Beclin 1 Promotes Endosome Recruitment of Hepatocyte Growth Factor Tyrosine Kinase Substrate to Suppress Tumor Proliferation

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ABSTRACT

Beclin 1 has nonautophagic 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 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.

Significance: Beclin 1 controls the trafficking fate of growth regulatory receptors to suppress tumor proliferation.

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 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 oncocytomas 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; ref. 10). This Beclin 1 core complex interacts in a mutually exclusive manner with either ATG14L/BARKOR (Atg14; Complex II) or UVRAG (Vps38; Complex I) 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 (MVE) where they are sequestered within intraluminal vesicles (ILV) 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 EGF receptor 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 this 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, New York, NY) and grown in DMEM containing 10% FBS (25). SUM-159 cells that were authenticated by short tandem repeat 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 Ham media supplemented with 5% FBS, 500 mmol/L HEPES, 1.5 mg/L insulin (UMass Medical School Institutional Animal Care and Use Committee).

Antibodies recognizing Beclin 1 (catalog no. 3738), ATG5 (catalog no. 2630), ATG13 (catalog no. 13468), p44/42 MAPK (ERK1/2; catalog no. 9102), pT202/Y204-MAPK (pERK1/2; catalog no. 4370), EGF (catalog no. 4267), pY1068-EGFR (catalog no. 3777), hepatocyte growth factor–regulated tyrosine kinase substrate (HRS, catalog no. 15087), AKT (catalog no. 9272), pT308-AKT (catalog no. 4056), cleaved caspase-3 (catalog no. 9661) and phospho-Histone H3 (catalog no. 9701), as well as mouse IgG1 (catalog no. 5415) and normal rabbit IgG (catalog no. 2729) were purchased from Cell Signaling Technology. Transferrin receptor (catalog no. 13-6800) and actin (catalog no. MA5-11869) antibodies were purchased from Invitrogen. pTyr antibody (catalog no. sc-7020) was purchased from Santa Cruz Biotechnology. Ki67 antibodies were purchased from Abcam (catalog no. 66155).

Autophagic flux assays

Cells were plated overnight and then incubated with complete DMEM containing 100 nmol/L rapamycin (Sigma, catalog no. R0395), 40 nmol/L bafilomycin (Sigma, catalog no. 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 × 106) were resuspended in 35 μL Matrigel (10 mg/mL; Trevigen; catalog no. 3432-005-01) immediately prior to injection into the third 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: 4/3π((L × H × W)/2).

Ex vivo tumor analysis

Following tumor dissection, equal size tumor slices were equilibrated in DMEM containing 10% FBS 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, catalog no. D5879), 5 μmol/L laptatinib (Selleckchem, catalog no. S1028), or 10 μmol/L PD98059 (Selleckchem, catalog no. S1177) for 48 hours. Tissues were either fast 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 hour in serum-free medium and then stimulated with human recombinant EGF (Sigma, catalog no.9944) prior to extraction. Cells were solubilized at 4°C in a 20 mmol/L Tris buffer, pH 7.4 containing 1% Nonidet P-40, 0.137 mol/L NaCl, 10% glycerol, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitors (Roche). Frozen tumors were
Cells were washed three times with cold Dulbecco PBS and 555 (50 ng/mL, Molecular Probes; catalog no. E35350) for 10 minutes. Bands were detected by chemiluminescence using a ferredoxin enzyme-linked immunosorbent assay (ELISA) kit (Roche). For immunoprecipitations, aliquots of cell or tumor extracts containing equivalent amounts of protein were precleared for 1 hour with nonspecific IgG and protein-A or -G sepharose beads (GE Healthcare) and then incubated for 3 hours 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 XR™ system (Bio-Rad Laboratories) and band intensities were quantified by densitometry using Image Lab (Beta 1; Bio-Rad Laboratories) or ImageJ. 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 hours and then treated with or without EGF-AlexaFluor 555 (50 ng/mL, Molecular Probes; catalog no. E35350) for 10 minutes. Cells were washed three times with cold Dulbecco PBS and fixed in 3.8% paraformaldehyde in Dulbecco PBS with 0.5% Tween (PBST) for 1 hour. Permeabilized cells were blocked for 1 hour using 3% BSA in PBST. Primary antibodies diluted in blocking buffer were added to cells and incubated at room temperature for 1 hour. 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 Technology) and the slides were viewed by confocal microscopy (Zeiss LSM710; 63× oil immersion objective). All images were adjusted equally for brightness/contrast using Adobe Photoshop.

IHC

Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated and antigen retrieval was performed in 10 mmol/L sodium citrate buffer, pH 6.0 with heating in a steamer for 1 hour. Tissues were incubated with 0.3% hydrogen peroxide to quench endogenous peroxides and then blocked using a Dual Avidin/Biotin Blocking Kit (Vector Laboratories, catalog no. SP-2001) followed by a 1-hour incubation in 1 × casein milk (Vector Laboratories, catalog no. SP-2050). Tissue sections were incubated with primary antibodies overnight followed by secondary antibody incubation with the elite ABC-HRP Kit (Vector Laboratories, catalog no. PK6101). Sections were developed with diaminobenzidine (DAB; Dako, catalog no. 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 the manufacturer's instructions (Promega, catalog no. G3250). Stained tissue sections were viewed and images captured on a Zeiss LSM 700 microscope. All images were adjusted equally for brightness/contrast using Adobe Photoshop.

qPCR

RNA was extracted from tumors using the RNeasy Kit (Qiagen, catalog no. 74134). cDNA was synthesized using a one-step cDNA kit (Biotool, catalog no. B22403). qRT-PCR was performed in a 20 μL reaction containing 0.5 μmol/L primers, 20 ng cDNA template, and 1 × SYBR Green Supermix (Biotool, catalog no. B2120). Primers were designed using the Harvard PrimerBank (Supplementary Table S1). Human R18S primers were used as a housekeeping control. qRT-PCR was performed using the Applied Biosystems QuantStudio 6 Flex apparatus. The ΔΔCt method was used to determine relative mRNA expression.

Results

Beclin 1 regulates endosomal HRS recruitment

Our previous in vitro studies demonstrated that Beclin 1 regulates insulin-like growth factor-1 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 HRS, which contains both an ubiquitin binding 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 ILVs of MVEs, 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 triple-negative breast cancer (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 using an in vivo permeabilization method. The HRS FYVE domain is recruited to the early endosome following EGF stimulation and the number of cytoplasmic HRS puncta increased markedly in Beclin 1–shRNA-positive puncta were observed in the cytoplasm of serum-starved cells, with a few puncta evident. After stimulation for 10 minutes, a number of oligomerization of the lectin domain of EGFR was 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 colocalized with

Statistical analysis

Statistical analysis between two groups was performed using the two-tailed unpaired Student 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. Fisher exact test was performed to determine MAPK enrichment in clusters.
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 (50 ng/mL) for 10 minutes. Cells were costained with HRS-specific antibodies. The data shown in the graph on the top right represent the mean ± SEM HRS puncta/cell (n = 17–25 cells). Scale bar, 10 μm. **B**, MDA-MB-231 LM2 cells expressing shGFP, shBECN1(#1), or shBECN1(#2) were stimulated with human EGF (50 ng/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 ± SEM of three independent experiments. **C**, MDA-MB-231 LM2 cells expressing shGFP, shATG5(#1), or shATG5(#2) were stimulated with human EGF (50 ng/mL) for the indicated time periods. Total cell extracts were immunoblotted with the indicated antibodies. Bottom, data shown represent the mean ± SEM of three independent experiments. *, P < 0.05; ***, P < 0.005.
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 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 cosegregated 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. On the basis of 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, subclusters 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 subclusters 1 and 11 identified several growth factors and hormone receptors (EGFR, IRIR, c-KIT, VEGFR2, phospho-HER3) and their downstream signaling intermediates (pY759-phospholipase C gamma2 (PLCγ2), pS664-protein kinase C delta (PKCd), 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 MAPK pathway components (receptors, kinases, and downstream substrates) that were included in the RPPA analysis revealed a significant enrichment for MAPK pathway activity in subcluster 1 and elevated pathway activity in subcluster 11 (Fig. 4D; 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. S2A and S6A), providing further evidence that the regulation of this signaling pathway by Beclin 1 may be independent of its regulation 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 was suppressed in another TNBC cell line SUM-159PT (Supplementary Fig. S1B). In contrast, EGFR-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 EGFR-stimulated recruitment of HRS to endosomes is regulated in a Beclin 1–dependent manner, but may be independent of autophagy.

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 with 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 B). 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.

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). EGFR-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, EGFR-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 EGFR-stimulated recruitment of HRS to endosomes is regulated in a Beclin 1–dependent manner, but may be independent of autophagy.

Beclin 1 Regulates Trafficking to Suppress Tumor Growth

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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 hours.
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 ± SEM positive nuclei/high powered field (hpf; five independent images/five tumors (n = 25). Scale bar, 50 μm. B, Representative images of TUNEL staining in tumors. The data shown in the graphs represent the mean ± SEM positive nuclei/hpf (three independent images/six tumors; n = 18). Scale bar, 50 μm. *, P < 0.05; n.s., nonsignificant.

Figure 3.

Beclin 1 regulates transferrin receptor-1 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, TFR1 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.
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(1–3) (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 subclusters). Red boxes identify subclusters with elevated expression patterns in shBECN1 tumors. B and C, Scatterplots of subcluster 1 (B) and subcluster 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. Bottom, data represent the mean ± 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. The data shown represent the mean ± 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 reprobed 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 ± SEM HRS phosphorylation of four tumors of each genotype and are shown as relative phosphorylation. *P < 0.05.
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 whether increased TFR1 expression contributes to the enhanced proliferation of shBECN1 tumors, LM2 cells were coinfected with shRNA targeting BECN1 and TFRC. Cells with a modest

Figure 5.

Proliferation of shBECN1 tumors is sensitive to inhibition of EGFR and ERK1/2 signaling. A, Immunoblot analysis of representative shGFP and shBECN1(t#1) tumors treated ex vivo for 48 hours with DMSO, lapatinib (Lap; 5 μmol/L), or PD98059 (PD; 10 μmol/L). The data shown in the graphs represent the mean ± 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, representative cells with reduced nuclear localization of ERK1/2. Scale bar, 50 μm. C, Representative images of hematoxylin and eosin or Ki67 staining of shGFP and shBECN1 tumors treated ex vivo as indicated. Bottom, data shown represent the mean ± SEM positive nuclei/hpf (three independent images/five tumors; n = 15). Scale bar, 50 μm. *P < 0.05; ***P < 0.005.
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 F). Tumor sections were analyzed for PH3 or TUNEL staining to determine whether the reduced growth observed upon suppression of TFR1 expression in the shBECN1 tumors was the result of decreased proliferation or

Figure 6.
Beclin 1 regulation of transferrin receptor expression promotes tumor proliferation. A, Scatterplot of subcluster 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. Right, data shown represent the mean ± SEM TFR1 expression from thirteen tumors of each genotype. C, TFRC mRNA expression. The data shown represent the mean ± 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 reprobed 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 TFR1 expression prior to injection. F, Expression of Beclin 1 and TFR1 in tumors. Right, data represent the mean ± SEM expression from six tumors of each genotype. G, Representative images of PH3 staining in tumors. Bottom, data shown represent the mean ± SEM positive nuclei/hpf (five independent images/five tumors; n = 25). Scale bar, 50 μm. H, Representative images of TUNEL staining in tumors. Right, data shown represent the mean ± SEM positive nuclei/hpf (three independent images/six tumors; n = 18). Scale bar, 50 μm. *P < 0.05; **P < 0.005; n.s., nonsignificant.
increased cell death, respectively (Fig. 6G and H). As we observed previously (Fig. 2), shBECN1 tumors exhibited increased PH3 staining compared with 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 plots (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). In 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 pathways.

Figure 7.

Beclin 1 regulates receptor trafficking through HRS. A, Kaplan–Meier plots showing the impact of HRS expression on the relapse-free survival 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.
Our demonstration that Beclin 1 controls the early endosome trafficking of EGFR and downstream activation of ERK1/2 increased in tumors upon reduction of Beclin 1 expression. This result is consistent with the fact that TNBC is frequently associated with elevated EGFR expression and activity (53). 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 patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Beclin 1 Regulates Trafficking to Suppress Tumor Growth

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Beclin 1 Promotes Endosome Recruitment of Hepatocyte Growth Factor Tyrosine Kinase Substrate to Suppress Tumor Proliferation

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