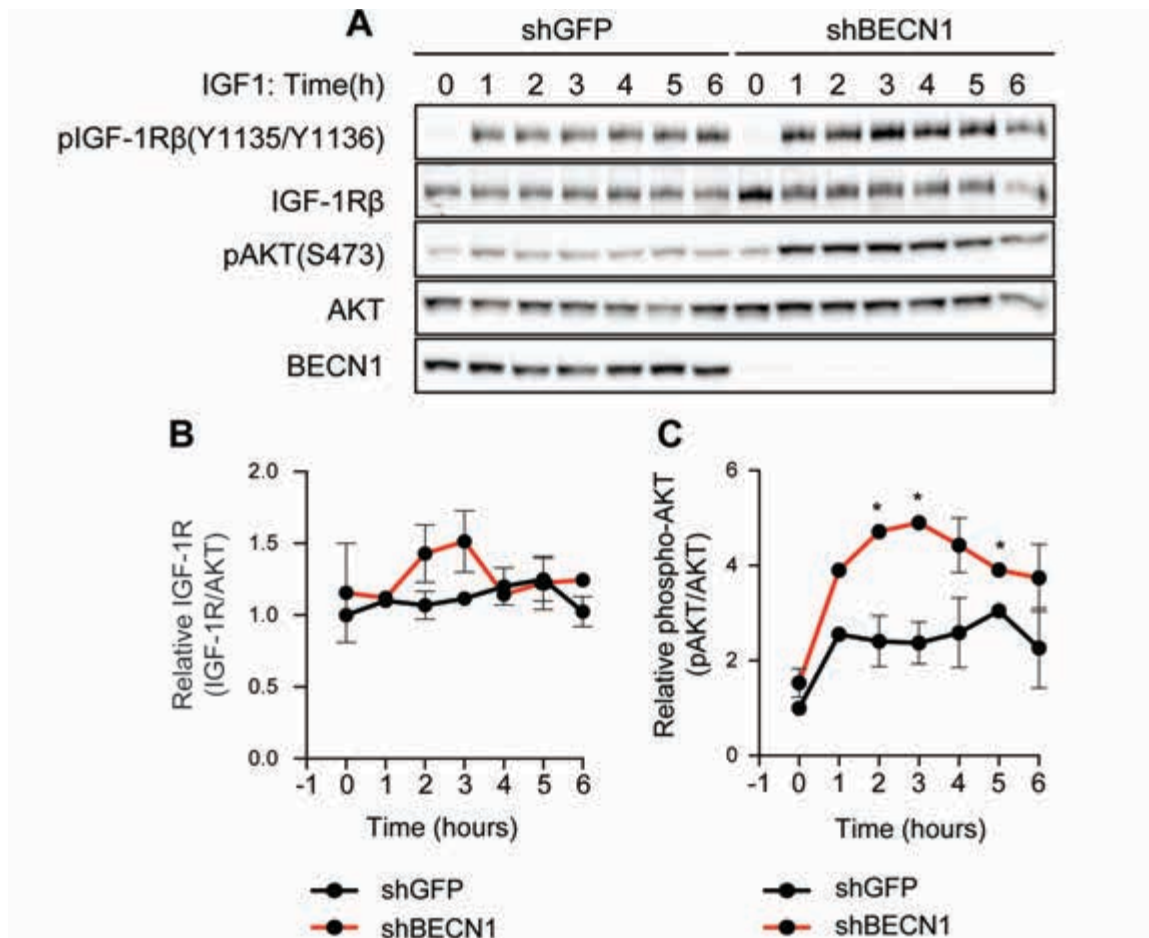


Figure 4.9. Regulation of IGF-1R degradation and signaling by Beclin 1.

MDA-MB-231 cells expressing shGFP or shBECN1 were pre-incubated with cycloheximide (50 μ g/ml) for 1hr and then stimulated with IGF-1 (100ng/ml) in the presence of cycloheximide for 0-6 hours. Cell extracts were immunoblotted with the indicated antibodies and IGF-1R protein (B) and phospho-AKT(S473) (C) levels were quantified by densitometry. The data shown represent the mean of two independent experiments. *, $p < 0.05$.

To determine if the effect of Beclin 1 on growth factor receptor degradation, AKT and MAPK activation involved its autophagic function, similar assays were performed after suppression of *ATG5*, an essential autophagy gene that is required for the formation and maturation of autophagosomes (155). Suppression of *ATG5* significantly impaired autophagy in response to serum deprivation, as monitored by LC3 II levels (Figure 3.6A). In contrast to the delayed degradation observed in the shBECN1 cells, the rate of EGFR degradation upon suppression of *ATG5* expression was similar to the control cells (Figure 4.10A and B). Furthermore, AKT and MAPK phosphorylation were neither elevated nor sustained in the absence of *ATG5* (Figure 4.10A, C and D). In addition, chemical inhibition of autophagy with the lysosomotropic agent chloroquine diphosphate also did not increase AKT phosphorylation at either site (Figure 4.11). Inhibition of autophagy was confirmed by assessing the levels of LC3-II which accumulated in cells treated with the inhibitor. Taken together, the data indicate that Beclin 1 regulates growth factor-stimulated AKT and MAPK activation through an autophagy-independent mechanism.

Beclin 1 regulates growth factor receptor-dependent PI3KC3 activation and endosomal maturation

Growth factor receptor signaling initiates at the cell surface and continues upon receptor internalization into early endosomes (156). To investigate further how Beclin 1 regulates signaling, receptor internalization was prevented using

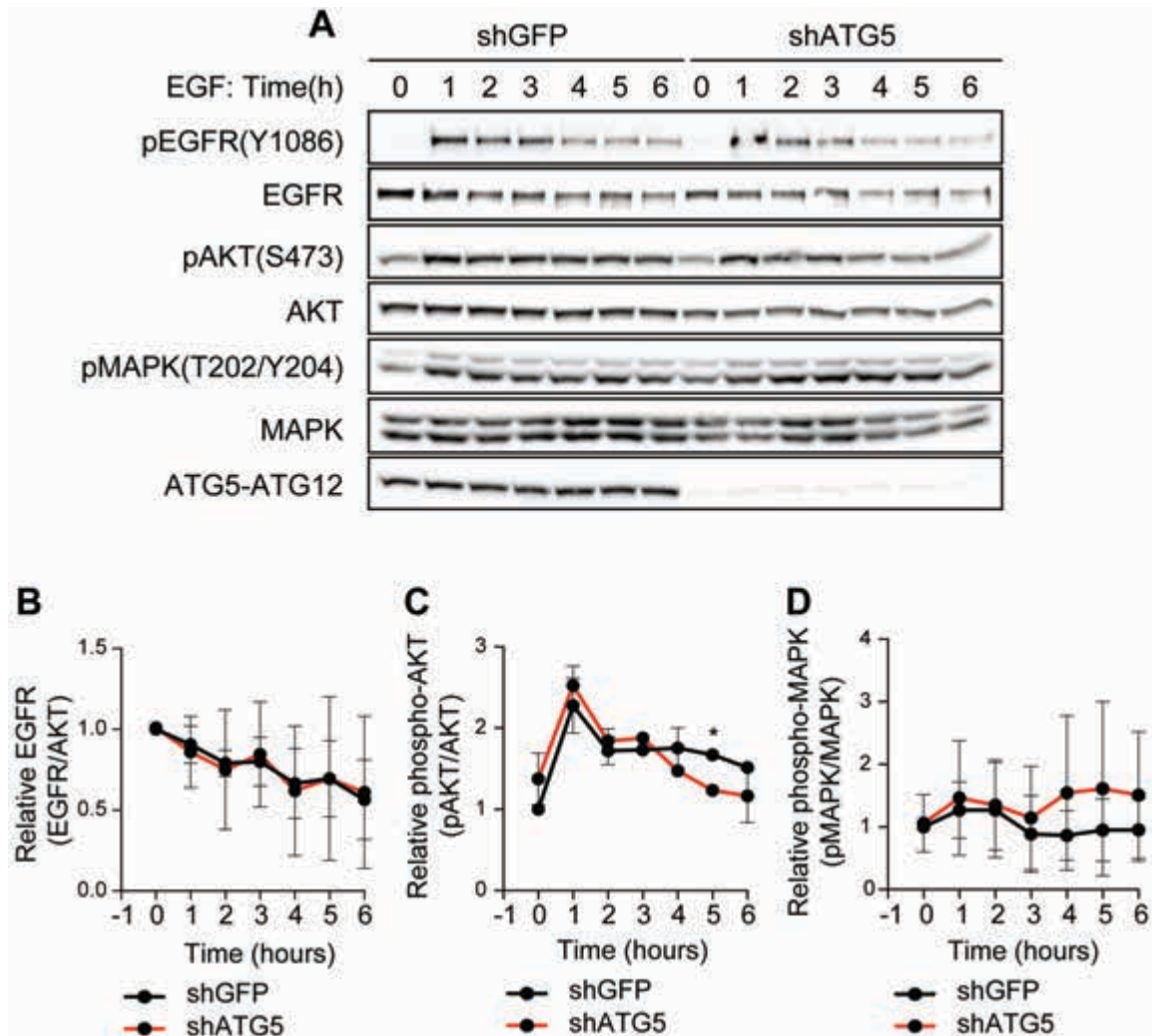


Figure 4.10. Regulation of receptor degradation and signaling by Beclin 1 is autophagy-independent.

MDA-MB-231 cells expressing shGFP or shATG5 were pre-incubated with cycloheximide (50 μ g/ml) for 1hr and then stimulated with EGF (50ng/ml) in the presence of cycloheximide for 0-6 hours. Cell extracts were immunoblotted with the indicated antibodies. EGFR protein (B), phospho-AKT(S473) (C) and phospho-MAPK(T202/Y204) (D) levels were quantified by densitometry. The data shown represent the mean of two independent experiments. *, $p < 0.05$.

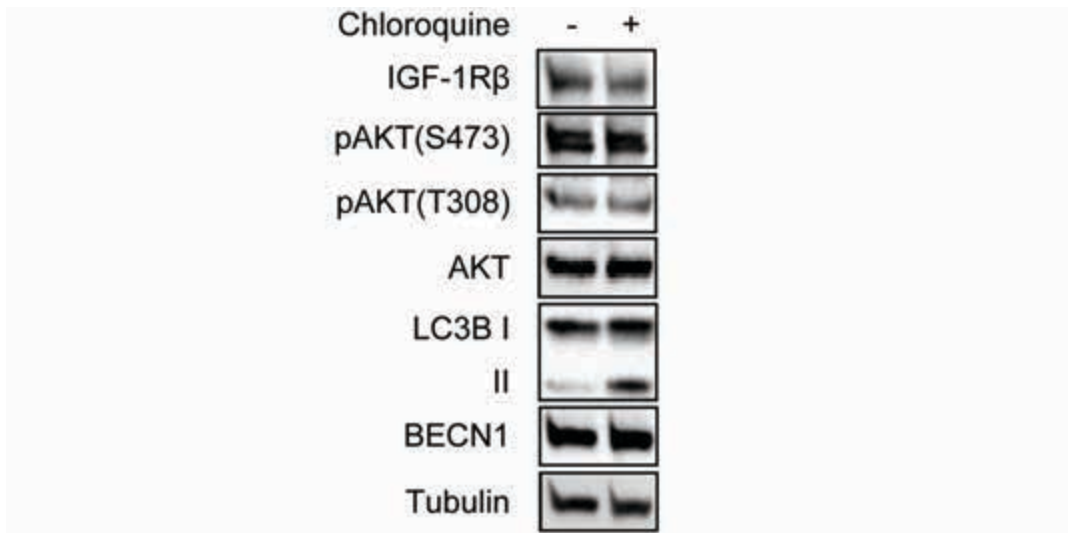


Figure 4.11. Chemical inhibition of autophagy does not enhance AKT activation.

MDA-MB-231 cells were treated with 30 μ M Chloroquine diphosphate for 4 hours. Cell extracts were immunoblotted with the indicated antibodies. Accumulation of the lower migrating, lipidated form of LC3B I, LC3B II, indicates inhibition of autophagy.

the clathrin inhibitor chlorpromazine (157). IGF-1-dependent activation of AKT was markedly diminished, but equivalent, in both the shGFP and shBECN1 cells in the presence of the clathrin inhibitor (Figure 4.12). This result suggested that Beclin 1 regulates growth factor receptor signaling post-internalization by controlling the trafficking of receptors from an early endosomal compartment that retains active signaling capacity (158). Recent studies have revealed that early endosomes, which are positive for the GTPase Rab5, are comprised of functionally distinct subpopulations of vesicles that differ in binding partners and signaling potential (159, 160). One such subpopulation is characterized by the presence of the adaptor protein APPL1, which binds both Rab5 and AKT and can regulate both AKT and MAPK activation (161-163). APPL1 also interacts with growth factor receptors including the EGFR (164) and insulin receptor (165). APPL1-positive (APPL1⁺) endosomes lack the lipid PI3P. When they mature from PI3P negative (PI3P⁻) endosomes to PI3P⁺ endosomes, APPL1 is displaced by FYVE-domain containing proteins, such as EEA1 and rabenosyn5, which bind competitively to both Rab5 and PI3P (160). In support of a role for Beclin 1 in regulating early endosome trafficking, knockout of Beclin 1 (ATG6) in the *Drosophila* fat body interferes with the movement of Rab5-positive vesicles from the plasma membrane to the perinuclear compartment (83).

To determine if suppression of Beclin 1 inhibits the maturation of PI3P⁻/APPL⁺ endosomes, thereby sustaining the activation of AKT and MAPK, shGFP

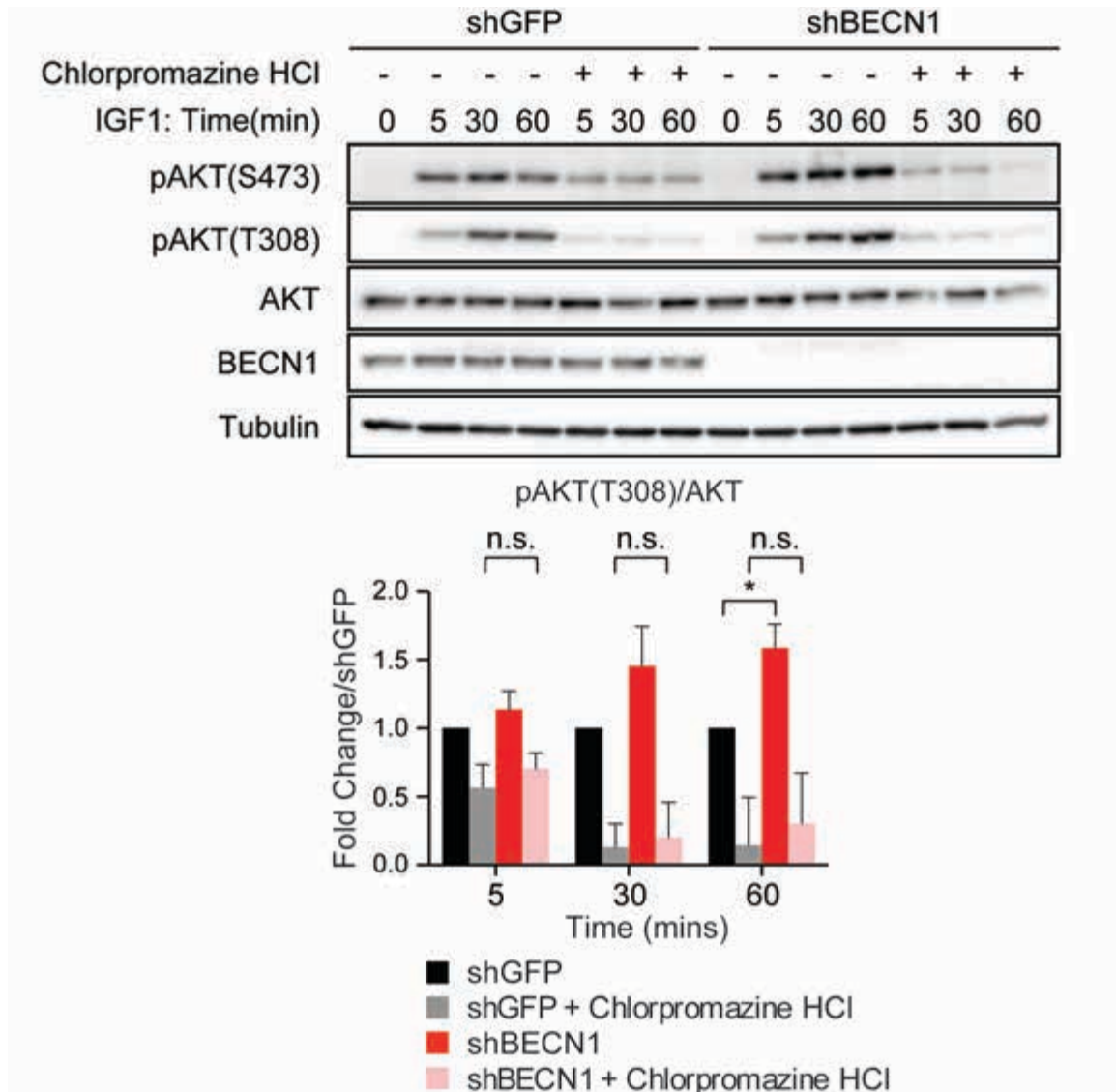


Figure 4.12. Beclin1 regulates growth factor signaling in early endosomes.

MDA-MB-231 cells expressing shGFP or shBECN1 were stimulated with IGF-1 (100ng/ml) for the indicated time periods in the presence or absence of the clathrin inhibitor chlorpromazine (10 μ g/ml). Cells treated with the inhibitor were also pre-treated for 1 hour with Chlorpromazine. Cell extracts were immunoblotted with the indicated antibodies. (B) Phospho-AKT(T308) levels were quantified by densitometry. The data shown represent the mean of four independent experiments. *, $p < 0.05$.

and shBECN1 cells were stained with APPL1-specific antibodies to evaluate the presence of APPL1⁺ endosomes. Images were captured using a confocal microscope and evaluated by counting the number of stained puncta in each cell. APPL1⁺ endosomes were more abundant and persisted over the time course of IGF-1 stimulation in the shBECN1 cells, compared with the shGFP cells (Figure 4.13A and B). In contrast, the number of APPL1⁺ endosomes increased modestly only at the very earliest time point of stimulation in shATG5 cells when compared to control shGFP cells (Figure 4.13A and B). Suppression of ATG5 significantly impaired autophagy in response to serum deprivation, as monitored by LC3 II levels (Figure 3.6A), whereas suppression of BECN1 resulted in only a modest reduction in LC3 II conversion (Figure 3.6B). The different results observed with Beclin 1 and ATG5 loss further supports our previous data and suggests that the regulation of early endosome trafficking involves Beclin 1 functions that are not exclusively related to its role in autophagy. Of note, suppression of APPL1 expression only modestly inhibited IGF-1-dependent signaling to AKT, indicating that it may contribute, but is not required for, activation of this signaling pathway (data not shown).

To investigate the role of Beclin 1 in early endosome receptor trafficking further, I quantified the residency time of growth factor receptors in APPL1⁺ endosomes using fluorophore-conjugated EGF to follow the EGFR in real-time using Total Internal Reflection Fluorescence (TIRF) microscopy (123). EGFR

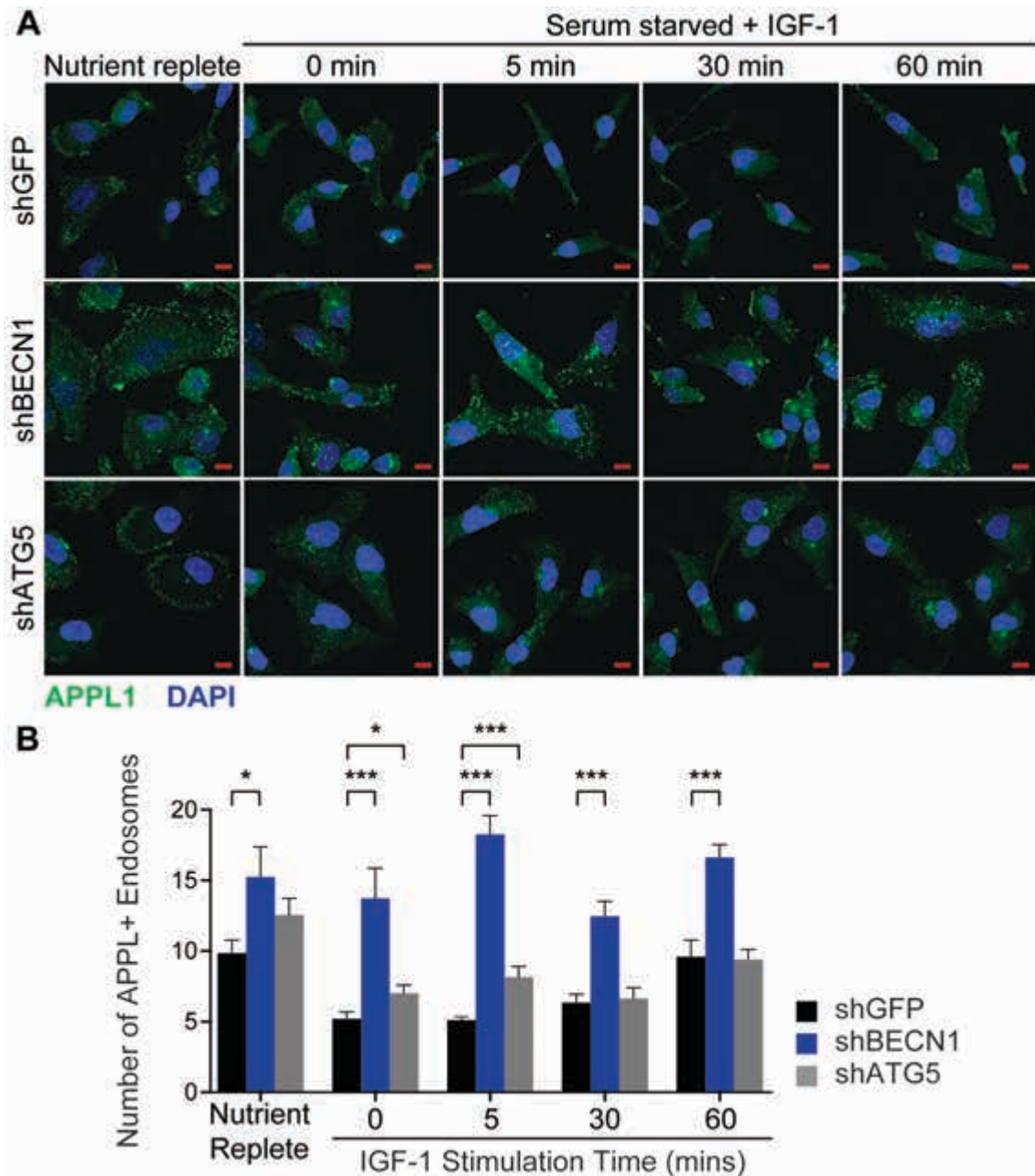


Figure 4.13. Beclin1 regulates APPL1⁺ endosomes.

(A) MDA-MB-231 cells expressing shGFP, shBECN1 or shATG5 were stimulated with IGF-1 (100ng/ml) for the time periods indicated. Cells were fixed and stained with antibodies that recognize APPL1 (green). Nuclei were stained with DAPI (blue). Scale bar, 10 μ m. (B) Quantification of APPL1⁺ endosomes shown in (A). The data shown represent the mean of 23-37 independent cells. *, $p < 0.05$; ***, $p < 0.0005$.

trafficking has been extensively studied using this approach and a corresponding fluorophore-conjugated IGF-1 is not available for these analyses. Control and shBECN1 cells were transfected with TagRFP-T-APPL1 and stimulated with fluorophore-conjugated EGF to track the EGFR. Cells were stimulated for 8 minutes and chased for another 12 minutes (Figure 4.14A). Images captured were processed to remove background and generate masks which were used to quantify the number of EGF and APPL1⁺ vesicles and the co-localization between the two. Only cells with similar APPL1 transfection efficiency and EGF uptake were used for quantification (Figure 4.14C). The extent of EGF and APPL1 co-localization was similar between the control and shBECN1 cells initially, but a significant increase in co-localization occurred over time in the shBECN1 cells and this co-localization persisted at an elevated level throughout the analysis (Figure 4.14B). These results are consistent with Beclin 1 regulating endosome maturation and suggest the possibility that PI3P⁺ endosomes are required for down-regulation of growth factor signaling.

If suppression of Beclin 1 decreases PI3P generation and therefore delays transition of PI3P⁻ endosomes to PI3P⁺ endosomes, a corresponding decrease in receptor co-localization with the latter should occur. To monitor PI3P⁺ endosomes, a GFP-tagged FYVE domain from the endosomal protein SARA (EGFP-FYVE) that binds PI3P was utilized. Generation of PI3P on early endosomes recruits EGFP-FYVE from the cytoplasm allowing quantitation of

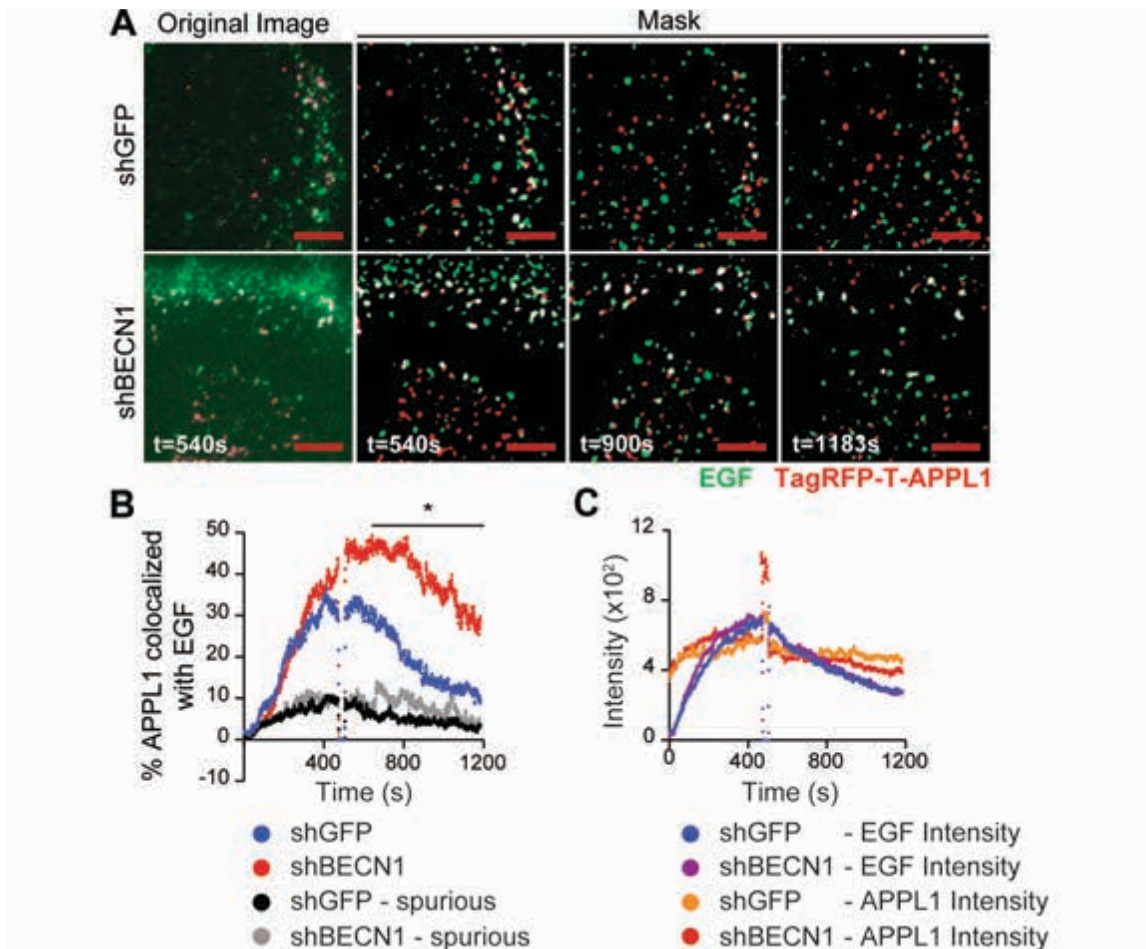


Figure 4.14. Beclin1 regulates growth factor-dependent early endosome maturation.

(A) MDA-MB-231 shGFP or shBECN1 cells expressing TagRFP-T-APPL1 (red) were exposed to Alexa-Fluor-488-EGF (200ng/ml) (green) and imaged using TSM Optical System for ~20 minutes. The far left panels are representative unprocessed images with background removed, while the remaining panels are overlapped masks of images from the individual time points after ligand stimulation. Co-localized signals are represented in white. (B) Percent of APPL1 colocalized with EGF in 100x100 pixels section of each cell (8 sections for shGFP and 7 sections for shBECN1) plotted over the indicated time interval. (C) Average pixel intensity of EGF and APPL1 in cells after ligand stimulation. Spikes represent the wash out of EGF. The data shown represent the mean of two independent experiments performed in duplicate or triplicate. Scale bar, 3 μ m. Spurious, non-specific co-localization. *, $p < 0.05$.

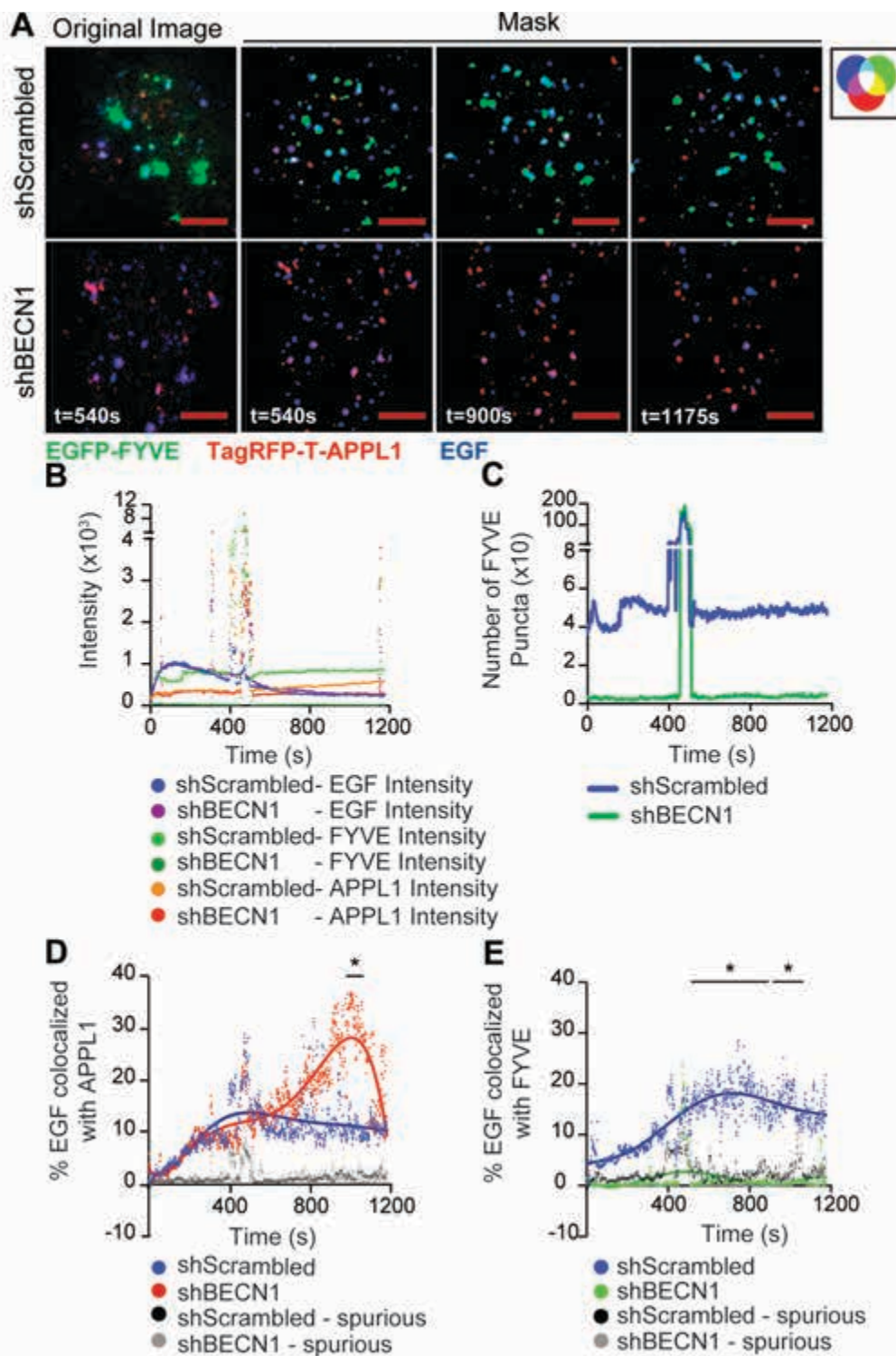


Figure 4.15. Beclin1 regulates growth factor-dependent transition to PI3P⁺ early endosomes.

Figure legend on next page.

Figure 4.15. Beclin1 regulates growth factor-dependent transition to PI3P⁺ early endosomes.

(A) shScrambled and shBECN1 cells expressing EGFP-FYVE (green) and TagRFP-T-APPL1 (red) were exposed to DyLight 650 labelled EGF (200ng/ml) (blue) and imaged using TISM Optical System for ~20 minutes. The far left panels are representative unprocessed images with background removed, while the remaining panels are overlapped masks of images from the individual time points after ligand stimulation. Co-localized signals are represented in colors indicated in the accompanying color wheel. Scale bar, 3 μ m. (B) Average pixel intensity of EGF, FYVE and APPL1 in cells after ligand stimulation. Spikes represent the wash out of EGF. (C) Number of FYVE⁺ puncta present in the TIRF zone at each time point. (D,E) Percent of EGF colocalized with APPL1 (D) and FYVE (E) in each cell (n=6). Masks were used to quantitate colocalization. The data shown represent the mean of three independent experiments (n=6). Spurious, non-specific co-localization. *, p < 0.05.

PI3P⁺ endosomes. EGFP-FYVE was co-transfected with TagRFP-T-APPL1 and the cells were stimulated with fluorophore-conjugated EGF (Figure 4.15A). TIRF images captured were processed to remove background and generate masks to quantify the number of endosomes and co-localization between them. Only cells with similar APPL1 and FYVE transfection efficiencies and EGF uptake were used for quantification (Figure 4.15B). As was observed in the cells expressing TagRFP-T-APPL1 alone, a significant increase in co-localization of EGF with APPL1 occurred over time in the shBECN1 cells (Figure 4.15D). In unstimulated cells, PI3P levels are low and EGFP-FYVE is found diffusely in the cytoplasm. Upon stimulation with EGF, EGFP-FYVE was recruited to endosomes in the TIRF zone in the control cells and their co-localization with EGF increased over time (Figures 4.15C and E). In contrast, EGFP-FYVE remained diffuse in the shBECN1 cells and very few positive endosomes were observed within the TIRF zone (Figures 4.15C and E). Rescue of Beclin 1 expression in the shBECN1 cells with TagRFP-T-BECN1, restored the presence of FYVE⁺ endosomes (Figure 4.16).

As an additional measure of PI3P⁺ endosome maturation, cells transfected with TagRFP-T-APPL1 and stimulated with fluorophore-conjugated EGF were fixed after 25 mins of ligand exposure and stained with antibodies specific for EEA1, a marker of Rab5⁺/PI3P⁺ early endosomes (Figure 4.17A). Once again, a significant increase in the amount of APPL1 that co-localized with

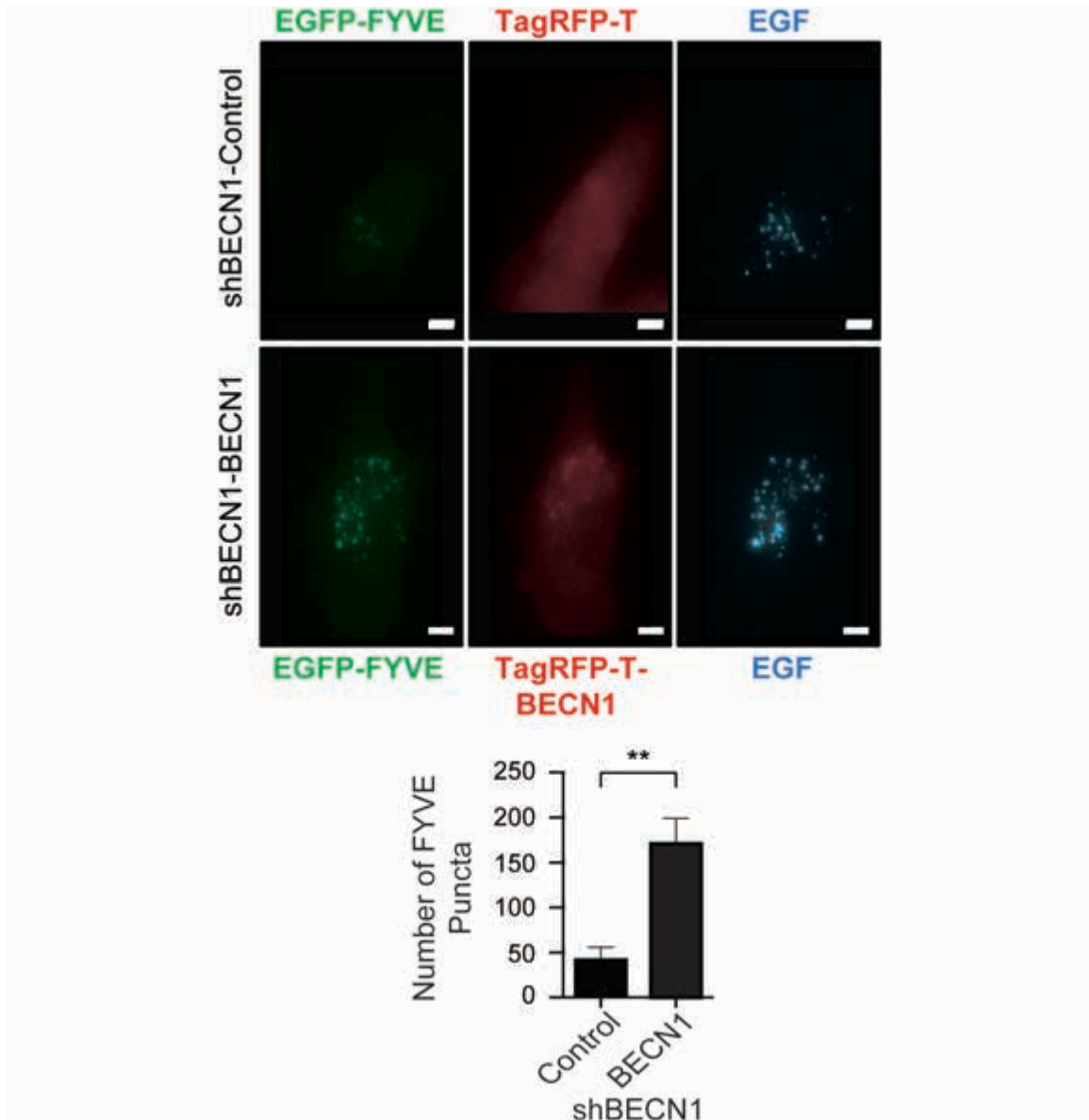


Figure 4.16. Rescue of Beclin 1 expression restores PI3P⁺ endosomes.

shBECN1 cells expressing either TagRFP-T or TagRFP-T-BECN1 (red) and EGFP-FYVE (green) were stimulated with DyLight 650 labelled EGF (200ng/ml) (blue) for 10 mins before fixation. The number of FYVE positive puncta in each cell was quantified. The data shown represents the mean of two experiments (n=11). Scale bar, 3 μ m. **, p < 0.005.

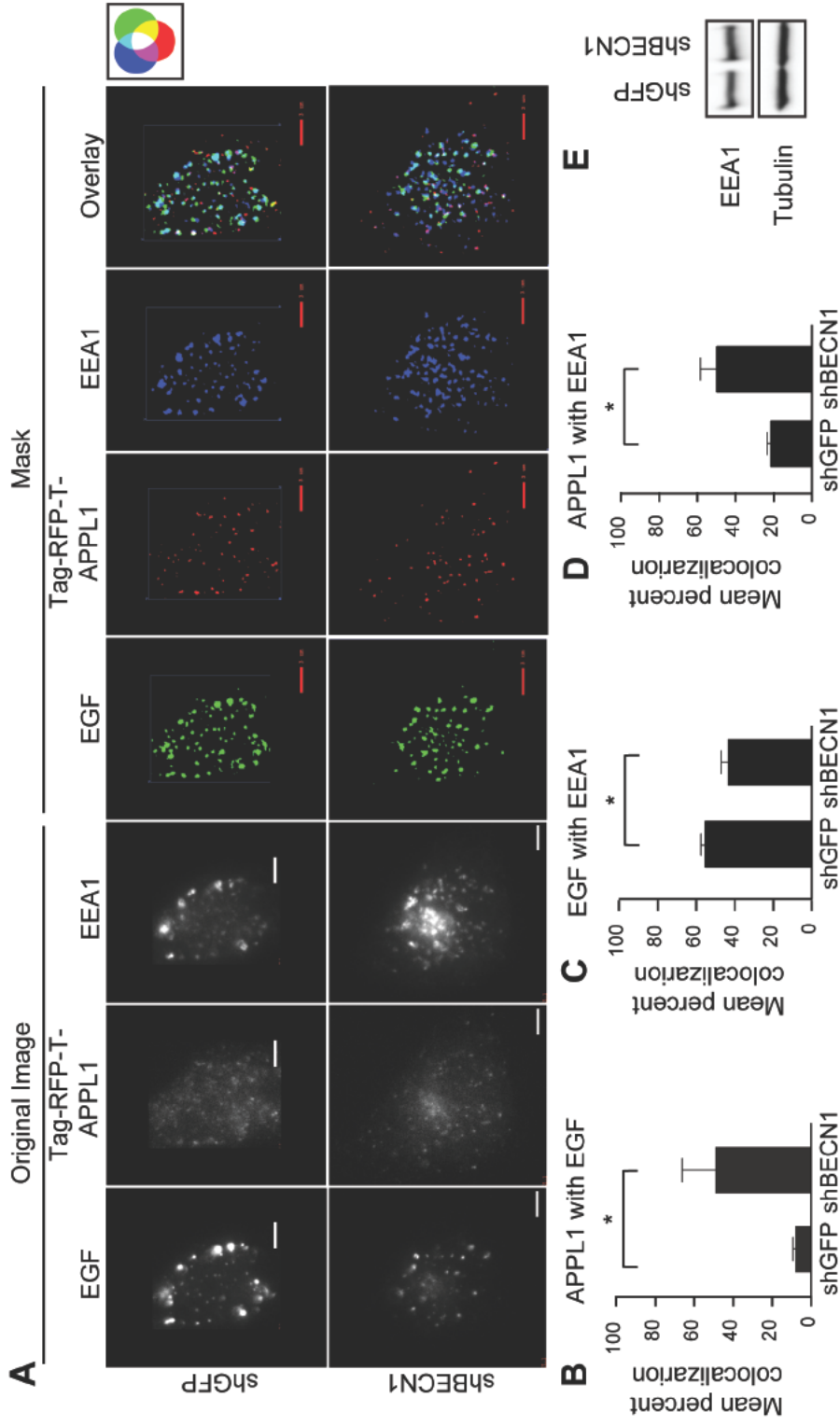


Figure 4.17. Beclin 1 controls the maturation of early endosomes.

Figure legend on next page.

Figure 4.17. Beclin 1 controls the maturation of early endosomes.

(A) Cells expressing TagRFP-T-APPL1 (red) were stimulated with Alexa-Fluor-488-EGF (200ng/ml) (green) for 25 mins, fixed and stained with antibodies that recognize EEA1 (blue). Original images without background noise (left) were processed to generate masks (right). Co-localized signals are represented by colors indicated in the accompanying color wheel. Scale bar, 3 μ m. (B,C,D) Quantification of percent co-localization between fluorophores in six independent cells. Percent of EGF colocalized with APPL1 (B) and EEA1 (C). Percent of APPL1 colocalized EEA1 (D). Masks were used to quantitate colocalization. (E) Immunoblots of cell extracts from (A). *, $p < 0.05$.

EGF was observed at this fixed time point in the shBECN1 cells (Figure 4.17B). A corresponding significant decrease in the co-localization of EGF with EEA1 was also revealed, confirming a delay in the transition of Rab5⁺/PI3P⁻ to Rab5⁺/PI3P⁺ endosomes (Figure 4.17C). Total EEA1 levels were equivalent in the shGFP and shBECN1 cells (Figure 4.17E). An increase in the co-localization of APPL and EEA1 was also detected upon Beclin 1 depletion (Figure 4.17D). This increase in hybrid endosomes that have not fully matured provides additional evidence for a role for Beclin 1 in the efficient transition of this endosomal population.

The increase in EGFP-FYVE⁺ endosomes in response to EGF stimulation and the absence of these endosomes in the shBECN1 cells indicate that Beclin 1 is required for the generation of PI3P in response to growth factor stimulation. To examine directly this role for Beclin 1 in the growth factor-dependent regulation of PI3P generation, I measured total cellular PI3P lipids in response to growth factor stimulation using a competitive ELISA assay. Stimulation of shGFP cells with either IGF-1 or EGF increased PI3P levels significantly (Figures 4.18A and B). Importantly, PI3P levels did not increase upon stimulation of the shBECN1 cells with either IGF-1 or EGF, which is consistent with the absence of EGFP-FYVE endosomes in the imaging experiments. Combined, my data support the hypothesis that Beclin 1 regulates PI3KC3 activation in response to growth factor stimulation and contributes to

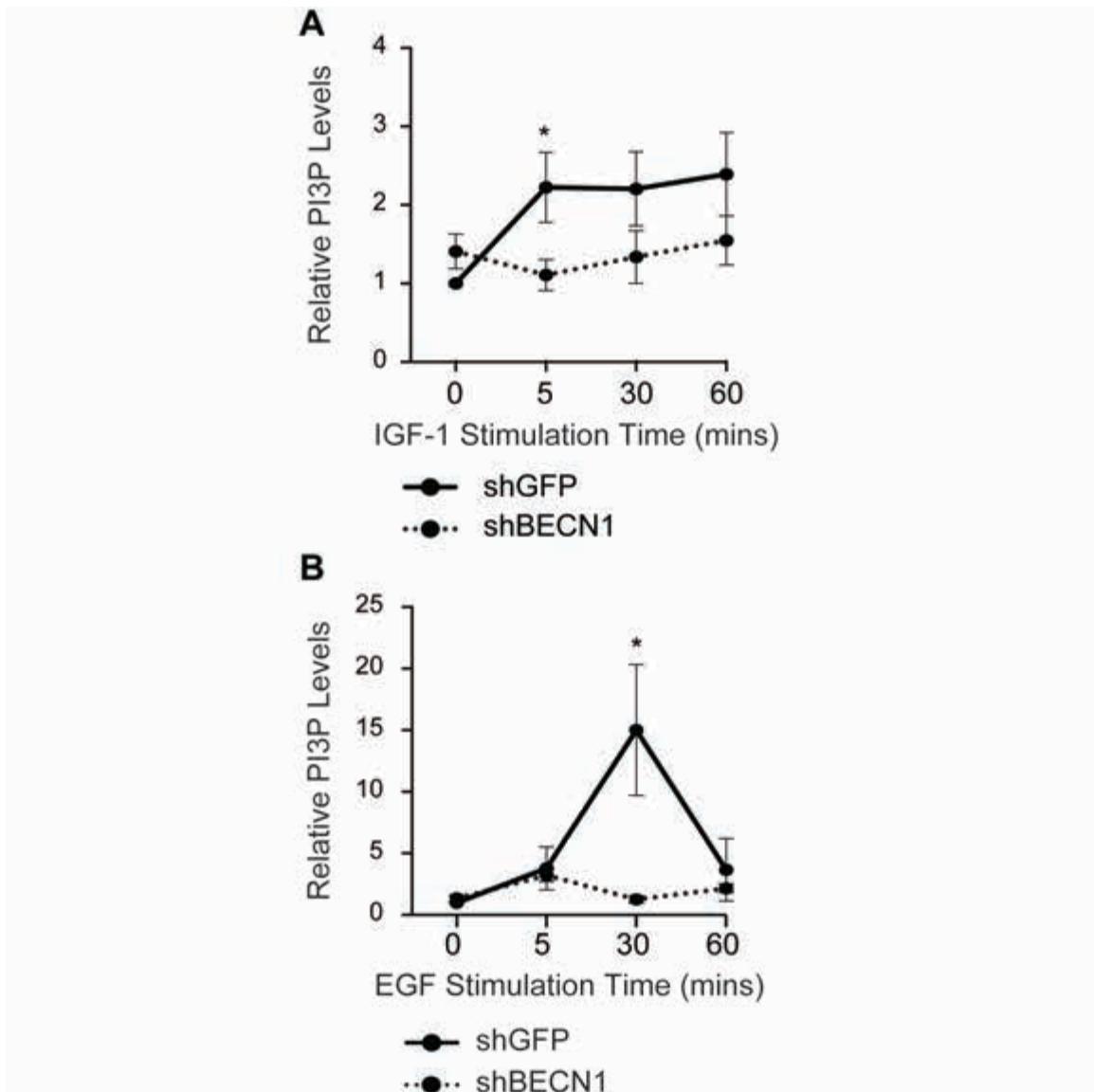


Figure 4.18. Beclin 1 controls PI3P levels.

(A,B) MDA-MB-231 cells expressing either shGFP or shBECN1 were serum-starved overnight and then stimulated with IGF-1 (100ng/ml) (A) or EGF (50ng/ml) (B) for the indicated time periods. Total PI3P lipids were extracted and the levels were measured by ELISA assay. The data shown represents the mean of two (EGF; n=4) or three (IGF-1; n=9) independent experiments. *, $p < 0.05$.

the dynamic changes in PI3P levels that accompany endosome maturation.

Beclin 1 negatively regulates invasion in breast carcinoma cell lines

AKT regulates migration and invasion in many tumor cell types, including breast carcinoma cells (166, 167). Therefore, I investigated whether loss of Beclin 1, and the resulting sustained activation of AKT, promotes migration or invasion. Suppression of Beclin 1 expression increased the invasion, but not migration, of MDA-MB-231 (Figure 4.19A) and SUM159 (Figure 4.19B) cells by two-four fold compared to control cells (shGFP) and restoration of Beclin 1 expression in the shBECN1 cells restored invasion to control levels (Figure 4.20). Importantly, inhibition of AKT activity using the inhibitor MK2206 suppressed the increased invasion of the MDA-MB-231 shBECN1 cells (Figure 4.21). The lack of an increase in migration in the shBECN1 cells suggests that the sustained AKT signaling that occurs upon loss of Beclin 1 selectively regulates pathways important for invasion, such as protease expression, and does not control general cell movement.

Beclin 1 inversely correlates with phosphorylated AKT and ERK in human tumors

To assess the impact of Beclin 1 on AKT and ERK activation in human breast tumors, we used cBioPortal to analyze the correlation between Beclin 1 expression and AKT and ERK phosphorylation in a cohort of breast cancer

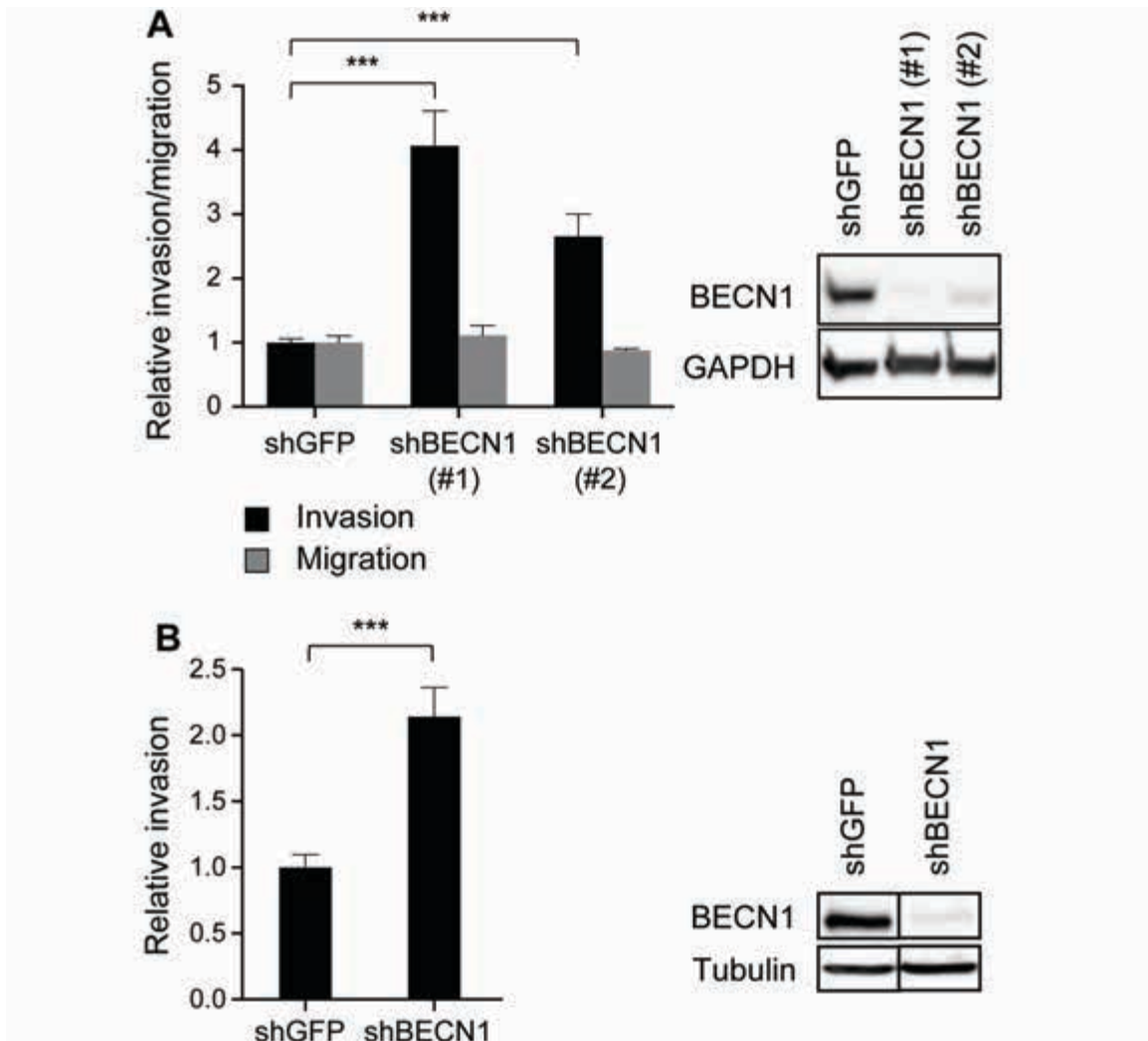


Figure 4.19. Beclin 1 regulates breast carcinoma cell invasion.

(A) MDA-MB-231 cells expressing shGFP, shBECN1 (#1) or shBECN1 (#2) were assayed for their ability to migrate (collagen 1) or invade (Matrigel). The data shown represent the mean of three (invasion; $n = 7$) and two (migration; $n = 5$) independent experiments performed in duplicate or triplicate. Inset, immunoblots showing Beclin 1 expression in the shBECN1 cells. (B) SUM159 cells expressing shGFP or shBECN1 were assayed for their ability to invade Matrigel. The data shown represent the mean of three independent experiments performed in triplicate ($n = 9$). Right, immunoblots showing Beclin 1 expression in the shBECN1 cells. Images are from the same immunoblot that was cropped to remove lanes. ***, $p < 0.0005$.

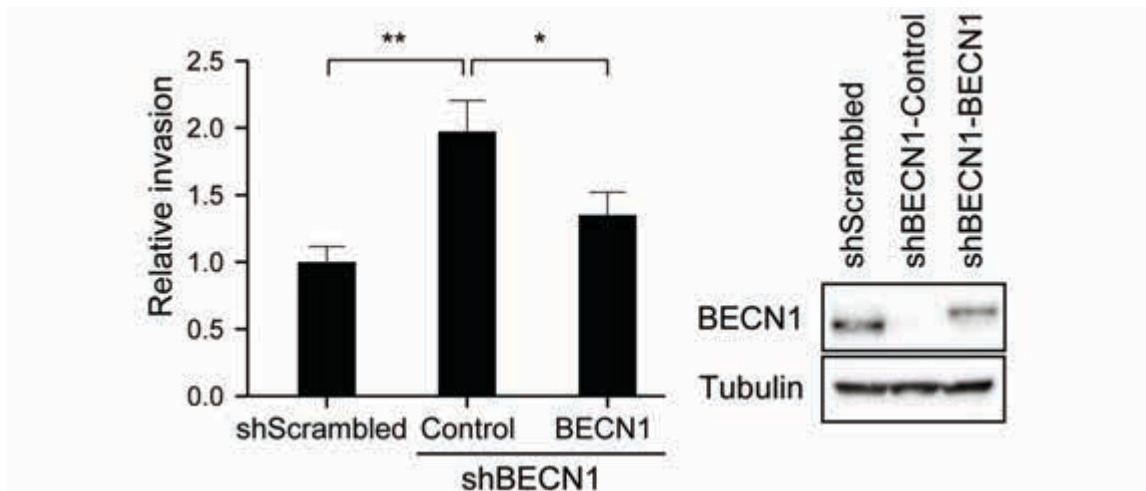


Figure 4.20. Rescue of Beclin 1 expression decreases enhanced invasion in Beclin 1-depleted cells.

MDA-MB-231 cells expressing shBECN1 or shBECN1 with restored FLAG-Beclin 1 expression were assayed for their ability to invade Matrigel. The data shown represent the mean of three independent experiments performed in triplicate. Inset, immunoblots showing Beclin 1 expression. *, $p < 0.05$; **, $p < 0.005$.

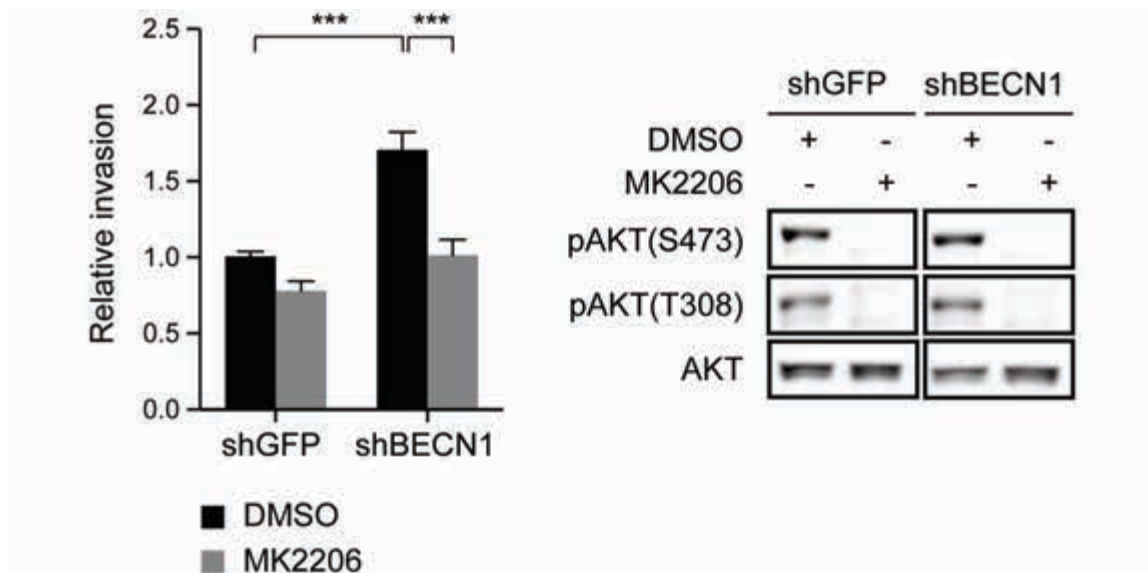


Figure 4.21. Inhibition of AKT signaling suppressed increased invasion in Beclin 1-deficient cells.

MDA-MB-231 cells were assayed for their ability to invade Matrigel after treatment with the AKT inhibitor MK2206 (0.5 μ M). The data shown represent the mean of four independent experiments performed in duplicate or triplicate ($n = 10$). Inset, immunoblots showing phospho-AKT(S473) and (T308) upon treatment with 0.5 μ M MK2206 for 1 hour. ***, $p < 0.0005$.

patients that had altered levels of Beclin 1 protein expression as measured by reverse phase protein array (RPPA) (168, 169). A statistically significant negative correlation between Beclin 1 and pAKT (S473; Pearson $r = -0.3612$) and pERK (T202; Pearson $r = -0.3904$) was observed (Figure 4.22).

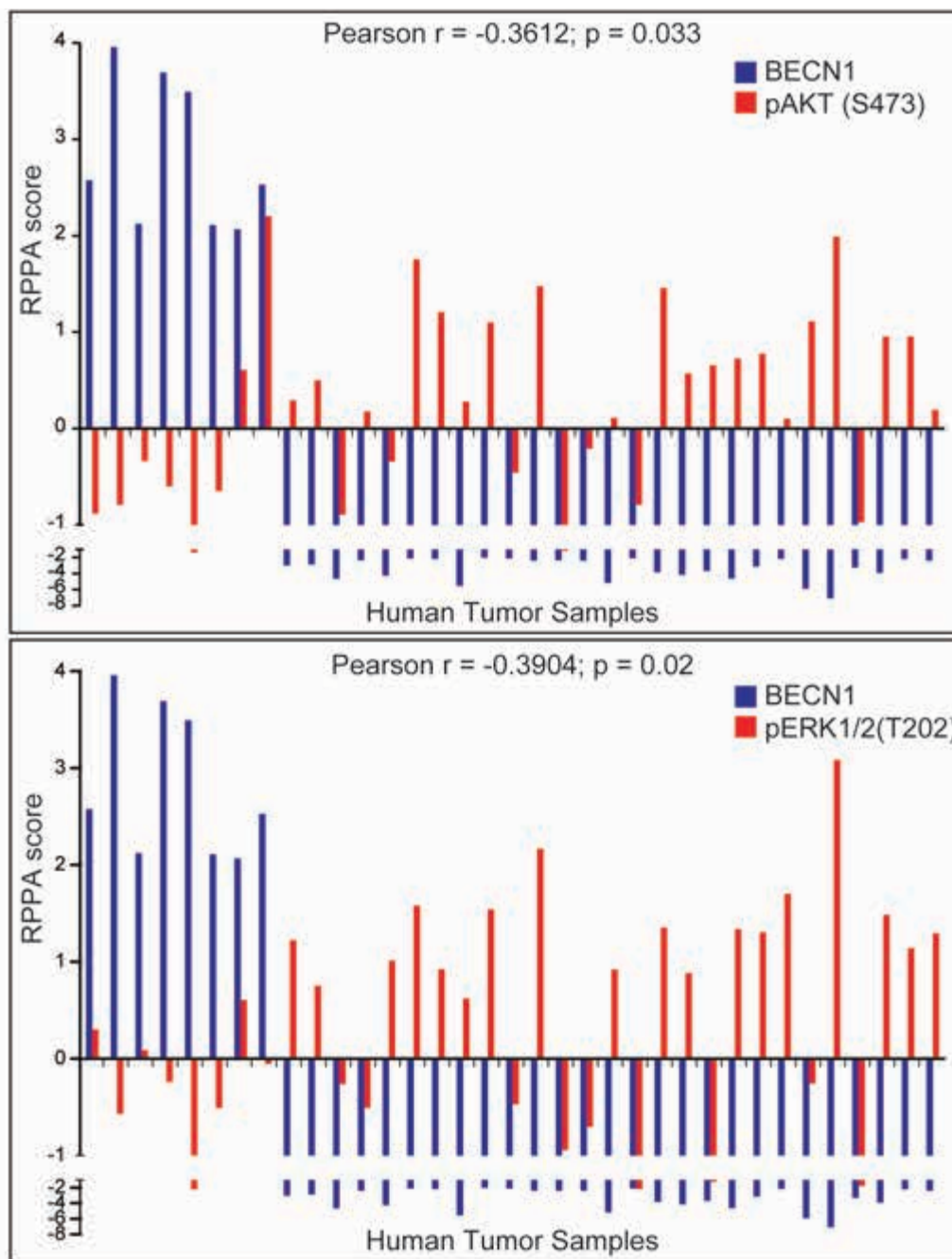


Figure 4.22. Inverse correlation of Beclin 1 expression and AKT and ERK phosphorylation in human breast tumors.

AKT and ERK phosphorylation were analyzed in a cohort of breast cancer patients that had altered levels of Beclin 1 protein expression using cBioPortal.

Discussion

In this study, I describe a novel mechanism of action for Beclin 1 in breast cancer involving its control of growth factor receptor signaling. Beclin 1 regulates PI3P lipid levels in response to growth factor stimulation to control the rate at which growth factor receptors transit through a signaling-competent early endosome compartment. In doing so, Beclin 1 controls the intensity and duration of growth factor stimulated AKT and ERK signaling. I demonstrate that suppression of *BECN1* sustains growth factor stimulated AKT and ERK activation in breast carcinoma cells *in vitro*, and that an inverse correlation between Beclin 1 expression and AKT and ERK phosphorylation is also observed in human breast tumors. Functionally, reduction of Beclin 1 expression enhances invasion in an AKT-dependent manner. Taken together, my data reveal a mechanism by which dysregulation of Beclin 1 expression or function contributes to malignant behavior.

Beclin 1 is an independent prognostic factor for overall survival (OS) and distant metastasis-free survival in breast cancer, with low levels of Beclin 1 expression predicting worse OS (97). Low Beclin 1 expression together with active WNT signaling also correlates with poor prognosis in breast cancer (146). However, the mechanism to explain how loss of Beclin 1 expression would contribute to aggressive tumor behavior remains unresolved. Autophagy, which is thought to support tumor progression by facilitating the survival of tumor cells

in stressful microenvironments that may lack sufficient oxygen and nutrients, is inhibited when Beclin 1 expression is decreased. My study provides evidence for an alternative mechanism of Beclin 1 action to explain how its loss promotes tumor progression. Specifically, we show that Beclin 1 loss enhances oncogenic signaling pathways such as AKT and MAPK that promote survival and invasion. In this direction, an autophagy-independent role for Beclin 1 loss has been suggested to contribute to the enhanced mammary stem and progenitor cell activity and tumorigenesis that is observed in *Wnt1/Becn1^{+/-}* mice (146). Taken together with the recent *Drosophila* study showing that Beclin 1 regulates Notch trafficking, these findings support the possibility that multiple receptor trafficking pathways may be impacted by Beclin 1 loss and that this combined dysregulation culminates in enhanced signaling to promote tumor progression (170).

I establish an important role for Beclin 1 in the regulation of early endosome maturation. Beclin 1 is required for the growth factor-stimulated generation of PI3P by PI3KC3, which facilitates the recruitment of FYVE domain containing proteins, such as EEA1, that promote endosome fusion and maturation (123, 171). Another FYVE-domain protein, Fab1/PIKfyve (FYVE-containing phosphatidylinositol 3-phosphate 5-kinase), is recruited to Rab5⁺/PI3P⁺ endosomes where it is activated in response to phosphorylation by AKT(172). PIKfyve phosphorylates PI3P to generate PI-3,5-P and promote endosome progression to multivesicular bodies where signaling is terminated. Reduced PI3P production in the absence of Beclin 1 would be expected to limit

the recruitment of PIKfyve and therefore inhibit maturation. Finally, Beclin 1 recruits UVRAG, which enhances PI3KC3 activation and PI3P production (16, 113). UVRAG also promotes early endosome maturation by activating C-VPS/HOPS to facilitate Rab7 recruitment and transition to the late endosome, a function that would also be lost in the absence of Beclin 1 (18).

My studies model the reduction of Beclin 1 expression that occurs in many tumors (97, 98). Other tumors, however, may exhibit a decrease in Beclin 1 function as a consequence of post-translational modifications, and this disruption would also be expected to impact growth factor receptor trafficking and signaling. For example, activated EGFR binds to Beclin 1 and promotes its phosphorylation on multiple tyrosine residues (32). These phosphorylation events disrupt the interaction of Beclin 1 with PI3KC3 and decrease its associated lipid kinase activity. Additionally, Beclin 1 is phosphorylated on S234 and S295 by AKT, which also disrupts PI3KC3 binding (31). These pathways likely function to regulate the rate of receptor trafficking and duration of signaling in normal cells, but when hyperactivated in tumors could further enhance their oncogenic signaling potential. In support of an involvement of this post-translational mechanism of regulation in cancer, expression of a Beclin 1 tyrosine phosphomimetic in NSCLC cells increased proliferation and stimulated enhanced tumor growth (32). Similarly, expression of a Beclin 1 mutant that is resistant to AKT phosphorylation inhibited Myr-AKT1-dependent tumorigenesis (31). Although these effects of the Beclin 1 phospho-mutants on tumor growth were

attributed to the impact of Beclin 1 on autophagy, dysregulated growth factor receptor signaling would also contribute to these phenotypes.

My study differs from previous work that has focused exclusively on the effect of Beclin 1 loss on autophagy and the contribution of this degradative pathway to cancer. I reveal an alternative mechanism to account for how Beclin 1 loss may impact tumor progression that involves enhancing the magnitude and duration of signals propagated through growth factor receptors, many of which have been identified as oncogenic drivers. Reduction of Beclin 1 expression in tumors would result in dysregulation of both growth factor receptor signaling and autophagy, and these pathways may work in concert to promote tumor progression.

Acknowledgements

This work was supported by National Institute of Health (NIH) grants CA142782 and CA177167 (LMS), DK60564 (SC) and CA159314 (EHB). We thank Lawrence M. Lifshitz and Clive Standley of the UMass Biomedical Imaging Group for their contributions to the programs used to analyze the TESM data. We thank Sha Zhu for assistance with cloning, Usha Acharya for assistance with the PI3P lipid measurements and Chung-Cheng Hsieh for statistical advice.

CHAPTER V

DISCUSSION

Summary

Autophagy is a complex process under the regulation of multiple signaling pathways that can either stimulate or repress the process. Dysregulation of some of these pathways is found to occur in a number of cancer types. Moreover, the autophagic process itself has also been shown to both inhibit and promote tumorigenesis. My study was designed to investigate the role of autophagy and one of its important regulators, Beclin 1, in breast cancer progression. Work presented in this thesis as well as preliminary data implicate a dependency of triple-negative breast cancer (TNBC) cell lines on autophagy. These cells exhibit higher levels of basal and induced autophagy when compared with luminal breast carcinoma cells. Moreover, both chemical and genetic inhibition of autophagy decreased the tumorigenicity of a TNBC cell line as measured by anchorage-independent growth. These findings are consistent with a recently published study that reported an increased sensitivity of TNBC cell lines to autophagy inhibition when compared with luminal and non-tumorigenic cell lines (129). Taken together, my results and the above-mentioned study implicate that TNBC tumors may potentially benefit from autophagy-targeted chemotherapy.

Interestingly, my study revealed different effects on glycolysis upon inhibition of two independent autophagic regulators – ATG5 and Beclin 1. Whereas suppression of ATG5 decreased glycolytic capacity, silencing Beclin 1 did not affect aerobic glycolysis. This result indicated a possible involvement of

alternative functions of Beclin 1 that counteract the loss of autophagy. The ATG5-independent, and hence presumably autophagy-independent, roles of Beclin 1 were further explored in the second part of my thesis.

Non-autophagy functions of Beclin 1 have been described in lower organisms and are now being investigated in mammals. However, the tumor suppressive function of Beclin 1 has been ascribed mostly to its role in autophagy. In my study I focused on contributions of alternative functions of this protein and uncovered a novel role for Beclin 1 in regulating growth factor-dependent signaling. Detailed analysis of the mechanism behind this regulation revealed a role for Beclin 1 in the maturation of early endosomes. As illustrated in Figure 5.1, I demonstrated that suppression of Beclin 1 expression inhibits PI3P production on early endosomes, which is necessary for maturation of these signaling-competent vesicles. As a result, termination of growth factor-dependent signaling is delayed. One of the consequences of the enhanced signaling that occurs upon Beclin 1 loss is increased breast carcinoma cell invasion.

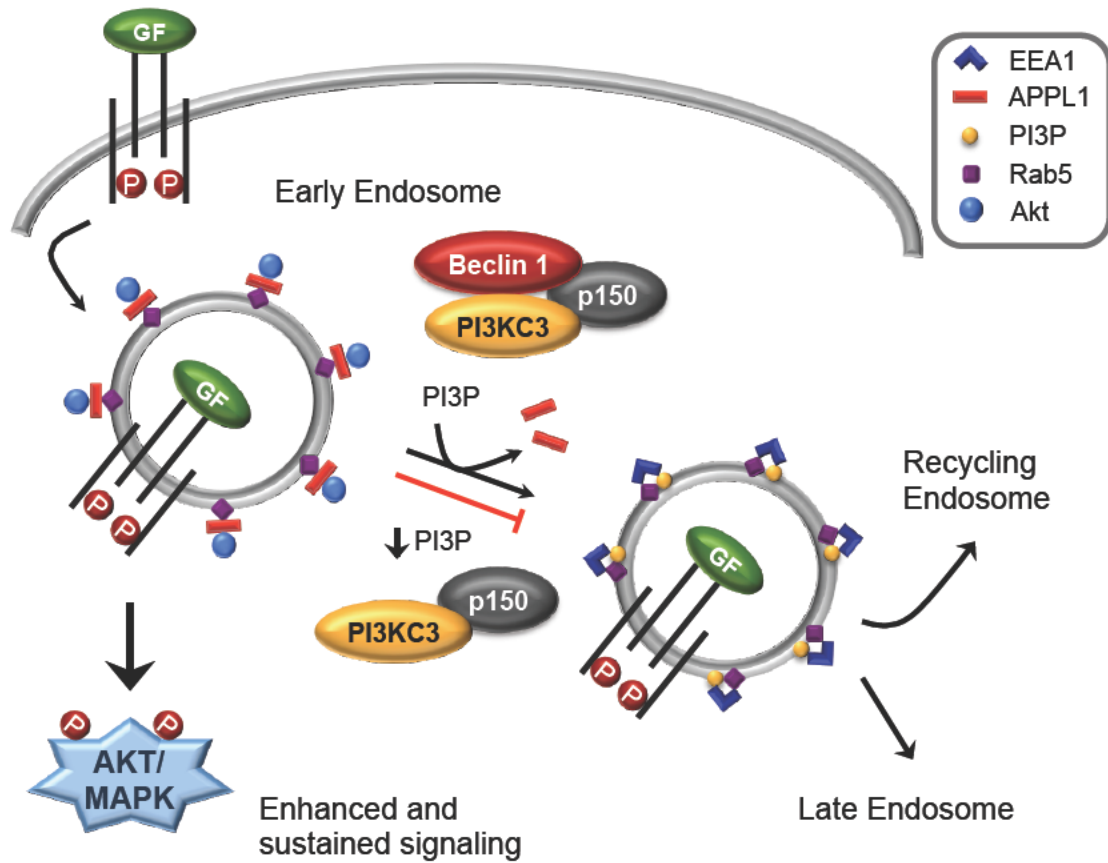


Figure 5.1. Model of Beclin 1's regulation of growth factor receptor signaling.

Beclin 1 activates PI3KC3 to generate the phospholipid PI3P. PI3P is required for maturation of Rab5⁺/PI3P⁻ early endosomes to Rab5⁺/PI3P⁺ endosomes. Loss of Beclin 1 decreases PI3P synthesis resulting in accumulation of signaling competent PI3P⁻ endosomes and hence enhanced signaling. GF, Growth Factor

Impact of Beclin 1 loss in cancer

Several studies have shown that autophagy facilitates tumor progression by helping tumor cells survive in their stressful microenvironment (126). Inhibition of key regulators of this degradative pathway, other than Beclin 1, reduce proliferation, anchorage-independent growth and glycolysis (135). Moreover, autophagy has also been shown to promote invasion in Ras-driven tumors by production and secretion of pro-migratory factors such as interleukin-6 (IL-6) (173). Suppression of autophagy genes decreased the invasive capacity of tumor cells, which could be rescued by treatment with conditioned media from autophagy-competent cells. Knockdown of ATG genes also reduced cell migration *in vitro* and pulmonary metastasis *in vivo* (173). The tumor-promoting function of autophagy is further supported by the study performed by Takamura *et. al.* who reported that systemic mosaic deletion of *Atg5* and liver-specific deletion of *Atg7* in mice results in development of benign adenomas only in the liver. These adenomas do not progress to adenocarcinomas indicating a requirement of autophagy for tumor progression (108). This result is in contrast to heterozygous deletion of *Becn1* in mice, which results in an increased rate of spontaneous malignancies (94). Decreased expression of Beclin 1 in multiple human tumors further supports the tumor suppressive role of Beclin 1, unlike other autophagy regulators. The above-mentioned contradictory studies suggest the possible contribution of additional non-autophagy functions of Beclin 1 to cancer.

In support of an autophagy-independent role for Beclin 1 in tumor suppression it has been proposed that since the anti-apoptotic protein Bcl-2 binds to Beclin 1, loss of the latter can promote cell survival and hence tumorigenesis. Interestingly, Beclin 1 as part of the PI3KC3 complex can also control the levels of the tumor suppressor p53 by regulating the stability and activity of the p53 deubiquitinating enzymes, USP10 and USP13. As a result, reduction in Beclin 1 expression results in reduced levels of p53. These findings indicate a potential mechanism that can promote the genomic instability that is observed upon loss of Beclin 1 (174). Since Beclin 1 has also been reported to be involved in regulating cytokinesis, loss of this protein may also directly result in genomic instability causing aneuploidy (19). My studies demonstrate an additional function of Beclin 1 that could contribute to tumor suppression, thus underscoring the importance of the long neglected autophagy-independent role of the protein. By regulating signals propagated through growth factor receptors, many of which have been identified as oncogenic drivers, Beclin 1 can exert its tumor suppressive function.

My data reveal that one of the functional outcomes of enhanced signaling in Beclin 1-deficient cells is an increase in invasive potential, a characteristic required for tumor cells to metastasize. In addition, Beclin 1-depleted cells are also able to maintain high glycolytic rates, unlike ATG5-depleted cells. Since activated AKT and MAPK are involved in cell growth, proliferation, survival and metabolism, hyper-activation of growth factor receptor-mediated signaling in

Beclin 1-depleted cells could be responsible for restoring glycolytic rates in these tumor cells. One of the mechanisms by which PI3K/AKT signaling controls glycolysis is by regulating localization of GLUT1 to the cell surface (122, 145). Enhanced PI3K/AKT signaling increases glycolysis that results in generation of lactic acid, a byproduct of glycolysis. Lactic acid can, in turn, facilitate tumor cell invasion by promoting the degradation of the extracellular matrix. Furthermore, suppression of *Glut1* expression in mouse mammary tumor cells has been reported to inhibit both glucose uptake and tumor cell invasion (122). These facts indicate that the elevated PI3K/AKT signaling in Beclin 1-depleted cells could result in increased GLUT1 expression on the cell surface, thereby promoting aerobic glycolysis and cancer cell invasion. This hypothesis can be further investigated by comparing aerobic glycolysis and invasion of shATG5 and shBECN1 cells upon treatment with AKT inhibitors as well as by silencing GLUT1 expression. My observations also implicate that the requirement of autophagy for maintaining the metabolic fitness of tumor cells could be counter-balanced by enhanced signaling in Beclin 1-depleted cells.

Beclin 1 deletion in cancer is monoallelic, indicating the possibility that low levels of autophagy still occur. My studies show that suppression of Beclin 1 enhances receptor-mediated signaling. Based on these facts it is possible that decreased Beclin 1 expression in tumors promotes progression and metastasis due to enhanced oncogenic signaling while maintaining low levels of autophagy to sustain cancer cells in the nutrient-depleted tumor microenvironment. To

further understand the interplay between the two functions of Beclin 1 - autophagy and modulation of receptor-mediated signaling in tumorigenesis - *in vivo* investigation is warranted. The effect of decreased expression rather than complete loss of Beclin 1 on tumor formation and metastasis should be studied. Mice with conditional, inducible, monoallelic deletion of *Becn1* in the mammary gland could be used for this analysis. These mice would allow for the investigation of tumor formation and progression upon heterozygous loss of *Becn1* in a specific tissue at a specific age, unlike previous studies that were performed with *Becn1*^{+/-} mice. Activation of AKT and MAPK as well as the presence of autophagosomes can be analyzed in the tumors to monitor autophagy and signaling. Furthermore, the contribution of the tumor microenvironment in supporting the growth and metastatic ability of Beclin 1-depleted cells should be assessed. Cancer-associated fibroblasts (CAFs) can undergo autophagy induced by the stressful tumor microenvironment resulting in the secretion of metabolites that can be taken up by Beclin 1-depleted cancer cells (175-177). Thus, the tumor microenvironment may counter-balance the inhibitory effects of decreased availability of intermediates for metabolic pathways and macromolecular biosynthesis by metabolically coupling with Beclin1-deficient cells.

Beclin 1 in human tumors

The tumor suppressive function of Beclin 1 has recently been challenged by a study that investigated the mutational status of *BECN1* in human tumor sequencing data obtained from The Cancer Genome Atlas (TCGA). This study found that *BECN1* was deleted only in conjunction with its proximal breast and ovarian tumor suppressor gene *BRCA1* in breast and ovarian cancers. Moreover, *BECN1* was not found to be mutated or focally deleted in other cancers (95). However, aberrant protein expression has been reported in several human tumors (96-98). Analysis of 378 ER⁺, HER2⁻ breast cancer patients revealed that patients with low Beclin 1 expression displayed decreased overall survival and distant metastasis-free survival implicating Beclin 1 as a negative prognostic factor (97). Moreover, comparison of 20 pairs of sporadic breast tumors with adjacent normal tissues revealed aberrant methylation of the promoter and intron 2 of *BECN1* in tumors expressing low levels of the protein. These data suggest that Beclin 1 expression can also be reduced by epigenetic regulation of the gene (96). Furthermore, overexpression of Beclin 1 in MCF7 cells reduces proliferation *in vitro* and tumorigenesis in nude mice (4). Above all, it is well established that heterozygous loss of *Becn1* in mice leads to spontaneous tumor formation (35, 94). Based on these facts, I would argue that Beclin 1 should still be considered a haploinsufficient tumor suppressor.

I demonstrated that loss of Beclin 1 can enhance growth factor-receptor signaling and promote invasion. Elevated AKT and MAPK signaling can activate the autophagy inhibitor mTORC1, thereby inhibiting autophagy. However, cancer cells require autophagy, especially in the poorly perfused interior sections of the tumor. Thus, it is possible that tumor cells could epigenetically silence *BECN1* to promote invasion and metastasis. Once at the secondary site, Beclin 1 expression could be restored, owing to the plasticity of epigenetic alterations, to promote growth of the metastatic lesion. In support of this hypothesis, my analysis of hypermethylation of *BECN1* in the basal MDA-MB-231, luminal MCF7 and immortalized normal mammary epithelial cell line MCF10A revealed that *BECN1* is epigenetically suppressed in the metastatic MDA-MB-231 cells but not in the non-metastatic MCF7 and MCF10A cells (Appendix Figure 1). Further *in vivo* studies are required to determine if suppression of Beclin 1 is essential to support invasion and metastasis of this autophagy-dependent cell line.

Analysis of human breast tumor data from cBioPortal for Cancer Genomics revealed a negative correlation between Beclin 1 expression and AKT and ERK phosphorylation in a cohort of breast cancer patients that had altered levels of Beclin 1 protein expression. My studies demonstrate that Beclin is involved in spatio-temporal regulation of receptor-mediated signaling. Through *in vitro* studies I have shown that suppression of Beclin 1 leads to increased AKT and MAPK activation. My findings provide a potential explanation for the negative correlation between Beclin 1 expression and AKT and ERK activation in breast

cancer. To confirm this, activation of AKT and MAPK pathways should be evaluated in tumors formed in mice upon depletion of Beclin 1 protein levels. Moreover to confirm the cause (low Beclin 1 expression) and effect (increased signaling) relationship, protein expression of Beclin 1 could be monitored upon treatment with AKT and/or MAPK inhibitors. Based on my results, loss of Beclin 1 enhances AKT and MAPK signaling. Hence, the potential use of inhibitors of these pathways to treat Beclin 1-deficient tumors should also be explored.

Regulation of Beclin 1 function by signaling pathway components

Signaling pathways that are frequently overexpressed/hyperactivated have been reported to regulate the function of Beclin 1 in autophagy. Multisite tyrosine phosphorylation of Beclin 1 by activated EGFR reduces PI3KC3 activity and autophagy. Interaction of activated EGFR with Beclin 1 increases its binding with the negative regulators Bcl-2 and Rubicon, and decreases PI3KC3 association with Beclin 1. Moreover, Beclin 1 tyrosine phosphomimetic mutant expression enhanced tumor growth and dedifferentiation of non-small-cell lung carcinoma xenografts (32). Similarly AKT-mediated phosphorylation of Beclin 1 inhibits autophagy. Additionally, expression of a non-phosphorylatable mutant of Beclin 1 suppressed AKT-mediated tumorigenesis and anchorage-independent growth and increased autophagy (31). In both of these reports, increased tumorigenesis was attributed to inhibition of autophagy. However, my findings also indicate a possible role for enhanced endosomal signaling in increased

tumorigenesis. Phosphorylation of Beclin 1 by aberrant activation of EGFR and AKT would lead to decreased PI3KC3 activity and hence delayed endosomal maturation. This would further enhance oncogenic signaling from signalosomes (signaling endosomes). Taken together, these data indicate that not only reduced expression of Beclin 1, but also its post-translational modification leading to decreased Beclin 1 function, could promote tumor development and progression. This hypothesis can be tested by analyzing the number of Rab5⁺/PI3P⁻ early endosomes (using markers such as APPL1) in human tumors with activated EGFR/AKT and phosphorylated Beclin 1 and comparing these tumors with Beclin 1 deficient tumors and normal tissue. *In vitro* expression of Beclin 1 phosphomimetics and their effect on signaling should also be explored. Based on my data, expression of Beclin 1 phosphomimetics would also enhance growth factor receptor signaling, similar to that seen upon suppression of Beclin 1 expression.

Role of Beclin 1 complexes in oncogenic signaling

The work presented in this dissertation provides new insight into the molecular mechanism by which Beclin 1 could function as a tumor suppressor. These findings give rise to additional questions. Future studies investigating the role of ATG14L and UVRAG on growth factor-dependent signaling should be performed to define the contribution of both complex I and II in tumorigenesis and metastasis. UVRAG, a component of complex II, enhances PI3KC3 activation

and PI3P production (16, 113). It also promotes early endosome maturation by activating C-VPS/HOPS to facilitate Rab7 recruitment and transition to the late endosome, a function that would also be lost in the absence of Beclin 1 (18). A role for UVRAG in controlling EGFR degradation has been previously reported. siRNA mediated depletion of UVRAG decreased EGFR receptor degradation (19). In addition, UVRAG is frequently monoallelically mutated in human colon carcinomas with microsatellite instability (113). All of these reports, together with my finding that ATG5 does not control receptor signaling, supports an autophagy-independent mechanism of regulation by Beclin 1 (complex II).

One of the caveats of inhibiting Beclin 1 is that it could affect both autophagy and endocytic vesicle trafficking. Hence, it is conceivable that inhibition of the autophagic pathway in Beclin 1-depleted cells may also contribute to increased oncogenic signaling. Although we did not observe any increase in the activation of AKT upon chemically inhibiting autophagy with chloroquine or genetically suppressing ATG5, inhibition of this process could delay degradation of growth factor receptor pathway components. In support of this possibility, the number of APPL⁺ endosomes increased modestly at the very earliest time point of stimulation in ATG5-deficient cells. This increase in signaling-competent early endosomes could result in a transient increase in growth factor-dependent signaling. To investigate this impact on signaling, a shorter-duration growth factor stimulation should be performed with ATG5-depleted cells and its effect on invasion and tumor progression should be

monitored. It is also plausible that the observed differences in growth factor receptor signaling in shBECN1 and shATG5 cells are due to inefficient suppression of autophagy in the latter. Therefore, the extent of autophagy inhibition in shBECN1 and shATG5 cells should be compared by quantitative analysis of this degradative process by methods such as transmission electron microscopy.

To further delineate the contribution of autophagy and vesicle trafficking in regulating growth factor receptor-mediated signaling, Beclin 1-depleted cells rescued with wild type Beclin 1 should be compared with shBECN1 cells rescued with Beclin 1 mutants that bind only UVRAG or ATG14L. Based on my findings and previous reports, complex II is involved in endocytic vesicle trafficking, a process known to regulate receptor-mediated signaling. Hence rescue of Beclin 1-deficient cells with the UVRAG-binding mutant would be predicted to restore signaling to control levels, whereas the ATG14L-binding mutant would not.

The impact of Beclin 1 on endosomal maturation

The role of Beclin 1 in endocytosis in lower organisms including *C. elegans* and *Drosophila* has been well documented (82, 83, 170). However, its function in mammals had been controversial. My data confirms the involvement of Beclin 1 in receptor endocytosis and contributes to a better understanding of the molecular mechanism by which receptor tyrosine kinases traffic, defects in which promote tumorigenesis (178). The requirement of Beclin 1 for maturation

of PI3P⁻ endosomes to PI3P⁺ endosomes have been supported by a very recently published study by McKnight *et. al.* (179) where they report the need for the Beclin 1-PI3KC3 complex II (containing UVRAG) but not complex I (containing ATG14L) for endosome maturation and EGFR down-regulation. They demonstrated that Beclin 1 associates with endosomes in neurons under normal conditions. Similar to my results, they too found that Beclin 1 deficiency reduced PI3KC3 activity and generation of PI3P, resulting in decreased PI3P⁺ endosome formation. Additionally, they also demonstrated that impaired PI3P⁺ endosome formation in Beclin 1-deficient cells could be rescued by over expression of UVRAG but not ATG14L, RUBICON or a UVRAG-binding deficient Beclin 1 mutant, implicating the involvement of complex II in endocytosis. These results support my findings that Beclin 1 controls endosomal maturation. Furthermore, my studies have also demonstrated that Beclin 1 modulates growth factor receptor signaling from early PI3P⁻ endosomes, resulting in increased invasive capabilities of Beclin 1-depleted cells and potentially increased glycolytic rates, both of which promote tumor progression. Hence, my results advance our understanding of Beclin 1 in cancer and perhaps other pathogenic conditions.

The phospholipid PI3P is involved in several important cellular functions including signaling, membrane trafficking and phagocytosis (180). Since Beclin 1 promotes PI3P production, decreased expression of this protein can affect all the above-mentioned processes. Moreover, receptor endocytosis is being used for

targeted drug delivery to the endolysosomal system (181). Since Beclin 1 regulates endosomal trafficking, decreased Beclin 1 expression may lead to reduced efficacy of such drugs.

Targeting autophagy for cancer treatment

The tumor suppressing and tumor promoting function of autophagy has been highlighted by a number of studies. Because of its involvement in tumor growth and progression, autophagy has been considered as a target for cancer therapy, which in turn has led to several clinical trials. However, autophagy can be cytoprotective and help cells survive therapy making them resistant or could also be cytotoxic as excessive autophagy can lead to cell death. Due to its paradoxical role in cancer, understanding the function of autophagy in a given cancer type is crucial before modulators of the process can be used for cancer treatment. My studies indicate that triple-negative breast carcinoma cells could be good candidates for autophagy-targeted therapy. Nonetheless targeting PI3KC3 or Beclin 1 to inhibit autophagy for cancer treatment may prove deleterious. Contradictory results were obtained by Kanzawa et. al. upon treatment of malignant glioma cells with 3-methyladenine, a PI3KC3 inhibitor, and Bafilomycin A1 which inhibits fusion between autophagosomes and lysosomes. Bafilomycin A1 sensitized glioma cells to chemotherapeutic drugs while 3-methyladenine rescued them (182). The results presented in this thesis could potentially explain these conflicting results. Inhibition of PI3KC3 could

enhance oncogenic survival signals thereby counteracting therapy-induced apoptosis.

Recently, PI3KC3 has also been gaining importance as an anticancer agent. PI3KC3 specific inhibitors are being developed to inhibit autophagy in cancer cells (183, 184). Though this treatment may be beneficial when used in combination with inhibitors of pathways such as AKT and MAPK, it could also lead to upregulation of other signaling pathways. The results of my study indicate that patients with autophagy-dependent triple-negative breast tumors could be good candidates for treatment with autophagy inhibitors. However, inhibition of PI3KC3 complex activity should be avoided to prevent progression of the disease. Since Beclin 1 is a regulator of endosomal signaling, its affect may be more global and encompass multiple signaling pathways. Thus, to target all the pathways, the possibility of rescuing Beclin 1-deficient cells by addition of exogenous PI3P may be explored in the future.

Overall significance

In the present study I have explored autophagy-dependent and independent functions of Beclin 1 in tumor progression. I (and others) have found that triple-negative breast carcinoma cells are potentially autophagy dependent and hence these tumors could be good candidates for treatment with autophagy inhibitors. I also identified a novel role for the essential autophagy gene Beclin 1 in modulating growth factor receptor trafficking and signaling. Beclin 1, by

regulating the residence time of growth factor receptors in early signaling-competent endosomes, controls the magnitude and duration of signals propagated. My studies reveal that loss of Beclin 1 could negatively affect two degradative pathways – autophagy and endocytosis, both of which could contribute to tumor progression.

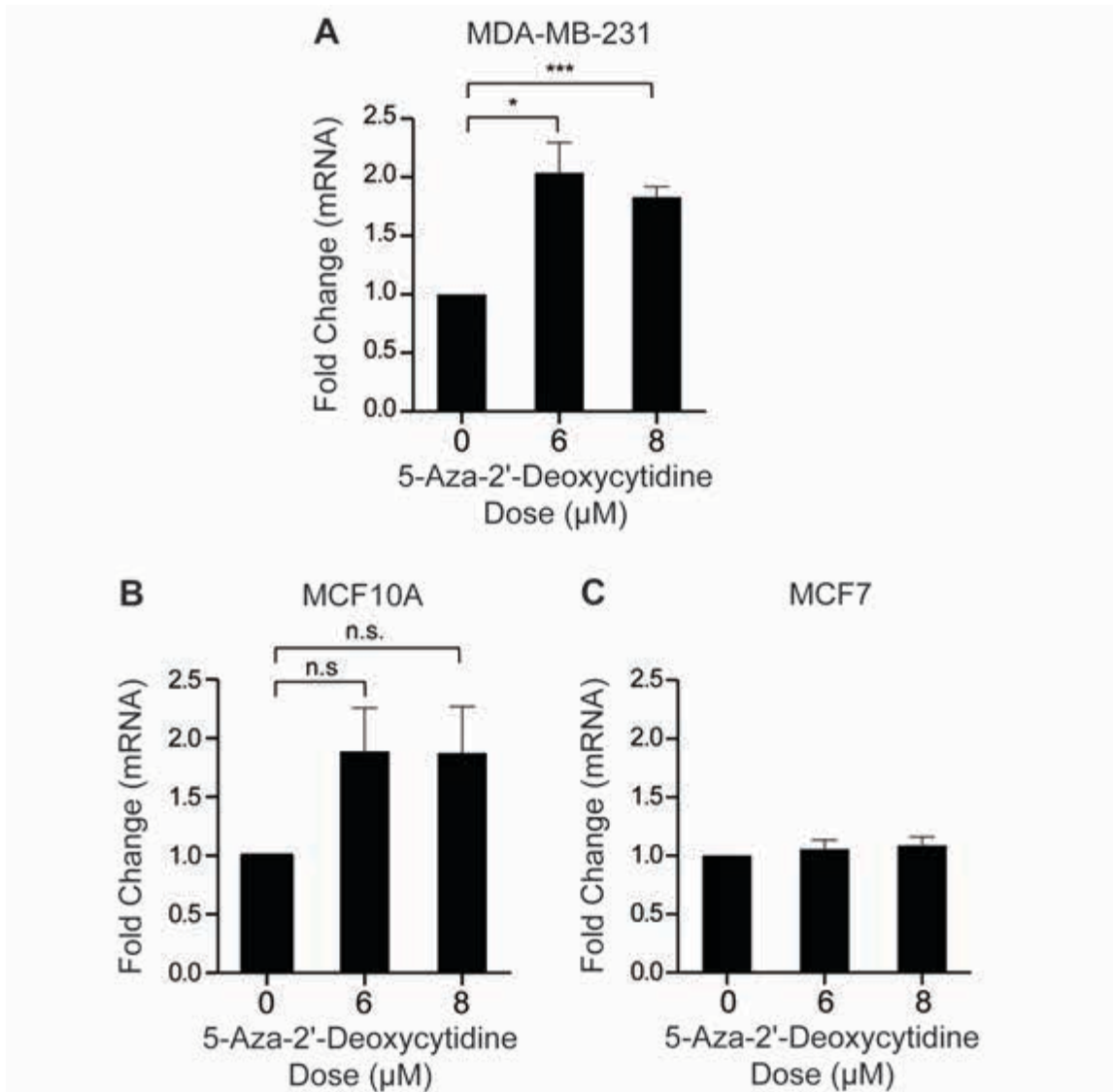
APPENDIX

ANALYSIS OF EPIGENETIC REGULATION OF BECN1 IN BREAST CARCINOMA CELLS

Beclin 1 expression is frequently downregulated in breast tumors both by allelic loss as well as aberrant DNA methylation. This protein maps to human chromosome 17q21 which is monoallelically deleted in 40% - 75% of ovarian, breast and prostate cancers (93). The 5' regulatory region from the promoter to part of intron 2 of *BECN1* is rich in CpG islands and is also epigenetically silenced in many breast tumors (96). A study performed by Aita *et. al.* reported allelic deletion of *BECN1* in 41% of breast carcinoma cell lines analyzed when compared to the normal immortalized mammary epithelial MCF10A cells. Their results indicate deletion of *BECN1* in MCF7 but not MDA-MB-231 cells (93). I sought to investigate whether *BECN1* was also epigenetically regulated in these cell lines belonging to different breast cancer subtypes.

To analyze hypermethylation of *BECN1* in the triple-negative MDA-MB-231, luminal MCF7 and immortalized normal mammary epithelial MCF10A cell lines, cells plated in triplicate were treated with 6 μ M and 8 μ M of 5-aza-2'-deoxycytidine for 72 hours. Control plates were treated with an equal volume of DMSO. Media was replaced each day with fresh media containing either DMSO or 5-aza-2'-deoxycytidine. After 72 hours, cells were harvested and RNA was extracted using the RNeasy kit (Qiagen). Superscript® II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA, which was subsequently utilized to quantify *BECN1* gene expression using SYBR® Green RT-PCR reagents (Applied Biosystems). *BECN1* expression was normalized to *actin*.

Inhibition of DNA methylation revealed that *BECN1* expression was enhanced by 1.8 – 2 fold upon treatment of MDA-MB-231 cells with 5-aza-2'-deoxycytidine. However, no significant difference in *BECN1* expression was detected in MCF7 and MCF10A cells treated with the inhibitor. These results indicate that Beclin 1 is epigenetically suppressed in the metastatic MDA-MB-231 cells, but not in the non-metastatic MCF7 and MCF10A cells. Although Beclin 1 is a key player in the autophagic process, I have also demonstrated that loss of this protein can promote invasion. Hence, it is possible that cancer cells epigenetically regulate *BECN1* expression during tumor progression to promote invasion and metastasis. Further *in vivo* studies are required to test this hypothesis.



Appendix Figure 1. Epigenetic regulation of Beclin 1.

MDA-MB-231 (A), MCF10A (B) and MCF7 (C) cells were treated with 5-aza-2'-deoxycytidine (Decitabine) at the concentrations indicated for 72 hours. RNA was extracted, reverse transcribed and *BECN1* mRNA levels were determined by qRT-PCR. The data shown represent the mean of four (MDA-MB-231; n=12) or three (MCF10A and MCF7; n=9) independent experiments. *, $p < 0.05$; ***, $p < 0.0005$; n.s, not significant.

BIBLIOGRAPHY

1. Seaman, M. N., Marcusson, E. G., Cereghino, J. L., and Emr, S. D. (1997) Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products. *The Journal of Cell Biology* **137**, 79–92
2. Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. (1998) Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 22284–22291
3. Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J. Virol.* **72**, 8586–8596
4. Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672–676
5. Wirawan, E., Lippens, S., Vanden Berghe, T., Romagnoli, A., Fimia, G. M., Piacentini, M., and Vandenabeele, P. (2012) Beclin1: A role in membrane dynamics and beyond. *autophagy* **8**, 6–17 [online] <http://www.landesbioscience.com/journals/autophagy/article/16645/>.
6. Oberstein, A., Jeffrey, P. D., and Shi, Y. (2007) Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J. Biol. Chem.* **282**, 13123–13132
7. Huang, W., Choi, W., Hu, W., Mi, N., Guo, Q., Ma, M., Liu, M., Tian, Y., Lu, P., Wang, F.-L., Deng, H., Liu, L., Gao, N., Yu, L., and Shi, Y. (2012) Crystal structure and biochemical analyses reveal Beclin 1 as a novel membrane binding protein. *Cell Res.* **22**, 473–489
8. Liang, X. H., Yu, J., Brown, K., and Levine, B. (2001) Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. *Cancer Res.* **61**, 3443–3449
9. Odorizzi, G., Babst, M., and Emr, S. D. (2000) Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends Biochem. Sci.* **25**, 229–235
10. Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001) Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep.* **2**, 330–335
11. He, C., and Levine, B. (2010) The Beclin 1 interactome. *Curr. Opin. Cell Biol.* **22**, 140–149

12. Kihara, A., Noda, T., Ishihara, N., and Ohsumi, Y. (2001) Two Distinct Vps34 Phosphatidylinositol 3-Kinase Complexes Function in Autophagy and Carboxypeptidase Y Sorting in *Saccharomyces cerevisiae*. *The Journal of Cell Biology* **152**, 519–530
13. Itakura, E., Kishi, C., Inoue, K., and Mizushima, N. (2008) Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Molecular Biology of the Cell* **19**, 5360–5372
14. Matsunaga, K., Morita, E., Saitoh, T., Akira, S., Ktistakis, N. T., Izumi, T., Noda, T., and Yoshimori, T. (2010) Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *The Journal of Cell Biology* **190**, 511–521
15. Fimia, G. M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P., Gruss, P., Piacentini, M., Chowdhury, K., and Cecconi, F. (2007) Ambra1 regulates autophagy and development of the nervous system. *Nature* **447**, 1121–1125
16. Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B.-H., and Jung, J. U. (2006) Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat. Cell Biol.* **8**, 688–699
17. Itakura, E., and Mizushima, N. (2009) Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. *autophagy* **5**, 534–536
18. Liang, C., Lee, J.-S., Inn, K.-S., Gack, M. U., Li, Q., Roberts, E. A., Vergne, I., Deretic, V., Feng, P., Akazawa, C., and Jung, J. U. (2008) Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat. Cell Biol.* **10**, 776–787
19. Thoresen, S. B., Pedersen, N. M., Liestøl, K., and Stenmark, H. (2010) A phosphatidylinositol 3-kinase class III sub-complex containing VPS15, VPS34, Beclin 1, UVRAG and BIF-1 regulates cytokinesis and degradative endocytic traffic. *Experimental Cell Research* **316**, 3368–3378
20. Takahashi, Y., Coppola, D., Matsushita, N., Cuaing, H. D., Sun, M., Sato, Y., Liang, C., Jung, J. U., Cheng, J. Q., Mulé, J. J., Pledger, W. J., and Wang, H.-G. (2007) Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat. Cell Biol.* **9**, 1142–1151
21. Erlich, S., Mizrachy, L., Segev, O., Lindenboim, L., Zmira, O., Adi-Harel, S., Hirsch, J. A., Stein, R., and Pinkas-Kramarski, R. (2007) Differential interactions between Beclin 1 and Bcl-2 family members. *autophagy* **3**, 561–568

22. Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., and Levine, B. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927–939
23. Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008) JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol. Cell* **30**, 678–688
24. Zalckvar, E., Berissi, H., Mizrachy, L., Idelchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R., and Kimchi, A. (2009) DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. *EMBO Rep.* **10**, 285–292
25. Tang, D., Kang, R., Livesey, K. M., Cheh, C.-W., Farkas, A., Loughran, P., Hoppe, G., Bianchi, M. E., Tracey, K. J., Zeh, H. J., and Lotze, M. T. (2010) Endogenous HMGB1 regulates autophagy. *The Journal of Cell Biology* **190**, 881–892
26. Strappazzon, F., Vietri-Rudan, M., Campello, S., Nazio, F., Florenzano, F., Fimia, G. M., Piacentini, M., Levine, B., and Cecconi, F. (2011) Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy. *EMBO J.* **30**, 1195–1208
27. Shi, C.-S., and Kehrl, J. H. (2010) TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy. *Science Signaling* **3**, ra42
28. Zhong, Y., Wang, Q. J., Li, X., Yan, Y., Backer, J. M., Chait, B. T., Heintz, N., and Yue, Z. (2009) Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat. Cell Biol.* **11**, 468–476
29. Sun, Q., Westphal, W., Wong, K. N., Tan, I., and Zhong, Q. (2010) Rubicon controls endosome maturation as a Rab7 effector. *Proceedings of the National Academy of Sciences* **107**, 19338–19343
30. Matsunaga, K., Saitoh, T., Tabata, K., Omori, H., Satoh, T., Kurotori, N., Maejima, I., Shirahama-Noda, K., Ichimura, T., Isobe, T., Akira, S., Noda, T., and Yoshimori, T. (2009) Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nature Publishing Group* **11**, 385–396
31. Wang, R. C., Wei, Y., An, Z., Zou, Z., Xiao, G., Bhagat, G., White, M., Reichelt, J., and Levine, B. (2012) Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation. *Science* **338**, 956–959
32. Wei, Y., Zou, Z., Becker, N., Anderson, M., Sumpter, R., Xiao, G., Kinch, L., Koduru, P., Christudass, C. S., Veltri, R. W., Grishin, N. V., Peyton, M.,

- Minna, J., Bhagat, G., and Levine, B. (2013) EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell* **154**, 1269–1284
33. Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T., and Mizushima, N. (2004) The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032–1036
34. Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., and Chiba, T. (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *The Journal of Cell Biology* **169**, 425–434
35. Yue, Z., Jin, S., Yang, C., Levine, A. J., and Heintz, N. (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15077–15082
36. Mizushima, N., and Komatsu, M. (2011) Autophagy: renovation of cells and tissues. *Cell* **147**, 728–741
37. Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T., and Thompson, C. B. (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* **120**, 237–248
38. Levine, B. (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* **120**, 159–162
39. Chiang, H. L., Terlecky, S. R., Plant, C. P., and Dice, J. F. (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246**, 382–385
40. Cuervo, A. M., and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* **273**, 501–503
41. Bandyopadhyay, U., Kaushik, S., Varticovski, L., and Cuervo, A. M. (2008) The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. *Mol. Cell. Biol.* **28**, 5747–5763
42. Sahu, R., Kaushik, S., Clement, C. C., Cannizzo, E. S., Scharf, B., Follenzi, A., Potolicchio, I., Nieves, E., Cuervo, A. M., and Santambrogio, L. (2011) Microautophagy of cytosolic proteins by late endosomes. *Dev. Cell* **20**, 131–139
43. Yang, Z., and Klionsky, D. J. (2010) Mammalian autophagy: core molecular machinery and signaling regulation. *Curr. Opin. Cell Biol.* **22**, 124–131

44. Ge, L., Melville, D., Zhang, M., and Schekman, R. (2013) The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife* **2**, e00947
45. Ge, L., and Schekman, R. (2014) The ER-Golgi intermediate compartment feeds the phagophore membrane. *autophagy* **10**, 170–172
46. Hailey, D. W., Rambold, A. S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P. K., and Lippincott-Schwartz, J. (2010) Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* **141**, 656–667
47. Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., Oomori, H., Noda, T., Haraguchi, T., Hiraoka, Y., Amano, A., and Yoshimori, T. (2013) Autophagosomes form at ER-mitochondria contact sites. *Nature* **495**, 389–393
48. Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., and Rubinsztein, D. C. (2010) Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nature Publishing Group* **12**, 747–757
49. Yen, W.-L., Shintani, T., Nair, U., Cao, Y., Richardson, B. C., Li, Z., Hughson, F. M., Baba, M., and Klionsky, D. J. (2010) The conserved oligomeric Golgi complex is involved in double-membrane vesicle formation during autophagy. *The Journal of Cell Biology* **188**, 101–114
50. Longatti, A., Lamb, C. A., Razi, M., Yoshimura, S.-I., Barr, F. A., and Tooze, S. A. (2012) TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *The Journal of Cell Biology* **197**, 659–675
51. Kim, I., Rodriguez-Enriquez, S., and Lemasters, J. J. (2007) Selective degradation of mitochondria by mitophagy. *Arch. Biochem. Biophys.* **462**, 245–253
52. Narendra, D., Tanaka, A., Suen, D.-F., and Youle, R. J. (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of Cell Biology* **183**, 795–803
53. Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131–24145
54. Kirkin, V., Lamark, T., Sou, Y.-S., Bjørkøy, G., Nunn, J. L., Bruun, J.-A., Shvets, E., McEwan, D. G., Clausen, T. H., Wild, P., Bilusic, I., Theurillat, J.-P., Øvervatn, A., Ishii, T., Elazar, Z., Komatsu, M., Dikic, I., and Johansen, T. (2009) A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol. Cell* **33**, 505–516

55. Thurston, T. L. M., Ryzhakov, G., Bloor, S., Muhlinen, von, N., and Randow, F. (2009) The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat. Immunol.* **10**, 1215–1221
56. Laplante, M., and Sabatini, D. M. (2012) mTOR signaling in growth control and disease. *Cell* **149**, 274–293
57. Noda, T., and Ohsumi, Y. (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273**, 3963–3966
58. Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Scaravilli, F., Easton, D. F., Duden, R., O’Kane, C. J., and Rubinsztein, D. C. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.* **36**, 585–595
59. Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.-L. (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* **4**, 648–657
60. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P. P. (2005) Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121**, 179–193
61. Furuta, S., Hidaka, E., Ogata, A., Yokota, S., and Kamata, T. (2004) Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene* **23**, 3898–3904
62. Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol.* **13**, 251–262
63. Gowans, G. J., Hawley, S. A., Ross, F. A., and Hardie, D. G. (2013) AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. *Cell Metab.* **18**, 556–566
64. Inoki, K., Zhu, T., and Guan, K.-L. (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577–590
65. Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E., and Shaw, R. J. (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* **30**, 214–226
66. Kim, J., Kundu, M., Viollet, B., and Guan, K.-L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Publishing Group* **13**, 132–141

67. Kim, J., Kim, Y. C., Fang, C., Russell, R. C., Kim, J. H., Fan, W., Liu, R., Zhong, Q., and Guan, K.-L. (2013) Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. *Cell* **152**, 290–303
68. Mercer, C. A., Kaliappan, A., and Dennis, P. B. (2009) A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *autophagy* **5**, 649–662
69. Hosokawa, N., Sasaki, T., Iemura, S.-I., Natsume, T., Hara, T., and Mizushima, N. (2009) Atg101, a novel mammalian autophagy protein interacting with Atg13. *autophagy* **5**, 973–979
70. Feng, Y., He, D., Yao, Z., and Klionsky, D. J. (2014) The machinery of macroautophagy. *Cell Res.* **24**, 24–41
71. Jung, C. H., Jun, C. B., Ro, S.-H., Kim, Y.-M., Otto, N. M., Cao, J., Kundu, M., and Kim, D.-H. (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Molecular Biology of the Cell* **20**, 1992–2003
72. Ganley, I. G., Lam, D. H., Wang, J., Ding, X., Chen, S., and Jiang, X. (2009) ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem.* **284**, 12297–12305
73. Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., Iemura, S.-I., Natsume, T., Takehana, K., Yamada, N., Guan, J.-L., Oshiro, N., and Mizushima, N. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Molecular Biology of the Cell* **20**, 1981–1991
74. Baehrecke, E. H. (2005) Autophagy: dual roles in life and death? *Nat. Rev. Mol. Cell Biol.* **6**, 505–510
75. Russell, R. C., Tian, Y., Yuan, H., Park, H. W., Chang, Y.-Y., Kim, J., Kim, H., Neufeld, T. P., Dillin, A., and Guan, K.-L. (2013) ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nature Publishing Group* **15**, 741–750
76. Tanida, I., Komatsu, M., Ueno, T., and Kominami, E. (2003) GATE-16 and GABARAP are authentic modifiers mediated by Apg7 and Apg3. *Biochem. Biophys. Res. Commun.* **300**, 637–644
77. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. *Nature* **395**, 395–398
78. Mizushima, N., Sugita, H., Yoshimori, T., and Ohsumi, Y. (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J. Biol. Chem.* **273**, 33889–33892

79. Mizushima, N., Noda, T., and Ohsumi, Y. (1999) Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *EMBO J.* **18**, 3888–3896
80. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19**, 5720–5728
81. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *The Journal of Cell Biology* **151**, 263–276
82. Ruck, A., Attonito, J., Garces, K. T., Núñez, L., Palmisano, N. J., Rubel, Z., Bai, Z., Nguyen, K. C. Q., Sun, L., Grant, B. D., Hall, D. H., and Meléndez, A. (2011) The Atg6/Vps30/Beclin 1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in *C. elegans*. *autophagy* **7**, 386–400
83. Shrivage, B. V., Hill, J. H., Powers, C. M., Wu, L., and Baehrecke, E. H. (2013) Atg6 is required for multiple vesicle trafficking pathways and hematopoiesis in *Drosophila*. *Development* **140**, 1321–1329
84. Zeng, X., Overmeyer, J. H., and Maltese, W. A. (2006) Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. *Journal of Cell Science* **119**, 259–270
85. Li, X., Garrity, A. G., and Xu, H. (2013) Regulation of membrane trafficking by signalling on endosomal and lysosomal membranes. *The Journal of Physiology* **591**, 4389–4401
86. Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., Waterfield, M. D., Backer, J. M., and Zerial, M. (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat. Cell Biol.* **1**, 249–252
87. Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.* **271**, 24048–24054
88. Song, X., Xu, W., Zhang, A., Huang, G., Liang, X., Virbasius, J. V., Czech, M. P., and Zhou, G. W. (2001) Phox homology domains specifically bind phosphatidylinositol phosphates. *Biochemistry* **40**, 8940–8944
89. Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005) Rab Conversion as a Mechanism of Progression from Early to Late Endosomes. *Cell* **122**, 735–749

90. White, E. (2012) Deconvoluting the context-dependent role for autophagy in cancer. *Nature Reviews Cancer* **12**, 401–410
91. Shen, H.-M., and Codogno, P. (2011) Autophagic cell death: Loch Ness monster or endangered species? *autophagy* **7**, 457–465
92. Choi, A. M. K., Ryter, S. W., and Levine, B. (2013) Autophagy in Human Health and Disease. *N Engl J Med* **368**, 651–662
93. Aita, V. M., Liang, X. H., Murty, V. V., Pincus, D. L., Yu, W., Cayanis, E., Kalachikov, S., Gilliam, T. C., and Levine, B. (1999) Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics* **59**, 59–65
94. Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E.-L., Mizushima, N., Ohsumi, Y., Cattoretti, G., and Levine, B. (2003) Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J. Clin. Invest.* **112**, 1809–1820
95. Laddha, S. V., Ganesan, S., Chan, C. S., and White, E. (2014) Mutational Landscape of the Essential Autophagy Gene BECN1 in Human Cancers. *Mol. Cancer Res.* **12**, 485–490
96. Li, Z., Chen, B., Wu, Y., Jin, F., Xia, Y., and Liu, X. (2010) Genetic and epigenetic silencing of the beclin 1 gene in sporadic breast tumors. *BMC Cancer* **10**, 98
97. Dong, M., Wan, X.-B., Yuan, Z. Y., Wei, L., Fan, X. J., Wang, T.-T., Lv, Y. C., Li, X., Chen, Z.-H., Chen, J., Lin, Q., Wen, J.-Y., Ma, X.-K., Liu, Q., and Wu, X. Y. (2013) Low expression of Beclin 1 and elevated expression of HIF-1 α refine distant metastasis risk and predict poor prognosis of ER-positive, HER2-negative breast cancer. *Med. Oncol.* **30**, 355
98. Wang, J., Pan, X.-L., Ding, L.-J., Liu, D.-Y., Da-Peng Lei, and Jin, T. (2013) Aberrant expression of Beclin-1 and LC3 correlates with poor prognosis of human hypopharyngeal squamous cell carcinoma. *PLoS ONE* **8**, e69038
99. Mathew, R., Karp, C. M., Beaudoin, B., Vuong, N., Chen, G., Chen, H.-Y., Bray, K., Reddy, A., Bhanot, G., Gelinas, C., Dipaola, R. S., Karantza-Wadsworth, V., and White, E. (2009) Autophagy suppresses tumorigenesis through elimination of p62. *Cell* **137**, 1062–1075
100. Wang, Y., Nartiss, Y., Steipe, B., McQuibban, G. A., and Kim, P. K. (2012) ROS-induced mitochondrial depolarization initiates PARK2/PARKIN-dependent mitochondrial degradation by autophagy. *autophagy* **8**, 1462–1476
101. Kurihara, Y., Kanki, T., Aoki, Y., Hirota, Y., Saigusa, T., Uchiyama, T., and Kang, D. (2012) Mitophagy plays an essential role in reducing

- mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. *Journal of Biological Chemistry* **287**, 3265–3272
102. Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.-S., Ueno, I., Sakamoto, A., Tong, K. I., Kim, M., Nishito, Y., Iemura, S.-I., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K., and Yamamoto, M. (2010) The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature Publishing Group* **12**, 213–223
 103. Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D. A., Jin, S., and White, E. (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* **10**, 51–64
 104. DeNardo, D. G., Johansson, M., and Coussens, L. M. (2008) Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev.* **27**, 11–18
 105. Smith, H. A., and Kang, Y. (2013) The metastasis-promoting roles of tumor-associated immune cells. *J. Mol. Med.* **91**, 411–429
 106. Kang, M. R., Kim, M. S., Oh, J. E., Kim, Y. R., Song, S. Y., Kim, S. S., Ahn, C. H., Yoo, N. J., and Lee, S. H. (2009) Frameshift mutations of autophagy-related genes ATG2B, ATG5, ATG9B and ATG12 in gastric and colorectal cancers with microsatellite instability. *J. Pathol.* **217**, 702–706
 107. Mariño, G., Salvador-Montoliu, N., Fueyo, A., Knecht, E., Mizushima, N., and López-Otín, C. (2007) Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J. Biol. Chem.* **282**, 18573–18583
 108. Takamura, A., Komatsu, M., Hara, T., Sakamoto, A., Kishi, C., Waguri, S., Eishi, Y., Hino, O., Tanaka, K., and Mizushima, N. (2011) Autophagy-deficient mice develop multiple liver tumors. *Genes Dev.* **25**, 795–800
 109. Karantza-Wadsworth, V., Patel, S., Kravchuk, O., Chen, G., Mathew, R., Jin, S., and White, E. (2007) Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev.* **21**, 1621–1635
 110. Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J. M., Dell'antonio, G., Mautner, J., Tonon, G., Haigis, M., Shirihai, O. S., Doglioni, C., Bardeesy, N., and Kimmelman, A. C. (2011) Pancreatic cancers require autophagy for tumor growth. *Genes Dev.* **25**, 717–729
 111. Fung, C., Lock, R., Gao, S., Salas, E., and Debnath, J. (2008) Induction

- of autophagy during extracellular matrix detachment promotes cell survival. *Molecular Biology of the Cell* **19**, 797–806
112. Chen, N., and Debnath, J. (2010) Autophagy and tumorigenesis. *FEBS Letters* **584**, 1427–1435
 113. Knævelsrud, H., Ahlquist, T., Merok, M. A., Nesbakken, A., Stenmark, H., Lothe, R. A., and Simonsen, A. (2010) UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy. *autophagy* **6**, 863–870
 114. Kim, M. S., Jeong, E. G., Ahn, C. H., Kim, S. S., Lee, S. H., and Yoo, N. J. (2008) Frameshift mutation of UVRAG, an autophagy-related gene, in gastric carcinomas with microsatellite instability. *Hum. Pathol.* **39**, 1059–1063
 115. Lee, J. W., Jeong, E. G., Soung, Y. H., Nam, S. W., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2006) Decreased expression of tumour suppressor Bax-interacting factor-1 (Bif-1), a Bax activator, in gastric carcinomas. *Pathology* **38**, 312–315
 116. Coppola, D., Oliveri, C., Sayegh, Z., Boulware, D., Takahashi, Y., Pow-Sang, J., Djeu, J. Y., and Wang, H.-G. (2008) Bax-interacting factor-1 expression in prostate cancer. *Clin Genitourin Cancer* **6**, 117–121
 117. Coppola, D., Khalil, F., Eschrich, S. A., Boulware, D., Yeatman, T., and Wang, H.-G. (2008) Down-regulation of Bax-interacting factor-1 in colorectal adenocarcinoma. *Cancer* **113**, 2665–2670
 118. Coppola, D., Helm, J., Ghayouri, M., Malafa, M. P., and Wang, H.-G. (2011) Down-regulation of Bax-interacting factor 1 in human pancreatic ductal adenocarcinoma. *Pancreas* **40**, 433–437
 119. Takahashi, Y., Hori, T., Cooper, T. K., Liao, J., Desai, N., Serfass, J. M., Young, M. M., Park, S., Izu, Y., and Wang, H.-G. (2013) Bif-1 haploinsufficiency promotes chromosomal instability and accelerates Myc-driven lymphomagenesis via suppression of mitophagy. *Blood* **121**, 1622–1632
 120. Hayakawa, A., Hayes, S. J., Lawe, D. C., Sudharshan, E., Tuft, R., Fogarty, K., Lambright, D., and Corvera, S. (2004) Structural basis for endosomal targeting by FYVE domains. *J. Biol. Chem.* **279**, 5958–5966
 121. Navaroli, D. M., Bellvé, K. D., Standley, C., Lifshitz, L. M., Cardia, J., Lambright, D., Leonard, D., Fogarty, K. E., and Corvera, S. (2012) Rabenosyn-5 defines the fate of the transferrin receptor following clathrin-mediated endocytosis. *Proceedings of the National Academy of Sciences* **109**, E471–80
 122. Pankratz, S. L., Tan, E. Y., Fine, Y., Mercurio, A. M., and Shaw, L. M.

- (2008) Insulin Receptor Substrate-2 Regulates Aerobic Glycolysis in Mouse Mammary Tumor Cells via Glucose Transporter 1. *Journal of Biological Chemistry* **284**, 2031–2037
123. Leonard, D., Hayakawa, A., Lawe, D., Lambright, D., Bellvé, K. D., Standley, C., Lifshitz, L. M., Fogarty, K. E., and Corvera, S. (2008) Sorting of EGF and transferrin at the plasma membrane and by cargo-specific signaling to EEA1-enriched endosomes. *Journal of Cell Science* **121**, 3445–3458
124. Jung, C. H., Ro, S.-H., Cao, J., Otto, N. M., and Kim, D.-H. (2010) mTOR regulation of autophagy. *FEBS Letters* **584**, 1287–1295
125. Guo, J. Y., Chen, H.-Y., Mathew, R., Fan, J., Strohecker, A. M., Karsli-Uzunbas, G., Kamphorst, J. J., Chen, G., Lemons, J. M. S., Karantza, V., Collier, H. A., Dipaola, R. S., Gelinias, C., Rabinowitz, J. D., and White, E. (2011) Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev.* **25**, 460–470
126. Lorin, S., Hamaï, A., Mehrpour, M., and Codogno, P. (2013) Autophagy regulation and its role in cancer. *Semin. Cancer Biol.* **23**, 361–379
127. Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A. L., Brown, P. O., and Botstein, D. (2000) Molecular portraits of human breast tumours. *Nature* **406**, 747–752
128. Prat, A., and Perou, C. M. (2011) Deconstructing the molecular portraits of breast cancer. *Molecular Oncology* **5**, 5–23
129. Maycotte, P., Gearheart, C. M., Barnard, R., Aryal, S., Mulcahy Levy, J. M., Fosmire, S. P., Hansen, R. J., Morgan, M. J., Porter, C. C., Gustafson, D. L., and Thorburn, A. (2014) STAT3-mediated autophagy dependence identifies subtypes of breast cancer where autophagy inhibition can be efficacious. *Cancer Res.*
130. Klionsky, D. J. *et al.* (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *autophagy* **8**, 445–544
131. Eskelinen, E.-L., Schmidt, C. K., Neu, S., Willenborg, M., Fuertes, G., Salvador, N., Tanaka, Y., Lüllmann-Rauch, R., Hartmann, D., Heeren, J., Figura, von, K., Knecht, E., and Saftig, P. (2004) Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. *Molecular Biology of the Cell* **15**, 3132–3145
132. Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., He, X., and Perou, C. M. (2010) Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* **12**, R68

133. Barnabas, N., and Cohen, D. (2013) Phenotypic and Molecular Characterization of MCF10DCIS and SUM Breast Cancer Cell Lines. *Int J Breast Cancer* **2013**, 872743
134. Warburg, O. (1956) On the origin of cancer cells. *Science* **123**, 309–314
135. Lock, R., Roy, S., Kenific, C. M., Su, J. S., Salas, E., Ronen, S. M., and Debnath, J. (2011) Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. *Molecular Biology of the Cell* **22**, 165–178
136. Guadamillas, M. C., Cerezo, A., and Del Pozo, M. A. (2011) Overcoming anoikis--pathways to anchorage-independent growth in cancer. *Journal of Cell Science* **124**, 3189–3197
137. Maycotte, P., Aryal, S., Cummings, C. T., Thorburn, J., Morgan, M. J., and Thorburn, A. (2012) Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. *autophagy* **8**, 200–212
138. Kim, M.-J., Woo, S.-J., Yoon, C.-H., Lee, J.-S., An, S., Choi, Y.-H., Hwang, S.-G., Yoon, G., and Lee, S.-J. (2011) Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. *Journal of Biological Chemistry* **286**, 12924–12932
139. Weinberg, F., Hamanaka, R., Wheaton, W. W., Weinberg, S., Joseph, J., Lopez, M., Kalyanaraman, B., Mutlu, G. M., Budinger, G. R. S., and Chandel, N. S. (2010) Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences* **107**, 8788–8793
140. Isakoff, S. J., Engelman, J. A., Irie, H. Y., Luo, J., Brachmann, S. M., Pearlman, R. V., Cantley, L. C., and Brugge, J. S. (2005) Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res.* **65**, 10992–11000
141. Sanchez, C. G., Ma, C. X., Crowder, R. J., Guintoli, T., Phommaly, C., Gao, F., Lin, L., and Ellis, M. J. (2011) Preclinical modeling of combined phosphatidylinositol-3-kinase inhibition with endocrine therapy for estrogen receptor-positive breast cancer. *Breast Cancer Res.* **13**, R21
142. Li, W.-W., Li, J., and Bao, J.-K. (2012) Microautophagy: lesser-known self-eating. *Cell. Mol. Life Sci.* **69**, 1125–1136
143. Kon, M., Kiffin, R., Koga, H., Chapochnik, J., Macian, F., Varticovski, L., and Cuervo, A. M. (2011) Chaperone-mediated autophagy is required for tumor growth. *Sci Transl Med* **3**, 109ra117
144. Saha, T. (2012) LAMP2A overexpression in breast tumors promotes cancer cell survival via chaperone-mediated autophagy. *autophagy* **8**, 1643–1656
145. Morandi, A., and Chiarugi, P. (2014) Metabolic implication of

- tumor:stroma crosstalk in breast cancer. *J. Mol. Med.* **92**, 117–126
146. Cicchini, M., Chakrabarti, R., Kongara, S., Price, S., Nahar, R., Lozy, F., Zhong, H., Vazquez, A., Kang, Y., and Karantza, V. (2014) Autophagy regulator BECN1 suppresses mammary tumorigenesis driven by WNT1 activation and following parity. *autophagy* **10**
 147. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* **260**, 88–91
 148. Stephens, L., Cooke, F. T., Walters, R., Jackson, T., Volinia, S., Gout, I., Waterfield, M. D., and Hawkins, P. T. (1994) Characterization of a phosphatidylinositol-specific phosphoinositide 3-kinase from mammalian cells. *Curr. Biol.* **4**, 203–214
 149. Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998) FYVE fingers bind PtdIns(3)P. *Nature* **394**, 432–433
 150. Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V., and Chawla, A. (1998) A functional PtdIns(3)P-binding motif. *Nature* **394**, 433–434
 151. Lindmo, K., and Stenmark, H. (2006) Regulation of membrane traffic by phosphoinositide 3-kinases. *Journal of Cell Science* **119**, 605–614
 152. Law, J. H., Habibi, G., Hu, K., Masoudi, H., Wang, M. Y. C., Stratford, A. L., Park, E., Gee, J. M. W., Finlay, P., Jones, H. E., Nicholson, R. I., Carboni, J., Gottardis, M., Pollak, M., and Dunn, S. E. (2008) Phosphorylated insulin-like growth factor-1/insulin receptor is present in all breast cancer subtypes and is related to poor survival. *Cancer Res.* **68**, 10238–10246
 153. Resnik, J. L., Reichart, D. B., Huey, K., Webster, N. J., and Seely, B. L. (1998) Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. *Cancer Res.* **58**, 1159–1164
 154. Park, H. S., Jang, M. H., Kim, E. J., Kim, H. J., Lee, H. J., Kim, Y. J., Kim, J. H., Kang, E., Kim, S.-W., Kim, I. A., and Park, S. Y. (2014) High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. *Modern Pathology*
 155. Kametaka, S., Matsuura, A., Wada, Y., and Ohsumi, Y. (1996) Structural and functional analyses of APG5, a gene involved in autophagy in yeast. *Gene* **178**, 139–143
 156. Miaczynska, M., Pelkmans, L., and Zerial, M. (2004) Not just a sink: endosomes in control of signal transduction. *Curr. Opin. Cell Biol.* **16**, 400–406
 157. Dutta, D., and Donaldson, J. G. (2012) Search for inhibitors of

- endocytosis: Intended specificity and unintended consequences. *Cellular Logistics* **2**, 203–208
158. Platta, H. W., and Stenmark, H. (2011) Endocytosis and signaling. *Curr. Opin. Cell Biol.* **23**, 393–403
 159. Hayakawa, A., Leonard, D., Murphy, S., Hayes, S., Soto, M., Fogarty, K., Standley, C., Bellve, K., Lambright, D., Mello, C., and Corvera, S. (2006) The WD40 and FYVE domain containing protein 2 defines a class of early endosomes necessary for endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 11928–11933
 160. Zoncu, R., Perera, R. M., Balkin, D. M., Pirruccello, M., Toomre, D., and De Camilli, P. (2009) A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. *Cell* **136**, 1110–1121
 161. Schenck, A., Goto-Silva, L., Collinet, C., Rhinn, M., Giner, A., Habermann, B., Brand, M., and Zerial, M. (2008) The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. *Cell* **133**, 486–497
 162. Mitsuuchi, Y., Johnson, S. W., Sonoda, G., Tanno, S., Golemis, E. A., and Testa, J. R. (1999) Identification of a chromosome 3p14. 3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. *Oncogene* **18**, 4891
 163. Lee, M.-H., Klein, R. L., El-Shewy, H. M., Luttrell, D. K., and Luttrell, L. M. (2008) The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/Ras-dependent pathway and stimulate cell growth. *Biochemistry* **47**, 11682–11692
 164. Jones, R. B., Gordus, A., Krall, J. A., and MacBeath, G. (2006) A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature* **439**, 168–174
 165. Ryu, J., Galan, A. K., Xin, X., Dong, F., Abdul-Ghani, M. A., Zhou, L., Wang, C., Li, C., Holmes, B. M., Sloane, L. B., Austad, S. N., Guo, S., Musi, N., DeFronzo, R. A., Deng, C., White, M. F., Liu, F., and Dong, L. Q. (2014) APPL1 potentiates insulin sensitivity by facilitating the binding of IRS1/2 to the insulin receptor. *7*, 1227–1238
 166. Dillon, R. L., Marcotte, R., Hennessy, B. T., Woodgett, J. R., Mills, G. B., and Muller, W. J. (2009) Akt1 and akt2 play distinct roles in the initiation and metastatic phases of mammary tumor progression. *Cancer Res.* **69**, 5057–5064
 167. Arboleda, M. J., Lyons, J. F., Kabbinar, F. F., Bray, M. R., Snow, B. E., Ayala, R., Danino, M., Karlan, B. Y., and Slamon, D. J. (2003) Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and

- ovarian cancer cells. *Cancer Res.* **63**, 196–206
168. Gao, J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C., and Schultz, N. (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science Signaling* **6**, p1
 169. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A. P., Sander, C., and Schultz, N. (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**, 401–404
 170. Lőrincz, P., Lakatos, Z., Maruzs, T., Szatmári, Z., Kis, V., and Sass, M. (2014) Atg6/UVRAG/Vps34-containing lipid kinase complex is required for receptor downregulation through endolysosomal degradation and epithelial polarity during *Drosophila* wing development. *Biomed Res Int* **2014**, 851349
 171. Lawe, D. C., Chawla, A., Merithew, E., Dumas, J., Carrington, W., Fogarty, K., Lifshitz, L., Tuft, R., Lambright, D., and Corvera, S. (2002) Sequential roles for phosphatidylinositol 3-phosphate and Rab5 in tethering and fusion of early endosomes via their interaction with EEA1. *J. Biol. Chem.* **277**, 8611–8617
 172. Er, E. E., Mendoza, M. C., Mackey, A. M., Rameh, L. E., and Blenis, J. (2013) AKT Facilitates EGFR Trafficking and Degradation by Phosphorylating and Activating PIKfyve. *Science Signaling* **6**, ra45–ra45
 173. Lock, R., Kenific, C. M., Leidal, A. M., Salas, E., and Debnath, J. (2014) Autophagy-dependent production of secreted factors facilitates oncogenic RAS-driven invasion. *Cancer Discov* **4**, 466–479
 174. Liu, J., Xia, H., Kim, M., Xu, L., Li, Y., Zhang, L., Cai, Y., Norberg, H. V., Zhang, T., Furuya, T., Jin, M., Zhu, Z., Wang, H., Yu, J., Li, Y., Hao, Y., Choi, A., Ke, H., Ma, D., and Yuan, J. (2011) Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell* **147**, 223–234
 175. Salem, A. F., Whitaker-Menezes, D., Lin, Z., Martinez-Outschoorn, U. E., Tanowitz, H. B., Al-Zoubi, M. S., Howell, A., Pestell, R. G., Sotgia, F., and Lisanti, M. P. (2012) Two-compartment tumor metabolism: autophagy in the tumor microenvironment and oxidative mitochondrial metabolism (OXPHOS) in cancer cells. *Cell Cycle* **11**, 2545–2556
 176. Ko, Y.-H., Lin, Z., Flomenberg, N., Pestell, R. G., Howell, A., Sotgia, F., Lisanti, M. P., and Martinez-Outschoorn, U. E. (2011) Glutamine fuels a vicious cycle of autophagy in the tumor stroma and oxidative mitochondrial metabolism in epithelial cancer cells: implications for

- preventing chemotherapy resistance. *cbt* **12**, 1085–1097
177. Capparelli, C., Guido, C., Whitaker-Menezes, D., Bonuccelli, G., Balliet, R., Pestell, T., Goldberg, A., Pestell, R., Howell, A., Sneddon, S., Birbe, R., Tsirigos, A., Martinez-Outschoorn, U., Sotgia, F., and Lisanti, M. (2012) Autophagy and senescence in cancer-associated fibroblasts metabolically supports tumor growth and metastasis via glycolysis and ketone production. *Cell Cycle* **11**, 2285–2302
 178. Parachoniak, C. A., and Park, M. (2012) Dynamics of receptor trafficking in tumorigenicity. *Trends Cell Biol* **22**, 231–240
 179. McKnight, N. C., Zhong, Y., Wold, M. S., Gong, S., Phillips, G. R., Dou, Z., Zhao, Y., Heintz, N., Zong, W.-X., and Yue, Z. (2014) Beclin 1 Is Required for Neuron Viability and Regulates Endosome Pathways via the UVRAG-VPS34 Complex. *PLoS Genet.* **10**, e1004626
 180. Falasca, M., and Maffucci, T. (2009) Rethinking phosphatidylinositol 3-monophosphate. *Biochim. Biophys. Acta* **1793**, 1795–1803
 181. Bareford, L. M., and Swaan, P. W. (2007) Endocytic mechanisms for targeted drug delivery. *Adv. Drug Deliv. Rev.* **59**, 748–758
 182. Kanzawa, T., Germano, I. M., Komata, T., Ito, H., Kondo, Y., and Kondo, S. (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ.* **11**, 448–457
 183. Ronan, B., Flamand, O., Vescovi, L., Dureuil, C., Durand, L., Fassy, F., Bachelot, M.-F., Lambertson, A., Mathieu, M., Bertrand, T., Marquette, J.-P., El-Ahmad, Y., Filoche-Romme, B., Schio, L., Garcia-Echeverria, C., Goulaouic, H., and Pasquier, B. (2014) A highly potent and selective Vps34 inhibitor alters vesicle trafficking and autophagy. *Nat. Chem. Biol.* **10**, 1013–1019
 184. Pasquier, B., El-Ahmad, Y., Filoche-Romme, B., Dureuil, C., Fassy, F., Abecassis, P.-Y., Mathieu, M., Bertrand, T., Benard, T., Barrière, C., Batti, El, S., Letaltec, J.-P., Sonnefraud, V., Brollo, M., Delbarre, L., Loyau, V., Pilorge, F., Bertin, L., Richepin, P., Arigon, J., Labrosse, J.-R., Clément, J., Durand, F., Combet, R., Perraut, P., Leroy, V., Gay, F., Lefrançois, D., Bretin, F., Marquette, J.-P., Michot, N., Caron, A., Castell, C., Schio, L., McCort, G., Goulaouic, H., Garcia-Echeverria, C., and Ronan, B. (2014) Discovery of (2S)-8-[(3R)-3-Methylmorpholin-4-yl]-1-(3-methyl-2-oxobutyl)-2-(trifluoromethyl)-3,4-dihydro-2H-pyrimido[1,2-a]pyrimidin-6-one: A Novel Potent and Selective Inhibitor of Vps34 for the Treatment of Solid Tumors. *J. Med. Chem.*