INTERFERON REGULATORY FACTOR-1 (IRF1) SIGNALING REGULATES APOPTOSIS AND AUTOPHAGY TO DETERMINE ENDOCRINE RESPONSIVENESS AND CELL FATE IN HUMAN BREAST CANCER

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By

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ABSTRACT

Interferon regulatory factor-1 (IRF1) is a nuclear transcription factor and pivotal regulator of cell fate in cancer cells. While IRF1 is known to possess tumor suppressive activities, the role of IRF1 in mediating apoptosis and autophagy in breast cancer is largely unknown. Here, we show that IRF1 inhibits antiapoptotic B-cell lymphoma 2 (BCL2) protein expression, whose overexpression often contributes to antiestrogen resistance. We proposed that directly targeting the antiapoptotic BCL2 members with GX15-070 (GX; obatoclax), a BH3-mimetic currently in clinical development, would be an attractive strategy to overcome antiestrogen resistance in some breast cancers. Inhibition of BCL2 activity, through treatment with GX, was more effective in reducing the cell density of antiestrogen resistant breast cancer cells versus sensitive cells, and this increased sensitivity correlated with an accumulation of autophagic vacuoles. While GX treatment promoted autophagic vacuole and autolysosome formation, p62/SQSTM1, a marker for autophagic degradation, levels accumulated. Moreover, GX exposure resulted in a reduction of cathepsin D and L protein expression that would otherwise digest autolysosome cargo. Taken together, these data highlight a new mechanism of GX-induced cell death that could be used to design novel therapeutic interventions for antiestrogen resistant breast cancer.
In addition to establishing a functional role for BCL2 members downstream of IRF1, we also provided evidence that this IRF1 mediates antiestrogen sensitivity in breast cancer through autophagy signaling. We demonstrated that IRF1 and autophagy-related protein 7 (ATG7) expression were inversely correlated \textit{in vivo} and in human breast cancer tissues. We then identified both ATG7 and the functionally related protein, beclin-1 (BECN1), as negative regulators of IRF1. Conversely, loss of IRF1 promoted antiestrogen resistance by increasing prosurvival autophagy, whereas combined ATG7 and IRF1 knockdown restored sensitivity to the antiestrogen fulvestrant (Faslodex, ICI 182780, ICI). Thus, inhibition of autophagy proteins, ATG7 and BECN1, enables IRF1-dependent and -independent signaling pathways and provides a novel therapeutic approach for inhibiting breast cancer cell proliferation.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>CHIP</td>
<td>C-terminus of Hsc70-interacting protein</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>dnIRF1</td>
<td>Dominant negative IRF1</td>
</tr>
<tr>
<td>E2</td>
<td>Beta-estradiol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>FAS</td>
<td>Faslodex</td>
</tr>
<tr>
<td>ICI</td>
<td>ICI 182,780</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane protein</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>M6P</td>
<td>Mannose-6-phosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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</table>
1.1. Breast Cancer Incidence and Risk Factors

Breast cancer is a major public health problem in the United States and many other parts of the world. Nearly one in every seven women will develop breast cancer in her lifetime, making it the second most common cancer among American women aside from non-melanoma skin cancer (1). For 2013, it is estimated that over 230,000 cases of breast cancer will be newly diagnosed and over 40,000 people will die from breast cancer in the United States alone (1). While breast cancer incidence rates from 2005-2009 have remained relatively stable, death rates associated with breast cancer have continued to decline (1). This trend largely reflects improvements in early detection and/or treatment. As these statistics have been disseminated nationwide, so has a search for clues to understand the etiology of this disease. Despite the discovery of key breast cancer susceptibility genes (i.e. BRCA1 and BRCA2) and other genetic risk factors, epidemiological data suggests that breast cancer risk may be more closely associated with environmental and lifestyle factors.

A woman’s lifestyle and health habits have been shown to be major contributors to breast cancer risk. One lifestyle factor influencing a women’s risk of developing breast cancer is her reproductive history. Women with no children or who had their first child after age 30 have an increased risk for breast cancer (2). This may be due to a decreased number of menstrual cycles a woman experiences during her lifetime. Today, women are having fewer children and having them later in life; thus, it is likely that the current trend may have a significant influence on breast cancer risk. Studies also suggest that women who
breastfeed may have a slightly lower risk of developing breast cancer. While this relationship has been difficult to study, breastfeeding for 1.5 – 2 years results in the greatest reduction in breast cancer risk (3). Similarly to pregnancy, the explanation for this effect may be due to a reduction in a woman’s total number of menstrual cycles.

Being overweight or obese after menopause is another risk factor for developing breast cancer. While obesity is commonly associated with diabetes and cardiovascular disease, weight gain is now being recognized as an established risk factor for breast cancer and other cancer types. Adiposity has been shown to influence breast cancer risk through the production of estrogen by adipose tissue. Up until menopause, estrogen is primarily synthesized by the ovaries; but once menopause occurs, most of a woman’s estrogen comes from peripheral tissue, such as adipose tissue (4, 5). Obesity has also been shown to influence breast cancer risk by increasing the expression of aromatase, an enzyme responsible for estrogen biosynthesis, and low-grade inflammation (6, 7). With obesity reaching epidemic proportions, compounds are now being developed to target inflammatory and metabolic pathways for the treatment of breast cancer (4). One possible therapeutic regimen worth pursuing is increased physical activity. Numerous studies suggest that exercise can inhibit inflammation and reduce breast cancer risk/recurrence (8, 9), offering a beneficial alternative to standard therapy.

Personal behaviors, such as alcohol consumption and what a woman chooses to eat, also influence breast cancer risk. Compared with non-drinkers, women who drink 2-3 alcoholic drinks a day increase their breast cancer risk by 32% (10). The biological
mechanism explaining this effect is thought to be due to an increase in sex hormone concentrations following excessive alcohol intake (11). Many studies have also investigated whether diet effects breast cancer risk; however, the results have been conflicting. While some studies have indicated that diet may play a role, others found no influence on breast cancer risk. Many studies have tried to link dietary fat to breast cancer risk (12, 13), but such studies are often complicated by other factors such as activity level and genetic factors. One example of a dietary component that may influence breast cancer risk is phytoestrogen consumption. Phytoestrogens are naturally occurring, biologically active compounds that modulate estrogen receptor (ER) activity and alter the transcription of growth promoting genes (14). Isoflavones, a class of phytoestrogens, are found in foods containing soy and have been shown to reduce breast cancer risk depending on timing and length of exposure (15, 16). Asian women, who consume large amounts of soy, experience a three-fold lower risk of breast cancer compared with women in the United States (17). Similar contradictory trends are reported for chemicals found in the environment. Some substances found in cosmetics, personal care products, pesticides (such as DDE), and polychlorinated biphenyls (PCBs) have estrogen-like properties, and in theory could affect breast cancer risk (18-20). At this time however, research does not show a clear link between breast cancer risk and exposure to these substances.

There are also breast cancer risk factors that a woman cannot change. Simply being a woman and aging significantly increases your risk of getting breast cancer. Moreover, about 5% to 10% of breast cancer cases are hereditary, indicating that a gene defect was
inherited from a parent (21). The most common causes of hereditary breast cancer result from mutations in the \textit{BRCA1} and \textit{BRCA2} genes (22). Both of these genes prevent breast cancer by preserving intact chromosome structure (23). If a mutated copy of \textit{BRCA1} or \textit{BRCA2} is inherited from either parent, an individual may have up to an 80% risk of developing breast cancer during their lifetime (22). Women who carry these mutations are also at increased risk for developing other cancers, particularly ovarian cancer. Variations in the \textit{ATM}, \textit{TP53}, \textit{CHEK2}, \textit{CDH1}, \textit{PTEN}, and \textit{STK11} genes also increase the risk of developing breast cancer (24-26). These mutations are much more rare and do not increase the risk of breast cancer as much as do the BRCA genes.

Breast cancer incidence and mortality rates also vary considerably among racial and ethnic groups. Overall, white women have a slightly higher risk for developing breast cancer as compared with African American women, Hispanic women, and other ethnic groups (1). However, African American women have a 16% higher death rate from breast cancer compared with white women (1). Furthermore, the proportion of women diagnosed with advanced disease is higher amongst African American, Hispanic, and American Indian natives when compared with Caucasian and Asian women (27). Factors known to contribute to racial disparities include differences in exposure to underlying risk factors, access to high quality healthcare, and timely diagnosis and treatment (27). The higher breast cancer incidence in white women compared with other ethnic groups is thought to reflect a combination of factors affecting diagnosis (more prevalent mammography in white women) and underlying disease conditions (later age at first birth and increased use of hormone replacement therapy among white women) (28).
In addition to family history and ethnicity, several other unchangeable risk factors influence a woman’s risk for developing breast cancer. Women with dense breasts (as measured by mammography) have a higher risk of developing breast cancer than women with less dense breasts (29). Breasts are considered dense when they have more glandular and fibrous tissue and less fatty tissue. Studies suggest that women with dense breast tissue in more than 60-75% of the breast are at a five times greater risk for developing breast cancer than those with no densities (29). Another interesting concept suggests that breast cancer risk might be pre-determined in utero (30). Alterations during fetal development, caused by either maternal diet or exposure to environmental hazards, can modify the epigenome and be maintained during the life of the child. These epigenetic changes may then alter one’s susceptibility to factors that can initiate breast cancer (30). Additional studies must be done to determine the effect of epigenetic modifications on breast cancer risk.

1.2. Endocrine Therapies for Breast Cancer

Over the past decade, many advances have been made in the treatment and prevention of breast cancer. Depending on the type of breast cancer and how far it has spread, treatment options may include surgery, chemotherapy, hormonal therapy, biologic therapy, and radiation. For some patients, participating in a clinical trial might be the best course of action because individuals have the chance to try new treatments and possibly benefit from them. Other options include breast cancer prevention measures, such as modifying one’s diet and lifestyle or eliminating certain risk factors (i.e., undergoing a preventive
bilateral mastectomy). Nonetheless, most people will get more than one type of treatment during the course of their disease. Of the patients entering the clinic for treatment, nearly 70% of them will have breast tumors that express the estrogen receptor-α (ERα) protein (31). ERα is considered a predictive factor for endocrine therapy responsiveness; thus, most patients will be treated with an antiestrogenic agent.

17β-estradiol (E2) is a naturally occurring estrogen that exerts its activity by binding to two estrogen receptors, ERα and ERβ. E2 has been shown to play significant roles in sexual development, reproduction, and other physiological processes in tissues throughout the body (32). Both subtypes of ERs are normally present in the mammary gland; however, the role of ERβ is less clear (31). Transcripts of the gene encoding the progesterone receptor (PgR) is ER-related and PgR expression is often interpreted as a marker for ER function. Tumors that express both ER and PgR are considered likely to be fully endocrine responsive. Furthermore, high expression of ERβ in breast tumors expressing the ERα and PgR has been associated with a favorable outcome (33). While ERα and ERβ share the majority of estrogen receptor elements (EREs), they differentially associate with transcription factors and coactivators/corepressors, thus leading to different patterns of gene transcription activation (31). Generally speaking, ERα seems to have a predominant role in cell proliferation, while ERβ is thought to have antiproliferative activity and oppose the actions of ERα in reproductive tissue (34). The predominant role of E2 and ERs in breast cancer has led to the development of a class of drugs that interfere with estrogen action termed endocrine therapy.
During estrogen synthesis, the enzyme aromatase is responsible for converting androgens into estrogens (35). As described previously, estrogen production ceases after menopause but other tissues continue to produce estrogen through the action of aromatase. When aromatase is blocked, estrogen levels in post-menopausal women can drop significantly, leading to growth arrest and/or apoptosis in hormone-responsive breast cancer cells. Thus, a major class of drugs used to treat breast cancer in post-menopausal are aromatase inhibitors (AIs). Randomized trials in the metastatic setting have shown greater efficacy of third generation AIs over the ER antagonist, Tamoxifen (TAM) (31). AIs, including anastrozole, exemestane and letrozole, have all demonstrated improved activity compared with TAM for the treatment of post-menopausal patients with hormone receptor positive breast cancer (36). A reason for explaining this superiority includes reduced dimerization and activation of ER following aromatase inhibition, which leads to greater abrogation of tumor growth (31). Despite AI efficacy, the response rate and duration of response is only slightly higher than TAM and resistance often occurs (31).

1.3. Antiestrogens

Since most breast cancers are fueled by estrogen, anti-estrogenic compounds agents are often used to treat the disease. Antiestrogen (hormonal) therapy works to control ER+ breast cancer primarily by blocking the effect of estrogen in breast tissue. There are two classes of antiestrogens currently used in the clinical setting: selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs) (31, 37). Tamoxifen (TAM) was the first SERM used in clinical practice for the treatment of early and advanced breast cancer (31). Since its development in the 1970’s, TAM has
continued to be used effectively to inhibit breast cancer growth in the clinic. TAM is a non-steroidal antiestrogen that exhibits mixed tissue dependent agonist/antagonist activity (38). TAM acts as an antagonist of the ER in breast tissue but behaves as an agonist in other tissues including the endometrium and bone (5). In the adjuvant setting, TAM significantly reduces the risk of recurrence and death in pre- and post-menopausal women with ER+ disease (39).

Five years of TAM is considered the standard duration for treatment in the adjuvant setting. Trials have also shown that TAM given in addition to chemotherapy improved survival for patients with early breast cancer (40). Recent reports suggest that the AI, anastrozole, significantly improves disease-free survival over TAM but shows no significant overall mortality benefit when compared with TAM. These results suggest that anastrozole should be the preferred treatment for post-menopausal women with localized ER+ breast cancer (41). Newer assays, such as the 21-gene recurrence score assay (Oncotype DX) are being developed to identify which patients will benefit from therapy and also assess the likelihood that a woman’s breast cancer will return (42).

While TAM significantly reduces invasive breast cancer in middle age and older women, it also has several adverse side effects. Among the most severe, TAM has been shown to increase the risk of developing endometrial cancer in some women (43). Furthermore, TAM is associated with reduced cognition and increased triglyceride concentration in the blood (44, 45). It is also thought that patients with variant forms of the CYP2D6 gene may not receive the full benefit of TAM because of too slow metabolism of TAM to its
active metabolite 4-hydroxytamoxifen (46, 47). However, TAM has been shown to protect against bone loss unlike the AI, anastrozole, which potentiates bone loss (5, 48).

An understanding of the structure-function relationships of TAM has led to the development of several TAM derivatives. TAM has been chemically modified either by altering the side chains to produce new TAM analogues such as toremifene, idoxifene, droloxifene, lasofoxifene and TAT-59, or by modifying the nonsteroidal triphenylethylene ring structure to produce a nonsteroidal fixed ring structure such as raloxifene and arzoxifene (37). All of these derivatives have been classified as SERMs because they exhibit mixed tissue dependent agonist/antagonist activity (37). Studies conducted to evaluate the effectiveness TAM analogues have shown that none are superior to TAM in the treatment and prevention of breast cancer. The direct comparison of TAM to raloxifene in the prevention trial P-2 (STAR) showed that both drugs were equally effective in reducing the onset of invasive and non-invasive breast cancer in post-menopausal women (49). However, after 7-years follow-up, raloxifene caused less toxicity, including fewer thromboembolic events and lower endometrial cancer rates than TAM (50).

Due to the partial agonist activity of SERMs, research has continued to focus on new compounds blocking the ER without agonist effects. The pure antiestrogen, Fulvestrant (Faslodex; ICI 182,780; ICI), is an estrogen receptor antagonist with no agonist effects. It works as a selective estrogen receptor down-regulator (SERD). Preclinical studies have shown that ICI inhibits TAM-resistant and TAM-sensitive human breast cancer cell lines
Furthermore, ICI has been shown to inhibit ER+ MCF-7 breast cancer cells to a greater extent than TAM but has no activity in the ER- cell line, MDA-MB-231 (52, 53). Clinically, ICI is recommended to postmenopausal women with ER+ metastatic breast cancer whose disease has progressed following antiestrogen therapy and is administered as a monthly intramuscular injection (54, 55). ICI is as effective as the AI, anastrozole, in post-menopausal women with advanced breast cancer whose tumors have progressed despite treatment with endocrine therapy (56, 57).

Studies evaluating the efficacy and tolerability of ICI versus TAM have shown that the two drugs have similar efficacy and are both well tolerated in patients with ER+ breast cancer (58). ICI, however, has demonstrated reduced cross-resistance with other endocrine treatments (55). Studies also demonstrate that ICI has no effect on the endometrium and does not lead to an increased risk of endometrial cancer following long-term exposure as seen with TAM (59). These factors, in addition to the route of administration and safety profile of ICI, may lead to improved patient compliance and better patient outcomes. While ICI is a useful treatment option for hormone-sensitive advanced breast cancer, the designation of ICI as a “pure” antiestrogen may be erroneous. ICI has been shown to exhibit estrogenic properties in some models, likely due to its ability to activate the transcriptional regulatory activities of ERβ (60, 61).

1.4. Mechanisms of Antiestrogen Action

As described previously, antiestrogens are divided into two major categories based on their mechanism of action. While both SERMs and SERDs are competitive inhibitors of
the binding of E2 to the ER, other differences are evident. Figure 1 below illustrates how antiestrogens can exploit the points of weakness during estrogen signaling. Estrogen normally diffuses through the plasma membrane where it binds to the ER (62). Upon ER binding, heat shock proteins dissociate and a change in conformation and dimerization occurs. The complex then interacts with estrogen response elements (ERE)s located in the promoter region of estrogen activated genes (63). TAM and its structural derivatives, however, compete with E2 for ER binding. The association of TAM with ER results in a receptor complex that is incompletely converted to the fully activated form. As a result of the altered receptor complex, ER gene activation is repressed (Figure 1.1). The SERD, ICI, can act similarly to TAM by displacing estrogen from the ER. However, once ICI binds to the ER, it forms an inactive complex that can bind EREs (64). ICI has also been shown to prevent ER dimerization, induce ER degradation through ubiquitination, and disrupt ER nucleocytoplasmic shuttling (65-67).

While TAM itself has relatively little affinity for its target protein, it is metabolized in the liver by cytochrome P450 isoforms CYP2D6 and CYP3A4 and converted into active metabolites, 4-hydroxytamoxifen (4-OHT) and N-desmethyl-4-hydroxytamoxifen. These metabolites have 30-100 times more affinity for the ER than TAM itself and compete with estrogen in the body for ER binding (68). In breast tissue, 4-OHT can act as an ER antagonist and inhibit the transcription of ER responsive genes. However, TAM metabolites can act as agonists in tissues such as uterus, liver, and bone, which can potentiate the risk of developing other types of cancer (69). Thus, the ability of TAM to induce antagonist versus agonist activity is tissue dependent (5).
Figure 1.1. Molecular model of antiestrogen action. Different antiestrogens act at several points of weakness in the ER-signal transduction pathway in the breast cell (adapted from MacGregor, *Pharm Rev*, 1998, ref. 59).

Once TAM is bound to the ER, it prevents coactivator binding but can also promote corepressor binding to the ER. This indicates that the activity resulting from ER/TAM binding is sensitive to the surrounding coactivators/corepressors in that tissue (70). Some examples of coactivator/corepressor proteins that bind to the ER/TAM complex include AIB1, NCoR, and SMRT (71, 72). TAM function is also regulated by variables such as growth factor signaling. High levels of ErbB2/HER2 have been shown in TAM-resistant
tumors; thus, TAM-induced prodeath signaling needs to overcome the prosurvival signaling from ErbB2 in order to be effective (72, 73). Expression of PAX2 is also required for the full anticancer effect of TAM. In the presence of high PAX2, the TAM/ER complex is able to suppress the pro-proliferative effects of ErbB2 (73).

1.5. Cell Death and Antiestrogens
While the primary function of antiestrogens is to prevent estrogen from binding to the ER and inhibit the activation of estrogen-responsive genes, both ER- and non-ER-mediated cell death mechanisms exist. The ER-mediated antiestrogenic mechanisms of TAM have been attributed to both cell cycle arrest and apoptosis (74-76). TAM induces apoptosis \textit{in vitro} (77), \textit{in vivo} (78), and in the clinical setting (76). Studies performed \textit{in vitro} also show that TAM decreases antiapoptotic B-cell lymphoma 2 (BCL2) family member expression (76). In breast cancer patients receiving three months of TAM, clinical response was associated with both increased apoptosis and decreased cell proliferation as measured by BCL2 (apoptosis) and Ki-S1 (mitosis) (76, 79). Antiestrogens also inhibit cell cycle progression by blocking the cyclin-CDK complex, cyclin D1-CDK4, and increasing expression of the CDK inhibitors, p27KIP1 and p21WAF1/CIP1 (80, 81).

Various signaling pathways also underlie antiestrogen-associated cell death that are not mediated by the ER (82). For example, TAM modulates protein kinase C (PKC) activity by inducing its translocation to the mitochondria. PKC then stimulates cytochrome \( c \) release and triggers apoptosis (83). TAM can also modulate the activity of the calcium binding protein, calmodulin, transforming growth factor-\( \beta \) (TGF-\( \beta \)), and the proto-
oncogene c-myc (82). Elevated caspase activity and reduced mitochondrial potential result from TAM exposure, suggesting that TAM-induced apoptosis results from changes in the mitochondria (84, 85). While several of these signaling pathways conclude in apoptosis, continuing research demonstrates that TAM-induced cell death involves other pathways including endoplasmic reticulum stress, autophagy, and oxidative stress (82, 86-88).

Recent reports suggest that TAM and its metabolite, 4-OHT, induce cytotoxic effects that are associated with autophagic induction and K-ras degradation (89). Autophagy is a lysosomal pathway that involves the formation of a double membrane around organelles known as autophagosomes. Autophagosomes fuse with lysosomes, resulting in the degradation of damaged or defective proteins/organelles via lysosomal hydrolases. If an insult is severe, autophagy becomes “pro-death” and is often referred to as programmed cell death-2 (90). Another study reports that dying MCF-7 cells following TAM and ICI exposure showed increased cytosolic autophagosome formation (91). However, more recent findings demonstrate that MCF-7 cells surviving TAM treatment exhibit increased autophagosome formation. This group hypothesized that autophagy-mediated cell death is dependent on the number of autophagosomes in each cell, resulting in a threshold limit (92). TAM may also induce the unfolded protein response (UPR), a stress signaling pathway that is cytoprotective if the stressor is mild, but pro-apoptotic if the stress is severe (93). These non-ER-mediated pathways have prompted the initiation of clinical trials using TAM to treat ER-negative malignancies.
The pathology of breast cancer is also associated with alterations in the body’s immune machinery. Interestingly, various antiestrogenic drugs have been demonstrated to affect the immune system. Depending on the menopausal status of a woman, TAM can either reduce natural killer (NK) cell activity or increase the number of NK cells (94). TAM can also increase the number of leukocytes including CD4+ and CD8+ lymphocytes in breast cancer patients following radiotherapy. While TAM can stimulate leukocyte activity, TAM decreases granulocyte ingestion, suggesting an inhibitory effect of TAM on phagocytic functions (95). ICI has been shown to inhibit dendritic cell differentiation, reduce the T cell differentiation marker CD154, and block the anti-inflammatory effect of glucocorticoids (96-98). Thus, enhancing our understanding of the effects of antiestrogenic drugs on immune function may bring us closer to developing new cancer therapeutics and overcoming drug resistance.

1.6. Antiestrogen Resistance

While antiestrogens are key modalities in the treatment of ER+ breast cancer, resistance to endocrine therapies remains a significant clinical problem. Two main categories of antiestrogen resistance have been described: de novo and acquired. Patients with tumors that respond and then recur in 0-3 years likely exhibit de novo resistance conferred by the biology of the pre-existing tumor cell population(s) at time of diagnosis. For an acquired resistant phenotype to occur, tumors must dynamically remodel their cell populations through various pharmacologic, immunological, and molecular events. Endocrine therapy will induce selective pressure and lead to the formation of new, drug-resistant populations (99). This is seen in patients with initially responsive tumors that eventually
recur following antiestrogen therapy (5, 100). The most common acquired resistance mechanism appears to be antiestrogen unresponsiveness, whereas the major *de novo* resistance phenotype is the absence of both ER and PgR (99). Furthermore, many ER+ tumors that fail to respond to TAM lack intracellular binding sites that prevent TAM from ever reaching the ER (5).

Even in breast tumors with varied receptor phenotypes, we fail to predict antiestrogen responsiveness. Approximately 75% of ER+/PgR+, 33% of ER+/PgR-, and 45% of ER-/PgR+ cases of metastatic breast cancers respond to TAM (5). Thus, it remains impossible to predict whether an individual patient will receive benefit from treatment and the magnitude or duration of any benefit. In addition to receptor phenotypes, several pharmacological phenotypes of endocrine resistance have been identified *in vitro* and *in vivo* (5). These phenotypes include: (i) estrogen-independent [breast cancers that become resistant to an AE but are still sensitive to AIs and *vice versa* (101)]; (ii) estrogen-inhibited [estrogen inhibits tumor growth after TAM withdrawal (102)]; (iii) TAM-stimulated [breast cancer cells that are unresponsive to TAM but sensitive to ICI (103)]; (iv) TAM and ICI crossresistant (104).

Whether the continued expression of ER is required for antiestrogen resistant tumor growth or survival is still controversial. Studies report that antiestrogenic agents inhibit ER expression in 25% of tumors (105). In a MCF-7 based model of antiestrogen resistance, microarray analysis confirmed that TAM resistant cells retain ER expression and a transcriptional response to E2 but are no longer growth stimulated by E2 (106).
Furthermore, only 25% of E2 regulated genes were maintained in TAM resistant cells compared with their MCF-7 parental cells (107). Some TAM resistant tumors are also responsive to AIs, suggesting these tumors retain some estrogen responsiveness (108). Thus, loss of ER expression does not seem to be a major mechanism driving acquired antiestrogen resistance.

Antiestrogen resistance is a complex phenotype with modifications in both genomic and non-genomic activities (5). Systems biology approaches are currently being used to understand the molecular changes that drive an acquired antiestrogen resistant phenotype. Previous approaches to determining the cause(s) of endocrine resistance have focused primarily on single gene/signaling pathways. However, research suggests that gene networks integrate components from multiple pathways and redundant signaling mechanisms (109). Microarray technologies have been vital in investigating the global expression changes within specific cellular and/or drug treatment contexts. Analyses of ER+ breast cancer cells exposed to E2 implicate an upregulation of growth factors, proliferation and cell-cycle related genes, with a concomitant downregulation of transcriptional repressors, pro-apoptotic, and antiproliferative genes (110, 111). We also identified key features of hormone resistant signaling using gene expression microarray experiments in antiestrogen sensitive (LCC1) versus resistant (LCC9) MCF-7 cells. Our network comprises genes from cell cycle functions, apoptosis, UPR, autophagy, all of which contribute to effective execution of the cell fate decision (90, 112).

We have identified that expression of antiapoptotic BCL2 family members drives
antiestrogen resistance in human breast cancer cells (113, 114). Antiestrogen resistant MCF7/LCC9 cells have greater BCL2 and BCL-W expression compared with sensitive MCF7/LCC1 cells (113). Furthermore, the small molecule BCL2 inhibitor, HA 14-1, significantly inhibits cell growth in LCC9 cells compared with LCC1 cells, suggesting antiestrogen resistant cells are more dependent on BCL2 for their survival (115). Reducing expression of antiapoptotic BCL2 members and survivin, with a concomitant increase in proapoptotic BCL2 protein expression, leads to an alteration in mitochondrial membrane permeability, release of cytochrome c, and ultimately apoptosis executed by selected caspases (116).

Understanding the role of autophagy in antiestrogen resistance is an area under active investigation. Upregulation of autophagy has been shown to protect cancer cells from various therapies, including radiotherapy and TAM, and promote cancer therapeutic drug resistance (90). Preclinical studies using chemical inhibitors of autophagy have validated the role of autophagy in stress and therapeutic resensitization of cancer cells. While chemical inhibitors often have off-target effects, siRNA targeting specific autophagy proteins may be beneficial in preclinical studies. Inhibiting autophagosome formation with 3-methyladenine (3-MA) or beclin-1 siRNA can significantly enhance antiestrogen-induced cell death in MCF-7 cells (92). Studies inhibiting autophagy with combined knockdown of Atg5, Atg7, and beclin-1 in TAM-sensitive and –resistant cell lines report that concurrent knockdown of autophagy with TAM treatment results in increased apoptotic cell death and overall reduced cell viability (117). Moreover, we show that 3-
MA and beclin-1 shRNA can restore antiestrogen sensitivity in the ICI-resistant, TAM crossresistant MCF7/LCC9 breast cancer cells (113).

As antiestrogen resistance signaling networks are being delineated, new mechanisms controlling gene expression, and the complexity of this regulation, have recently been discovered. Estrogen regulated gene expression has been shown to be altered by E2-regulated microRNAs (118, 119). Using microRNA specific microarrays, 97 TAM regulated microRNAs were found as differentially expressed in antiestrogen resistant LY2 cells compared with MCF-7 control cells. Putative target genes of these microRNAs include ERα, BCL2, and ERBB3 (120). Studies have also investigated how small nucleotide polymorphisms (SNPs) contribute to TAM responsiveness. Analysis of genome-wide association studies identified 54 signature genes containing SNPs whose differential expression was able to distinguish between TAM treated and untreated patients (121, 122). These data suggest that the recent advances in technology could enhance our understanding of endocrine signaling in breast cancer.

1.7. Interferon Regulatory Factor-1 Mediates Antiestrogen Sensitivity

Dr. Clarke’s laboratory has established interferon regulatory factor-1 (IRF1) as a key regulatory node in the gene network controlling endocrine responsiveness and breast cancer cell fate. The chromosomal region of IRF1 is located at 5q31.1 and is commonly deleted in human breast tumors (123-125). IRF1 was initially discovered because it could transcriptionally activate type I interferons. It is well established that interferons can sensitize breast cancer cells to TAM (126, 127). More recently, IRF1 was identified as a
nuclear transcription factor that is activated by many immune effector molecules including the type I and type II (IFN-γ) interferons, tumor necrosis factor-α (TNF-α), retinoic acid, interleukin-1 (IL-1), IL-6, and in response to viral infection (128). Upon activation, IRF1 alters the transcriptional activity of genes involved in immunomodulation, antiviral responses, and tumor suppression (128, 129). The ability of IRF1 to induce apoptosis has contributed to IRF1 often being described as a tumor suppressor gene (130).

Following these initial observations, Dr. Clarke’s laboratory confirmed the functional involvement of IRF1 in breast cancer using a dominant negative approach (dnIRF1). Whereas IRF1 reduces cell proliferation in vitro and tumorigenesis in vivo, dnIRF1 increases cell proliferation and tumor incidence through inhibition of caspases 3/7 and 8 (131). Gene expression microarrays between antiestrogen-responsive (MCF7/LCC1) and -resistant (MCF7/LCC9) human breast cancer cell lines report that IRF1 is downregulated in resistant cells. Furthermore, IRF1 signals to apoptosis and is a key mediator of cell death in response to the antiestrogens, TAM and ICI (116, 132, 133). These findings suggest that loss of IRF1 may contribute to endocrine resistance through its ability to induce cell death (134). Therefore, we aimed to examine the role of IRF1 and its integrated signaling through BCL2 family members within a broader signaling network to regulate cell fate.

1.8. Hypothesis and Aims

The following hypothesis provided the basis for which the research presented in this dissertation was based.
We hypothesize that IRF1 regulates ER-positive breast cancer cell fate primarily by coordinating/integrating the balance of prodeath and prosurvival BCL2 family member activities, and those of other downstream effectors.

This hypothesis was tested according to two specific aims:

**Aim 1**: We will determine the functional role of antiapoptotic BCL2 members in the modulation of AE-induced apoptosis and autophagy downstream of IRF1.

**Aim 2**: We will determine how IRF1 regulates apoptotic and/or autophagy signaling using *in vitro, in vivo*, and human breast cancer models.

Experiments designed to address these aims are presented here within.
CHAPTER 2: EFFECT OF THE BCL2 INHIBITOR, GX15-070, ON APOPTOSIS AND AUTOPHAGY IN BREAST CANCER CELL LINES


With permission from Molecular Cancer Therapeutics

2.1. Introduction

2.1.1. Antiestrogen resistance

Approximately two-thirds of newly diagnosed invasive breast tumors express the ERα protein (99) and most will be treated with an endocrine therapy such as an antiestrogen or aromatase inhibitor. Antiestrogens can inhibit ER function and/or expression, blocking the ER-regulated signaling that induces breast cancer cell survival and proliferation (135). Tamoxifen (TAM), a selective estrogen receptor modulator (SERM), is the most frequently prescribed antiestrogen and is effective in increasing overall survival and reducing the incidence of ER+ disease in high-risk women (136). The selective estrogen receptor downregulator (SERD) fulvestrant (Faslodex, ICI 182780, ICI), does not exhibit the partial agonist activities of some SERMs and is often an effective treatment option following relapse on TAM or an AI (137, 138). Despite the widespread clinical efficacy
of antiestrogens in the treatment of ER+ breast cancers, approximately half of these women will exhibit *de novo* or acquired resistance to endocrine therapies (139).

### 2.1.2. BCL2 family members

Breast cancer cells can acquire resistance to antiestrogens through changes in molecular signaling that affect cell proliferation and death. The BCL2 family of proteins are central regulatory proteins comprising antiapoptotic members (BCL2, BCL-W, BCL-XL, MCL1, and A1) and proapoptotic members (BAX, BAK, and BH3-only proteins) (Figure 2.1) (140). BH3-only members interact with the core antiapoptotic BCL2 proteins to promote apoptosis by activating BAX and/or BAK, which leads to downstream cytochrome *c* release (141). BCL2 also interacts with beclin-1 (BECN1), a critical regulator of autophagy that facilitates autophagosome production (142). Prior to systemic therapy, BCL2 overexpression often correlates with ERα and is usually a favorable prognostic indicator (143). However, BCL2 levels fall in tumors that respond to 3 months of TAM therapy, whereas BCL2 expression is high in tumors that remain 3 months after TAM (76, 144). Thus, targeting the antiapoptotic BCL2 family members may be a useful strategy to overcome antiestrogen resistance in some breast cancers.
Figure 2.1. Core BCL2 family members and their conserved domains. The BCL2 family shares one or more of the four characteristic BCL2 homology (BH) domains (named BH1, BH2, BH3, and BH4). Adapted from Youle, *Nat Rev Mol Cell Biol*, 2008, ref. 138.

2.1.3. BH3 mimetics

Recently, a new series of small molecules that mimic BH3-only proteins has been generated, constituting a new class of potentially useful drugs. By mimicking BH3-only proteins (such as NOXA, PUMA, BID, BAD, BIM), antiapoptotic BCL2 members can be sequestered, thus allowing BAK and BAX to activate the intrinsic apoptotic pathway. Among the BH3-mimetics, GX15-070 (GX; obatoclax) is an indole bipyrrrole compound that can inhibit all known prosurvival BCL2 family members (145, 146). GX is currently under investigation in Phase II clinical trials for the treatment of leukemia, lymphoma, myelofibrosis, and mastocytosis (147, 148). Previous studies also report that GX
overcomes resistance to Lapatinib, a tyrosine kinase inhibitor often used in HER2 amplified breast cancer (149). While GX can induce mitochondrial apoptosis (150), the precise molecular mechanism(s) of cell death by GX is unclear. Several reports suggest that GX may induce autophagy and other forms of death as an alternate mechanism to caspase-dependent apoptosis (149, 151, 152).

2.1.4. Autophagy and lysosomal function

Autophagy enables cells to degrade and recycle proteins and organelles through a catabolic process occurring primarily in lysosomes. When a cell becomes stressed, autophagic ‘self-eating’ allows for the digestion of damaged or unnecessary subcellular material, which can be recycled to maintain homeostasis (90). However, if the stress cannot be resolved, autophagy persists at high levels and may become pro-death. While autophagy was initially described as a cell death mechanism (programmed cell death-2), it remains unclear whether autophagy is an independent cell death pathway, acts as a backup system when apoptosis is impaired, or is used as a survival mechanism under cellular stress and persists even when an irreversible cell death process has been activated. Since the discovery of the autophagy (Atg) proteins, this process has received great attention and has been implicated in a variety of pathological conditions. While autophagy is a complex, multistep process, recent advances into this phenomenon have emphasized the role of lysosomes in autophagy regulation, which can ultimately determine cancer cell survival.

Rapidly dividing cancer cells are dependent on autophagy and effective lysosomal
function. However, malignant transformation or toxic insults to the cellular milieu can lead to a series of alterations in lysosomal function. These changes include increased lysosomal biogenesis and proteolytic activity, altered trafficking of lysosomes, and increased sensitivity to the lysosomal cell death pathway. Accordingly, expression and function of lysosomal hydrolases are increased and facilitate the promotion of invasive growth and drug resistance that can lead to a poor prognosis (153). Furthermore, lysosomal proteases can be released into the cytosol and trigger apoptosis or lysosomal cell death, providing a rationale for cancer therapies destabilizing the lysosomal membrane (154).

2.1.5. Normal function of lysosomes

Lysosomes are acidic membrane-enclosed organelles that function to maintain cellular homeostasis by controlling the turnover of macromolecules. Containing about 50 different hydrolytic enzymes, lysosomes are responsible for breaking down all types of biological polymers delivered to them by endocytosis, autophagy, and phagocytosis (155). Lysosomal enzymes exert their maximal activity at low pH (pH 4-5), which is maintained by a vacuolar ATPase that pumps protons from the cytosol to the lysosomal lumen (156). To maintain membrane integrity, heavily glycosylated membrane proteins, lysosomal-associated membrane protein-1 and -2 (LAMP1 and LAMP2), which reside in the lysosome membrane, resist digestion from acidic hydrolases (157). When the lysosomal material is degraded, the cargo is either recycled back to the cytosol or directed to the extracellular space through secretory vesicles (exocytosis) (158). During
autophagy, lysosomes facilitate energy retrieval by degrading damaged or unnecessary proteins or entire organelles, leading to cell death or cell survival (90).

Lysosomes are morphologically heterogeneous and originate from a complex pathway that requires the biosynthetic and endocytic pathways (158). This process involves the inward budding of the limiting membrane, which results in numerous internal vesicles. These vesicles function as sorting stations for proteins and lipids that are either recycled back to the plasma membrane or are intended for lysosomal degradation (158). At this point, the early endosomes mature into late endosomes and finally to lysosomes. The entire process of lysosomal biogenesis occurs within approximately 30 minutes and requires the continuous substitution of newly synthesized components to fuel this dynamic system.

2.1.6. Lysosomal hydrolases

During lysosomal biogenesis, lysosomes receive a number of essential hydrolases. These enzymes are synthesized in the rough endoplasmic reticulum and processed in the Golgi complex, where many of them are tagged with mannose-6-phosphate (M6P). The glycosylated enzymes are then targeted to early endosomes expressing the specific M6P receptor (159). Upon maturation, the hydrolases are released into late endosomes and continue into lysosomes, where the low pH is the optimal working condition for hydrolases. Acidification is also a necessary step in facilitating the dissociation of the M6P-receptor-ligand complexes and proteolytic processing that activates numerous hydrolases (159). Cathepsins are among the best-studied lysosomal hydrolases and are
classified into three subgroups according to their active site amino acid: 1) cysteine: B, C, H, F, K, L, O, S, V, W, Z; 2) aspartic: D and E; or 3) serine: G (160). Several members of the cathepsin family, specifically aspartic protease cathepsin D and cysteine cathepsins B and L, are overexpressed in several cancers and have been implicated in tumor spread, invasion, and metastasis (161, 162). Recently, these cathepsins have been shown to mediate lysosomal cell death pathways and are discussed further below.

2.1.7. Cathepsin D

Overexpression of cathepsin D in human cancer was first reported in the 1980s and more recent studies confirm these findings in most solid cancers (160, 161). Unlike cathepsins B and L, cathepsin D requires a more acidic pH to be proteolytically active (pH 4.5-5.0). Furthermore, no endogenous inhibitors of cathepsin D have been found in mammals (160). Studies conducted in ER+ breast cancer cell lines revealed that cathepsin D is tightly regulated by estrogen and some growth factors (i.e. IGF1, EGF), which have been shown to stimulate both its mRNA and protein expression (163, 164). In addition, cathepsin D plays an essential role in tumor progression by stimulating cancer cell proliferation, fibroblast outgrowth and angiogenesis, and has also been shown to inhibit tumor apoptosis. This protease is now being used as a prognostic factor for poor prognosis in breast cancer and correlates with an increased incidence of clinical metastasis. Interestingly, when cathepsin D is mutated in its catalytic site, it still induces cancer cells growth, suggesting an alternative mechanism for cathepsin D and its different peptide forms.
2.1.8. Cathepsins B and L

Cathepsins B and L are cysteine proteases that belong to the papain subfamily of cysteine proteases. Located predominantly in endolysosomal vesicles, both cathepsins B and L are constitutively expressed and participate in normal protein turnover (165). However, their increased expression has been widely reported in many cancer types, including cancer cells themselves, as well as tumor-associated fibroblasts and macrophages (158). In particular, cathepsin B expression tends to localize near the edge of tumor cells, consistent with the idea that it enhances metastasis and invasion. While increased cathepsin B protein expression correlates with higher mRNA levels of the \textit{CTSB} gene, studies also suggest that the oncogenes \textit{Ras}, \textit{Src}, or \textit{ERBB2} enhance the levels of \textit{CTSB}. Furthermore, non-invasive breast cancer cells overexpressing ErbB2 upregulate cathepsins B and L and become highly invasive in a three-dimensional invasion model. Cathepsin L has also been shown to increase tumor cell migration by reducing cell-cell adhesion and degrading components of the extracellular matrix (158).

In addition to the strong tumor promoting activities of cathepsins B and L, these cysteine cathepsins have been reported to destabilize the lysosomal membrane by cleaving LAMP1 and LAMP2, leading to decreased stress tolerability and sensitization to lysosome-targeting drugs (158). Oncogenic transformation has also been shown to result from cathepsin L activity in the nucleus. Using an isoform of cathepsin L that lacks the signal peptide directing it to mature lysosomes, cathepsin L migrates to the nucleus where it cleaves transcription factors (i.e. CDP/Cux) and alters cell cycle progression (158). While these studies confirm the roles of cathepsins B and L in tumor development, they
also demonstrate their overlapping roles and redundant activity in cancer. For instance, in the MMTV-PyMT mouse model of mammary carcinoma, tumor cells deficient in cathepsin B upregulate cathepsin Z on the cell surface. Furthermore, cathepsins B, L, and S must be genetically deleted to significantly increase apoptosis of RIP1-Tag2 tumors (165). Thus, the development of broad-spectrum cysteine cathepsin inhibitors as cancer therapeutics may be of significant potential value.

We examined the effects of GX15-070 and an antiestrogen using two separate models of antiestrogen resistance in breast cancer (104). Comparisons were made between MCF-7 (ER+, estrogen-dependent, antiestrogen-sensitive) and MCF7/RR [ER+, estrogen-independent, tamoxifen-resistant; ICI-sensitive, derived from MCF-7 cells selected against tamoxifen (166, 167)] cells and between estrogen-independent MCF7/LCC1 [ER+, antiestrogen-sensitive, derived by in vivo selection of MCF-7 cells (168)] and MCF7/LCC9 [ER+, estrogen-independent, ICI-resistant; tamoxifen crossresistant, derived from MCF7/LCC1 cells selected against ICI (104)] cells. In this report, we show that inhibiting antiapoptotic BCL2 family expression with GX15-070 induces cell death in antiestrogen-resistant breast cancer cell lines through the completion of apoptosis and a cathepsin-mediated inhibition of autophagy. These findings have important clinical implications and provide a mechanistic rationale for the use of GX15-070 in combination with an antiestrogen for the treatment of ER+ breast cancers.
2.2. Materials and Methods

2.2.1. Cell culture and reagents

MCF-7 human breast cancer cells were provided by Dr. Marvin Rich (Karmanos Cancer Institute, Detroit, MI); MCF7/RR, LCC1, and LCC9 cells were established as previously described (104, 166, 168). MCF7 and MCF7/RR cells were cultured in improved Minimal Essential Media (IMEM; Invitrogen) with phenol red and supplemented with 5% FBS. LCC1 and LCC9 cells were routinely grown in phenol red–free IMEM supplemented with 5% charcoal-stripped calf serum. Cells were authenticated by DNA fingerprinting and tested regularly for Mycoplasma infection. GX15-070 was purchased from Selleck Chemicals; ICI 182780 (Faslodex; fulvestrant) and Z-VAD-FMK from Tocris Bioscience; Bafilomycin A1 (BAF) from EMD Biosciences; 4-hydroxytamoxifen (tamoxifen), hydroxychloroquine (HCQ), BAPTA-AM, and 3-methyladenine (3-MA) were from Sigma-Aldrich. The cathepsin L (CTSL1; 1-napthalenesulfonyl-Ile-Trp-aldehyde) and D (Ac-Leu-Val-Phe-aldehyde) inhibitors were from Enzo Life Sciences and Bachem, respectively.

2.2.2. Cell proliferation

Cells were seeded at a density of 5 x 10^3 per well in 96-well plates and, 24 hours later, treated with the indicated concentration of GX15-070 (GX) or vehicle control. Cells were incubated with drug for 48 hours or 6 days, with media containing either drug or vehicle being replaced every 3 days. Following treatment, cells were stained with a crystal violet staining solution as previously described (131). Sodium citrate buffer was used to extract the dye, and absorbance was measured at 550 nM using a microplate reader (Bio-Rad).
Cell density was calculated from the crystal violet assay.

2.2.3. RNA interference

ATG7 and BECN1 siRNA were from Cell Signaling and Origene, respectively. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

2.2.4. Western blot analysis

Western blot analysis LCC1, LCC9, MCF7, and MCF7/RR cells were plated in 6-well dishes and the following day treated with 100 nM GX ± 100 nM ICI/TAM, 100 nM ICI/TAM or vehicle control. Lysates were harvested 48 hours later for protein analysis as previously described (136). Protein expression was measured by probing proteins with the following antibodies overnight at 4°C: ATG7, BECN1, CTSB, CTSD, LC3B, PARP (Cell Signaling); p62 (BD Transduction Labs); BCL2 (Enzo Life Sciences); and CTSL1 (eBioscience). To confirm equal loading of the gels, membranes were reprobed for β-actin (Santa Cruz Biotechnology).

2.2.5. Reverse transcription PCR

RNA was extracted using Trizol (Invitrogen). 2 µg RNA was used from each sample as a template for cDNA synthesis with the High Capacity RNA-to-cDNA kit (Invitrogen). PCR amplification was performed using the following primers (purchased from Integrated DNA Technologies): CTSB: 5′-GCCGCGAGCTCATGTCAGCTCTGGCGGCTCC-3 (forward) and 5′-
ATTATCCCGGGTTAGATCTTTTCCCAGTACTG-3’ (reverse), CTSD: 5’-

GTGCCCTGCCAGTCAGCGTCGTCAG-3’ (forward) and 5’-

CCTGCTCAGGTAGAAGGAGAAGATG-3’ (reverse), and CTSL1: 5’-

GACTCTGAGGAATCCTATCCA-3’ (forward) and 5’-

AAGGACTCATGACCTGCATCAA-3’ (reverse).

2.2.6. Apoptosis and autophagosome formation assays

Cells were plated in 6-well tissue culture plates one day before treatment with 100 nM GX ± 100 nM ICI/TAM, 100 nM ICI/TAM, or vehicle control. 48 hours post treatment, cells were harvested and stained as described in the Enzo Life Sciences Annexin V-FITC Apoptosis Detection Kit for flow cytometry. Accumulation of autophagic vesicles was measured using a modified monodansylcadaverine according to the manufacturer’s instructions (Cyto-ID Autophagy detection kit, Enzo). Stained cells were detected and appropriate signals measured by fluorescence-activated cell sorting (LCCC FACS Shared Resource).

2.2.7. Autophagosome Maturation

LCC9 cells (1 x 10^5) were seeded onto 18 x 18 mm glass coverslips and 24 hours later were transfected with LC3 tagged with a green fluorescent protein (GFP) and/or a p62 cDNA tagged with a red fluorescent protein (RFP). An mRFP-GFP tandem fluorescent-tagged LC3 vector (Addgene) was used to assess whether GX inhibited autophagic flux (169). The following day, cells were treated with 500 nM GX, 100 nM ICI, or 5 nM BAF in CCS-IMEM. The pH of autolysosomes was measured in LCC9 cells using the
LysoSensor Green dye (Invitrogen) as indicated by the supplier. 24 hours post treatment, cells were fixed, mounted on coverslips, and visualized as previously described (116).

2.2.8. Orthotopic xenografts in athymic nude mice

LCC1 or LCC9 cells were injected orthotopically into the mammary fat pads of five-week-old ovariectomized athymic nude mice as previously described (170). Mice were sacrificed after 9 weeks; tumors were removed at necropsy, fixed in neutral buffered formalin, and processed using routine histological methods.

2.2.9. Immunohistochemistry

Five-micron sections from LCC1 and LCC9 paraffin embedded tissues were stained with mouse anti-BCL2 (Dako, 1:150) antibody as previously described (170). A computer-assisted counting technique with a grid filter to select cells was used to quantify the immunohistochemical staining of BCL2 (171).

2.2.10. Electron microscopy

Following 24 hours of treatment with 500 nM GX or vehicle control, LCC9 cells were harvested and fixed in a glutaradehyde/paraformaldehyde solution. Embedding sectioning and staining were performed as previously described (172). After fixation, cells were double stained with uranyl acetate and lead citrate. Electron micrographs of ultrathin sections (90 nm) were viewed at a magnification of 10,000/15,000x with a JEOL JM1010 transmission electron microscope.
2.2.11. Cathepsin activity

Cathepsin activity was determined using the commercial assay provided by Biovision according to the manufacturer’s protocol. Cells were seeded in 10 cm\(^2\) dishes 24 hours prior to treatment with 100 nM GX ± 100 nM ICI, 100 nM ICI, or vehicle control. 48 hours post treatment, cathepsin activity was measured using 10 mM cathepsin B (Ac-RR-AFC) or L substrate (Ac-FR-AFC). A fluorometer (Tecan) was used to quantify the cleavage of synthetic substrate of cathepsins B and L. Cathepsin activity was expressed as relative fluorescence units (RFU) per microgram protein.

2.2.12. Isolation of cytosolic enriched fraction

LCC1 and LCC9 cells were seeded in 10 cm\(^2\) dishes 24 hours prior to treatment with 100 nM GX ± 100 nM ICI, 100 nM ICI, or vehicle control. 48 hours post treatment, cells were harvested with trypsin, washed, and resuspended in cytosol extraction buffer according to the Panomics Nuclear Extraction kit instructions.

2.2.13. Statistical analysis

The statistical significance of differences between two groups was analyzed by two-tailed Student’s \(t\) tests. For multiple group comparisons, Bonferroni's Multiple Comparison Test was applied following one-way ANOVA. Results were considered to be significantly different at \(P < 0.05\). Statistical analysis was carried out using the Prism version 5.0 software.
2.3. Results

2.3.1. Breast cancer models for antiestrogen resistance

A series of cell lines were derived from MCF-7 cells to investigate antiestrogen resistance in breast cancer. Two distinct models were developed through *in vitro* and *in vivo* selection: TAM-resistance (MCF7/RR) and ICI/TAM-cross-resistance (sensitive MCF7/LCC1 and resistant MCF7/LCC9). The TAM-resistance model, known as MCF7/RR cells, was generated by selecting MCF-7 cells against increasing concentrations of TAM (Figure 2.2). MCF7/RRs retain ER expression, grow independently of estrogen, are resistant to TAM but remain sensitive to ICI (166). The ICI/TAM-cross-resistance model includes LCC variants that were established from an estrogen-independent variant of MCF-7 cells (MCF7/MIII) initially selected for growth in ovariectomized nude mice (Figure 2.2). MCF7/LCC1 cells were selected from a subpopulation of MIII cells and are estrogen-independent, retain ER expression, and are sensitive to antiestrogens (51). The antiestrogen resistant MCF7/LCC9 cell line was established by selecting MCF7/LCC1 against increasing concentrations of ICI. Cross-resistance to TAM occurred early in the selection stage of MCF7/LCC9 cells (104). MCF7/LCC9 cells retain some ER expression, grow independently of estrogen, and are resistant to both ICI and TAM (104) (Figure 2.2).
Figure 2.2. Models of antiestrogen resistance. TAM-resistant breast cancer cells (MCF7/RR) were derived from MCF-7 cells. The ICI/TAM-cross-resistant MCF7/LCC9 cells were derived from MCF7/LCC1 cells (which are derivatives of MCF-7 cells).
2.3.2. *Endogenous BCL2 expression in breast cancer cell lines can be targeted with the BH3 mimetic, GX*

Endogenous BCL2 protein expression was measured in MCF7, MCF7/RR, LCC1, and LCC9 breast cancer cells (Figure 2.3A). Increased BCL2 expression was observed in estrogen-regulated MCF7 cells and estrogen-independent, antiestrogen-resistant LCC9 cells (Figure 2.3A). We also measured total BCL2 protein by immunohistochemistry in LCC1 and LCC9 mammary tumor xenografts (Figure 2.3B). Data from these studies revealed that LCC9 tumors had significantly higher BCL2 expression compared with LCC1 tumors (Figure 2.3C; \( P = 0.0005 \)). Given that GX is a BH3 mimic that inhibits all known antiapoptotic BCL2 members (structure shown in Figure 2.3D), we sought to determine the effect of GX on antiestrogen sensitive and resistant breast cancer cells.

2.3.3. *GX alone and in combination with an antiestrogen inhibits breast cancer cell density through apoptosis*

Increasing concentrations of GX (0, 0.1, 0.5, 1, and 10 µM) inhibited both antiestrogen sensitive (LCC1 and MCF7) and resistant (LCC9 and MCF7/RR) cell density after 48 hours (Figures 2.4A and B). When combined with the antiestrogen TAM over the course of 6 days, GX had an additive effect on inhibiting the relative cell density of MCF7 and MCF7/RR cells (Figure 2.4D), while no significant additive effect was observed in the LCC1 and LCC9 cells with GX+ICI (Figure 2.4C). We also measured Annexin V stained cells following GX exposure and observed that GX enhanced apoptosis consistent with its on-target effects (Figures 2.5A and B). These data suggested that GX enhanced apoptosis-mediated cell death in antiestrogen sensitive and resistant breast cancer cells.
Figure 2.3. BCL2 expression in breast cancer cell lines. A, MCF7, MCF7/RR, LCC1, and LCC9 cells were grown in 6-well plates under basal conditions for 48 hrs. Whole cell lysates were prepared and immunoblotted against BCL2; β-actin serves as the loading control. Densitometric analysis from ≥ 3 separate experiments. B and C, Immunohistochemical staining of LCC1 and LCC9 tumor xenografts was performed for BCL2 (B) and quantified using immunoscore (C) as described in Materials and Methods. ***P < 0.001 versus LCC1. D, chemical structure of GX15-070.
Figure 2.4. **GX and an antiestrogen inhibit breast cancer cell density.** A-B, LCC1, LCC9, MCF7, and MCF7/RR cells were seeded in 96-well tissue culture dishes 24 hours before treatment with GX at the indicated concentration. 48 hours post treatment, cells were stained with crystal violet and cell density was determined with a plate reader at wavelength 550 nm. C-D, LCC1, LCC9, MCF7, and MCF7/RR cells were plated in 96-well tissue culture plates and treated with the indicated drug(s) for 6 days (refed with medium containing vehicle or drug on day 3) prior to measuring cell proliferation as in A-B. Data are presented as relative cell density and represent the mean ± SEM for ≥ 3 independent experiments; *$P < 0.05$, ***$P < 0.001$ versus vehicle/control.
Figure 2.5. GX induces apoptosis in breast cancer cells. A and B, LCC1, LCC9, MCF7, and MCF7/RR cells were seeded in 6-well tissue culture plates 24 hours prior to treatment with vehicle control, 100 nM ICI/TAM, 100 nM GX, or ICI/TAM + GX. 48 hours post treatment, apoptosis was quantified using flow cytometry analysis of annexin V-FITC-stained cells.
2.3.4. **GX induces autophagosome formation**

While GX enhanced apoptosis, the pan caspase inhibitor Z-VAD-FMK failed to abrogate killing by GX ± an antiestrogen (Figure 2.6A). Furthermore, Z-VAD-FMK failed to reduce the number of apoptotic cells following GX exposure, suggesting that GX-mediated apoptosis occurred via a caspase independent mechanism (Figure 2.6B). Since cell viability was not affected by a pan caspase inhibitor and because others have linked autophagic cell death to GX, we determined whether GX either alone or in combination with an antiestrogen could induce autophagosome formation in breast cancer cells. We measured the initial phase of autophagy in response to GX ± antiestrogen treatment by selectively labeling autophagosomes with a modified monodansylcadaverine. GX alone and in combination with an antiestrogen significantly increased the number of autophagic vacuoles in antiestrogen sensitive and resistant cell lines (Figures 2.7A and B; \( P = 0.0001 \)).

2.3.5. **GX induces BECN1-dependent autophagosome formation, but autophagy is not required for GX toxicity**

We next explored the effect of GX on autophagy regulation by measuring the formation of LC3-II, which participates in elongation of the autophagosome membrane (90). In LCC9 cells, 48 hours of GX ± an antiestrogen significantly induced the formation of LC3-II (Figure 2.8A; \( P = 0.0005 \)). When autophagy was blocked by RNAi targeting BECN1, a critical regulator of autophagy (90), LC3-II cleavage was significantly reduced (Figure 2.8A; \( P = 0.0005 \)).
Figure 2.6. Caspase inhibition fails to abrogate GX-mediated apoptosis. A, LCC9 cells were treated with 50 µM of the pan caspase inhibitor Z-VAD-FMK (ZVAD) 30 min before treatment with 500 nM GX ± 100 nM ICI or 100 nM ICI for 48 hours. Cell proliferation was assessed using crystal violet staining. Data are presented as relative density and represent the mean ± SEM for ≥ 3 independent experiments; *P < 0.05. D, The effect of ZVAD on ICI and GX-induced PARP cleavage was assessed by SDS–PAGE. β-actin was used as a loading control. B, LCC9 cells were treated as in A. 48 hours post treatment, apoptosis was quantified using flow cytometry analysis of annexin V-FITC-stained cells. Data are presented as annexin V-FITC-stained cells relative to vehicle control and represent the mean ± SEM for ≥ 3 independent experiments.
Figure 2.7. GX induces autophagosome formation. A, LCC1, LCC9, MCF7, and MCF7/RR cells were seeded in 6-well tissue culture plates 24 hours before treatment with the indicated drug(s). 48 hours post treatment, autophagosome formation was detected as described in Materials and Methods. Data are presented as percentage of total cells positive for green fluorescence and represent the mean ± SEM for ≥ 3 independent experiments; *P < 0.05 versus control/vehicle experiment.
In contrast, the increase in autophagosome formation observed after GX exposure was not suppressed by the addition of RNAi targeting ATG7, a protein necessary for the formation of the preautophagosomal structure (90) (Figure 2.8B). To assess whether GX-induced autophagosome formation was calcium-dependent, LCC9 cells were transfected with a GFP tagged version of LC3, and the following day treated with 500 nM GX and 10 µM of the calcium chelator BAPTA-AM. Formation of LC3-GFP aggregates increased with GX and BAPTA + GX treatment, suggesting that GX-mediated LC3 punctae formation was largely independent of calcium signaling (Figure 2.8C).

To determine whether inhibition of autophagy could suppress GX-mediated cell death, we used several autophagy inhibitors in combination with GX ± ICI. LCC1 and LCC9 cells were treated with 500 nM GX ± 100 nM ICI, 100 nM ICI, or a combination of ICI, GX, and one of the autophagy inhibitors, 3-MA (5 mM; early stage inhibitor), HCQ (10 µM; late stage inhibitor), or Bafilomycin A1 (5 nM; BAF; late stage inhibitor). 3-MA slightly suppressed activity of the drug combination in LCC1 ($P = 0.04$) but not LCC9 cells (Figure 2.8D). Since 3-MA inhibits autophagy by blocking autophagosome formation via inhibition of type III Phosphatidylinositol 3-kinases (PI3K), these data suggested that GX-induced autophagy was independent of PI3K. BAF, which results in the accumulation of autophagosomes, promoted drug combination lethality in LCC1 and LCC9 cells (Figure 2.8D; $P = 0.0006$ and $P = 0.003$, respectively), while HCQ+GX+ICI reduced cell viability equally to GX+ICI (Figure 2.8D). Despite BECN1-dependent LC3-
Figure 2.8. GX-mediated autophagosome formation is dependent on BECN1. A-B, LCC9 cells were transfected with BECN1 siRNA (siBECN1), ATG7 siRNA (siATG7), or control siRNA (siCtrl) 24 hours before treatment with GX ± ICI, ICI, or vehicle control. 48 hours post treatment, cells were lysed and protein expression was measured by probing proteins with the indicated antibodies; β-actin served as the loading control. Representative images from 3 independent experiments. C, LCC9 cells transfected with LC3-GFP were treated with 500 nM GX ± 10 µM BAPTA-AM for 24 hours. Cells were fixed, permeabilized, stained with DAPI, and visualized by confocal microscopy. D, LCC1 and LCC9 cells were treated with GX, ICI, and the indicated autophagy inhibitor for 48 hours. Cell viability was assessed using a crystal violet assay. Data are presented as relative density and represent the mean ± SEM for ≥ 3 independent experiments, *P < 0.05, ***P < 0.001 between indicated experimental groups.
II processing following GX exposure, GX reduced equally the cell density of LCC9 cells transfected with control or BECN1 siRNA (Figure 2.9).

2.3.6. **GX induces autophagic vacuole and lysosome formation in antiestrogen resistant LCC9 breast cancer cells.**

To confirm our observation that GX induces autophagosome formation, we used electron microscopy to examine LCC9 cells treated with GX. Untreated control cells appeared to have a normal cytoplasm with few autophagic vacuoles (Figure 2.10; arrows). 24 hours of GX exposure resulted in an accumulation of lysosomes (marked “L”) and autophagic vacuoles (Figure 2.10B). Under higher magnification, autophagic vesicles were observed to have typical double-membrane boundaries containing electron dense material (Figure 2.10B). Mitochondria (marked “M”) were also enlarged in the GX treated cells (Figure 2.10B), suggesting that the cell was in the later stages of apoptosis (173). The electron micrographs revealed that GX increased lysosome and autophagic vacuole formation, leading us to examine proteins involved in autolysosome formation and degradation.

2.3.7. **GX treatment results in the accumulation of LC3-II and p62 proteins.**

We next investigated the role of GX on later events of the autophagy process. In mammalian cells, p62/sequestosome-1 (SQSTM1) is implicated in autophagic cargo recognition and is lost in the final stages of autophagy during autolysosome degradation (90). Using western blot analysis, we found an accumulation of p62 protein in antiestrogen sensitive and resistant breast cancer cells following treatment with GX (Figure 2.11A). By immunofluorescence, we measured LC3 punctae and p62 protein
Figure 2.9. Knockdown of BECN1 by siRNA does not prevent GX-mediated inhibition of cell density. LCC9 cells were reverse transfected with siRNA targeting BECN1 or scrambled siRNA, and 24 hours later, cells were exposed to 500 nM GX. 48 hours post treatment, cells were stained with crystal violet and cell density was determined with a plate reader at wavelength 550 nm. Data are presented as relative density and represent the mean ± SEM for ≥ 3 independent experiments.
Figure 2.10. GX induces autophagosome and lysosome formation in antiestrogen resistant LCC9 cells. A, electron microscopy images of vehicle (control) LCC9 treated breast cancer cells. The cytoplasm appears normal and contains few autophagic vacuoles. B, Following 24 hours of 500 nM GX exposure, the number of autophagic vacuoles in LCC9 cells increased. Swollen mitochondria (marked “M”) and an increase in the number of lysosomes (marked “L”) were specifically observed in cells treated with GX.
expression in LCC9 cells treated with 100 nM ICI, 500 nM GX, ICI+GX, or the late stage autophagy inhibitor, 5 nM BAF. DAPI staining shows the location of the nuclei; when merged with LC3-GFP and red fluorescent p62 cDNA, the levels of LC3 punctae and p62 were elevated in GX and BAF treated cells compared with both vehicle and ICI-treated cells (Figure 2.11B). Accumulation of LC3 punctae and p62 suggested that GX functioned as a downstream autophagy inhibitor.

2.3.8. **GX blocks autophagic degradation through attenuation of cathepsin activity.**

In the final stages of autophagy, mature autolysosomes are subjected to proteolytic degradation, leading to a reduced level of autophagic contents and substrates such as p62 (174). Therefore, we focused on the lysosomal hydrolases cathepsins B (CTSB), D (CTSD), and L (CTSL1), as potential targets of GX. 48 hours of GX exposure resulted in a 5-fold inhibition of CTSD and CTSL1 protein expression in LCC1, LCC9, and MCF7 cells (Figure 2.12 A-B). While GX reduced CTSB modestly in LCC1 cells, ICI alone stimulated expression of the three cathepsins in antiestrogen sensitive cells (Figure 2.12A). We further examined the mechanism of GX by measuring cathepsin mRNA expression in LCC1 and LCC9 cells. CTSB, CTSD, and CTSL1 mRNA levels remained unchanged after GX±ICI exposure (Figure 2.13).

We next assessed whether cathepsin activity was attenuated by GX by measuring activity of B and L in LCC1 and LCC9 cells exposed to 100 nM GX ± 100 nM ICI, 100 nM ICI, or vehicle control. GX ± ICI significantly inhibited CTSL1 activity in LCC1 and LCC9 cells after 48 hours (Figure 2.12C; \( P = 0.0001 \) and \( P = 0.0001 \), respectively). While GX
Figure 2.11. GX results in LC3 and p62 accumulation. A, LCC1, LCC9, MCF7, and MCF7/RR cells were seeded in 6-well plates 24 hours before treatment with the indicated drug(s). 48 hours post treatment, cells were lysed and the indicated proteins were detected by immunoblot; β-actin served as the loading control. Representative images from ≥ 3 independent experiments. B, LCC9 cells transfected with LC3-GFP and a p62 cDNA tagged with a red fluorescent protein were treated with 100 nM ICI, 500 nM GX, ICI + GX, 5 nM BAF, or vehicle control. 24 hours post treatment, the cells were fixed, permeabilized, stained for DAPI, and visualized by confocal microscopy.
Figure 2.12. GX inhibits autophagic degradation through attenuation of cathepsin activity. A-B, LCC1, LCC9, MCF7, and MCF7/RR cells were treated with the indicated amount of GX ± ICI/TAM, ICI/TAM, or vehicle control for 48 hours and expression of the indicated proteins were detected by immunoblot; β-actin served as the loading control; n=3 independent experiments. C-D, LCC1 and LCC9 cells were assayed for cathepsin L and B activity as described in Materials and Methods. Data represent the mean fluorescence unit relative to the vehicle control for ≥3 independent experiments, *P < 0.05, **P <0.01, ***P < 0.001 versus control/vehicle treatment.
Figure 2.13. Cathepsin mRNA expression is unaffected by GX exposure. A-B, To determine whether GX affects cathepsin mRNA expression, LCC1 and LCC9 cells were plated in 10 cm² dishes 1 day before treatment with vehicle control, 100 nM ICI, 100 nM GX, or ICI + GX. 48 hours post treatment, RNA was collected using Trizol. RNA was used from each sample as a template for cDNA synthesis, and qRT-PCR was performed using primers for CTSB, CTSD, and CTSL1. GADPH was used as an internal control. Data are presented as fold change to vehicle control treated cells and represent the mean ± SEM for ≥ 3 independent experiments.
slightly reduced CTSB activity in LCC1 cells ($P = 0.004$), CTSB activity was unaffected by GX in LCC9 cells (Figure 2.12D). Without a specific substrate, activity for CTSD could not be determined. To further elucidate how GX affects autophagosome maturation, we transfected LCC9 with an mRFP-GFP tandem tagged LC3. Typically, GFP-LC3 is degraded by hydrolases following autophagosome-lysosome fusion (169); however, GX induced an accumulation of mRFP and GFP, suggesting that a reduction in cathepsin protein expression inhibited the degradation of GFP (Figure 2.14).

Acidic pH is required for cathepsin activity (160), so we determined whether GX affected lysosomal pH. Merged fluorescence images of LCC9 cells stained with a red fluorescent dye to measure acidic organelles and a green dye to measure pH show as yellow and indicate an acidic environment (Figure 2.15). GX clearly induced an acidic pH suitable for cathepsin activity when compared with vehicle treated control cells (Figure 2.15). These results indicate that inhibition of cathepsin protein expression by GX inhibits autophagic flux, and these changes were not mediated by changes in lysosomal pH. Another possible explanation for the reduction in cathepsin levels was that GX increased the secretion of cathepsins into the cytosol. Thus, we measured cathepsin protein expression from LCC1 and LCC9 whole cell lysates and cytosolic fragments and found that GX had no effect on cathepsin secretion into the cytosol (Figures 2.16A and B).
Figure 2.14. GX inhibits the autophagic flux of LCC9 breast cancer cells. LCC9 cells were transfected with an mRFP-GFP tandem fluorescent-tagged LC3 1 day before treatment with 500 nM GX, 2 nM tunicamycin, or vehicle control. 24 hours post treatment, the cells were fixed, permeabilized, stained for DAPI, and visualized by confocal microscopy.
Figure 2.15. GX has no effect on lysosomal pH. LCC9 cells were incubated with CellLight LysoTracker Red (30 particles per cell) ± 500 nM GX for 24 hours. 15 min. prior to fixation, cells were incubated with a LysoSensor Green dye. Merged (yellow) images are indicative of an acidic pH.
Figure 2.16. GX has no effect on cathepsin secretion into the cytosol. A-B, LCC1 and LCC9 cells were seeded in 6-well plates 24 hours before treatment with the indicated drug(s). 48 hours post treatment, whole cell lysates and cytosolic fractions were collected as described in Materials and Methods. Western blot analysis was used to measure the expression of indicated proteins; β-actin served as the loading control. Representative images from 3 independent experiments.
2.3.9. *Inhibition of cathepsin L and D results in breast cancer cell death by blocking autophagosomal degradation.*

We next sought to determine whether known inhibitors of cathepsins L and D would also reduce breast cancer cell growth through the inhibition of autophagosomal lysis. Relative cell proliferation of LCC1 and LCC9 cells was inhibited by 20 µM of the CTSL1 (1-Napthalenesulfonyl-Ile-Trp-Aldehyde) and 50 µM of the CTSD inhibitor (Ac-Leu-Val-Phe-aldehyde) alone and in combination with 100 nM ICI after 48 and 24 hours, respectively (Figures 2.17A and B; $P = 0.0001$). Cell death was accompanied by an accumulation of p62 in antiestrogen sensitive and resistant cells, which is indicative of inhibition of autophagic flux (Figures 2.18A and B). CTSL1 and CTSD inhibition also increased autophagosome formation in both LCC1 and LCC9 cells (Figures 2.18C and D; $P = 0.0001$). Taken together, these data provide strong evidence that GX inhibits autophagic degradation through a CTSL1 and CTSD dependent mechanism, which causes cancer cells to lose their ability to recycle subcellular components through autophagy and so also to restore metabolic homeostasis.
Figure 2.17. Cathepsin L and D inhibition reduces breast cancer cell density. A-B, LCC1 and LCC9 cells were incubated with 20 µM of CTSL1 inhibitor (A) or 50 µM of CTSD inhibitor (B) ± 100 nM ICI, 100 nM ICI, or vehicle control for 48 or 24 hours, respectively. Cell proliferation was assessed as changes in cell density measured using crystal violet staining. Data are presented as relative density and represent the mean ± SEM for ≥ 3 independent experiments; ***P < 0.001 versus control/vehicle experiment.
Figure 2.18. Cathepsin L and D mediate autolysosomal membrane component degradation. A-B, Indicated proteins were detected by immunoblot; β-actin served as the loading control; representative images from ≥ 3 independent experiments. C-D, LCC1 and LCC9 cells were incubated with 20 µM of CTSL1 inhibitor (C) or 50 µM of CTSD inhibitor (D) ± 100 nM ICI, 100 nM ICI, or vehicle control for 48 or 24 hours, respectively. Cells were detected for autophagosome formation by measuring a modified monodansylcadaverine probe by flow cytometry. Data are presented as percentage of total cells positive for green fluorescence and represent the mean ± SEM for ≥ 3 independent experiments; **P < 0.01, ***P < 0.001 versus control/vehicle experiment.
2.4. Discussion

Increased expression of BCL2 and/or BCLW plays a role in antiestrogen resistance by allowing cells to evade apoptosis (113). Using multiple cell lines and different endocrine therapies, we showed that GX, a small molecule pan-inhibitor of antiapoptotic BCL2 family members, potentiated cell death in both antiestrogen sensitive and resistant human breast cancer cells. While reports describe apoptosis and autophagy as the major forms of death induced by GX (146, 150, 151), the downstream mediators of GX-induced autophagy have yet to be elucidated. We showed that GX’s anticancer efficacy was due to the blockade of antiapoptotic BCL2 family members and an increase in autophagy initiation without the complete digestion of autolysosomes; perhaps one form of apparent autophagic cell death. Furthermore, GX inhibited the protein expression of cathepsins D and L that would ultimately limit cells from effectively recycling cargo that could be used to fuel cell metabolism and restore metabolic homeostasis.

2.4.1. GX inhibits breast cancer cell proliferation through caspase- and PARP-independent mechanisms

Using multiple cell lines affected differently by estrogen, we were able to establish a role for GX in antiestrogen resistant breast cancer. BCL2 and CTSD are both estrogen-regulated genes (161, 175), so assays were conducted with or without estrogen to determine whether the effects of GX were mediated by ER signaling. We hypothesized that GX would inhibit the proliferation of cells that express high levels of antiapoptotic BCL2. While BCL2 members are known to regulate apoptosis (113), several antiapoptotic BCL2 members also affect autophagy through their interaction with
BECN1 (142). In the antiestrogen resistant LCC9 cells, we observed a strong induction of apoptosis and autophagy compared with their LCC1 parental cells following GX treatment alone. We noted a similar induction of apoptosis and autophagy in MCF7 cells compared with their TAM-resistant MCF7/RR derived cells. Thus, ER+ breast tumors that have high BCL2 expression appear to be good candidates for GX ± antiestrogen treatment.

GX appeared to induce apoptosis as measured by annexin V localization to the outer leaflet of the plasma membrane (Figure 2.5). To determine whether GX-mediated cell death was caspase-dependent, we treated cells with GX in combination with the pan caspase inhibitor Z-VAD-FMK. While Z-VAD-FMK inhibited PARP cleavage, it did not influence the effects of GX on cell density and annexin V translocation (Figure 2.6). Thus, we hypothesized that GX killed breast cancer cells by a caspase-independent cell death mechanism. Z-VAD-FMK can potentiate necrotic cell death in certain cell types (176), suggesting that the drug combination killing could have resulted from off-target effects of Z-VAD-FMK. Further studies investigating the effects of Z-VAD-FMK+GX on metabolic regulators and necroptosis are in progress.

2.4.2. GX-mediated autophagosome formation is dependent on BECN1 but independent of ATG7

Recent studies have associated increased autophagy with endocrine resistance and imply that autophagy provides one mean for cells to delay an apoptotic cell death (109, 113, 177, 178). However, when autophagy persists at high levels, it is often associated with
cell death (90). The BH3 mimetic GX can induce both apoptosis and autophagy (150, 151). Therefore, we expected to see both apoptosis and autophagy implicated in GX-induced lethality in antiestrogen sensitive and resistant breast cancer cells. Indeed, GX promoted autophagic vacuole and lysosome formation in LCC9 cells (Figures 2.7 and 2.10). However, this was accompanied by an accumulation of p62 (Figure 2.11), which suggests an impaired ability to degrade contents of autophagic vesicles. We also observed swollen mitochondria in GX treated cells (Figure 210). Previous reports characterize mitochondria swelling with release of cytochrome c in the later stages of apoptosis (173).

GX-induced LC3 processing has been suggested to depend on ATG7 (150). However, we showed that LC3-II formation following GX exposure was dependent on BECN1 and independent of ATG7 (Figures 2.8A and B). BECN1 contains a BH3 domain and its ability to initiate autophagy is inhibited by antiapoptotic BCL2 family members (142). It is possible that inhibition of antiapoptotic BCL2 members by GX released free BECN1, allowing for BECN1-dependent autophagosome formation. However, GX reduced cell density in the presence of an autophagy inhibitor (Figure 2.8D) and BECN1 siRNA (Figure 2.9), suggesting that GX toxicity promoted cell death through an unknown, PI3K, BECN1, and ATG7 independent mechanism. These different cell death mechanisms show the plasticity of breast cancer cell signaling to regulate cell fate in response to endocrine-based stress.
2.4.3. *GX inhibits autophagic cargo degradation*

To determine the precise role of GX in autophagy maturation, we systematically measured the downstream events of autophagy. Previous reports suggest that defective autophagic degradation, reflected by the accumulation of undigested autophagosomes and p62 protein, may contribute to cell death induced by a combination of GX and lapatinib (179). However, the mechanism by which GX inhibits autophagy is unclear. In the present study, we showed that degradation of the autophagy substrate, p62, and clearance of autolysosomes is blunted in GX-treated cells. This is further supported by data illustrating that GX prevents the degradation of GFP and mRFP-tagged LC3, suggesting GX inhibits autophagic flux (Figure 2.14). This led us to focus on studying cathepsins B and L, which are known to degrade autolysosome contents (180), and CTSD that is tightly regulated by estrogen (161) and often overexpressed in ER+ breast cancers (181).

2.4.4. *GX blocks autophagic cargo degradation by inhibiting cathepsin D and L protein expression*

Our data showed that expression of cathepsins D, L, and to a lesser extent B, protein was suppressed in GX treated cells (Figure 2.12). Importantly, cathepsins B, D, and L have each been implicated in tumor invasion, and metastasis (161, 162). Consistent with the protein expression data, we also reported reduced proteolytic activity of CTSB and CTSL1 (Figure 2.12). Despite decreases in protein expression, we did not observe any changes in cathepsin B, D, or L mRNA expression following GX exposure (Figure 2.13). Increased secretion of cathepsin D and L could contribute to reduced intracellular levels; however, we measured cytosolic cathepsin expression following GX treatment and found
no difference in cathepsin protein expression when compared with vehicle treated cells (Figure 2.16). Thus, it is possible that GX modulated the post-translational modification of cathepsins, which, in turn, attributed to the increase in LC3-II and p62 protein levels. To mimic the effects of GX, we suppressed cathepsins D and L with a chemical inhibitor and treated cells with ICI. As with GX treatment, we detected an accumulation of autophagosomes in cells with depressed CTSL1 and CTSD activity (Figure 2.18). This observation is consistent with the ability of CTSL1 to degrade lysosomal membrane components (180). Since CTSD and CTSL1 are implicated in breast cancer progression, inhibition of cathepsin activity using GX or other strategies may be clinically beneficial.

In summary, our results showed that inhibition of antiapoptotic BCL2 expression by GX effectively reduced breast cancer cell growth alone and additively with an antiestrogen. Mechanistically, GX induced apoptosis by freeing up BH3-only BCL2 members and increased autophagic vacuole formation in breast cancer cells. Despite the increase in autophagosome formation with GX exposure, the decision for the cell to undergo cell death was driven by GX’s ability to impair cathepsin protein expression, which led to the accumulation of autophagosomes and autolysosomes but interrupted completion of degradation of autophagic vacuoles. This study provides strong data supporting the potential use of GX and an endocrine therapy for the treatment of ER+ breast cancer cells with detectable BCL2, CTSD, and CTSL1 expression.
Adapted from Publication: Schwartz-Roberts, J. L., Katherine L. Cook, Chun Chen, Ayesha N. Shajahan-Haq, Margaret Axelrod, Anni Wärri, Rebecca B. Riggins, Bassem R. Haddad, Bhaskar V. Kallakury, William T. Baumann, and Robert Clarke. “IRF1 signaling regulates the switch between autophagy and apoptosis to determine breast cancer cell fate.”

3.1. Introduction

3.1.1. Structure of the IRF1 gene/protein

IRF1 was initially characterized for its role in the transcriptional activation of type I interferon (IFN) genes. During the study on the regulation of the IFN-β gene by a virus, a factor that was called IRF1 was found to bind to the IFN-β gene promoter and to regulate its transcription (182). Ten splice variants of IRF1 have been identified and are labeled as splice patterns 1-10 (183). IRF1 is now recognized as an essential player in many facets of the immune response and oncogenesis (184). Since the discovery of IRF1 in 1988, there are now nine known IRF family members in humans and mice: IRF1, IRF2, IRF3, IRF4 (also known as PIP, LSIRF, or ICSAT), IRF5, IRF6, IRF7, IRF8 (ICSBP), and IRF9 (ISGF3γ) (184). Each IRF contains a well-conserved N-terminal DNA-binding domain (DBD) of approximately 120 amino acids and five conserved tryptophan repeats (183). The IRF DBD has a helix-turn-helix architecture that recognizes a specific DNA
sequence corresponding to the IFN-stimulated response element (ISRE;G(A)AAAG/CT/CGAAAG/CT/C) (185).

A single point mutation (P325A) in the C-terminal region of IRF1 (Multifunctional-1; Mf1; residues 301-325) increases both IRF1’s ability to regulate its own transcription and rate of degradation (186). We have also reported a novel single nucleotide polymorphism in the IRF1 gene (A4396G). IRF1-A4396G is more frequent in human breast cancer cell lines than in the general population and is more frequently expressed in African American than Caucasian women (187).

Figure 3.1. Domain structures of IRF1 and IRF2. IRF1 and IRF2 are composed of a DNA-binding domain (DBD; N-terminus) and a regulatory domain (C-terminus). The DBD is characterized by five tryptophan residues each separated by 10-18 amino acids. IRF1 and IRF2 also contain an IRF association domain (IAD). For IRF1, activity depends on phosphorylation, whereas IRF2 contains a repression domain (red). The size of each IRF1 (in amino acids; aa) is also indicated. C, carboxyl terminus; N, amino terminus.
Subsequent to the identification of IRF1, a structurally similar molecule, IRF2, was isolated by its ability to cross-hybridize with the IRF1 cDNA (Figure 3.1) (188). The two factors show 62% homology in their N-terminal regions (spanning the first 154 residues), whereas the rest of the family members exhibit only 25% similarity (188). While IRF1 is characterized by an abundance of acidic amino acids and serine-threonine residues in its carboxy-terminal region (transcriptional activation domain), IRF2 is relatively rich in basic residues (188). When activated by IFN signaling, both IRF1 and IRF2 bind to the same DNA element, known as IRF-E, which is almost identical to the interferon-stimulated response element (ISRE) (183). Despite their similar DBDs, these two factors are functionally distinct. IRF1 mRNA is dramatically upregulated after upon viral infection or IFN stimulation (188). A high level of IRF1, in turn, results in the induction of endogenous IFN-α and IFN-β in a variety of cell types, while IRF2 represses IRF1 transcriptional activation (189). The IRF1 protein is also very unstable (half-life ~30 min) compared with IRF2 (half-life ~8 hrs) (190). These findings suggest respective functional activator and repressor roles for IRF1 and IRF2 for regulation of the IFN-α/β genes. Further studies demonstrated markedly diverse roles for IRF family members including how they contribute to the regulation of key functions in the development of immune cells and in the control of oncogenesis.

3.1.2. Role of IRF1 in IFN signaling

IRF1 is expressed at low levels in unstimulated cells and is activated by many cytokines including type I (IFNα, IFNβ, and others) and II (IFNγ) interferons, tumor necrosis factor-α (TNF-α), retinoic acid, interleukin-1 (IL-1), IL-6, and viral infection (184).
Initial signaling is mediated through the Janus-activated kinase-signal transducer and activator of transcription (JAK/STAT1) pathway, leading to activation of the IRF1 promoter by the Stat and NF-κB transcription factors (183, 191). When a signal is transduced through the IFN receptor, phosphorylated STAT1 translocates to the nucleus where it induces the transcription of primary IFN\(\gamma\) response genes (183). Stat1-deficient cells no longer respond to IFN stimulation by inducing IRF1 expression (192).

3.1.3. Regulation of IRF1 expression

As IRF1 is a short-lived protein; rapid changes in steady state levels occur in response to stimuli such as DNA damage or viral infection (190, 193). The precise mechanism regulating IRF1 stability is unknown, but IRF1 is polyubiquitinated by the E3 ubiquitin ligase and then degraded by the 26S proteasome (194). In unstressed cells, IRF1 is chaperoned by the E3 ligase and the C-terminus of Hsc70-interacting protein (CHIP). In stressed cells, a complex forms between CHIP and IRF1, leading to an increase in ubiquitination of IRF1 and a decrease in its steady state levels (195). Additionally, the C-terminal region of IRF1 (Mf1) was identified as the regulatory domain that modulates target gene expression and determines the rate of IRF1 protein degradation. Without this enhancer region, the IRF1 protein becomes more resistant to both degradation and ubiquitination in proliferating cells (193). IRF1 is also serine-phosphorylated by casein kinase II (CKII), protein kinase A (PKA), and protein kinase C (PKC) at two clustered regions (between amino acids 138-150 and 219-231), which may also have an effect on IRF1 regulation (196). Recent evidence suggests that IRF1 is also a target of the microRNA, miR-23a (197).
IRF1 protein turnover and activation is also regulated by its multifunctional Mf1 domain. Recruitment of Hsp70 to the Mf1 domain leads to the further recruitment of Hsp90, which results in an increase in endogenous IRF1 protein (198). IRF1 is a member of a class of proteins considered to be unstructured (199), which allows it to interact with multiple proteins (200). In addition to NPM, which can inhibit IRF1 function, the interaction between IRF1 repressors, YB-1 (Y-box protein) and TRIM28 (tripartite motif-containing 28), which are both overexpressed in various cancer types, has also been reported (200). The presence of multiple IRF1 regulating proteins and its short protein half-life suggest the presence of several redundant regulatory interactions, often the mark of a central functional activity for the control of critical cellular functions. Perhaps this is not surprising, given IRF1’s ability to affect cell fate decisions.

3.1.4. Biological functions of IRF1

IRF1 has remarkable functional diversity and controls the transcription of genes involved in mediating antiviral, immunomodulatory, and antiproliferative effects (184). Events downstream of IRF1 activation include changes in major histocompatibility complex (MHC) class I and interferon expression, inducible nitric oxide synthase (iNOS) expression, the development of CD8+ T cells, induction of IL-12 and T helper differentiation, and natural killer (NK) development (201). In addition to having critical functions in the development and activation of immune cells, IRF1 is also involved in cell cycle regulation and apoptosis in response to a variety of stressors (202). For instance, IRF1 coordinates expression of the immunoproteasome (203), regulates human
telomerase activity (204, 205), and controls vital aspects of DNA damage repair (206, 207). IRF1 can regulate signaling that leads to the induction of apoptosis (130), which it can achieve with or without induction of either p21\textsuperscript{cip1} (202) or p27\textsuperscript{kip1} (208), and through the activation of caspases (CASP)-1 (206), CASP-3 (131), CASP-7 (209), CASP-8 (206), and/or Fas ligand (210). IRF1 also induces apoptosis in a p53-dependent or –independent manner (130, 206). Thus, IRF1 is capable of functioning in multiple cellular contexts. For example, p53 is frequently mutated in many cancers including 30% of breast cancers but this loss of p53 function does not necessarily abrogate IRF1’s capacity to regulate cell fate decisions (211).

### 3.1.5. Tumor suppressive activities of IRF1

A critical facet of IRF1’s function in host defense is regulation of oncogenesis. The first studies to highlight IRF1’s role in tumor suppression and cell cycle control were established using \textit{IRF1-/−} mouse embryonic fibroblasts (MEFs). \textit{IRF1-/−} MEFs are deficient in their ability to undergo DNA-damage cell cycle arrest, a phenotype similar to that observed in MEFs lacking the tumor suppressor p53 (202). Furthermore, transcriptional induction of the cell-cycle inhibitor, p21 (WAF1, CIP1), following gamma irradiation is dependent on both IRF1 and p53 (202). Several other reports have also elucidated the involvement of IRF1 in cell growth effects (212, 213). IRF1 can eliminate precancerous cells through apoptosis induced by DNA damage or other stimuli. For instance, wild type MEFs require two or more oncogenic mutations for transformation, whereas a “single hit” with c-Ha-ras induces transformation in \textit{IRF1-/−} MEFs (130). In this situation, apoptosis is dependent upon both p53 and IRF1. In contrast, DNA damage-
induced apoptosis in mitogenically activated mature T lymphocytes is dependent on IRF1 but independent of p53 (206). These studies demonstrate that IRF1 can mediate two crucial compounds of neoplastic progression: apoptosis and cell cycle arrest (130, 131).

3.1.6. IRF1 in breast cancer

The IRF1 locus at chromosome 5q31.1 was initially reported to be lost in a substantial proportion of leukemia and preleukemic myelodysplasia cells (214). IRF1 is also frequently deleted (loss of heterozygosity) in esophageal (215) and gastric carcinoma (point mutation) (216). Approximately 11% of sporadic breast cancers exhibit the loss of chromosome 5q12-31, the most frequent chromosome loss detected by comparative genomic hybridization (CGH) (124). Other studies report that approximately 30% of neoplastic breast tissues have loss of IRF1 by immunohistochemical staining when compared with normal breast epithelium (217). Furthermore, high grade ductal carcinoma in situ (DCIS) or node-positive invasive ductal cancers were less likely to express IRF1 and were much more likely to have higher oncogenic IRF2 protein than normal cells (217). CGH also shows IRF1 loss of heterozygosity (LOH) in 50% of BRCA1 mutated breast cancers tumors (125, 218). These results are consistent with the hypothesis that loss of IRF1 expression in some breast cancers contributes to the loss of appropriate growth-control.

Allelic loss of IRF1 is detected in 32% of women with breast cancer (12/37 breast tissue specimens) (219). This loss of heterozygosity is associated with an increased risk of recurrence and risk of death in the cases studied, strongly implicating a tumor suppressive
role for the \textit{IRF1} gene in breast cancer (219). Analyses of two publically available ONCOMINE cancer gene microarray datasets also imply an important tumor suppressive role for \textit{IRF1} in many sporadic breast cancers. Protein expression studies show that in breast tumors the most prevalent location of IRF1 is within the cytosol (90%); this location suggests a transcriptionally inactive form of IRF1 (220). In contrast, 51% of the reported tumors expressed IRF1 in the nucleus (in more than 50% of the tumor cells), consistent with a potential to represent a transcriptionally active form (220). Thus, some breast tumors may differentially regulate the activation of IRF1 by controlling its subcellular localization. These observations are consistent with a study reporting higher levels of IRF1 protein in adjacent normal breast epithelium when compared with high-grade ductal carcinoma \textit{in situ} or lymph node-positive invasive ductal carcinoma of the breast (125). Collectively, these studies imply that some cells may bypass the growth inhibitory mechanisms of IRF1 by down-regulating its expression (220, 221). This observation is supported by the evidence that reduced IRF1 expression in breast cancer cells is associated with low caspase activity, apoptosis, and ultimately, increased cell survival (116, 131, 134).

\textbf{3.1.7. \textit{IRF1} and antiestrogen sensitivity}

To identify key genes contributing to the endocrine resistant phenotype in breast cancer cells, the transcriptomes of MCF7/LCC1 (AE sensitive) and MCF7/LCC9 (AE resistant) cells were analyzed by gene expression microarrays. The nuclear transcription factor, IRF1, appeared as a differentially expressed gene upregulated in the MCF7/LCC1 and downregulated in the MCF7/LCC9 cells (134). A dominant-negative IRF1 (dnIRF1),
which lacks the carboxy terminal transactivation domain, produces opposing effects of IRF1 and reduces antiestrogen sensitivity in MCF7 and T47D breast cancer cells through an inability of IRF1 to induce a caspase cascade (131, 133, 222). In this chapter, we elucidated the role of IRF1 in breast cancer progression through regulating BCL2 members and functionally related proteins, such as beclin-1 (BECN1). This pathway is significant because neoplastic breast tissue has lower expression of the tumor suppressor IRF1 than normal tissue (217, 220) and preliminary data show a significant inverse relation between the IRF1 and BCL2 mRNAs in breast cancers (p=0.038). Recent studies show that a low dose of the inflammatory cytokine, interferon-γ (IFNγ) is sufficient to restore IRF1 expression and ICI sensitivity in antiestrogen resistant cells through inhibition of BCL2 (116).

3.1.8. IRF1 in glucose metabolism

IRF1 is implicated in glucose metabolism and diabetes. In vascular smooth muscle cells (VSMCs), overexpression of IRF1 accelerates cell proliferation under high glucose conditions (223). In contrast, down regulation of IRF1 depresses the proliferative ability of VSMCs. It is possible that IRF1 is associated with enhanced proliferation of VSMCs in diabetic vascular diseases and could be a potential target for disease treatment (223). Another report suggests that an increase in the angiotensin II receptor, AT2R, is associated with an increase in IRF1 in hyperglycemic rats and in high glucose-treated HK2 cells (224). Silencing of IRF1 with siRNA in HK2 cells prevents glucose-induced AT2R upregulation (224). Thus, IRF1 is a regulator of AT2R expression in hyperglycemia and may be beneficial to the diabetic kidney. IRF1 activation has also been shown to
inhibit cell growth in baby hamster kidney cells by altering energetic metabolism (225). However, how glucose regulates IRF1 expression is still unclear. Since there is a strong link between, obesity, diabetes, and cancer, understanding IRF1’s role in glucose metabolism is necessary for cancer prevention and treatment.

3.1.9. Interplay between various forms of cell death: apoptosis and autophagy

Precisely how breast cancer cells die following endocrine therapy is unclear. When a cell makes the decision to die, it must activate a programmed cell death (PCD) pathway such as apoptosis (PCD1), autophagy (PCD2), or necrosis (PCD3). For endocrine therapies, cell death by apoptosis (133, 226) and autophagy (227) are consistently reported in vitro; the extent to which necrotic cell death (228) occurs is less certain. Emerging evidence suggests a more intimate relationship between signaling to apoptosis and autophagy, implying the use of similar or related signaling molecules for cell death crosstalk. BCL2 members are known regulators of apoptosis (229) but also play critical roles in autophagy (142). Several antiapoptotic BCL2 members inhibit BECN1, a critical regulator of autophagy that facilitates autophagosome production (142). Thus, competitive interactions by proapoptotic BAD, BID, BIK, NOXA (PMAIP1), PUMA, and BMF can promote autophagy by effectively sequestering BECN1 inhibitors and releasing free BECN1 to act elsewhere (90).

3.1.10. Autophagy in immunity and inflammation

Apoptosis is widely implicated as a cell death pathway in antiestrogen sensitive cells, whereas the autophagic process has been shown to facilitate breast cancer resistance to
endocrine, cytotoxic, and molecularly targeted agents (90, 109, 230). Autophagy is a major catabolic process used by cells to turnover superfluous or damaged proteins and organelles, likely promoting tumor growth by providing cells with the necessary nutrients to maintain homeostasis (90). Embryonic mice lacking the autophagy related 5 (Atg5) gene have increased inflammation in tissue with impaired clearance of apoptotic cells, raising the possibility that autophagy has a role in the prevention of inflammation and autoimmunity (231). Other autophagy proteins have a major effect on the regulation of inflammatory transcriptional responses. Increased levels of p62/sequestosome-1 (SQSTM1), which accumulate when autophagy is disrupted, activate pro-inflammatory NF-κB signaling (232). Mice with low levels of the autophagy protein, ATG16L1, show enhanced transcription of pro-inflammatory cytokines and adipokines in intestinal immune epithelial cells, suggesting a role for autophagy in regulating inflammation (233).

A critical decision point in the determination of cell fate in response to endocrine therapies appears to be affected by the balance between autophagy (prosurvival) and apoptosis (prodeath) (90). The activities of IRF1 in multiple cell types suggests that it may be a key component in regulating the cell fate decision and perhaps a critical regulator of the switch between apoptosis and autophagy. Thus, we investigated the biological function of IRF1 in autophagy in the context of endocrine responsiveness in breast cancer. We established an important (inverse) link between ATG7 and IRF1 expression/function, which we confirmed using immunohistochemical studies in human breast carcinomas and tissue from Atg7 +/- mice. We also showed that inhibition of the
autophagy proteins ATG7 and BECN1 promoted IRF1 signaling and induced apoptotic cell death. Conversely, inhibition of IRF1 prolonged cell survival and promoted autophagy through both increased BECN1 and suppressed mTOR signaling. Thus, our data suggests that IRF1 inhibits breast cancer cell growth through its ability to regulate both autophagy and apoptosis. To better understand the molecular interactions, we constructed an influence diagram of the novel signaling and then used this as a guide to build mathematical models using our experimental data. Model simulations suggested additional experimentation to distinguish whether ATG7 knockout promotes endocrine responsiveness through either IRF1-dependent or -independent signaling. Collectively, these data indicate a major role for IRF1 in regulating cell fate decisions in breast cancer and establish an IRF1-independent path as an additional signaling component in ATG7-mediated cell death.

3.2 Materials and Methods

3.2.1. Cell culture, reagents, and small interfering RNA (siRNA) treatments

MCF7, T47D, BT-474, and MDA-MB-231 cells were maintained in improved minimal essential media (IMEM) with phenol red and supplemented with 5% fetal bovine serum (Life Technologies). MCF7/LCC1 (LCC1) and MCF7/LCC9 (LCC9) cells were grown in phenol red-free IMEM supplemented with 5% charcoal-stripped calf serum. All cells were maintained in a humidified atmosphere at 37°C and 95% air/5% CO₂. ATG7 (SignalSilence; Cell Signaling Technology), BECN1, STAT1, and ERα (three unique siRNAs for each target; OriGene), IRF1 (Silencer Select; Life Technologies), or control (Ctrl) siRNA were transfected in cells using Lipofectamine RNAiMAX (Life
Technologies) according to the manufacturer’s instructions. ERα and ATG7 cDNA were from Origene; ICI 182,780 (ICI; Faslodex; Fulvestrant) from Tocris Bioscience; Hydrochloroquine (HCQ), 3-methyladenine (3-MA), N-acetylcysteine (NAC), paclitaxel (PAC), and doxorubicin (DOX) were from Sigma-Aldrich; JAK inhibitor 1 (2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one) from EMD Millipore.

3.2.2. Lentiviral plasmid creation and infection; creation of stable cell lines

The IRF1 and dnIRF1 inserts were amplified using KOD Hot Start DNA Polymerase (Novagen) from pcDNA3.1 plasmids (Invitrogen) using the primers 5’-CACCATGGACTACAAGGACGACGATGACAAGGGATCCATGCCCATCACTCGGATGCG (IDT), which includes the Flag tag sequence and forward TOPO recognition site and 5’-GACACTAATCAGCTGGG. As a control, lacZ was cloned into pENTR™/D-TOPO plasmid (Invitrogen) and grown in kanamycin. After successful sequencing, inserts were cloned into pLenti-puro/TO/V5-DEST (received from Andrew Aplin, Albany Medical College) using Gateway® LR ClonaseTM II Enzyme Mix (Invitrogen) and grown in ampicillin to select for recombination. After directional analysis using EcoRV, virus plasmids were transfected into Platinum-A Retroviral Packaging Cells (CellBioLabs) and grown for 72 hours. Subsequently, the supernatent was added to MCF7, MCF7/LCC1, MCF7/LCC9, T47D, and MDA-MB-231 cells for 24-48 hours before being selected for with puromycin.
3.2.3. Cell proliferation

Cells were transfected with IRF1, ATG7, BECN1, or Ctrl siRNA and seeded at a density of 0.2 x 10^6 per well in 24-well plates. One day after plating, cells were treated with the indicated concentration of ICI, PAC, DOX, or vehicle control. Cells were incubated with ICI or PAC/DOX for 6 days or 48 hours, respectively, with media containing either drug or vehicle being replaced every 3 days. Cells were then stained with a crystal violet staining solution as previously described (131). Sodium citrate buffer was used to extract the dye, and absorbance was measured at 550 nM using a microplate reader (Bio-Rad). Cell density was calculated from the crystal violet assay.

3.2.4. Microarray analysis

Total RNA was extracted using TRIzol from three passages each of MCF7/IRF1 and MCF7/control (vector only). High quality total RNA was labeled and hybridized to Affymetrix U133 A GeneChips using the manufacturer’s protocols. Expression data were normalized by the Robust Multichip Average (RMA) method as implemented in Bioconductor (http://bioconductor.org). Student t-test p value and fold change were calculated by comparing MCF7/control to MCF7/IRF1. 1048 genes with \( P < 0.05 \) (univariate, two tailed), fold change \( \geq 1.45 \), and signal intensity \( \geq \log_2(10) \) in both control and experimental groups were identified as differentially expressed genes.

3.2.5. Western blot analysis

Lysates were harvested from transfected cell monolayers as previously described (170). Membranes were probed for proteins of interest at 4°C overnight using the following
antibodies (1:1000): ATG5, ATG7, BECN1, cleaved Caspase 7, IGF1R, LC3B, mTOR, P-4EBP1 (Thr37/46), P-STAT1, STAT1, STAT2 (Cell Signaling Technology); IRF1 and p62 (BD Biosciences); ERα (Vector Labs); and LAMP2 (Enzo Life Sciences). Following incubation, membranes were exposed to polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) for 1 hour at room temperature. Reactive products were visualized by chemiluminescence, and quantified by densitometry using Quantity One Software (Version 4.6.9; Bio-Rad). To confirm equal loading of the gels, membranes were reprobed for β-actin (1:1000; Santa Cruz Biotechnology).

3.2.6. Isolation of mitochondrial enriched fraction

LCC1 and LCC9 cells were seeded in 10 cm² dishes 24 hours prior to treatment with 100 nM ICI or vehicle control. 48 hours post treatment, cells were homogenized, suspended in lysis buffer, and centrifuged (14,000 x g, 15 min, 4°C) to pellet mitochondria. Mitochondria were resuspended and stored in mitochondria storage buffer according to the Qiagen Qproteome Mitochondria Isolation kit instructions.

3.2.7. MMP, autophagosome formation, ROS, and glucose uptake assays

LCC1 and LCC9 cells were reverse transfected with ATG7, BECN1, IRF1, or Ctrl siRNA and plated in 6-well tissue culture plates. The following day, cells were treated with 100 nM ICI or vehicle control. Cells were harvested 48 hours later and stained as described in the Mitochondrial Permeability Detection Kit for flow cytometry (Enzo). Accumulation of autophagic vesicles was measured using a modified monodansylcadaverine according to the manufacturer’s instructions (Enzo Cyto-ID
Autophagy detection kit). Total reactive oxygen species (ROS) were stained according to Enzo’s Total ROS Detection Kit instructions. To determine how IRF1 affects glucose uptake, cells were stained with a fluorescently-labeled deoxyglucose analog in accordance with the Glucose Uptake Cell-Based Assay kit (Cayman Chemical). Stained cells were detected and appropriate signals measured by fluorescence-activated cell sorting (LCCC FACS Shared Resource).

3.2.8. Autophagosome maturation and localization studies

LCC1 cells (1 x 10⁵) were reverse transfected with IRF1, ATG7, BECN1, or Ctrl siRNA and seeded onto 18 x 18 mm glass coverslips. The following day, IRF1 siRNA cells were transfected with LC3 tagged with a green fluorescent protein (GFP). 24 hours later, cells were treated with 500 nM ICI or vehicle, and then fixed and stained for IRF1 as previously described (116). IRF1 and ERα were also measured in LCC1 cells following knockdown of ATG7, BECN1, or IRF1 to determine localization.

3.2.9. Mathematical modeling and data fitting

Mathematical modeling was performed by collaborators at Virginia Tech. They used Matlab (version 7.9.0) to build the mathematical model and perform simulations. An ordinary differential equation (ODE) formalism that allowed them to capture the complex dependencies of species in a signaling network (234-236) was used to capture the phenomenological behaviors observed in experiments. Further details on the mathematical modeling can be found in Schwartz-Roberts, et al., 2013 (under review at Science Signaling).
3.2.10. In vivo experiments

All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of animals in biomedical research. Female Atg7 +/− and wild-type (WT) mice (237) (Harlan, USA) were treated with medroxyprogesterone acetate (MPA, DepoProvera; Pfizer) and 7,12-dimethylbenz(a)anthracene (DMBA; Sigma) to induce mammary tumors, as described earlier (238). Mice were euthanized when tumors reached 10% of the mouse body size or when a single tumor volume reached 1000 mm³; mammary tumors and organs were removed, fixed in neutral buffered formalin, and processed using routine histological methods.

3.2.11. Human tissue microarrays (TMAs)

The TMAs were obtained from the Familial Cancer Registry (FCR) at Georgetown University Lombardi Comprehensive Cancer Center. These TMAs contained 88 cores obtained from breast tumors of FCR participants (2 cores/subject) with a known BRCA1/2 mutation status (i.e., know BRCA1/2 mutation carriers and non-carriers). Histologic grade, estrogen receptor, progesterone receptor, and HER2 status were known for each core. The histologic diagnosis was confirmed at the time of the immunohistochemistry scoring, by a pathologist. Patient/tumor characteristics are described in the table below.
3.2.12. Immunohistochemistry

Five-micron sections from paraffin embedded human TMAs and mouse mammary tissue were stained with mouse anti-IRF1 (1:100; BD Biosciences), mouse anti-BCL2 (1:150; Dako), rabbit anti-PUMA (1:250; Cell Signaling), or rabbit anti-APG7 (1:130; Santa Cruz Biotechnology) antibodies as previously described (170). Slides were exposed to biotinylated anti-mouse or anti-rabbit IgG secondary antibodies (Vectastain ABC reagent and DAB chromagen; Dako; Denmark). All assessments of immunohistochemical staining in human tissue were performed by a pathologist blinded both to the clinical and molecular data and to patient outcome. Cytoplasmic and nuclear staining of BCL2, PUMA, ATG7, and IRF1 were described in terms of intensity (0: negative, 1+: weak, 2+: intermediate, 3+: strong) and distribution (0: negative, +1: <10%, 2+: 11-50%; 3+: >50%). A simplified total immunohistochemistry score (the sum of the intensity and distribution scores) was calculated for each core. Total immunohistochemistry scores were used to determine BCL2, PUMA, ATG7, and IRF1 prevalence. For the in vivo staining, a computer-assisted counting technique with a grid filter to select cells was used to quantify the immunohistochemical staining of IRF1 (171). Positive cells were expressed as a percentage of the total cell number examined (100 cells sampled from each tissue site within each breast tumor section).

3.2.13. Statistical analysis

Prism 5.0 was used for statistical analysis. Differences between two groups were analyzed by Student t tests, and multiple group comparisons were assessed by one-way ANOVA followed by Bonferroni post hoc tests. The Pearson correlation coefficient was
calculated to measure the correlation between ATG7 and IRF1 in human tumor tissue. Differences were considered statistically significant at $P < 0.05$.

3.3. Results

3.3.1. IRF protein expression in antiestrogen sensitive and resistant breast cancer cell lines

Members of the IRF protein family are critical in eliciting immune responses and are also involved in tumorigenesis (239). Gene expression microarrays between antiestrogen-responsive (MCF7/LCC1) and -resistant (MCF7/LCC9) human breast cancer cell lines report that IRF1 is downregulated in resistant cells, suggesting that loss of IRF1 may contribute to endocrine resistance through its ability to induce cell death (116, 131, 132). Thus, we investigated whether expression of IRF1 and other tumor suppressive IRFs were differentially regulated in antiestrogen sensitive and resistant breast cancer cell lines. We found that IRF1 was downregulated in both the resistant MCF7/RR and LCC9 cell lines compared with their sensitive controls (Figure 3.2). Furthermore, IRF5 expression was downregulated in the MCF7/RRs while IRF7 expression was reduced in the LCC9s compared with their sensitive controls (Figure 3.2). These data suggest that IRFs may be differentially regulated in breast cancer cell lines and have overlapping roles in tumor suppressive signaling.

3.3.2. PUMA is an IRF1 target gene that is upregulated following ICI treatment

IRF1 induces cell death through apoptosis (206), so we investigated proapoptotic BCL2 expression to determine which members may be implicated in AE sensitivity. The BH3-
only member PUMA (p53 upregulated modulator of apoptosis) was upregulated in cells overexpressing IRF1 and was induced following treatment with ICI (Figure 3.3A). To further delineate a mechanistic role for ICI-induced PUMA expression, we determined the effects of PUMA knockdown on ICI-induced apoptosis. MCF-7 cells overexpressing IRF1 (MCF7/IRF1) or lacZ control (MCF7/lacZ) were treated with either non-targeting siRNA (siCtrl) or PUMA specific siRNA (siPUMA) in the presence of ICI or vehicle control for 48 hours. Induction of apoptosis was determined by Annexin V-FITC labeling and analyzed by flow cytometry. Figure 3.3B shows that PUMA knockdown attenuated the apoptotic response to ICI compared with cells transfected with Ctrl siRNA. The level of apoptosis in MCF7/IRF1 cells is shown as fold change relative to MCF7/lacZ vehicle control from at least three separate experiments (Figure 3.3B). Collectively, these data illustrate that PUMA plays a critical role in the apoptotic signaling cascade induced by IRF1.
Figure 3.2. IRF protein expression in antiestrogen sensitive and resistant breast cancer cell lines. MCF7, MCF7/RR, LCC1, and LCC9 cells were grown under basal conditions for 48 hours. Western blot analysis of protein homogenates was used to measure the expression of the indicated proteins. β-actin served as the loading control; representative images from 3 independent experiments.
Figure 3.3. PUMA expression is activated by IRF1 and mediates the apoptotic response to ICI. A, MCF7/IRF1, MCF7/dnIRF1, and MCF7/lacZ cells were treated with 100 nM ICI or vehicle control for 48 hours. Western blot hybridization of treated protein homogenates was used to measure IRF1 and PUMA expression. β-actin served as the loading control; representative images from 3 independent experiments. B, MCF7/lacZ cells (gray bars) and MCF7/IRF1 cells (black bars) were transfected with Ctrl (siCtrl) or PUMA (siPUMA) siRNA and treated with 100 nM ICI for 48 hours. Apoptosis was quantified using flow cytometry analysis of annexin V-FITC-stained cells. *$P < 0.05$. Data are presented as annexin V-FITC-stained cells relative to MCF7/lacZ vehicle control and represent the mean ± SEM for ≥ 3 independent experiments.
3.3.3. **PUMA expression is higher in normal versus neoplastic breast tissue**

To determine whether IRF1 and downstream PUMA expression were altered in human breast cancer compared to normal tissue, we measured IRF1 and PUMA expression by immunohistochemistry from a patient diagnosed with invasive ductal breast carcinoma. Ductal carcinoma and normal surrounding tissue were stained with polyclonal IRF1 and monoclonal PUMA antibodies. Immunohistochemistry staining patterns and intensity of IRF1 and PUMA are shown in Figure 3.4. PUMA expression is significantly higher in normal vs. neoplastic breast tissue (Fig 3.4); this is consistent with lower expression of PUMA in invasive ductal carcinoma (240). In contrast, IRF1 was expressed in both normal and neoplastic breast tissue (Figure 3.4). While very preliminary, this observation suggests that loss of PUMA expression is an important step in the progression of breast cancer.

3.3.4. **Antiapoptotic BCL2 and IRF1 are inversely correlated in breast cancer cell lines**

We proposed that IRF1 coordinates BCL2 family member activity in a central role that ultimately determines both the extent and nature of cell fate decisions. To determine how IRF1 regulates BCL2 *in vitro*, we used both dnIRF1 and IRF1 siRNA as tools to knockdown IRF1. In MCF-7 and LCC1 cells stably overexpressing IRF1, dnIRF1, or lacZ control, BCL2 and BCLW expression was upregulated in dnIRF1 cells (Figure 3.5). However, BCL2 and BCLW were differentially expressed between cell types: BCLW was upregulated in MCF7/dnIRF1 cells whereas BCL2 was elevated in LCC1/dnIRF1 cells (Figure 3.5). While BCLW was reduced with 100 nM ICI treatment in IRF1 and lacZ cells, ICI had no effect on BCL2 expression (Figure 3.5).
Figure 3.4. PUMA expression is reduced in neoplastic versus normal breast tissue. Immunohistochemical staining of human invasive ductal carcinoma and adjacent normal tissue was performed for IRF1 and PUMA as further described in Materials and Methods.
Figure 3.5. BCL2 and BCLW are differentially expressed in dnIRF1 breast cancer cell lines. MCF7 and LCC1 cells stably expressing IRF1, dnIRF1, or lacZ were treated with 100 nM ICI for 48 hours. Western blot hybridization of treated protein homogenates was used to measure IRF1, BCL2, and BCLW expression. β-actin served as the loading control; representative images from 3 independent experiments.
In LCC1 and LCC9 cells transfected with IRF1 siRNA, both BCL2 and MCL1 were upregulated compared with cells transfected with Ctrl siRNA (Figure 3.6A-B). We observed an approximate 4-fold increase in BCL2 expression when IRF1 was knocked down with siRNA (Figure 3.6B; \( P = 0.01 \)). These data strongly support a functional link between IRF1 induction and antiapoptotic BCL2 family member expression in ER+ breast cancer cell lines.

3.3.5. IRF1 localizes to the mitochondria

Although commonly found in the cytoplasm, IRF1 is known to localize to the nucleus, especially when activated by agents such as IFN\( \gamma \) (241) or retinoic acid (242). However, no literature on IRF1 translocation to the mitochondria exists. Since BCL2 resides in the outer mitochondrial membrane (243) and its expression is suppressed by IRF1, we investigated the possible association of IRF1 with the mitochondria in human breast cancer cells. Using confocal microscopy, we visualized cytoplasmic IRF1 (green) overlapping with areas stained with mitotracker red (Figure 3.7A) in LCC1 cells. We further investigated this phenomenon by collecting mitochondrial-enriched fractions of LCC1 and LCC9 cells and measuring IRF1 protein expression (Figure 3.7B). Mitochondrial fractions of LCC1 and LCC9 cells treated with 100 nM ICI or vehicle control both contained IRF1 expression (Figure 3.7B). These lysates were also probed for \( \beta \)-tubulin (cytosolic marker) and COXIV (mitochondrial marker) to determine the purity of the fractionation. Thus, our novel observation suggests that IRF1 may reside and possibly regulate the activities of other proteins in the mitochondria of breast cancer cells.
Figure 3.6. BCL2 and MCL1 expression is upregulated in LCC1 and LCC9 cells transfected with IRF1 siRNA. A, LCC1 and LCC9 cells transfected with IRF1 (siIRF1) or Ctrl (siCtrl) siRNA were treated with 100 nM ICI for 48 hours. Western blot hybridization of protein homogenates was used to measure IRF1, BCL2, and MCL1 expression. β-actin served as the loading control; representative images from 3 independent experiments. B, Densitometric analysis of BCL2 expression from ≥ 3 independent experiments.
**Figure 3.7. IRF1 localizes to the mitochondria.** A, LCC1 cells were incubated with mitotracker red, fixed, permeabilized, stained for IRF1 (green) and DAPI, and then visualized by confocal microscopy. B, LCC1 and LCC9 cells were seeded in 6-well plates 24 hours before treatment with the indicated drug(s). 48 hours post treatment, whole cell lysates (W), cytosolic (C), and mitochondrial (M) fractions were collected as described in Materials and Methods. Western blot analysis was used to measure the expression of indicated proteins; β-tubulin served as the loading control. Representative images from ≥ 3 independent experiments.
3.3.6. Breast cancer cell lines overexpressing dnIRF1 are sensitive to BCL2-mediated growth inhibition

Cells expressing dnIRF1 or transfected with IRF1 siRNA have increased levels of antiapoptotic BCL2 (Figures 3.5 and 3.6). These cells may be dependent on antiapoptotic BCL2 members for their survival and may evade cell death following treatment with antiestrogens. We tested this hypothesis using the pan BCL2 inhibitor, obatoclax (GX; GX15-070). Figure 3.8 shows that MCF7/dnIRF1 and LCC1/dnIRF1 cells are resistant to 100 nM ICI alone, but sensitized to ICI following treatment with 100 nM GX after 6 days. Interestingly, GX alone inhibited the growth of LCC1/dnIRF1 but not MCF7/dnIRF1 cells, suggesting a differential regulation of BCL2 between these two cell types (Figure 3.8).

3.3.7. IRF1 expression in familial breast tumors

The chromosomal region of IRF1 is lost in 11% of sporadic breast cancer (124) and over 85% of tumors from women carrying inherited BRCA1 mutations (125). To evaluate the role of IRF1 in human breast tumors, we obtained tissue microarrays containing core-needle biopsies from 107 patients with familial breast cancers (BRCA1/2 mutation carriers and non-carriers) and measured IRF1 protein expression. We asked where IRF1 localized and whether it was expressed in patterns consistent with its known functions and putative gene expression network. The patient/tumor characteristics of the breast tissue microarrays are described in Table 3.1.
Figure 3.8. AE resistant MCF7/dnIRF1 and LCC1/dnIRF1 cells are sensitive to treatment with an AE and obatoclax (GX). MCF7/dnIRF1 and LCC1/dnIRF1 were seeded in 96-well tissue culture dishes and the following day treated with 100 nM GX ± 100 nM ICI, 100 nM ICI, or vehicle control. Cells were refed with media containing drug/vehicle every three days. Six days post treatment, cells were stained with crystal violet and cell density was determined with a plate reader at wavelength 550 nm. Data are presented as relative cell density and represent the mean ± SEM for ≥ 3 independent experiments; ***$P < 0.001$ versus vehicle/control.
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Table 3.1. Patient/tumor characteristics of human breast cancer tissue microarrays.

Abbreviations: DCIS, ductal carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor.
Since only nuclear IRF1 is likely to be transcriptionally active, we scored nuclear IRF1 staining; reflecting its role as a tumor suppressor gene, we expected to find only a modest proportion of tumors with high levels of nuclear staining (220). We found that the primary form of IRF1 was cytoplasmic (expressed in 80% of cases), while nuclear IRF1 was only detected in 12% (13/107) of tumors (Figure 3.9A). We also examined IRF1 expression in tissue specimens from women with DCIS, invasive breast cancer, or normal breast tissue. Patients with DCIS had greater cytoplasmic IRF1 expression compared with normal and invasive breast cancer tissue (Figure 3.9B). However, no difference in nuclear IRF1 expression was detected between DCIS or invasive breast cancer compared with normal tissue (Figure 3.9B).

We also established that breast tumors with BRCA1 mutations (BR1) had significantly higher nuclear IRF1 protein expression compared with non-mutation carriers (FN) (Figure 3.10A). Since IRF1 regulates BCL2, we next examined BCL2 expression in familial breast tumors. BCL2 expression was significantly decreased in BRCA1 mutated tumors as compared with non-mutation and BRCA2 mutation (BR2) carriers (Figure 3.10B). These data suggested that IRF1-mediated regulation of BCL2 is an important signaling component in tumors from patients carrying BRCA mutations.
Figure 3.9. Immunohistochemical staining of IRF1 in human breast cancer. A, Cytoplasmic and nuclear staining was assessed by a pathologist and scored with an intensity of 0, 1, 2, 3 and a distribution of 0, 1, 2, 3. The sum of the intensity and distribution scores equated to the total immunohistochemistry score. Average immunohistochemistry score of cytoplasmic and nuclear IRF1. B, Average immunohistochemistry score of IRF1 in normal, DCIS, and invasive breast cancer tissue.
Figure 3.10. Nuclear IRF1 expression is increased and BCL2 expression is decreased in tumors from patients carrying BRCA1 mutations. A-B (top panel), Representative images of IRF1 and BCL2 immunohistochemistry in 107 breast carcinomas from patients carrying BRCA1 (BR1), BRCA2 (BR2), mutations or non-mutation carriers (full negative; FN). A-B (bottom panel), Cytoplasmic and nuclear staining was assessed by a pathologist and scored with an intensity of 0, 1, 2, 3 and a distribution of 0, 1, 2, 3. The sum of the intensity and distribution scores equated to the total immunohistochemistry score. Total immunohistochemistry scores were used to determine IRF1 and BCL2 prevalence in familial breast cancer.
3.3.8. Nuclear IRF1 and ATG7 expression are inversely correlated in vivo and in human breast carcinomas

To investigate the possibility that IRF1 might be involved in autophagy, we used immunohistochemistry to examine IRF1 expression in mammary tumors from wild-type (WT) mice and mice with a mutated Atg7 allele (+/-); Atg7 null mice (-/-) are not viable (237). Mammary tumors, spleen, and kidney from Atg7 +/- mice exhibited increased nuclear IRF1 staining (arrows in Figure 3.11A) compared with their WT controls (Figure 3.11; P < 0.001). We then examined whether nuclear IRF1 protein expression was also associated with ATG7 protein expression in the human disease by staining tissue microarrays (TMAs) containing n=107 cores from breast tumors. We found that >96% of samples (90/93) with positive ATG7 expression had low or undetectable nuclear IRF1 expression (Figures 3.12A and B). Conversely, >70% of samples (10/14) with nuclear IRF1 expression had low ATG7 expression. An inverse correlation was found between ATG7 and nuclear IRF1 expression in human breast cancer (Figure 3.12B; r = -0.28, P < 0.01). We then examined the expression of IRF1 and ATG7 in ER+ and ER- human breast tumors using immunohistochemistry. ER status was identified in 52 out of the 107 human breast cancer cores. We found that ER+ tumors exhibited significantly greater ATG7 (P < 0.001) and decreased nuclear IRF1 (P < 0.01) expression compared with ER- tumors (Figure 3.12C). Taken together, these observations suggest that nuclear IRF1 expression is elevated in ER- breast tumors and that this increase is correlated with reduced ATG7 expression.
Figure 3.11. *Atg7* heterozygote mice exhibit increased nuclear IRF1 expression in mammary tumors and organs compared with wild-type mice. A, Representative immunohistochemical staining of IRF1 in wild-type (WT) and *ATG7* +/− mouse mammary tumors (top panel), kidney (middle panel), and spleen (bottom panel) tissue. B, Positive cells were quantified using ImageJ software. n=3; *P < 0.05*, **P < 0.01**, ***P < 0.001 versus wild-type/control.
Figure 3.12. Nuclear IRF1 and ATG7 are inversely correlated in human breast cancer. A, Representative immunohistochemical staining of ATG7 (left) and IRF1 (right) in matched human breast cancer tissues. Arrows indicate nuclear IRF1 staining. B, Tissue sections of 107 breast cancer cores (two per patient) were immunostained for the expression of ATG7 and IRF1 and their correlation was analyzed by Pearson’s rank correlation ($P < 0.01$). C, Average immunohistochemical score of ATG7 and nuclear IRF1 expression in ER+ and ER- breast tumors. n=52; **$P < 0.01$, ***$P < 0.001$ between indicated groups.
3.3.9. ATG7 and BECN1 knockdown stimulates IRF1 expression

Since a putative link was established between IRF1 and ATG7, we used various inhibitors and siRNA targeting ATG7 and other key autophagy proteins in breast cancer cell lines and measured IRF1 protein expression. Antiestrogen sensitive LCC1 cells were treated with vehicle (control), 100 nM ICI, 5 mM 3-methyladenine (3-MA), 10 µM hydroxychloroquine (HCQ), or reverse transfected with ATG5, ATG7, or beclin-1 (BECN1) siRNA for 48 hours before total protein was harvested (Figure 3.13). ATG5 and ATG7 are involved in autophagosome membrane formation and elongation, and BECN1 (also known as the mammalian ortholog of yeast ATG6), interacts with Class III PI3K (Vsp34) to mediate the localization of other autophagy proteins to the preautophagosomal membrane (90, 231). Using western blot hybridization, levels of IRF1 were measured in breast cancer cell lines with deficient autophagy and were found to be significantly elevated in LCC1 cells with reduced ATG7 and BECN1 (Figure 3.13; \( P < 0.001 \)). We further examined IRF1 expression following ATG7 (Figure 3.14A) and BECN1 (Figure 3.14B) knockdown in other ER+ breast cancer cell lines, including MCF7 (antiestrogen-sensitive), LCC9 (antiestrogen-resistant), T47D (antiestrogen-sensitive, p53 mutant), and BT-474 (antiestrogen-sensitive, HER2 amplified). We observed that ATG7 and BECN1 knockout cells (except ATG7 knockout BT-474 cells) had elevated expression of IRF1 compared with control cells (Figures 3.14A and B). Furthermore, we determined that ATG7 and BECN1 siRNA induced apoptosis in LCC1 and LCC9 cells as measured by an increase in mitochondrial membrane permeability (Figure 3.14C).
Figure 3.13. Knockdown of ATG7 and BECN1 induces IRF1 expression in LCC1 cells. Representative Western blot images of IRF1, ATG5, ATG7, and BECN1 in LCC1 cells treated with vehicle (control), 100 nM ICI, 5 mM 3-MA, 10 μM HCQ, or transfected with ATG5, ATG7, or BECN1 siRNA for 48 hours. β-actin served as the loading control. n=3 independent experiments.
However, the ER- cell line, MDA-MB-231, did not exhibit enhanced IRF1 expression following transfection with ATG7 or BECN1 siRNA (Figure 3.14D). To better understand the relationship between ATG7 and IRF1 protein expression, we overexpressed ATG7 in LCC1 cells and found that ectopic expression of ATG7 significantly reduced IRF1 expression (Figure 3.14E; \( P < 0.05 \)). Taken together, these results suggest that ER+ breast cancer cells use autophagy machinery (ATG7 and BECN1) to regulate IRF1 expression and block apoptosis.

### 3.3.10. IRF1 localizes to the nucleus following ATG7 and BECN1 knockdown and inhibits BCL2 expression

To examine further the relationships among IRF1, ATG7/BECN1, and ER status, we first used confocal microscopy to establish the subcellular localization of IRF1 and ER\(\alpha\) following ATG7 and BECN1 knockdown. ER+ LCC1 cells were transfected with IRF1, ATG7, BECN1, or control (Ctrl) siRNA for 48 hours and stained for IRF1 (green) and ER\(\alpha\) (red); DAPI staining indicates nuclear localization (Figure 3.15A). In LCC1 control cells, IRF1 and ER\(\alpha\) were predominantly localized in the nucleus and a significant amount of each co-localized (Figure 3.15A). Transfection with ATG7 or BECN1 siRNA enhanced the nuclear expression of IRF1; however, ER\(\alpha\) was no longer detected in nuclei with IRF1 knockdown (Figure 3.15A). The association between ER\(\alpha\) and IRF1 was further investigated by measuring basal ER\(\alpha\) and IRF1 protein levels in MDA-MB-231 (231-wt), and MDA-MB-231 cells transfected with a full-length ER\(\alpha\) cDNA (231-ER) using western blot hybridization. 231-ER cells had decreased IRF1 expression compared with their 231-wt counterparts (Figure 3.15B).
Figure 3.14. ATG7 and BECN1 knockdown induces IRF1 expression in ER+ breast cancer cell lines. A-B, MCF7, LCC9, T47D, and BT-474 were transfected with Ctrl, ATG7 siRNA (A) or BECN1 siRNA (B) and the following day, treated with 100 nM ICI for 48 hours. Western blot hybridization was used to measure IRF1, ATG7, and BECN1 protein expression. C, Mitochondrial permeability assay measured by flow cytometry in LCC1 and LCC9 cells transfected with ATG7, BECN1, or control (Ctrl) siRNA. D, IRF1 expression was measured in MDA-MB-231 cells transfected with ATG7, BECN1, or Ctrl siRNA. E, LCC1 cells were transfected with ATG7 cDNA or empty vector and treated with 100 nM ICI for 48 hours. The expression of IRF1 and ATG7 were analyzed by Western blot. β-actin served as the loading control. n=3 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001 versus control/vehicle experiment.
We were then interested in measuring the effect of ERα knockdown on IRF1 protein expression. LCC1 cells were transfected with a small hair RNA (shRNA) targeting ERα or control shRNA. The following day, cells were treated with 100 nM ICI for 72 hours and protein homogenates were collected for Western blot hybridization. These samples were established and validated for ERα as previously described (244). While no difference in full-length IRF1 (48 kDa) was detected between ERα and control shRNA groups, ERα shRNA caused an IRF1 splice variant (~28 kDa) to form (Figure 3.15C). Interestingly, no IRF1 splice variants of this size have been cited in the literature before; thus, further research is necessary to understand the functional role of ER-mediated IRF1 degradation/splicing.

BCL2 expression is induced by 17β-estradiol, whereas BCL2 promoter activity is reduced by IRF1 (116). To determine whether IRF1 regulated BCL2 following suppression of autophagy, we measured BCL2 protein expression in LCC1 and LCC9 cells transfected with ATG7, BECN1, or Ctrl siRNA. Knockdown of either ATG7 or BECN1 significantly increased IRF1 expression and inhibited BCL2 expression in both LCC1 and LCC9 cells (Figures 3.16A and B). While antiestrogen-resistant LCC9 cells have higher basal BCL2 expression compared with LCC1 cells, BCL2 levels fell when either ATG7 or BECN1 was knocked down (Figures 3.16A and B). These data strongly support a functional link between ER-mediated IRF1 induction and BCL2 repression in autophagy-deficient breast cancer cells.
Figure 3.15. IRF1 localizes to the nucleus following ATG7 and BECN1 knockdown.

A, LCC1 breast cancer cells transfected with IRF1, ATG7, BECN1, or Ctrl siRNA were used to determine IRF1 and ERα localization by electron microscopy. B, MDA-MB-231 cells were transfected with ERα or control cDNA and treated with vehicle or 100 nM ICI for 48 hours. C, LCC1 cells transfected with ERα or control shRNA were treated with vehicle or 100 nM ICI for 72 hours. Western blot hybridization of protein homogenates was used to measure ERα and IRF1 expression. β-actin or β-tubulin served as the loading control. n=3 independent experiments.
Figure 3.16. ATG7 and BECN1 knockdown reduces BCL2 protein expression. A-B, LCC1 and LCC9 cells with transfected with Ctrl, ATG7 (A) or BECN1 siRNA (B) and treated with 100 nM ICI for 48 hours. Western blot hybridization of protein homogenates was used to measure IRF1, ATG7, BECN1, STAT1, P-STAT1, and BCL2. β-actin served as the loading control. Representative image of at least 3 independent experiments.
Because IRF1 can be activated by the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (128), we next investigated whether inhibition of this pathway would block IRF1 expression in LCC1 cells deficient in ATG7 and BECN1. Despite inhibition of STAT1 and STAT2 with STAT1 siRNA and a JAK inhibitor (JI1; C19H16FN3O), IRF1 remained elevated in ATG7 (Figure 3.17A) and BECN1 knockdown cells (Figure 3.17B). IRF1 gene expression is also dependent on reactive oxygen species (ROS) formation (245). We found that LCC1 cells transfected with ATG7 siRNA had increased levels of ROS compared with control-transfected cells (Figure 3.18A). However, treatment with 5 mM N-acetylcysteine (NAC), an antioxidant that suppresses ROS, had no effect on IRF1 suppression in ATG7 deficient cells (Figure 3.18B). Collectively, these data demonstrate that ATG7 and BECN1 regulate IRF1 through an ER-mediated but STAT1/2 and ROS independent mechanism.

3.3.11. Knockdown of IRF1 stimulates autophagy

To further investigate the relationships among IRF1, ATG7, and BECN1, we knocked down IRF1 in antiestrogen-sensitive and -resistant breast cancer cells and determined its effect on autophagy. LCC1 and LCC9 cells were transfected with IRF1 or Ctrl siRNA and treated with vehicle or 100 nM ICI for 48 hours. Consistent with previous reports (116), we found increased levels of BCL2 when IRF1 was repressed (Figure 3.19A). Western blotting also demonstrated that the total expression of LC3-II (the lipidated form of LC3 found in autophagosome membranes) was increased in LCC1 and LCC9 cells expressing little to no IRF1 (Figure 3.19A). Furthermore, p62/sequestosome-1
Figure 3.17. Silencing ATG7 and BECN1 induces IRF1 expression independently of STAT1. A-B, LCC1 cells were transfected with Ctrl, ATG7/BECN1, STAT1, ATG7/BECN1+STAT1 siRNA and treated with vehicle 100 nM ICI, or 15 nM JAK inhibitor (JI1) for 48 hours. Protein homogenates were probed for the indicated proteins. β-actin served as the loading control. n=3 independent experiments.
Figure 3.18. Silencing ATG7 and BECN1 induces IRF1 expression independently of ROS. A, LCC1 cells transfected with Ctrl or ATG7 siRNA were treated with 100 nM ICI for 48 hours. Total ROS production was quantified using flow cytometry. Data are presented as total ROS-stained cells (green fluorescence) relative to vehicle control and represent the mean ± SEM for ≥ 3 independent experiments. B, LCC1 and LCC9 cells were transfected with Ctrl or ATG7 siRNA and treated with 5 mM of the ROS inhibitor, N-acetyl-L-cysteine (NAC) for 48 hours. Protein homogenates were collected and probed for the indicated proteins. β-actin served as the loading control. n=3 independent experiments.
(SQSTM1; an autophagic cargo marker degraded in the final stages of autophagy) was reduced in LCC1 cells transfected with IRF1 siRNA (Figure 3.19A).

To confirm our observation that breast cancer cells with reduced IRF1 undergo enhanced autophagy, we used both confocal microscopy and flow cytometry to measure autophagic vacuole formation. LCC1 and LCC9 cells transfected with IRF1 or Ctrl siRNA were treated with vehicle or 100 nM ICI, and 48 hours later, labeled with a modified monodansylcadaverine to measure autophagosome formation. While ICI slightly enhanced autophagosome formation ($P < 0.05$), cells transfected with IRF1 siRNA had significantly higher numbers of autophagic vacuoles ($P < 0.001$) compared with Ctrl siRNA treated cells (Figure 3.19B). By immunofluorescence, we measured LC3 punctae formation in LCC1 cells transfected with IRF1 or Ctrl siRNA (Figure 3.19C). IRF1 knockdown cells showed elevated LC3 punctae compared with control cells, further indicating that loss of IRF1 leads to increased autophagy. In contrast, overexpression of IRF1 in LCC9 cells resulted in an accumulation of p62, decreased LAMP2 (a critical protein involved in autophagosome/lysosome fusion), and increased cleaved caspase 7 (an apoptotic marker) (Figure 3.19D). Taken together, these results suggest that IRF1 inhibits autophagy through modulation of autophagic vacuole and lysosome fusion.

**3.3.12. IRF1 knockdown increases BECN1 and suppresses IGF1R and mTOR signaling**

To identify autophagy effectors downstream of IRF1 action, we performed gene expression microarray analysis on MCF7 cells transfected with IRF1 cDNA and control
Figure 3.19. **IRF1 knockdown induces autophagy.** A, LCC1 and LCC9 cells were transfected with IRF1 siRNA and treated with 100 nM ICI for 48 hours. Western blot analysis of protein homogenates was used to measure expression of IRF1, BCL2, LC3B, and p62. B, Autophagosome formation assay performed in LCC1 and LCC9 cells transfected with IRF1 or Ctrl siRNA and treated with 100 nM ICI. Data are presented as percentage of total cells positive for green fluorescence; n=3; *P < 0.05, ***P < 0.001 versus control/vehicle experiment. C, LCC1 cells were transfected with IRF1/Ctrl siRNA and LC3-GFP and then treated with ICI. The following day, cells were fixed, permeabilized, stained for IRF1 and DAPI, and visualized by confocal microscopy. D, LCC9 cells were transfected with IRF1 or control (empty vector) cDNA and treated with 100 nM ICI. Representative Western blot images of indicated proteins. β-actin served as the loading control. n=3 independent experiments.
cells (MCF7 cells transfected with an empty vector). Interestingly, overexpression of IRF1 decreased insulin-like growth factor 1 receptor [IGF1R; mediates cancer cell growth and survival through the PI3K-AKT pathway (246)] and increased eukaryotic translation initiation factor 4E binding protein 1 [EIF4EBP1; 4E-BP1; mediates protein translation by various stimuli (247)] mRNA expression (Figure 3.20; \( P < 0.05 \) and \( P < 0.01 \), respectively).

We then measured the expression of selected autophagy and growth factor proteins in LCC1 and LCC9 cells transfected with IRF1 or Ctrl siRNA (Figures 3.21A-C). The essential autophagy protein, BECN1, contains an ISRE in its promoter and is negatively regulated by IRF1 in LCC1 and LCC9 cells (Figures 3.21A-C). Mammalian target of rapamycin (mTOR) inhibits the initiation of autophagy and is downstream of IGF1R (90). Knockdown of IRF1 with siRNA inhibited IGF1R and mTOR protein expression (Figures 3.21A-C). Furthermore, LCC1 and LCC9 cells transfected with IRF1 siRNA have reduced phosphorylated 4E-BP1, which leads to its dissociation from eukaryotic translation initiation factor 4E (eIF4E) and activation of mRNA translation (Figures 3.21A-C). These findings suggest that IRF1 may inhibit autophagy through alteration of the IGF1R/mTOR and BECN1 pathway.

**3.3.13. IRF1 knockdown does not affect glucose uptake**

Since loss of IRF1 altered IGF1R expression in LCC1 and LCC9 cells, we next determined whether silencing IRF1 mediated glucose uptake. LCC1 cells were transfected with IRF1 or Ctrl siRNA for 48 hours and then cultured in glucose free
Figure 3.20. IGF1R mRNA is downregulated and 4E-BP1 mRNA is upregulated in MCF7 cells overexpressing IRF1. IGF1R and 4E-BP1 mRNA expression as measured by microarray analysis in IRF1-overexpressing or control (vector only) MCF7 cells. The graph represents IGF1R and 4E-BP1 mRNA levels (fold change) as compared with MCF7 control cells. Data represent the mean ± SEM for 3 independent experiments.
Figure 3.21. Knockdown of IRF1 alters BECN1, IGF1R/mTOR protein expression, and downstream signaling. LCC1 and LCC9 cells were transfected with IRF1 or Ctrl siRNA and treated with vehicle or 100 nM ICI for 48 hours. Western blot analysis of protein homogenates was used to measure the expression of the indicated proteins. β-actin served as the loading control. B-C, Densitometric analysis from (A). n=3; *P < 0.05 versus control/vehicle experiment.
medium containing vehicle or 50 µM apigenin (a flavonoid that inhibits glucose transport) for 3 hours. Silencing IRF1 in LCC1 cells did not alter glucose uptake, while apigenin had a modest effect in reducing glucose uptake (Figure 3.22A). We also examined whether silencing IRF1 had any effect on altering expression of the glucose receptor, Glut1 (Figure 3.22B). LCC1 cells with reduced IRF1 expression also had reduced Glut1 expression, which was not further altered by ICI treatment (Figure 3.22B). These data suggested that IRF1 might play a small role in facilitating glucose transport across the plasma membrane of human breast cancer cells.

3.3.14. Mathematical modeling of IRF1-related signaling data

We next determined how silencing ATG7 and/or IRF1 affects ICI responsiveness in antiestrogen sensitive breast cancer cells. Loss of IRF1 expression contributes to antiestrogen resistance and a dominant-negative IRF1 eliminates the ICI-induced apoptotic response in antiestrogen sensitive breast cancer cells (133). To confirm that IRF1 siRNA had the same effect on ICI responsiveness, we measured the effect of IRF1 knockdown on ICI-mediated inhibition of cell density using crystal violet assays. Western blot analysis confirmed that IRF1 and ATG7 successfully inhibited expression of the appropriate target (Figures 3.23A and B). LCC1 cells transfected with IRF1 were markedly less sensitive to growth inhibition by 10-1000 nM ICI treatment compared with Ctrl siRNA transfected cells (Figure 3.23A; $P < 0.05$). Cells were then transfected with ATG7 siRNA alone and in combination with IRF1 siRNA to determine whether ATG7 knockdown could resensitize IRF1-deficient cells to ICI. While IRF1 siRNA blocked ICI responsiveness, LCC1 cells were growth inhibited by ATG7 siRNA alone, which was
Figure 3.22. Knockdown of IRF1 by siRNA does not affect glucose uptake. A, LCC1 cells were transfected with siRNA targeting IRF1 or scrambled siRNA for 48 hours and then cultured in glucose free media containing vehicle or 50 µM apigenin. After three hours of treatment, 150 µg/ml 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG) was added to each well of cells. After 15 minutes of incubation, the amount of 2-NBDG taken up by cells from different treatments was analyzed by flow cytometry. Glucose uptake is represented by 2-NBDG uptake and represent the mean ± SEM for ≥ 3 independent experiments. B, Western blot analysis of protein homogenates was used to measure Glut1 expression. β-actin served as the loading control; n=3 independent experiments.
further decreased by 100 nM ICI treatment (Figure 3.23B). LCC1 cells with silenced IRF1 and ATG7 had restored sensitivity to 100 nM ICI compared with cells transfected with IRF1 siRNA (Figure 3.23B; \( P < 0.001 \)).

To capture and quantitatively understand the experimental facts detailed above, we created a mathematical model based on the key molecular components (ER\( \alpha \), IRF1, ATG7, IGF1R) and their interactions. Parameter values for the model were chosen to minimize the difference between model simulations of proliferation and the experimental values. We found that two different models (‘full’ and ‘partial’) could fit the experimental data. The ‘full model’ predicted that ICI decreased proliferation through an IRF1-independent path (due to the direct effect of ER\( \alpha \) on proliferation) and a number of IRF1-dependent paths (Figure 3.24A; left panel). The ‘partial model’ suggested that proliferation was controlled through IRF1-dependent paths only (Figure 3.24B; left panel). Parameter sets can be found for both scenarios to fit the experimental data quite well (Figures 3.24A and B; middle and right panels). This led us to further examine whether the resensitization by ATG7 knockdown was due solely to the IRF1-dependent path or required an IRF1-independent path.

3.3.15. Silencing ATG7 restores ICI responsiveness in an IRF1-independent manner

To distinguish between the ‘full’ and ‘partial’ models as described above, we simulated both models under conditions of a more severe knockdown of IRF1. In the ‘full model’, a higher concentration of IRF1 siRNA resulted in ICI effectively inhibiting cell proliferation in cells with joint IRF1 and ATG7 knockdown (Figure 3.25A). However, in
Figure 3.23. Knockdown of IRF1 and ATG7 restores ICI sensitivity in LCC1 breast cancer cells. A-B, Top panel: Western blot analysis of LCC1 cells transfected with Ctrl, IRF1, ATG7, or IRF1+ATG7 siRNA. β-actin served as the loading control. n=3. A, Bottom panel: LCC1 cells transfected with Ctrl or IRF1 siRNA were treated with vehicle control, 1 nM, 10 nM, 100 nM, or 1000 nM ICI for 6 days and cell density measured by crystal violet. B, Bottom panel: LCC1 cells transfected with Ctrl, IRF1, ATG7, or IRF1+ATG7 siRNA were treated with vehicle control or 100 nM ICI for 6 days before crystal violet assay. Relative cell densities of each case are plotted. n=3 independent experiments; *P < 0.05.
Figure 3.24. Mathematical modeling of proliferation data. A and B, Left panel: Wiring diagrams of the ‘full’ and ‘partial’ model. Middle and right panels: Average simulation results for the ‘full’ and ‘partial’ models. Both model simulations could reproduce data from B.
the ‘partial model’, ICI was ineffective in reducing cell proliferation in LCC1 cells with silenced IRF1 and ATG7 (Figure 3.25B). We then performed an additional set of experiments using an increased concentration of IRF1 siRNA to determine the underlying signaling in this situation. While siIRF1 alone killed cells at this concentration, it can be seen that ICI was effective when both IRF1 and ATG7 were knocked down, lending support to the ‘full model’ (Figure 3.25C). Thus, our findings support a model in which ATG7 knockdown causes ERα to exit the nucleus and reduce proliferation not only by activating nuclear IRF1, resulting in cell death, but also through an IRF1-independent effect on proliferation (Figure 3.25D). Overall, these data imply that an IRF1-independent path from ERα to proliferation is an important component in the resensitization of IRF1 knockdown cells to ICI by also knocking down ATG7.

3.3.16. Knockdown of IRF1 mediates sensitivity to the cytotoxic drug, doxorubicin, but not paclitaxel

We observed a significant difference in the responsiveness of LCC1 cells transfected with IRF1 siRNA to ICI compared with cells transfected with Ctrl siRNA. To determine whether the observed alteration was seen with chemotherapeutic agents, we treated LCC1 cells transfected with Ctrl or IRF1 siRNA with increasing doses of doxorubicin (DOX; anthracycline family) or paclitaxel (PAC; taxane family) (Figures 3.26A and B). Surprisingly, knockdown of IRF1 significantly reduced the growth inhibitory effects of DOX (Figure 3.26A) at all concentrations but not with PAC treatment (Figure 3.26B) in LCC1 cells. These data suggested that IRF1 mediates sensitivity to the cytotoxic drug DOX but not PAC.
Figure 3.25. Silencing ATG7 mediates cell proliferation in an IRF1-independent way. A, Simulations of the full model predicted that ICI was still effective in reducing cell proliferation in LCC1 cells transfected with IRF1 and ATG7 siRNA. B, Simulations of the partial model predicted ICI was ineffective in the case of IRF1+ATG7 knockdown. Both models were simulated under increased concentrations of IRF1 siRNA. C, To select the best model, LCC1 cells were transfected with Ctrl, ATG7 siRNA, or three times the concentration of IRF1 siRNA. Cells were treated with 100 nM ICI for 6 days and cell density measured by crystal violet. Relative cell densities of each case are plotted. n=3 independent experiments; ***P < 0.001 versus indicated experimental groups. D, Model of how ATG7 knockdown resensitizes IRF1-deficient breast cancer cells to death.
Figure 3.26. IRF1 mediates sensitivity to the cytotoxic drug, DOX, but not PAC. A-B, LCC1 cells transfected with Ctrl or IRF1 siRNA were seeded in 96-well tissue culture dishes 24 hours before treatment with DOX or PAC at the indicated concentration. 48 hours post treatment, cells were stained with crystal violet and cell density was determined with a plate reader at wavelength 550 nm. Data are presented as relative cell density and represent the mean ± SEM for ≥ 3 independent experiments; ***P < 0.001 between indicated experimental groups.
3.3.17. Knockdown of epidermal growth factor receptor (EGFR) reduces IRF1 protein expression

Previous reports suggest that EGFR activates a module of IFN-associated genes, including IRF1 (248). To determine whether IRF1 protein expression was dependent on EGFR signaling, we knocked down EGFR using siRNA and measured IRF1 protein levels. Using the EGFR siRNA, we detected a 50% reduction in EGFR protein expression; IRF1 protein levels were also significantly reduced (Figures 3.27A and B). Furthermore, we did not observe any changes in EGFR expression following treatment with ICI. Since we established an association between IRF1 and BCL2, we examined the effect of EGFR knockdown on BCL2 protein levels. While loss of EGFR did not affect BCL2 expression in LCC1 cells, we noted a reduction in BCL2 protein levels in LCC9 cells (Figures 3.27A and B). These data suggested that EGFR mediates IRF1 protein expression and may play a role in BCL2 regulation.
Figure 3.27. Silencing EGFR using siRNA reduces IRF1 protein expression. A, LCC1 and LCC9 cells transfected with EGFR (siEGFR) or Ctrl (siCtrl) siRNA were treated with 100 nM ICI for 48 hours. Western blot hybridization of protein homogenates was used to measure IRF1 and BCL2 expression. β-actin served as the loading control; representative images from 3 independent experiments. B, Densitometric analysis of IRF1 expression from ≥ 3 separate experiments.
3.4 Discussion

The results presented here reveal that IRF1 is a critical signaling protein that contributes to the switch between apoptosis and autophagy to determine breast cancer cell fate. We found that nuclear IRF1 and ATG7 expression were inversely correlated in human breast carcinomas and regulated by estrogen receptor signaling. We also established that knockdown of ATG7 and BECN1 induced nuclear IRF1 expression and increased mitochondrial membrane permeability. Furthermore, loss of IRF1 alone was sufficient to increase prosurvival autophagy. This function was mediated through the regulation of BECN1 and IGF1R/mTOR signaling. Using our experimental data, we built a mathematical model suggesting that ATG7 resensitized IRF1-deficient breast cancer cells to ICI treatment through both an IRF1-dependent and -independent pathway that involves other ER-regulated genes that can also modify cell fate outcomes. Redundancy in the regulation of cell fate decisions likely contributes to the challenge in finding new treatments for many recurrent breast cancers.

3.4.1. IRF1 expression in human breast cancer

Recent studies confirm that upregulation of autophagy can protect breast cancer cells from antiestrogens (90). Previous studies also report that IRF1 mediates the antiproliferative and proapoptotic effects in cancer cells (123, 131-133, 249). Thus, we investigated the relationship between nuclear IRF1 and the key autophagy protein, ATG7, in vivo, and in human breast cancer. Atg7-deficient mice exhibited increased nuclear IRF1 staining in DMBA-induced mammary tumors, kidney, and spleen, suggesting a broad role for IRF1 in autophagy signaling. Immunohistochemical staining
of IRF1 in human breast tumors was broadly consistent with a smaller study by Zhu et al., further supporting a tumor suppressive role for IRF1 (220). ATG7 expression was detected in 96% (90/93) of tumors and was inversely correlated with nuclear IRF1 expression ($r = -0.28$, $P < 0.01$). Since autophagy has a prosurvival role in breast cancer, ATG7 expression could enhance aggressiveness of breast cancer cells and their ability to adapt to apoptotic stimuli. Furthermore, ATG7 could be negatively regulating the activation state of IRF1 to determine breast cancer aggressiveness and response to therapy. Interestingly, we found that ER- tumor cells expressed detectable (1+ and higher) nuclear IRF1 staining compared with ER+ breast tumor cells. These findings suggested that some breast tumors may differentially regulate the activation state/subcellular localization of IRF1 through modulation of ATG7 and ERα.

3.4.2. Inhibition of autophagy proteins induces IRF1

We are the first to show that silencing the autophagy proteins, ATG7 and BECN1, induced IRF1 and restored drug sensitivity to ICI. Previous studies have reported that nude mice with $atg5$-deficient thymi showed massive inflammatory infiltrates, suggesting autophagy negatively regulated T cell activation (250, 251). We did not observe IRF1 induction with other autophagy inhibitors: 3-MA, HCQ, or ATG5 siRNA. Hence, ATG7 and BECN1 could be targeting different innate immune signaling molecules, specifically IRF1. We also found little to no detectable IRF1 induction in BT-474 or MDA-MB-231 cells when either ATG7 or BECN1 was silenced (Figures 3.12 and 3.13). BT-474 cells have amplified HER2, and in 3T3 cells transduced with Her2/neu, IRF1 became constitutively active (128). Thus, it is possible that BT-474 cells have constitutively
active IRF1, and knockdown of ATG7 with siRNA had no additional effect on IRF1.
MDA-MB-231 cells do not express ERα; thus, IRF1 induction by ATG7 and BECN1 in ER+ cells may be due in part to ERα action.

Our data illustrated that knockdown of ATG7 and BECN1 caused ERα to exit, and IRF1 to enter, the nucleus (Figure 3.14A). It was possible that ERα was inactivated and IRF1 was activated in response to ATG7 and BECN1 knockdown. We confirmed ERα regulation of IRF1 in the ER- cell line, MDA-MB-231, by overexpressing ERα and finding reduced IRF1 expression compared with control cells (Figure 3.14B). However, the role of altered receptor coactivator or corepressor expression in this situation is still unclear. For instance, the transcription factor p300 can regulate IRF1 promoter activity and acts synergistically with ERα (252). Examining the role of other coactivators/corepressors in this situation is undergoing. We also found that the upstream regulation of IRF1 following ATG7 and BECN1 knockdown was independent of STAT1 and ROS (Figures 3.16 and 3.17). Thus, it is likely that IRF1 was activated through another stimulator. IRF1 also inhibited BCL2 protein expression when either ATG7 or BECN1 was silenced (Figure 3.15). This is clinically important because TAM-resistant breast tumors have sustained BCL2 expression (76, 144). In the previous chapter, we showed that inhibiting BCL2 with the BH3 mimic, GX15-070 (obatoclax), in combination with an antiestrogen successfully inhibited breast cancer cell growth through modulation of apoptosis and autophagy (253). By knocking down prosurvival autophagy, we discovered that IRF1 is a shared protein between the apoptotic and autophagy pathways and is a critical regulator of breast cancer cell fate.
3.4.3. IRF1 knockdown enhances prosurvival autophagy

While IRF1 promotes apoptosis, we established that loss of IRF1 enhanced autophagy in human breast cancer cell lines. We investigated BECN1 protein expression since it contains an interferon regulated response element and is regulated by BCL2 (142) and found that IRF1 inhibited autophagy by reducing BECN1 (Figure 3.20). We also discovered that IRF1 might be an important regulator of LAMP2, a selective receptor for the import and degradation of proteins in the lysosome (Figure 3.18) (254). These data suggested that IRF1 might play a role in enhancing cell death by preventing autophagic cargo degradation. To further delineate how IRF1 inhibited the initiation of autophagy, we performed gene expression microarray analysis to find downstream targets transcriptionally regulated by IRF1. We were particularly struck by the altered expression of IGF1R (Figure 3.19), which is implicated in autophagy signaling and can also promote endocrine resistance (81). While IRF1 decreased IGF1R mRNA (Figure 3.19), IRF1 was necessary for the stability of IGF1R protein (Figure 3.20). It is possible that IRF1 is involved in IGF1R’s normal turnover or stability through protein-protein interactions. We then determined whether downstream signaling of IGF1R was effected by IRF1. Consistent with the reduction in IGF1R, both mTOR and phosphorylated 4E-BP1 were also reduced when IRF1 was silenced (Figure 3.20), indicating that IRF1 suppressed IGF1R/mTOR-mediated autophagy. Studies also report that IRF1 inhibits the autophagic response in lipopolysaccharide-stimulated macrophages by modulating the activation of mTOR (255).
3.4.4. Mathematical modeling of IRF1 signaling data

Using our experimental data, we presented two mathematical models fitting the observation that silencing ATG7 or BECN1 can block the effect of IRF1 siRNA on ICI responsiveness. To select the most correct model, an experiment for which the models predicted different results was identified and run, illustrating the usefulness of mathematical modeling to help evaluate complex biological behaviors and prioritize experiments. The new experimental results supported the view that ATG7 knockdown could resensitize IRF1-deficient cells to ICI through an IRF1-independent mechanism. This resensitization could be due to a switch from autophagy to apoptosis and be mediated by other proteins that are shared between pathways, such as BCL2 (256). It is also possible that silencing ATG7 activated other cell death pathways such as necroptosis or an ER-mediated stress pathway (257, 258). Our co-localization studies also supported the modeling by showing that ERα left the nucleus and stimulated nuclear IRF1 expression following ATG7 knockdown.

3.4.5. Agents to restore IRF1 in the clinical setting

Previous reports suggest that high mRNA levels of IRF1 are a marker for good prognosis for breast cancer patients given neoadjuvant therapy (259, 260). Given that IRF1 plays a pivotal role in mediating antiestrogen sensitivity, restoring IRF1 expression or controlling its modulation may be useful for the treatment of ER+ breast tumors. Currently, mixed results have been shown with IFNγ as an antitumor agent in the clinic (128). Recently, the natural compound baicalein has been shown to increase IRF1 activity and inhibit cancer cell growth (261). Using compounds that activate IRF1, such as baicalein, to
restore its activity could lead to better prognosis in breast cancer. We now show that inhibiting key autophagy proteins, ATG7 and BECN1 also induces IRF1, which could provide a possible therapeutic option in the future. This novel ATG7/IRF1 axis provides new insight into the mechanisms underlying antiestrogen resistance, and restoration of IRF1 may be a potential therapeutic strategy for the treatment of breast cancer in the future.
CHAPTER 4: DISCUSSION

4.1. Antiapoptotic BCL2 expression contributes to antiestrogen resistance

We established a crucial role for BCL2 in the modulation of antiestrogen-induced autophagy and apoptosis signaling pathways downstream of IRF1. BCL2 has previously been implicated in antiestrogen sensitivity by allowing resistant cells to evade apoptosis (113). A major challenge in breast cancer management is to identify patients who will benefit endocrine therapy. BCL2 is a favorable prognostic marker in early-stage breast cancer (almost exclusively in ER+ disease) but contributes to resistance and metastasis following therapy (144, 262, 263). As death signals persist during tumor progression, cancer cells may become addicted to these survival mechanisms and be in a state of “BCL2 dependence.” Thus, identifying BCL2 expression in human breast cancer and targeting its expression with anti-BCL2 therapy could save many women from unnecessary cytotoxic drug treatment.

Based on solid evidence that BCL2-like proteins promote cancer cell survival (264), we investigated the functional role of BCL2 downstream of IRF1 using the small molecule BH3 mimetic, obatoclax (GX). We chose to use GX in our study based on both preliminary data and its potential for use in the clinic. First, GX is the only small molecule antagonist of BCL2 designed to inhibit all relevant members of the antiapoptotic BCL2 family of proteins, including the dominant member MCL1 (265). After testing the effects of GX and other BCL2 inhibitors, ABT-737 and HA14-1, on breast cancer cell proliferation, we discovered that GX was the only agent able to inhibit proliferation using a clinically relevant concentration. Secondly, we chose to use GX
based on its potential in the clinic. Numerous Phase I and II clinical trials are ongoing with GX for the treatment of lung cancers and various forms of leukemia and lymphomas (266). GX has also been fairly well tolerated with the main side effects including sleepiness, euphoria, loss of coordination, and oxygen desaturation (less than normal levels of oxygen carried in the blood) (267).

4.2. GX promotes cancer cell death through several mechanisms

Here, we identified that inhibition of BCL2 using the BH3 mimetic, GX, induced apoptosis and inhibited autophagic cargo degradation (253). First, we identified that GX promoted assembly of autophagosomes as demonstrated by the conversion of LC3-I to LC3-II. This engagement of autophagy was required for LC3 lipidation, as genetic silencing of BECN1 inhibited GX-triggered accumulation of autophagosomal membranes (Figure 2.8). Second, GX prevented degradation of the autophagy cargo protein, p62, which is normally degraded in the final stages of autophagy (Figure 2.11). Third, GX inhibited cathepsins D and L protein expression that would otherwise digest autophagic cargo (Figure 2.12). Thus, by dissecting the steps of autophagy, we provided new insight into the mechanisms of GX-induced non-apoptotic cell death. Furthermore, we identified that GX-mediated inhibition of autophagic cargo degradation was a key event connecting autophagy to cell death via apoptosis.

Cathepsin D is a well-characterized lysosomal hydrolase often dysregulated in human breast cancer patients (268). Our results indicate that GX inhibited cathepsins D and L protein expression, supporting the use of cathepsins D and possibly L as biomarkers for
GX sensitivity. Thus, further studies are necessary to confirm cathepsin D and L expression and/or activity as reliable biomarkers for GX treatment. This study also suggested that cathepsin inhibitors might be potentially useful in the clinic. Recently, another BCL2-related protein was proposed as a biomarker for chemotherapy in endocrine resistant breast cancers. Stone et al., found that BCL2 hypermethylation could provide a useful biomarker for cytotoxic therapy (269). Hypermethylation of the exon 2 region of BCL2 correlated with reduced BCL2 protein expression and provided a stronger predictor for patient survival when compared with BCL2 gene expression (269).

Although GX successfully inhibited breast cancer cell growth in vitro, we identified a major off-target effect of the drug. Many drugs, such as GX, are designed to target a specific protein or pathway but often interact with off-target proteins. To effectively block antiapoptotic BCL2 members and combine it with antiestrogen therapy, we need to design specific inhibitors that can be used at low concentrations. One of the most promising BH3 mimetics currently in clinical development for anticancer therapy is ABT-737 and its orally bioactive analogue, navitoclax (ABT-263). ABT-737 has subnanomolar affinities for BCL2, BCL-W, and BCL-XL (270). A recent study by Vaillant et al., showed that ABT-737 in combination with TAM was more efficacious than TAM alone at reducing tumor burden and extending survival in ER+ patient-derived xenograft models (271). Interestingly, ABT-737 counteracted tamoxifen-induced endometrial hyperplasia, suggesting that this deleterious effect of TAM may relate to BCL2 (271). However, ABT-737 does not target MCL1, which may result in resistance to ABT-737 through upregulation of MCL1. It is critical, therefore, that anti-BCL2
therapy exhibits a fine-tuned inhibition of MCL1 and other antiapoptotic BCL2 family members.

GX was recently described to trigger another mode of cell death called necroptosis, a programmed form of necrosis (272). This occurred through the assembly of a necrosome (comprising a distinct set of signaling proteins including RIP1, RIP3, and Caspase 8) on autophagosomes following GX exposure. Furthermore, RIP1 was required for GX-induced cell death downstream of autophagosome formation. This study, in addition to our own, highlights the diverse effects of GX and other small molecule drugs on tumor development. We must look beyond apoptosis as the mechanism of cell death by BCL2 inhibitors. BCL2 interacts with many proteins, including BECN1, RAD9, and can also regulate calcium homeostasis (273-275). Thus, in order to treat patients effectively, we must understand the molecular events that occur upon treatment with GX alone or in combination with other drugs.

4.3. Applications of GX study to the clinic

In the clinic, chemotherapy, anti-HER2, and endocrine therapies often fail to completely eradicate breast tumors. Targeting BCL2 in ER+ tumors might enhance the efficacy of these predominately proapoptotic treatments. Our data suggests that two drugs are often more effective than one and may in fact be the only strategy than can offer a cure for many cancers. Furthermore, effective cancer treatment will have to be administered using drug combinations that target distinct signaling pathways. Improving our understanding of the BCL2 network and describing the effects of inhibitors will be necessary to fully
exploit BCL2 dependence. This new information will then allow us to design predictive biomarkers and companion tests that are necessary to implement BCL2 inhibitors in an approach to personalized medicine.

4.4. Enhancing IRF1 activity for anticancer therapy

The IRF1 activator, IFNγ, was thought to have great promise as an immunomodulatory drug. Clinical trials using this cytokine started in 1986 and focused on side effects and dose escalation (276). Later trials then determined its therapeutic benefit against cancer and infections. The most common side effect of IFNγ includes “flu-like” symptoms and other common side effects such as fatigue, rash, diarrhea and nausea (276-278). The majority of clinical trials involving IFNγ have been performed using Actimmune or IFNγ-1b, a genetically engineered form of IFNγ. While in vitro studies show IFNγ has antitumor effects, is anti-angiogenic, and inhibits cell proliferation, there have been mixed results as to the efficacy of IFNγ in the treatment of various cancers (276, 279).

However, our understanding of interferon function is becoming much more refined and will lead us to tailor treatments to optimize effectiveness. A recent study found that when IFNβ binds to a cell, it transmits a signal that seems linked to some of the toxic side effects of interferon therapy (280). This provides a promising avenue to pursue more selective activation of interferon action. New approaches are also improving how cytokines are used for therapeutic application. Bansal et al., delivered IFNγ to fibroblasts and pericytes to directly inhibit their fibrotic activity using a platelet-derived growth
factor-beta binding carrier (281). This is one example of how novel therapeutic compounds can be designed to directly target cytokines to cancer cells.

Other such agents are known to induce IRF1 activation. Retinoids (vitamin A derivatives) and baicalein (a type of flavonoid) have shown preclinical utility as therapeutic agents in breast cancer (261, 282). Previous work from our lab showed that IRF1 was induced 7-fold by treatment with 9-cis retinoic acid (133). Baicalein, a phytoestrogen similar to genistein, enhances IRF1 activity and causes tumor suppression of cancer cells \textit{in vitro} and \textit{in vivo} (261). Thus, further investigation will be able to determine the potential clinical uses of these natural compounds for the treatment of breast cancer.

4.5. IRF1 expression in human breast cancer

We explored the expression levels of IRF1 and other functionally related proteins (PUMA, ATG7) in human breast cancer specimens from women diagnosed at our institution. Since IRF1 is a tumor suppressor, we expected only a small amount of activated IRF1 to be in the nucleus. Further, we expected that the cytosolic form would predominate and only a small proportion of tumors would contain high nuclear IRF1 staining. Consistent with this hypothesis, we found that 80% (86/107) of samples had cytoplasmic IRF1 and only 12% (13/107) of tumors had detectable nuclear IRF1 staining. Our findings were broadly consistent with the Zhu \textit{et al.} study reporting that 90% (38/42) of tumors expressed cytoplasmic IRF1 while 51% of cases exhibited high nuclear IRF1 staining (220). While the Zhu \textit{et al.} study only examined 42 human breast tumor cases, 80% of these tumors expressed the ER\(\alpha\). Therefore, the variation in these percentages
was likely due to sample size and ERα expression differences, both of which may affect IRF1 expression. Furthermore, Zhu et al. proposed that cytosolic IRF1 was inactive, while our data suggested that cytosolic IRF1 may also play a role modifying proteins near the mitochondria. Unfortunately, we could not assess whether nuclear or cytosolic IRF1 expression affected drug responsiveness or outcome due to a lack of clinical data.

Another study by Doherty et al. established that IRF1 was differentially expressed in high-grade DCIS and node-positive invasive cancer compared with normal adjacent tissue (217). Their data implied that IRF1 was lost during breast cancer progression. However, we found no difference in IRF1 expression levels between normal, DCIS, and invasive breast cancer tissue. It was possible that IRF1 expression levels were altered by drug treatment and since therapy data was unavailable, we cannot come to a clear conclusion. Another factor that could affect IRF1 expression is the amount of IFNγ at the tumor site. If there were a lack of IFNγ, or also inactivation of the receptor components or the signal transduction pathway elements, IRF1 would fail to be induced. Thus, further investigations investigating the differences in IRF1 expression among tumor types are warranted.

We are the first to examine ATG7 expression and determine whether it was correlated with IRF1 in human breast cancer. ATG7 expression was detected in 96% (90/93) of tumors and was inversely correlated with nuclear IRF1 expression ($r = -0.28, P < 0.01$). Since autophagy has a prosurvival role in breast cancer, ATG7 expression could enhance the aggressiveness of breast cancer cells and their ability to adapt to apoptotic stimuli.
Furthermore, ATG7 could be negatively regulating the activation state of IRF1 to determine breast cancer aggressiveness and response to therapy. In general, these results suggest that some breast tumors may differentially regulate the activation state of IRF1 through modulation of ATG7.

We also examined BCL2 and PUMA expression in human breast cancer and found no direct correlations with IRF1. We did note, however, that nuclear IRF1 expression was elevated whereas BCL2 was reduced in tumors with BRCA1 mutations. While low BCL2 characterized most BRCA1-associated breast carcinomas, this trait was not shared with BRCA2-associated tumors. Our data was consistent with the Freneaux et al. study suggesting that BRCA1-associated tumors have high apoptotic rates resulting from reduced BCL2 expression (283). We now showed that increased nuclear IRF1 could also contribute to the elevated rates of apoptosis. Sporadic breast tumors, on the other hand, often have high BCL2 expression, which is often associated with a favorable prognosis (283). Future studies investigating how BRCA1 functions to activate IRF1 and reduce BCL2 are warranted.

Interestingly, we found that ER- tumor cells expressed detectable (1+ and higher) nuclear IRF1 staining compared with ER+ breast tumor cells. Previous reports from our laboratory showed that while there was no IRF1 regulation by antiestrogens in the ER-cell line, MDA-MB-231, IRF1 was induced by the chemotherapeutic agent, doxorubicin, in these cells (133). These data suggested that DNA damage pathways remain intact in ER- breast cancer cells and can signal to IRF1. In ER+ cells, IRF1 regulation by
antiestrogens is mediated through ERα; when ERα is silenced or perturbed this signaling axis alters the responsiveness to antiestrogens. Therefore, it may be that patients with ER+ breast tumors with low IRF1 still respond to some chemotherapeutic agents, because signaling to IRF1 with these agents can occur independently of ERα expression.

4.6. IRF1 as a predictor of responsiveness to therapy

The studies contained here support IRF1 as a regulator of antiestrogen sensitivity and provide evidence that the antiproliferative effects are mediated by apoptosis and autophagy. While clinical outcome data was unavailable for the IRF1 stained human TMAs used in our study, our in vitro data supports the use of IRF1 as a potentially useful biomarker of antiestrogen sensitivity in breast cancer. Several lines of evidence also suggest that IRF1 predicts responsiveness to various forms of therapy. Kolacinska et al. showed that elevated IRF1 gene expression measured before neoadjuvant therapy was predictive for a complete response to chemotherapy or hormonal therapy (259). Further, IRF1 appears to play a central role in influencing different tumor phenotypes. Melanoma cell lines with high inducible IRF1 exhibited increased apoptosis, whereas tumor cells with low inducible IRF1 activation were associated with tumor aggressiveness and poor prognosis (284). Therefore, measuring IRF1 expression before treatment may be a successful strategy for identifying patients who will benefit from therapy.

Interestingly, we found that IRF1 mediated drug sensitivity to the cytotoxic drug, doxorubicin but not paclitaxel (Figure 3.26). While doxorubicin inhibits cancer cell growth by intercalating DNA and blocking DNA synthesis (285), paclitaxel functions by
interfering with the normal breakdown of microtubules (286). It is likely that these different mechanisms of action were contributing to the observed differences in breast cancer cell growth. IRF1 is involved in response pathways following DNA damage (206); therefore, we hypothesized that breast cancer cells with silenced IRF1 were unable to induce cell death in response to doxorubicin treatment due to a lack of DNA damage signaling. Paclitaxel treated cells, on the other hand, most likely underwent cell death through an IRF1-independent mechanism.

Currently, the only biomarkers that predict for the successful use of antiestrogens are the presence of the ER and/or PgR. While an ER+/PR+ phenotype predicts that approximately 75% of breast tumors will initially respond to antiestrogen therapy, nearly 50% of these tumors eventually recur (109). Due to this high rate of recurrence, it is unlikely that one mechanism alone is sufficient to induce acquired resistance. Rather, it is likely the dysregulation of an entire gene network that imparts this phenotype. Here, we show that IRF1 is a good potential biomarker for sensitivity to antiestrogenic agents. Other genes have been identified that correlate with poor prognosis, including p53, EGFR, and HER2 (287-289). Developing tumor biomarker tests will be critical for personalized medicine, which means that patients get treatments that benefit them specifically. Finding and using additional markers in the clinic will more specifically define antiestrogen resistance and better predict breast cancer survival.
4.7. IRF1 regulation of BCL2 family members

Most of IRF1’s antiproliferative properties are thought to occur via apoptosis (116, 130, 131). IRF1 has been shown to activate apoptosis by binding to and upregulating PUMA transcription in gastric cancer (290). Previous studies from our laboratory also indicate that IRF1 and BCL2 are inversely correlated in human breast tumors. Our results showed that IRF1 inhibited antiapoptotic BCL2, BCLW, and MCL1 expression while upregulating proapoptotic PUMA protein expression in antiestrogen sensitive and resistant breast cancer cell lines (Figures 3.3-3.6). These findings further link the tumor immune response to apoptosis in cancer cells. IRF1 regulation of BCL2 family members is clearly an important mechanism used by the immune surveillance system to rid the body of cancer cells.

BCL2 acts as an antiapoptotic regulator by localizing mainly to the mitochondria (291). While investigating IRF1 and BCL2, we found a novel population of IRF1 in mitochondria of LCC1 and LCC9 cells (Figure 3.7). While validation of IRF1 localization is necessary, we hypothesized that mitochondrial IRF1 acts to further regulate cancer cell proliferation through interacting with BCL2 family members. Isolation of mitochondria followed by immunoprecipitation of IRF1 and Western blotting for possible protein targets may reveal new important information. If IRF1 is localized to the mitochondria, it could be working to activate apoptosis via BCL2 regulation or modulating autophagy to promote cell death via proteins such as BECN1.
4.8. Association of IRF1 and IFNγ with autophagy

Recent developments reveal a crucial role for inflammatory proteins, including IRF1 and IFNγ, in the autophagy pathway. Consistent with our findings suggesting that knockdown of IRF1 induces prosurvival autophagy in breast cancer cells, Zhang et al. showed that IRF1 knockout mice exhibit increased autophagy compared with wild-type mice in response to lipopolysaccharide, an autophagy stimulator (255). Furthermore, the authors found reduced mTOR in IRF1 knockout macrophages that was mediated by nitric oxide (255). Other studies reported that IFNγ and its downstream signaling molecules induced autophagy. Situations like these occur during pathogen degradation, apoptotic corpse clearance, and protection against microbial toxins/factors (292). IFNγ has also been shown to induce autophagy by stimulating the cleavage of ATF6, a transcription factor that activates genes in the endoplasmic reticulum stress response (293).

Recent developments suggest that apoptosis may not account for all of IRF1’s growth inhibitory effects (294). We found that specific autophagy proteins can also regulate IRF1 activation. Since only ATG7 and BECN1 were found to negatively regulate IRF1 activation, it is possible that different autophagy proteins are designed to target different immune signaling proteins. ATG7 and BECN1 may be markers for particular stressors, which would lead to abnormal autophagy and IRF1 activation. It would also be of importance to further investigate whether this regulation occurs through autophagy-dependent or -independent mechanisms. Since IRF1 mediates mTOR activity, we also plan to investigate how knockdown of mTOR using siRNA effects IRF1 protein
expression. In general, these findings suggested that autophagy proteins act to achieve a balance between activation and inactivation of innate immune signaling.

4.9. Systems biology approach to studying IRF1 and autophagy signaling

Systems biology is an emerging field where complex biological systems are explained using a holistic perspective rather than the traditional reductionist approach (295). The fundamental premise of systems biology is to further understand our knowledge of molecular networks by gathering information on both its parts and dynamic interactions (296). We used a systems biology approach to study IRF1 and autophagy signaling by integrating computational modeling with quantitative experiments. Using mathematical modeling, we were able to predict two possible scenarios by which knockdown of ATG7 results in cancer cell death. Then, using quantitative experiments, we tested the two scenarios and determined that ATG7 knockdown induced cell death through an IRF1-independent mechanism. This approach allowed us to interpret the network as whole rather than just using laboratory techniques to study the visible parts of the system.

Systems biology approaches have also been used to discover that many tumor suppressor genes and oncogenes are involved in autophagy regulation. By studying cancer signaling networks, PTEN and Akt, were established as key regulators of autophagy (297, 298). A comprehensive study by Caron et al. helped establish that the mTOR signaling network is an attractive network target for cancer therapy and expanded our knowledge about autophagic related pathways in cancer (299). Studies like these will help us further understand the mechanisms of cancer and help us predict potential drug targets.
4.10. Future directions

Based upon the results presented in this body of work, it is clear that apoptotic and autophagy signaling play crucial roles in IRF1-mediated inhibition of breast cancer cell growth. A relationship between IRF1, antiapoptotic BCL2 family members, autophagy, and antiestrogen sensitivity likely exists and warrants further investigation. Questions that remain and would build upon our current findings are discussed below:

4.10.1. What are the effects of GX in vivo?

To consider GX as a future anticancer drug to combine with antiestrogens, in vivo studies using GX alone and in combination with ICI/TAM are necessary. These studies would firmly establish the cell death mechanisms by which GX inhibits breast cancer cell proliferation. Using an established preclinical tumor model, we would measure the effect of GX ± ICI/TAM on human breast cancer cell growth and validate our in vitro assays. Some anticancer drugs may be harmful to normal functioning cells or organs, so it is also vital that future studies investigate the effects of GX on non-malignant cells. Using a cell lines such as MCF-10A, a normal mammary gland cell, we could determine whether GX exposure results in a similar cell death mechanism or has no effect on healthy versus cancerous breast cancer cell lines. GX treatment has been used to treat other malignancies and has been well tolerated in the clinic. Therefore, future studies would involve testing GX alone or in combination with an antiestrogen in Phase I clinical trials.
4.10.2. Can IRF1 be used as a biomarker for antiestrogen sensitivity in human breast cancer?

Predicting whether a patient will respond to endocrine therapy is an important area of study in breast cancer research. It is vital that standard and novel biomarkers be tested in order to look at the heterogeneity of breast cancer. While our work supports IRF1 as a possible biomarker for antiestrogen sensitivity, further studies must be performed to validate IRF1 in this role. Thus, future studies would involve collecting data from human tumor tissue with known clinical outcome and assessing IRF1 expression. A recent study by Kolacinska et al., found that IRF1 gene expression significantly differed between breast cancer samples according to pathologic response (259). While studies like these are still preliminary, they are important in demonstrating that immune signatures correlated well with good prognosis. We would like to collect and analyze more human samples for IRF1 to successfully identify patients most likely to benefit from treatment.

4.10.3. What is the role of mitochondrial IRF1?

Regulation of apoptosis is mediated by the balance of pro- and anti-apoptotic BCL2 family members at the outer mitochondrial membrane (300). Our studies show that IRF1 regulates BCL2 protein expression and can be found in mitochondrial fractions of cell lysates. A role for mitochondrial IRF1 has yet to be established; thus, future studies are necessary in light of our interesting findings. Since IRF1 is known to bind to promoter sequences and regulate transcription, it may be that IRF1 is regulating mitochondrial DNA or other associated proteins, such as BCL2. Using various imaging techniques, we could examine precisely where in the mitochondria IRF1 is located. Furthermore, we
could determine how IRF1 expression affects mitochondrial gene expression using a mitochondrial gene chip array. As no literature has been published on this observation, we would be the first to highlight a novel role for IRF1 at the mitochondria.

4.10.4. What are the roles of other IRFs in breast cancer?

While this work focused primarily on IRF1 expression in human breast cancer, other IRFs have also been implicated in the progression of breast cancer. The IRF family of transcription factors consists of nine members, each of which contain a well-conserved DBD (128). Since a critical facet of IRF1’s function in host defense is regulation of oncogenesis, it is no surprise that other IRFs also possess tumor suppressive activity. IRF5 and IRF7 have recently emerged as other family members that regulate tumor suppression (301, 302). Using human breast cancer specimens, a recent study demonstrated that down regulation of IRF5 correlated with ER/PgR- tumor expression and increased invasiveness. Furthermore, overexpression of IRF5 inhibited breast cancer cell growth in vitro and in vivo through sensitization to DNA damage (301). IRF7, on the other hand, is associated with prolonged bone metastasis-free survival in breast cancer, but its expression does not affect primary breast cancer cell growth (302).

These studies demonstrate the importance of understanding IRF-driven innate immune pathways in the progression of cancer. Future studies will be required to determine the value of IRF signatures in predicting how patients will respond to therapy. It is possible that IRFs utilize both redundant and unique signaling pathways. Therefore, it is important that we learn more about IRF function so that their immunosurveillance capabilities can
be exploited for therapy. Results from our studies showed that both IRF5 and IRF7 protein expression were downregulated in antiestrogen resistant versus sensitive breast cancer cell lines (Figure 3.2). Thus, focusing our effects on understanding the role of these two IRFs would be highly relevant to the development of antiestrogen resistance.

IRF family members also do not function alone and are known to cooperate with other family members and other transcription factors (184). The relationship between IRF1 and NF-κB (Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1) is of particular interest because they exert opposing effects on cell growth and survival but are activated by a common set of stimuli (184). Understanding how IRFs and other transcription factors cooperate and antagonize each other is another important question to be addressed.

4.10.5. What is the relationship between ERα and IRF1?

We found that IRF1 and ERα failed to co-localize when ATG7 or BECN1 was silenced with siRNA (Figure 3.15A). While we identified a novel relationship between IRF1 and ERα, how ATG7 or BECN1 signals to ERα and regulate IRF1 remains unknown. To further investigate this relationship, we knocked down ERα to determine the effect on IRF1 protein expression. The molecular weight of wild-type IRF1 is 48 kDa and studies have indicated the modifications, such as SUMOylation, can increase the molecular weight of IRF1 (57). We identified an IRF1 band at 28 kDa, and while no literature has been published on this variant, it is possible that the 28 kDa-IRF1 is associated with a mutated IRF1 identified previously by our laboratory (187). Future studies include
genotyping this splice variant and determining whether it matches with our previous work.

4.10.6. What is the role of IRF1 in EGFR signaling?

EGFR is an important receptor tyrosine kinase involved in the regulation of cancer cell survival, proliferation, and motility (248). In addition to IRF1, microarray analyses performed in our lab established EGFR as a differentially regulated gene during the transition to antiestrogen resistance. Both IRF1 and EGFR were expressed twofold higher in LCC1 cells compared with LCC9 cells (99). It is possible that low levels of EGFR in LCC9 cells fails to induce IRF1, which remains low in these cells, or vice versa. While our studies investigating the relationship between EGFR and IRF1 are still developing, previous studies have shown that EGFR activates IRF1 through activation of STAT1 and STAT3 (248). It has also been suggested that crosstalk between ERs, EGFR, and IGFR is critical for resistance to endocrine therapies (303). Thus, it would be of interest to investigate EGFR regulation of IRF1 in both ER+ and ER- breast cancer cell lines to further define the role of ERα in this signaling axis. It may be possible that cells with high EGFR expression, such as MDA-MB-231 cells, induce IRF1 to promote downstream cell death pathways independently of ERα. This mechanism may also be part of a larger immune response that could better predict patient survival. Thus, the assessment of IRF1 and EGFR signaling would provide interesting clues to the development of growth factor and immune cross talk.
4.11. Conclusions

This work has addressed the critical problem of antiestrogen resistance in breast cancer by establishing IRF1 as a key determinant of cell survival that signals to BCL2 family members and autophagy proteins in response to antiestrogen therapy. While IRF1 is known to have tumor suppressive activities in breast cancer, how autophagy and apoptosis are regulated in the context of endocrine responsiveness was previously unknown. We now show that IRF1 affects endocrine responsiveness by acting through apoptotic and autophagy pathways and establish a new paradigm for combining endocrine and anti-BCL2 therapy. We further established that antiestrogen resistant breast cancer cells are sensitized to the BH3 mimetic, obatoclax, which acts by promoting apoptosis and blocking autophagic cargo degradation to induce cell death. These findings have broad implications for overcoming antiestrogen resistance in breast cancer and offer unique insights into how breast cancer cells evade cell death. In conclusion, this work has made significant contributions to the development of immune, apoptotic, and autophagy signaling in the context of endocrine responsiveness in breast cancer and proposes new, intriguing questions for future studies.
CHAPTER 5: BIBLIOGRAPHY


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