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

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Abstract

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

INTRODUCTION

Beclin 1 is a haploinsufficient tumor suppressor that is associated with poor prognosis in a number of cancer types (1,2). In breast cancer, reduced Beclin 1 expression is an independent predictor of poor overall patient survival (3). Heterozygous loss of Beclin 1 (*BECN1*^{+/-}) promotes mammary tumorigenesis in response to parity and enhances WNT1-driven mammary tumor progression (4). The majority of studies that have investigated Beclin 1 function in cancer have focused on its role in regulating macroautophagy (hereafter referred to as autophagy). Autophagy is a conserved homeostatic and stress response

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pathway by which damaged proteins and organelles are engulfed within a double membrane vesicle and degraded upon fusion with lysosomes to prevent cytotoxicity and recycle macromolecules for energy supply (5). While a role for autophagy in suppressing tumor initiation has been supported by experimental studies (6), a paradoxical requirement for autophagy function in tumor progression has also been revealed (7). For example, knockout of *Atg5*, an essential autophagy gene that is required for the elongation and closure of the autophagosome, enhances tumor initiation in a *Kras* mouse model of pancreatic cancer, but these tumors remain benign and do not progress to invasive cancer (8). Moreover, *Kras/p53*-driven lung tumors revert to benign oncocytoomas upon acute knockout of *Atg7*, another essential autophagy gene important for autophagosome elongation (9). These outcomes contrast with the enhanced tumor growth and progression observed in mice when Beclin 1 expression is reduced (1,2,4). The requirement of autophagy for the development and maintenance of malignant tumors conflicts with the role of Beclin 1 as a tumor suppressor, and this discrepancy underscores the likelihood that alternative functions of Beclin 1 are involved in its regulation of tumor progression.

Autophagy-independent functions of Beclin 1 have been less studied in the context of cancer, although growing evidence supports their involvement in tumor suppression. Beclin 1 (Atg6/Vps30) regulates membrane trafficking events through its interaction with p150 (Vps15) and the lipid kinase class III phosphatidylinositol-3 kinase (PI3KC3/Vps34) (10). This Beclin 1 core complex interacts in a mutually exclusive manner with either ATG14L/BARKOR (Atg14; Complex I) or UVRAG (Vps38; Complex II) to regulate distinct vesicular trafficking functions (11). Complex I regulates autophagy and Complex II regulates autophagy-independent functions including vacuolar protein sorting, cytokinesis, phagocytosis, fluid phase endocytosis and endolysosomal receptor trafficking (11-14). Beclin 1, UVRAG and another Complex II-specific binding partner BIF-1 each suppress xenograft tumor growth when overexpressed, a finding not reported for ATG14L (15-17). This selective regulation supports a unique role for Beclin 1 and Complex II in cancer.

One mechanism by which Beclin 1 may regulate tumor growth and progression is through the control of endolysosomal trafficking, which plays an important role in controlling the outcomes of cell surface receptor function. For growth factor receptors, ligand binding initiates internalization and entry into the early endosome compartment, which is required for the activation of some signaling pathways (18). Other receptors, such as the transferrin receptor (TFR1), are internalized constitutively in a ligand-independent manner (19). Once internalized into early endosomes, receptors are sorted to either late endosomes/multivesicular endosomes (MVEs) where they are sequestered within intraluminal vesicles (ILVs) for signal termination and subsequent degradation upon fusion with the lysosome (20), or to the recycling endosomes for return to the cell surface (21). Beclin 1, UVRAG and BIF-1 have been reported to regulate the rate at which the epidermal growth factor receptor (EGFR) is degraded after stimulation with its ligand EGF (12,22). In previous work, we showed that Beclin 1 regulates phosphatidylinositol-3 phosphate (PI3P) production in response to growth factor stimulation and promotes the transition of PI3P-negative (PI3P⁻) early endosomes to PI3P⁺ endosomes (23,24). By doing so, Beclin 1 controls the length of time that growth factor receptors remain in the PI3P⁺ signaling competent compartment and consequently determines the duration of growth regulatory signals (24). The fact that Beclin

1 expression inversely correlates with AKT and ERK phosphorylation in human breast tumors is indicative that this Beclin 1-dependent regulation of growth factor receptor signaling occurs in human cancer (24).

Despite knowledge that Beclin 1 has been implicated in growth factor receptor signaling and trafficking, much remains to be learned about the mechanism by which this occurs. PI3P is necessary for the recruitment of FYVE (Fab1p, YOTB, Vac1p, EEA1) or PX (Phox homology) domain containing effector proteins that control the trafficking fate of cargo within the endocytic pathway (23). However, specific PI3P-interacting proteins that are regulated by Beclin 1 have not been identified. Moreover, the existing data on Beclin 1 regulation of trafficking were derived from *in vitro* studies and the impact of Beclin 1 on receptor trafficking and signaling *in vivo*, and the effect on tumor behavior, has not been demonstrated. In the current study, we demonstrate that Beclin 1 regulates the trafficking and function of growth factor and nutrient receptors that drive tumor cell proliferation *in vivo* by a mechanism that appears to be autophagy independent. These findings provide novel insight into the mechanism by which Beclin 1 regulates receptor function and how loss of Beclin 1 expression contributes to tumor progression.

MATERIALS AND METHODS

Cells, antibodies and reagents.

MDA-MB-231 LM2 4175 human breast cancer cells were purchased from the laboratory of Joan Massague (Memorial Sloan Kettering Cancer Center, Cornell University) and grown in DMEM media containing 10% FBS (25). SUM-159 cells that were authenticated by STR profiling at the University of Arizona Genetics Core in August 2017 were a kind gift from Art Mercurio (UMass Medical School, Worcester, MA) and grown in F12 Hams media supplemented with 5% FBS, 500mM HEPES, 1.5mg Insulin and 1mg/mL hydrocortisone. Upon receipt of cells, expanded stocks were frozen down and fresh knockdown cells were generated after two months in culture. Cells tested negative for mycoplasma using the Morwell MD Biosciences EZ PCR Mycoplasma Test Kit (cat# 409010; April 2019). Stable knockdown cell lines were generated using lentiviral vectors containing shRNAs that target human *BECN1* (TRCN0000033550, TRCN0000033552), *ATG5* (TRCN0000151963, TRCN0000151474), *ATG13* (TRCN0000172704, TRCN0000427008) and *TFRC* (TRCN0000057660) (Open Biosystems, Lafayette, CO, USA). pLKO.1 puromycin containing shRNA that targets green fluorescent protein (*GFP*) was purchased through Addgene (Cat# 30323). For dual expression, shRNAs were sub-cloned into a pLKO.1 neomycin vector (Addgene; cat # 13425) using EcoRI and MfeI sites. For restoration of Beclin 1 expression, FLAG-Beclin 1 with silent mutations that disrupt shRNA targeting was subcloned into the pCDH-puro lentiviral vector (24). Stable cell lines were selected with 2ug/ml of puromycin (Gold Bio), 0.5ug/ml G418 Neomycin (Gold Bio), or both.

Antibodies recognizing Beclin 1 (cat# 3738), ATG5 (cat# 2630), ATG13 (cat# 13468), p44/42 MAPK (ERK1/2; cat#9102), pT202/Y204-MAPK (pERK1/2; cat# 4370), EGFR (cat# 4267), pY1068-EGFR (cat# 3777), HRS (cat# 15087), AKT (cat# 9272), pT308-AKT (cat# 4056), cleaved Caspase-3 (cat# 9661) and phospho-Histone H3 (cat# 9701), as well as mouse IgG1 (cat# 5415) and normal rabbit IgG (cat# 2729) were purchased from

Cell Signaling Technologies (CST) (Danvers, MA). Transferrin receptor (cat# 13–6800) and actin (cat# MA5-11869) antibodies were purchased from Invitrogen (Carlsbad, CA). pTyr antibody (cat# sc-7020) was purchased from Santa Cruz Biotechnology (Dallas, TX). LC3B (cat# L7543), Tubulin (cat# T5168) and pY334-HRS (cat# SAB4504231) were purchased from Sigma (St. Louis, MO). Ki67 antibodies were purchased from Abcam (Cambridge, UK; cat# 66155).

Autophagic flux assays.

Cells were plated overnight and then incubated with complete DMEM containing 100nM Rapamycin (Sigma; cat# R0395), 40nM Bafilomycin (Sigma; cat# B1793), or both for 8 hours. Cell extracts containing equivalent amounts of total protein were analyzed for LC3I to LC3II conversion by immunoblotting.

Orthotopic *in vivo* assays.

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

***Ex vivo* tumor analysis.**

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

Reverse Phase Protein Array.

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

Immunoprecipitation and Immunoblotting.

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

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

Whole cell or tumor extracts containing equivalent amounts of protein or immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously (26). Bands were detected by chemiluminescence using a ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA) and band intensities were quantified by densitometry using Image Lab (Beta 1; Bio-Rad Laboratories) or Image J. Only signals within a linear range were used for quantitation and signals were normalized to total protein and/or housekeeping genes.

Immunofluorescent staining.

Subconfluent, adherent cells plated on glass coverslips were serum starved for 2 hrs and then treated with or without EGF-Alexafluor 555 (50ng/ml; Molecular Probes; cat# E35350) for 10 minutes. Cells were washed three times with cold Dulbecco's PBS and fixed in 3.8% paraformaldehyde in Dulbecco's PBS with 0.5% Tween (PBST) for 1 hr. Permeabilized cells were blocked for 1 hr using 3% BSA in PBST. Primary antibodies diluted in blocking buffer were added to cells and incubated at room temperature for 1 hr. Secondary antibodies were diluted in the same buffer and cells were incubated at room temperature for an additional 30 minutes. Cells were washed three times with PBST after each antibody incubation. Coverslips were then mounted on glass slides using Prolong Gold containing DAPI (Cell signaling) and the slides were viewed by confocal microscopy (Zeiss LSM700; 63X oil immersion objective). All images were adjusted equally for brightness/contrast using Adobe Photoshop.

Immunohistochemistry.

Formalin-fixed, paraffin-embedded tissue sections (5uM) were deparaffinized and rehydrated, and antigen retrieval was performed in 10mM sodium citrate buffer, pH 6.0 with heating in a steamer for 1hr. Tissues were incubated with 0.3% Hydrogen peroxide to quench endogenous peroxides and then blocked using a dual avidin/biotin blocking kit (Vector Laboratories; cat # SP-2001) followed by a 1-hour incubation in 1x casein milk (Vector Laboratories; cat# SP-2050). Tissue sections were incubated with primary antibodies overnight followed by secondary antibody incubation with the elite ABC-HRP kit (Vector Laboratories; cat# PK6101). Sections were developed with diaminobenzidine (DAB) (Dako; cat# K3468) and then counterstained with hematoxylin. Stained tumor sections were viewed on an Olympus BX41 light microscope (Olympus). Images were captured with an Evolution MPCOLOR camera (Media Cybernetics). TUNEL staining was performed according to manufacturer's instructions (Promega, cat# G3250). Stained tissue sections were viewed and images captured on a Zeiss LSM-700 microscope. All images were adjusted equally for brightness and contrast using Adobe Photoshop.

Quantitative Polymerase Chain Reaction (qPCR).

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

Statistical Analysis.

Statistical analysis between two groups was performed using the two-tailed unpaired student's t-test. Statistical analysis was performed using Prism7, Graphpad. A two-sided p-value of <0.05 was considered to indicate statistical significance. K means clustering was performed in MATLAB using the built-in function 'kmeans' using the distance metric squared Euclidean. Fischer's exact test was performed to determine MAPK enrichment in clusters.

RESULTS

Beclin 1 regulates endosomal HRS recruitment

Our previous *in vitro* studies demonstrated that Beclin 1 regulates Insulin-like growth factor-1 (IGF-1R) and EGFR receptor trafficking and signaling by controlling the activation of VPS34 and generation of PI3P (24). Ligand-dependent receptor activation stimulates the production of PI3P and this increase is inhibited when Beclin 1 expression is suppressed (24). Reduced PI3P levels result in delayed receptor degradation, but the mechanism of this regulation is not known. A primary signal for sorting receptors that are destined for lysosomal degradation is receptor ubiquitination (27). Ubiquitinated receptors are recognized by the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), which contains both an ubiquitin binding (UIM) domain and a FYVE domain (28-30). The HRS FYVE domain recognizes PI3P in the early endosomal membrane and is required for its recruitment to these vesicles (31). In cells treated with wortmannin to reduce PI3P levels and inhibit HRS recruitment to the early endosome, activated receptors escape sorting into intraluminal vesicles (ILVs) of multivesicular endosomes (MVE), a step prior to lysosomal degradation, and their signaling and expression are prolonged (32,33).

We hypothesized that suppression of Beclin 1 sustains growth factor receptor expression and signaling because HRS recruitment to the early endosome is limited, allowing receptors to escape sorting to ILVs and delay degradation. To investigate this potential mechanism of Beclin 1 function, we used a variant of MDA-MB-231 cells (hereafter referred to as LM2 cells) because Beclin 1 expression is elevated in these cells when compared across a panel of TNBC cells (34). Cells were generated that stably express shRNA targeting either *GFP* (control), *BECN1* or *ATG5*. This knockdown approach was taken to mimic the reduction, but not complete loss, of Beclin 1 expression that is commonly observed in human tumors (3). Beclin 1 expression was restored in the *shBECN1* cells using a construct in which silent mutations were introduced into the region of *BECN1* targeted by the shRNA to control for

specificity of the knockdown (24). To visualize the recruitment of HRS to endosomes, cells were treated with EGF-AlexaFluor 555 (EGF-555) to stimulate and monitor trafficking of the EGFR and co-stained with HRS-specific antibodies (Fig. 1A). HRS localization was primarily diffuse in the cytoplasm of serum-starved cells, with a few puncta evident. After stimulation for 10 min, a similar number of EGF-555 positive puncta were detected in all cells, supporting an equivalent level of EGFR activation. The number of cytoplasmic HRS puncta increased markedly in *shGFP* and *shATG5* cells after stimulation, and these puncta co-localized with EGF-555. Significantly fewer HRS puncta were induced by EGF stimulation in *shBECN1* cells, but rescue of Beclin 1 expression restored HRS puncta formation.

To investigate further the Beclin 1-dependent regulation of HRS, we evaluated the tyrosine phosphorylation of HRS in response to EGF stimulation. HRS is phosphorylated in response to EGFR activation and this phosphorylation event requires PI3P-mediated recruitment of HRS to endosomes, making it a surrogate marker for HRS endosome localization (28,33,35). EGF-stimulated HRS phosphorylation (pY334-HRS) decreased in cells expressing shRNA targeting two different sites within *BECN1* when compared with *shGFP* cells (Fig. 1B) and the reduced HRS phosphorylation in *shBECN1* cells was increased upon rescue of Beclin 1 expression (Supplementary Fig S1A). HRS phosphorylation was also reduced when Beclin 1 expression was suppressed in another TNBC cell line SUM-159PT (Supplementary Fig S1B). In contrast, EGF-stimulated HRS phosphorylation was modestly enhanced in *shATG5* cells (Fig. 1C) when compared with *shGFP* cells. A similar increase in HRS phosphorylation was observed when expression of another autophagy gene, ATG13, was suppressed (Supplementary Fig S1C). These results support that the EGF-stimulated recruitment of HRS to endosomes is regulated in a Beclin 1-dependent manner, but may be independent of autophagy.

Beclin 1 regulates tumor proliferation.

To investigate whether Beclin 1/HRS-dependent regulation of receptor trafficking impacts tumor growth, shRNA-modified LM2 cells were injected into the mammary fat pad (mfp) of NOD/SCID mice. *shBECN1* cells expressing reduced Beclin 1 grew at an increased rate, and the final tumor volume was significantly greater when compared with *shGFP* control tumors (Fig. 2A). Rescue of Beclin 1 expression (*shBECN1:Beclin 1*) significantly diminished tumor growth rate and size (Fig. 2A), confirming the specificity of the Beclin 1 knockdown. In contrast to the enhanced tumor growth observed upon suppression of Beclin 1 expression, the growth rate and final volume of *shATG5* (Fig. 2B) and *shATG13* (Supplementary Fig. S2A) tumors was similar to *shGFP* tumors.

To determine the extent to which autophagy was inhibited in the knockdown cells, autophagic flux was examined by treating cells with either rapamycin to inhibit mTOR and stimulate autophagy, bafilomycin A1 to inhibit lysosomal degradation, or both together (36). A similar reduction in LC3-I to LC3-II conversion was evident in the *shBECN1*, *shATG5* (Fig. 2C) and *shATG13* (Supplementary Fig. S2B) cells, demonstrating that autophagy inhibition was comparable in these cells. Moreover, autophagic flux was restored to control

shGFP levels in the *shBECN1:Beclin 1* rescue cells (Fig. 2D). Importantly, the reduction in autophagy observed *in vitro* was maintained *in vivo* (Fig. 2A and 2B).

Tumor sections were analyzed for either Phospho-Histone H3 (PH3) to assay proliferation or TUNEL staining to assay cell death. *shBECN1* tumors exhibited increased PH3 staining compared to *shGFP* and *shBECN1:Beclin 1* tumors (Fig. 3A). In contrast, no differences in TUNEL staining were detected (Fig. 3B). The cell death results were further validated by cleaved Caspase 3 staining, which also revealed low, but similar, levels of apoptosis in the tumors (Supplementary Fig. S3). Both PH3 and TUNEL staining were equivalent in the *shGFP* and *shATG5* tumors, reflecting their similar growth rates (Fig. 3A and 3B). Taken together, our results support the conclusion that the enhanced tumor growth observed for *shBECN1* tumors does not result from decreased autophagy alone and that alternative functions of Beclin 1 are involved in its regulation of tumor cell proliferation.

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

To explore further the hypothesis that Beclin 1 regulates tumor proliferation through the control of endocytic receptor trafficking, we performed an unbiased high-throughput, quantitative reverse phase protein array (RPPA) to assess the expression of 302 proteins and phosphoproteins that have important functions in cancer (37). This array included many growth factor receptors and downstream signaling effectors that have been implicated in tumor proliferation. Tumor lysates from three tumors of each genotype (*shGFP*, *shBECN1* and *shBECN1:Beclin 1*) were analyzed by RPPA. Unsupervised hierarchical clustering of the Z-scored data revealed segregation of the *shBECN1* tumors from the *shGFP* and *shBECN1:Beclin 1* tumors, with the exception of one *shGFP* tumor that co-segregated with the *shBECN1* tumors (Supplementary Fig. S4A). K means clustering was used as an unbiased approach to identify changes in expression patterns that are unique to *shBECN1* tumors. Based on an analysis of the root-mean-square error (RMSE) we selected 18 clusters (K=18) as having the optimal balance between the similarity of the signaling profiles within each cluster while maintaining a small overall number of clusters (Supplementary Fig. S4B). Of the 18 distinct expression patterns that were identified, sub-clusters 1 and 11 contained proteins and phosphoproteins that exhibited increased expression in *shBECN1* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Fig. 4A).

Analysis of sub-clusters 1 and 11 identified several growth factor (GF) and hormone receptors (EGFR, IR β , c-KIT, VEGFR2, phosphoHER3) and their downstream signaling intermediates (pY759-phospholipase C gamma2 (PLC γ 2), pS664-protein kinase C delta (PKC δ), and pS116-PEA-15) that were increased in *shBECN1* tumors (Fig. 4B; Supplementary Fig. S4C). In addition, pT202/Y204-extracellular regulated kinases 1/2 (pERK1/2), major regulators of cell cycle progression, as well as ERK1/2 substrates (pS383-ELK1 and pS318/S321-FOXO3A) were also increased in *shBECN1* tumors (Fig. 4C; Supplementary Fig. S4C). Analysis of all mitogen activated protein kinase (MAPK) pathway components (receptors, kinases and downstream substrates) that were included in the RPPA analysis revealed a significant enrichment for MAPK pathway activity in sub-cluster 1 and elevated pathway activity in sub-cluster 11 (Fig. 4D and Supplementary Fig. S4D). In contrast, increased PI3K/AKT pathway activity was not evident in the *shBECN1* tumors by

RPPA analysis, indicating a selective activation of the MAPK signaling pathway in these tumors.

Immunoblot analysis of additional tumors (n=7) confirmed increased EGFR expression and activation of ERK1/2 in *shBECN1* tumors when compared with *shGFP* and *shBECN1:Beclin 1* tumors (Fig. 4E). This analysis also suggested that EGFR is preferentially localized within a signaling competent compartment in *shBECN1* tumors. Specifically, relative EGFR activation, as measured by phosphorylation of Y1068-EGFR, a GRB2 binding site, was similar across all tumors, but downstream ERK1/2 phosphorylation was significantly increased (Fig. 4E). pT308-AKT levels were not elevated in the *shBECN1* tumors, confirming the RPPA findings that PI3K/AKT signaling is not enriched in these tumors. Increased EGFR expression and pERK1/2 activity and equivalent AKT activity were also validated in a second cohort of *shGFP* and *shBECN1* tumors (Supplementary Fig. S5). In contrast, MAPK pathway activity was not elevated in *shATG5* or *shATG13* tumors (Supplementary Figs. S6A and S2A), providing further evidence that the regulation of this signaling pathway by Beclin 1 may be independent of its regulation of autophagy.

EGFR mRNA levels were not significantly different across the three tumor genotypes indicating that Beclin 1 regulates EGFR at the level of protein expression (Fig. 4F). To examine the hypothesis that this regulation occurs through EGFR endolysosomal trafficking, HRS tyrosine phosphorylation was assessed in the tumors. Overall HRS phosphorylation was lower in the tumors than detected after acute EGF stimulation *in vitro*. However, reduced HRS phosphorylation was detected in the *shBECN1* tumors when compared with *shBECN1:Beclin 1* tumors (Fig. 4G). These results support our conclusion that Beclin 1 regulates HRS function *in vivo* to control receptor trafficking.

To assess the functional contribution of the EGFR/ERK signaling pathway to the enhanced proliferation observed in *shBECN1* tumors, *shGFP* and *shBECN1* tumor slices were incubated *ex vivo* for 48 hrs in the presence of either the EGFR/HER2 dual inhibitor Lapatinib or PD98059, an inhibitor of MEK, the upstream regulator of ERK1/2 activation (38-40). Pathway activity in the *ex vivo* tissue slices and inhibition of activity by the drugs were confirmed by immunoblotting tumor extracts (Fig. 5A). EGFR activity (pY1068-EGFR) was inhibited significantly by Lapatinib in both *shGFP* and *shBECN1* tumors. MEK activity, as measured by pT202/Y204-ERK1/2 levels, was also inhibited significantly in both tumor genotypes by PD98059, but pEGFR levels remained the same in the presence of this drug. ERK1/2 phosphorylation was not inhibited in response to Lapatinib treatment, which may reflect the fact that these tumors express constitutively active mutant Ras that acts downstream of the EGFR and sustains ERK1/2 activation in the presence of this drug (41). ERK1/2 function is regulated at both the level of activation (phosphorylation) and localization, with transition from the cytoplasm to the nucleus required for growth factor-dependent cell cycle entry (42). Therefore, we assessed the localization of ERK1/2 in tumor sections treated with Lapatinib (Fig. 5B). Homogeneous staining was evident in the DMSO treated tumors, indicating that ERK1/2 was present in both the cytoplasm and nucleus. In contrast, treatment with Lapatinib resulted in a decrease in nuclear staining (white arrows), indicating that ERK1/2 function was inhibited by this drug treatment.

Tumor morphology was maintained during the *ex vivo* culture period as evidenced by similar H&E staining in tumors that were immediately fixed (untreated) or incubated *ex vivo* (DMSO) (Fig. 5C). The *ex vivo* tumor sections were analyzed for Ki67 expression by IHC staining to assess proliferation. *shBECN1* tumors exhibited increased Ki67 staining when compared with *shGFP* tumors, indicating that the enhanced proliferation observed *in vivo* was maintained during the *ex vivo* incubation period. Ki67 expression was reduced significantly in the *shBECN1* tumors in response to both Lapatinib and PD98059 treatment (Fig 5C). Although a similar trend was observed for *shGFP* tumors, the decrease in Ki67 staining was not significant for either drug, suggesting that the enhanced EGFR/ERK signaling that occurs in *shBECN1* tumors renders their proliferation more dependent upon this signaling pathway and more sensitive to inhibition by these drugs.

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

Additional analysis of our RPPA data revealed that TFR1 expression was significantly upregulated in *shBECN1* tumors (Fig. 6A). The ability of cells to proliferate requires not only a growth factor stimulus but also the appropriate metabolic conditions to support the anabolic processes that must occur for a cell to divide (43). Iron is an essential nutrient cofactor for enzymes that are involved in DNA synthesis and cell cycle and it is required for proliferation (44,45). Extracellular iron is bound by transferrin and transported into cells by endocytic trafficking of TFR1 (19). TFR1 expression correlates with proliferative capacity and receptor levels are elevated in tumor cells to satisfy the increased iron demand of these rapidly dividing cells (44). Increased expression of TFR1 in *shBECN1* tumors and restoration of expression to *shGFP* levels in *shBECN1:Beclin 1* tumors was confirmed by immunoblotting (n=13 tumors) (Fig. 6B). Similar to EGFR mRNA expression, *TFRC* mRNA levels were equivalent across the tumor genotypes (Fig. 6C), indicating that increased TFR1 expression in *shBECN1* tumors also occurs at the level of protein expression. TFR1 protein expression did not increase in *shATG5* tumors (Supplementary Fig. S6B), supporting the possibility that the upregulation of TFR1 expression in *shBECN1* tumors occurs in an autophagy-independent manner.

The link between Beclin 1 and TFR1 was unexpected because TFR1 is typically sorted in the early endosome for constitutive recycling back to the cell surface. As a result of this recycling, expression remains constant. Alternatively, TFR1 can be ubiquitinated by members of the membrane associated RING-CH (MARCH) family of ubiquitin ligases and this ubiquitination targets TFR1 for lysosomal degradation (46). We hypothesized that TFR1 is ubiquitinated in the tumor microenvironment and TFR1 levels increase in tumors with low Beclin 1 expression because these ubiquitinated receptors escape HRS-mediated sorting to the lysosome for degradation. In support of this mechanism of regulation by Beclin 1, elevated TFR1 expression was associated with increased ubiquitination in *shBECN1* tumors (Fig. 6D).

To determine if increased TFR1 expression contributes to the enhanced proliferation of *shBECN1* tumors, LM2 cells were co-infected with shRNA targeting *BECN1* and *TFRC*. Cells with a modest suppression of TFR1 expression, resulting in expression levels equivalent to the levels observed in *shGFP* cells, were selected for further *in vivo* analysis.

Restoration of TFR1 expression to control *shGFP* tumor levels inhibited the enhanced tumor growth observed in cells expressing *shBECN1* alone (Fig. 6E and 6F). Tumor sections were analyzed for PH3 or TUNEL staining to determine if the reduced growth observed upon suppression of TFR1 expression in the *shBECN1* tumors was the result of decreased proliferation or increased cell death, respectively (Fig. 6G and 6H). As we observed previously (Fig. 2), *shBECN1* tumors exhibited increased PH3 staining compared to *shGFP* tumors and no differences in TUNEL staining were detected. *shBECN1:shTFRC* tumors exhibited PH3 and TUNEL staining equivalent to *shGFP* tumors, indicating that Beclin 1-dependent control of TFR1 expression contributes to tumor cell proliferation.

We infer from our receptor trafficking and *in vivo* data that low HRS expression in human tumors should be associated with poor patient outcomes. To assess the significance of HRS expression in human breast cancer, the impact of HRS expression on patient outcomes was analyzed using Kaplan-Meier plotter (47). Low HRS expression significantly correlated with reduced relapse-free survival (RFS) when all breast cancer subtypes were analyzed together, and this significance was maintained upon analysis of only Basal subtype tumors (Fig. 7A). By contrast, HRS expression did not correlate with RFS in HER2 positive tumors. This lack of significant correlation likely reflects the fact that HER2 is not downregulated by HRS-dependent sorting to the lysosome and therefore the expression and activity of these receptors would not be enhanced if HRS expression was reduced (48). The inverse association of HRS with RFS supports that the control of receptor trafficking is important for the suppression of tumor progression.

DISCUSSION

We demonstrate that Beclin 1 regulates endocytic receptor trafficking by a mechanism that may be independent from its regulation of autophagy, and conclude that this function of Beclin 1 contributes to its role as a tumor suppressor. Specifically, we show that Beclin 1 regulates the endosomal recruitment of HRS, which is essential in the sorting of receptors for signal silencing and degradation. When Beclin 1 expression is reduced in tumors, early endosome recruitment of HRS is diminished and expression and activation of receptors that would normally be sorted for degradation persists (Fig. 7B). A consequence of this prolonged expression and function is increased tumor proliferation. By RPPA analysis, we identified two independent growth regulatory receptors that contribute to enhanced proliferation when Beclin 1 expression levels are decreased. EGFR expression and function are elevated and downstream ERK1/2 activation is increased, and this enhanced activity renders tumor proliferation more sensitive to drugs that target this signaling pathway. Expression of the iron transporter TFR1 is also increased in tumors when Beclin 1 expression is low and this nutrient receptor supports enhanced tumor cell proliferation. Importantly, increased tumor proliferation and elevated EGFR and TFR1 expression were not observed when autophagy was reduced to an equivalent extent by suppression of other autophagy genes. Taken together, our data provide insight into how reduced Beclin 1 expression in tumors contributes to progression.

Our demonstration that Beclin 1 controls the early endosome recruitment of HRS to impact receptor sorting identifies a novel mechanism by which the expression and functional

outcomes of cell surface receptors can be regulated. This regulation can be mediated through either changes in Beclin 1 expression, which occurs in tumors and is modeled in our current studies, or function, such as through post-translational modifications of Beclin 1 that disrupt its interactions with PI3KC3. For example, phosphorylation of Beclin 1 by EGFR or AKT inhibits its interaction with PI3KC3, resulting in decreased lipid kinase activity (49,50). Ubiquitination of Beclin 1 also reduces PI3KC3 activation by targeting Beclin 1 for proteasomal degradation (51). These modifications of Beclin 1 inhibit PI3P production, which prevents HRS recruitment and delays receptor sorting to the lysosome. Beclin 1 post-translational modifications likely regulate the duration of receptor signaling and expression in normal cells in response to physiological stimuli, and may further alter receptor trafficking when these pathways are activated in tumors. Our data demonstrate an important role for Beclin 1/HRS regulation of EGFR trafficking in TNBC. However, many additional growth regulatory receptors are regulated by endolysosomal trafficking and would be impacted by Beclin 1 expression. As one example, in *Drosophila*, Atg6 (the *Drosophila* homolog of Beclin 1) regulates Notch and Wingless signaling pathways through the control of lysosomal receptor degradation (52). In *Atg6* mutant flies, receptor signaling is sustained which results in cell polarity and developmental defects. Future studies are warranted to determine if the expression and activity of other receptors that are downregulated by endolysosomal trafficking are enhanced in tumors upon reduction of Beclin 1 expression and if this mechanism of regulation contributes to their oncogenic properties.

Although Beclin 1 has been implicated as a tumor suppressor, the mechanisms involved have not been well characterized (1,2). Our conclusion that Beclin 1 controls the endocytic trafficking of growth factor and nutrient receptors that drive tumor proliferation provides novel insight into this problem. Importantly, this mechanism of action may explain conflicting reports on the role of Beclin 1 as a tumor suppressor. We discovered that the expression and function of the EGFR and downstream activation of ERK1/2 increased in *shBECN1* tumors and that this enhanced signaling promoted tumor proliferation. This result is consistent with the fact that TNBC is frequently associated with elevated EGFR expression and activity (53). However, we posit that the functional impact of Beclin 1 loss in an individual tumor will likely reflect the level of addiction to a specific receptor signaling pathway and whether it is controlled by HRS and endocytic trafficking. For example, heterozygous Beclin 1 loss enhances tumor development and growth in a mouse mammary tumor model driven by WNT-1, which acts through Frizzled receptors (4). EGFR, TFR1 and the Frizzled receptors are cell surface receptors whose expression and function are regulated by endolysosomal trafficking (19,54,55). In contrast, mammary tumorigenesis and growth are not enhanced by heterozygous loss of Beclin 1 in mouse models driven by either the polyoma-middle T oncogene (PyMT) or HER2 (56). PYMT is a cytoplasmic protein that regulates activation of PI3K, MAPK and Src signaling pathways independently of upstream receptor regulation (57). Therefore, disruption of HRS-mediated endocytic sorting would not be anticipated to enhance signaling and promote tumor growth in this model. Although HER2 is a surface receptor that is internalized into the endocytic pathway, it is not targeted for degradation but instead is preferentially sorted to the recycling endosome. In fact, heterodimerization of HER2 with EGFR inhibits EGFR degradation and promotes recycling to the cell surface (48). Therefore, disruption of the signals that promote receptor sorting to

the endolysosomal pathway would not be expected to enhance HER2 expression or function, and tumor growth would not be promoted by loss of Beclin 1 expression. In this regard, HRS expression is not predictive of outcomes in HER2 positive tumors.

Our implication of Beclin 1 in the regulation of TFR1 expression is novel and significant for understanding how Beclin 1 affects tumor proliferation. Iron is an essential nutrient for cell growth and proliferation and enhanced iron metabolism is commonly observed in tumors to support their rapid proliferation (44). In breast cancer, iron levels are increased when compared with normal breast tissue and an iron-regulatory gene signature is prognostic for patient outcome (58). As TFR1 is the major source of iron uptake into cells, regulating its expression is key to maintaining iron homeostasis. TFR1 expression can be regulated in an iron-dependent manner at the level of mRNA stability through the binding of iron-responsive proteins-1 (IRP-1) and IRP-2 to elements in the 3' untranslated region (59). However, TFR1 protein expression can also be regulated through ubiquitination and sorting to the lysosome for degradation, a mechanism that allows for the acute regulation of metabolically available iron, or the labile iron pool (46). Our finding that Beclin 1 regulates TFR1 expression at the level of protein expression and that increased TFR1 ubiquitination is observed in *shBECN1* tumors can be explained by decreased HRS endosomal recruitment that allows ubiquitinated TFR1 to escape sorting to the lysosome. Collectively our results provide a novel mechanism by which Beclin 1 regulates both growth factor (EGFR) and nutrient receptors (TFR1) that are important for cell proliferation, and demonstrate how coordinated dysregulation of these pathways upon loss of Beclin 1 expression drives tumor proliferation.

Our study reveals opportunities for the clinical management of tumors with low Beclin 1 expression. We observed that *shBECN1* tumors were more sensitive to inhibition of proliferation by EGFR and MEK inhibitors than control tumors, indicating a greater dependence of these tumors on the enhanced EGFR/ERK signaling that occurs when Beclin 1 expression is reduced. Although EGFR expression is frequently upregulated in TNBC, clinical trials of EGFR inhibitors in these patients have not shown overall efficacy (60). Screening of patients with low Beclin 1 expression could identify subgroups of patients that would be more sensitive to these drugs, as well as inhibitors of other receptors that are regulated by trafficking, to improve outcomes. TFR1 is also of clinical interest both as a drug target and because of its potential for drug delivery (44). Tumors expressing elevated levels of TFR1, such as we observed in *shBECN1* tumors, would be more sensitive to the inhibition of iron-uptake by antibodies that block TFR1 function or iron chelators (61,62). In addition, transferrin-chemotherapeutic drug conjugates that are transported intracellularly by endocytosis of the TFR1 would be more effective in tumors that express low levels of Beclin 1 and elevated TFR1 (63). Tumors with reduced Beclin 1 expression are also anticipated to be more sensitive to drugs that stimulate ferroptosis, an iron-dependent mechanism of cell death, due to their increased iron uptake (64). Given that Beclin 1 expression is frequently decreased across many human tumors, Beclin 1 could be a clinically relevant biomarker for many cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* 2003;100:15077–82 [PubMed: 14657337]
2. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003;112:1809–20 [PubMed: 14638851]
3. Tang H, Sebti S, Titone R, Zhou Y, Isidoro C, Ross TS, et al. Decreased BECN1 mRNA Expression in Human Breast Cancer is Associated with Estrogen Receptor-Negative Subtypes and Poor Prognosis. *EBioMedicine* 2015;2:255–63 [PubMed: 25825707]
4. Cicchini M, Chakrabarti R, Kongara S, Price S, Nahar R, Lozy F, et al. Autophagy regulator BECN1 suppresses mammary tumorigenesis driven by WNT1 activation and following parity. *Autophagy* 2014;10:2036–52 [PubMed: 25483966]
5. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 2004;6:463–77 [PubMed: 15068787]
6. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev* 2007;21:1621–35 [PubMed: 17606641]
7. Liu J, Debnath J. The Evolving, Multifaceted Roles of Autophagy in Cancer. *Adv Cancer Res* 2016;130:1–53 [PubMed: 27037750]
8. Yang A, Rajeshkumar NV, Wang X, Yabuuchi S, Alexander BM, Chu GC, et al. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Cancer Discov* 2014;4:905–13 [PubMed: 24875860]
9. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev* 2013;27:1447–61 [PubMed: 23824538]
10. Stack JH, Herman PK, Schu PV, Emr SD. A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *EMBO J* 1993;12:2195–204 [PubMed: 8387919]
11. Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol* 2001;152:519–30 [PubMed: 11157979]
12. Thoresen SB, Pedersen NM, Liestol K, Stenmark H. A phosphatidylinositol 3-kinase class III sub-complex containing VPS15, VPS34, Beclin 1, UVRAG and BIF-1 regulates cytokinesis and degradative endocytic traffic. *Exp Cell Res* 2010;316:3368–78 [PubMed: 20643123]
13. Shravage BV, Hill JH, Powers CM, Wu L, Baehrecke EH. Atg6 is required for multiple vesicle trafficking pathways and hematopoiesis in *Drosophila*. *Development* 2013;140:1321–9 [PubMed: 23406899]
14. Galluzzi L, Green DR. Autophagy-Independent Functions of the Autophagy Machinery. *Cell* 2019;177:1682–99 [PubMed: 31199916]

15. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402:672–6 [PubMed: 10604474]
16. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* 2006;8:688–99 [PubMed: 16799551]
17. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 2007;9:1142–51 [PubMed: 17891140]
18. Platta HW, Stenmark H. Endocytosis and signaling. *Curr Opin Cell Biol* 2011;23:393–403 [PubMed: 21474295]
19. Transferrin Kawabata H. and transferrin receptors update. *Free Radic Biol Med* 2019;133:46–54 [PubMed: 29969719]
20. Dobrowolski R, De Robertis EM. Endocytic control of growth factor signalling: multivesicular bodies as signalling organelles. *Nat Rev Mol Cell Biol* 2011;13:53–60 [PubMed: 22108513]
21. Grant BD, Donaldson JG. Pathways and mechanisms of endocytic recycling. *Nat Rev Mol Cell Biol* 2009;10:597–608 [PubMed: 19696797]
22. Runkle KB, Meyerkord CL, Desai NV, Takahashi Y, Wang HG. Bif-1 suppresses breast cancer cell migration by promoting EGFR endocytic degradation. *Cancer Biol Ther* 2012;13:956–66 [PubMed: 22785202]
23. Raiborg C, Schink KO, Stenmark H. Class III phosphatidylinositol 3-kinase and its catalytic product PtdIns3P in regulation of endocytic membrane traffic. *FEBS J* 2013;280:2730–42 [PubMed: 23289851]
24. Rohatgi RA, Janusis J, Leonard D, Bellve KD, Fogarty KE, Baehrecke EH, et al. Beclin 1 regulates growth factor receptor signaling in breast cancer. *Oncogene* 2015;34:5352–62 [PubMed: 25639875]
25. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436:518–24 [PubMed: 16049480]
26. Zhu S, Ward BM, Yu J, Matthew-Onabanjo AN, Janusis J, Hsieh CC, et al. IRS2 mutations linked to invasion in pleomorphic invasive lobular carcinoma. *JCI Insight* 2018;3(8): e97398
27. Polo S, Di Fiore PP, Sigismund S. Keeping EGFR signaling in check: ubiquitin is the guardian. *Cell Cycle* 2014;13:681–2 [PubMed: 24526125]
28. Komada M, Kitamura N. Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton protein with a structurally conserved putative zinc finger domain. *Mol Cell Biol* 1995;15:6213–21 [PubMed: 7565774]
29. Petiot A, Faure J, Stenmark H, Gruenberg J. PI3P signaling regulates receptor sorting but not transport in the endosomal pathway. *J Cell Biol* 2003;162:971–9 [PubMed: 12975344]
30. Komada M, Kitamura N. The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. *J Biochem* 2005;137:1–8 [PubMed: 15713877]
31. Raiborg C, Bremnes B, Mehlum A, Gillooly DJ, D'Arrigo A, Stang E, et al. FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. *J Cell Sci* 2001;114:2255–63 [PubMed: 11493665]
32. Futter CE, Collinson LM, Backer JM, Hopkins CR. Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol* 2001;155:1251–64 [PubMed: 11756475]
33. Urbe S, Mills IG, Stenmark H, Kitamura N, Clague MJ. Endosomal localization and receptor dynamics determine tyrosine phosphorylation of hepatocyte growth factor-regulated tyrosine kinase substrate. *Mol Cell Biol* 2000;20:7685–92 [PubMed: 11003664]
34. Ringner M, Fredlund E, Hakkinen J, Borg A, Staaf J. GOBO: gene expression-based outcome for breast cancer online. *PLoS One* 2011;6:e17911 [PubMed: 21445301]
35. Bache KG, Raiborg C, Mehlum A, Madshus IH, Stenmark H. Phosphorylation of Hrs downstream of the epidermal growth factor receptor. *Eur J Biochem* 2002;269:3881–7 [PubMed: 12180964]
36. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 2016;12:1–222 [PubMed: 26799652]

37. Akbani R, Becker KF, Carragher N, Goldstein T, de Koning L, Korf U, et al. Realizing the promise of reverse phase protein arrays for clinical, translational, and basic research: a workshop report: the RPPA (Reverse Phase Protein Array) society. *Mol Cell Proteomics* 2014;13:1625–43 [PubMed: 24777629]
38. Roife D, Dai B, Kang Y, Perez MVR, Pratt M, Li X, et al. Ex Vivo Testing of Patient-Derived Xenografts Mirrors the Clinical Outcome of Patients with Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res* 2016;22:6021–30 [PubMed: 27259561]
39. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002;21:6255–63 [PubMed: 12214266]
40. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A* 1995;92:7686–9 [PubMed: 7644477]
41. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011;121:2750–67 [PubMed: 21633166]
42. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J* 1999;18:664–74 [PubMed: 9927426]
43. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009;324:1029–33 [PubMed: 19460998]
44. Torti SV, Torti FM. Iron and cancer: more ore to be mined. *Nat Rev Cancer* 2013;13:342–55 [PubMed: 23594855]
45. Puig S, Ramos-Alonso L, Romero AM, Martinez-Pastor MT. The elemental role of iron in DNA synthesis and repair. *Metallomics* 2017;9:1483–500 [PubMed: 28879348]
46. Fujita H, Iwabu Y, Tokunaga K, Tanaka Y. Membrane-associated RING-CH (MARCH) 8 mediates the ubiquitination and lysosomal degradation of the transferrin receptor. *J Cell Sci* 2013;126:2798–809 [PubMed: 23606747]
47. Györfy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* 2010;123:725–31 [PubMed: 20020197]
48. Worthylake R, Opresko LK, Wiley HS. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. *J Biol Chem* 1999;274:8865–74 [PubMed: 10085130]
49. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, et al. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science* 2012;338:956–9 [PubMed: 23112296]
50. Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, Xiao G, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell* 2013;154:1269–84 [PubMed: 24034250]
51. Platta HW, Abrahamsen H, Thoresen SB, Stenmark H. Nedd4-dependent lysine-11-linked polyubiquitination of the tumour suppressor Beclin 1. *Biochem J* 2012;441:399–406 [PubMed: 21936852]
52. Lorincz P, Lakatos Z, Maruzs T, Szatmari Z, Kis V, Sass M. 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;2014:851349 [PubMed: 25006588]
53. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 2004;10:5367–74 [PubMed: 15328174]
54. Rives AF, Rochlin KM, Wehrli M, Schwartz SL, DiNardo S. Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the *Drosophila* wing. *Dev Biol* 2006;293:268–83 [PubMed: 16530179]
55. Bakker J, Spits M, Neeffjes J, Berlin I. The EGFR odyssey - from activation to destruction in space and time. *J Cell Sci* 2017;130:4087–96 [PubMed: 29180516]

56. Lozy F, Cai-McRae X, Teplova I, Price S, Reddy A, Bhanot G, et al. ERBB2 overexpression suppresses stress-induced autophagy and renders ERBB2-induced mammary tumorigenesis independent of monoallelic Becn1 loss. *Autophagy* 2014;10:662–76 [PubMed: 24492513]
57. Webster MA, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton M, Tortorice CG, et al. Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. *Mol Cell Biol* 1998;18:2344–59 [PubMed: 9528804]
58. Miller LD, Coffman LG, Chou JW, Black MA, Bergh J, D'Agostino R Jr., et al. An iron regulatory gene signature predicts outcome in breast cancer. *Cancer Res* 2011;71:6728–37 [PubMed: 21875943]
59. Kim HY, Klausner RD, Rouault TA. Translational repressor activity is equivalent and is quantitatively predicted by in vitro RNA binding for two iron-responsive element-binding proteins, IRP1 and IRP2. *J Biol Chem* 1995;270:4983–6 [PubMed: 7890603]
60. Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 2005;23:2445–59 [PubMed: 15753456]
61. Crepin R, Goenaga AL, Jullienne B, Bougherara H, Legay C, Benihoud K, et al. Development of human single-chain antibodies to the transferrin receptor that effectively antagonize the growth of leukemias and lymphomas. *Cancer Res* 2010;70:5497–506 [PubMed: 20530676]
62. Whitnall M, Howard J, Ponka P, Richardson DR. A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. *Proc Natl Acad Sci U S A* 2006;103:14901–6 [PubMed: 17003122]
63. Tortorella S, Karagiannis TC. Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. *J Membr Biol* 2014;247:291–307 [PubMed: 24573305]
64. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 2017;171:273–85 [PubMed: 28985560]

Figure 1: Beclin 1 regulates early endosome recruitment of HRS.

(A) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1*(#1), *shBECN1*(#1):*Beclin 1* or *shATG5* were serum starved and then stimulated with EGF-AlexaFluor 555 (50ng/ml) for 10 minutes. Cells were co-stained with HRS-specific Abs. The data shown in the graph on the top right represent the mean $\pm$ SEM HRS puncta/cell ($n = 17 - 25$ cells). Scale bar = 10 μ M. (B) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1*(#1) or *shBECN1*(#2) were stimulated with human EGF (50ng/ml) for the indicated time periods. Total cell extracts were immunoblotted with the indicated antibodies. The data shown in the graph below represent the mean $\pm$ SEM of three independent experiments. (C) MDA-MB-231 LM2 cells expressing *shGFP*, *shATG5*(#1) or *shATG5*(#2) were stimulated with human EGF

(50ng/ml) for the indicated time periods. Total cell extracts were immunoblotted with the indicated antibodies. The data shown in the graph below represent the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$; ***, $p < 0.005$

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Figure 2: Beclin 1 regulates breast tumor growth.

(A and B) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1*(#1) or *shBECN1*(#1):*Beclin 1* (A) or *shGFP* and *shATG5*(#2) (B) were grown as orthotopic xenografts in NOD-SCID mice. Insets, Beclin 1 (A) and ATG5 (B) expression prior to injection. Expression of Beclin 1 and LC3II/I in tumors is shown in the immunoblots. The data shown in the graphs to the right of the immunoblots represent the mean $\pm$ SEM expression of seven (A) or six (B) tumors. (C and D) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1*(#1) or *shATG5*(#2) (C) or *shGFP*, *shBECN1*(#1) or *shBECN1*(#1) with restored Beclin 1 expression (*shBECN1:Beclin 1*) (D) were assayed for autophagic flux. The

data shown in the graphs on the right represent the mean $\pm$ SEM LC3II/LC3I ratio of three independent experiments. *, $p < 0.05$ relative to *shGFP*, #, $p < 0.05$ relative to *shBECN1*.

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Figure 3: Beclin 1 regulates tumor proliferation but not survival.

(A) Representative images of Phospho-histone H3 (PH3) staining in tumors. The data shown in the graphs represent the mean $\pm$ SEM positive nuclei/high powered field (hpf; five independent images/five tumors; $n = 25$). Scale bar = 50uM. (B) Representative images of TUNEL staining in tumors. The data shown in the graphs represent the mean $\pm$ SEM positive nuclei/hpf (three independent images/six tumors; $n = 18$). Scale bar = 50uM. *, $p < 0.05$

Figure 4: RPPA analysis identifies enhanced EGFR/ERK1/2 signaling pathway activity in shBECN1 tumors.

(A) K means clustering analysis of RPPA data from three *shGFP* (1-3), *shBECN1* (4-6) and *shBECN1*(#1):*Beclin 1* (7-9) tumors. Log² data was converted to Z-scores to perform K-means clustering analysis. Images represent consensus plots for K=18 (18 sub-clusters). Red boxes identify sub-clusters with elevated expression patterns in *shBECN1* tumors. (B-C) Scatter-plots of sub-cluster 1 (B) and sub-cluster 11 (C) highlighting growth factor/hormone receptors and ERK1/2 signaling pathway activity. (D) Enrichment analysis for a MAPK signaling signature. Dotted line represents $-\log_{10}(1.3)$ which indicates a p value of 0.05. (E) Immunoblots of representative *shGFP*, *shBECN1* and *shBECN1*:*Beclin 1* tumors.

The data shown in the graph below represent the mean $\pm$ SEM expression of seven tumors from each genotype. Data are shown as fold change in expression relative to *shGFP* tumors. (F) Relative mRNA expression was determined by real-time quantitative PCR (RQ-PCR). The data shown represent the mean $\pm$ SEM mRNA expression of five (*shGFP* and *shBECN1:Beclin 1*) or four (*shBECN1*) tumors. (G) Tumor extracts from representative *shBECN1* and *shBECN1:Beclin 1* tumors were immunoprecipitated with HRS-specific antibodies and immunoblotted with antibodies specific for phosphotyrosine (pTyr). The blot was stripped and re-probed with HRS-specific antibodies. Lanes from the same immunoblot were merged as indicated by the black line. The data shown in the graph represent the mean $\pm$ SEM HRS phosphorylation of four tumors of each genotype and are shown as relative phosphorylation. *, $p < 0.05$.

Figure 5: Proliferation of *shBECN1* tumors is sensitive to inhibition of EGFR and ERK1/2 signaling.

(A) Immunoblot analysis of representative *shGFP* and *shBECN1*(#1) tumors treated *ex vivo* for 48 hrs with DMSO, Lapatinib (Lap; 5uM), or PD98059 (PD; 10uM). The data shown in the graphs represent the mean $\pm$ SEM expression of eight tumors of each genotype. (B) Immunofluorescent staining for ERK1/2 expression in representative *ex vivo* tumors treated with DMSO or Lapatinib. Arrows indicate representative cells with reduced nuclear localization of ERK1/2. Scale bar = 50uM. (C) Representative images of H&E or Ki67 staining of *shGFP* and *shBECN1* tumors treated *ex vivo* as indicated. The data shown in the graph below represent the mean $\pm$ SEM positive nuclei/hpf (three independent images/five tumors; n = 15). Scale bar = 50uM. *, $p < 0.05$; ***, $p < 0.005$

Figure 6: Beclin 1 regulation of transferrin receptor (TFR1) expression promotes tumor proliferation.

(A) Scatter-plot of sub-cluster 1 from the K means clustering analysis of RPPA data highlighting TFR1 expression in the triplicate tumors of each genotype. (B) Immunoblots of representative *shGFP*, *shBECN1*(#1) or *shBECN1*(#1):*Beclin 1* tumors. The data shown in the graph on the right represent the mean $\pm$ SEM TFR1 expression from thirteen tumors of each genotype. Data are shown as fold change in expression relative to *shGFP* tumors. (C) *TFRC* mRNA expression. The data shown represent the mean $\pm$ SEM *TFRC* expression from five tumors of each genotype. (D) Tumor extracts from representative *shGFP* and *shBECN1* tumors were immunoprecipitated with TFR1-specific antibodies and

immunoblotted with antibodies specific for Ubiquitin (Ub). The blot was stripped and re-probed with TFR1-specific antibodies. Lanes from the same immunoblot were merged as indicated by the black line. (E) MDA-MB-231 LM2 cells expressing *shGFP*, *shBECN1(#1)* or *shBECN1(#1):shTFRC* were assayed for tumor growth as orthotopic xenografts in NOD-SCID mice. Inset, Beclin 1 and TRF1 expression prior to injection. (F) Expression of Beclin 1 and TFR1 in tumors. The data shown in the graph on the right represent the mean $\pm$ SEM expression from six tumors of each genotype. (G) Representative images of PH3 staining in tumors. The data shown in the graph represent the mean $\pm$ SEM positive nuclei/hpf (five independent images/five tumors; n = 25). Scale bar = 50uM. (H) Representative images of TUNEL staining in tumors. The data shown in the graph represent the mean $\pm$ SEM positive nuclei/hpf (three independent images/six tumors; n = 18). Scale bar = 50uM. *, p<0.05; ***, p<0.005

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Figure 7: Beclin 1 regulates receptor trafficking through HRS.

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