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IL-1 induces p62/SQSTM1 and autophagy in ER α ⁺/PR⁺ BCa cell lines concomitant with ER α and PR repression, conferring an ER α ⁻/PR⁻ BCa-like phenotype

A.F. Nawas¹, S. Narayanan¹, R. Mistry¹, S.E. Thomas-Jardin¹, J. Ramachandran¹, J. Ravichandran¹, E. Neduvellil¹, K. Luangpanh¹, N. A. Delk¹

A.F. Nawas: Afshanfathima.Nawas@utdallas.edu; S. Narayanan: nshrinath1994@gmail.com; R. Mistry: Ragini.Mistry@utdallas.edu; S.E. Thomas-Jardin: set110020@utdallas.edu; J. Ramachandran: janani.ramachandran@risd.org; J. Ravichandran: Jananisree.Ravichandran@utdallas.edu; E. Neduvellil: ebin.nedu@gmail.com; K. Luangpanh: Krisha.Luangpanh@utdallas.edu; N. A. Delk: nikki.delk@utdallas.edu

¹Biological Sciences Department, University of Texas at Dallas, 800 West Campbell Road, Richardson, TX 75080

Abstract

Estrogen receptor alpha (ER α)^{low/-} tumors are associated with breast cancer (BCa) endocrine resistance, where ER α low tumors show a poor prognosis and a molecular profile similar to triple negative BCa tumors. Interleukin-1 (IL-1) downregulates ER α accumulation in BCa cell lines, yet the cells can remain viable. In kind, IL-1 and ER α show inverse accumulation in BCa patient tumors and IL-1 is implicated in BCa progression. IL-1 represses the androgen receptor (AR) hormone receptor in prostate cancer (PCa) cells concomitant with the upregulation of the prosurvival, autophagy-related protein, Sequestome-1 (p62/SQSTM1; hereinafter, p62); and given their similar etiology, we hypothesized that IL-1 also upregulates p62 in BCa cells concomitant with hormone receptor repression. To test our hypothesis, BCa cell lines were exposed to conditioned medium from IL-1-secreting bone marrow stromal cells (BMSCs), IL-1, or IL-1 receptor antagonist (IL-1RA). Cells were analyzed for the accumulation of ER α , progesterone receptor (PR), p62, or the autophagosome membrane protein, Microtubule-Associated Protein 1 Light Chain 3 (LC3), and for p62-LC3 interaction. We found that IL-1 is sufficient to mediate BMSC-induced ER α and PR repression, p62 and autophagy upregulation, and p62-LC3 interaction in ER α ⁺/PR⁺ BCa cell lines. However, IL-1 does not significantly elevate the high basal p62 accumulation or high basal autophagy in the ER α ⁻/PR⁻ BCa cell lines. Thus, our observations imply that IL-1 confers a prosurvival ER α ⁻/PR⁻ molecular phenotype in ER α ⁺/PR⁺ BCa cells that may be dependent on p62 function and autophagy and may underlie endocrine resistance.

CORRESPONDING AUTHOR Nikki A. Delk, nikki.delk@utdallas.edu.

AUTHORS' CONTRIBUTIONS

AFN was responsible for experimental design, optimization, execution, and data analysis and prepared the manuscript. SN, JR, JR, EN, KL assisted with cell culture, western blot analysis and immunostaining. RM was responsible for optimization, execution, and analysis of immunoprecipitation results. SETJ assisted with execution and optimization of RT-QPCR and proof-read manuscript. NAD is corresponding author. All authors read and approved the final manuscript.

DISCLOSURE STATEMENT

The authors declare that they have no competing interests

Keywords

p62/SQSTM1; autophagy; estrogen receptor alpha; interleukin-1; endocrine resistance

1. INTRODUCTION

Estrogen receptor alpha (ER α) and progesterone receptor (PR) are nuclear hormone receptors that drive the proliferation and survival of breast cancer (BCa) cells, where PR is also an ER α target gene^{1,2}. Around 70% of BCa patients have tumors positive for ER α and PR (ER α ⁺/PR⁺) at diagnosis; therefore ER α is a therapeutic target for BCa¹. ER α -targeting endocrine therapies include estrogen deprivation (ovarian ablation or aromatase inhibitors), selective estrogen receptor modulators (SERMs; e.g., tamoxifen) and selective estrogen receptor downregulators (SERDs; e.g., fulvestrant) that block ER α activity and/or reduce ER α protein accumulation³. Unfortunately, reportedly 40–50% of BCa patients will eventually acquire resistance to ER α -targeting therapy¹. Studies suggest that endocrine therapy resistance is due to aberrant ER α activity as a result of ER α mutations, posttranslational modifications, altered chromatin binding, or estrogen-independent growth factor signaling¹. ER α reduction or loss is also a mechanism of endocrine resistance^{1,4}. ER α ^{low/-} tumors are observed in 10–30% of endocrine resistant BCa patients^{1,4} and ER α ^{low/-} tumors are predictive of endocrine resistance and poor prognosis as compared to BCa patients with higher ER α tumor levels^{5–9}.

Tumor inflammation positively correlates with ER α reduction or loss in BCa patient tumors and multiple different inflammatory cytokines have been shown to reduce ER α levels, thus, implicating inflammation in BCa endocrine resistance⁴. For example, the interleukin-1 (IL-1) inflammatory cytokine family has been shown to repress ER α levels^{10–12} and IL-1 inversely correlates with ER α in BCa patient tumors^{13–15}, making IL-1 a likely culprit contributing to BCa endocrine resistance.

The major IL-1 family members are IL-1 alpha (IL-1 α) and IL-1 beta (IL-1 β), both of which bind to the cell surface IL-1 Receptor 1 (IL-1R1)¹⁶. IL-1 is elevated in BCa tumor tissue and patient serum and correlates with relapse and poor prognosis^{17–20}. Functionally, IL-1 signaling induces expression of pro-metastatic genes, angiogenic proteins, and growth factors that promote tumor progression²¹. Particularly in BCa, IL-1 can enhance progression through the induction of metastasis and cachexia²⁰. The IL-1 receptor antagonist (IL-1RA) blocks IL-1 signaling¹⁶ and IL-1RA has been shown to reduce breast cancer xenograft tumor volume and bone metastasis *in vivo*²². As such, IL-1RA is being investigated in clinical trials ([ClinicalTrials.gov](https://clinicaltrials.gov)) as adjuvant therapy for multiple cancer types including BCa, to, in part, block IL-1 tumorigenic functions such as angiogenesis.

We have previously shown that IL-1 β downregulates androgen receptor (AR) mRNA and protein in prostate cancer (PCa) cell lines²³. AR is a nuclear hormone receptor that promotes PCa growth and proliferation and, thus, is a PCa therapeutic target²⁴. However, as with ER α ^{low/-} BCa tumors, PCa tumors enriched in AR^{low/-} cells are associated with hormone therapy resistance^{24,25}. Furthermore, as with BCa, IL-1 also promotes PCa metastasis to bone²⁶ and IL-1 is implicated in PCa hormone therapy resistance²⁷. Therefore, given the

etiological similarities between BCa and PCa, we hypothesize that the IL-1 mechanisms that promote resistance to hormone receptor-targeting therapies are similar in BCa and PCa.

We found that IL-1 signaling downregulates AR in PCa cell lines concomitant with the induction of several prosurvival, pro-growth pathways that may contribute to treatment resistance (Thomas-Jardin et al., in press), such as p62 signaling²⁸. p62 is a multi-domain scaffold protein that has been shown to be cytoprotective for multiple different cancer types^{29–31} and p62 mediates various prosurvival signaling cascades, including autophagy³². Autophagy-dependent p62 function involves p62 sequestration of cytoplasmic proteins and organelles into the autophagosome for degradation and biomolecule recycling³². Cancer cells utilize autophagy to survive cellular stresses such as low nutrient availability, genotoxic stress, chemotherapy, and radiation³³. Not surprisingly, p62 overexpression^{34–38} or elevated autophagy^{39,40} in BCa patient tumors is associated with poor prognosis. Therefore, we investigated the effect of IL-1 on p62 and autophagy concomitant with ER α and PR loss in BCa cell lines.

Here, we characterize ER α ⁺/PR⁺ BCa cell lines, MCF7 and T47D, and ER α ⁻/PR⁻ BCa cell lines, BT549 and MDA-MB-231. We find that ER α ⁻/PR⁻ BCa cell lines have high basal p62 accumulation and autophagy flux relative to ER α ⁺/PR⁺ BCa cell lines. We show that IL-1 represses ER α and PR in hormone receptor positive BCa cell lines concomitant with p62 upregulation, autophagy induction, and p62 interaction with the autophagosome membrane protein, LC3. Thus IL-1-induced p62-autophagy signaling might be an underlying mechanism promoting BCa tumor cell survival when hormone receptor accumulation is repressed.

2. MATERIALS AND METHODS

2.1 Cell Culture:

Breast cancer cell lines (MCF7, T47D, MDA-MB-231), prostate cancer cell lines (LNCaP, C4-2, PC3, DU145), and bone marrow stromal cell lines (HS-5, HS-27a) were grown in a 37°C, 5.0% (v/v) CO₂ incubator in DMEM medium (Gibco; 1185–076) supplemented with 10% FB Essence (Seradigm; 3100–500), 0.4 mM L-glutamine (L-glut; Gibco/Invitrogen, 25030081), and 10 U/ml penicillin G sodium and 10 mg/ml streptomycin sulfate (pen-strep; Gibco/Invitrogen, 15140122). BT549 breast cancer cell line was grown in RPMI-1640 media (Hyclone; SH30027.01) supplemented with 10% FB essence, L-glut, Pen-strep.

2.2 Conditioned Media (CM) Treatment:

Culture medium (CM) was removed from BCa cell lines, HS-5 or HS-27a cells and replaced with fresh DMEM or RPMI supplemented with 10% FB Essence. After 3 days incubation, the conditioned DMEM or RPMI media was collected, filtered and stored at –80°C. For CM treatment experiments, cells were grown in BCa CM (control CM), HS-5 CM, or HS-27a CM for 3 days.

2.3 Cytokines and Drugs:

Human recombinant IL-1 α , IL-1 β and IL-1RA were purchased from R&D systems (200-LA/CF; 201-LB-005; 280-RA/CF) and resuspended in 0.1% bovine serum albumin (BSA) in 1X phosphate buffered saline (PBS). Chloroquine (CQ) diphosphate aqueous solution was purchased from Invitrogen (P-36239). Cells were treated with 25ng/mL IL-1 or vehicle control (0.1% BSA in 1X PBS) added to DMEM or RPMI growth media for 3 days. For the IL-1RA experiments, cells grown in DMEM were pre-treated with 200 ng/mL IL-1RA in for 1 day and then the medium replaced with fresh DMEM or HS-5 CM with or without IL-1RA for an additional 3 days.

2.4 Western Blot and Antibodies:

Protein was isolated from cells using NP40 lysis buffer (0.5% NP-40 (US biological; N3500), 50mM Tris (pH 7.5), 150mM NaCl, 3mM MgCl₂, 1 $\times$ protease inhibitors (Roche; 05892953001). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific; 23227). For western blot analysis, equal protein concentrations were loaded onto and separated in 12% or 16% (w/v) sodium dodecyl sulfate polyacrylamide gel (40% acrylamide/bis-acrylamide solution; Bio-Rad; 161-0148). Proteins were transferred from the gel to 0.45 μ m pore size nitrocellulose membrane (Maine manufacturing; 1215471) and total protein visualized using Ponceau S (Amresco; K793). The membrane was blocked with 2.5% (w/v) BSA (Fisher; BP 1600-1) in 1 $\times$ TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20). Primary and secondary antibodies were diluted in 2.5% BSA in 1 $\times$ TBST. Protein blot bands were visualized using Clarity Western ECL Substrate (BioRad; 1705061) and imaged using Amersham Imager 600 (GE). *Primary antibodies*: ER α (Cell signaling technology; 8644), LC3B (Novus Biologicals; NB600-1384), p62/SQSTM1 (Santa Cruz Biotechnology; sc-28359), SOD2 (Abgent; AP7579a) and β -actin (Abcam; ab8226). *Secondary antibodies*: sheep anti-mouse (Jackson ImmunoResearch Laboratories; 515-035-062), goat anti-rabbit (Sigma-Aldrich; A6154). Western blot densitometry was performed using Image J and graphed, where protein bands were first normalized to β -actin and then relative protein levels normalized to the lowest protein/ β -actin ratio for a given protein.

2.5 RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-QPCR):

RNA was extracted using GeneJET RNA Purification kit (Thermo-Fisher; K0732) as per manufacturer's instructions. cDNA was synthesized using iScript Reverse Transcription Supermix (Biorad; 170-8841). RT-QPCR reactions were performed using the iTaq Universal SYBR Green Supermix (Biorad; 172-5125) as per manufacturer's instructions using BioRad CFX Connect. *ER α* , *PR*, *p62* or *SOD2* cycle times were normalized to the *β -actin*. Relative mRNA levels were calculated using 2^{-CT} method. *5'-3' Primer sequences*: *ER α* Forward, ACAAGGGAAGTATGGCTATGGA; *ER α* Reverse, GGTCTTTTCGTATCCACCTTTC; *PR* Forward, ACATGGTAGCTGTGGGAAGG; *PR* Reverse, GCTAAGCCAGCAAGAAATGG; *p62* Forward, AAATGGGTCCACCAGGAACTGGA; *p62* Reverse, TCAACTTCAATGCCAGAGGGCTA; *SOD2* Forward, GGCCTACGTGAACAACCTGA; *SOD2* Reverse, GTTCTCCACCACCGTTAGGG; *β -Actin* Forward, GATGAGATTGGCATGGCTTT; *β -Actin* Reverse, CACCTTCACCGGTCCAGTTT

2.6 Immunoprecipitation:

Cells were lysed in NP40 lysis buffer and equal protein concentrations incubated with 2 μ g of LC3B antibody (Novus; NB100–2220) or rabbit IgG (Santa Cruz Biotechnology; sc-2027). Immunoprecipitation (IP) was performed using Pierce Magnetic co-IP kit (Thermo Scientific; 88804) per manufacturer's instructions.

2.7 Immunofluorescence:

Cells were fixed and permeabilized with 100% methanol at -20°C for 30 minutes. Fixed cells were blocked with 2.5% BSA in 1 $\times$ PBS at room temperature for at least 30 minutes. Antibodies were diluted in 2.5% BSA in 1 $\times$ PBS. Cells were incubated in primary antibody overnight at 4°C , washed with 1 $\times$ PBS, and then incubated with fluorescently labeled secondary antibody overnight at 4°C in the dark. *Primary antibodies:* LC3B (Novus Biologicals; NB600–1384), p62/SQSTM1 (Santa Cruz Biotechnology; sc-28359). *Fluorescently labeled secondary antibodies:* Alexafluor 488, goat anti-mouse (Invitrogen; A11001), Alexafluor 568, goat anti-rabbit (Invitrogen; A11061). Immunostained cells were imaged at 10x, 20x, and 100x magnification using a Nikon epifluorescence microscope. Images were analyzed using Nikon NIS Elements. Cell counts were performed for five representative microscopy fields at 10x magnification for each treatment. The cell count average and standard deviation of 3 biological replicates per treatment was calculated.

2.8 Statistical analysis:

Statistical significance between two groups was tested using Student's *t* test. Error bars represent standard deviation (STDEV) of at least $n = 3$ biological replicates (Bio Rep) and asterisks denotes statistical significance (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$).

3. RESULTS

3.1 ER α^{-} /PR $^{-}$ BCa cell lines have high basal p62 accumulation and autophagy flux

We previously discovered that AR $^{-}$ PCa cell lines have high basal p62 and/or LC3-II relative to AR $^{+}$ PCa cell lines²⁹. LC3-II is the membrane-conjugated form of Microtubule Associated Protein 1 Light Chain 3 (LC3) that comprises the autophagosome double membrane³². When autophagy is induced, free LC3 (LC3-I) is conjugated to phosphatidylethanolamine lipid to form LC3-II³². p62 binds LC3-II to sequester proteins and organelles into the autophagosome for subsequent degradation and recycling to maintain cellular homeostasis³². To determine if p62 and LC3-II accumulation might be stratified by hormone receptor status in BCa cells we compared p62 and LC3 mRNA and/or protein in ER α^{-} /PR $^{-}$ versus ER α^{+} /PR $^{+}$ cell lines. RT-QPCR and western blot reveal that relative to the ER α^{+} /PR $^{+}$ BCa cell lines, MCF7 and T47D, the ER α^{-} /PR $^{-}$ BCa cell lines, BT549 and MDA-MB-231, have high basal p62 mRNA (Fig. 1A) and protein (Fig. 1B) and high basal LC3-II protein (Fig. 1B). Thus, as we previously discovered for AR $^{-}$ versus AR $^{+}$ PCa cell lines²⁹, p62 and LC3-II are also basally high in ER α^{-} /PR $^{-}$ BCa cell lines relative to ER α^{+} /PR $^{+}$ BCa cell lines.

p62 binds to inner membrane LC3-II to sequester cargo into the autophagosome³², subjecting both p62 and LC3-II to autophagy-mediated degradation. Therefore, while

increased LC3-II levels can indicate active autophagosome formation, elevated p62 and LC3-II protein levels could also be due to a block in autophagosome fusion with the lysosome or a block in autolysosomal degradation⁴¹. To determine if the high basal p62 and LC3-II in the ER α ⁻/PR⁻ BCa cell lines is due to a block in autophagy, we treated BT549 and MDA-MB-231 BCa cell lines with the lysotropic drug, chloroquine (CQ). When autophagosomes fuse with lysosomes to form autolysosomes, the lysosomal hydrolases degrade the vesicle cargo. CQ increases lysosomal pH, thereby preventing degradation of autolysosomal cargo, including p62 and inner membrane LC3 (LC3-II), contained in autolysosomes. If autophagy flux is functional, then CQ will prevent autolysosomal turnover, causing a cellular accumulation of autophagosomes, autolysosomes and their cargo (e.g. p62 and LC3-II). Thus, cellular sensitivity to CQ indicates that autophagosome formation, lysosomal fusion, and autolysosomal degradation are functional⁴¹. Indeed, CQ increases p62 and LC3-II protein accumulation in the BT549 and MDA-MB-231 ER α ⁻/PR⁻ BCa cell lines (Fig. 4C), demonstrating that autophagy flux is functional in these BCa cell lines, where the high basal LC3-II (Fig. 1B) likely reflects enhanced autophagy flux and the high basal p62 is likely due to constitutive *p62* gene expression (Fig. 1A). Taken together, we observe similar p62 and LC3-II expression and accumulation patterns in hormone receptor negative versus positive BCa cell lines, as we previously observed for hormone receptor negative versus positive PCa cell lines²⁹. Thus, BCa and PCa may have evolved a similar regulation and functional requirement for p62 and autophagy that is stratified by hormone receptor status.

3.2 Bone marrow stromal cell paracrine factors upregulate p62 and autophagy in ER α ⁺/PR⁺ BCa cell lines

Bone-derived immune cells infiltrate tumors and secrete inflammatory cytokines that promote tumor progression both in the primary tumor and metastatic niche.⁴² Furthermore, BCa and PCa preferentially metastasize to bone. Thus, in investigating the paracrine effects of the HS-5 and HS-27a bone marrow stromal cell lines (BMSC) on PCa tumor cell biology, we discovered that HS-5 BMSCs, but not HS-27a BMSCs, secrete factors that induce p62 and autophagy in AR⁺ PCa cell lines^{28,43,44}. HS-5 BMSCs are secretory, while HS-27a BMSCs represent a structural component of the bone, where the HS-5-secreted milieu includes IL-1 α and IL-1 β ⁴⁵. To determine if ER α ⁺/PR⁺ BCa cell lines respond similarly to HS-5 BMSC paracrine factors, we treated MCF7 and T47D BCa cell lines with condition medium (CM) from the HS-5 or HS-27a BMSC cell lines. RT-QPCR and western blot revealed that HS-5 CM, but not HS-27a CM, induces p62 mRNA (Fig. 2A) and protein (Fig. 2B) and upregulates LC3-I and/or LC3-II accumulation (Fig. 2B) in MCF7 and T47D cell lines. Furthermore, p62 and LC3-II levels increase in the presence of CQ (Fig. 2C), indicating that autophagy is functional in the presence of HS-5 CM. Thus, HS-5 BMSCs secrete paracrine factors that have a similar effect on p62 and autophagy regulation in both hormone receptor positive BCa and PCa cell lines, suggesting p62 and autophagy are regulated through conserved mechanisms in both cancer types.

3.3 HS-5 BMSC paracrine factors repress hormone receptors in hormone receptor positive BCa cell lines

Given that high basal p62 and autophagy flux are associated with ER α ⁻/PR⁻ BCa cell lines (Fig. 1A), we predicted HS-5 BMSCs might induce ER α and PR repression concomitant with p62 and autophagy induction in ER α ⁺/PR⁺ BCa cell lines. Our rationale is based on our previous observations that HS-5 CM represses AR concomitant with p62 and autophagy induction in AR⁺ PCa cell lines^{23,44}. Treatment with HS-5 CM represses ER α and PR mRNA and protein (Fig. 2A & B). HS-5 CM can also repress AR mRNA and protein in BCa cell lines (data not shown). Interestingly, we could not detect HS-5 CM-mediated repression of mRNA levels or protein accumulation for ER beta (ER β) or glucocorticoid hormone receptors in BCa cell lines (data not shown). Thus, HS-5 BMSC paracrine regulation of AR, ER α , and PR hormone receptors may be through a conserved mechanism.

3.4 IL-1 signaling mediates HS5 BMSC paracrine regulation of p62, autophagy, and hormone receptor accumulation in ER α ⁺/PR⁺ BCa cell lines.

We found that IL-1 alone²³ or in the HS-5 CM milieu⁴⁶ is sufficient to repress AR and induce p62 mRNA and protein accumulation in PCa cell lines. To determine if IL-1 signaling is sufficient to mediate HS-5 BMSC paracrine modulation of ER α , PR, p62, and/or autophagy in ER α ⁺/PR⁺ BCa cell lines, we blocked IL-1 signaling in MCF7 and T47D cells with the IL-1 receptor antagonist (IL-1Ra). Our IL-1Ra treatment does not restore ER α mRNA levels (Fig. 3A), but does attenuate HS-5-induced repression of ER α protein accumulation (Fig. 3B). Thus, other factors in HS-5 CM may regulate ER α mRNA levels and IL-1 may also regulate ER α protein stability. IL-1Ra does, however, restore both PR mRNA and protein in HS-5 CM-treated cells (Fig. 3A & B), which may be due to IL-1Ra-restored ER α protein that can subsequently transactivate PR expression. Finally, while IL-1Ra attenuates HS-5-induced LC3-II accumulation in both MCF7 and T47D cells (Fig. 3B), only T47D cells show IL-1Ra attenuation of HS-5-induced p62 mRNA expression and protein accumulation. Hence, MCF7 and T47D have differential sensitivity to the HS-5 CM milieu and additional HS-5 paracrine factors likely regulate p62 levels. Taken together, our data suggests that IL-1 signaling is sufficient, but not strictly necessary, to mediate HS-5 BMSC paracrine repression of hormone receptors and induction of p62 and autophagy.

3.5 IL-1 α and IL-1 β are sufficient to repress nuclear receptors and induce p62 and autophagy in nuclear hormone receptor positive BCa cell lines

IL-1 α and IL-1 β are the major IL-1 family members and signal through the IL-1R1 receptor¹⁶. IL-1 α and IL-1 β show similar biological activity, but do have some distinct functions¹⁶. Therefore, to determine if each cytokine can individually repress hormone receptors or induce p62 or autophagy in BCa cells, we treated MCF7 and T47D BCa cell lines with IL-1 α and IL-1 β . Both IL-1 α and IL-1 β can repress ER α and PR mRNA and protein, induce p62 mRNA and protein, and induce LC3-I and/or LC3-II accumulation in MCF7 and T47D cells, while ER α ⁻/PR⁻ BT549 and MDA-MB-231 BCa cells lines show only a modest or no response to IL-1 α or IL-1 β (Fig. 4A & B). To determine if BT549 and MDA-MB-231 cells can respond to IL-1 and to ensure IL-1 treatment efficacy, we assayed the induction of the IL-1 target gene, mitochondrial *Superoxide Dismutase 2* (*SOD2*). IL-1 α

and IL-1 β induce SOD2 mRNA and protein in both hormone receptor negative and positive BCa cell lines (Fig. 4A & B), indicating that ER α ⁻/PR⁻ BCa cells are indeed responsive to IL-1 signaling, while IL-1-mediated regulation of p62 and autophagy is specific to ER α ⁺/PR⁺ BCa cells. It is also possible that the high basal levels of p62 and LC3 in ER α ⁻/PR⁻ BCa cell lines masks any IL-1 effect on p62 induction or autophagy flux. Finally, p62 and LC3-II levels increase in the presence of CQ, indicating that autophagy is functional in the presence of IL-1 in ER α ⁺/PR⁺ MCF7 and T47D cell lines and ER α ⁻/PR⁻ BT549 and MDA-MB-231 cell lines (Fig. 4C, Fig. 6C, data not shown). Taken together, IL-1 α and IL-1 β are sufficient to regulate hormone receptor accumulation, p62 accumulation, and autophagy induction in ER α ⁺/PR⁺ BCa cells.

3.6 p62 and LC3 interact in hormone receptor negative BCa cell lines and in response to IL-1 in hormone receptor positive BCa cell lines

p62 interacts with LC3-II to sequester protein and organelles into the autophagosome for degradation³². Given that hormone receptor negative BCa cell lines have high basal p62 and LC3 (Fig. 1) and IL-1 induces p62 and LC3-II accumulation in hormone receptor positive BCa cell lines (Fig. 4), we performed immunoprecipitation to determine if p62 and LC3 are interacting in these contexts. p62 and LC3 form a complex in ER α ⁻/PR⁻ BT459 and MDA-MB-231 BCa cell lines under basal growth conditions (Fig. 5A), while IL-1 promotes p62-LC3 interaction in HS-5 CM-treated (Fig. 5B) and IL-1-treated (Fig. 5C) ER α ⁺/PR⁺ MCF7 and T47D BCa cell lines.

We also co-immunostained IL-1-treated cells for p62 and LC3, and in support of our immunoprecipitation results, we see that p62 and LC3 co-localize as distinct puncta in MCF7 cells (Fig. 6A) and p62-LC3 colocalization is enriched in IL-1-treated MCF7 cell populations (Fig. 6B & C). Unlike for MCF7 cells, we did not detect p62-LC3 puncta fluorescence in T47D cells above background signal, likely because T47D cells accumulate less total LC3 than MCF7 cells (Fig. 1B) and MCF7 cells show a more robust response to IL-1 for p62 and LC3 induction (Fig. 4A & B). Nevertheless, MCF7 co-immunostaining suggests that IL-1 promotes p62-LC3 interaction.

Given the hormone receptor-stratified similarities between BCa and PCa cell lines, we also investigated p62-LC3 interaction under basal growth conditions in the AR⁻ PCa cell lines, PC3 and DU145, and in response to IL-1 β treatment in AR⁺ PCa cell lines, LNCaP and C4-2. As observed for ER α ⁻/PR⁻ BT459 and MDA-MB-231 BCa cell lines, p62 and LC3 interact under basal growth conditions in AR⁻ PC3 cells (Fig. 5A). DU145 cells do not perform canonical autophagy and, therefore, do not form LC3-II⁴⁷; therefore, as expected, we do not detect an interaction between p62 and LC3 in DU145 cells (Fig. 5A). As observed for ER α ⁺/PR⁺ MCF7 and T47D BCa cell lines, p62 and LC3 interact in response to IL-1 β in AR⁺ LNCaP and C4-2 cells (Fig. 5C). Of note, because our IL-1 treatment does not appear to induce autophagy flux in AR⁺ PCa cell lines (data not shown), as a positive control we also treated the cells with CQ to prevent basal p62 and LC3 turnover and maintain p62 and LC3 interaction.

Taken together, our data suggest an autophagy-dependent function for p62 in ER α ⁻/PR⁻ BCa cell lines that is induced by IL-1 in ER α ⁺/PR⁺ BCa cell lines, and this function is likely

conserved in PCa cells. Studies are underway to definitively determine the mechanistic function(s) of p62 in hormone receptor negative versus positive BCa and PCa cell lines and in response to IL-1.

4. DISCUSSION

ER α ⁻ BCa tumors, such as triple negative tumors, are innately resistant to ER α -targeting endocrine therapy and a subpopulation of endocrine resistant ER α ⁺ BCa patients have or evolve tumors with low or no ER α accumulation^{1,4}. Undoubtedly, tumor cells with low or no ER α elicit compensatory survival and growth pathways to compensate for ER α loss and to evade endocrine therapy, but the underlying molecular survival mechanisms are still being explored.

The Kabos lab showed that HS-5 BMSCs repress *ER α* expression and, when co-cultured with MCF7 cells, reduce tumor sensitivity to tamoxifen *in vivo*, possibly through compensatory EGFR or HER2 signaling⁴⁸. We show that IL-1 signaling mediates HS-5 BMSC repression of ER α and PR levels concomitant with p62 upregulation and autophagy induction in ER α ⁺/PR⁺ BCa cell lines (Fig. 2 & 3); and we contend that IL-1-induced p62 upregulation and autophagy induction also contribute to BCa cell survival, growth, and endocrine resistance when hormone receptors are repressed. In support of this notion, knockout or silencing of p62 or autophagy have been shown to prevent breast cancer initiation, progression, or metastasis in mouse models^{37,38,49} and *in vitro* studies demonstrate that autophagy is cytoprotective against endocrine therapy⁵⁰⁻⁵².

IL-1 inversely correlates with ER α in BCa patient tumors¹³⁻¹⁵, is elevated in BCa tumor tissue and patient serum, and correlates with relapse and poor prognosis¹⁷⁻²⁰. However, it will be important to determine if IL-1 levels can be used to identify patients that will and/or have acquired endocrine resistance due to low or no ER α accumulation. These patients could benefit from IL-1Ra adjuvant therapy to (re)sensitize their tumors to endocrine therapy. Furthermore, while p62 overexpression³⁴⁻³⁸ or elevated autophagy^{39,40} are known to be associated with poor prognosis in BCa, a correlation between IL-1, p62, and autophagy in BCa endocrine resistance has yet to be determined. If such a correlation exists, these patients would also benefit from p62- and autophagy-targeting therapies.

Based on our data showing that ER α ⁻/PR⁻ BCa cell lines are insensitive to IL-1-induced p62 accumulation or autophagy flux (Fig. 4), their high basal levels (Fig. 1) suggest that ER α ⁻/PR⁻ BCa cells have evolved a requirement for p62 and autophagy that could be therapeutically exploited. Indeed, p62³⁴ and autophagy⁵³ are elevated in triple negative BCa patients and autophagy is cytoprotective for triple negative BCa cells⁵³⁻⁵⁵.

Finally, IL-1 induces p62-LC3 interaction (Fig. 5 & 6), but the autophagy-dependent p62 function in BCa progression or endocrine resistance is unknown. As such, diverse roles for p62 in BCa initiation and progression are constantly being discovered. For example, p62 interaction with LC3 and KEAP1 may support BCa mammosphere formation through NRF2 signaling⁵⁶, p62 interaction with VANGL2 may promote BCa tumor growth through non-canonical WNT signaling⁵⁷, and p62 interaction with VIMENTIN may mediate BCa cell

metastasis³⁸. Thus, given the multifunctional nature of p62, it is likely that IL-1 promotes both autophagy-dependent and –independent p62 functions in BCa cells. Experiments are underway to delineate IL-1-induced p62 functions in BCa that may contribute to endocrine resistance.

5. CONCLUSION

Our current and previous^{28,43,44} published reports suggest that p62 and autophagy regulation and function are conserved and stratified by receptor status in BCa and PCa cells. Given the similarities in BCa and PCa etiology, our previous and current data reveals an important, seemingly conserved, role for IL-1 in treatment resistance and aggressive disease – the repression of hormone receptors concomitant with the upregulation of prosurvival proteins. IL-1, p62, and autophagy signaling could each be exploited to prevent and treat progressive disease for both BCa and PCa; and the FDA approved and experimental therapies in cancer clinical trials targeting IL-1⁵⁸, p62⁵⁹, and autophagy^{60–62} emphasize the importance of these pathways in driving cancer progression.

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REFERENCES

1. Dixon JM. Endocrine Resistance in Breast Cancer. *New J Sci* [Internet] 2014;2014:1–27. Available from: <https://www.hindawi.com/archive/2014/390618/>
2. Daniel AR, Hagan CR, Lange CA. Progesterone receptor action: defining a role in breast cancer. *Expert Rev Endocrinol Metab* [Internet] 2011;6:359–69. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3156468&tool=pmcentrez&rendertype=abstract> [PubMed: 21857868]
3. Barrios C, Forbes JF, Jonat W, Conte P, Gradishar W, Buzdar A, Gelmon K, Gnant M, Bonnetterre J, Toi M, Hudis C, Robertson JFR. The sequential use of endocrine treatment for advanced breast cancer: where are we? *Ann Oncol Off J Eur Soc Med Oncol* [Internet] 2012;23:1378–86. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22317766>
4. Murray JI, West NR, Murphy LC, Watson PH. Intratumoural inflammation and endocrine resistance in breast cancer. *Endocr Relat Cancer* [Internet] 2015;22:R51–67. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25404688> [PubMed: 25404688]
5. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* [Internet] 1999;17:1474–81. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10334533> [PubMed: 10334533]
6. McGuire WL. Steroid receptors in human breast cancer. *Cancer Res* [Internet] 1978;38:4289–91. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/698967> [PubMed: 698967]
7. Kuukasjärvi T, Kononen J, Helin H, Holli K, Isola J. Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *J Clin Oncol* [Internet] 1996;14:2584–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8823339> [PubMed: 8823339]

8. Prabhu JS, Korlimarla A, Desai K, Alexander A, Raghavan R, Anupama C, Dendukuri N, Manjunath S, Correa M, Raman N, Kalamdani A, Prasad M, et al. A Majority of Low (1–10%) ER Positive Breast Cancers Behave Like Hormone Receptor Negative Tumors. *J Cancer* [Internet] 2014;5:156–65. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24563670> [PubMed: 24563670]
9. Gloyeske NC, Dabbs DJ, Bhargava R. Low ER+ Breast Cancer: Is This a Distinct Group? *Am J Clin Pathol* [Internet] 2014;141:697–701. Available from: <https://academic.oup.com/ajcp/article-lookup/doi/10.1309/AJCP34CYSATWFDPO> [PubMed: 24713741]
10. Huang J, Woods P, Normolle D, Goff JP, Benos PV, Stehle CJ, Steinman RA. Downregulation of estrogen receptor and modulation of growth of breast cancer cell lines mediated by paracrine stromal cell signals. *Breast Cancer Res Treat* [Internet] 2017;161:229–43. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27853906> [PubMed: 27853906]
11. Danforth DN, Sgagias MK. Interleukin 1 alpha blocks estradiol-stimulated growth and down-regulates the estrogen receptor in MCF-7 breast cancer cells in vitro. *Cancer Res* [Internet] 1991;51:1488–93. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/1997187> [PubMed: 1997187]
12. Jiménez-Garduño AM, Mendoza-Rodríguez MG, Urrutia-Cabrera D, Domínguez-Robles MC, Pérez-Yépez EA, Ayala-Summano JT, Meza I. IL-1 β induced methylation of the estrogen receptor ER α gene correlates with EMT and chemoresistance in breast cancer cells. *Biochem Biophys Res Commun* [Internet] 2017;490:780–5. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0006291X17312482> [PubMed: 28645612]
13. Chavey C, Bibeau F, Gourgou-Bourgade S, Burlincho S, Boissière F, Laune D, Roques S, Lazennec G. Oestrogen receptor negative breast cancers exhibit high cytokine content. *Breast Cancer Res* 2007;9:R15–R15. [PubMed: 17261184]
14. Singer CF, Hudelist G, Gschwanter-Kaulich D, Fink-Retter a, Mueller R, Walter I, Czerwenka K, Kubista E. Interleukin-1 α protein secretion in breast cancer is associated with poor differentiation and estrogen receptor α negativity. *Int J Gynecol Cancer* [Internet] 2006;16 Suppl 2:556–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17010072>
15. Miller LJ, Kurtzman SH, Anderson K, Wang Y, Stankus M, Renna M, Lindquist R, Barrows G, Kreutzer DL. Interleukin-1 family expression in human breast cancer: interleukin-1 receptor antagonist. *Cancer Invest* [Internet] 2000;18:293–302. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10808364> [PubMed: 10808364]
16. Garlanda C, Dinarello CA, Mantovani A. The Interleukin-1 Family: Back to the Future. *Immunity* [Internet] 2013;39:1003–18. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S1074761313005153> [PubMed: 24332029]
17. Soria G, Ofri-Shahak M, Haas I, Yaal-Hahoshen N, Leider-Trejo L, Leibovich-Rivkin T, Weitzenfeld P, Meshel T, Shabtai E, Gutman M, Ben-Baruch A. Inflammatory mediators in breast cancer: coordinated expression of TNF α & IL-1 β with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. *BMC Cancer* [Internet] 2011;11:130. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21486440> [PubMed: 21486440]
18. Pantschenko A, Pushkar I, Anderson K, Wang Y, Miller L, Kurtzman S, Barrows G, Kreutzer D. The interleukin-1 family of cytokines and receptors in human breast cancer: Implications for tumor progression. *Int J Oncol* [Internet] 2003;23:269–84. Available from: <http://www.spandidos-publications.com/10.3892/ijo.23.2.269> [PubMed: 12851675]
19. Al-Hassan AA. Prognostic Value of Proinflammatory Cytokines in Breast Cancer. *J Biomol Res Ther* [Internet] 2013;01. Available from: <https://www.omicsonline.org/open-access/prognostic-value-of-proinflammatory-cytokines-in-breast-cancer-2167-7956.1000104.php?aid=10308>
20. Esquivel-Velázquez M, Ostoa-Saloma P, Palacios-Arreola MI, Nava-Castro KE, Castro JJ, Morales-Montor J. The Role of Cytokines in Breast Cancer Development and Progression. *J Interf Cytokine Res* [Internet] 2015;35:1–16. Available from: <http://online.liebertpub.com/doi/abs/10.1089/jir.2014.0026>
21. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J Transl Med* [Internet] 2006;4:48. Available from: <http://translational-medicine.biomedcentral.com/articles/10.1186/1479-5876-4-48> [PubMed: 17096856]

22. Holen I, Lefley D V, Francis SE, Rennicks S, Bradbury S, Coleman RE, Ottewell P. IL-1 drives breast cancer growth and bone metastasis *in vivo*. *Oncotarget* [Internet] 2016;7. Available from: <http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&nop=view&npath%5B%5D=12289> [PubMed: 26683705]
23. Chang MA, Patel V, Gwede M, Morgado M, Tomasevich K, Fong EL, Farach-Carson MC, Delk NA. IL-1B Induced p62/SQSTM1 and Represses Androgen Receptor Expression in Prostate Cancer Cells. *J Cell Biochem* 2014;115:2188–97. [PubMed: 25103771]
24. Pienta KJ, Bradley D. Mechanisms underlying the development of androgen-independent prostate cancer. *Clin Cancer Res* 2006;12:1665–71. [PubMed: 16551847]
25. Hoang DT, Iczkowski KA, Kilari D, See W, Nevalainen MT. Androgen receptor-dependent and -independent mechanisms driving prostate cancer progression: Opportunities for therapeutic targeting from multiple angles. *Oncotarget* [Internet] 2015;8:3724–45. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27741508> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5356914>
26. Liu Q, Russell MR, Shahriari K, Jernigan DL, Lioni MI, Garcia FU, Fatatis A. Interleukin-1 β promotes skeletal colonization and progression of metastatic prostate cancer cells with neuroendocrine features. *Cancer Res* [Internet] 2013;73:3297–305. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23536554> [PubMed: 23536554]
27. Staverosky JA, Zhu X, Ha S, Logan SK. Anti-androgen resistance in prostate cancer cells chronically induced by interleukin-1 β . *Am J Clin Exp Urol* [Internet] 2013;1:53–65. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25374900> [PubMed: 25374900]
28. Chang MA, Patel V, Gwede M, Morgado M, Tomasevich K, Fong ELL, Farach-Carson MCC, Delk NA. IL-1 β Induces p62/SQSTM1 and Represses Androgen Receptor Expression in Prostate Cancer Cells. *J Cell Biochem* [Internet] 2014;115:2188–97. Available from: <http://doi.wiley.com/10.1002/jcb.24897> [PubMed: 25103771]
29. Chang MA, Morgado M, Warren CR, Hinton C V., Farach-Carson MC, Delk NA. P62/SQSTM1 is required for cell survival of apoptosis-resistant bone metastatic prostate cancer cell lines. *Prostate* 2014;74:149–63. [PubMed: 24122957]
30. Nihira K, Miki Y, Ono K, Suzuki T, Sasano H. An inhibition of p62/SQSTM1 caused autophagic cell death of several human carcinoma cells. *Cancer Sci* 2014;105:568–75. [PubMed: 24618016]
31. Zhou J, Ren F, Shu G, Liu G, Liu D, Zhou J, Yuan L. Knockdown of p62/sequestosome 1 attenuates autophagy and inhibits colorectal cancer cell growth. *Mol Cell Biochem* 2014;385:95–102. [PubMed: 24065390]
32. Puissant A, Fenouille N, Auberger P. When autophagy meets cancer through p62/SQSTM1. *Am J Cancer Res* [Internet] 2012;2:397–413. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3410580&tool=pmcentrez&rendertype=abstract> [PubMed: 22860231]
33. Hu Y-L, Jahangiri A, Delay M, Aghi MK. Tumor cell autophagy as an adaptive response mediating resistance to treatments such as antiangiogenic therapy. *Cancer Res* [Internet] 2012;72:4294–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22915758> [PubMed: 22915758]
34. Luo R-Z, Uan Z, Xi S. Accumulation of p62 is associated with poor prognosis in patients with triple-negative breast cancer. 2013;883–8.
35. Rolland P, Madjd Z, Durrant L, Ellis IO, Layfield R, Spendlove I. The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. *Endocr Relat Cancer* 2007;14:73–80. [PubMed: 17395976]
36. Thompson HGR, Harris JW, Wold BJ, Lin F, Brody JP. p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells. *Oncogene* 2003;22:2322–33. [PubMed: 12700667]
37. Xu L-Z, Li S, Zhou W, Kang Z, Zhang Q, Kamran M, Xu J, Liang D, Wang C-L, Hou Z, Wan X, Wang H-J, et al. p62/SQSTM1 enhances breast cancer stem-like properties by stabilizing MYC mRNA. *Oncogene* [Internet] 2017;36:304–17. Available from: <http://www.nature.com/doifinder/10.1038/ncr.2016.202> [PubMed: 27345399]
38. Li S-S, Xu L-Z, Zhou W, Yao S, Wang C-L, Xia J-L, Wang H-F, Kamran M, Xue X-Y, Dong L, Wang J, Ding X-D, et al. p62/SQSTM1 interacts with vimentin to

enhance breast cancer metastasis. *Carcinogenesis* [Internet] 2017;38:1092–103. Available from: <http://academic.oup.com/carcin/article/38/11/1092/4139685/p62SQSTM1-interacts-with-vimentin-to-enhance> [PubMed: 28968743]

39. Sun WY, Kim HM, Koo JS. Expression of autophagy-related proteins in metastatic breast cancer of different site. *Int J Clin Exp Pathol* [Internet] 2016;9:7040–9. Available from: <http://www.ijcep.com/files/ijcep0022520.pdf>
40. Ueno T, Saji S, Sugimoto M, Masuda N, Kuroi K, Sato N, Takei H, Yamamoto Y, Ohno S, Yamashita H, Hisamatsu K, Aogi K, et al. Clinical significance of the expression of autophagy-associated marker, beclin 1, in breast cancer patients who received neoadjuvant endocrine therapy. *BMC Cancer* [Internet] 2016;16:230. Available from: <http://www.biomedcentral.com/1471-2407/16/230> [PubMed: 26984766]
41. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008;4:151–75. [PubMed: 18188003]
42. Multhoff G, Molls M, Radons J. Chronic inflammation in cancer development. *Front Immunol* 2012;2:1–17.
43. Chang MA, Morgado M, Warren CR, Hinton C V, Farach-Carson MC, Delk NA. p62/SQSTM1 is required for cell survival of apoptosis-resistant bone metastatic prostate cancer cell lines. *Prostate* [Internet] 2014;74:149–63. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24122957> [PubMed: 24122957]
44. Delk NA, Farach-Carson MC. Interleukin-6: A bone marrow stromal cell paracrine signal that induces neuroendocrine differentiation and modulates autophagy in bone metastatic PCa cells. *Autophagy* 2012;8:650–63. [PubMed: 22441019]
45. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood* 1995;85:997–1005. [PubMed: 7849321]
46. Thomas-Jardin SE, Kanchwala MS, Jacob J, Merchant S, Meade RK, Gahnmim NM, Nawas AF, Xing C, Delk NA. Identification of an IL-1-induced gene expression pattern in AR+ PCa cells that mimics the molecular phenotype of AR– PCa cells. *Prostate* [Internet] 2018;78:595–606. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29527701> [PubMed: 29527701]
47. Ouyang DY, Xu LH, He XH, Zhang YT, Zeng LH, Cai JY, Ren S. Autophagy is differentially induced in prostate cancer LNCaP, DU145 and PC-3 cells via distinct splicing profiles of ATG5. *Autophagy* 2013;9:20–32. [PubMed: 23075929]
48. Brechbuhl HM, Finlay-Schultz J, Yamamoto TM, Gillen AE, Cittelty DM, Tan A-C, Sams SB, Pillai MM, Elias AD, Robinson WA, Sartorius CA, Kabos P. Fibroblast Subtypes Regulate Responsiveness of Luminal Breast Cancer to Estrogen. *Clin Cancer Res* [Internet] 2017;23:1710–21. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27702820> [PubMed: 27702820]
49. 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. [PubMed: 21764854]
50. Yu X, Luo A, Liu Y, Wang S, Li Y, Shi W, Liu Z, Qu X. MiR-214 increases the sensitivity of breast cancer cells to tamoxifen and fulvestrant through inhibition of autophagy. *Mol Cancer* [Internet] 2015;14:208. Available from: <http://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-015-0480-4> [PubMed: 26666173]
51. Lui A, New J, Ogony J, Thomas S, Lewis-Wambi J. Everolimus downregulates estrogen receptor and induces autophagy in aromatase inhibitor-resistant breast cancer cells. *BMC Cancer* [Internet] 2016;16:487. Available from: <http://bmccancer.biomedcentral.com/articles/10.1186/s12885-016-2490-z> [PubMed: 27421652]
52. Samaddar JS, Gaddy VT, Duplantier J, Thandavan SP, Shah M, Smith MJ, Browning D, Rawson J, Smith SB, Barrett JT, Schoenlein P V. A role for macroautophagy in protection against 4-hydroxytamoxifen-induced cell death and the development of antiestrogen resistance. *Mol Cancer Ther* [Internet] 2008;7:2977–87. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18790778> [PubMed: 18790778]
53. Lefort S, Joffre C, Kieffer Y, Givel A-M, Bourachot B, Zago G, Bieche I, Dubois T, Meseure D, Vincent-Salomon A, Camonis J, Mechta-Grigoriou F. Inhibition of autophagy as a new

means of improving chemotherapy efficiency in high-LC3B triple-negative breast cancers. Autophagy [Internet] 2014;10:2122–42. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25427136> [PubMed: 25427136]

54. Liu Z, He K, Ma Q, Yu Q, Liu C, Ndege I, Wang X, Yu Z. Autophagy inhibitor facilitates gefitinib sensitivity in vitro and in vivo by activating mitochondrial apoptosis in triple negative breast cancer. PLoS One [Internet] 2017;12:e0177694. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28531218> [PubMed: 28531218]
55. Liang DH, Choi DS, Ensor JE, Kaiparettu BA, Bass BL, Chang JC. The autophagy inhibitor chloroquine targets cancer stem cells in triple negative breast cancer by inducing mitochondrial damage and impairing DNA break repair. Cancer Lett 2016;376:249–58. [PubMed: 27060208]
56. Ryoo I- G, Choi B- H, Kwak M- K. Activation of NRF2 by p62 and proteasome reduction in sphere-forming breast carcinoma cells. Oncotarget [Internet] 2015;6:8167–84. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4480743&tool=pmcentrez&rendertype=abstract> [PubMed: 25717032]
57. Puvirajesinghe TM, Bertucci F, Jain A, Scerbo P, Belotti E, Audebert S, Sebbagh M, Lopez M, Brech A, Finetti P, Charafe-Jauffret E, Chaffanet M, et al. Identification of p62/SQSTM1 as a component of non-canonical Wnt VANGl2–JNK signalling in breast cancer. Nat Commun [Internet] 2016;7:10318. Available from: <http://www.nature.com/doi/10.1038/ncomms10318> [PubMed: 26754771]
58. Lust JA, Lacy MQ, Zeldenrust SR, Dispenzieri A, Gertz MA, Witzig TE, Kumar S, Hayman SR, Russell SJ, Buadi FK, Geyer SM, Campbell ME, et al. Induction of a Chronic Disease State in Patients With Smoldering or Indolent Multiple Myeloma by Targeting Interleukin 1 β -Induced Interleukin 6 Production and the Myeloma Proliferative Component. Mayo Clin Proc 2009;84:114–22. [PubMed: 19181644]
59. Ponomarenko DM, Klimova ID, Chapygina YA, Dvornichenko VV., Zhukova NV, Orlova RV, Manikhas GM, Zyryanov AV, Burkhanova LA, Badrtdinova II, Oshchepkov BN, Filippova EV, et al. Safety and efficacy of p62 DNA vaccine ELENAGEN in a first-in-human trial in patients with advanced solid tumors. Oncotarget [Internet] 2015;8:53730–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28388548><http://www.oncotarget.com/abstract/16574>
60. Rosenfeld MR, Ye X, Supko JG, Desideri S, Grossman SA, Brem S, Mikkelsen T, Wang D, Chang YC, Hu J, McAfee Q, Fisher J, et al. A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. Autophagy 2014;10:1359–68. [PubMed: 24991840]
61. Boone BA, Bahary N, Zureikat A, Moser AJ, Normolle D, Wu W-C, Singhi AD, Bao P, Bartlett D, Liotta L, Espina V, Loughran P, et al. Safety and Biologic Response of Pre-operative Autophagy Inhibition with Gemcitabine in Patients with Pancreatic Adenocarcinoma. Ann Surg Oncol 2015;22:4402–10. [PubMed: 25905586]
62. Rangwala R, Leone R, Chang YC, Fecher LA, Schuchter LM, Kramer A, Tan K-S, Heitjan DF, Rodgers G, Gallagher M, Piao S, Troxel AB, et al. Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. Autophagy 2014;10:1369–79. [PubMed: 24991839]

Figure 1. Basal $ER\alpha$, PR, p62 and LC3 levels in $ER\alpha^{-}/PR^{-}$ versus $ER\alpha^{+}/PR^{+}$ BCa cell lines. (A) RT-QPCR and (B) western blot analysis were performed for the $ER\alpha^{-}/PR^{-}$ BCa cell lines, BT549 and MDA-MB-231, and the $ER\alpha^{+}/PR^{+}$ BCa cell lines, MCF7 and T47D, grown in BCa CM for 3 days. BT549 and MDA-MB-231 cells have little or no detectable $ER\alpha$ and PR mRNA (A) or protein (B) relative to MCF7 and T47D cells. BT549 and MDA-MB-231 cells have higher basal p62 or LC3-II mRNA (A) and/or protein (B) compared to MCF7 and T47D cells. Error bars = $\pm$ STDEV of 3 biological replicates; mRNA levels were normalized to T47D for *ERα* and *p62* QPCR and normalized to MCF7 for *PR* QPCR. For western blot densitometry, MCF7 was set at 1. β -actin is the western blot loading control.

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Figure 3. IL-1Ra attenuates HS-5 BMSC modulation of ERα, PR, p62 and LC3 in ERα⁺/PR⁺ BCa cells.

Hormone receptor positive BCa cell lines were pretreated with 200 ng/mL Interleukin-1 receptor antagonist (IL-1Ra) for 1 day followed by treatment with HS-5 CM ± 200 ng/mL IL-1Ra for an additional 3 days. DMEM (control CM) served as control for HS-5 CM. Treated cells were analyzed for mRNA (A) or protein accumulation (B) by QPCR or western blot, respectively. IL-1Ra can attenuate HS-5 CM modulation of ERα, PR, p62 or LC3 mRNA (A) and/or protein (B) in MCF7 and T47D cells. The attenuation of HS-5 CM-induced SOD2 protein is a control for IL-1Ra efficacy. Error bars = ± STDEV of 4 biological replicates (Bio Rep); p-value = * 0.05, ** 0.005, *** 0.0005. NS = not statistically significant. mRNA fold change is normalized within each cell line to its control and protein fold change is normalized to the lowest protein/β-actin ratio for a given protein. β-actin is western blot loading control.

Figure 4. IL-1 α or IL-1 β modulate ER, PR, p62 and LC3 in ER α ⁺/PR⁺ BCa cells. ER α ⁺/PR⁺ BCa cell lines, MCF7 and T47D, and ER α ⁻/PR⁻ BCa cell lines, BT549 and MDA-MB-231, were treated for 3 days with vehicle control (0.1% BSA in PBS), IL-1 α (25ng/mL) or IL-1 β (25ng/mL) in the absence (A, B) or presence (C) of 20 μ M chloroquine (CQ). Cells were analyzed for mRNA by RT-QPCR (A) or protein by western blot (B, C). IL-1 α or IL-1 β downregulate ER α and PR mRNA (A) and protein (B) and induce p62 and LC3 mRNA (A) and/or protein (B) and in MCF7 and T47D cells, but does not modulate p62 or LC3 levels in BT549 or MDA-MB-231 cells. IL-1 α - or IL-1 β -induced SOD2 mRNA and protein accumulation serve as positive controls for treatment efficacy. (C) CQ blocks autophagy-mediated degradation of p62 and LC3-II in IL-1 α - or IL-1 β -treated cells. Error bars = $\pm$ STDEV of 4 biological replicates (Bio Rep); p-value = * 0.05, ** 0.005, *** 0.0005. mRNA fold change is normalized within each cell line to its control and protein fold change is normalized to the lowest protein/ β -actin ratio for a given protein. β -actin is the western blot loading control.

Figure 5. p62-LC3 interaction in hormone receptor negative or positive BCa cell lines.

LC3 was immunoprecipitated from untreated ER α ⁻/PR⁻ BCa cell lines, BT549 and MDA-MB-231, AR⁻ PCa cell lines, PC3 and DU145 (A), treated ER α ⁺/PR⁺ BCa cell lines, MCF7 and T47D (B, C), or treated AR⁺ PCa cell lines, LNCaP and C4-2 (C). Western blot analysis was performed on the immunoprecipitation elution to detect LC3 and p62. (A) BT549, MDA-MB-231, and PC3 cell lines show LC3-p62 interaction under basal growth conditions. LC3-p62 interaction is not detected in DU145 cells, which do not accumulate LC3-II. HS-5 CM (B) or IL-1 α or IL-1 β (C) induce LC3-p62 interaction in MCF7, T47D LNCaP, and C4-2 cells. (B) IL-1RA attenuates HS-5 CM induced LC3-p62 interaction in MCF7 and T47D cells. IgG serves as control for LC3 antibody. * = Non-specific band.

Figure 6. p62 and LC3 co-localization in control and IL-1 treated MCF7 cells.

Vehicle control or IL-1 (25 ng/mL) treated MCF7 cells grown in the absence or presence of CQ (20 μ M) for 3 days were co-immunostained for p62 (FITC; green) and LC3 (Texas Red, red). Nuclei are stained with DAPI (blue). (A) p62 and LC3 puncta co-localize under each growth condition. (B, C) Images show merged p62 (FITC), LC3 (Texas Red), and DAPI fluorescence. IL-1 increases p62-LC3 co-localization in the total cell population (images and graph); and in the presence of CQ, 100% of the cell population accumulates co-localized p62 and LC3, indicating that autophagy flux is functional under both basal and IL-1 treatment conditions. Cells were imaged at 100x (A; scale bar = 10 μ M) and 20x (C;

scale bar = 100 μ M) magnification. Error bars = $\pm$ STDEV of 3 biological replicates; 12,000 < n < 22,000 total cells counted; p-value = * 0.05, ** 0.005, *** 0.0005.