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BECLIN-1 regulates 3D to 2D cancer cell growth and adhesion.

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INTRODUCTION

1. Introduction

1.1. Breast cancer (BC)

Breast cancer (BC) is the leading cause of cancer-related deaths among women worldwide, second only to lung cancer in overall cancer mortality (Ferlay et al, 2024). While the incidence of BC continues to rise globally, particularly in countries with widespread access to screening programs, the mortality rates remain disproportionately high in regions with limited access to early detection and treatment. This disparity highlights significant healthcare inequalities and emphasizes the urgent need for better access to resources, especially in low- and middle-income countries (Sun *et al.*, 2017).

Several factors, including lifestyle factors such as obesity, delayed childbirth, and the use of postmenopausal hormone therapy, have been linked to an increased risk. Women with a family history of BC or those who have previously undergone chest radiation are also at a heightened risk of developing the disease (Sun *et al.*, 2017).

Breast carcinogenesis is a multifaceted process driven by both genetic mutations and environmental influences. It starts with genetic changes that transform normal cells into cancerous ones, progressing through stages such as hyperplasia, premalignant lesions, and eventually invasive carcinoma. This transformation involves both inherited genetic mutations and somatic alterations (Sun *et al.*, 2017).

The most notable genetic mutations associated with BC are found in the *BRCA1* and *BRCA2* genes. These tumor suppressor (TS) genes are vital for DNA repair, and mutations in both significantly elevate the risk of developing breast and ovarian cancers. Women with *BRCA1* mutations have a 65% lifetime risk of BC by age 70, while those with *BRCA2* mutations face a 45% risk. However, these mutations account for only about 5-10% of all BC cases(Akram, 2017). On the other hand, *BECN1*, which is an important TS gene, is linked to tumor susceptibility and is deleted in about 30% of BCs. In sporadic BC, lower levels of *BECN1* mRNA, rather than changes in the nearby *BRCA1* gene, increase the risk of more aggressive cancer types and are linked to poorer patient outcomes (Wijshake *et al.*, 2021; Tang *et al.*, 2015).

Several studies have identified other driver mutations, such as *TP53*, *CHEK2*, *PALB2*, and *ATM*, that also contribute to BC risk but they are less common than *BRCA* mutations. These genes play roles in DNA repair and cell cycle regulation, and their mutations can increase the likelihood of cells becoming cancerous (Xiong *et al.*, 2025).

Oncogenes like *HER2* and *EGFR* are also involved in BC development by promoting cell growth and survival. *HER2* amplification, which occurs in about 20% of BCs, is associated with more aggressive tumors and a poorer prognosis. Likewise, overexpression of *EGFR* in BC is linked to worse clinical outcomes and suggests that targeting these pathways may offer potential therapeutic benefits(Sun *et al.*, 2017).

In addition to these major mutations, smaller genetic changes, such as single-nucleotide polymorphisms (SNPs), can increase BC risk. These changes typically affect genes involved in estrogen metabolism, such as *CYP17* and *CYP19*. When several of these genetic variations occur together, the risk of BC is notably higher (Xiong *et al.*, 2025).

Because of the revolutionary work of Perou, Sørlie, and colleagues, BC has been classified into four clinically relevant molecular subtypes: luminal A, luminal B, HER2-enriched, and basal-like. Histological evidence has further contributed to the division of these subtypes by steroid hormone receptor (estrogen receptor (ER), progesterone receptor (PR), and HER2 status, as well as tumor cell proliferation measured by Ki67. They are subdivided as follows: luminal A-like subtype (ER+ or PR+, or both, HER2-, low proliferation); luminal B-like subtype (ER+ or PR+, or both, HER2-, high proliferation); HER2 subtype, non-luminal (HER2+ and ER- and PR- negative) or luminal (HER2+ and ER+ or PR+, or both); basal-like subtype (HER2- and ER- and PR-; triple-negative BC (TNBC)) (Perou *et al.*, 2000) (Sørlie *et al.*, 2001). These subtypes display notable differences in their incidence, steroid hormone receptor status, prognosis, and response to treatment (Table 1) (Watkins, 2019). In terms of prognosis, the Luminal A subtype has the most favorable, as evidenced by numerous patient datasets in early BC studies.

Subtype	Immunohistochemistry	Prognosis
Luminal A	• Estrogen receptor positive • Progesterone receptor positive • HER-2 negative	Good
Luminal B	• Estrogen receptor positive • Progesterone receptor positive • HER-2 positive or negative	Fair
HER-2 enriched	• Estrogen receptor negative • Progesterone receptor negative • HER-2 positive	Poor
Basal-like / TBN	• Estrogen receptor negative • Progesterone receptor negative • HER-2 negative	Poor

Table 1. Breast cancer molecular subtypes

The diagnosis of BC involves a combination of screening techniques, imaging, biopsy, and molecular profiling. Initial screening typically includes mammography and ultrasonography, with MRI used in specific cases like dense breasts or high-risk patients. Biopsy is the gold standard for confirming diagnosis, most commonly via ultrasound-guided core needle biopsy, which provides critical information on tumor grade, hormone receptor status, and HER-2 expression, guiding treatment choices(Bhushan, Gonsalves and Menon, 2021; Łukasiewicz et al., 2021).

In addition to histopathological evaluation, genomic tests like Oncotype DX, MammaPrint, and PAM50 have improved BC diagnosis and management. Oncotype DX evaluates 21 genes to assess recurrence risk in ER-positive BC and help guide decisions about chemotherapy. Similarly, MammaPrint analyzes 70 genes and predicts recurrence risk for both ER-positive

and ER-negative BC, allowing for more tailored treatments, particularly in early stages(Cardoso et al., 2016).

The utility of tumor markers has also expanded in BC diagnosis. These include proteins such as HER2, Ki67, ER, and PR, which are crucial for assessing tumor biology, prognosis, and treatment response (Łukasiewicz et al., 2021). Other markers, such as microRNAs (i.e., miR-21, miR-10b), have been identified as potential biomarkers for early detection, prognosis, and monitoring during treatment (Jelski, Okrasinska and Mroczko, 2025).

Additionally, clinical history and physical examination remain fundamental, while breast self-examination helps women detect abnormalities early. Technological advances, including AI and Convolutional Neural Networks (CNNs), are improving diagnostic accuracy and reducing human error, particularly in image analysis (Yan, Li and Wu, 2023; Al-Karawi et al., 2024).

Overall, a combination of imaging, biopsy, genomic profiling, and advanced technologies provides a comprehensive approach to BC diagnosis and management.

1.2. Autophagy

Autophagy is a highly conserved intracellular degradation process that maintains cellular homeostasis by degrading damaged or unnecessary components within lysosomes. This process, essential for cellular quality control and adaptation to stress, was initially thought to be a non-selective bulk degradation system but is now recognized to have remarkable specificity in cargo selection(Kroemer, Mariño and Levine, 2010)(Kumar *et al.*,2021). Autophagy is classified into three main types: macroautophagy, the most extensively studied, involves the formation of double-membrane vesicles called autophagosomes that engulf cytoplasmic material and fuse with lysosomes for degradation; microautophagy occurs through the direct invagination of cytoplasmic components into lysosomes without vesicle formation; and chaperone-mediated autophagy (CMA), a selective pathway, relies on chaperone proteins like HSC70 to recognize and transport specific proteins across the lysosomal membrane via LAMP2A (S. Liu *et al.*, 2023). While macroautophagy has been the primary focus of research due to its significant role in cellular metabolism, stress response, and disease regulation, growing evidence suggests that microautophagy and CMA

also contribute to crucial physiological and pathological processes, including aging, cancer, and neurodegeneration. Understanding the interplay between these autophagic pathways can help us better understand their impact on health and disease (Cassidy and Narita, 2022).

Autophagy has a dual role in cancer. In the early stages, it helps prevent tumor formation by maintaining cell health. It removes damaged proteins and organelles, supports the cell during stress, and maintains a balanced internal environment. However as cancer progresses, autophagy has been shown to help cells survive harsh conditions such as hypoxia, nutrient deprivation as well as chemotherapy (Yun *et al.*, 2020).

Established tumors often activate autophagy to adapt to nutrient limitation. This helps them grow rapidly even in harsh environments. Because of this, cancer cells can become "autophagy-dependent" to maintain their survival and progression; in this case, the autophagy will play role as a promoter for the cancer cells in advanced stages (Debnath, Gammoh and Ryan, 2023).

Autophagy is tightly regulated by the mechanistic target of rapamycin (mTOR), a serine/threonine kinase that functions as a key metabolic sensor. Belonging to the phosphatidylinositol 3-kinase-related kinase (PI3KK) superfamily, mTOR is an evolutionarily conserved protein that exists in two distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Of these, mTORC1 plays a central role in the regulation of autophagy, primarily by detecting changes in both extracellular and intracellular nutrient levels, particularly amino acids, as well as integrating signals from growth factors, cellular energy status, and oxygen availability (Rabanal-Ruiz, Otten and Korolchuk, 2017).

In nutrient-rich conditions, where amino acids and growth factors are plentiful, mTORC1 remains localized at the lysosomal membrane and actively inhibits autophagy. This inhibition is achieved through the phosphorylation of key components of the ULK1 complex, such as ULK1 itself and ATG13. Phosphorylation of ULK1 reduces its kinase activity, while that of ATG13 disrupts its functional role in complex assembly, collectively preventing the initiation of autophagy. In contrast, during periods of nutrient scarcity, mTORC1 activity diminishes and dissociates from the lysosome, leading to the dephosphorylation and activation of the

ULK1 complex. This activation facilitates the formation of the autophagy initiation complex, thereby triggering autophagic processes (Meng et al., 2021).

The initiation of autophagy is triggered by the assembly of the ULK1 complex, which includes ULK1, ATG13, FIP200 (Focal Adhesion Kinase Family Interacting Protein of 200kDa), and ATG101(Grasso, Renna and Vaccaro, 2018). ATG13 also interacts with Atg9, playing a role in directing Atg9-containing vesicles to the pre-autophagosomal structure (Levine and Kroemer, 2019).

Following the formation of this complex, ULK1 phosphorylates the Ambra1 protein, an event that promotes the recruitment of the BECLIN-1-PI3KC3 complex, also known as the BECLIN-1-Vps34 complex, which comprises BECLIN-1, Vps34, Vps15, and ATG14L. BECLIN-1 functions as a scaffold for the PI3KC3 complex, enabling the targeted localization of autophagy-related proteins to the phagophore membrane. This scaffold protein contains a BH3 domain that can be bound by anti-apoptotic proteins BCL-2 and BCL-XL, which interfere with the formation of the BECN1-Vps34 complex. Additionally, phosphorylation of Vps34 disrupts its interaction with BECN1. However, the presence of AMBRA1, Vps15, and ATG14 enhances the interaction between Vps34 and BECN1 (Levy, Towers, and Thorburn, 2017).

Vps34 plays a central role in synthesizing phosphatidylinositol 3-phosphate (PI3P), a lipid signal essential for recruiting effector proteins such as WIPI and DFCP1 to the autophagosome initiation site (Y. Liu *et al.*, 2023). The accumulation of PI3P-binding proteins at this location facilitates the recruitment of additional autophagy-related proteins (ATGs), which are required for the elongation and closure of the autophagosomal membrane (Kocaturk et al., 2019).

The elongation of the autophagosome membrane relies on two ubiquitin-like conjugation systems: the ATG12–ATG5–ATG16L complex and the ATG8 system, which involves microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3) conjugated to phosphatidylethanolamine (PE). In the first pathway, ATG12 is covalently linked to ATG5 through a process mediated by ATG7, functioning as an E1-like ubiquitin-activating enzyme, and ATG10, acting as an E2-like ubiquitin-conjugating enzyme. The resulting ATG12–ATG5 complex associates with ATG16L to form a multimeric complex that facilitates the second

conjugation system, where LC3 is linked to PE. Initially, LC3 is synthesized as Pro-LC3 and is cleaved by the cysteine protease ATG4 to generate LC3-I. This form is subsequently conjugated to phosphatidylethanolamine with the coordinated action of ATG7, ATG3 (an E2-like enzyme), and the ATG12–ATG5–ATG16L complex, which serves a role analogous to an E3 ligase. The lipidated form, known as LC3-II, becomes embedded in the autophagosomal membrane, playing a critical role in the biogenesis and elongation of the autophagosome (Gómez-Virgilio et al., 2022).

In addition to their involvement in the formation and maturation of autophagosomes, the ATG8/LC3/GABARAP family of proteins also functions as a crucial link between autophagic cargo and the core autophagy machinery. These proteins facilitate selective recognition and efficient sequestration of cargo into autophagosomes (Li et al., 2021).

In particular, LC3-II facilitates the attachment of cargo through adaptor proteins such as p62, which binds ubiquitinated substrates and directs them toward degradation via autophagy (Ascenzi et al., 2021).

Once the autophagosome is fully formed, it undergoes a maturation process that includes the encapsulation of cargo and subsequent fusion with the lysosome. This step is tightly regulated and involves several key molecular players, including SNARE proteins, Rab GTPases, and tethering factors. The fusion between the autophagosome and lysosome is mediated by two distinct SNARE complexes: STX17–SNAP29–VAMP8 and YKT6–SNAP29–STX7. Tethering factors, particularly the HOPS (homotypic fusion and vacuole protein sorting) complex, assist in this process by capturing vesicles and facilitating the proximity of SNARE proteins to the target membranes during intracellular trafficking. The HOPS complex specifically supports autophagosome-lysosome fusion by interacting directly with STX17, promoting the assembly of the SNARE machinery required for membrane fusion (Chen et al., 2023).

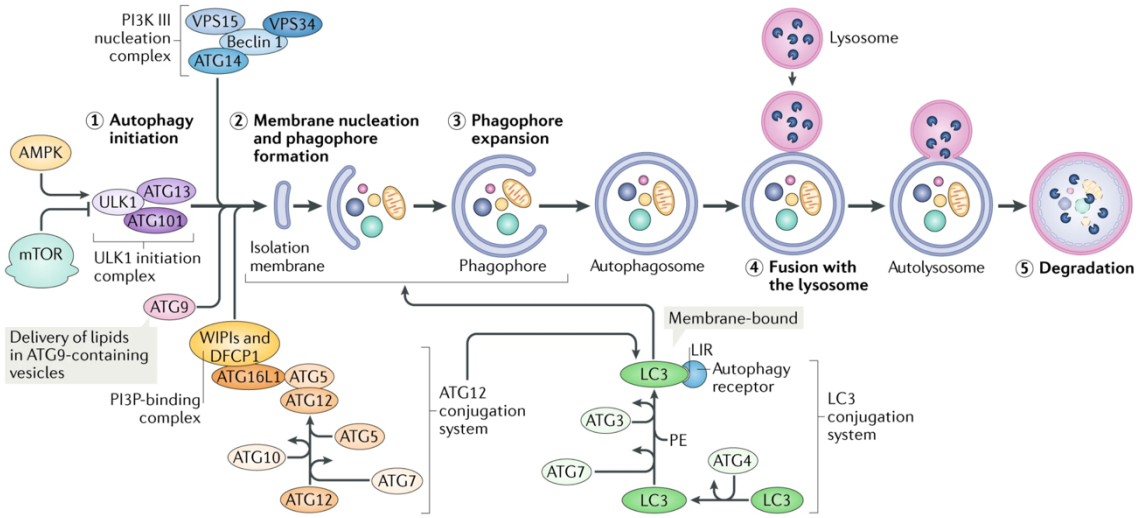

Figure 1. An illustration showing the process and key regulatory machinery of autophagy. adapted from (Hansen, Rubinsztein and Walker, 2018).

1.3. The gene: *BECN-1*

BECLIN-1 (*BECN1*), known as BCL2-interacting coiled-coil protein, was the first mammalian protein indicated to be involved in autophagy. In yeast, it is known as ATG6 or VPS30 (He *et al.*, 2013). The ground-breaking research paper published by the Levine lab described BECN1 as a BCL2 binding protein connecting the two fundamental pathways of autophagy and apoptosis (Liang *et al.*, 1998).

BECN1, located on chromosome 17q21 in proximity to *BRCA1*, is frequently mono-allelically deleted in various types of cancer, including breast tumors, ovarian tumors, and prostate tumors. This suggests that the deletion of *BECN1* may play a role in the development of multiple cancer types (Liang *et al.*, 1999). This pattern supports its classification as a haploinsufficient tumor suppressor, where the loss of a single gene copy contributes to tumor development (Liang et al., 1999Tang *et al.*, 2015). Animal studies using Becn1$^{+/-}$ mice have shown a higher incidence of spontaneous tumors, including basal-like mammary carcinomas (Cicchini *et al.*, 2014).

Functionally, BECLIN-1 is vital for both the initiation and regulation of autophagy and is a key focus in cancer research. It forms two major complexes: PI3KC3 Complex I, which initiates autophagosome formation, and PI3KC3 Complex II, which supports autophagosome

maturation. Autophagy can be inhibited by Rubicon, which blocks the function of Complex II. Additionally, BECLIN-1 activity is regulated through its interaction with anti-apoptotic proteins Bcl-2 and Bcl-xL, which bind to its BH3 domain and suppress autophagy. Under stress conditions, such as starvation, phosphorylation by enzymes like JNK1, DAPK, ROCK1, and MK2/3 disrupts this inhibitory binding, allowing autophagy to proceed. Conversely, the kinase Mst1 enhances the interaction between BECLIN-1 and Bcl-2, maintaining autophagy suppression (Figure 2) (Vega-Rubin-de-Celis, 2019).

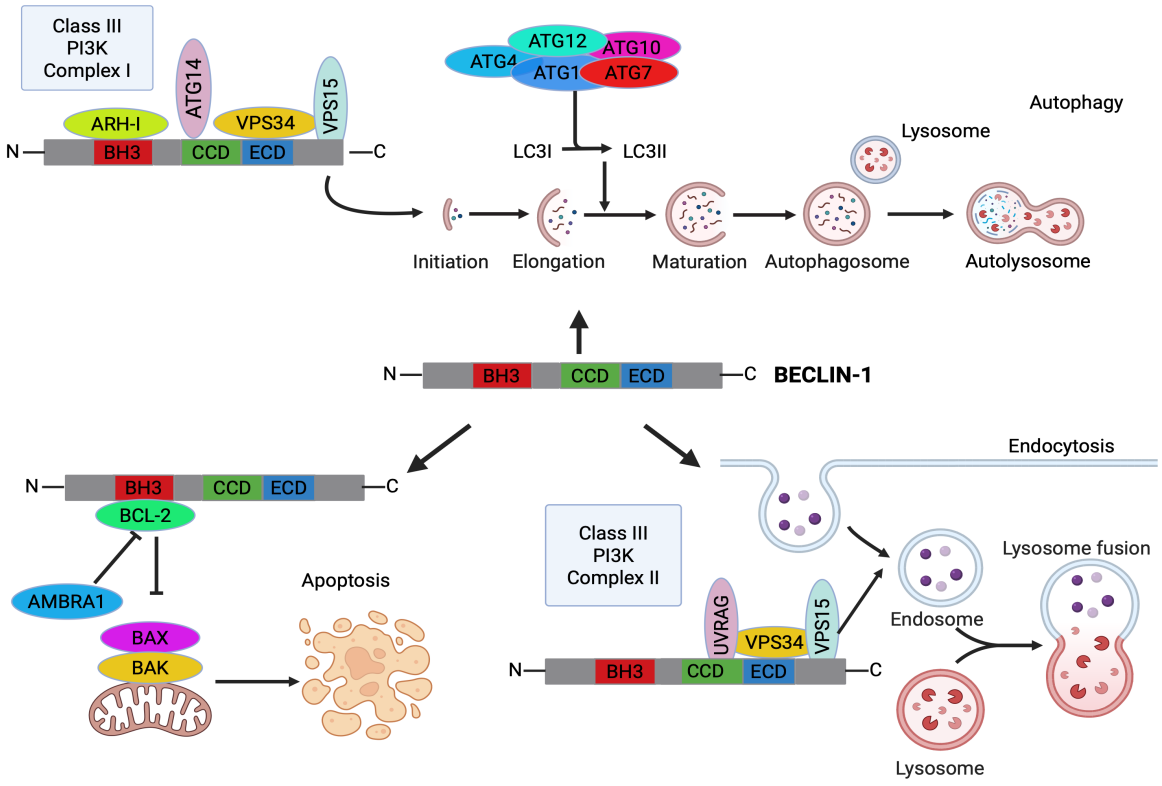

Figure 2. An illustration indicating that BECLIN-1 plays roles in Autophagy, Apoptosis and Endocytic trafficking (Maheshwari *et al.*, 2025).

i) BECLIN-1 *Structure:*

BECLIN-1 is a 450-amino-acid protein that plays a key role in autophagy, the process by which cells recycle their components. It has three main regions. The first is the N-terminal region (residues 1–150), which is flexible and contains sites where it can be phosphorylated. This region also includes a BH3 domain that allows BECLIN-1 to interact with BCL-2 family proteins, which are important for cell survival. The second region is the coiled-coil (CC) domain (residues 144–269), which helps BECLIN-1 bind to other proteins like UVRAG or ATG14. Sometimes, BECLIN-1 can bind to itself through this domain, forming unstable pairs, but when it binds to UVRAG or ATG14, the resulting complexes are more stable. The BECLIN-1-UVRAG complex is especially stable and may be favored under certain conditions. The third region is the BARA domain (residues 269–450), which overlaps with what's called the Evolutionarily Conserved Domain (ECD). This part of the protein is important for attaching to cellular membranes and for interacting with the VPS34 lipid kinase, which is necessary for autophagy. Specific amino acids in the BARA domain (F359, F360, and W361) help BECLIN-1 bind to membranes. If this region is mutated, BECLIN-1 can't bind VPS34, leading to problems with autophagy and loss of its tumor-suppressing function. Additionally, a protein called RUBICON can bind to the BARA domain and block BECLIN-1 from interacting with membranes, which reduces autophagy. Overall, the structure of BECLIN-1 allows it to interact with different proteins and membranes, enabling it to play its central role in autophagy (figure 3) (Tran, Fairlie and Lee, 2021; Ye *et al.*, 2023).

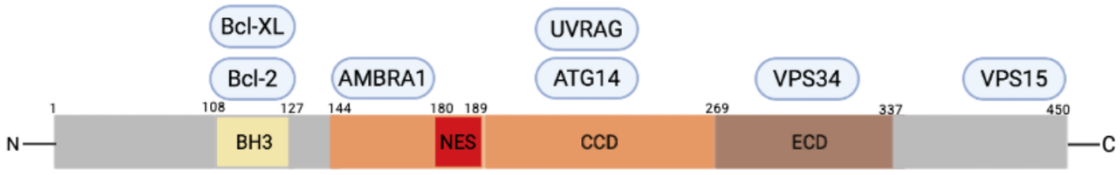

Figure 3. The structure of BECLIN-1. BECLIN-1 structure contains three identifiable domains, such as BH3 motif, CCD and ECD (Maheshwari *et al.*, 2025).

ii) *BECN1* Post-Transcriptional Modifications

The DNA sequencing of AML cells performed by Niu and colleagues demonstrated the existence of a 3' alternative splice *BECN1* variant, in which exon 11 is skipped, resulting in a truncated C terminus (Niu *et al.*, 2014). Another group discovered an additional *BECN1* splice variant that lacks both exon 10 and 11 and is designated *BECN1* short isoform (*BECN1*s). It has been reported in multiple cell types. Whereas *BECNI*s is unable to initiate macroautophagy, its expression supports mitophagy. It was later shown that *BECN*1s selectively associates with the outer mitochondrial membrane to drive PINK1-PRKN/PARKIN-dependent mitophagy (Cheng *et al.*, 2015). Our group subsequently identified two additional splice variants, *BECN1*β and *BECN1***γ**. *BECN1*β lacks exons 5, 6, 10, and 11, while *BECN1***γ** lacks exons 5, 6, and 11. They found that overexpression of the BECN1β isoform reduces autophagy with a dominant negative effect over the endogenous *wild-type BECN1,* while overexpression of *BECN1***γ** has little effect on autophagy (Maheshwari *et al.*, 2022).

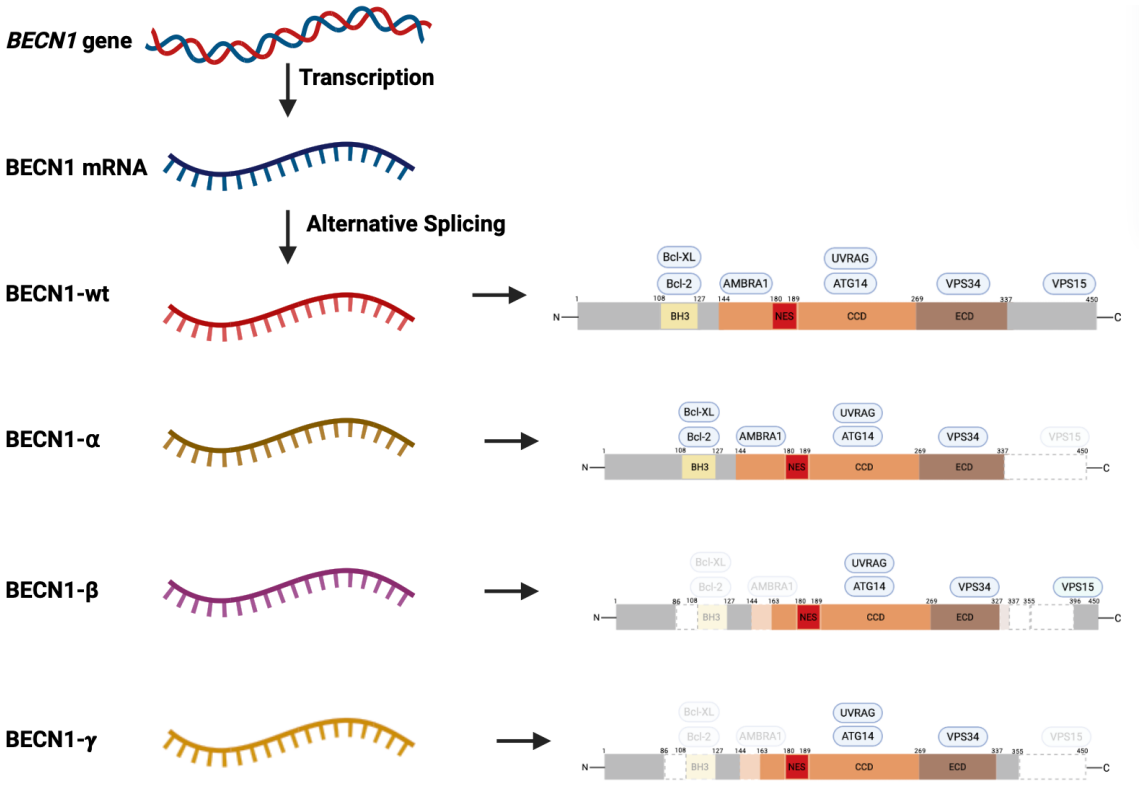

Figure 4. BECN1 (wild-type or wt) and the alternatively spliced isoforms Alpha (α), Beta (ß), and Gamma (ɣ)(Maheshwari *et al.*, 2025)

iii) BECLIN-1 Post-Translational Modifications:

Post-translational modifications (PTMs) are essential regulatory mechanisms that significantly influence protein function. These chemical modifications, which occur after protein synthesis, are critical for maintaining proper protein structure and activity in both normal and disease contexts. It is estimated that between 50% and 90% of proteins in human cells undergo various types of PTMs (Ramazi and Zahiri, 2021). Classical modifications such as phosphorylation, ubiquitination, methylation, and acetylation, as well as more recently characterized nonclassical modifications like glycation, malonylation, and succinylation, play pivotal roles in cellular processes. These modifications can modulate protein activity, stability, localization, and signal transduction of proteins under physiological and pathological conditions.

Increasing evidence indicates that PTMs are deeply involved in the regulation of the tumor microenvironment, including processes such as immune cell activation, metabolic reprogramming, and tumor cell proliferation. Alterations in PTMs of key oncogenes, tumor suppressor genes, or other critical regulatory proteins can directly impact cancer initiation, progression, treatment response, and patient prognosis (Teng et al., 2023).

BECLIN-1 is a key protein in the regulation of autophagy, and its function is tightly controlled by post-translational modifications, mainly phosphorylation and ubiquitination. These modifications can either promote or inhibit autophagy, depending on the type and location of the modification. Phosphorylation of BECLIN-1 by kinases such as ULK1 and AMPK (at sites like Ser15 and Ser90) activate autophagy. It does so by enhancing BECLIN-1's interaction with the VPS34 complex and reducing its binding to the inhibitory protein BCL2. On the other hand, phosphorylation by AKT at Ser234 and Ser295 suppresses autophagy. This happens because AKT increases BECLIN-1's binding to 14-3-3 proteins, which block its pro-autophagic activity. Similarly, tyrosine phosphorylation by kinases like EGFR or HER2 reduces BECLIN-1's ability to form complexes necessary for autophagy, leading to its inhibition. Ubiquitination also plays a dual role. Lys63-linked ubiquitination (by ligases like TRAF6 or TRIM50) activates autophagy by stabilizing BECLIN-1 and enhancing its activity. In contrast, Lys48-linked ubiquitination can lead to BECLIN-1

degradation and inhibit autophagy. Enzymes such as A20 and USP14 can remove ubiquitin chains, helping to maintain the proper balance of BECLIN-1 activity. These modifications allow cells to finely adjust autophagy in response to stress, nutrients, and growth signals (Kim et al., 2011; Russell et al., 2013; Hill, Wrobel and Rubinsztein, 2019).

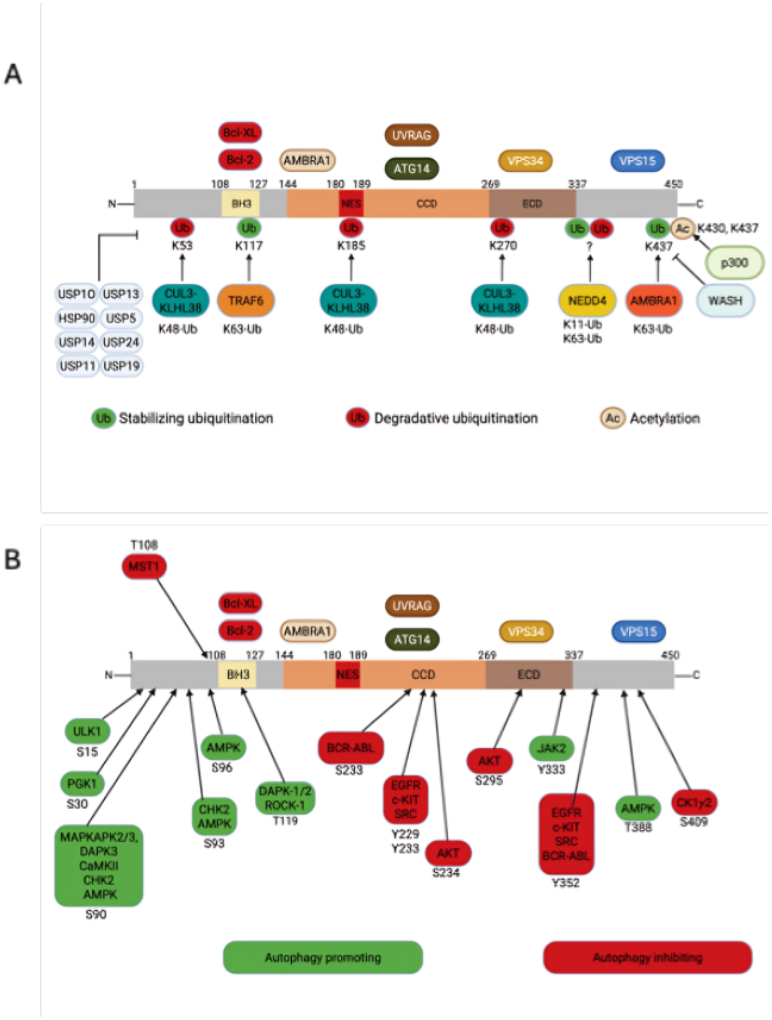

Figure 5. The illustration above shows site-specific post-translational modification sites on the BECLIN-1 protein. The picture graphically represents the modifications related to ubiquitination, acetylation (**A**), and phosphorylation (**B**) of the BECLIN-1 protein function (Maheshwari *et al.*, 2025).

1.4. Metastasis and Autophagy

Metastasis is a multistep and highly complex process in which cancer cells disseminate from the primary tumor and establish secondary lesions in distant organs (Van Denderen and Thompson, 2013; Guan, 2015). It represents the major cause of BC related mortality, accounting for nearly 90% of deaths, and is particularly associated with aggressive subtypes such as triple-negative and HER2-positive disease (Chaffer and Weinberg, 2011; Bachmann *et al.*, 2015; Jin and Mu, 2015).

Recent studies demonstrate that autophagy is a key modulator of metastatic behavior in BC. Similar to its role in other malignancies, its impact is highly context-dependent, capable of either suppressing or promoting the metastatic process depending on disease stage (Dower *et al.*, 2018). In early carcinogenesis, basal autophagy generally acts as a tumor suppressor by maintaining cellular integrity, removing damaged organelles and oncoproteins, and supporting anti-tumor immune surveillance (Deretic, Saitoh and Akira, 2013; Ma *et al.*, 2013). However, as tumors progress, autophagy undergoes a functional shift and acts as a pro-survival mechanism that actively promotes metastasis (Galluzzi *et al.*, 2015; Huang *et al.*, 2018). This function is critical for allowing BC cells to resist lethal microenvironmental stresses, including hypoxia, anoikis, and starvation, thereby supporting the survival of circulating tumor cells, maintaining the viability of cancer stem cells, and contributing to therapeutic resistance(Degenhardt *et al.*, 2006; Niklaus *et al.*, 2021).

Given its pro-survival shift in advanced disease, autophagy has emerged as a promising therapeutic target for metastatic BC, as pharmacological inhibition or knockdown of autophagy-related genes can sensitize tumor cells to stress and induce cell death (Zhou, Rucker and Zhou, 2016). Increasing evidence further demonstrates that autophagy inhibition can enhance the efficacy of existing therapies, including endocrine treatments, HER2-directed agents, or mTOR pathway inhibitors, with combined approaches overcoming treatment resistance and improving clinical response in metastatic disease (Alayev *et al.*, 2015; Caswell-Jin *et al.*, 2018).

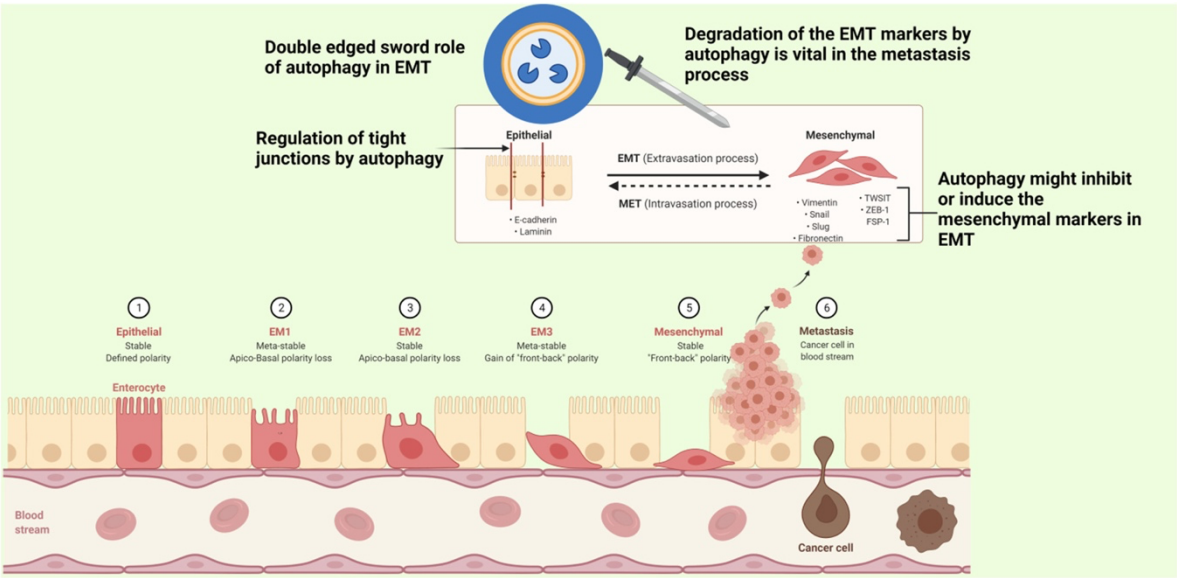

Figure 6. The illustration above highlights the roles of autophagy in metastasis (Gundamaraju *et al.*, 2022).

AIM OF THE STUDY

2. Aim of the study:

A defining hallmark of cancer progression is the ability of tumor cells to invade surrounding tissues and disseminate to distant organs, ultimately giving rise to metastatic lesions. The metastatic cascade is a highly dynamic and inefficient process that requires tumor cells to overcome multiple biological barriers, including local invasion, survival under detachment-induced stress (anoikis resistance), adaptation to altered extracellular matrix interactions, and successful colonization of secondary sites. Autophagy has emerged as a critical regulator of these processes.

This study aims to investigate the relationship between autophagy and metastatic potential in BC by analyzing BECLIN-1 expression across different cell models. Using the 3D culture environment and subsequently re-plating them into conventional two-dimensional (2D) culture, we compare non-tumorigenic (MCF-10A), low-metastatic (MCF-7), and highly metastatic (MDA-MB-231) breast cell lines to determine how BECLIN-1 influences cellular adaptation and survival during metastatic dissemination and colonization.

MATERIALS AND METHODS

3. Materials and methods

3.1. Cell culture and treatments

To investigate the alternative splicing of the BECN1 gene, three breast-derived cell lines were employed: MDA-MB-231, MCF-10A, and MCF-7. MDA-MB-231 cell line represents a highly aggressive TNBC and was originally established from a metastatic pleural effusion obtained from a 51-year-old White female diagnosed with mammary adenocarcinoma. In contrast, MCF-10A is a non-tumorigenic breast epithelial cell line, derived in 1984 from the mammary tissue of a 36-year-old White female with fibrocystic breast disease, and is commonly used as a model of normal breast epithelium.

The MCF-7 cell line, representative of luminal A estrogen receptor–positive (ER^+) BC, was derived in 1970 from the pleural effusion of a 69-year-old White female with metastatic breast adenocarcinoma. MCF-7 cells maintain functional hormone receptor pathways, display low metastatic potential, and are widely utilized as a model of hormone-responsive BC. All cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

MDA-MB-231 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% horse serum, 1% L-glutamine, 1% penicillin/streptomycin, 20 ng/mL epidermal growth factor (EGF), 10 µg/mL insulin from bovine pancreas, 50 µg/mL hydrocortisone, and 100 ng/mL cholera toxin.

MCF-7 cells, representing luminal A hormone-responsive BC, were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and non-essential amino acids (NEAA). The MEM formulation provides additional metabolic support required for optimal proliferation and maintenance of the epithelial characteristics of MCF-7 cells.

All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown until approximately 80% confluence before being harvested for experimental procedures. To ensure adequate nutrient supply, the medium was changed every 48 hours.

Prior to each assay, cells were washed with phosphate-buffered saline (PBS) and detached using a 100× trypsin solution (Sigma) dissolved in Puck's Buffer. Trypsin facilitates cell detachment by cleaving anchoring proteins, enabling resuspension of the monolayer and subsequent replacing.

3.2. Spheroid-forming assay

For 3D multicellular spheroid formation, cells were cultured in specialized 12-well plates that were pre-coated with 5 mg/mL poly (2-hydroxyethyl methacrylate) (poly-HEMA, cod. P3932; Sigma-Aldrich) to inhibit cell adhesion. To prepare the coating, a poly-HEMA stock solution (120 mg/mL) was made by dissolving the polymer in 95% ethanol and rotating it overnight at 50 °C. The following day, the stock solution was diluted in 95% ethanol to obtain the working concentration of 5 mg/mL. The wells were coated with this solution and left to dry completely under a biological safety hood.

After coating, cells were seeded at a density of 100,000 cells/cm² and cultured for four days post-treatment. Fresh culture medium, supplemented with Spautin-1 (10 µM) was gently added every 48 hours. The development of spheroids was monitored at each time point using a phase-contrast microscope. Spheroid areas were measured using ImageJ software (version 1.52) and expressed in arbitrary units (A.U.).

For the 3D-to-2D transition experiment, MCF-10A, MCF-7 and MDA-MB-231 cells were first grown as mammospheres in poly-HEMA-coated 6-well plates for four days. On the fourth day, the 3D aggregates were collected by centrifugation and then reseeded onto standard adherent 6-well plates. Their ability to adhere and proliferate in 2D conditions was observed over 48 hours through microscopic imaging. After this incubation period, the cells were lysed in NADOC lysis buffer for subsequent Western blot analysis.

3.3. Bradford assay

The Bradford assay was used to measure the protein concentration in cultured cells. Once the cells reached the desired confluency, they were lysed using 300 µL of NADOC lysis buffer per P35 Petri dish. This buffer contained 0.2% NADOC (a detergent that disrupts cell membranes), a Protease Inhibitor Cocktail (1000x), and phosphatase inhibitors (Na_3VO_4 and

NaF) to preserve protein integrity. The cells were incubated with the lysis buffer on ice for 30 minutes, after which the lysates were subjected to sonication to ensure thorough homogenization and release of cellular proteins.

For the Bradford assay, a standard curve was generated using serial dilutions of bovine serum albumin (BSA) (Table 2). Experimental samples were prepared by diluting 5 µL of the cell lysate in 45 µL of distilled water, according to the established protocol. Then, 950 µL of Bradford reagent was added to each sample, followed by incubation for 30 minutes at 37 °C. Protein concentration was measured by recording absorbance at 595 nm using the NanoDrop™ 2000 Spectrophotometer and standard cuvettes.

Standard Curve	**BSA 0.2mg/ml**	**H_2O**	**Lysis Buffer**	**Sample**	**Bradford Solution**
1µg A	5µL	40µL	5µL	/	950µL
2µg B	10µL	35µL	5µL	/	950µL
3µg C	15µL	30µL	5µL	/	950µL
4µg D	20µL	25µL	5µL	/	950µL
5µg E	25µL	20µL	5µL	/	950µL
6µg F	30µL	15µL	5µL	/	950µL
BLANK	/	45µL	5µL	/	950µL
SAMPLES	/	45µL	/	5µL	950µL

Table 2. Protein Dosage with Bradford Assay.

3.4. Western blot assay

Cell monolayers or spheroids were grown in sterile 6-well plates, either uncoated or coated with poly-HEMA to prevent adhesion. Following treatment, cells were washed with cold phosphate-buffered saline (PBS) and lysed using a NADOC lysis buffer composed of 0.2 % NADOC, supplemented with a Protease Inhibitor Cocktail and phosphatase inhibitors (Na_3VO_4 and NaF). The lysates were then sonicated, and protein concentrations were determined using the Bradford assay. For each sample, 25 µg of total protein was mixed with 5× loading buffer and heated at 95 °C for 10 minutes.

Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, run first at 80 V for 30 minutes, then at 120 V. After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad) using a wet transfer system at 100 V for 2 hours. Membranes were blocked with 5% skim milk for one hour at room temperature and then incubated overnight at 4 °C with the appropriate primary antibodies. The next day, membranes were incubated with HRP-conjugated secondary antibodies (anti-rabbit IgG-HRP or anti-mouse IgG-HRP; Bio-Rad) for at least one hour.

Detection of protein bands was performed using a luminol-based chemiluminescent substrate (PerkinElmer Inc., Waltham, MA, USA), and signals were visualized with the ChemiDoc XRS Imaging System (Bio-Rad). Densitometric analysis of the bands was carried out using Quantity One Software (Bio-Rad).

3.5. Antibodies

For immunoblotting analysis, we used a rabbit polyclonal anti-BECN1 antibody (PA5-96649, Invitrogen), which targets the amino acid region 1–280 of the BECN1 protein. Additionally, a mouse monoclonal anti-β-Actin antibody (A5441, Sigma-Aldrich) was used as a cytoplasmic loading control, along with a mouse monoclonal anti-Histone H3 antibody (61475, Active Motif) for nuclear protein detection.

3.6. Bioinformatic analysis

All clinical data and gene copy number variation data were retrieved from the cBioPortal database (https://www.cbioportal.org, last accessed on 1 October 2025). Two large independent, publicly available breast cancer cohorts were analyzed: BRCA-METABRIC and TCGA-BRCA.

Scatter plots were employed to show the correlation between the expression of relevant biomarkers in the patient cohort. Regression was estimated by calculating Spearman's correlation coefficient (r) and the relative *p*-values.

Statistical analyses were performed by R (v.4.4.2; R Foundation for Statistical Computing, Vienna, Austria). Kaplan-Meier curves were visualized from the 'survival' package (v.3.8-3).

3.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, CA, USA).

RESULTS

4. Results

4.1. *BECN1* expression is negatively correlated with pro-EMT genes and positively correlated with genes related to adhesion.

To investigate the potential role of *BECN1* in regulating metastasis in breast cancer, we examined the relationship between *BECN1* mRNA expression and key genes associated with the epithelial–mesenchymal transition (EMT). Using the TCGA-BRCA dataset accessed through cBioPortal, we performed correlation analyses between *BECN1* expression and established pro-EMT markers, including *VIM, SNAI1,* and *MMP1*. Notably, *BECN1* expression was negatively correlated with these pro-EMT genes (Figure 5A), suggesting that *BECN1* may antagonize EMT-associated transcriptional programs in breast cancer. Interestingly, it showed a positive correlation with cell adhesion molecules, including *CDH1, ITGB1,* and PTK2 (Figure 5B), hinting that *BECN1* may help maintain a more epithelial, less invasive state.

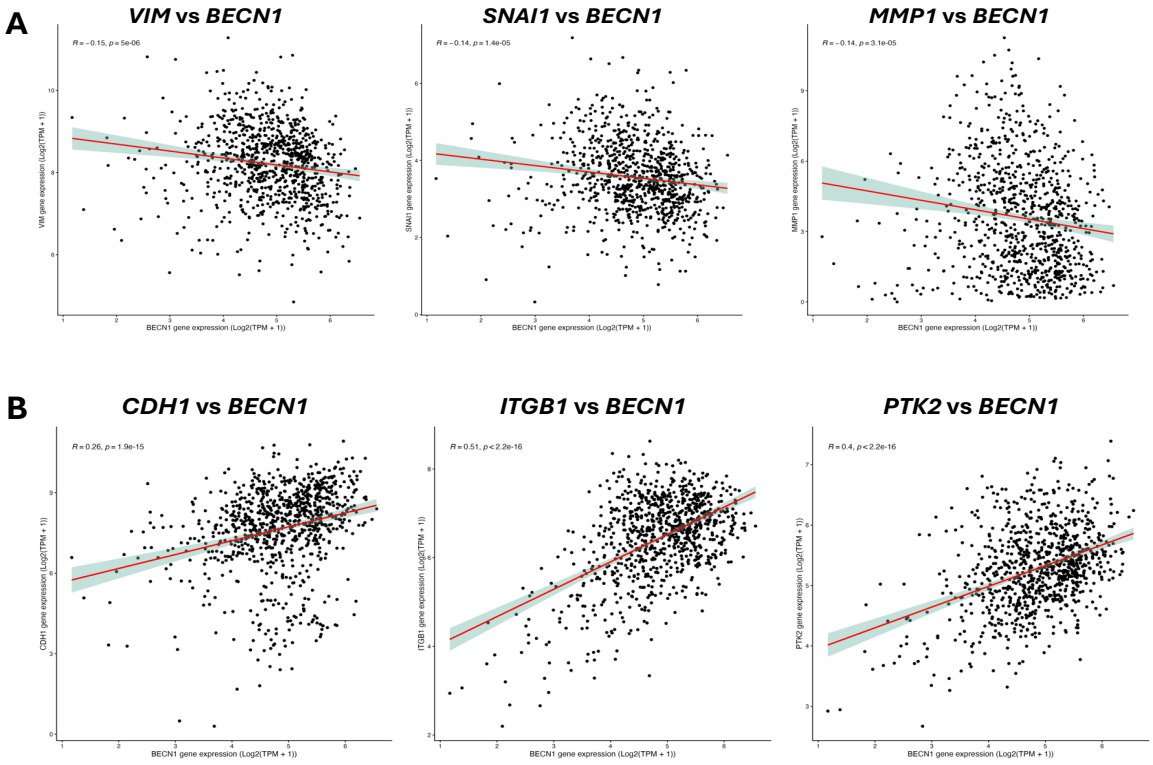

Figure 5 . Correlation of BECN1 expression with EMT-related and adhesion-related genes in the TCGA-BRCA breast cancer cohort.

4.2. 2D-to-3D-to-2D shift reveals differential metastatic competence among breast cell lines

To mimic critical stages of the metastatic process in vitro, the breast cell lines MCF-10A, MCF-7, and MDA-MB-231 were subjected to a sequential 2D-to-3D-to-2D transition assay. After forming 3D spheroids, cells were re-plated onto standard 2D adherent surfaces. This step was essential to assay their metastatic competence by measuring key functional characteristics: their capacity for attachment, cellular spreading, and the formation of secondary colonies. MCF-10A spheroids maintained their spherical form up to Day 4 of transfer, failing to adhere or produce secondary colonies (Figure 6). In contrast, MCF-7 spheroids showed partial spreading and limited secondary colony formation after reattachment, reflecting their intermediate metastatic capability. MDA-MB-231 cells demonstrated the most aggressive behaviors, rapidly attaching, extensively spreading, and forming robust secondary colonies consistent with their highly metastatic nature.

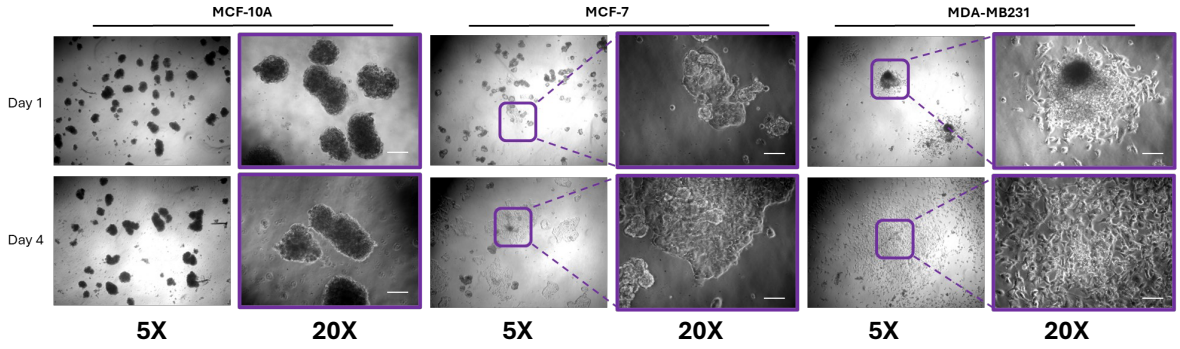

Figure 6. Differential metastatic ability among breast cell lines. MCF-10A, MDA-MB-231, and MCF-7 were cultured as spheroids for 4 days and shifted back to adherent conditions to observe secondary colony formation for 4 days. Phase contrast Images were taken at Day 1 and Day 4. Images captured at 5× and 20× magnification (scale bar = 100 µm).

4.3. 3D-to-2D switch reverses BECLIN-1 levels in TNBC MDA-MB-231 cells.

We investigated how the shift between different culture conditions would impact autophagy. To understand the changes in autophagy, we analyzed by western blotting the relative expression of BECLIN-1 and LC3II/LC3I ratios during the shift from 2D to 3D and then back to 2D cell culture.

In MCF-10A, BECLIN-1 levels are maintained similarly across 2D, 3D, and 3D-to-2D conditions. However, we do observe a slight decrease in the autophagy flux from 2D to 3D as shown by the decrease in the LC3II/LC3I ratio, which subsequently rises sharply (5.5) after the 3D-2D switch (Figure 7).

In MCF-7, BECLIN-1 expression follows the same trend as MCF-10A, remaining relatively stable during the shift from 2D to 3D and upon replating of the spheroids back to 2D (3D-2D). The LC3II/LC3I ratio increases only slightly from 2D (3.7) to 3D (4.1) but then drops upon the 3D-2D switch (2.6).

In comparison with the earlier cell lines, with MDA-MB231, the BECLIN-1 levels decreased significantly in the 3D model, then rose again after the 3D-2D transition. However, the autophagy flux did not change significantly across the different culture conditions.

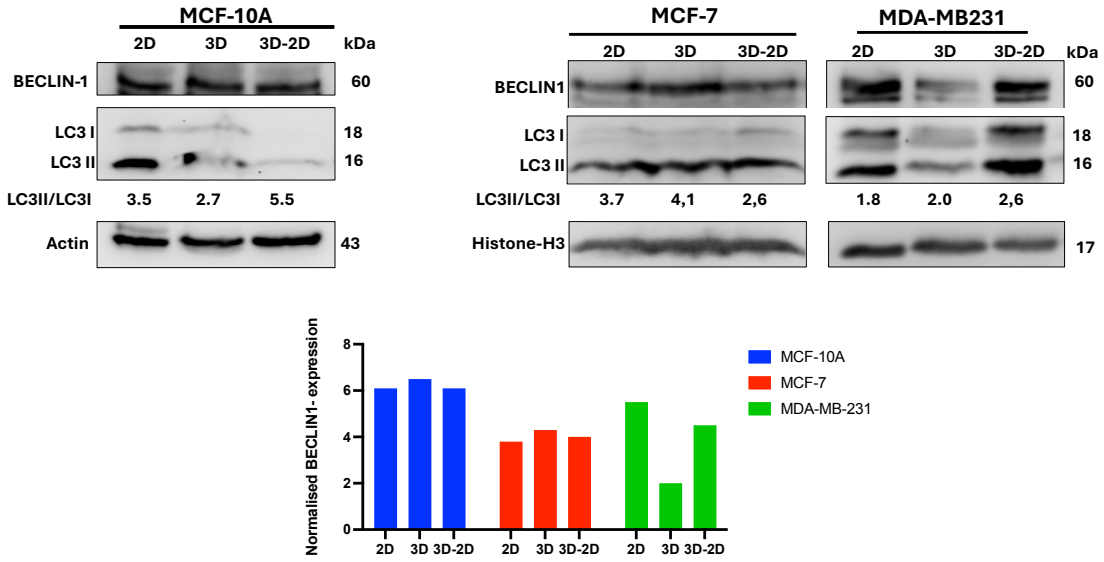

Figure 7. Reversal of BECLIN-1 levels in MDA-MB-231 cells. MCF-10A, MDA-MB-231, and MCF-7 were cultured as spheroids for 4 days and shifted back to adherent conditions to observe secondary colony formation for 4 days. BECLIN-1 and LC3 in MCF-10A, MCF-7 and MDA-MB-231 cells cultured in 2D, 3D and 3D to 2D (3D-2D). BECLIN-1, LC3 and Histone (H3) as a loading control was analyzed by immunoblotting of cell homogenates. Densitometric analysis of the immunoblotting was represented as BECLIN-1/Histone (H3), BECLIN-1/β-Actin and LC3 II/LC3 I ratios as a measure of autophagosome formation.

4.4. Spautin-1 induces preferential cytotoxicity in BECLIN-1-deficient BC cells

To further investigate the role of BECLIN-1 in breast cancer cell survival, MDA-MB231 cells (with diploid *BECN1*) and MCF-7 cells (which specifically harbor a monoallelic deletion of the *BECN1* gene) were exposed for 24 hours to 10 μM Spautin-1. Spautin-1 promotes BECLIN-1 ubiquitination and subsequent proteasomal degradation, thereby preventing the formation of the VPS34-BECLIN-1 complex, which is essential for autophagosome formation. Upon treatment, pronounced differences in survival were observed between the two cell lines: MDA-MB-231 cells maintained their normal cell morphology and viability under both conditions, demonstrating resistance to Spautin-1-induced cytotoxicity (Figure 8). In contrast, MCF-7 cells exhibited substantial loss of viability, with widespread cell death and morphological deterioration, suggesting a heightened sensitivity to disruption of BECLIN-1-mediated autophagy (Figure 8).

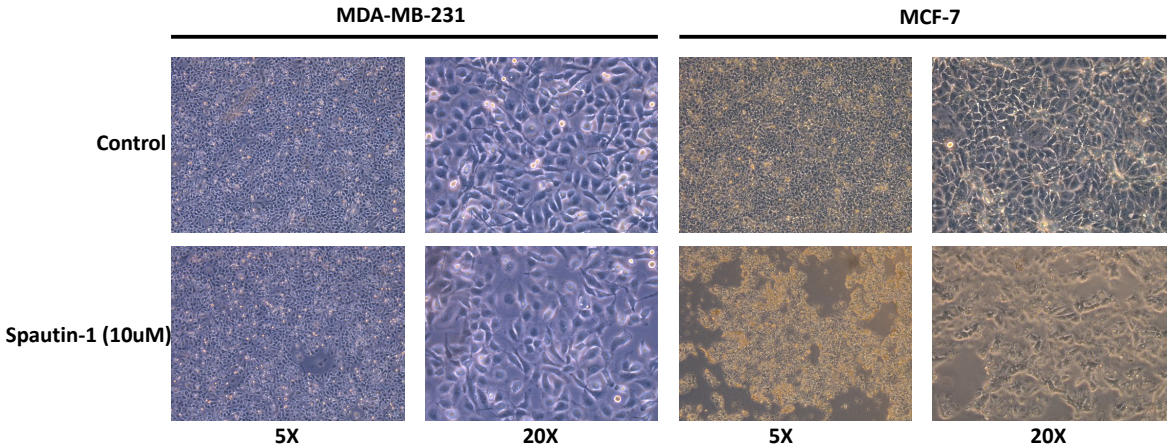

Figure 8. Spautin-1 treatment reduces the viability of BECLIN-1-deficient MCF-7 cells. MDA-MB-231 cells were treated with or without 10uM for 24 hours. The phase contrast images were taken at the end of the treatment. Magnification = 5x and 20x.

4.5. BECLIN-1 depletion impairs 3D growth of MDA-MB-231 mammospheres

Since MCF-7 cells exhibited heightened sensitivity to Spautin-1, we continued with MDA-MB231 for the subsequent experiments. To assess the role of BECLIN-1 in the growth and maintenance of BC cell spheroids, MDA-MB-231 mammospheres were cultured in suspension and treated with 10 μM Spautin-1 for four days. In untreated control conditions, mammospheres exhibited continuous growth from day 0 to day 4 (Figure 9A). In contrast, Spautin-1 treatment led to a marked reduction in mammosphere size and viability, reflecting compromised 3D growth (Figure 9A). Western blot analysis confirmed that Spautin-1 treatment decreased BECLIN-1 expression levels, along with only slight changes in the LC3II/LC3I ratio. These results collectively demonstrate that BECLIN-1 is essential for the sustained proliferation and survival of MDA-MB-231 mammospheres (Figure 9B).

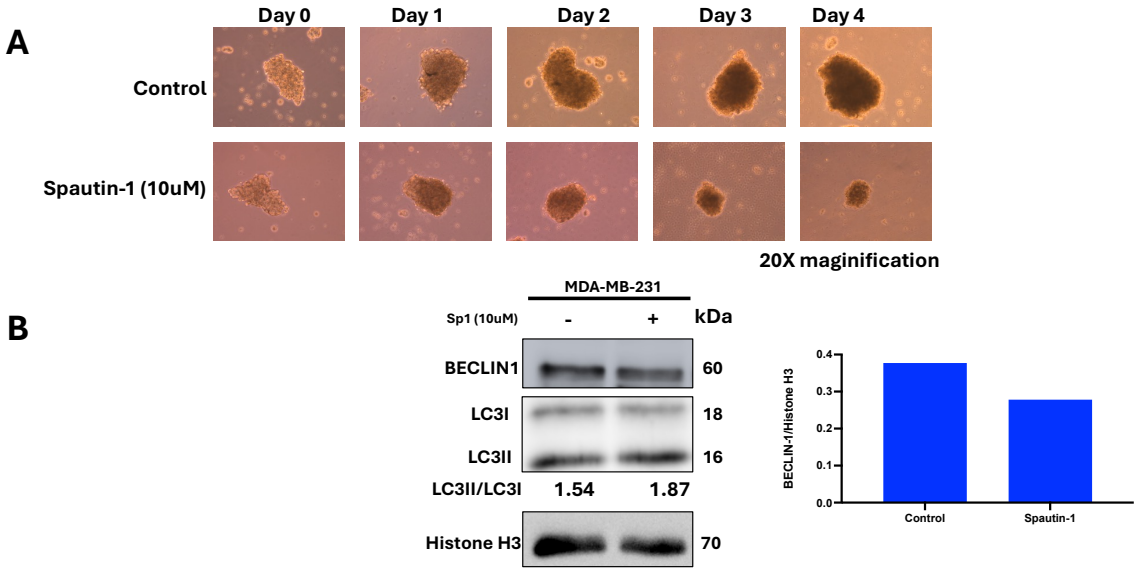

Figure 9. Spautin-1 impairs the growth of MDA-MB-231 mammospheres. (A) MDA-MB-231 spheroids were cultured with or without 10μM spautin-1 for 4 days. Images were taken at time points Day 1, Day 2, Day 3, and Day 4. Magnification = 20x. (B) BECLIN-1, LC3, and Histone (H3) as a loading control were analyzed by immunoblotting of cell homogenates. Densitometric analysis of the immunoblotting was represented as BECLIN-1/Histone (H3) and LC3 II/LC3 I ratios as a measure of autophagosome formation.

4.6. BECLIN-1 is required for reattachment and growth of MDA-MB-231 mammospheres in 2D Conditions:

To evaluate how BECLIN-1 depletion influences the ability of MDA-MB-231 mammospheres to reattach and grow under 2D adherent conditions, spheroids treated with or without Spautin-1 were transferred for reattachment analysis over two days. The untreated spheroids maintained the ability to reattach and form secondary colonies at both day 5 and day 6, indicating preserved adhesive and proliferative capacity (Figure 10). In contrast, Spautin-1–treated spheroids exhibited greatly impaired reattachment and limited colony expansion, underscoring a deficiency in adherence and 2D outgrowth when BECLIN-1 protein is reduced. This result further illustrates that partial BECLIN-1 depletion compromises not only the spheroid growth but also the reattachment when transferred from non-adherent to adherent conditions.

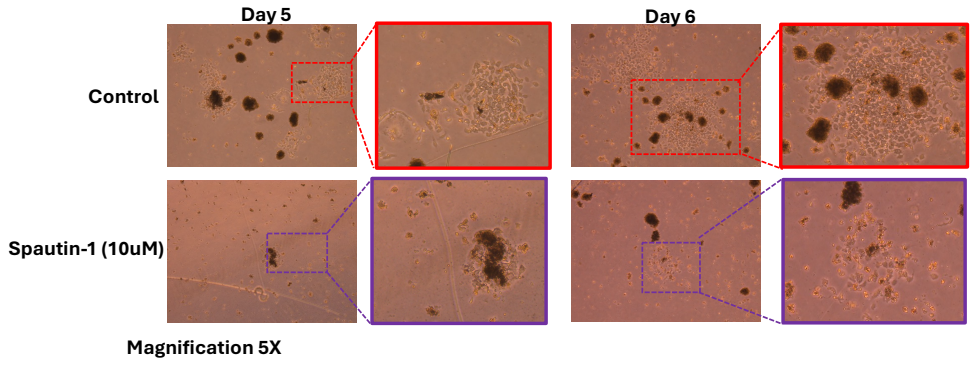

Figure 10. Partial depletion of BECLIN-1 by Spautin-1 impairs reattachment and 2D outgrowth of MDA-MB-231 mammospheres. Images were taken at time points 24h, 48h, 72h and 96h. Magnification = 20x.

4.7. Shallow deletion of *BECN1* confers an overall poor prognosis in Breast Cancer patients.

As reported above, *BECN1* is located on chromosome 17q21, a region frequently deleted in human breast cancers, and is a well-known haploinsufficient tumor suppressor. So, we decided to evaluate the clinical significance of *BECN1* copy number status in breast cancer. We interrogated the CBioPortal database (https://www.cbioportal.org) to download copy number variation data of *BECN1* from two breast cancer datasets, Molecular Taxonomy of Breast Cancer International Consortium (BRCA-METABRIC) and The Cancer Genome Atlas (TCGA-BRCA). Patients from both cohorts were stratified based on *BECN1* CNV status into diploid and shallow deletion, and Kaplan-Meier survival analyses were performed. In the METABRIC dataset, *BECN1* shallow deletion was associated with a significantly worse overall survival compared to diploid *BECN1* status ($p < 0.001$), with the TCGA-BRCA dataset showing the same trend, although not significantly ($p = 0.65$). Our findings indicate that the loss of *BECN1* indeed leads to a more aggressive disease phenotype.

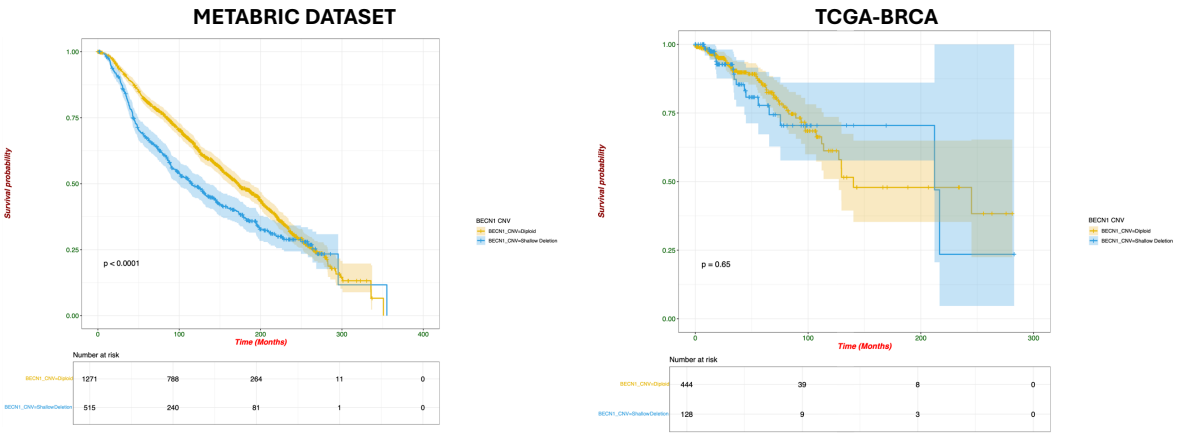

Figure 11. Survival curves based on *BECN1* CNV status. Comparison of overall survival between *BECN1* diploid and shallow deletion groups across two major clinical datasets. Statistical significance is observed in the METABRIC cohort ($p < 0.001$), while TCGA-BRCA shows a consistent non-significant trend ($p = 0.65$).

DISCUSSION

5. Discussion

The relationship between autophagy and cancer progression is characterized by functional duality, shifting from suppressing to promoting tumors depending on the cellular environment and disease stage (Li, He and Ma, 2020). Metastasis in cancer remains the major cause of death from cancer. Although the role of autophagy in metastasis is being brought to light, it is still unclear whether autophagy is pro- or antimetastatic (Mowers, Sharifi and Macleod, 2017). In this study, we focused on BECLIN-1 (*BECN1*), which is a pivotal regulator of autophagy, to understand its specific impact on breast cancer metastasis. By combining bioinformatic analysis with adherent and non adherent cell culture conditions of breast cancer cell lines, we aimed to explore how this autophagy regulator influences the metastatic process in Breast Cancer.

Our initial look at the TCGA-BRCA dataset revealed a distinct pattern: *BECN1* expression appears to act as a brake on the EMT program. We found that when *BECN1* levels are high, pro-invasive genes like *VIM*, *SNAI1*, and *MMP1* tend to remain suppressed. Conversely, genes responsible for cell-to-cell adhesion, such as *CDH1*, *ITGB1*, and *PTK2*, showed a positive correlation. Essentially, a cell with high *BECN1* expression maintains an epithelial profile and a less invasive phenotype, aligning with the tumor suppressor role of autophagy.

Current research favors 3D cultures for their ability to mimic realistic biochemical and biomechanical microenvironments (Esposito et al Int J Cell Biol 2023). These models recreate in vivo-like oxygen and nutrient gradients that impact on autophagy. We utilized a 2D to 3D to 2D transition model to stimulate the metabolic and physical stresses of the metastatic cascade, specifically detachment, cluster migration and reattachment (Secomandi et al. 2024). Consistent with their known phenotypes, the highly aggressive MDA-MB-231 cells demonstrated the fastest and more efficient reattachment, while the less metastatic MCF-7 attached less efficiently. The benign MCF-10A cells showed no reattachment, validating the model's ability to distinguish metastatic potential. We may hypothesize that benign cells once transitioning to the suspended phase lose the ability to reattach in a secondary site.

Interestingly, BECLIN-1 modulation during the 2D-3D-2D transition was cell line dependent. While BECLIN-1 expression remained stable in the benign MCF-10 and MCF-7 lines, the aggressive MDA-MB-231 line exhibited a significant, downregulation of BECLIN-1 while growing in the spheroid phase, which was subsequently restored upon reattachment. Notably, autophagic flux remained constant despite these fluctuations in BECLIN-1 levels suggesting that in advanced breast cancer, BECLIN-1 may facilitate survival through non-canonical, autophagy-independent pathways rather than its traditional role in the autophagic machinery.

We also assessed the consequences of inhibiting the BECLIN-1 using Spautin-1. In 2D cell culture, the MCF-7 cells, characterized by lower baseline *BECN1* levels, were highly sensitive, showing significant cell death compared to MDA-MB-231 cells, which appeared more resistant. This was an indication of high vulnerability, where cells with BECLIN-1 haploinsufficiency appear more dependent on their remaining autophagic machinery for survival. Furthermore, when we depleted BECLIN-1 in MDA-MB-231 mammospheres, their capacity for 3D growth and 2D reattachment was severely compromised. This confirms that BECLIN-1 is essential for tumor cells to withstand the stresses associated with metastatic outgrowth.

Finally, clinical data from the METABRIC and the TCGA-BRCA cohorts reinforce these findings. Patients with a shallow deletion of the *BECN1* gene faced poorer overall survival outcomes. This bridges the gap between our laboratory observations and clinical reality, confirming that the loss of *BECN1* is a significant driver of disease aggressiveness.

In summary, *BECN1* plays a multifaceted role in breast cancer. While its loss drives cells toward a more invasive (EMT) state, the protein itself remains vital for helping those same cells survive the physiological rigors of metastasis. These findings suggest that therapeutic strategies targeting autophagy-related pathways must be carefully tailored to the specific genetic status of *BECN1* within the tumor.

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