Interaction between Her2 and Beclin-1 Proteins Underlies a New Mechanism of Reciprocal Regulation*

Jie Han†1, Wen Hou†1, Caisheng Lu‡, Leslie A. Goldstein§, Donna B. Stolz§, Simon C. Watkins§, and Hannah Rabinowich†2

From the Departments of Pathology and †Cell Biology and Physiology, University of Pittsburgh School of Medicine and the University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213

Background: Beclin-1 is one of the essential autophagic proteins. This study identified a novel complex between breast carcinoma Her2 and Beclin-1 that is disrupted by lapatinib, a Her2-tyrosine kinase inhibitor. Lapatinib thwarts the reciprocal cross-regulation between Her2 and Beclin-1, impacting cellular autophagy and Her2 signaling. The findings elucidate a hitherto unknown association between lapatinib-induced autophagy and the disruption of Her2-Beclin-1 complex.

Results: This study identified a novel complex between breast carcinoma Her2 and Beclin-1 that is disrupted by lapatinib, a Her2-tyrosine kinase inhibitor. This study identified a novel complex between breast carcinoma Her2 and Beclin-1 that is disrupted by lapatinib, a Her2-tyrosine kinase inhibitor. Lapatinib thwarts the reciprocal cross-regulation between Her2 and Beclin-1, impacting cellular autophagy and Her2 signaling. The findings elucidate a hitherto unknown association between lapatinib-induced autophagy and the disruption of Her2-Beclin-1 complex.

Conclusion: Lapatinib thwarts the reciprocal cross-regulation between Her2 and Beclin-1, impacting cellular autophagy and Her2 signaling. The findings elucidate a hitherto unknown association between lapatinib-induced autophagy and the disruption of Her2-Beclin-1 complex.

Significance: The findings elucidate a hitherto unknown association between lapatinib-induced autophagy and the disruption of Her2-Beclin-1 complex.

Beclin-1 is a key regulator of autophagy that functions in the context of two phase-specific complexes in the initiation and maturation of autophagosomes. Its known interacting proteins include autophagy effectors, Bcl-2 family members, and organelle membrane anchor proteins. Here we report a newly identified interaction between Beclin-1 and the protein tyrosine kinase receptor Her2. We demonstrate that in Her2-expressing breast carcinoma cells that do not succumb to lapatinib, this Her1/2 inhibitor disrupts the cell surface interaction between Her2 and Beclin-1. The data suggest that the ensuing autophagic response is correlatively associated with the release of Beclin-1 from its complex with Her2 and with the subsequent increase in cytosolic Beclin-1. Upon its interaction with Her2, Beclin-1 up-regulates the phosphorylation levels of Her2 and Akt. The Beclin-1 evolutionarily conserved domain is required both for the interaction of Beclin-1 with Her2 and for the increased Her2 and Akt phosphorylation. These findings shed new light on mechanisms involved in lapatinib-mediated autophagy in Her2-expressing breast carcinoma cell lines and in Beclin-1 signaling in these cells.

Macrouptosis is a cellular process that directs the trafficking of cytosolic proteins and organelles to lysosomes for degradation (1). The process involves the formation of autophagosomes, double-membrane vesicles that engulf proteins and organelles destined for degradation and recycling. Constitutive levels of autophagy are required for maintaining homeostasis, whereas up-regulation of this process serves to promote cell survival under different conditions of cellular stress, including nutrient deprivation, protein aggregate formation, pathogen infection, endoplasmic reticulum stress, and more (2–4). In recent years, it has become apparent that anticancer cytotoxic drugs that do not culminate in cell death induce autophagy in the treated tumor cells (1). Thus, induction of autophagy has been demonstrated in tumor cells that did not succumb to treatments by radiation (5–8), chemotherapy (9–12), death receptor-targeting cytokines (13–16), and certain anticancer tyrosine kinase inhibitors (TKIs)3 (17, 18). As stress-mediated autophagy promotes the survival of tumor cells under unfavorable conditions, autophagy has been considered a cellular mechanism of drug resistance. Indeed, inhibition of autophagy is being assessed as a therapeutic approach to expand the cell death response to those tumor cells that invoke autophagy to overcome anticancer treatment-mediated stress (9, 19–22).

The current study focuses on autophagy mediated by lapatinib (LP), a small molecule TKI, that targets two members of the EGFR family: EGFR and Her2. The EGFR family has four members, EGFR/Her1, Her2, Her3, and Her4, all of which except Her2 are known to bind ligands. Ligand binding to the extracellular domain of members of this family causes receptor homo- or heterodimerization and tyrosine kinase activation (23). Signal triggering via any of the EGFRs leads to the activation of a network of signaling cascades that are involved in cell proliferation and tumorigenesis. Although ligands for Her2 are not known, it has a key role in EGFR family signaling through its heterodimerization with other family members. LP is an ATP competitor that reversibly binds to the ATP binding pocket in the kinase domain of Her2 or EGFR. It specifically interacts with a non-active conformation of the kinase domain, preventing its activation for the duration of the complex (24).

3 The abbreviations used are: TKI, tyrosine kinase inhibitor; LP, lapatinib; EGFR, epidermal growth factor receptor; BBD, Bcl-2 binding domain; CCD, coiled-coil domain; ECD, evolutionarily conserved domain; PepA, pepstatin A; LC3, light chain 3; IP3, inositol 1,4,5-trisphosphate receptor; TLR, Toll-like receptor; ERα, estrogen receptor α; AVd, advanced vesicle; IP, immunoprecipitation; p, phospho.
Her2-Beclin-1 Complex in LP-induced Autophagy

Recent studies have demonstrated that TKIs (18), including LP (25), induce cytoprotective autophagy in tumor cells that do not succumb to the TKI toxicity. These findings suggest that the inhibition of this cell survival pathway may offer a strategy to overcome multiple molecular mechanisms involved in TKI resistance (26, 27). The molecular mechanisms that give rise to an autophagic response in drug-resistant tumor cells have not yet been fully elucidated.

Beclin-1 is an essential autophagic protein that functions in the context of Complex I (Vps34, Vps15, Atg14) in the signaling events of autophagosome formation and in the context of Complex II (UVRAG (UV radiation resistance-associated gene), Rubicon) in autophagosome maturation (28–30). Beclin-1 is a haploinsufficient tumor suppressor gene whose heterozygous deletion in mice increases the incidence of spontaneous tumors (31). Paradoxically, Beclin-1 is also involved in the tumorigenicity of breast cancer (BrCA) stem cells (32). Additionally, specific phosphorylation of Beclin-1 reverses its tumor suppression function to oncogenesis-driving activity (33).

In the current study, we identified a novel complex between BrCA Her2 and Beclin-1 that is disrupted by LP treatment. Our findings suggest an association between LP-mediated release of Beclin-1 from its sequestration by Her2 and the onset of autophagy in LP-resistant, Her2-expressing BrCA cells. We also obtained evidence that Beclin-1 enhances the phosphorylation level and potentially the signaling capability of Her2.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies—**Lapatinib was purchased from LC Laboratories; pepstatin A (PepA) and DAPI were from Sigma; and E64D was from Calbiochem. Antibodies against β-tubulin (sc-9104), Beclin-1 (sc-11427), β-actin (sc-47778), LAMP2 (sc-18822), Her2 (sc-284), p-Her2 (sc-12352), Akt (sc-8312), and p-Akt (sc-7985) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Vps34 (ab5451) was from Abcam; anti-MAP-LC3 antibodies for immunoblotting were from AnaSpec (San Jose, CA); and anti-MAP-LC3 antibody (sc-28266) for immunostaining was from Santa Cruz Biotechnology. The secondary antibodies HRP-conjugated anti-rabbit and anti-mouse IgG were from Thermo Scientific. Alexa Fluor 488- and 647-conjugated anti-rabbit and anti-mouse Ig were from Invitrogen.

**Cell Lines—**The human BrCA cell lines MCF7, SKBR3, MDA-MB-361, and BT474 were purchased from ATCC. All experiments were performed with freshly cultured cells from the original shipment. Her2-positive 4T1 and NT-5.1 murine breast carcinoma cells were provided by Dr. Eliezer Gorelik (University of Pittsburgh). 4T1 cells were originally derived from sporadic BrCa in Balb/c mice (34) and were stably transfected with Her2. NT-5.1 cells were derived from Her2/neu transgenic FVB/N mice (35). The tumor cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 20 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. LP-resistant BT474 cells were selected by multiple cycles of increasing LP doses (0.01–3 μM) that were applied during 6–8 weeks in cycles of 3-day treatment and 1 week of recovery.

**Cell Survival Assays—**Clonogenic assays were performed with methylcellulose-based semisolid medium (MethoCult H4230, StemCell Technologies) according to the manufacturer’s protocol. In brief, after treatment, the cells were washed, suspended in MethoCult medium, and cultured in triplicates (300 cells/3 ml) in 35-mm Petri dishes. The cultures were maintained at 37 °C in 5% CO2 for 35 days, and colonies were counted using an inverted microscope and gridted scoring dishes. Cyt fluorometric analyses of apoptosis were performed by co-staining with propidium iodide and fluorescein isothiocyanate-annexin V conjugates (BD Biosciences).

**Molecular Cloning of Human Beclin-1—**Total RNA was isolated from Jurkat T-cells using RNA STAT-60 reagent (Tel-Test, Inc.). Reverse transcription was carried out with 5 μg of total RNA using an oligo(dT)12–18 primer and SuperScript II RNase H− reverse transcriptase (Invitrogen). PCR was performed using the Expand high fidelity PCR system kit (Roche Applied Science). A Beclin-1 amplicon containing its open reading frame (ORF) was generated with the following primer pair that extends from six nucleotides into its 5′-untranslated region (UTR) through the ORF and extends 179 bases into the 3′UTR (forward and reverse): 5′-CGGGATCTCAGGATGG-GAAAGGTCTAAG-3′ and 5′-ACGGTGAGCTCAGTGG-GAAAGATCTGTCAC-3′. The putative full-length Beclin-1 amplicon was size-selected using a 1% agarose gel, and DNA was purified with the QiAquick gel extraction kit (Qiagen). The purified amplicon was digested with the restriction enzymes BamHI and SalI and ligated into a plasmid vector, pCDNA4/TO (Invitrogen), that had been previously digested with BamHI and Xhol. Following transformation (Escherichia coli TOP 10F’, Invitrogen), plasmids from randomly picked colonies underwent automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) to confirm sequence integrity.

**Generation of N-terminal 3×FLAG Beclin-1—**Utilizing the full-length WT Beclin-1 plasmid described above as a template, we carried out PCR as above with an N-terminal 3×FLAG-encoding forward primer: 5′-CGGGATCCGCTCCATGGACTACAAGAAGCATGAGGTATTATAAGGATCATGACA-3′ and the same reverse primer utilized above. The putative full-length Beclin-1 amplicon was size-selected using a 1% agarose gel, and DNA was purified with the QiAquick gel extraction kit (Qiagen). The purified amplicon was digested with the restriction enzymes BamHI and SalI and ligated into a plasmid vector pCR3.1. After transformation (E. coli DH5α, Invitrogen), random colonies were sequenced as above.

**Production of N-terminal 3×FLAG Beclin-1 Deletion Mutants—**Three N-terminal 3×FLAG Beclin-1 deletion mutants, including ΔBcl-2 binding domain (ΔBBBD), Δcoiled-coil domain (ΔCCD), and the Δevolutionarily conserved domain (ΔECD), were generated in a two-step procedure by overlap extension using the PCR method. Using the WT Beclin-1 cDNA clone described previously as a template, PCR was performed in the first step as above with the following sets of forward and reverse primer pairs: ΔBBBD (ΔMet-88 through Thr-150), N-terminal 3×FLAG primer from above, and
5’-GACGGTGGCCCTGGCTGGGCGATGATCT-3’ and 5’-CCCCAGCGACGCTCAAGTCACTGAA-AAT-3’ and the reverse primer for WT Beclin-1; ΔCDD (ΔLeu-144 through Val-269), N-terminal 3×FLAG primer and 5’-GGTTGCAATGAAGATCTGATCCTGACAAG-3’ and 5’-TGACAGATCTTTTTTATTGCAATGCTTC-CACATCGG-3’ and the reverse primer for WT Beclin-1; ΔEDC (ΔAsp-244 through Ser-337), N-terminal 3×FLAG primer and 5’-AGACTCCAGATACAGCCTGCTCGTCTG-GTTT-3’ and 5’-CAGCTGGAGCTGATCTGAGCTGCTGACAGC-3’ and the reverse primer for WT Beclin-1. In the second round of PCR, 0.5 μl of each cognate PCR reaction was combined using the N-terminal 3×FLAG primer and reverse primer for WT Beclin-1 as the outside primers. All putative deletion mutant amplicons were processed as described for generating the WT Beclin-1 cDNA and ligated into the plasmid vector pCR3.1. After transformation of DH5α cells, random colonies were sequenced as described above to confirm all mutations.

**Transfection and RNAi**—All siRNAs as well as the matching non-targeting controls were obtained as siGENOME SMARTpool or ON-TARGETplus SMARTpool siRNAs from Dharmacon. These reagents consist of four distinct RNA oligoduplexes per target or non-target. Additional individual Beclin-1 siRNAs and their appropriate negative controls were obtained from Invitrogen. RNAi for each gene included three individual Invitrogen Stealth Select siRNAs and their matching Stealth negative control. All knockdown experiments were repeated with at least two distinct siRNAs per target with similar results. Transfection of siRNA was performed with Oligofectamine according to the manufacturer’s transfection protocol (Invitrogen). Transient transfections were carried out with Lipofectamine LTX and Plus reagent (Invitrogen) according to the manufacturer’s instructions. Cell treatments were applied 24 h after transfection.

**Cell Microscopy and Image Acquisition**—Confocal images were obtained with an Olympus Fluoview 1000 confocal microscope and the companion software FV10-ASW1.6. Images were acquired with the use of the same setting at a resolution of 1020 × 1024 pixels. Morphometric measurements were performed using MetaMorph (Universal Imaging) on at least 50 cells per condition. Endogenous LC3 puncta were monitored by two measures: (i) manual counting of dot number per cell and (ii) determination of cumulative dot area per cell area. Cumulative dot area and cell area were determined by MetaMorph on images where the set threshold eliminated the detection of low or non-puncta staining.

For electron microscopy, cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) followed by 1% OsO₄. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1011CX electron microscope (JEOL, Peabody, MA). Images were acquired digitally. At least 50 cells per treatment were utilized for quantification.

**Immunoblotting and Immunoprecipitation**—Cell lysates were prepared with 1% NP40, 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The immuno blotting and immunoprecipitation procedures were described in our previous publications (13, 36–38). All immunoprecipitations were controlled by a sham procedure with non-specific matching immunoglobulin. Quantification of scanned protein bands was performed by the US-SCAN-IT Gel software (Silk Scientific, Inc., Orem, UT).

**Determination of Autophagic Flux**—Assessment of autophagic flux was performed by quantifications of autophagic markers in the absence or presence of predetermined saturating concentrations of the cathepsin inhibitors E64D and pepstatin A. Autophagic markers included autophagosome number (transmission electron microscopy), LC3 puncta number and relative cell area (confocal microscopy), and LC3-II protein level (immunoblotting).

**Statistical Analysis**—All immunoblot analyses are representative of at least three experiments. Images are representative of more than 50 cells from at least three repeats. Quantifications by MetaMorph were performed on at least 50 cells per treatment, and the results were confirmed in three independent experiments. Numerical data are presented as means ± S.E. of at least 50 cells used for each quantification. Statistical analysis was performed by GraphPad Prism V software, utilizing the nonparametric test, Mann-Whitney U.

**RESULTS**

**Induction of Cytoprotective Autophagy in LP-treated BrCA Cells**—LP is a reversible dual inhibitor of EGFR and Her2 that has also been reported to mediate off-target antitumor activity (39). To investigate the autophagic capability of LP, we utilized BrCA cell lines with amplified expression of Her2 (BT474, SKBR3, and MDA-MB-361) as well as the MCF7 BrCA cell line that expresses low levels of Her2 and EGFR. As the majority of the BT474 cells (but not of SKBR3 or MDA-MB-361) are LP-sensitive, we selected by multiple LP treatment cycles a monoclonal LP-resistant cell line that allows the analysis of those cells that do not succumb to the toxic effects of LP. Presumably, such a cell line is composed of multiple clones, each representing an LP resistance mechanism(s) that exists in the WT cell line. The relative resistance to LP of the BT474 selected cells was confirmed by their increased cell survival in a long term clonogenic assay (Fig. 1A). We also determined that cisplatin-selected MCF7 cells (15) were cross-resistant to LP. When freshly treated with LP, the resistant BrCA cell lines, LP-selected BT474 cells and cisplatin-selected MCF7 cells, did not succumb to the drug toxicity, but instead exhibited up-regulation of multiple autophagic markers. In comparison, LP treatment of WT BT474 cells (not preselected for LP resistance) generated a mixed population of apoptotic, necrotic, and autophagic cells (data not shown). As assessed by transmission electron microscopy, LP treatment of preselected, resistant cells induced the formation of autophagosomes and autophagolysosomes (Fig. 1, B and C). In a combined treatment by LP and saturating concentrations of the cathepsin inhibitors, E64D/PepA, there was a further increase in the number of autophagolysosomes and in their size and density, confirming the induction by LP of an autophagic flux (Fig. 1, D and E, results for BT474 are shown).

---

4 J. Han and H. Rabinowich, unpublished results.
The addition of E64D/PepA changed the appearance of the autophagolysosomes/advanced vesicles (AVds), allowing the distinction between potentially active and inhibited AVds (Fig. 1, D and E). LP-treated BrCA cells, including BT474, SKBR3, and MCF7, exhibited enhanced punctate appearance of endogenous LC3 as assessed by confocal microscopy (Fig. 2) or by quantitative image cytometry (Cellomics ArrayScan, data not shown). The development of an autophagic flux was confirmed by a confocal assessment of endogenous LC3 puncta in BrCA cells treated by LP in the presence E64D/PepA. The autophagic flux was monitored by the increase in the number of endogenous LC3 puncta per cell (Fig. 2, B, E, and H) or by their cumulative area relative to the corresponding cell area (Fig. 2, C, F, and I). The occurrence of an autophagic flux in LP-treated BrCA cells was confirmed by each of the monitoring assays in each of the BrCA cell lines tested. The LP-mediated increase in formation of autophagolysosomes was further demonstrated by increased co-localization of LC3 and LAMP2 puncta (Fig. 2, G and J).

Additionally, LP-treated cells exhibited increased conversion of LC3-I to its lipidated form LC3-II, currently the best marker for membrane-associated LC3. The enhanced LC3-II accumulation by a combined LP and E64D/PepA treatment, relative...
FIGURE 2. Altered expression of endogenous LC3 puncta in LP-treated BrCA cells. LP-selected BT474 cells (A–C), SKBR3 cells (D–F), and cisplatin-selected MCF7 cells (G–J) were treated with LP in the absence or presence of E64D/PepA (E/P). Expression patterns for LC3 and LAMP2 were assessed by confocal microscopy (A, D, and G) and quantified by MetaMorph. Scale bars: A and D, 30 μm; G, 50 μm. The LC3 dot number per cell was determined by manual counting (B, E, and H), whereas the cumulative LC3 dot area × 10^-4 per cell area was determined by MetaMorph (C, F, and I). The percentage of LC3-LAMP2 co-localization (LC3 over LAMP2) was quantified by MetaMorph. All quantifications were performed on at least 50 cells per treatment in each of three independent experiments with equivalent results; ***, p < 0.0001 (Mann-Whitney U).
to E64D/PepA alone, confirmed the occurrence of an autophagic flux in these LP-treated cells (Fig. 3).

The cytoprotective nature of the autophagic response to LP was not only demonstrated by their clonogenic survival, but also by the shift of the LP cell response from autophagy to apoptosis upon inhibition of autophagy. Thus, a marked increase in annexin V-positive cells was observed in LP-resistant BrCA cells treated by the combination of LP with Beclin-1 siRNA (Fig. 4, BT474, 26% ; SKBR3, 21%; MCF7, 47%). This series of experiments documents the induction of cytoprotective autophagy by LP and the involvement of Beclin-1 in this stress adaptation response of BrCA cells.

**Interaction of Beclin-1 with Her2**—To investigate the autophagic response to LP in Her2-expressing cells, we assessed its effect on the Her2 expression pattern. A significant co-localization between Her2 and Beclin-1 was detected at the cell surface of both BT474 and SKBR3 cells (Fig. 5, A and B). However, within 15 min of exposure to LP, both BT474 and SKBR3 cells lost the surface expression of Her2 and its surface co-localization with Beclin-1. An extended treatment by LP revealed a significant cytosolic accumulation of Beclin-1 and its precursor, LC3-I, in these cells.

**FIGURE 3.** Induction of autophagic flux as assessed by LC3-II level in LP-treated BrCA cells. A–H, accumulation of LC3-II was tested in the absence or presence of saturating concentrations of E64D/PepA (E/P) (10 μg/ml each). The immune-probed protein bands (A, C, E, and G) were quantified as described under “Experimental Procedures,” and the ratio to control treatment is presented in the corresponding charts (B, D, F, and H). A and B, LP-selected BT474 re-treated with LP (2 μM, 16 h). C and D, LP-treated SKBR3 cells (2 μM, 6 h). E and F, LP-treated cisplatin-resistant MCF7 cells (5 μM, 16 h). G and H, LP-treated 4T1 and NT5.1, Her2-positive murine BrCA cell lines were obtained from Her2 transgenic mouse (2 μM, 16 h). Please note that in certain cell lines, only LC3-II, and not its precursor, LC3-I, is detected.

**FIGURE 4.** Beclin-1 knockdown shifts the response of LP-resistant cells from autophagy to apoptosis. A–F, Beclin-1 knockdown by distinct siRNAs induces LP apoptosis susceptibility in LP-selected BT474 cells (A and B); SKBR3 cells (C and D); and cisplatin-selected MCF7 cells (E and F). B, D, and F, evidence for Beclin-1 RNAi in cells utilized in A, C, and E, respectively. Different Beclin-1 siRNA targeting sequences 1 and 2 are indicated.
To characterize the nature of the interaction between Her2 and Beclin-1, we assessed by immunoprecipitation their potential presence within the same complex. Co-immunoprecipitation of Beclin-1 and Her2 confirmed the existence of such a complex between either endogenously expressed Her2 and Beclin-1 or endogenous Her2 and transfected Beclin-1 (Fig. 6, A–C). In confirmation of the results obtained by immunostaining, LP treatment significantly disrupted the Her2-Beclin-1 complex, as demonstrated by a reduction in their co-immunoprecipitation in the presence of this inhibitor (Fig. 6, D and E).

To map the potential Beclin-1 interaction site(s) with Her2, we generated 3×FLAG (N terminus)-tagged WT Beclin-1 and its corresponding deletion mutants, including a deletion of the coiled-coil domain (Beclin-1ΔCCD), of the Bcl-2 binding domain (Beclin-1ΔBBD), and of the evolutionarily conserved domain (Beclin-1ΔECD). These 3×FLAG-tagged Beclin-1 variants were transfected into BT474 or SKBR3 cells and subjected to immunoprecipitation by either anti-FLAG or anti-Her2 antibodies. In both cell lines, the deletion of the Beclin-1 ECD inhibited the immunoprecipitation of the Her2-Beclin-1 complex (Fig. 7, A–C). As the interaction between Beclin-1 and Her2 is present under basal conditions, but disrupted by LP concomitant with its autophagy induction, these findings suggest that LP interferes with sequestration of Beclin-1 by Her2.

Impact of Beclin-1 on Her2 Signaling—To investigate the impact of Beclin-1 on Her2 signaling, we transfected Beclin-1 into three Her2-expressing BrCA cell lines, including MDA-MB-361, SKBR3, and BT474, and assessed the phosphorylation levels of Her2 and Akt in the presence of LP or a control vehicle. Relative to the control vector, Beclin-1 overexpression increased the phosphorylation levels of both Her2 and Akt (Fig. 8, A, C, and E, lane 1′ versus lane 1) in each of the tested Her2-expressing BrCA cell lines (Fig. 8, A–F). Moreover, in cells overexpressing Beclin-1, the negative impact of LP treatment on the expression levels of p-Her2 or p-Akt was attenuated (Fig. 8, A, C, and E, lanes 2′ and 3′ versus lanes 2 and 3). This attenuation may be related to an increased phosphorylation level in the
presence of Beclin-1, to a reduced access of LP to Her2 in the presence of Beclin-1, or to a combination of these impacts. Although Beclin-1 appears to reduce the access of LP to Her2 (based on attenuated Her2 dephosphorylation by LP in the presence of Beclin-1), LP effectively disrupts the Her2-Beclin-1 complex, and overexpression of Beclin-1 does not provide protection against LP to LP-sensitive cells. The increased phosphorylation of Her2 and Akt was not observed when Beclin-1/H9004ECD was transfected into MDA-MB-361, SKBR3, or BT474 cells (Fig. 8, G and H). Likewise, the attenuation of LP dephosphorylation activity on p-Her2 or p-Akt was not detected with the ECD-deficient Beclin-1. These findings suggest that the ECD of Beclin-1 is required for the interaction between Beclin-1 and Her2, for the impact of Beclin-1 on the signaling capability of Her2, and for attenuating the dephosphorylation activity of LP on either p-Her2 or p-Akt.

**DISCUSSION**

The current study describes a heretofore unknown interaction between Beclin-1 and Her2. The data suggest that the Her2-Beclin-1 complex is present at the cell surface of Her2-expressing BrCA cells; that the Her2-Beclin-1 complex is disrupted by LP treatment, which concomitantly induces adaptive autophagy in BrCA cells that are resistant to the death-inducing effect of this TKI; and that the interaction with Beclin-1 increases the phosphorylation of Her2 and attenuates the Her2 dephosphorylation activity of LP. These findings further suggest that under basal conditions, Beclin-1 may impact the Her2 signaling network. The disruption of this Her2-Beclin-1 complex may contribute to the cell autophagic response by both interfering with the Her2 survival signaling (cellular stress) in combination with the release of Beclin-1 from potential sequestration.

There is a significant dichotomy between LP-sensitive and LP-resistant BrCA with regard to LP-mediated autophagy. BrCA cells that are LP-sensitive are being eliminated in response to LP treatment by various mechanisms of cell death. In contrast, BrCA cells that respond in autophagy carry primary resistance to LP, potentially mediated by specific mutations in their Her2 kinase domain. Such an autophagic response is cyto-

---

**FIGURE 7. The ECD of Beclin-1 is required for its interaction with Her2.** BT474 or SKBR3 cells were transfected (Tx) with 3×FLAG WT Beclin-1 or with the indicated deletion mutants of Beclin-1 (ΔECD, ΔBBD, and ΔECD), each with an N-terminal 3×FLAG tag. A–C, the cells were then subjected to IP of endogenous Her2 (A and C) or of an exogenous 3×FLAG Beclin-1 variant (B). The asterisks indicate unidentified protein bands.

**FIGURE 8. Increased expression of Beclin-1, but not Beclin-1ΔECD, enhances the phosphorylation levels of Her2 and Akt and reduces the access of LP to Her2.** A–F, MDA-MB-361 cells (A and B), SKBR3 cells (C and D), and WT BT474 cells (E and F) were transfected with vector control or WT Beclin-1 and treated with LP (6 h, 1 or 2.5 μM (A–D) and 0.25 or 0.5 μM (E and F). The cell lysates were assessed by immunoblotting for the expression of the indicated proteins. Results of one representative experiment of three performed for each cell line are shown. The expression levels of p-Her2 and p-Akt were quantified, and the percentages of increases in Beclin-1 transfected cells versus vector-transfected cells are presented in the corresponding charts (B, D, and F). G and H, the Beclin-1 ECD domain is required for the observed Her2 and Akt phosphorylation changes. MDA-MB-361 cells (G), SKBR3 cells (G), and WT BT474 cells (H) were transfected with vector control or Beclin-1ΔECD and treated with LP, as described above for the corresponding cell line. The asterisks indicate unidentified protein bands.
Beclin-1 (42). Binding of Beclin-1 to ER inhibition level remains unchanged despite overexpression of Beclin-1. However, overexpression of Beclin-1 may contribute to the complex stability, facilitating the Beclin-1-dependent and, thus, increases with Beclin-1 overexpression, but is independent of the interaction of Her2 with Beclin-1. In such a case, the Beclin-1 ΔECD mutant loses its capability of inducing Her2 phosphorylation because of changes in the protein that are not directly related to its ability to interact with Her2.

The Beclin-1-mediated increase in the Her2 phosphorylation level suggests that Beclin-1 contributes to the signaling and, potentially, the oncogenic activity of Her2. These findings are inconsistent with the established tumor suppressor activity of Beclin-1. The tumor suppressor activity of Beclin-1 has been deduced from the monoallelic deletion of Beclin-1 in multiple cancers, including BrCA, and from the increased incidence of spontaneous tumors in heterozygous BECN1+/− mice (31, 47). Beclin-1 tumor suppression may relate to its autophagic role, yet autophagy may both enhance and inhibit the tumorigenesis process. Thus, autophagic degradation of damaged cellular components generated by genetic alterations and chromosomal instability plays an anti-tumorigenesis role, whereas the cytoprotective function of autophagy may have the opposite effect of enhanced tumorigenesis (48, 49). Furthermore, in contrast to established BrCA tumors where tumorigenesis is associated with reduced Beclin-1 expression, tumorigenesis of BrCA stem cells has been demonstrated to be Beclin-1-dependent (32). In addition, specific Akt phosphorylation of Beclin-1 shifts its activity from suppression to progression of the oncogenic process (33). Thus, the tumor suppressor activity of Beclin-1 may depend on the phase of the tumor development, specific Beclin-1 phosphorylation, and the overall network of Beclin-1 cellular interactions.

This study is the first to report the interaction between Beclin-1 and a member of the EGFR family. Although Beclin-1 has been identified as an essential participant in the EGFR endocytic pathway (50), the exact mechanism that underlies its involvement in the EGFR degradation remains unknown. The potential involvement of pre-existing complexes between Beclin-1 and other EGFR members and the cross-talk between their signaling networks and the autophagic response will be the focus of future studies.

REFERENCES

1. Kroemer, G., Mariño, G., and Levine, B. (2010) Autophagy and the integrated stress response. Mol. Cell 40, 280–293
2. Moreau, K., Luo, S., and Rubinsztein, D. C. (2010) Cytoprotective roles for autophagy. Curr. Opin. Cell Biol. 22, 206–211
3. Mariño, G., Mdeo, F., and Kroemer, G. (2011) Autophagy for tissue homeostasis and neuroprotection. Curr. Opin. Cell Biol. 23, 198–206
4. Shen, S., Kepp, O., and Kroemer, G. (2012) The end of autophagic cell death? Autophagy 8, 1–3
5. Ito, H., Daido, S., Kanzawa, T., Kondo, S., and Kondo, Y. (2005) Radiation-induced autophagy is associated with LC3, and its inhibition sensitizes malignant glioma cells. Int. J. Oncol. 26, 1401–1410
6. Apel, A., Herr, I., Schwarz, H., Rodemann, H. P., and Mayer, A. (2008) Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res. 68, 1485–1494
7. Chaouchouy, H., Ohnseit, P., Toulany, M., Kehlbach, R., Multhoff, G., and Rodemann, H. P. (2011) Autophagy contributes to resistance of tumor
Her2-Beclin-1 Complex in LP-induced Autophagy

cells to ionizing radiation. Radiat. Oncol. 99, 287–292
8. Chen, Y. S., Song, H. X., Lu, Y., Li, X., Chen, T., Zhang, Y., Xue, J. X., Liu, H., Kan, B., Yang, G., and Fu, T. (2011) Autophagy inhibition contributes to radiation sensitization of esophageal squamous carcinoma cells. Dis. Esophagus 24, 437–443
9. Amaravadi, R. K., Yu, D., Lum, J. J., Bui, T., Christophorou, M. A., Evan, G. I., Thomas-Tikhonenko, A., and Thompson, C. B. (2007) Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. 117, 326–336
10. Levy, J. M., and Thorburn, A. (2011) Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol. Ther. 131, 130–141
11. Li, J., Hou, N., Faried, A., Tsutsumi, S., Takeuchi, T., and Kuwano, H. (2009) Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann. Surg. Oncol. 16, 761–771
12. Livesey, K. M., Tang, D., Zeh, H. J., and Lotze, M. T. (2009) Autophagy inhibition in combination cancer treatment. Curr. Opin. Investig. Drugs 10, 1209–1219
13. Han, J., Hou, W., Goldstein, L. A., Lu, C., Stolz, D. B., Yin, X. M., and Rabinowich, H. (2008) Involvement of protective autophagy in TRAIL resistance of apoptosis-defective tumor cells. J. Biol. Chem. 283, 19665–19677
14. Herrero-Martín, G., Høyer-Hansen, M., García-García, C., Fumarola, C., Farkas, T., López-Rivas, A., and Jáättelä, M. (2009) TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. EMBO J. 28, 677–685
15. Hou, W., Han, J., Lu, C., Goldstein, L. A., and Rabinowich, H. (2008) Enhancement of tumor-TRAIL susceptibility by modulation of autophagy. Autophagy 4, 940–943
16. Mills, K. K., Reginato, M., Debnath, J., Queenan, B., and Brugge, J. S. (2004) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. Proc. Natl. Acad. Sci. USA. 101, 3438–3443
17. Gorzálezany, Z., Gilad, Y., Amihai, D., Hammel, I., Sagi-Eisenberg, R., and Merimsky, O. (2011) Combining an EGFR directed tyrosine kinase inhibitor with autophagy-inducing drugs: a beneficial strategy to combat non-small cell lung cancer. Cancer Lett. 310, 207–215
18. Han, W., Pan, H., Chen, Y., Sun, J., Wang, X., Li, J., Ge, W., Feng, L., Lin, X., Wang, X., Wang, X., and Jin, H. (2011) EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. PLoS One 6, e18691
19. Amaravadi, R. K., Lippincott-Schwartz, J., Yin, X. M., Weiss, W. A., Takebe, N., Timmer, W., DiPaolo, R. S., Lotze, M. T., and White, E. (2011) Principles and current strategies for targeting autophagy for cancer treatment. Clin. Cancer Res. 17, 654–666
20. Bellodi, C., Lidonnici, M. R., Hamilton, A., Helgason, G. V., Soliera, A. R., Ronchetti, M., Galavotti, S., Young, K. W., Selmi, T., Yacob, R., Van Etten, R. A., Donato, N., Hunter, A., Dinsdale, D., Tirrò, E., Vigneri, P., Nicotera, P., Dyer, M. J., Holyoke, T., Salomoni, P., and Calabretta, B. (2009) Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J. Clin. Invest. 119, 1109–1123
21. Calabretta, B., and Salomoni, P. (2011) Inhibition of autophagy: a new strategy to enhance sensitivity of chronic myeloid leukemia stem cells to tyrosine kinase inhibitors. Leuk. Lymphoma 52, Suppl. 1, 54–59
22. Janku, F., McConkey, D. J., Hong, D. S., and Kurzrock, R. (2011) Autophagy as a target for anticaner therapy. Nat. Rev. Clin. Oncol. 8, 528–539
23. Holbro, T., and Hynes, N. E. (2004) ErbB receptors: directing key signaling networks throughout life. Annu. Rev. Pharmacol. Toxicol. 44, 195–217
24. Shan, Y., Eastwood, M. P., Zhang, X., Kim, E. T., Arkhipov, A., Dror, R. O., Jumper, J., Kuriyan, J., and Shaw, D. E. (2012) Oncogenic mutations counteract intrinsic disorder in the EGFR kinase and promote receptor dimerization. Cell 149, 860–870
25. Huang, H. L., Chen, Y. C., Huang, Y. C., Yang, K. C., Pan, H. Y., Shih, S. P., and Chen, Y. J. (2011) Lapatinib induces autophagy, apoptosis, and megakaryocytic differentiation in chronic myelogenous leukemia K562 cells. PloS one 6, e29014
Joza, N., Vitale, I., Morselli, E., Tailler, M., Castedo, M., Maiuri, M. C., Molgò, J., Szabadkai, G., Lavandero, S., and Kroemer, G. (2009) The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. *Cell Death Differ.* 16, 1006–1017

45. Yue, Z., Horton, A., Bravin, M., Delager, P. L., Selimi, F., and Heintz, N. (2002) A novel protein complex linking the δ2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. *Neuron* 35, 921–933

46. Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402, 672–676

47. Yue, Z., Jin, S., Yang, C., Levine, A. J., and Heintz, N. (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15077–15082

48. Dikic, I., Johansen, T., and Kirkin, V. (2010) Selective autophagy in cancer development and therapy. *Cancer Res.* 70, 3431–3434

49. Mathew, R., and White, E. (2011) Autophagy in tumorigenesis and energy metabolism: friend by day, foe by night. *Curr. Opin. Genet. Dev.* 21, 113–119

50. Thoresen, S. B., Pedersen, N. M., Liestøl, K., and Stenmark, H. (2010) A phosphatidylinositol 3-kinase class III sub-complex containing VPS15, VPS34, Beclin 1, UVRAG, and BIF-1 regulates cytokinesis and degradative endocytic traffic. *Exp. Cell Res.* 316, 3368–3378

---

**Her2-Beclin-1 Complex in LP-induced Autophagy**