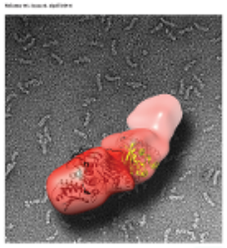
Autophagy



Autophagy

ISSN: 1554-8627 (Print) 1554-8635 (Online) Journal homepage: https://www.tandfonline.com/loi/kaup20

ERBB2 overexpression suppresses stress-induced autophagy and renders ERBB2-induced mammary tumorigenesis independent of monoallelic *Becn1* loss

Fred Lozy, Xiaofeng Cai-McRae, Irina Teplova, Sandy Price, Anupama Reddy, Gyan Bhanot, Shridar Ganesan, Alexei Vazquez & Vassiliki Karantza

To cite this article: Fred Lozy, Xiaofeng Cai-McRae, Irina Teplova, Sandy Price, Anupama Reddy, Gyan Bhanot, Shridar Ganesan, Alexei Vazquez & Vassiliki Karantza (2014) ERBB2 overexpression suppresses stress-induced autophagy and renders ERBB2-induced mammary tumorigenesis independent of monoallelic *Becn1* loss, Autophagy, 10:4, 662-676, DOI: 10.4161/auto.27867

To link to this article: https://doi.org/10.4161/auto.27867

Autophagy 10:4, 662–676; April 2014; © 2014 Landes Bioscience

ERBB2 overexpression suppresses stress-induced autophagy and renders ERBB2-induced mammary tumorigenesis independent of monoallelic *Becn1* loss

Fred Lozy,[1,2] Xiaofeng Cai-McRae,[1,2] Irina Teplova,[1,2] Sandy Price,[1,2] Anupama Reddy,[1] Gyan Bhanot,[1,2] Shridar Ganesan,[1,2,3] Alexei Vazquez,[1,2] and Vassiliki Karantza[1,2,3,*]

[1]Rutgers University; State University of New Jersey; New Brunswick, NJ USA; [2]Rutgers Cancer Institute of New Jersey; New Brunswick, NJ USA; [3]Division of Medical Oncology; Department of Internal Medicine; Rutgers Robert Wood Johnson Medical School; Piscataway, NJ USA

Keywords: autophagy, ERBB2, breast cancer, MMTV-*Neu*, MMTV-*PyMT*, trastuzumab

Abbreviations: AKT1, v-akt murine thymoma viral oncogene homolog 1; ATG, autophagy-related; BA, basal-like; BafA1, bafilomycin A_1; BCL2, B-cell CLL/lymphoma 2; BECN1, Beclin 1; CASP3, caspase 3; CQ, chloroquine; EGFP, enhanced green fluorescent protein; EM, electron microscopy; ER, estrogen receptor; RB1CC1/FIP200, RB1-inducible coiled-coil 1; ERBB2, v-erb-b2 avian erythoblastic leukemia viral oncogene homolog 2; ERBB3, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3; ERBB4, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4; IHC, immunohistochemistry; iMMECs, immortalized mouse mammary epithelial cells; MAP1LC3B (LC3B), microtubule-associated protein 1 light chain 3 beta; MAPK1, mitogen-activated protein kinase 1; MAPK3, mitogen-activated protein kinase 3; MG, mammary gland; MKI67, marker of proliferation Ki-67; MMTV, mouse mammary tumor virus; PALB2, partner and localizer of BRCA2; PI3K, phosphoinositide 3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; pMMECs, primary mouse mammary epithelial cells; PTEN, phosphatase and tensin homolog; PyMT, polyoma middle T; RAS (KRAS and HRAS), Kirsten and Harvey rat sarcoma viral oncogene homolog; SlG, salivary gland; SQSTM1/p62, sequestosome 1; TOP2A, topoisomerase (DNA) II alpha 170 kDa; TP53, tumor protein 53; TZB and tzb, trastuzumab; UT, untreated

Defective autophagy has been implicated in mammary tumorigenesis, as the gene encoding the essential autophagy regulator BECN1 is deleted in human breast cancers and *Becn1*$^{+/-}$ mice develop mammary hyperplasias. In agreement with a recent study, which reports concurrent allelic *BECN1* loss and ERBB2 amplification in a small number of human breast tumors, we found that low *BECN1* mRNA correlates with ERBB2-overexpression in breast cancers, suggesting that *BECN1* loss and ERBB2 overexpression may functionally interact in mammary tumorigenesis. We now report that ERBB2 overexpression suppressed autophagic response to stress in mouse mammary and human breast cancer cells. ERBB2-overexpressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ immortalized mouse mammary epithelial cells (iMMECs) formed mammary tumors in nude mice with similar kinetics, and monoallelic *Becn1* loss did not alter ERBB2- and PyMT-driven mammary tumorigenesis. In human breast cancer databases, ERBB2-expressing tumors exhibit a low autophagy gene signature, independent of *BECN1* mRNA expression, and have similar gene expression profiles with non-ERBB2-expressing breast tumors with low *BECN1* levels. We also found that ERBB2-expressing BT474 breast cancer cells, despite being partially autophagy-deficient under stress, can be sensitized to the anti-ERBB2 antibody trastuzumab (tzb) by further pharmacological or genetic autophagy inhibition. Our results indicate that ERBB2-driven mammary tumorigenesis is associated with functional autophagy suppression and ERBB2-positive breast cancers are partially autophagy-deficient even in a wild-type *BECN1* background. Furthermore and extending earlier findings using tzb-resistant cells, exogenously imposed autophagy inhibition increases the anticancer effect of trastuzumab on tzb-sensitive ERBB2-expressing breast tumor cells, indicating that pharmacological autophagy suppression has a wider role in the treatment of ERBB2-positive breast cancer.

*Correspondence to: Vassiliki Karantza; Email: karantva@cinj.rutgers.edu
Submitted: 06/21/2013; Revised: 01/12/2014; Accepted: 01/14/2014
http://dx.doi.org/10.4161/auto.27867

Introduction

Autophagy is a dynamic self-catabolic cellular process, whereby proteins and organelles are targeted to lysosomes for degradation. Autophagy is upregulated during periods of stress and maintains cell viability by enabling basic biomolecule and energy recycling. Under regular growth conditions, basal autophagy preserves cellular homeostasis by mediating degradation of misfolded proteins and aged or damaged organelles, thus mitigating cell damage.[1-4]

Defective autophagy has been implicated in tumorigenesis, as the essential autophagy gene, *BECN1*, is commonly deleted in breast, ovarian, and prostate cancers.[5] BECN1 is essential for autophagosome formation and, when ectopically expressed in partially autophagy-deficient human MCF7 breast cancer cells, it restores functional autophagy and suppresses tumorigenesis.[6] *becn1*$^{-/-}$ mice die early in embryogenesis, while aging *Becn1*$^{+/-}$ mice are tumor-prone, developing lymphomas and carcinomas of the lung and liver.[7,8] Furthermore, mammary tissues from *Becn1*$^{+/-}$ mice display preneoplastic, hyperproliferative changes, but no spontaneous mammary carcinomas.[7] The seemingly paradoxical association between increased tumorigenesis and dysfunction and/or loss of a survival mechanism can be reconciled by the findings that autophagy defects render cells susceptible to metabolic stress and DNA damage, thus enhancing tumor necrosis, inflammation and genomic instability, which in turn accelerate tumorigenesis.[4,9,10] However, autophagy may also act as a tumor-promoting mechanism by supporting cancer cell survival, as it is readily induced in hypoxic tumor regions and in response to chemotherapy and radiation.[11-13]

Although allelic *BECN1* loss has been implicated in the pathophysiology of breast cancer,[5] its specific role(s) in tumor initiation and progression have not been determined. A recent study reveals significant association between *BECN1* deletion and *ERBB2* amplification,[14] thus providing evidence for lower BECN1 expression in a particular breast cancer subtype.[15]

ERBB2/HER2/neu (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) is a member of the HER family of tyrosine kinases, along with EGFR (epidermal growth factor receptor), ERBB3, and ERBB4. In normal cells, a variety of extracellular ligands bind to HER receptor heterodimers, leading to activation of pathways that control growth, differentiation, motility, and adhesion.[16-20] Deregulation of these signaling networks occurs frequently in cancer, as exemplified by *ERBB2* gene amplification in breast cancer and by constitutive EGFR activation in lung and colon cancers.[21-25] ERBB2 overexpression results in aberrant signaling of the PI3K-AKT1 and MAPK1/3 pathways, which in turn are associated with malignant transformation,[26] and ERBB2-positive breast malignancies are characterized by aggressive nature, poor clinical outcome, and chemotherapy resistance.[27] In addition to *ERBB2* amplification, further genomic changes are commonly required for ERBB2-induced tumorigenesis, as abnormal ERBB2 signaling leads to apoptosis in cells carrying wild-type *TP53*.[28,29]

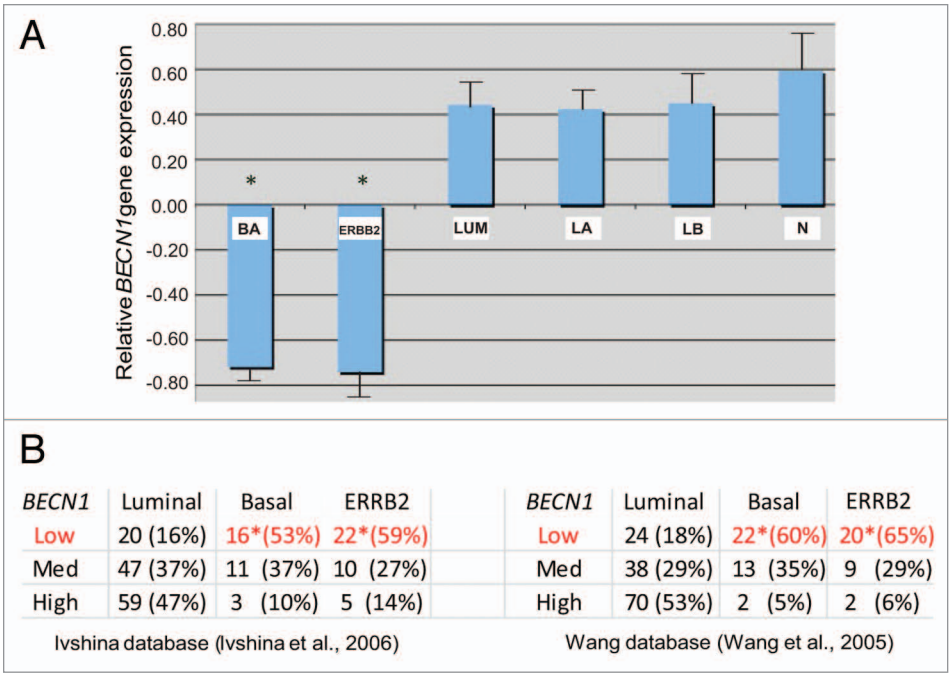
Interestingly, the human *BECN1* and *ERBB2* genes are both located on chromosome 17, specifically at the 17q12 and 17q21 locuses, respectively, which are characterized by frequent genomic instability events, such as *ERBB2-TOP2A* amplification and allelic loss events,[30-32] in human tumors. In a small number of breast tumors examined by fluorescence in situ hybridization, genomic *BECN1* loss correlates with *ERBB2* amplification and this result has been confirmed in 2 independent, public copy number microarray data sets.[14] Furthermore, breast cancers with concurrent *BECN1* deletion and *ERBB2* amplification were also characterized by alterations in the *TP53*, *PTEN,* and *PIK3CA* genes.[14] However, despite the reported association between *BECN1* loss and ERBB2-positive breast cancer, the role of *BECN1* in ERBB2-induced mammary tumorigenesis has not yet been investigated. Quite intriguingly, ERBB2-positive tumors resistant to the humanized mouse monoclonal ERBB2 antibody trastuzumab (tzb) upregulate basal autophagy and are resensitized to treatment by autophagy inhibition,[33] thus implicating autophagy induction in development of treatment resistance and the high relapse rates observed in patients with metastatic ERBB2-positive breast cancer.

To determine the role of *BECN1* deficiency in ERBB2-positive breast cancer pathogenesis and treatment, we investigated the impact of ERBB2 overexpression on the functional status of autophagy in immortalized mouse mammary epithelial cells and human breast cancer cell lines under metabolic stress. We also investigated the effect of monoallelic *Becn1* loss on mammary tumorigenesis in the MMTV-*Neu* and MMTV-*PyMT* mouse tumor models. We now report that ERBB2 overexpression does not affect basal autophagy, but suppresses stress-induced autophagy in mammary tumor cells, even in a wild-type *Becn1* background. Furthermore, monoallelic *Becn1* deletion does not alter the tumorigenicity of ERBB2-expressing iMMECs in nude mice in vivo and does not impact spontaneous mammary tumorigenesis in the MMTV-*Neu* and MMTV-*PyMT* mouse models. We also found that low *BECN1* expression correlates with the ERBB2-positive and basal-like human breast cancer subtypes and that ERBB2-positive breast tumors, independently of *BECN1* mRNA levels, are likely functionally autophagy-deficient, as determined by gene expression profiling. Finally, both genetic and pharmacological autophagy inhibition enhance the response of tzb-sensitive, ERBB2-positive breast cancer cells to trastuzumab, indicating that autophagy modulation may improve the therapeutic efficacy of standard treatment in ERBB2-positive breast cancer.

Results

Low *BECN1* expression correlates with ERBB2-positive and basal-like breast cancer subtypes

Given the recently reported association between genetic *BECN1* loss and ERBB2 amplification in a small number of human breast tumors,[14] we investigated larger human breast cancer gene profiling databases to determine whether *BECN1* expression correlates in any way with particular breast cancer subtypes. Using 3 independent DNA microarray databases[34-36] totaling 254 breast cancer specimens, we discovered that both ERBB2-positive and basal-like breast tumors commonly exhibit



B

BECN1	Luminal	Basal	ERRB2
Low	20 (16%)	16*(53%)	22*(59%)
Med	47 (37%)	11 (37%)	10 (27%)
High	59 (47%)	3 (10%)	5 (14%)

Ivshina database (Ivshina et al., 2006)

BECN1	Luminal	Basal	ERRB2
Low	24 (18%)	22*(60%)	20*(65%)
Med	38 (29%)	13 (35%)	9 (29%)
High	70 (53%)	2 (5%)	2 (6%)

Wang database (Wang et al., 2005)

Figure 1. Low *BECN1* mRNA levels correlate with the ERBB2-positive and triple negative breast cancer subtypes. (**A**) Relative expression of *BECN1* mRNA in basal (BA), luminal (LUM), and ERBB2-amplified (ERBB2) breast cancer subclasses and normal breast tissue (N) from samples analyzed in Wang et al. and Richardson et al. The luminal class is shown further subdivided into luminal A (LA) and luminal B (LB) subclasses. The mean expression of *BECN1* in the total sample set is shown normalized to 0. (**B**) The relative number of samples falling into the lower, middle, and high tertiles of *BECN1* gene expression in BA, LUM and ERBB2 breast cancer subclasses in an independent set of samples from Ivshina et al. and Wang et al. is shown. Statistically significant subclasses (*) indicating a *P* value < 0.05 (Student *t* test).

low *BECN1* expression, whereas estrogen receptor (ER)-positive tumors are characterized by higher *BECN1* mRNA levels (**Fig. 1A and B**, $P < 0.05$).

ERBB2 overexpression in mammary tumor cells does not affect basal autophagy, but suppresses autophagy induction in response to metabolic stress

To examine the effect of ERBB2 overexpression on the functional status of autophagy, we used our previously described mouse mammary epithelial cell model,[37] and transfected *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs with a plasmid expressing wild-type human ERBB2. While the transfection efficiencies were similar for *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs as previously described,[4] we obtained several ERBB2-expressing *Becn1*$^{+/+}$ iMMEC lines (7 out of 13 antibiotic-resistant colonies), but only one ERBB2-expressing *Becn1*$^{+/-}$ iMMEC line (1 out of 13 antibiotic-resistant colonies) (**Fig. 2A**), possibly indicating that ERBB2 overexpression is not well tolerated in partially autophagy-deficient *Becn1*$^{+/-}$ iMMECs and is, thus, negatively selected for in vitro. Similar results were obtained when the experiment was repeated, at which time one more ERBB2-expressing *Becn1*$^{+/-}$ iMMEC line was recovered.

Given the recently documented upregulation of basal autophagy in mutant RAS-expressing mouse and human cancer cell lines and the resultant dependence of RAS-mutant tumors on autophagy for growth,[38] we hypothesized that the low recovery rate of ERBB2-expressing *Becn1*$^{+/-}$ cell lines might be secondary to a requirement for high functional autophagy in ERBB2-positive breast tumor cells. To investigate how ERBB2 overexpression impacts autophagy, we used stably and transiently ERBB2-expressing iMMEC and human breast cancer cell lines. First, BCL2-expressing, apoptosis-deficient *Becn1*$^{+/+}$ iMMECs stably expressing EGFP-LC3B and either a human wild-type ERBB2 plasmid or vector control were subjected to nutrient deprivation (Hanks treatment) in the absence or presence of the autophagic flux inhibitor, bafilomycin A_1 (BafA1). Autophagy induction was quantified by fluorescence microscopy, as previously described.[4,39,40] As shown in **Figure 2B and C**, ERBB2-overexpressing apoptosis-deficient *Becn1*$^{+/+}$ iMMECs exhibited highly attenuated puncta formation in response to nutrient deprivation compared with vector-expressing *Becn1*$^{+/+}$ iMMECs. This effect was observed even in the presence of BafA1 at a concentration (25 nM) that inhibits autophagic flux without affecting cell viability (**Fig. S1**), thus indicating that ERBB2 overexpression suppresses stress-induced autophagy in apoptosis-defective iMMECs (**Fig. 2C**, $P < 0.05$). This result was verified by LC3B immunoblotting to follow the conversion of LC3B-I to LC3B-II.[41] In the absence of BafA1, LC3B-II increased over time in vector-expressing *Becn1*$^{+/+}$ iMMECs under nutrient deprivation, indicating autophagy induction. In the presence of BafA1, LC3B-II was stabilized and displayed higher levels compared with non-BafA1 conditions, in agreement with LC3B-II accumulation in association with autophagic flux inhibition. In contrast, in ERBB2-expressing iMMECs, total LC3B protein levels and LC3B-I to LC3B-II conversion, normally observed in wild-type iMMECs under nutrient deprivation, were suppressed both in the absence and presence of BafA1 (**Fig. 2D and E**), thus indicating that this result was not secondary to ERBB2-promoted acceleration of autophagic flux. The autophagy adaptor, SQSTM1/p62, commonly degraded during the process of autophagy exhibited higher protein levels in apoptosis-deficient ERBB2-expressing *Becn1*$^{+/+}$ iMMECs compared with vector-expressing *Becn1*$^{+/+}$ iMMECs, also indicating a suppression of the autophagic process (**Fig. 2D and E**).[39] The same result was obtained using a different metabolic stressor and quantifying autophagy by electron microscopy (EM). In this case, apoptosis-deficient *Becn1*$^{+/+}$ iMMECs stably expressing ERBB2 under low (1%) oxygen and glucose-deprivation conditions showed decreased number of autophagosomes compared with their non-ERBB2-expressing *Becn1*$^{+/+}$ counterparts (**Fig. 2F and G**).

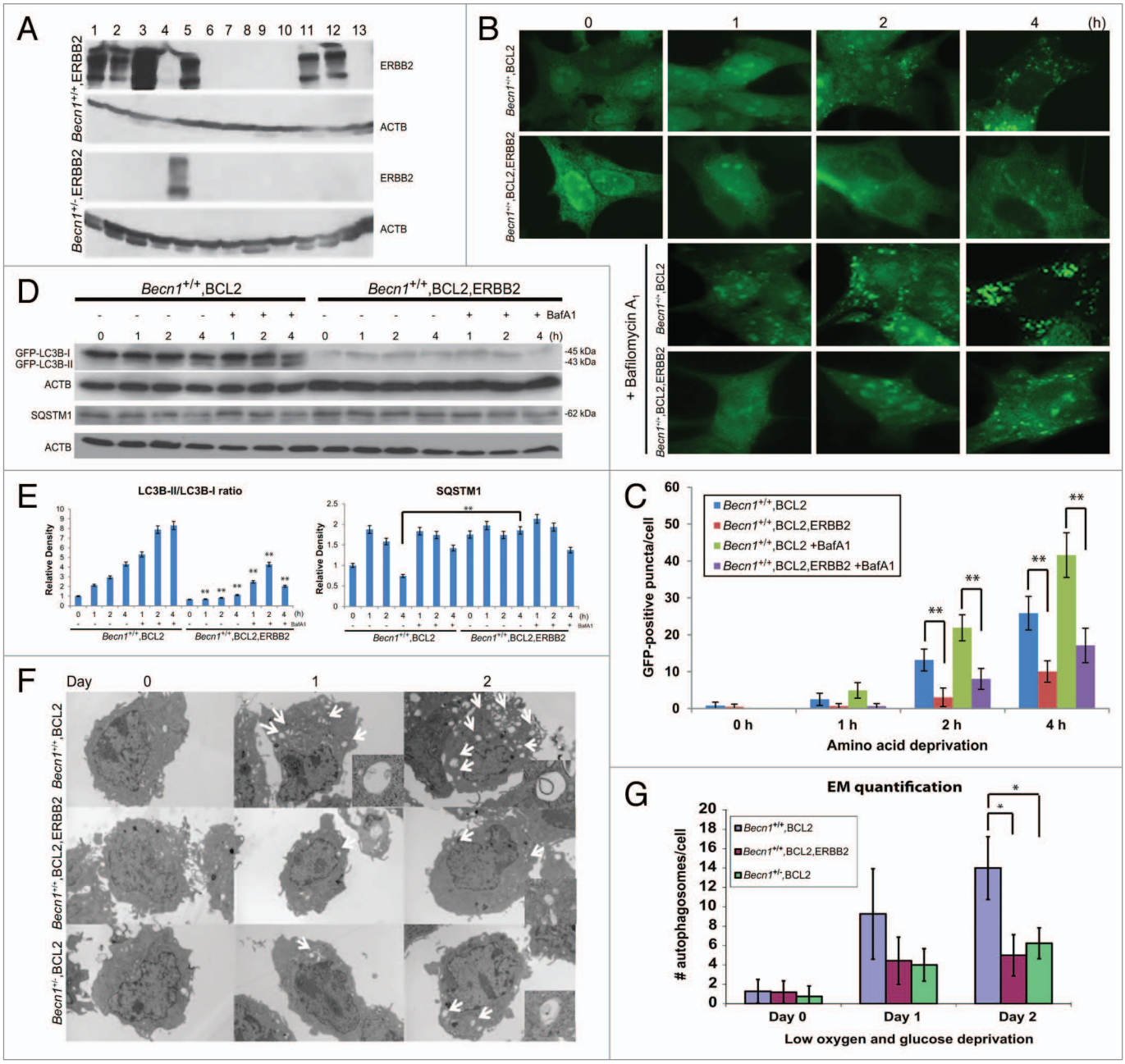


Figure 2. Stable ERBB2 overexpression inhibits stress-induced autophagy in mouse mammary tumor cells. (**A**) Autophagy-competent *Becn1*$^{+/+}$ and partially autophagy-defective *Becn1*$^{+/-}$ iMMECs were stably transfected with a human wild-type ERBB2-expressing plasmid conferring resistance to G418. Seven out of 13 *Becn1*$^{+/+}$ G418^{R}-colonies and 1 out of 13 *Becn1*$^{+/-}$ G418^{R}-colonies overexpressed ERBB2, as shown by ERBB2 and ACTB western blots of whole cell protein lysates. (**B**) GFP-fluorescence microscopy of BCL2- and EGFP-LC3B-expressing *Becn1*$^{+/+}$ iMMECs, also stably expressing an ERBB2 or vector-control plasmid, under nutrient deprivation (Hanks medium) without or with bafilomycin A_1 (BafA1, 25 nM). (**C**) Autophagy quantification of (**B**) based on number of GFP-fluorescent puncta per cell, as imaged by GFP-fluorescence microscopy. Each data point is an average of triplicate experiments ± SD after quantifying puncta in one hundred cells per experiment. ***P* value < 0.01. (**D**) GFP, SQSTM1 and ACTB western blots of whole cell protein lysates from apoptosis-deficient, EGFP-LC3B-expressing, and vector- or ERBB2-expressing *Becn1*$^{+/+}$ iMMECs under nutrient deprivation without (-) and with (+) BafA1. ERBB2-expressing iMMECs exhibited lower GFP-LC3B expression than vector-expressing iMMECs after stable GFP-LC3B transfection, so protein lysates from ERBB2-expressing iMMECs were loaded at 2× the amount of vector-expressing lysates to better visualize lower GFP-LC3B levels in these cells. (**E**) Densitometric analysis of LC3B-II/LC3B-I ratio and SQSTM1 protein bands, as normalized to ACTB, using ImageJ. LC3B levels were corrected for 2× loading of ERBB2-expressing lysates. (**F**) Electron micrographs of *Becn1*$^{+/+}$ iMMECs expressing BCL2 (top row), *Becn1*$^{+/+}$ iMMECs expressing BCL2 and ERBB2 (middle row), and *Becn1*$^{+/-}$ iMMECs expressing BCL2 (bottom row) under metabolic stress (1% oxygen and no glucose). Images were taken at 3800×. White arrows indicate autophagic structures. Inlayed zoomed images show autophagosomes. (**G**) Autophagosome quantification of (**F**). Each data point is an average of the number of autophagosomes in 25 cells ± SD. **P* value < 0.05.

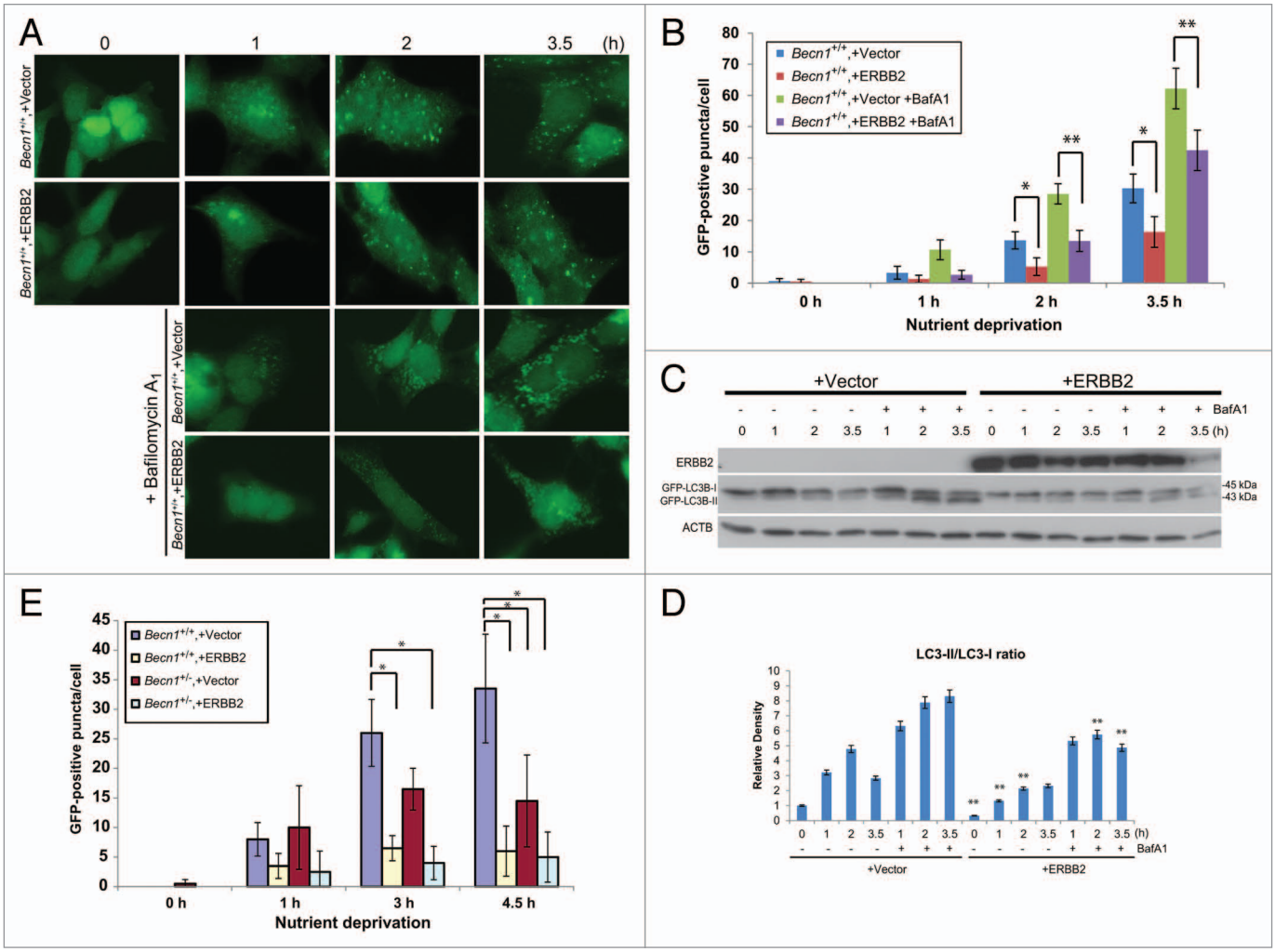


Figure 3. Transient ERBB2 overexpression inhibits stress-induced autophagy in *Becn1*$^{+/+}$ iMMECs to the level observed in partially autophagy-defective non-ERBB2-expressing *Becn1*$^{+/-}$ iMMECs. (**A**) GFP-fluorescence microscopy of EGFP-LC3B-expressing *Becn1*$^{+/+}$ iMMECs transiently transfected with a ERBB2-expressing or vector control plasmid under nutrient deprivation conditions without or with bafilomycin A_1 (BafA1, 25 nM). (**B**) Autophagy quantification of (**A**) based on number of GFP-fluorescent puncta per cell. Each data point is an average of triplicate experiments ± SD after quantifying puncta in 100 cells per experiment. **P* value < 0.05; ***P* value < 0.01. (**C**) GFP and ACTB western blots of whole cell protein lysates from *Becn1*$^{+/+}$ iMMECs transiently expressing ERBB2 under nutrient deprivation without and with BafA1. (**D**) Densitometric analysis of LC3B-II/LC3B-I ratio, as normalized to ACTB, using ImageJ. (**E**) EGFP-LC3B-expressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs transiently transfected with a ERBB2-expressing or vector control plasmid were subjected to nutrient deprivation, and autophagy was quantified by the number of GFP-fluorescent puncta per cell. Each data point is an average of triplicate experiments ± SD after quantifying puncta in 100 cells per experiment. **P* value < 0.05; ***P* value < 0.01.

Interestingly, the level of autophagy induction in metabolically stressed ERBB2-expressing *Becn1*$^{+/+}$ iMMECs was similar to that of vector-expressing *Becn1*$^{+/-}$ iMMECs (**Fig. 2F and G**), confirming that ERBB2 overexpression renders mammary epithelial cells partially autophagy-deficient under stress.

To further investigate the impact of ERBB2 overexpression on stress-induced autophagy in an alternate system and in an apoptosis-competent background, we used a transient ERBB2 expression system.[40] To this intent, *Becn1*$^{+/+}$ iMMECs stably overexpressing EGFP-LC3B were transiently transfected with a ERBB2-expressing or vector control plasmid and, after overnight recovery in regular culture medium, were incubated in Hanks medium for up to 3.5 h. Similar to the results described above (**Fig. 2B–E**), transient ERBB2 overexpression did not affect basal autophagy, but suppressed autophagy induction in wild-type iMMECs in response to nutrient deprivation (**Fig. 3A–D**, $P < 0.01$). This result was confirmed by decreased LC3B-I to LC3B-II conversion in iMMECs transiently overexpressing ERBB2 in both the absence and presence of BafA1 (**Fig. 3C and D**). ERBB2 overexpression did not affect expression of the essential autophagy regulators BECN1 and ATG7 (**Fig. S2**), but resulted in decreased conversion of endogenous LC3B-I to LC3B-II (**Fig. 3C**), indicating that ERBB2-promoted suppression of the autophagic response to stress was not associated with alterations in ATG expression.

Finally, when EGFP-LC3B-expressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs were transiently transfected with a ERBB2-expressing or vector control plasmid and subjected to nutrient starvation,

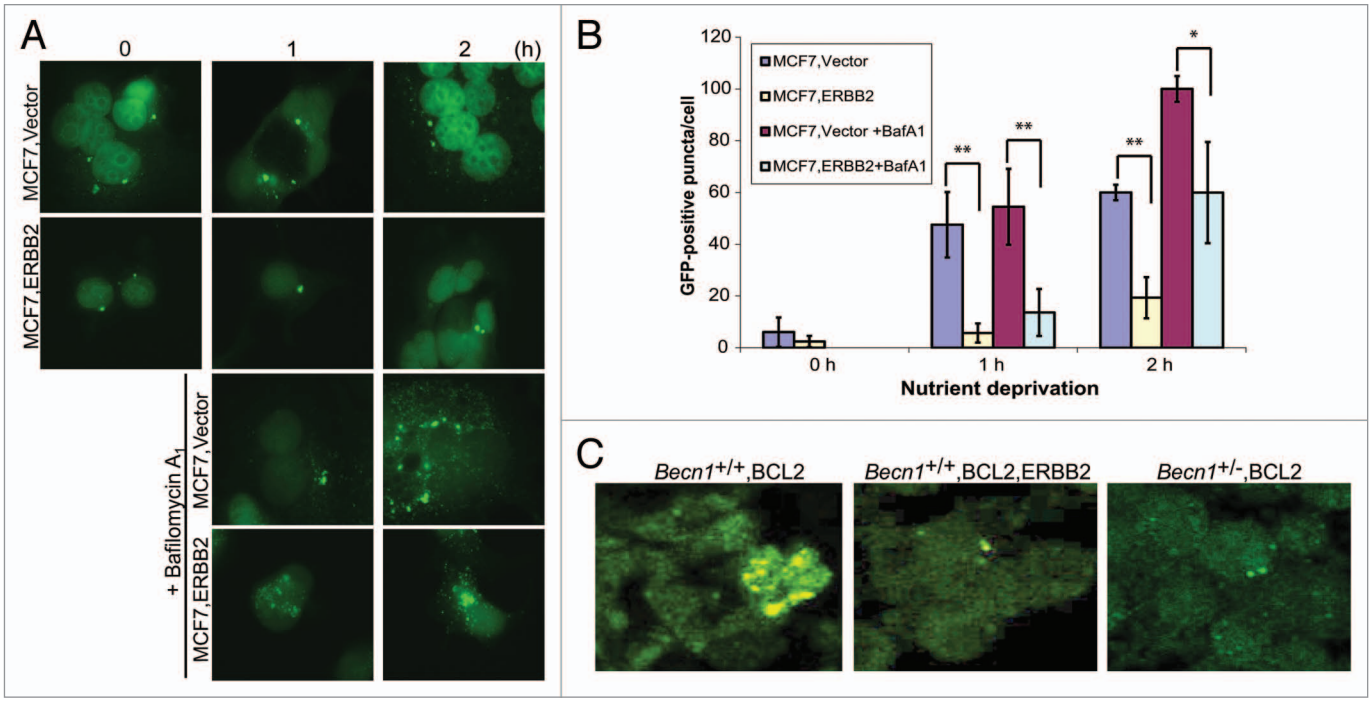


Figure 4. Transient ERBB2 overexpression inhibits stress-induced autophagy in human breast cancer cells. (**A**) GFP-fluorescence microscopy of EGFP-LC3B-expressing MCF7 cells transiently transfected with a ERBB2-expressing or vector control plasmid under nutrient deprivation conditions for 0, 1, and 2 h without and with bafilomycin A_1 (BafA1, 25 nM). (**B**) Autophagy quantification of (**A**) based on number of GFP-fluorescent puncta per cell. Each data point is an average of triplicate experiments ± SD after quantifying puncta in 100 cells per experiment. **P* value < 0.05; ***P* value < 0.01. (**C**) GFP-fluorescence confocal microscopy of tumor cell plaques dissected 24 h post orthotopic implantation of BCL2-expressing *Becn1*$^{+/+}$ (left panel), BCL2- and ERBB2-expressing *Becn1*$^{+/+}$ (middle panel), and BCL2 expressing *Becn1*$^{+/-}$ (right panel) iMMECs in nude mice.

ERBB2 overexpression in either *Becn1*$^{+/+}$ or *Becn1*$^{+/-}$ iMMECs induced similar number of autophagic puncta to those observed in vector-transfected *Becn1*$^{+/-}$ iMMECs (**Fig. 3E**, $P < 0.05$), indicating that ERBB2 expression rendered mammary epithelial cells autophagy-defective, independent of allelic *Becn1* status.

The effect of ERBB2 overexpression on the functional status of autophagy in human breast cancer cells was examined by transfection of stably EGFP-LC3B-expressing MCF7 cells with an ERBB2-expressing or vector control plasmid. Similar to the iMMEC results described above (**Fig. 2**; **Fig. 3**), transient ERBB2 expression did not alter basal autophagy levels in MCF7 cells, but suppressed autophagy induction in response to nutrient starvation (**Fig. 4A and B**). Inhibition of autophagic flux by bafilomycin A_1 resulted in higher GFP puncta accumulation per cell in vector- compared with ERBB2-expressing MCF7 cells (**Fig. 4A and B**, $P < 0.05$), again indicating that the ERBB2 signaling pathway decreases autophagy induction in response to stress and, thus, suggesting that ERBB2-positive breast tumors may be functionally autophagy-defective, independent of *BECN1* expression.

To examine the effect of ERBB2 overexpression on the functional status of autophagy in vivo, BCL2-, ERBB2- and EGFP-LC3B-expressing *Becn1*$^{+/+}$ iMMECs, as well as BCL2-expressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs, were orthotopically implanted into the mammary fat pad of nude mice. Plaques were dissected 24 h post iMMEC implantation and LC3B translocation was qualitatively evaluated using fluorescence confocal microscopy. Similar to **Figures 2D and 3E**, ERBB2-overexpressing *Becn1*$^{+/+}$ mammary cells exhibited similar number and size of GFP-LC3B puncta to *Becn1*$^{+/-}$ cells in vivo, but fewer and smaller in size puncta than *Becn1*$^{+/+}$ cells (**Fig. 4C**).

Monoallelic *Becn1* loss does not alter ERBB2- and PyMT-driven mammary tumorigenesis

To investigate whether allelic *Becn1* status impacts ERBB2-induced mammary tumorigenesis, ERBB2-overexpressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs were orthotopically implanted in NCR nude female mice. The kinetics of allograft mammary tumor formation were independent of *Becn1* status (**Fig. 5A**), indicating that, in our mouse mammary epithelial model,[37] monoallelic *Becn1* loss does not alter ERBB2-induced mammary tumorigenesis. Mammary tumors generated by ERBB2-overexpressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs exhibited similar ERBB2, MKI67 (Ki67) and cleaved CASP3 levels, indicating that ERBB2-overexpressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMEC-generated tumors were similar in oncogene expression and in cell proliferation and death rates. However, given lower total LC3B expression, but similar SQSTM1 levels in *Becn1*$^{+/-}$ compared with *Becn1*$^{+/+}$ tumors (**Fig. 5B**), differences in the functional status of autophagy could not be reliably determined.

The functional interactions between the ERBB2 and autophagy pathways were further studied by crossing *Becn1*$^{+/-}$ mice to 2 well-characterized mouse mammary tumor models, namely the MMTV-*Neu*[42] and MMTV-*PyMT*[43] models, which show cosegregating tumor gene expression profiles when compared with other mouse mammary tumor models.[44] Similar to the iMMEC

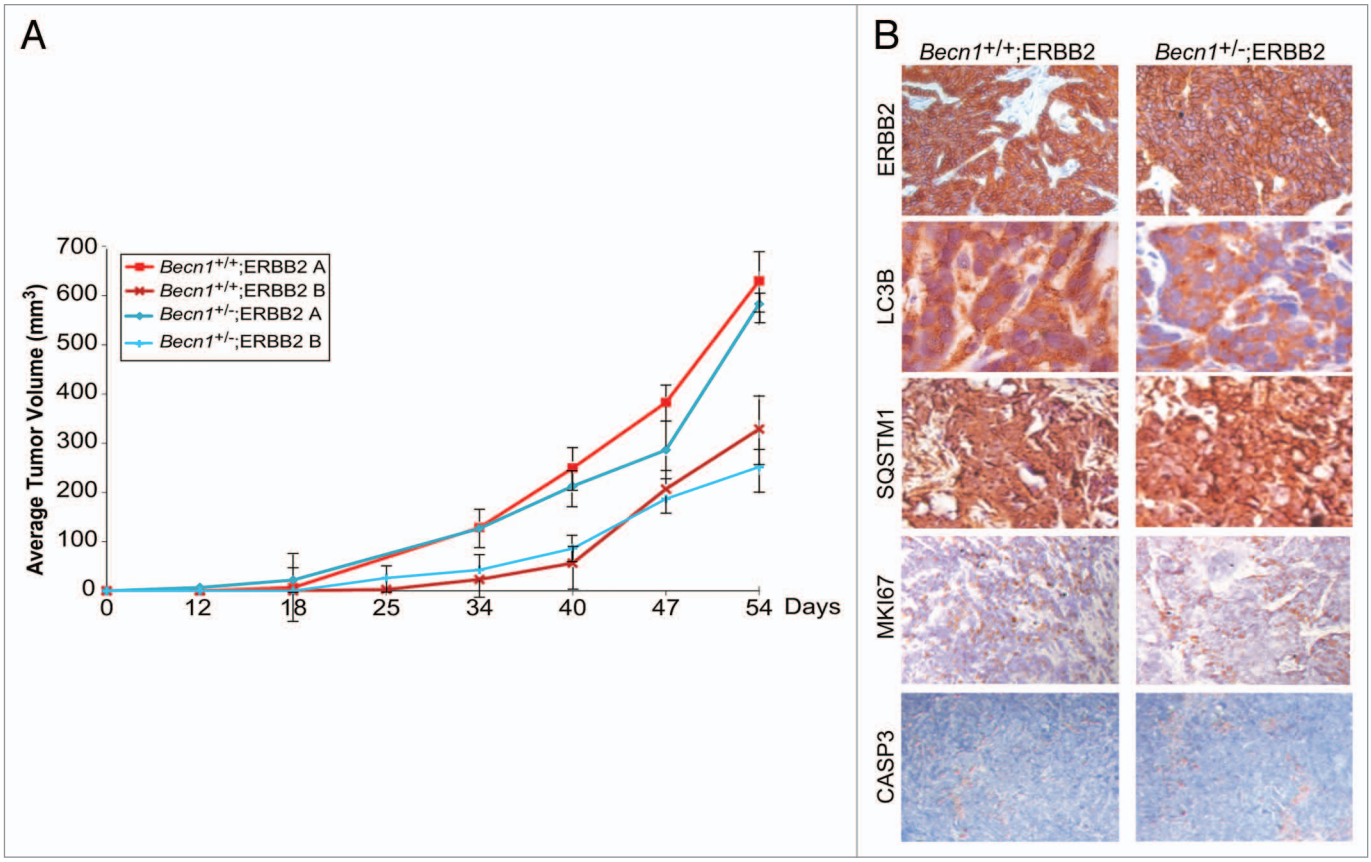


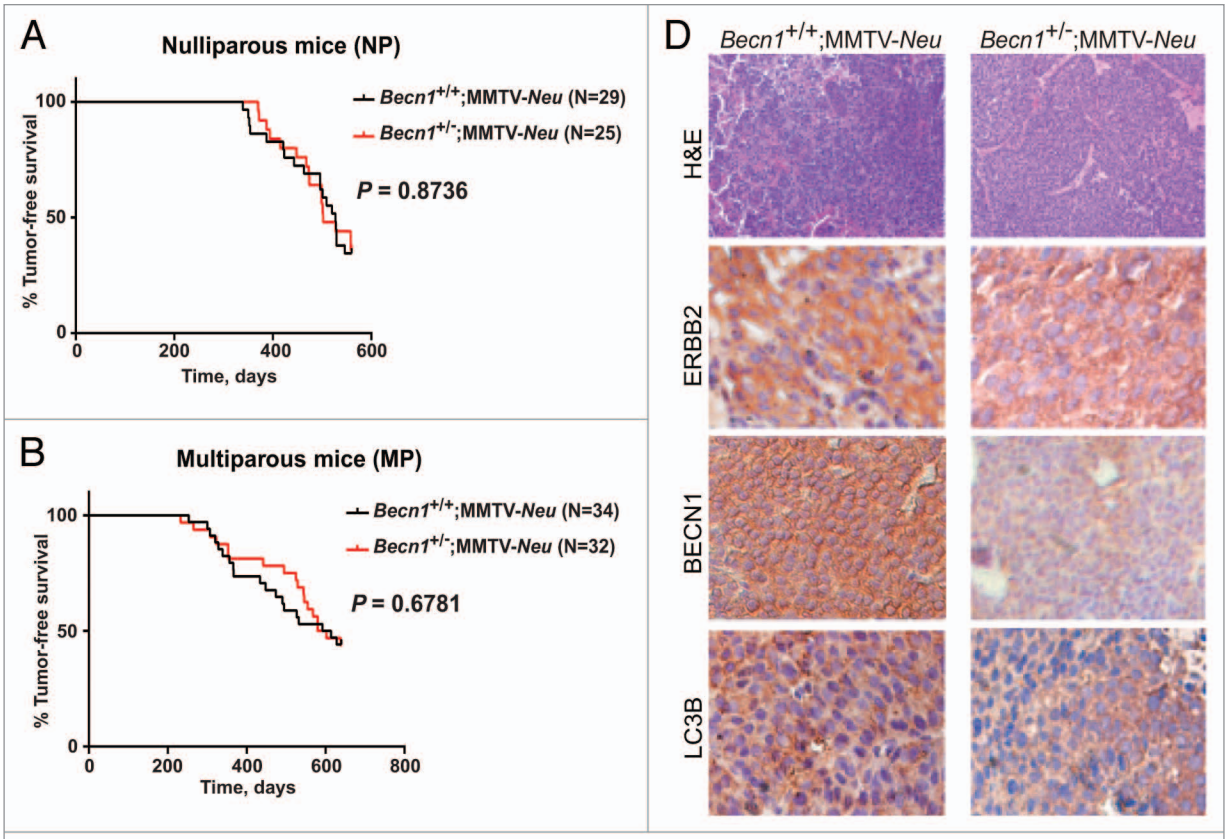
Figure 5. ERBB2-expressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMECs have similar tumor-forming capacities in nude mice. (**A**) Independent ERBB2-overexpressing *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ iMMEC lines (e.g., **A and B**) were bilaterally implanted into the 3rd mammary fat pads of nude mice. Mice were monitored for tumor growth. Each data point represents the average volume of iMMEC-generated mammary tumors in 5 mice (2 tumors per mouse) per genotype ± SD (**B**) Representative images of ERBB2, LC3B, SQSTM1, MKI67, and cleaved CASP3 expression, as determined by IHC, in ERBB2-expressing *Becn1*$^{+/+}$ (*Becn1*$^{+/+}$;ERBB2) and ERBB2-expressing *Becn1*$^{+/-}$ (*Becn1*$^{+/-}$;ERBB2) iMMEC-generated allograft mammary tumors from (**A**).

studies described above (**Fig. 5A**), monoallelic *Becn1* loss did not alter the incidence, latency or multiplicity of ERBB2-induced mammary tumors, independent of parity status (**Fig. 6A**). Similar ERBB2, but lower BECN1 and LC3B, levels were observed in *Becn1*$^{+/-}$;MMTV-*Neu* compared with *Becn1*$^{+/+}$;MMTV-*Neu* mammary glands (**Fig. 6B**).

It is of interest to note that the cross between *Becn1*$^{+/-}$[8] and MMTV-*Neu* mice resulted in a mixed C57BL/6:FVB (50:50) background and mammary tumors arose with greater latency and lower penetrance in *Becn1*$^{+/+}$;MMTV-*Neu* mice than in the FVB/N MMTV-*Neu* model,[42] in agreement with earlier reports that the C57BL/6 background suppresses ERBB2-induced mammary tumor formation.[45,46] To generate a *Becn1*$^{+/-}$ mouse model that is more readily amenable to mammary tumorigenesis studies, we changed the genetic background of the *Becn1*$^{+/-}$ mice from C57BL/6[8] to FVB/N. Rather than repeating the lengthier cross with MMTV-*Neu* mice, we instead crossed FVB *Becn1*$^{+/-}$ mice to the MMTV-*PyMT* mouse model, which develops mammary tumors in all mammary glands within 6 to 8 wk[43] and is frequently used as a surrogate model for ERBB2-driven mammary tumorigenesis, as NEU- and PyMT-induced mammary tumors exhibit cosegregating gene expression signatures and high ERBB2 expression.[47] Similar to the cross between *Becn1*$^{+/-}$ and MMTV-*Neu* mice (**Fig. 6A**), *Becn1* heterozygosity did not impact PyMT-induced mammary tumorigenesis (**Fig. 7A**). Compared with *Becn1*$^{+/+}$;MMTV-*PyMT* mammary tumors, *Becn1*$^{+/-}$;MMTV-*PyMT* tumors exhibited lower BECN1 expression, but comparable ERBB2, LC3B, SQSTM1 and MKI67 levels (**Fig. 7B**), indicating that cell proliferation and likely functional autophagy status in *PyMT*-driven mammary tumors were not affected by monoallelic *Becn1* deletion.

Autophagy inhibition enhances the response of tzb-responsive human breast cancer cells to trastuzumab

Our findings that ERBB2-positive breast tumors often exhibit low *BECN1* expression (**Fig. 1**), ERBB2 overexpression suppresses stress-induced autophagy in mammary tumor cells in vitro and in vivo (**Figs. 2–4**), and *Becn1* heterozygosity does not impact ERBB2-induced mammary tumorigenesis in the mouse tumor models examined (**Figs. 5–7**) suggest that, in contrast to mutant RAS-driven tumors,[38,48-50] ERBB2-overexpressing cancer cells do not depend on high functional autophagy levels for growth. It is possible, however, that the suppressed, but not absent, autophagic potential is still essential for ERBB2-positive cancer cell survival under stress and that further autophagy inhibition may promote tumor cell death. To investigate this clinically significant hypothesis, we examined whether pharmacological



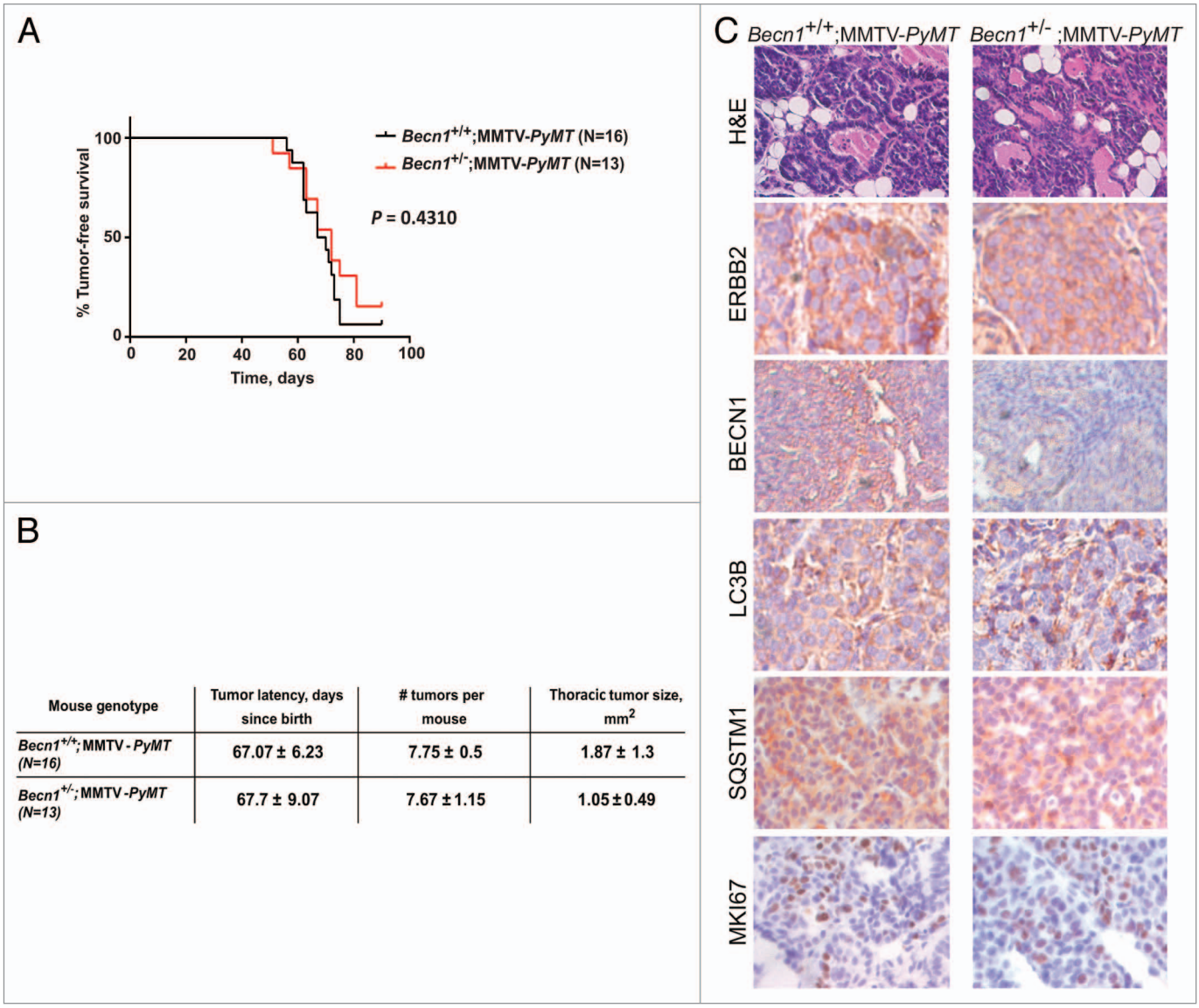
C

Mouse genotype	Tumor frequency %	Tumor latency, days	# tumors per mouse	Tumor distribution, %			
				MG 1/SIG	MG 2/3	MG 4	MG 5
Becn1$^{+/+}$;MMTV-*Neu*(NP)	65.5	458 ± 72	1.16 ± 0.37	13.6	50	18	13.6
Becn1$^{+/-}$;MMTV-*Neu* (NP)	64	465 ± 62	1.38 ± 0.5	13.6	50	18	13.6
Becn1$^{+/+}$;MMTV-*Neu*(MP)	55	407.6 ± 98.5	1.24 ± 0.44	9.5	43	33	14
Becn1$^{+/-}$;MMTV-*Neu* (MP)	65	439.6 ± 116.7	1.06 ± 0.25	23.5	47	12	29

Figure 6. Monoallelic *Becn1* deletion does not affect ERBB2-driven mammary tumorigenesis. (**A**) Kaplan-Meier curve depicting percentage of tumor-free virgin (nulliparous-NP) mice over a period of 600 d post birth. (**B**) Kaplan-Meier curve depicting percentage of tumor-free retired breeder (multiparous-MP) mice over a period of 600 d post birth. (**C**) Table summarizing tumor frequency, latency, multiplicity, and anatomical distribution per genotype. MG, mammary gland; SlG, salivary gland. (**D**) Representative images of hematoxylin and eosin (H&E) and ERBB2, BECN1, and LC3B expression by IHC in mammary tumors from *Becn1*$^{+/+}$;MMTV-*Neu* and *Becn1*$^{+/-}$;MMTV-*Neu* virgin mice. Mice used: *Becn1*$^{+/-}$ (C57BL/6); MMTV-*Neu* (FVB/N).

or genetic autophagy inhibition increased sensitivity of the ERBB2-positive human breast cancer cell line, BT474, to the humanized mouse monoclonal ERBB2 antibody trastuzumab.[51] Previous reports have shown that tzb-sensitive BT474 cancer cells exhibit low levels of basal autophagy and fail to upregulate autophagy in response to stress to the levels of other human breast cancer cell lines,[33,52,53] further supporting our finding that ERBB2 overexpression suppresses autophagy. As shown in **Figure 8A**, trastuzumab inhibited BT474 cell growth at 48 and 72 h of treatment ($P < 0.05$ and < 0.01, respectively), whereas the lysosomotropic agent and indirect autophagy inhibitor chloroquine (CQ), at a concentration that blocks autophagic flux (25 μM, **Fig. 8B and C**), had minimal effect on BT474 cell growth. The combination of trastuzumab and CQ showed statistically significant enhanced antitumor effect relative to the single agent trastuzumab (**Fig. 8A**), indicating that pharmacological autophagy inhibition with CQ augments the therapeutic efficacy of trastuzumab on tzb-sensitive BT474 breast cancer cells ($P < 0.05$). Near-complete *BECN1* knockdown with siRNA (**Fig. 8E**) did not affect BT474 cell growth, but increased the antitumor effect of trastuzumab at 72 h (**Fig. 8D**, $P < 0.05$), indicating that targeted suppression of BECN1 expression also impacts ERBB2-positive breast cancer cell responsiveness to trastuzumab.

ERBB2-positive human breast tumors exhibit a low autophagy gene signature independent of *BECN1* mRNA status



Mouse genotype	Tumor latency, days since birth	# tumors per mouse	Thoracic tumor size, mm^2
Becn1$^{+/+}$;MMTV-*PyMT* (N=16)	67.07 ± 6.23	7.75 ± 0.5	1.87 ± 1.3
Becn1$^{+/-}$;MMTV-*PyMT* (N=13)	67.7 ± 9.07	7.67 ± 1.15	1.05 ± 0.49

Figure 7. Monoallelic *Becn1* loss does not impact PyMT-driven mammary tumorigenesis. (**A**) Kaplan-Meier curve depicting percentage of tumor-free virgin mice over a period of 100 d post birth. (**B**) Table summarizing tumor latency, multiplicity, and size. (**C**) Representative images of H&E and ERBB2, BECN1, LC3B, SQSTM1, and MKI67 expression by IHC in mammary tumors from *Becn1*$^{+/+}$;MMTV-*PyMT* and *Becn1*$^{+/-}$;MMTV-*PyMT* mice. Mice used: *Becn1*$^{+/-}$ (FVB/N); MMTV-*PyMT* (FVB/N).

Given our initial observation that low *BECN1* expression in human breast tumors correlates with the ERBB2 and basal-like subtypes in independent, but rather small, gene profiling databases (**Fig. 1**), we examined whether this finding holds true in much larger and unrelated breast cancer cohorts.[54-57] Tumors were clustered into 4 subgroups based on *BECN1* expression levels [high (BECN1+) vs. low (BECN1–) for *BECN1* levels above or below the mean across samples, respectively] and reported ERBB2 status [positive (ERBB2+) vs. negative (ERBB2–)] and their expression profiles were compared regarding hormone receptor status, autophagy-related gene expression, and gene signatures of metabolic pathways (**Fig. S3**) previously reported to be affected by functional autophagy status.[38,48,49,58-60] This analysis confirmed that ERBB2-positive and triple negative breast tumors commonly express low levels of *BECN1* mRNA (**Fig. 9**, **Fig. S4**, P = 5.70E-18). We also discovered that ERBB2-positive tumors, independent of *BECN1* expression and very similar to non-ERBB2-expressing *BECN1*-low tumors, exhibited low expression of autophagy-regulated genes, possibly indicating functional autophagy suppression in ERBB2-positive breast cancers even when *BECN1* is highly expressed (**Fig. 9**, **Fig. S4**, P = 2.60E-03). It is of great interest and worthy of further investigation that, similar to non-ERBB2-expressing *BECN1*-low tumors and in contrast to non-ERBB2-expressing *BECN1*-high tumors, ERBB2-positive breast tumors showed decreased fatty acid β-oxidation and oxidative phosphorylation gene signatures, independent of *BECN1* expression. Intriguingly, ERBB2-positive breast cancers seem to have glycolysis and cell

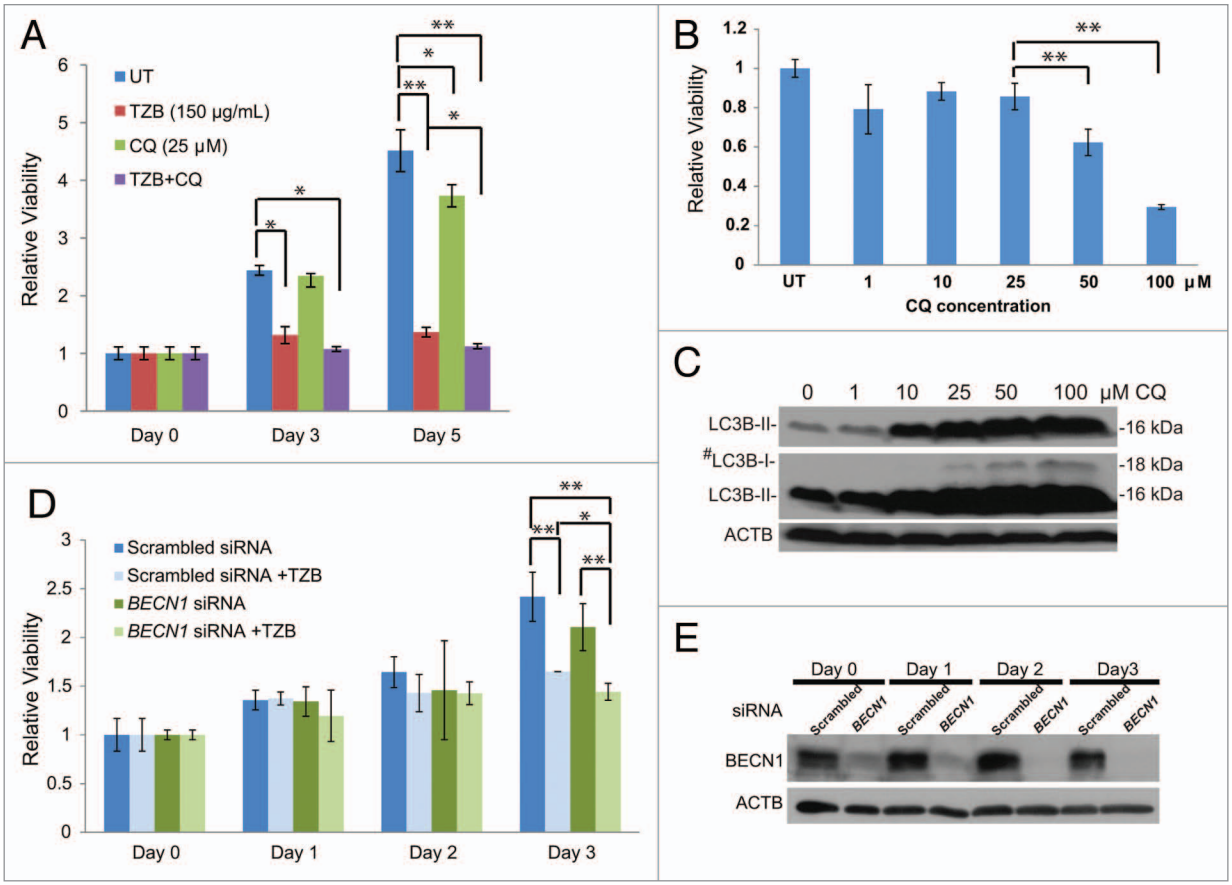


Figure 8. Autophagy inhibition sensitizes tzb-responsive ERBB2-positive breast cancer cells to trastuzumab. (**A**) Viability assays of BT474 cells treated with trastuzumab (TZB, 150 μg/ml), chloroquine (CQ, 25 μM), or combination of both for 0, 3, and 5 d. (**B**) Viability of BT474 after 3 d of treatment with increasing CQ concentrations. (**C**) LC3B western blot of BT474 cells treated with increasing CQ concentrations. #middle panel is higher exposure of top panel (**D**) Viability assays of BT474 cells treated with TZB (150 μg/ml) for 0 to 3 d, starting at 24 h after transfection with *BECN1* or scrambled siRNA. (**E**) BECN1 immunoblot confirms target knockdown by siRNA during 0 to 3 d of treatment. *P* values were calculated using paired Student *t* test. Each data point is an average of triplicate experiments ± SD. **P* value < 0.05; ***P* value < 0.01.

proliferation gene signatures between the significantly upregulated and significantly downregulated patterns observed in non-ERBB2-expressing *BECN1*-low (i.e., mostly triple negative) and non-ERBB2-expressing *BECN1*-high (i.e., mostly hormone receptor-positive) tumors, respectively (**Fig. 9**, **Fig. S4**, *P* = 1.60E-05 and *P* = 3.10E-06).

Discussion

Role of autophagy in mammary tumorigenesis

The human epidermal growth factor receptor and tyrosine kinase ERBB2 has been widely studied because of its strong transforming potential, its role in the pathogenesis of breast cancer, and its use as a therapeutic target in patients with ERBB2-positive breast tumors. ERBB2 amplification is likely an early genetic event in mammary tumorigenesis, as it is commonly observed in ductal carcinoma in situ, in the absence of invasive disease.[61] ERBB2 status remains constant as disease progresses to invasive and then metastatic stages.[62-65] *Becn1* has been identified as a haplo-insufficient tumor suppressor, as *Becn1*$^{+/-}$ mice develop mammary hyperplasias, lymphomas, and lung and liver carcinomas, which retain a wild-type *Becn1* allele.[4,7] Epithelial cells with autophagy defects, including *Becn1* heterozygosity, exhibit susceptibility to metabolic stress, which is accompanied by DNA damage and increased genomic instability, in turn likely driving cancer progression.[4] In our study, and in agreement with earlier work,[14] ERBB2 overexpression and low *BECN1* mRNA levels are positively correlated in human breast cancers (**Fig. 1**), indicating that many ERBB2-positive breast malignancies may be functionally autophagy-deficient. In support of this hypothesis, we found that ERBB2 axis activation suppresses stress-induced autophagy (**Figs. 2–4**), suggesting that ERBB2-positive premalignant and malignant breast lesions may exhibit decreased autophagic potential, even if *BECN1* is genomically intact. Given the contribution of allelic *Becn1* loss to DNA damage and genomic instability,[4] it is conceivable that, by functionally suppressing autophagy, early ERBB2 activation may lead to further ERBB2 amplification and, thus, a positive feedback loop maintaining and increasing the protumorigenic function of the ERBB2 axis. In this case, defective autophagy, but not necessarily allelic *BECN1* deletion, may indeed play a role in ERBB2-positive breast cancer, particularly during tumor initiation.

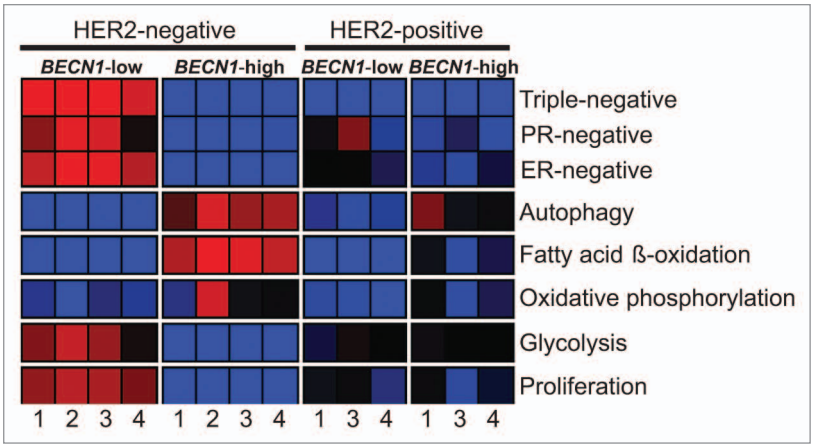


Figure 9. ERBB2-positive breast cancers have, independent of *BECN1* expression, gene expression signatures similar to those of non-ERBB2-expressing breast cancers with low *BECN1* mRNA levels. The heatmap reports gene set enrichment analysis (GSEA) for selected gene signatures (rows) in breast cancer cohorts defined by ERBB2 and *BECN1* status. The color indicates the enrichment (Fischer exact test) of samples with gene signature upregulation (red, *P*+, enrichment < 0.05), downregulation (blue, *P*–, enrichment < 0.05), or no difference (black, *P*+, enrichment ≥ 0.05 and *P*–, enrichment ≥ 0.05) within a cohort subgroup relative to the remaining samples in the cohort. Gene signature lists and statistical analysis are provided in **Figures S3 and S4**, respectively. Databases: 1) Decremoux et al. 2011, 2) Hatzis et al. 2011 (ERBB2-negative breast cancers only), 3) Servant et al. 2012, and 4) Sabatier et al. 2011.

Recent studies support the concept that autophagy suppression is important for aberrant tyrosine kinase-induced tumorigenesis, such as those mediated by AKT1 and EGFR,[66,67] which inhibit autophagy by phosphorylation of BECN1. Upon expression of a BECN1 mutant resistant to phosphorylation in cancer cell lines, autophagy could not be inhibited and tyrosine kinase-mediated xenograft tumor formation in nude mice was suppressed.[66,67] It would be very informative to further investigate the role of autophagy in ERBB2-induced tumorigenesis by generating a transgenic mouse model that combines ERBB2 overexpression and a constitutively activated or non-suppressible autophagy status. As it is evident from our in vitro and in vivo allograft and spontaneous tumorigenesis studies, unless autophagy is ectopically induced or engineered to be in a "nonsuppressible state," activation of the ERBB2 axis suppresses the autophagic response to stress and renders ERBB2-positive breast cancer cells autophagy-deficient, thus providing an explanation for why partial *Becn1* deficiency does not impact ERBB2-driven mammary tumor formation (**Figs. 5–7**).

Our bi-transgenic mouse models combining monoallelic *Becn1* loss and ERBB2 or PyMT activation under the MMTV-promoter join the efforts to study the role of defective autophagy in mammary tumorigenesis using mammary tumor-prone mouse models.[68,69] In the first such publication, mammary gland-targeted deletion of the positive autophagy regulator *Rb1cc1/Fip200* suppresses mammary tumor initiation and progression in the MMTV-*PyMT* model, in association with defective autophagy in tumor cells, as indicated by accumulation of ubiquitinated protein aggregates and SQSTM1, deficient LC3B conversion, and increased number of abnormal-appearing mitochondria.[68] In a more recently published study, monoallelic *Becn1* loss suppresses mammary tumor formation driven by *Palb2* deletion in mammary epithelial cells (MECs) in a wild-type *Trp53* background, but fails to impact tumorigenesis induced by combined MEC-specific *Palb2* and *Trp53* loss,[69] suggesting that the role of allelic *Becn1* status in mammary tumorigenesis is greatly influenced by other oncogenic events. The results mentioned above, including our studies, once more indicate that the role of autophagy in breast cancer is complex and warrants further investigation.

Autophagy modulation for ERBB2-positive breast cancer treatment

Our treatment studies (**Fig. 8**) demonstrate that pharmacological inhibition of autophagy increases sensitivity of tzb-responsive breast cancer cells to trastuzumab, indicating that the functionally reduced autophagy status in ERBB2-overexpressing breast cancer cells still preserves cell viability and provides protection against chemotherapy. This finding is in agreement with previously published studies, which implicated autophagy in resistance to breast cancer treatment, as autophagy inhibition by CQ or silencing of *Atg* genes resensitized tzb-resistant SKBR3 cells and hormone-resistant MCF7 cells to trastuzumab and tamoxifen, respectively.[33,70]

Tumor cell addiction to autophagy

The suppressive effect of ERBB2 overexpression on the functional status of autophagy is in sharp contrast to the upregulation of basal autophagy and the strong dependence of RAS-mutant tumors on autophagy for growth.[38,48-50,58] RAS-mediated adhesion-independent transformation is dependent on autophagy, as autophagy inhibition reduced glycolytic capacity and attenuated cell proliferation and transformation.[48] Furthermore, RAS-expressing cells have high basal autophagy to maintain a functional mitochondrial pool and meet energy demands imposed by oncogenic RAS, as autophagy suppression decreased tumor cell survival under starvation and abrogated tumorigenesis in nude mice, in association with depletion of oxidative phosphorylation and tricarboxylic acid cycle intermediates.[38,49] Whereas the aforementioned work[38,48-50,58] suggests an oncogene-induced requirement for autophagy induction during tumorigenesis, our present studies indicate that "autophagy addiction" is not a generalized phenomenon in cancer pathophysiology, but its activation is instead specific to particular oncogenic events.

Despite the differences in autophagy functional status in RAS-mutant and ERBB2-positive tumors and the potentially discrete roles of defective autophagy in RAS- and ERBB2-driven tumor initiation and maintenance, our studies reveal a common role for autophagy in resistance to cancer therapy. Similar to the sensitization of different tumor types to standard anticancer agents,[13,71-73] tzb-responsive breast cancer cells were rendered more sensitive to trastuzumab by pharmacological and genetic autophagy suppression, thus further supporting use of

autophagy inhibitors in combination with conventional cancer therapies.

Autophagy functional status in ERBB2-positive breast tumors

Our finding that ERBB2 activation suppresses stress-induced autophagy in breast cancer cells in vitro and in vivo (**Figs. 2–4**) is in agreement with our analysis of human breast tumor DNA microarray data showing that ERBB2-expressing breast cancers exhibit lower expression of autophagy-related genes (**Figs. S3 and S4**), independent of *BECN1* expression levels (**Fig. 9**). It is, thus, likely that ERBB2-positive breast tumors are functionally autophagy-defective and, similar to *Becn1*$^{+/-}$ iMMECs,[74] sensitive to oxidative and endoplasmic reticulum (ER) stress-inducing agents; this hypothesis will be investigated in subsequent studies.

An interesting and thought-provoking finding from our gene expression analysis is the striking downregulation of glycolysis and proliferation gene signatures in non-ERBB2-expressing *BECN1*-high breast cancers, which are highly enriched in hormone receptor-positive tumors and also exhibit high and relative upregulation of fatty acid β-oxidation and oxidative phosphorylation gene signatures, respectively. ERBB2-positive breast cancers, independent of *BECN1* expression, are characterized by a relative upregulation of glycolysis and proliferation gene signatures compared with non-ERBB2-expressing *BECN1*-high (mostly hormone receptor-positive) tumors, but not to levels observed in non-ERBB2-expressing *BECN1*-low (mostly triple negative breast) tumors. Upregulation of glycolysis in association with ERBB2 activation has been reported before.[75-77] However, the high expression of glycolysis-related genes in conjunction with a low autophagic gene signature is surprising considering that, in RAS-mediated transformation, defective autophagy reduces glycolytic capacity.[48] It is possible that breast tumors with high functional autophagy do not rely on glycolysis for meeting their metabolic demands, as fatty acid β-oxidation and oxidative phosphorylation can be sustained at high levels in autophagy-maintained healthy mitochondria. In contrast, low *BECN1* expression, and likely defective autophagy and deregulation of mitochondrial homeostasis, correlates with significant suppression of fatty acid β-oxidation and oxidative phosphorylation, as previously reported,[38,60] thus forcing the cancer cell metabolic machinery toward glycolysis. The relationship between autophagy regulation and metabolic reprogramming is obviously quite complex,[58] and further studies are needed to explore the metabolic profiles of the different breast cancer subtypes and incorporate the knowledge acquired in the design of more effective therapeutic regimens.

Materials and Methods

Cell line generation and culture conditions

Primary mouse mammary epithelial cells (pMMECs) from *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$ mice[8] were immortalized to generate iMMEC cell lines, which were then engineered to stably express BCL2, EGFP-LC3B or wild-type human ERBB2, as previously described.[4] The BT474 (HTB-20) cell line was obtained from American Type Culture Collection. Hank's balanced salt solution (Life Technologies, 14025-092) was used for nutrient-deprivation studies. Bafilomycin A_1 (BafA1; Sigma-Aldrich, B1793) was used at a concentration of 25 nM.

Fluorescence and electron microscopy

Autophagy was quantified by quantification of EGFP-LC3B puncta per cell using fluorescence microscopy, using an Olympus IX51 fluorescent microscopy system at 60× magnification. One hundred cells per cell line were evaluated for number of EGFP-LC3B puncta per cell at each time point. Three independent experiments were performed, and the average number of GFP-fluorescent puncta per cell with standard deviation for each cell line at each time point is presented. For EM, cells were fixed in a 2.5% glutaraldehyde, 4% paraformaldehyde, 8 μM calcium chloride, 0.1 M cacodylate, pH 7.4 fixative buffer. Electron microscopy was performed with a JEOL 1200EX electron microscope at 3800× magnification. Statistical analysis (2-tailed Student *t* test) was performed by Excel's Data Analysis ToolPak (Microsoft, www.microsoft.com).

Western blotting and immunohistochemistry

Western blotting using whole-cell protein extracts and immunohistochemistry (IHC) were performed as previously described.[78] Antibodies used were against ERBB2 (Cell Signaling, 2165); Ki67 (Leica Microsystems, NCL-L-Ki67-MM1); LC3B (Novus Biologicals, NB100-2331); BECN1/Beclin1 (Santa Cruz Biotechnology, sc-11427); SQSTM1 (p62, Enzo Life Sciences, BML-PW9860); ATG7 (A2856), ACTB/β-Actin (Sigma-Aldrich, A4527). Cleaved CASP3 IHC was performed by Rutgers Cancer Institute of New Jersey Tissue Analytical Services. Densitometry analysis was performed by ImageJ.[79]

Tumorigenicity assays

Orthotopic mammary gland implantation of iMMECs[4] and trangenic mouse tumorigenicity studies were performed according to Institutional Animal Care and Use Committee-approved protocols. C57BL/6 *Becn1*$^{+/-}$ mice were crossed to MMTV-*Neu* mice [FVB/N-Tg(MMTVneu) 202 Mul/J] (The Jackson Laboratory, 002376). To circumvent suppression of ERBB2-induced mammary tumorigenesis by the C57BL/6 background,[45] C57BL/6 *Becn1*$^{+/-}$ mice were backcrossed into the FVB/N background for 10 generations. FVB *Becn1*$^{+/-}$ mice were subsequently crossed to MMTV-PyMT mice.[80] Progeny cohorts of all resultant genotypes were observed for spontaneous mammary tumor formation by weekly palpation. Kaplan-Meier survival curves and subsequent *P* values (2-tailed logrank test) were generated using GraphPad Prism version 5.0 for Windows (GraphPad Software, www.graphpad.com).

Cell viability assays

Cells were plated in 6-well plates at medium density, so as to ensure nonconfluency after 5 d of vehicle treatment. Media and drugs were changed after 3 d. Cell viability was assessed using the trypan blue exclusion method automated by a Vi-Cell (Beckman Coulter). Trastuzumab (Herceptin) was supplied as an aqueous solution at a concentration of 25 mg/mL and it was a generous gift from the Rutgers Cancer Institute of New Jersey clinical pharmacy. Chloroquine diphosphate salt was purchased from Sigma-Aldrich (C6628). Statistical analysis

(2-tailed Student *t* test) was performed by Excel's Data Analysis ToolPak.

Gene expression analysis

For the data shown in **Figure 1**, gene expression array data from early stage breast cancers published by Wang et al. and by Richardson et al. (which included some normal breast samples) were combined and analyzed.[35,36] The data set of Richardson et al., obtained on U133-Plus Affymetrix arrays, was made compatible with that of Wang et al., obtained on Affymetrix U133A arrays, by restricting it to the probe sets of the U133A chip and processing it with the mas5 software available at http://www.bioconductor.org.[81] The distance-weighted discrimination method was used for systematic source and batch bias adjustment in the 2 data sets.[82] Breast cancers were classified into basal-like cancers (BA), ERBB2-positive, Luminal (LUM), Luminal A (LA) and Luminal B (LB) by robust consensus clustering.[83] The average expression of each gene across all samples was normalized to 0. The mean relative expression of probes corresponding to gene of interest in each subtype was calculated and graphed. Statistical analysis (Student *t* test) was performed by Excel's Data Analysis ToolPak (Microsoft, www.microsoft.com).

For the data shown in **Figure 9**, 4 reported breast cancer data sets were downloaded from Gene Expression Omnibus (GEO): de Cremoux et al.[54] (226 breast tumors, Affymetrix U133 Plus 2.0 Array, GEO series GSE26639), Hatzis et al.[56] (508 ERBB2+ breast tumors, Affymetrix U133A Array, GEO series GSE25066), Servant et al.[57] (343 breast tumors, Illumina HumanWG-6 v3.0 expression bead chip Array, GEO series GSE30682) and Sabatier et al.[55] (266 breast tumors, Affymetrix U133 Plus 2.0 Arrays, GEO series GSE21653). All the Affymetrix data sets were processed using the justRMA function in R Bioconductor, obtaining a log2 expression values. For the Servant et al. data set assayed with an Illumina array, log2 of the reported variance stabilized expression values were used.[57] The samples were classified into ERBB2-positive (ERBB2+) or ERBB2-negative (ERBB2–) based on the reported ERBB2 amplification status. The samples were classified into *BECN1*-high (Beclin+) or *BECN1*-low (Beclin-) depending on whether *BECN1* expression (probe 208945_s_at) was above or below the mean across samples. Gene expression signatures were analyzed using Gene Set Enrichment Analysis,[84] obtaining a quantification of the statistical significance for upregulation (*P*+) or downregulation (*P*–) for each signature and sample pair. A sample was said to have a signature significantly upregulated if $P+ < 0.05$, significantly downregulated if $P- < 0.05$, and no significant change otherwise. The complete lists of gene signatures are listed in **Figure S3**. Statistical analysis is listed in **Figure S4**.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Z Yue and S Jin (Mount Sinai School of Medicine and Rutgers Robert Wood Johnson Medical School, respectively; *Becn1*$^{+/-}$ mice), E Lattime (Rutgers Cancer Institute of New Jersey; human wild-type ERBB2-expressing plasmid), R Patel (Rutgers Robert Wood Johnson Medical School) for assistance with electron microscopy, N Goldsmith-Kane (Rutgers University) for assistance with confocal microscopy, and the Rutgers Cancer Institute of New Jersey Tissue Analytical Services. We are grateful to the New Jersey Commission on Cancer Research (Predoctoral Fellowship to FL), NIH-NCI (R00 grant to VK) and Damon Runyon Cancer Research Foundation (Clinical Investigator Award to VK) for their generous financial support.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/27867

References

1. Dunn WA Jr. Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol 1994; 4:139-43; PMID:14731737; http://dx.doi.org/10.1016/0962-8924(94)90069-8
2. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005; 169:425-34; PMID:15866887; http://dx.doi.org/10.1083/jcb.200412022
3. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000; 290:1717-21; PMID:11099404; http://dx.doi.org/10.1126/science.290.5497.1717
4. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 2007; 21:1621-35; PMID:17606641; http://dx.doi.org/10.1101/gad.1565707
5. Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999; 59:59-65; PMID:10395800; http://dx.doi.org/10.1006/geno.1999.5851
6. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402:672-6; PMID:10604474; http://dx.doi.org/10.1038/45257
7. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112:1809-20; PMID:14638851
8. 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; PMID:14657337; http://dx.doi.org/10.1073/pnas.2436255100
9. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 2006; 10:51-64; PMID:16843265; http://dx.doi.org/10.1016/j.ccr.2006.06.001
10. Amaravadi RK, Thompson CB. The roles of therapy-induced autophagy and necrosis in cancer treatment. Clin Cancer Res 2007; 13:7271-9; PMID:18094407; http://dx.doi.org/10.1158/1078-0432.CCR-07-1595
11. Mathew R, White E. Why sick cells produce tumors: the protective role of autophagy. Autophagy 2007; 3:502-5; PMID:17611387
12. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer 2007; 7:961-7; PMID:17972889; http://dx.doi.org/10.1038/nrc2254
13. Chen N, Karantza V. Autophagy as a therapeutic target in cancer. Cancer Biol Ther 2011; 11:157-68; PMID:21228626; http://dx.doi.org/10.4161/cbt.11.2.14622
14. Negri T, Tarantino E, Orsenigo M, Reid JF, Gariboldi M, Zambetti M, Pierotti MA, Pilotti S. Chromosome band 17q21 in breast cancer: significant association between beclin 1 loss and HER2/NEU amplification. Genes Chromosomes Cancer 2010; 49:901-9; PMID:20589936; http://dx.doi.org/10.1002/gcc.20798
15. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, et al. Molecular portraits of human breast tumours. Nature 2000; 406:747-52; PMID:10963602; http://dx.doi.org/10.1038/35021093
16. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 1995; 378:390-4; PMID:7477376; http://dx.doi.org/10.1038/378390a0
17. Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. EMBO J 1998; 17:719-31; PMID:9450997; http://dx.doi.org/10.1093/emboj/17.3.719

18. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 1995; 378:394-8; PMID:7477377; http://dx.doi.org/10.1038/378394a0
19. Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development. Nature 1995; 378:386-90; PMID:7477375; http://dx.doi.org/10.1038/378386a0
20. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, et al. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 1995; 269:230-4; PMID:7618084; http://dx.doi.org/10.1126/science.7618084
21. Pierce JH, Arnstein P, DiMarco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of erbB-2 in human mammary epithelial cells. Oncogene 1991; 6:1189-94; PMID:1713661
22. Liu Y, el-Ashry D, Chen D, Ding IY, Kern FG. MCF-7 breast cancer cells overexpressing transfected c-erbB-2 have an in vitro growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity in vivo. Breast Cancer Res Treat 1995; 34:97-117; PMID:7647336; http://dx.doi.org/10.1007/BF00665783
23. Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn PA Jr. Epidermal growth factor receptor family in lung cancer and premalignancy. Semin Oncol 2002; 29(Suppl 4):3-14; PMID:11894009; http://dx.doi.org/10.1053/sonc.2002.31520
24. Hirsch FR, Franklin WA, Veve R, Varella-Garcia M, Bunn PA Jr. HER2/neu expression in malignant lung tumors. Semin Oncol 2002; 29(Suppl 4):51-8; PMID:11894014; http://dx.doi.org/10.1053/sonc.2002.31523
25. Yarom N, Jonker DJ. The role of the epidermal growth factor receptor in the mechanism and treatment of colorectal cancer. Discov Med 2011; 11:95-105; PMID:21356164
26. Ménard S, Pupa SM, Campiglio M, Tagliabue E. Biologic and therapeutic role of HER2 in cancer. Oncogene 2003; 22:6570-8; PMID:14528282; http://dx.doi.org/10.1038/sj.onc.1206779
27. Tai W, Mahato R, Cheng K. The role of HER2 in cancer therapy and targeted drug delivery. J Control Release 2010; 146:264-75; PMID:20385184; http://dx.doi.org/10.1016/j.jconrel.2010.04.009
28. Daly JM, Jannot CB, Beerli RR, Graus-Porta D, Maurer FG, Hynes NE. Neu differentiation factor induces ErbB2 down-regulation and apoptosis of ErbB2-overexpressing breast tumor cells. Cancer Res 1997; 57:3804-11; PMID:9288791
29. Giani C, Casalini P, Pupa SM, De Vecchi R, Ardini E, Colnaghi MI, Giordano A, Ménard S. Increased expression of c-erbB-2 in hormone-dependent breast cancer cells inhibits cell growth and induces differentiation. Oncogene 1998; 17:425-32; PMID:9696035; http://dx.doi.org/10.1038/sj.onc.1201954
30. Ellsworth RE, Ellsworth DL, Patney HL, Deyarmin B, Love B, Hooke JA, Shriver CD. Amplification of HER2 is a marker for global genomic instability. BMC Cancer 2008; 8:297; PMID:18854030; http://dx.doi.org/10.1186/1471-2407-8-297
31. Mano MS, Rosa DD, De Azambuja E, Ismael GF, Durbecq V. The 17q12-q21 amplicon: Her2 and topoisomerase-IIalpha and their importance to the biology of solid tumours. Cancer Treat Rev 2007; 33:64-77; PMID:17113234; http://dx.doi.org/10.1016/j.ctrv.2006.10.001
32. Futreal PA, Söderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW. Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Res 1992; 52:2624-7; PMID:1568230
33. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. PLoS One 2009; 4:e6251; PMID:19606230; http://dx.doi.org/10.1371/journal.pone.0006251
34. Ivshina AV, George J, Senko O, Mow B, Putti TC, Smeds J, Lindahl T, Pawitan Y, Hall P, Nordgren H, et al. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. Cancer Res 2006; 66:10292-301; PMID:17079448; http://dx.doi.org/10.1158/0008-5472.CAN-05-4414
35. Wang Y, Klijn J, Zhang Y, Atkins D, Foekens J. Gene expression profiles and prognostic markers for primary breast cancer. Methods Mol Biol 2007; 377:131-8; PMID:17634613; http://dx.doi.org/10.1007/978-1-59745-390-5_7
36. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell 2006; 9:121-32; PMID:16473279; http://dx.doi.org/10.1016/j.ccr.2006.01.013
37. Karantza-Wadsworth V, White E. A mouse mammary epithelial cell model to identify molecular mechanisms regulating breast cancer progression. Methods Enzymol 2008; 446:61-76; PMID:18603116; http://dx.doi.org/10.1016/S0076-6879(08)01604-2
38. Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, Kamphorst JJ, Chen G, Lemons JM, Karantza V, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 2011; 25:460-70; PMID:21317241; http://dx.doi.org/10.1101/gad.2016311
39. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010; 140:313-26; PMID:20144757; http://dx.doi.org/10.1016/j.cell.2010.01.028
40. Lee HK, Myers RA, Marzella L. Stimulation of autophagic protein degradation by nutrient deprivation in a differentiated murine teratocarcinoma (F9 12-1a) cell line. Exp Mol Pathol 1989; 50:139-46; PMID:2646143; http://dx.doi.org/10.1016/0014-4800(89)90063-4
41. Mizushima N. Methods for monitoring autophagy. Int J Biochem Cell Biol 2004; 36:2491-502; PMID:15325587; http://dx.doi.org/10.1016/j.biocel.2004.02.005
42. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992; 89:10578-82; PMID:1359541; http://dx.doi.org/10.1073/pnas.89.22.10578
43. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992; 12:954-61; PMID:1312220
44. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007; 8:R76; PMID:17493263; http://dx.doi.org/10.1186/gb-2007-8-5-r76
45. Lifsted T, Le Voyer T, Williams M, Muller W, Klein-Szanto A, Buetow KH, Hunter KW. Identification of inbred mouse strains harboring genetic modifiers of mammary tumor age of onset and metastatic progression. Int J Cancer 1998; 77:640-4; PMID:9679770; http://dx.doi.org/10.1002/(SICI)1097-0215(19980812)77:4<640::AID-IJC26>3.0.CO;2-8
46. Rowse GJ, Ritland SR, Gendler SJ. Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. Cancer Res 1998; 58:2675-9; PMID:9635596
47. Desai KV, Xiao N, Wang W, Gangi L, Greene J, Powell JI, Dickson R, Furth P, Hunter K, Kucherlapati R, et al. Initiating oncogenic event determines gene-expression patterns of human breast cancer models. Proc Natl Acad Sci U S A 2002; 99:6967-72; PMID:12011455; http://dx.doi.org/10.1073/pnas.102172399
48. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell 2011; 22:165-78; PMID:21119005; http://dx.doi.org/10.1091/mbc.E10-06-0500
49. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev 2011; 25:717-29; PMID:21406549; http://dx.doi.org/10.1101/gad.2016111
50. Kim MJ, Woo SJ, Yoon CH, Lee JS, An S, Choi YH, Hwang SG, Yoon G, Lee SJ. Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. J Biol Chem 2011; 286:12924-32; PMID:21300795; http://dx.doi.org/10.1074/jbc.M110.138958
51. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. Nat Rev Clin Oncol 2012; 9:16-32; PMID:22124364; http://dx.doi.org/10.1038/nrclinonc.2011.177
52. Xiao D, Bommareddy A, Kim SH, Sehrawat A, Hahm ER, Singh SV. Benzyl isothiocyanate causes FoxO1-mediated autophagic death in human breast cancer cells. PLoS One 2012; 7:e32597; PMID:22457718; http://dx.doi.org/10.1371/journal.pone.0032597
53. Gong C, Bauvy C, Tonelli G, Yue W, Deloménie C, Nicolas V, Zhu Y, Domergue V, Marin-Esteban V, Tharinger H, et al. Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. Oncogene 2013; 32:2261-72, 1-11; PMID:22733132; http://dx.doi.org/10.1038/onc.2012.252
54. de Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, Tran-Perennou C, Barbaroux C, Servant N, Vacher S, Sigal-Zafrani B, et al. Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. BMC Cancer 2011; 11:215; PMID:21631949; http://dx.doi.org/10.1186/1471-2407-11-215
55. Sabatier R, Finetti P, Cervera N, Lambaudie E, Esterni B, Mamessier E, Tallet A, Chabannon C, Extra JM, Jacquemier J, et al. A gene expression signature identifies two prognostic subgroups of basal breast cancer. Breast Cancer Res Treat 2011; 126:407-20; PMID:20490655; http://dx.doi.org/10.1007/s10549-010-0897-9
56. Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A, Vidaurre T, Holmes F, Souchon E, Wang H, et al. A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. JAMA 2011; 305:1873-81; PMID:21558518; http://dx.doi.org/10.1001/jama.2011.593
57. Servant N, Bollet MA, Halfwerk H, Bleakley K, Kreike B, Jacob L, Sie D, Kerkhoven RM, Hupé P, Hadhri R, et al. Search for a gene expression signature of breast cancer local recurrence in young women. Clin Cancer Res 2012; 18:1704-15; PMID:22271875; http://dx.doi.org/10.1158/1078-0432.CCR-11-1954
58. Lozy F, Karantza V. Autophagy and cancer cell metabolism. Semin Cell Dev Biol 2012; 23:395-401; PMID:22281437; http://dx.doi.org/10.1016/j.semcdb.2012.01.005

59. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112:1809-20; PMID:14638851
60. Vasko R, Goligorsky MS. Dysfunctional lysosomal autophagy leads to peroxisomal oxidative burnout and damage during endotoxin-induced stress. Autophagy 2013; 9:442-4; PMID:23328407; http://dx.doi.org/10.4161/auto.23344
61. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007; 26:6469-87; PMID:17471238; http://dx.doi.org/10.1038/sj.onc.1210477
62. Tsuda H, Akiyama F, Terasaki H, Hasegawa T, Kurosumi M, Shimadzu M, Yamamori S, Sakamoto G. Detection of HER-2/neu (c-erb B-2) DNA amplification in primary breast carcinoma. Interobserver reproducibility and correlation with immunohistochemical HER-2 overexpression. Cancer 2001; 92:2965-74; PMID:11753973; http://dx.doi.org/10.1002/1097-0142(20011215)92:12<2965::AID-CNCR10156>3.0.CO;2-A
63. Latta EK, Tjan S, Parkes RK, O'Malley FP. The role of HER2/neu overexpression/amplification in the progression of ductal carcinoma in situ to invasive carcinoma of the breast. Mod Pathol 2002; 15:1318-25; PMID:12481013; http://dx.doi.org/10.1097/01.MP.0000038462.62634.B1
64. Carlsson J, Nordgren H, Sjöström J, Wester K, Villman K, Bengtsson NO, Ostenstad B, Lundqvist H, Blomqvist C. HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. Br J Cancer 2004; 90:2344-8; PMID:15150568
65. Park K, Han S, Kim HJ, Kim J, Shin E. HER2 status in pure ductal carcinoma in situ and in the intraductal and invasive components of invasive ductal carcinoma determined by fluorescence in situ hybridization and immunohistochemistry. Histopathology 2006; 48:702-7; PMID:16681686; http://dx.doi.org/10.1111/j.1365-2559.2006.02403.x
66. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J, Levine B. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 2012; 338:956-9; PMID:23112296; http://dx.doi.org/10.1126/science.1225967
67. Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, Xiao G, Kinch L, Koduru P, Christudass CS, Veltri RW, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. Cell 2013; 154:1269-84; PMID:24034250; http://dx.doi.org/10.1016/j.cell.2013.08.015
68. Wei H, Wei S, Gan B, Peng X, Zou W, Guan JL. Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. Genes Dev 2011; 25:1510-27; PMID:21764854; http://dx.doi.org/10.1101/gad.2051011
69. Huo Y, Cai H, Teplova I, Bowman-Colin C, Chen G, Price S, Barnard N, Ganesan S, Karantza V, White E, et al. Autophagy opposes p53-mediated tumor barrier to facilitate tumorigenesis in a model of PALB2-associated hereditary breast cancer. Cancer Discov 2013; 3:894-907; PMID:23650262; http://dx.doi.org/10.1158/2159-8290.CD-13-0011
70. Qadir MA, Kwok B, Dragowska WH, To KH, Le D, Bally MB, Gorski SM. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. Breast Cancer Res Treat 2008; 112:389-403; PMID:18172760; http://dx.doi.org/10.1007/s10549-007-9873-4
71. Janku F, McConkey DJ, Hong DS, Kurzrock R. Autophagy as a target for anticancer therapy. Nat Rev Clin Oncol 2011; 8:528-39; PMID:21587219; http://dx.doi.org/10.1038/nrclinonc.2011.71
72. Rosenfeldt MT, Ryan KM. The multiple roles of autophagy in cancer. Carcinogenesis 2011; 32:955-63; PMID:21317301; http://dx.doi.org/10.1093/carcin/bgr031
73. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 2011; 10:1533-41; PMID:21878654; http://dx.doi.org/10.1158/1535-7163.MCT-11-0047
74. Kongara S, Kravchuk O, Teplova I, Lozy F, Schulte J, Moore D, Barnard N, Neumann CA, White E, Karantza V. Autophagy regulates keratin 8 homeostasis in mammary epithelial cells and in breast tumors. Mol Cancer Res 2010; 8:873-84; PMID:20530580; http://dx.doi.org/10.1158/1541-7786.MCR-09-0494
75. Suárez E, Bach D, Cadefau J, Palacin M, Zorzano A, Gumá A. A novel role of neuregulin in skeletal muscle. Neuregulin stimulates glucose uptake, glucose transporter translocation, and transporter expression in muscle cells. J Biol Chem 2001; 276:18257-64; PMID:11278386; http://dx.doi.org/10.1074/jbc.M008100200
76. Zhang D, Tai LK, Wong LL, Chiu LL, Sethi SK, Koay ES. Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. Mol Cell Proteomics 2005; 4:1686-96; PMID:16048908; http://dx.doi.org/10.1074/mcp.M400221-MCP200
77. Walsh A, Cook RS, Rexer B, Arteaga CL, Skala MC. Optical imaging of metabolism in HER2 overexpressing breast cancer cells. Biomed Opt Express 2012; 3:75-85; PMID:22254170; http://dx.doi.org/10.1364/BOE.3.000075
78. Nelson DA, Tan TT, Rabson AB, Anderson D, Degenhardt K, White E. Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. Genes Dev 2004; 18:2095-107; PMID:15314031; http://dx.doi.org/10.1101/gad.1204904
79. Rasband W. ImageJ. Bethesda, MD: U.S. National Institute of Health, 1997–2012
80. Guy CT, Muthuswamy SK, Cardiff RD, Soriano P, Muller WJ. Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. Genes Dev 1994; 8:23-32; PMID:7507074; http://dx.doi.org/10.1101/gad.8.1.23
81. Bioconductor: http://www.bioconductor.org
82. Benito M, Parker J, Du Q, Wu J, Xiang D, Perou CM, Marron JS. Adjustment of systematic microarray data biases. Bioinformatics 2004; 20:105-14; PMID:14693816; http://dx.doi.org/10.1093/bioinformatics/btg385
83. Alexe G, Dalgin GS, Scanfeld D, Tamayo P, Mesirov JP, DeLisi C, Harris L, Barnard N, Martel M, Levine AJ, et al. High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. Cancer Res 2007; 67:10669-76; PMID:18006808; http://dx.doi.org/10.1158/0008-5472.CAN-07-0539
84. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102:15545-50; PMID:16199517; http://dx.doi.org/10.1073/pnas.0506580102