FBXL16 PROMOTES BREAST CANCER CELL GROWTH AND DIMINISHES FULVESTRANT RESPONSIVENESS BY STABILIZING ERα PROTEIN

A Dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by

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ABSTRACT

Shah, Krushangi Nirav, Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2022. FBXL16 promotes breast cancer cell growth and diminishes fulvestrant responsiveness by stabilizing ERα protein.

Breast cancer is the most prevalent cancer in women. Approximately 75% of breast cancers are estrogen receptor alpha positive (ER+) and are treatable with endocrine therapies and/or CDK inhibitors. However, endocrine therapy (ET) resistance and metastasis are major obstacles in advanced ERα+ breast cancer (ER+ BCa) therapeutics. Upregulated oncogenic ERα activity plays critical role in progression of ER+ BCa. One essential mechanism of regulating ERα signaling is the ubiquitination-dependent proteasomal degradation of ERα. Owing to its direct effect on ERα degradation, fulvestrant is a first-line FDA-approved ET for metastatic and locally advanced breast cancer and a second-line drug for treatment of unresponsive ER+ BCa progression. Unfortunately, intrinsic or acquired resistance to fulvestrant develops in majority of patients with advanced ER+ BCa. The mechanism underlying fulvestrant resistance is still largely unknown. In the current study, we have identified F-Box and Leucine-Rich Repeat Protein 16 (FBXL16) as a novel positive regulator of oncogenic ERα signaling. F-box proteins are major components of the SCF (SKP1-CUL1-F-box) E3 ubiquitin ligases that mediate protein ubiquitination. FBXL16 does not show detectable interaction with CUL1 and is a poorly studied F-box protein. Our lab has recently discovered that FBXL16 upregulates several oncoproteins targeted by SCF-E3 ligases, including c-myc and β-catenin. However, little is known about the roles
of FBXL16 in cancer. By data-mining of cancer-related databases and immunohistological analysis of BCa tissue microarrays, we found that FBXL16 is highly upregulated in invasive ductal and lobular carcinomas. There is a strong positive correlation between FBXL16 expression and ERα status, implying its important role in ER⁺ BCa. Our study reveals that FBXL16 stabilizes ERα and decreases ERα ubiquitination thereby promoting ERα-mediated transcription and breast cancer cell proliferation. Specifically, we identified that FBXL16 decreases estradiol-induced ERα degradation by antagonizing an E3-ubiquitin ligase, FBXO45. Moreover, FBXL16 silencing downregulates protein stability of constitutively active mutant ERα-Y537S and restricts proliferation of cells expressing ERα-Y537S. Silencing of FBXL16 accelerates fulvestrant-mediated ERα degradation and increases fulvestrant efficacy in inhibiting cell growth. In conclusion, our findings identify FBXL16 as a novel regulator of ERα signaling and a potential therapeutic target for treating advanced ER⁺ BCa.
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INTRODUCTION

ER+ Breast cancer and current treatment strategies

Breast cancer (BCa) is the second leading cause of cancer related deaths among women, causing half a million deaths worldwide (Jemal et al., 2010; Lukong, 2017; Bray et al., 2018). Breast tumors display high heterogeneity with a wide range of phenotypic and genotypic profiles, resulting in varied clinical features in patients (Almendro and Fuster, 2011). Based on the expression status of hormone receptors, including estrogen receptor (ER) and progesterone receptor (PR), and other genes, breast carcinomas are broadly classified into three subtypes: ER-positive (ER+), human epidermal growth factor receptor 2 enriched (HER-2+), and triple-negative (ER/PR/HER2-) BCas. HER2 is amplified and overexpressed in about 20% of BCas, which are more aggressive and fast-growing, and thus tend to have a worse prognosis (Mitri, Constantine, and O’Regan, 2012; Rybárová et al., n.d.). Monoclonal antibodies targeting HER-2 receptor, like trastuzumab (Herceptin®) and pertuzumab (Perjeta®), or tyrosine kinase receptor inhibitors, such as lapatinib (Tykerb®), neratinib (Nerlynx®), and Tucatinib (Tykysa®) effectively work against HER-2+ BCas by blocking the growth factor signaling that promotes tumor growth and progression (von Minckwitz et al., 2011, 2017; Echavarria et al., 2017; Murthy et al., 2020). Triple-negative tumors present challenges in terms of their high metastatic potential and receptor negativity which limits treatment options due to having no upfront therapeutic target (Hudis and Gianni, 2011; Tomao et al., 2015). About 10-20% of BCas are triple-
negative type tumors that are primarily treated with chemotherapeutic interventions, radiation, and/or surgical removal. Recent advancements in biomarker discovery, therapeutic regimen trials, and immunotherapy led to a series of latest FDA approvals like talazoparib (Talzenna®), sacituzumab (Trodelvy®) which hold promise for a better prognostic outcome for patients with triple-negative breast tumors (Hoy, 2018; Gunev Eskiler, 2019; Goldenberg and Sharkey, 2020, Anon., 2020). The third subtype of BCa, the ER+ subtype accounts for 65-70% of all BCAs (Nagaraj, Ellis, and Ma, 2012; Wang and Liu, 2015; Dai et al., 2015). ER+ primary tumors develop under the stimulation of the hormone estrogen (17β-estradiol), which exerts its proliferative effect through estrogen receptor α (ERα). As an important transcription factor, ERα is the key to cell growth in ER+ BCa (Heldring et al., 2007; Green and Carroll, 2007). Current treatment strategies for ER+ tumors focus on blocking ERα signaling through targeted endocrine therapies. Approved endocrine therapies work through different mechanisms: (i) reduce biosynthesis of estrogen by aromatase inhibitors like letrozole (Femara®), anastrozole (Arimidex®), and exemestane (Aromasin®); (ii) competitive inhibition of ERα signaling by selective ER modulators (SERMs) like tamoxifen (Nolvadex®) and raloxifene (Evista®); (iii) degradation of ERα by selective degraders like Fulvestrant (Faslodex®) (Cardoso et al., 2019; Visvanathan et al., 2019). Despite being highly efficacious in improving overall survival, 15% of tumors are intrinsically resistant to endocrine therapy, and a large subset of patients (~30%) develop resistance, leading to tumor recurrence and even metastasis to other organ sites (Encarnacion et al., 1993; Chang, 2012). Alterations in ERα signaling and/or other signaling pathways are considered to promote endocrine therapy resistance, disease progression, and metastasis.
ERα signaling in BCa cells

ERα is a member of the nuclear receptor superfamily which includes steroid receptors, thyroid receptors, and retinoid receptors. Structurally, ERα consists of two transactivation domains (AF1 and AF2), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) (Kumar et al., 1987) (Figure 1A). AF1 which is located on the N-terminus is responsible for ligand-independent ERα activity (Tora et al., 1989). The DNA-binding domain has a consensus palindromic sequence that causes receptor dimerization and DNA binding. The hinge region contains a nuclear localization sequence that facilitates the nuclear transport of ERα. The ligand-binding domain has another nuclear localization sequence followed by an AF2 region on the C-terminus which mediates ligand-dependent activation of ERα (Osborne et al., n.d.). ERα has important physiological role during development and puberty (Daniel, Silberstein, and Strickland, 1987; Mallepell et al., 2006). Estrogen/ERα signaling is critical for mammary gland development. ERα knockout mice show an underdeveloped mammary duct structure that lacks terminal end buds and extensions (Korach et al., 1996). However, ERα overexpression in breast tissue presents an increased risk for developing BCa, indicating its role in BCa initiation and progression (Khan et al., 1994, 1999; Frech et al., 2005). Ligand dependent ERα signaling is initiated with the binding of the hormone estradiol (E2) to ERα leading to the dissociation of ERα from chaperone proteins. Upon E2 binding, ERαs dimerize and translocate to the nucleus. Once in the nucleus, ligand-bound ERα binds to estrogen response element (ERE) binding sites in the promoter region of E2 target genes (classical pathway). Besides, ERα can regulate gene transcription without direct DNA binding by interacting with other transcription factors (AP-1, SP1, p300, NF-κβ) that are bound to their target gene
promoters (indirect interaction with DNA-nonclassical pathway) (Beato, 1989; Tsai and O’Malley, 1994; Carroll et al., 2006, p.). Instantaneously, different domains of ERα, including activation function domains (AF1 & AF-2), LBD domains, and DBD domains get phosphorylated at various sites, which are important for the recruitment of transcriptional co-factors and the assembly of the transcription machinery (Kumar et al., 1987; Kato et al., 1995). These cofactors include steroid receptor coactivators (SRCs) such as SRC-3, methyltransferases (i.e., CARM1), and acetyltransferases (like p300), which help with remodeling the chromatin for RNA polymerase to bind and initiate gene transcription (Figure 1B). Eventually, induction of target gene expression, including cyclin D1 and c-myc, promotes cell cycle progression and growth of ER+ cancer cells (Horwitz et al., 1996; Hanstein et al., 1996; Yi et al., 2017a; Osborne et al., n.d.). In addition to the estrogen-dependent activity of ERα, growth factor signaling through PI3k/Akt/mTOR or MAPK pathway leads to the phosphorylations of ERα and its coactivators at different sites and activates ERα-mediated transcription in a ligand-independent manner (Weigel and Zhang, 1998; Schiff et al., 2004, p.; VanHook, 2010).

Regulation of ERα signaling in BCa

ERα signaling is often perturbed by oncogenic signals leading to cancer progression, treatment resistance, and/or metastasis. Some of the key molecular mechanisms identified for dysregulation of ERα signaling are: (i) hyperactivation of growth factors and other ligand-independent pathways, (ii) imbalance in the expression of coregulators, (iii) mutations in the ligand-binding domain (LBD) of ERα, and (iv) alterations in ERα expression (Osborne et al., 2003; Zilli et al., 2009; Dixon, 2014; Zhou and Slingerland,
The possibility and clinical cases where amplified mitogenic stimulus may activate a particular growth factor signaling pathway and feed into ERα mediated transcription are well studied. Therapeutics like alpelisib (PI3K inhibitor), everolimus and temsirolimus (mTOR inhibitors), parthenolide (NF-κβ inhibitor), and olaparib (PARP inhibitor) have been developed to successfully block amplified growth factor signaling for the treatment of endocrine-resistant ER+ BCa (Rani et al., 2019). In addition, blockade of tumor growth by CDK4/6 inhibitors (palbociclib, abemaciclib) has proven to be effective in the treatment of metastatic ER+ BCa (Finn et al., 2009). Similarly, altered expression and activity of co-repressors and co-activators and the subsequent changes in their association with ERα at the target gene also lead to dysregulation in transcription complex assembly and ERα mediated transcription (deConinck, McPherson, and Weigel, 1995; Pinzone et al., 2004). Many important coregulators of ERα have been identified and investigated as biomarkers and potential treatment strategies for ER+ BCa (Cottone et al., 2001). Of note, a retrospective analysis showed SRC-3, a coactivator of ERα, as a prognostic biomarker in ER+ invasive breast carcinoma cases (Narbe et al., 2019). Another study stated that ER+ BCa patients with low expression of an ERα corepressor, NCOR1, exhibited resistance to tamoxifen treatment, therefore suggesting that NCOR1 expression is a predictive marker for tamoxifen sensitivity in ER+ BCa cases (Girault et al., 2003). Moreover, ERα mutations and alterations in ERα expression are long-standing challenges in the treatment of advanced ER+ BCa. Frequent mutations like Y537S and D538G in the LBD are shown to impair the binding of estradiol, tamoxifen, or fulvestrant to the receptor, making the tumor hormone-independent and irreversible to treatment (Toy et al., 2013a, 2017a).
A **Functional domains of ERα protein**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-1</td>
<td>185</td>
<td>250</td>
</tr>
<tr>
<td>DBD</td>
<td>250</td>
<td>312</td>
</tr>
<tr>
<td>Hinge</td>
<td>312</td>
<td>546</td>
</tr>
<tr>
<td>LBD/AF-2</td>
<td>546</td>
<td>595</td>
</tr>
</tbody>
</table>

(B) Schematic representation of ERα signaling in the BCa cell.

Estradiol induces ERα signaling by binding to ERE or other transcription factors (genomic). Upon binding of ERα to the promoter region, transcription coactivators like SRC-3, CARM1, and p300 assemble with ERα. CARM1 as a methyltransferase and p300 being an acetyltransferase mediate the transfer of methyl (Me) group on arginine (R) residues and acetyl (Ac) group on lysine (K) residues on histones, respectively, which help
in chromatin remodeling followed by RNA polymerase binding and gene transcription. Besides, ERs can mediate rapid non-genomic E2 action through interacting with and activating intracellular kinases such as MAPK, PI3K, and c-SRC (Adapted from (Yi et al., 2017b; Pham et al., 2019).
Most importantly, ERα expression itself is thought to be a predictor of treatment response and disease progression (Johnston et al., 1995a; Gutierrez et al., 2005a). Epigenetic changes and post-translational modifications like ubiquitination, SUMOylation, neddylation, phosphorylation, and palmitoylation regulate ERα protein expression, stability, and transcription activity (Lonard et al., 2000a; Reid et al., 2003a; Pedram et al., 2007; Karamouzis et al., 2008; Zhou and Slingerland, 2014; Anbalagan and Rowan, 2015; Jia et al., 2019). In addition, there are interplays among different posttranslational modifications of ERα. Of particular note, ubiquitination of ERα is regulated by other post-translational modifications like phosphorylation, neddylation, and acetylation. Moreover, ERα transactivation is shown to be linked to its proteasomal degradation since proteasome inhibitors that increase ERα protein stability can also impair transcription at certain sites (Lonard et al., 2000a; Reid et al., 2003a). Explicably, targeting ubiquitination and proteasomal degradation of ERα is one of the highly sought-after strategies to modulate ERα signaling.

Modulation of ERα stability by Ubiquitin Proteasome System (UPS)

The UPS is a primary protein degradation mechanism in eukaryotes. The ubiquitination process comprises of three main steps: (1) Activation of ubiquitin molecule by E1 ubiquitin-activating enzyme; (2) ubiquitin conjugation to an E2 ubiquitin-conjugating enzyme; (3) transfer of activated ubiquitin to the substrate by an E3 ubiquitin ligase. There are more than 700 E3 ligases identified in humans which are classified into three major classes: HECT type, RING-finger type, and RBR (Ring-betweenRING-RING) type. It is insinuated that ubiquitination and the subsequent degradation of ERα after the transcription
cycle are preceded by the recruitment of E3 ligase to the EREs of ER\(\alpha\) target genes along with transcriptional coactivators. This suggests that ER\(\alpha\) turnover is tightly controlled for the subsequent transcription cycle (Nawaz et al., 1999a; Reid et al., 2003a). Different E3 ligases like breast cancer type 1 susceptibility protein (BRCA1), Mouse Double Minute 2 (MDM2), Human Papilloma Virus E6-Associated Protein (E6AP), S-phase associated kinase-associated protein 2 (SKP2), Carboxy Terminus Of Hsp70-Interacting Protein (CHIP) are shown in BCa cell lines to ubiquitinate ER\(\alpha\) either in ligand-dependent or ligand-independent fashion. For example, E6AP is preferentially associated with E2-ligated ER\(\alpha\). Estrogen-mediated phosphorylation at Y537 residue of ER\(\alpha\) facilitates E6AP binding followed by transcription and ER\(\alpha\) degradation (Nawaz et al., 1999c; Sun et al., 2012a). MDM2 and CHIP are preferentially, but not exclusively, recruited to unliganded ER\(\alpha\). MDM2 is reported as a coactivator of ER\(\alpha\), whereas the coregulator function of CHIP is unknown (Saji et al., 2001a, p. 2; Fan, Park, and Nephew, 2005a; Duong et al., 2007, p. 2). ER\(\alpha\) is also shown to be a substrate of BRCA1-BARD1 \textit{in-vitro} (Eakin et al., 2007a). However, BRCA1 functions as either coactivator or corepressor under different contexts (Hashizume et al., 2001; Fan et al., 2002a; Calvo and Beato, 2011a, p. 1) (Table 1).

An important class of drugs for BCa treatment, SERDs (i.e. fulvestrant, AZD9496, bazedoxifene, and RAD1901), are designed to degrade ER\(\alpha\) through UPS (Yeh et al., 2013a; Wardell et al., 2013; Lai et al., 2015; Savi et al., 2015a; Garner et al., 2015). However, specific E3 ligase for SERDs-mediated ER\(\alpha\) degradation is not yet identified. Fulvestrant, an FDA-approved drug, increases the ER\(\alpha\) turnover rate and limits the pool of existing ER\(\alpha\) protein for subsequent transactivation cycles. This mechanism of action of
Fulvestrant has proven to be more effective than other endocrine therapies. Even though selective degradation of ERα is a successful strategy, fulvestrant has poor oral bioavailability. Thus, a growing interest has developed in designing a chemical strategy called PROTAC (Proteolysis-targeting chimera) to selectively degrade ERα (Lin, Xiang, and Luo, 2020). Briefly, PROTAC consists of two small molecules joined by a linker. One of the small molecules is designed to bind to the target protein while the other small molecule binds to and brings together the E3 ligase for the target protein enabling degradation of the target protein by utilizing the existing cellular machinery. So far, two different groups have developed PROTAC against ERα with a ligand for von Hippel–Lindau tumor suppressor (VHL) as an E3 ligase for ERα. These PROTACs were demonstrated to degrade ERα with an IC50 as low as 0.3 nM in MCF7 cells and showed antitumor activity in ER⁺ BCa cell lines (Jiang et al., 2018; Kargbo, 2019). VHL is previously shown to ubiquitinate ERα and HIF1α in normal conditions and ERα also in hypoxic conditions (Jung et al., 2012). A collaborative attempt from Arvinas and Pfizer led to the development of the first-ever commercial PROTAC targeting ERα, ARV-471, for the treatment of ER⁺/HER⁻ advanced BCa. ARV-471 significantly inhibited tumor growth in a PDX advanced tumor model with Y537S ESR1 mutation. The combination treatment of ARV-471 and CDK 4/6 inhibitor (Palbociclib) showed an additive effect in restricting tumor formation in the PDX model (Flanagan et al., 2019). Currently, ARV-471 development has successfully transitioned to phase 2 clinical trials. In addition to identifying E3 ligases for ERα, it is also important to investigate novel coactivators of ERα which can be therapeutically targeted using other approaches (RNAi, CRISPR) for ER⁺ BC treatment.
Interestingly, a series of E3 ligase family proteins have been recently discovered to function as coactivators of ERα signaling by stabilizing ERα protein. RING-finger family E3 ligases RNF8 and RNF31 were shown to stabilize ERα by promoting ERα monoubiquitination (Zhu et al., 2014b, p. 31; Wang et al., 2017a, p. 8). Other ligases, TRIM56 and SMURF1, promote ERα stability through K63-linked ubiquitination which likely blocks K48-mediated polyubiquitination of ERα (Yang et al., 2018a; Xue et al., 2019a, p. 56) (Table 1). Cullin-RING (Really Interesting New Gene) ligase family is the major class of E3 ligases, including the SKP1-Cullin-F-box protein (SCF) complex subfamily and the Anaphase-promoting complex (APC), out of which the SCF E3 ligase subfamily is the largest (Metzger et al., 2014; Nguyen, Wang, and Xiong, 2017). Several SCF-E3 ligases function as oncogenes or proto-oncogenes by playing key roles in modulating cell proliferation, survival, and migration/invasion (Jackson and Eldridge, 2002, p. 3; Cardozo and Pagano, 2004; Jia and Sun, 2011, p. 3). Thus, many research groups have attempted to develop small molecule inhibitors selectively targeting an SCF E3, especially after the FDA approval of Bortezomib (Velcade®), a general proteasome inhibitor for the treatment of multiple myeloma and lymphoma, and subsequent approvals of other proteasome inhibitors (Richardson et al., 2005; Kuhn et al., 2007; Chauhan et al., 2011).
Table 1: List of E3 ligases that regulate ERα expression and ubiquitination

<table>
<thead>
<tr>
<th>E3 Ligase</th>
<th>Class of E3 ligase</th>
<th>Mechanism of ERα ubiquitination</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>RING-type E3 ligase</td>
<td>Co-repressor; Evidence of in-vitro ubiquitination by BRCA1/BARD1 complex</td>
<td>(Hashizume et al., 2001, p. 1; Fan et al., 2002b, p. 1; Eakin et al., 2007b, p. 1)</td>
</tr>
<tr>
<td>MDM2</td>
<td>RING-type E3 ligase</td>
<td>Co-activator; Ligand-dependent and ligand-independent ubiquitination of ERα</td>
<td>(Saji et al., 2001a, p. 2; Duong et al., 2007, p. 2)</td>
</tr>
<tr>
<td>E6AP</td>
<td>HECT-type E3 ubiquitin ligase</td>
<td>Transcriptional activation followed by ERα degradation upon E6AP binding</td>
<td>(Nawaz et al., 1999d; Sun et al., 2012b)</td>
</tr>
<tr>
<td>SKP2</td>
<td>RING-type E3 ligase</td>
<td>Ubiquitination of ERα specifically in G1/S and S phase of cell cycle</td>
<td>(Bhatt et al., 2012a, p. 2; Zhou et al., 2014)</td>
</tr>
<tr>
<td>CHIP</td>
<td>Modified RING-type E3 ligase</td>
<td>Represses ERα mediated transcription and promotes ERα degradation</td>
<td>(Fan et al., 2005a)</td>
</tr>
<tr>
<td>RNF8</td>
<td>RING-type E3 ligase</td>
<td>Co-activator; increases ERα protein stability by promoting ERα monoubiquitination</td>
<td>(Wang et al., 2017a, p. 8)</td>
</tr>
<tr>
<td>RNF31</td>
<td>RING-type E3 ligase</td>
<td>Co-activator; increases ERα protein stability by inducing ERα monoubiquitination</td>
<td>(Zhu et al., 2014b, p. 31)</td>
</tr>
<tr>
<td>TRIM56</td>
<td>RING-type E3 ligase</td>
<td>Increases ERα protein stability by promoting K63 linked polyubiquitination and decreasing K48 linked polyubiquitination</td>
<td>(Xue et al., 2019a, p. 56)</td>
</tr>
<tr>
<td>SMURF1</td>
<td>HECT-type E3 ubiquitin ligase</td>
<td>Increases ERα protein stability by inhibiting K48 polyubiquitination</td>
<td>(Yang et al., 2018a, p. 1)</td>
</tr>
</tbody>
</table>
F-box proteins and their molecular function in regulating protein ubiquitination

In the SCF complex, F-box proteins are the substrate recognition components that recruit substrates for ubiquitination by E3 ligases (Kipreos and Pagano, 2000). F-box proteins are mainly categorized into three sub-groups based on the presence of the characteristic domains for substrate binding: FBXWs (with WD40 domains), FBXLs (containing leucine-rich repeats), and FBXOs (with other domains) (Figure 2A). Cullin1, another essential component of the SCF E3 ligase complex, acts as a scaffolding protein bridging the SKP1 via the N-terminus and E2 (ubiquitin-conjugating enzyme) as well as RBX1 via the C-terminus. F-box proteins interact with cullin1 through the adaptor protein SKP1. Post-translational modifications occur on the substrates in response to the stimulus which primes them for recognition by a specific F-box protein. Ultimately, the functional SCF E3 ligase complex assembles and initiates ubiquitination for proteasomal degradation of a substrate by utilizing ubiquitin molecule activated and conjugated by E1 and E2 enzymes, respectively (Figure 2B). Most frequently, phosphorylation or glycosylation in the substrate degron directs its ubiquitination and degradation. For instance, FBXW7 and FBXW1 (β-TRCP) bind to specific consensus phosphodegron sequences in their substrates, while ligases like FBXO2, FBXO6, FBXO44 recognize glycosylated substrates (Nash et al., 2001; Yoshida et al., 2002, 2003, p. 2; Orlicky et al., 2003; Glenn et al., 2008). F-box proteins are known to be involved in key vital cellular processes like cell proliferation, differentiation, cell division, survival, migration, and invasion. As such, many F-box proteins are shown to perturb cancer hallmark pathways, including cell cycle regulation, apoptosis, DNA damage response, and epithelial-mesenchymal transition. Depending on the domains they possess, different F-box proteins have specificity towards
a unique subset of substrates. Thereby, F-box proteins may act as either tumor suppressors or oncoproteins depending on the substrates to be targeted. Some of the extensively studied F-box proteins are FBXW7, SKP2, and β-TRCP (FBXW1). FBXW7 acts as a tumor suppressor by inducing the degradation of many oncoproteins like cyclin E, c-myc, mTOR, MCL1, c-Jun (Welcker et al., 2004; Wei et al., 2005; Zhang and Koepp, 2006; Mao et al., 2008; Ren et al., 2013). On the other hand, SKP2 is identified primarily as an oncoprotein due to its action on tumor suppressors like p21, p27, YAP1, and FOXO1 (Huang et al., 2005, p. 1; Yao et al., 2018; Hume et al., 2021, p. 2). The role of β-TRCP as a tumor suppressor or an oncoprotein is found to be context-dependent. Most importantly, β-TRCP degrades β-catenin which is a key oncogenic regulator of Wnt signaling, in which β-TRCP functions as a tumor suppressor (Hart et al., 1999). While the oncogenic function of β-TRCP is still arguable, a series of studies have shown that β-TRCP induces the degradation of tumor suppressors like Iκβα and FOXO3 (Xia et al., 2009; Tsai et al., 2010, p. 3). A list of a few known F-box proteins and their roles in cancers are summarized in Table 2.
Figure 2: SCF E3 ligase complex.

(A) Classification of F-box proteins. F-box proteins are classified into three subgroups depending on the substrate recognizing domains they possess: (i) 10 FBXW proteins containing WD40 domains, (ii) 22 FBXL proteins that possess leucine-rich repeats (LRR), (iii) 37 FBXO proteins harboring other domains

(B) F-box proteins induce protein ubiquitination and degradation. F-box proteins interact with SKP1 and assemble in the functional E3 ligase complex with SKP1 and cullin1. F-box proteins through their unique substrate recognition domains bring in the substrate to the SCF E3 ligase for ubiquitination and degradation by the proteasomal system. Ub: ubiquitin.
Table 2: Known substrates of F-box proteins and their functions related to cancer

<table>
<thead>
<tr>
<th>F-box protein</th>
<th>Substrates</th>
<th>Function (molecular signaling pathways)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor suppressors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBXW7</td>
<td>Cyclin E, c-myc, mTOR, NOTCH1, c-Jun, MCL1</td>
<td>Cell survival, cell cycle progression, proliferation, mTOR pathway, Apoptosis</td>
<td>(Welcker et al., 2004; Wei et al., 2005; Zhang and Koepp, 2006; Mo et al., 2007; Mao et al., 2008; Ren et al., 2013)</td>
</tr>
<tr>
<td>FBXL2</td>
<td>FOXM1, cyclin D, AURKB</td>
<td>Cell cycle checkpoints, cell proliferation, cytokinesis</td>
<td>(Chen et al., 2012, 2013, p. 2; Ueda et al., 2020, p. 1)</td>
</tr>
<tr>
<td>FBXO11</td>
<td>HIF1α, BCL6, SNAIL</td>
<td>HIF1α pathway, B-cell differentiation, EMT</td>
<td>(Duan et al., 2012, p. 11; Jin et al., 2015, p. 11; Ju et al., 2015, p. 11)</td>
</tr>
<tr>
<td><strong>Potential oncoproteins</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SKP2 (FBXL1)</td>
<td>Akt, p21, p27, RELN, FOXO1, YAP1</td>
<td>Cell survival, cell cycle checkpoints, apoptosis, transcription of hippo signaling genes</td>
<td>(Huang et al., 2005, p. 1; Chan et al., 2012; Yao et al., 2018; Du et al., 2019; Hume et al., 2021, p. 2)</td>
</tr>
<tr>
<td>FBXL20</td>
<td>PUMA, BAX, E-cadherin, SET</td>
<td>Apoptosis, cell invasion, EMT, cell viability</td>
<td>(Zhu et al., 2012, 2014a, p. 20; Manne et al., 2021, p. 20)</td>
</tr>
<tr>
<td><strong>Oncoprotein but tumor suppressor in some cases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-TRCP1/2</td>
<td>p53, Iκβα, β-catenin, c-myc, lipin 1, FOXO3</td>
<td>p53 pathway, cell viability/survival (Wnt signaling), NF-κβ pathway, anti-apoptotic, fatty acid synthesis, autophagy</td>
<td>(Winston et al., 1999; Hart et al., 1999; Xia et al., 2009; Tsai et al., 2010, p. 3; Popov et al., 2010; Shimizu et al., 2017)</td>
</tr>
<tr>
<td><strong>Ambiguous (unclear)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBXO45</td>
<td>N-cadherin, PAR-4, p73, ERα, FBXW7</td>
<td>Cell invasion, Apoptosis, neuronal differentiation, cell proliferation</td>
<td>(Pes chiaroli et al., 2009a, p. 45; Chen et al., 2014, p. 45; Han et al., 2016a; Richter et al., 2020, p. 45; Na et al., 2020, p. 45)</td>
</tr>
<tr>
<td>FBXW8</td>
<td>MAP4K1, IRS1</td>
<td>Cell proliferation differentiation</td>
<td>(Xu et al., 2008; Wang et al., 2014, p. 8)</td>
</tr>
</tbody>
</table>
Regulation of ERα stability by F-box proteins

SKP2, also known as FBXL1, is an F-box protein that mediates ERα ubiquitination and degradation upon E2 stimulation and functions as a coactivator of ERα (Bhatt et al., 2012b; Zhou et al., 2014). Phosphorylation of ERα at S294 by p38-MAPK mediates SKP2 binding and ERα degradation (Bhatt et al., 2012). In addition, another study identified SKP2 as a late-acting ERα coactivator that binds to ERα upon cyclin E-CDK2-mediated ERαS341 phosphorylation and induces ERα ubiquitination and degradation (Zhou et al., 2014, p. 2).

Another F-box protein FBXO45, a known E3 ligase for TP73, was first identified as an estrogen-inducible gene (Yoshida, 2005; Peschiaroli et al., 2009b, p. 45). A recent study indicated that FBXO45 interacts with ERα and induces its degradation upon treatment with Tissue Selective Estrogen Complex (TSEC, a formulation possessing ERα antagonistic activity) (Han et al., 2016a). While nearly 70 different F-Box proteins have been identified in humans, it is unclear whether any of F-Box proteins plays a positive role in regulating ERα protein stability. Our preliminary data suggest that FBXL16 is one such coregulator that is upregulated in ER+ BCa and may positively regulate ERα protein stability and activity.

Role of FBXL16 in development and cancer

FBXL16 is an understudied F-box protein. Genetic screening of F-box proteins showed that specific knockdown of FBXL16 in the eye, wings, or notum (thorax) region in drosophila caused lethal phenotype or detrimental developmental defects in the few that escaped lethality (Dui et al., 2012). Global knockout of FBXL16 in mice shows a perinatal lethal phenotype (Dickinson et al., 2016a), indicating the pivotal role of FBXL16 in...
development. A few reports identified FBXL16 expression in the brain and related diseases. A study analyzing gene expression of attention deficit hyperactivity disorder (ADHD) associated genes observed increased expression of FBXL16 in the female subgroup of ADHD meta-analysis data. Moreover, FBXL16 expression was downregulated in early and mid-prenatal stages and subsequently kept increasing after these stages. FBXL16 expression in the ADHD dataset clustered together with the MTRN gene which is important for axial network formation in the brain as well as glial cell development (Alonso-Gonzalez et al., 2019). High expression of FBXL16 was observed in a rat model for Parkinson’s disease (Qin et al., 2016). On the other hand, FBXL16 expression was reported to be significantly lower in glioblastoma tumors as compared to normal brain tissue (Mamoor, 2020). FBXL16 knockdown promoted the differentiation of mouse embryonic stem cells (ESC) along cardiomyocyte lineage (Honarpour et al., 2014a, p. 16). In another study, FBXL16 expression along with seven other genes is found to be increased 100-fold during lactation (Anantamongkol et al., 2009).

Little is known about the role of FBXL16 in cancers. A study reported that FBXL16 is a transcription target of E2F1, an oncogenic transcriptional factor (Sato et al., 2010a). Additionally, silencing of p16INK4a or p14ARF, both of which are cell cycle inhibitors and tumor suppressors, was shown to lead to upregulation of FBXL16 mRNA level in HeLa cells (Sato et al., 2010a). Moreover, FBXL16 was suggested to be a potential inhibitor of protein phosphatase 2A (PP2A), a tumor suppressor in multiple human cancers (Honarpour et al., 2014a, p. 16).
Role of FBXL16 in regulating protein ubiquitination and stability

FBXL16 contains an N-terminal F-box motif, a proline-rich region, and seven leucine-rich repeat domains in the C-terminus which are involved in protein-protein interaction (Figure 3). While many F-Box proteins like FBW7 and FBXL1 (SKP2) are known to form an SKP1-Cullin1-F-Box (SCF) E3 ligase complex for degrading their substrates, FBXL16 doesn’t show detectable interaction with CUL1 (Honarpour et al., 2014a; Liu et al., 2018a). Surprisingly, our lab has recently discovered that FBXL16 upregulates SCF E3 ligase substrates like c-myc, SRC-3, and β-catenin. We found that FBXL16 stabilizes c-myc protein by antagonizing FBW7-mediated ubiquitination and degradation of c-myc (Morel, Shah, and Long, 2020a, p. 16). Consistent with our findings, another study also saw a reduction in SRC-3 protein levels upon FBXL16 knockdown (Yang and Jing, 2021, p. 3). They further demonstrated that FBXL16 promotes cell proliferation and reduces autophagy in BCa cells by activating the SRC-3-Akt signaling pathway. Even though FBXL16 does not interact with cullin 1, a study reported that exogenously overexpressed FBXL16 directly binds to HIF1α and induces ubiquitination and degradation of HIF1α in triple-negative BCa cells. However, the interaction sites between FBXL16 and HIF1α have so far not been identified and the detailed mechanism of how FBXL16 degrades HIF1α is unclear. It is yet to be understood if FBXL16 interacts with SKP1 and any other cullin isoforms to make a functional SCF complex to initiate ubiquitination of HIF1α. Overall, the negative relationship of FBXL16 with tumor suppressor genes (p16, p14, and PP2A) and its role in stabilizing oncoproteins like c-myc and SRC-3 led us to evaluate its expression status in different cancers (Figure 3).
FBXL16 contains a proline-rich region in the N-terminus followed by an F-box motif that interacts with SKP1. It possesses seven leucine-rich repeat domains on the C-terminus which interact with the substrate. FBXL16 might inhibit the PP2A B55 subunit and negatively regulates its activity (Honarpour et al., 2014b). Additionally, a recent study from our lab suggests that FBXL16 stabilizes oncoproteins like c-myc, SRC-3, and β-catenin by inhibiting their protein ubiquitination (Morel, Shah, and Long, 2020b). Moreover, exogenously overexpressed FBXL16 was shown to induce HIF1α protein ubiquitination in triple-negative BCa (TNBC) cell lines (Kim et al., 2021).
Preliminary data for the putative role of FBXL16 in BCa

To identify the expression of FBXL16 in various cancers, we performed an extensive search of online patient datasets on different cancer databases. Interestingly, in cBioPortal for the Cancer Genomics database, FBXL16 was found to be altered (gene amplification and/or high level of mRNA) in 14% of invasive breast carcinomas (Figure 4A).

We further checked FBXL16 expression levels in different molecular subtypes of breast cancer and noticed a strong positive correlation between ER status and FBXL16 expression in breast tumors. FBXL16 was preferentially upregulated in ER+ breast tumors (luminal A and B) as compared to normal breast tissue samples whereas there wasn’t a significant upregulation of FBXL16 expression in HER2-enriched or basal-like (triple-negative) subtypes. These findings suggest that FBXL16 may play important roles in ERα signaling and ER+ BCa progression (Figure 4B).

As ERα is a transcription factor, we first hypothesized that ERα regulates FBXL16 expression. To investigate that, we silenced ERα in ER+ BCa cell lines MCF7 and T47D growing under regular culture conditions (in the absence of E2 stimulation). We found that knockdown of ERα did not cause a significant change in FBXL16 mRNA or protein levels (Figure 5A, 5B). Next, we tested the effect of E2 stimulation on FBXL16 expression. MCF-7 cells were cultured in charcoal-stripped serum (CSS) media for 2 days to deprive hormone signaling and then were treated with E2 (100nM) for up to 48 hours. E2 stimulation did not alter FBXL16 protein levels (Figure 5C). These observations suggest that FBXL16 expression is not regulated by ERα signaling. We then tested whether FBXL16 has a role in regulating ERα. We silenced FBXL16 in MCF7 and T47D cells. Interestingly, ERα levels were downregulated with FBXL16 silencing and there was a
corresponding decrease in phosphorylation at serine 118 of ERα (Figure 6A-B). This preliminary data suggests that FBXL16 may regulate ERα protein levels and thereby ERα mediated gene transcription and cell proliferation.
Figure 4: FBXL16 gene expression analysis in breast invasive carcinoma.

(A) **FBXL16 expression is altered in invasive breast carcinoma** (data source: cBioPortal database- TCGA dataset). (B) **FBXL16 mRNA expression is significantly upregulated in ER+ breast carcinoma samples** (data source: GEPIA2- BRCA dataset, One-way ANOVA- p<0.01). Breast tissue samples in the BRCA dataset are segregated based on molecular subtypes: Basal-type (ER-negative), HER2+ (ER-negative), Luminal A (ER-positive), and Luminal B (ER-positive), indicated in red. FBXL16 mRNA expression in each subtype was compared to the normal breast tissue (in gray). The number of tumor samples for each subtype is referred to as Num(T) and the number of normal tissue samples is referred to as Num (N). FBXL16 is expressed significantly higher in luminal A and luminal B (ER-positive) as compared to normal breast tissue.
ERα (ESR1) was transiently silenced in T47D and MCF7 cells using siRNA against ERα. A non-targeting siRNA was used as a negative control (siControl). After 48 hours of transfection, FBXL16 mRNA (A) and protein (B) levels were detected using RT-qPCR and western blotting, respectively. (C) MCF-7 cells were cultured in charcoal-stripped serum for 2 days and then treated with E2 (100 nM) for different times. FBXL16 protein expression was analyzed using immunoblotting. Here, c-myc is used as positive control being an E2 inducible gene. Estradiol stimulation did not induce FBXL16 protein expression.

Figure 5: ERα silencing does not affect FBXL16 mRNA & protein levels in BCa cells.
Figure 6: Silencing of FBXL16 downregulates ERα protein levels and phosphorylation at s118 site.

FBXL16 was silenced in MCF7 (A) and T47D (B) cells using siRNA against FBXL16 (siFBXL16). The non-targeting control siRNA (siControl) was used as a negative control. The knockdown of FBXL16 and protein levels of ERα and S118 phosphorylated-ERα (p-ERα (S118)) were analyzed using western blotting.
OBJECTIVES

BCa is the second leading cause of cancer-related death in women. One in every eight women in the U.S. is estimated to get diagnosed with invasive BCa during their lifetime and about 75% of these BCa cases are ERα positive (ER+). The prime challenges in treating ER+ BCa are therapeutic resistance and metastasis. In most cases, dysregulated ERα signaling plays a pivotal role in ER+ BCa progression and endocrine therapy resistance. Post-translational modifications especially ubiquitination is one of the major mechanisms that regulate ERα protein expression and its transcriptional activity. Thus, identifying positive regulators of estrogen receptor expression and protein stability will help in developing better therapeutics for advanced ER+ BCa.

In the current study, we are focusing on FBXL16, a new positive regulator of ERα. A previous study from our lab has revealed that FBXL16 increases the stability and expression levels of several oncoproteins including c-myc, β-catenin, and SRC-3 in lung cancer cells (Morel et al., 2020a). Interestingly, data from publicly available BCa datasets indicate a significant upregulation of FBXL16 in invasive ductal and lobular breast tumors, and FBXL16 upregulation occurs preferentially in ER+ breast tumors and correlates with poor survival of breast cancer patients. These results imply that FBXL16 may play an important role in ERα-signaling mediated BCa growth. The main goal of this project is to investigate the role of FBXL16 in regulating ERα expression, transcriptional activity, and ERα signaling-mediated cancer cell growth. Specifically, we hypothesize that FBXL16 upregulates ERα protein level and transcriptional activity by decreasing its
polyubiquitination, thereby promoting BCa cell proliferation and endocrine therapy resistance. FBXL16 is a relatively understudied F-box protein whose activity and functions are largely unknown. We further postulate that FBXL16 stabilizes ERα by antagonizing a specific SCF-E3 ligase that mediates ERα ubiquitination and proteasomal degradation. To test this hypothesis, we pursued the following specific aims:

Specific Aim 1: Determine the role of FBXL16 in regulating ERα protein expression and transcriptional activity.

Specific Aim 2: Determine if FBXL16 stabilizes ERα by decreasing its proteasomal degradation.

Specific Aim 3: Determine the roles of FBXL16 in promoting BCa cell growth and altering endocrine therapeutic response.
SIGNIFICANCE

A growing number of studies reported that alterations in ERα protein degradation and stability are implicated in sustaining an elevated level of ERα that augments the transcription of genes involved in BCa cell proliferation and survival (Lonard et al., 2000a; Duong et al., 2007; Jia et al., 2019). Taking advantage of this aspect, a category of drugs called SERDs has been designed to degrade ERα for the treatment of BCa (Yeh et al., 2013a; Savi et al., 2015a). Yet, alterations in the regulation of ERα by UPS result in an excess cellular repertoire of ERα, which eventually makes cells resistant to SERDs and other endocrine therapies (Zilli et al., 2009; Tang et al., 2016; Tecalco-Cruz and Ramírez-Jarquín, 2017). Extensive research efforts have been focused so far on identifying E3 ligases that ubiquitinate and degrade ERα (Hashizume et al., 2001; Fan et al., 2005a; Duong et al., 2007; Sun et al., 2012a). Conversely, studies identifying proteins and mechanisms which can lead to increased ERα expression and activity remain relatively underrepresented (Zhu et al., 2014b, p. 31; Yang et al., 2018a, p. 1; Xue et al., 2019a).

This study aims to define the novel role of FBXL16 in stabilizing ERα and its functional impact on BCa cell proliferation and endocrine therapy resistance. This study will not only enrich our understanding of ERα protein degradation and stability and may identify FBXL16 as a new prognostic factor and/or therapeutic target of ER⁺ BCa.

The developmental role of FBXL16 in the brain and in differentiating embryonic stem cells into cardiomyocyte lineage has been established (Honarpour et al., 2014a). Recently our
lab identified an important biochemical function of FBXL16 in stabilizing oncoproteins like c-myc, β-catenin, and SRC-3 in lung cancer cell lines (Morel et al., 2020a). Previous reports suggest FBXL16 has oncogenic roles (Sato et al., 2010a, p. 16; Honarpour et al., 2014a; Morel et al., 2020a, p. 1). Yet, the role of FBXL16 is largely unknown in cancer and this is the first-ever study to explore the functional consequences of FBXL16 in BCa in the context of functional ERα signaling. This study is innovative as it identifies FBXL16 as the first F-Box protein that upregulates ERα protein level and signaling, thereby promoting BCa cell growth.

Unlike other F-box proteins such as FBW7 and SKP2, FBXL16 does not form a functional E3 ligase complex with CUL1 (Honarpour et al., 2014a; Morel et al., 2020a). Moreover, to date, it is unclear whether or not FBXL16 possesses E3 ligase activity. It is likely that FBXL16 interacts with other cullin isoforms to form a functional E3 ligase and thus has a completely different set of substrates. The current study sheds new light on the counteracting roles of FBXL16 and FBXO45 in regulating estradiol-induced ERα degradation. The expression of proteins involved in regulating ERα stability generally correlates with ERα protein levels and its transcription activity. This implies that findings from this research can be extrapolated in proposing FBXL16 as a potential therapeutic target candidate and biomarker for ER⁺ BCa. Besides identifying molecular functions of FBXL16 in ER⁺ BCa, we also explore the clinical significance of targeting FBXL16 in advanced treatment-resistant BCa by investigating its role in regulating the protein stability of constitutively active ERα mutant protein and its impact on affecting fulvestrant responsiveness in ER⁺ BCa.
MATERIALS AND METHODS

I. Materials

Table 3: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Growth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>Human breast carcinoma (isolated from pleural effusion)</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human breast adenocarcinoma (derived from pleural effusion)</td>
<td>IMEM</td>
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<tr>
<td>BT-474</td>
<td>Human breast carcinoma (isolated from a solid, invasive ductal carcinoma)</td>
<td>DMEM</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>Human breast adenocarcinoma (derived from pleural effusion cells)</td>
<td>RPMI 1640</td>
</tr>
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<tr>
<td>BT-549</td>
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<td>RPMI 1640</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical adenocarcinoma</td>
<td>DMEM</td>
</tr>
<tr>
<td>293T</td>
<td>Human embryonic kidney cells</td>
<td>DMEM</td>
</tr>
<tr>
<td>Antibody</td>
<td>Description</td>
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<tr>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>Anti-FBXL16</td>
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<td>GeneTEX (GTX31424)</td>
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<td>Anti-ERα</td>
<td>rabbit, monoclonal (Immunogen-synthetic peptide corresponding to amino acids 13-32 (C-ALLHQIQGNELEPLNRPLK) of human ERα)</td>
<td>Millipore (04-820)</td>
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<td>Anti-ERα</td>
<td>mouse, monoclonal (Immunogen-recombinant estrogen receptor alpha protein)</td>
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<td>Rabbit</td>
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</tr>
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</table>
### Table 5: Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’ → 3’)</th>
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</thead>
<tbody>
<tr>
<td>FBXL16-reverse</td>
<td>5’- TGC ATC TGT TCA AGC ATA ACC T -3’</td>
</tr>
<tr>
<td>FBXL16-forward</td>
<td>5’- AAG GGT GTC AAA GCC ATG AG -3’</td>
</tr>
<tr>
<td>ESR1- reverse</td>
<td>5’- CGA GAT GAT GTA GCC AGC AG -3’</td>
</tr>
<tr>
<td>ESR1- forward</td>
<td>5’- TTG CTC CTA ACT TGC TCT TGG -3’</td>
</tr>
<tr>
<td>pS2- reverse</td>
<td>5’- GAT CCC TGC AGA AGT GTC TAA AA -3’</td>
</tr>
<tr>
<td>pS2- forward</td>
<td>5’- CCC CTG GTG CTT CTA TCC TAA -3’</td>
</tr>
<tr>
<td>CCND1- reverse</td>
<td>5’- CCA CTT GAG CTT GTT CAC CA -3’</td>
</tr>
<tr>
<td>CCND1- forward</td>
<td>5’- GCC GAG AAG CTG TGC ATC -3’</td>
</tr>
<tr>
<td>c-myc- reverse</td>
<td>5’- TTC CTG TTG GTG AAG CTA AC -3’</td>
</tr>
<tr>
<td>c-myc- forward</td>
<td>5’- TTT TTC GGG TAG TGG AAA AC -3’</td>
</tr>
<tr>
<td>FBXO45- reverse</td>
<td>5’- GGC ATG TTG AAA AGC ACG TA -3’</td>
</tr>
<tr>
<td>FBXO45- forward</td>
<td>5’- GAT GAG AAC AGC GAG GTG TG -3’</td>
</tr>
<tr>
<td>GAPDH- reverse</td>
<td>5’- GCC CAA TAC GAC CAA ATC C -3’</td>
</tr>
<tr>
<td>GAPDH- forward</td>
<td>5’- AGC CAC ATC GCT CAG ACA C -3’</td>
</tr>
<tr>
<td>FBXL16-KpnI-F</td>
<td>5’- CCAGGTACCATGTCAGGCCCCGGGATC -3’</td>
</tr>
<tr>
<td>(Cloning PCR primer)</td>
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<tr>
<td>FBXL16-KpnI-R</td>
<td>5’- CCTGGTACCCTACTCAATGACGAGGCAGCGG -3’</td>
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<tr>
<td>(Cloning PCR primer)</td>
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<tr>
<td>pSIH1-H1-Puro</td>
<td>5’-AATGTCTTTGGATTTGGGAATCTTATAT-3’</td>
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<tr>
<td>shRNA-F (PCR primer, sequencing primer)</td>
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<tr>
<td>pSIH1-H1-Puro</td>
<td>5’-TGGTCTAACAGAGGAGAACCAGTA-3’</td>
</tr>
<tr>
<td>shRNA-R (PCR primer)</td>
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II. Methods

Plasmids and cloning

The FBXL16 expression vector was generated by PCR amplifying the coding region of FBXL16 from FBXL16 EST clone as a template (Dharmacon MHS6278–202807943, Accession: BC036680, Clone ID: 5262152) using the PCR primers FBXL16-KpnI-F and FBXL16-KpnI-R. The PCR product was cloned into the pSG5-KF2M1 vector using the KpnI restriction site to generate the pSG5-Flag-FBXL16 (Morel et al., 2020b). Retrovirus expression plasmids, pMSCV-eGFP, and pMSCV-FBXL16 were received from Dr. Wade Harper at Harvard Medical School (Tan et al., 2013). pMT-HA ubiquitin was described previously (Li et al., 2007). pCDNA-HA-ERα WT plasmid was purchased from Addgene (#49498). pCMV-flag-ERα, pCMV-flag-ERαY537S, pCMV-flag-ERαD538G were provided by Dr. Charles Foulds at Baylor College of Medicine. The estrogen-response element reporter plasmid, pERE-E1b-LUC is described in (Nawaz et al., 1999b). For the lentiviral overexpression ERα construct, ERα was digested from pcDNA-HA-ERα using Apal and NheI sites, and the ERα insert was cloned into the pLJM1-eGFP backbone using EcoRI and NheI restriction sites. pCMV-FBXO45 was made in Dr. Hideyuki Okano’s lab at Keio University, Japan and was a kind gift from Dr. Sang Jun Han at Baylor College of Medicine.

pSIH-sh-Luc and pSIH-shFBXL16 were generated by cloning luciferase control shRNA and FBXL16 shRNA into pSIH1-H1-Puro shRNA Expression Lentivector (System Biosciences, SI500A-1) following the manufacturer's protocol. In brief, the pSIH-H1 lentivector was linearized using EcoRI/BamHI restriction digestion. The shRNAs against FBXL16 were designed to have BamHI and EcoRI restriction site overhang sequences for
directional cloning of shRNA in the digested backbone. The FBXL16 shRNA sequences are as follows:

Forward-
GATCCACGGACACTGGCCTCAGCTACTTCTGGTCAGATAGCTGAGGCCAGT
GTCCGTTTTTTG
Reverse-
GGTGCCTGTGACCGGAGTCGATGAAGGACAGTCTATCGACTCCGGTCACAGG
CACAAAAACTTTAA

The sequences for luciferase control shRNA are as follows:

Forward-
GATCCGTTGGTTGTTAGTACTAATCCTATTTTGAGCAGATGAAATAGGGTT
GGTACTAGCAACGCACTTTTTTG
Reverse-
GCACGCAACAATCATGATTAGGATAAACAATTCGTCTACTTTATCCCAACCAG
GATCGTTGCGTGGAAAAACCTTTA

The shRNA template oligonucleotides were phosphorylated using T4 Polynucleotide Kinase and annealed to improve cloning efficiency. Lastly, these shRNA templates were ligated in the linearized pSIH backbone. The ligated products were transformed in E. coli and the clones with the target shRNA template were identified using PCR followed by the sequencing analysis. The PCR primers are mentioned in Table 5.
Cell culture

The cell lines listed in Table 3 were maintained using Dulbecco’s Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 growth medium or Improved MEM-Richter’s modified (IMEM) media (as indicated in Table 3), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Penicillin-streptomycin). All the culture media and supplements were purchased from Gibco and Corning.

Production of lentiviral particles and generation of stable cell lines with knockdown of FBXL16

Lentiviral particles containing shRNA were produced in 293T cells by co-transfecting lentiviral packaging plasmids, psPAX2, which expresses gag, pol, tat, and rev genes, and pMD2.G, which encodes for Vesicular Stomatitis Virus Glycoprotein (VSV-G), along with non-targeting shLuc or shFBXL16 cloned into the pSIH lentiviral vector. The pseudovirus particles were harvested 48 hours after transfection and concentrated using PEG-it Virus Precipitation Solution (System Biosciences, LV810A), following the manufacturer’s instructions. Stable knockdown of FBXL16 in T47D cells containing WT ERα (T47D TWT) or mutant ERα (T47D Y537S) was achieved by lentiviral transduction of short hairpin RNA (shRNA) specific to FBXL16. Polybrene (4 µg/mL) was used to facilitate viral infection in the cells. Cells with stable expression of luciferase pSIH-H1 (T47D-shLuc) served as a control cell line. Cells transduced with lentivirus were selected using puromycin to generate stable cells with FBXL16 knockdown.
Lentiviral particles for ERα expression were produced in 293T cells by co-transfecting psPAX2 and pMD2.G packaging plasmids with a pLJM1 vector containing ERα cDNA. Lentiviral particles produced using pLJM1-EV (empty vector) were used as a negative control. For the cell growth rescue experiment, pLJM1-ERα or pLJM1-EV lentivirus were transiently transduced in T47D cells with 4 µg/mL polybrene along with siRNA transfection for FBXL16 silencing (described in the transient transfection).

**Generation of cell lines harboring mutations in ERα protein**

These cell lines were a kind gift from Dr. Steffi Oesterreich at University of Pittsburgh Medical Center. Briefly, ESR1 Y537S mutation in T47D was introduced by CRISPR-Cas9 genome editing (Bahreini *et al.*, 2017). ERα sequence flanking Y537S was utilized to design sgRNA using the webtool [http://crispr.mit.edu](http://crispr.mit.edu). The sgRNA sequence was cloned into a PX458 plasmid also coding for Cas9, green fluorescent protein (GFP), and tracrRNA. This resulting plasmid was co-transfected with 70 bp oligos which are designed based on the target site. GFP+ cells were selected by fluorescence-activated cell sorting (FACS) and the mutation was confirmed by Sanger sequencing and ddPCR.

For MCF7 cells containing Y537S mutation were generated using adeno-associated virus (AAV) (Bahreini *et al.*, 2017)(Wang *et al.*, 2016). Site-directed mutagenesis was used to generate mutation in the adenovirus construct at the target sequence. The resulting adenovirus construct was co-transfected with pHelper, pRC (Agilent) plasmids in 293T cells to produce the virus. After virus infection in MCF7 cells, neomycin-resistant clones were selected using a PCR screening assay (Gelsomino *et al.*, 2016).
**Generation of retroviral particles and transient retrovirus transduction**

For retroviral particles generation, 4µg pMSCV-eGFP or pMSCV-FBXL16 vectors were co-transfected with retroviral packaging particles, 2µg pMSCV-VSVG and 2µg pMSCV-GagPol in 293T cells using Lipofectamine 3000. After 6 hours, 5ml DMEM with 20% FBS was added. 48 hours post-transfection, the retrovirus was harvested and filtered using a 0.45µm syringe filter to remove cell debris. The virus was precipitated by overnight incubation of the supernatant with RetroX concentrator (Takara, 631455) at 4°C. The sample was then centrifuged at 1500g for 45 minutes, the supernatant was carefully aspirated, and the pellet was resuspended in 150µl of cold DPBS.

For transient transduction experiments, T47D cells were transduced with retrovirus expressing either 5µl of pMSCV-flag-eGFP or pMSCV-flag-FBXL16 retrovirus with 4 µg/mL polybrene in a 12-well plate for 2 days.

**Transient transfection**

Transient plasmid transfections for overexpression studies were performed using Lipofectamine 3000 (Invitrogen, L3000015) or Fugene 6 (Promega, E2691) reagent. siRNA transfections in BCa cell lines were performed using DharmaFECT 1 transfection reagent (Dharmacon, T-2001) following the manufacturer’s instructions. 50nM of Hs_FBXL16_8 FlexiTube siRNA (Qiagen SI04287276) was used to target FBXL16, and AllStars Negative Control siRNA (Qiagen SI03650318) 50nM was used as a non-silencing control. SKP2 was silenced using Hs_SKP2_5 FlexiTube siRNA (Qiagen SI00287819). For ERα silencing, Silencer Select Predesigned siRNA (Ambion, 4427037). Silencer Select
negative control siRNA (Ambion, 4390843) was used as a negative control for ERα siRNA. Unless stated otherwise, SKP2 and ERα siRNAs were used at 30nM concentration.

Immunohistochemistry

To determine the FBXL16 protein expression in breast tissues, BCa tissue microarray was used (US Biomax, BR1921c). The tissue microarray contained paraffin-embedded (FFPE) breast carcinoma and adjacent normal breast tissue, containing 80 cases of each invasive ductal carcinoma and invasive lobular carcinoma, 21 adjacent normal tissue, 4 cancer adjacent tissue, and 7 normal tissues. Tissue slides were deparaffinized using xylene and rehydrated in gradient concentration series of ethanol with two washes in distilled water for 5 minutes each. Antigen retrieval was performed by treating the slides in citrate-based antigen unmasking solution pH 6.0 (Vector Laboratories, H-3300) for 12 min at 90 °C using an electric pressure cooker (Cuisinart-Model, CPC-6). Permeabilization was done by incubation in PBS containing 0.3% Triton X-100 (Sigma, T8787) for 45 minutes and followed by blocking with 10% normal serum in PBS with 1% BSA for 1 hour to mask non-specific antibody binding sites. The slide was incubated with the antibody against FBXL16 in the blocking solution (1:250 dilution) overnight at 4 °C. The slide was then washed with PBS for 5 minutes followed by secondary antibody incubation with biotinylated HRP-conjugated antibody (Vector Laboratories, BA-1000) at room temperature for 1 hour. Freshly prepared diaminobenzidine (DAB- Acros Organics, 112090250) solution (1.7mM DAB in 50mM Tris (pH 7.6) with 0.05% hydrogen peroxide) was used along with the Vectastain ABC kit (Vector Laboratories, PK-6100) to develop the slide. The slide was then counter-stained with hematoxylin, cleaned, and mounted.
Western blotting

Whole-cell extracts were prepared using EBC lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM cOmplete protease inhibitors (Roche, 11697498001), and 1 mM phosphatase inhibitor cocktail III (Sigma-Aldrich, P0044)] for 10 minutes on ice. Following the incubation, adherent cells were scraped off. Samples were transferred to microcentrifuge tubes and incubated for 10 more minutes on ice. Cell debris was removed by centrifuging the samples at 14,000 RPM for 10 minutes. Equivalent concentrations of protein were resolved using either 8% (for ubiquitination assays) or 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline with tween 20 (TBS-T) for 30 minutes. Afterward, the membranes were incubated overnight with the primary antibodies at 4°C followed by 1-hour incubation with the appropriate HRP-conjugated secondary antibodies at room temperature. The primary and secondary antibodies are listed in Table 4. The chemiluminescence was detected by adding Pierce ECL chemiluminescence substrate (ThermoFisher, 32106). β-actin or GAPDH were used as a loading control in the Western blotting experiments.

Immunoprecipitation assays

293T cells were co-transfected with pCMV-flag-ERα and pSG5-HA-FBXL16. For detection of interaction with endogenous ERα, MCF7 cells were transfected with pSG5-HA-FBXL16. Two days post-transfection, the cells were lysed with EBC lysis buffer as described under Western blotting. Up to 1mg of equivalent protein concentrations were used for immunoprecipitation. Lysates were precleared for 1 hour using 20µl Protein A
Affinity gel beads (Sigma-Aldrich, P6486). Flag-tagged ERα proteins were immunoprecipitated using anti-flag affinity agarose beads (Sigma-Aldrich, F2426) for 3 hours. For MCF7 cell lysates, overexpressed FBXL16 was precipitated using HA-tagged affinity agarose beads (Sigma-Aldrich, E6779). As a control, cell lysates were incubated with mouse IgG on Protein A Affinity gel beads. Afterward, the beads were washed 3 times with EBC lysis buffer for 10 minutes per wash and the proteins were eluted from the beads in 2X SDS sample buffer. The proteins were resolved on SDS-PAGE gels and detected using antibodies indicated in the specific experiment by following the western blotting procedure.

**Protein degradation and stability assays**

Protein stability assays were performed by inhibiting de novo protein synthesis using cycloheximide. Cycloheximide (CHX) is a translation elongation inhibitor that binds to the 60S subunit of the ribosome and immobilizes the ribosome thereby stalling protein translation (Godchaux, Adamson, and Herbert, 1967; McKeehan and Hardesty, 1969). Cells were transiently transfected with siRNA for 48 hours as indicated in respective experiments and treated with 100μg/mL CHX (Sigma-Aldrich, C7698) for different times as indicated. For overexpression, cells were transiently transduced with pMSCV-eGFP or pMSCV-FBXL16 retrovirus, and 48 hours after the transduction, cells were treated with 100μg/mL CHX for indicated time points. Similarly, cells with stable knockdown of FBXL16 were seeded in a 24-well plate. 24 hours after plating, cells were treated with 100μg/mL CHX and harvested at different time points as indicated. ERα protein levels at each time point were analyzed by western blot and normalized to that of β-actin loading control. The normalized ERα protein level at the 0-hour time point was arbitrarily set as 1.
and the protein half-life was calculated with GraphPad Prism 6 software using the one-phase exponential decay model.

For ligand-induced ERα degradation, cells were transiently silenced as described for 48 hours and treated with either estradiol (10nM) (Sigma, E4389) or fulvestrant (100nM) (MCE, HY-13636) for the respective experiment. ERα protein levels at each time point were analyzed by western blot and normalized to that of β-actin or GAPDH loading control. ERα protein levels were made relative to the zero-minute time point and the half-life was calculated as described above.

**Ubiquitination assay**

HeLa cells were co-transfected with pMT123-HA-Ubiquitin, pCMV-Flag-ERα, and either pSG5-HA-EV or pSG5-HA-FBXL16 as described. After 30-32 hours of transfection, HeLa cells were treated with 20μM of MG132 (Calbiochem, 474790) for 6 hours. Cells were then lysed in a modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM cOmplete Protease Inhibitors, and 1 mM phosphatase inhibitor mixture III) containing 25 mM N-ethylmaleimide (Sigma-Aldrich, E3876). Ubiquitinated proteins were then immunoprecipitated using anti-HA affinity gel beads (Sigma-Aldrich, E6779) with rotation at 4°C. Lysates incubated with an equivalent amount of non-specific IgG antibody on protein-A gel beads (Sigma-Aldrich, P6486) were used as the negative control for the experiment. Following incubation, bead complexes were washed three times with the lysis buffer and subjected to immunoblot analysis. The ubiquitinated form of ERα was detected using an ERα antibody.
RNA extraction and RT-qPCR

For gene expression analysis, cells were transiently transfected with siRNA as indicated. Total RNA was extracted from cells using Trizol reagent (Ambion, 15596018), and reverse transcription (RT) was carried out using SuperScript IV VILO Master Mix (Invitrogen, 11756500) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was performed using the TaqMan Probe system (Roche) with TaqMan Universal Master Mix II (Applied Biosystems, 4440040) on the 7500 Real-Time PCR instrument (Applied Biosystems). The details on primer sequences are included in Table 5. GAPDH was used as the internal control. Relative expression to the normalizer sample was calculated using the ΔΔCT method.

Luciferase reporter assay

HeLa cells were transfected with estrogen-responsive reporter construct (pERE-LUC) along with mammalian expression vectors for ERα (pcDNA-HA-ERα) and FBXL16 or empty vector (pSG5-flag-FBXL16). Briefly, cells were seeded in phenol red-free charcoal-stripped serum-containing DMEM media in a 24-well plate and transfected with expression vectors. After 24 hours after transfection, cells were treated with vehicle (water) or estradiol (20 nM) for 8 hours. Luciferase assay was performed using Pierce™ Firefly Luc One-Step Glow Assay Kit (Thermo, 16197) according to the manufacturer’s instructions.

Cell proliferation assay

Cells were plated in a 96-well plate and transfected with siRNA as mentioned previously. Cell proliferation was assessed using the FluoReporter Blue Fluorometric dsDNA
Quantitation Kit (Invitrogen, F2962) following the manufacturer’s instructions. The kit utilizes Hoescht 33258 fluorescent dye to measure double-stranded DNA amount as an indicator of the number of live cells present in each well. Briefly, at a defined time point, the culture plate was frozen. The cells were freeze-thawed, following which Hoechst dye was added, and fluorescence (excitation 360 nm and emission 460 nm) was measured.

**Soft agar colony formation assay**

Anchorage-independent colony formation assay was performed using ultrapure noble agar (ThermoFisher, J10907). T47D cells expressing wild type (TWT) or mutant (Y537S) ERα with stable knockdown of FBXL16 were plated at a cell seeding density of 3000 cells/well for cells grown in regular culture condition or 5000 cells/well for charcoal-stripped serum (CSS) condition in a 24-well plate. Cells were plated in 0.3% agarose in a complete medium containing 1 μg/μl of puromycin with a supporting bottom layer of 0.6% agarose in a complete medium and were allowed to grow at 37 °C humidified cell culture incubator for indicated time points. Spent old media was replaced with fresh complete media containing 1 μg/μl of puromycin every 2 days. For the fulvestrant responsiveness (IC50) experiment, cells were plated in agar using media containing CSS. Treatment was performed every 2 days with 20 pM estradiol plus varying concentrations of freshly prepared fulvestrant solution in CSS culture media. Nitro blue tetrazolium chloride tablets (EMS, 19035) were used to make colony staining solution by making 100mg/ml intermediate dilution in dimethylformamide (DMF). Cell colonies in agarose were then stained with a final concentration of 1 mg/ml of nitro blue tetrazolium chloride solution in 1X PBS overnight. Cell colony formation was quantified using Image J software. IC50
calculations were performed using GraphPad Prism 6 software using the nonlinear regression equation for ‘dose-response inhibition’. The values at individual concentrations were compared for significance using the Two-way ANOVA test.

**Lung metastasis assay**

For the lung metastasis experiments, SCID/ Beige mice were injected with T47D-ERαY537S-shLuc or T47D-ERαY537S-shFBXL16 cells (5 mice each) at a density of $1 \times 10^6$ cells via tail vein. The tail vein injection was performed in Dr. Bin Fang’s lab at Baylor College of Medicine. After 1 month of inoculation, mice were sacrificed, and the lungs were harvested. The formation of tumor nodules in the lungs was analyzed by hematoxylin/eosin staining of paraffin-embedded lung sections. H&E-stained lung images were taken at 50X magnification and the number of tumor nodules and foci in the lungs were counted.

**Statistics**

Statistical analyses were performed using GraphPad Prism 6 software program. All experiments were repeated at least three times and the representative figure for each experiment was shown. Data are represented as mean ± SEM (standard error of the mean). Statistically significant differences between the groups were determined using Student's $t$-test, one-way analysis of variance (ANOVA), or two-way ANOVA as indicated in the figure legends. Differences were considered statistically significant at $p \leq 0.05$ (*) $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.)
RESULTS

I. Regulation of ERα expression and transcriptional activity by FBXL16 in BCa

FBXL16 expression is upregulated and is positively correlated to ERα expression in BCa tissue samples and cell lines

From our investigation of FBXL16 expression in several cancers, we observed a drastic increase in FBXL16 expression in ER+ breast carcinomas. To identify the role of FBXL16 in BCa, we further analyzed mRNA levels of FBXL16 in BCa patient samples from publicly available microarray datasets using the Oncomine database (Rhodes et al., 2004). We compared the expression of FBXL16 in breast carcinoma samples in both lobular and ductal subtypes with that of normal breast tissue samples. FBXL16 mRNA is significantly overexpressed in invasive lobular (n=148) and invasive ductal (n=1556) breast tumors in comparison to normal breast (n=144) tissue samples in the Curtis dataset (Curtis et al., 2012) (Figure 7A, B). These findings corroborated with the data from the TCGA dataset that also show significant upregulation of FBXL16 gene expression in invasive lobular (n=36) (Figure 7C) and invasive ductal carcinoma (n=389) (Figure 7D) versus the normal breast tissue samples (n=61) (Cancer Genome Atlas Network, 2012). Additionally, we had noticed that the upregulation of FBXL16 transcript levels is selective in luminal A and luminal B subtypes (ER+ subtypes) (Figure 4B). To reaffirm the upregulation of FBXL16 mRNA expression in ER+ BCa, ERα status (negative or positive- determined by IHC) of 1108 BCa samples in TCGA firehose legacy dataset was plotted against FBXL16 mRNA levels.
Figure 7: FBXL16 mRNA expression is upregulated in both invasive ductal breast carcinoma and invasive lobular breast carcinoma samples from Curtis and TCGA datasets.

Normalized log2 intensities are median centered and plotted for each group. (A) comparison of FBXL16 expression in normal breast tissue (n=144) versus invasive lobular breast carcinoma tissue (n=148) in Curtis BCa dataset; (B) comparison of FBXL16 expression in normal breast tissue (n=144) versus invasive ductal breast carcinoma tissue (n=1556) in Curtis BCa dataset; (C) comparison of FBXL16 expression in normal breast tissue (n=61) versus invasive lobular breast carcinoma tissue (n=36) in TCGA BCa dataset; (D) comparison of FBXL16 expression in normal breast tissue (n=61) versus invasive
lobular breast carcinoma tissue (n=389) in TCGA BCa dataset; (data source: Oncomine database, *student’s t-test, P-value* included under each graph).
The expression of FBXL16 was found to be significantly higher in ER+ BCa tissues as compared to ER- BCa samples (Figure 8) (Cerami et al., 2012; Gao et al., 2013).

To verify the findings from publicly available online datasets and confirm the upregulation of FBXL16 at the protein level in clinical samples, we obtained a commercially available BCa tissue microarray containing 192 breast tissue samples with known ERα status and stained the samples with FBXL16 antibody by immunohistochemical (IHC) analysis. Each sample was scored for FBXL16 staining intensity. The scoring and interpretation of IHC staining were performed by following the intensity scoring system of 0-3+ (Kurosumi, 2007; Arihiro et al., 2007, Anon., n.d.). Quantification of FBXL16 staining confirmed the increase in FBXL16 protein expression level in invasive ductal and lobular carcinoma as compared to adjacent normal breast tissue samples. Additionally, we segregated the tumor samples into two groups, ER+ and ER-, based on ERα IHC status. We observed significantly higher FBXL16 staining in ER+ BCa samples versus ER- BCas (Figure 9).

Moreover, we analyzed expression levels of FBXL16 and ERα proteins in different BCa cell lines to ascertain the co-occurrence of these two proteins. We collected cell lysates from ERα positive (ER+) cell lines (MCF7, T47D, and BT474) and ERα negative (ER-) cell lines (SKBR3, BT549, MDA-MB-231) (Dai et al., 2017). We observed that FBXL16 is expressed in cell lines expressing ERα protein (Figure 10). These results corroborate a positive correlation between FBXL16 and ERα in ER+ BCa.
Figure 8: FBXL16 mRNA expression is significantly higher in ERα+ breast tumors than ERα- BCas.

BCa tissues are categorized in ERα positive and ERα negative groups based on immunohistochemical analysis. FBXL16 mRNA expression is compared in a box and whisker plot in ERα- BCa specimens (n=238) as compared to ERα+ BCa samples (n=812). The gray box represents the upper and the lower quartile of the data spread, and the middle line shows the median for the data samples (two-tailed student’s t-test p<0.0001).
Figure 9: Invasive BCa tissues display upregulation of FBXL16 protein expression level, and its expression is significantly higher in ER⁺ invasive breast carcinomas.

The top panel shows representative images of immunohistochemical staining of FBXL16 protein in BCa tissue microarray (US Biomax: BR1921c). Scale bar, 200 µm. FBXL16 staining was quantified using a common intensity scoring system of 0-3+ (Kurosumi, 2007) and score was represented as mean ± SEM on the scatter plots. The bottom left panel shows the quantification of FBXL16 IHC staining demonstrating increased average staining of FBXL16 in invasive ductal and lobular tissue samples as compared to adjacent normal breast tissue (One way ANOVA, *** p<0.001). The bottom right panel shows average FBXL16 IHC staining scores in BCa tissue samples with different ER status (ER⁺ versus ER⁻, Student’s t-test, ** p<0.01).
Figure 10: FBXL16 protein expression is correlated to ERα expression in BCa cell lines.

Western blot analysis of FBXL16 and ERα protein expression in MCF7, T47D, and BT474 cell lines (ER⁺ group) and SKBR3, BT549, and MDA-MB-231 cells lines (ER⁻ group).
FBXL16 positively regulates ERα and SRC-3 protein levels in ER⁺ BCa cell lines

Given the strong positive correlation between FBXL16 expression and ERα protein levels in BCa and no effect of ERα in regulating FBXL16 expression (Figure 5), we next postulated if FBXL16 has a role in regulating ERα protein expression. To elucidate the regulation of ERα by FBXL16, we silenced FBXL16 in ER⁺ BCa cell lines BT474, T47D, and MCF7. In all these ER⁺ BCa cell lines, knockdown of FBXL16 led to a concomitant decrease in ERα protein levels (Figure 11). Our lab previously reported that FBXL16 upregulates SRC-3 protein levels in lung cancer cell lines (Morel et al., 2020b, p. 16), so we also looked at the effect of FBXL16 on SRC-3 which is a known co-activator of ERα signaling. FBXL16 silencing downregulated SRC-3 protein levels also in ER⁺ BCa cell lines (Figure 11). To confirm these findings, we overexpressed either eGFP (control) or FBXL16 in BT474 and T47D cells by transient retroviral transduction. Cell line transduced with eGFP retrovirus served as a control for FBXL16 overexpression. Indeed, as compared to eGFP, transient overexpression of FBXL16 elevated ERα and SRC-3 protein levels (Figure 12).

FBXL16 regulates ERα expression in a ligand-independent manner

Estradiol (E2) not only activates ERα-mediated gene transcription but also induces ERα poly-ubiquitination and turnover, indicating that the estradiol-induced ERα transcription activation cycle is followed by its degradation (Nirmala and Thampan, 1995; Lonard et al., 2000b; La Rosa, Marino, and Acconcia, 2011). As the ERα protein level is regulated by the ligand estradiol, we wanted to determine whether the regulation of FBXL16 on the ERα protein level is affected by estradiol. Here, we cultured T47D cells in media containing
Figure 11: Silencing of FBXL16 reduced ERα protein levels.

Three ER⁺ cell lines, BT474, T47D and MCF7 cells were transfected with 50nM non-targeting control siRNA (siControl) or siRNA targeting FBXL16 (siFBXL16). Three days post-transfection, the levels of FBXL16, ERα, and SRC-3 proteins were analyzed by Western blotting. Fold change in ERα and SRC-3 protein levels (normalized to GAPDH) relative to the siControl condition is plotted in separate graphs (Two-way ANOVA, ***-p<0.001).
Figure 12: Overexpression of FBXL16 upregulates ERα and SRC-3 protein levels.

ER⁺ cell lines, BT474, and T47D were transiently transduced with control or FBXL16-expressing retrovirus. 2 days post retrovirus transduction, ERα and SRC-3 protein levels were analyzed by immunoblotting. Fold change in ERα and SRC-3 protein levels, (normalized to GAPDH) relative to eGFP control condition is plotted in separate graphs (Two-way ANOVA, **-p<0.01 and *- p<0.05).
charcoal-stripped serum from which lipophilic materials like hormones and steroids were removed. FBXL16 was then transiently silenced using siRNA. 48 hours after siRNA transfection, cells were treated with either vehicle (water) or E2 (10nM) for 24 hours. FBXL16 silencing had the same effect of reducing ERα protein levels in cells with either vehicle or E2 treatment, suggesting that the regulation of ERα protein levels by FBXL16 is ligand-independent (Figure 13).

**FBXL16 promotes ERα transcriptional activity**

We next investigated if positive regulation of ERα and SRC-3 by FBXL16 results in increased ERα transcriptional activity. Hence, we performed a luciferase reporter assay to determine the effect of FBXL16 on ERα transcriptional activity. ERα constructs and the luciferase reporter construct containing estrogen-response elements (ERE-Luc) were overexpressed with either the control vector or FBXL16, and cells were then treated with vehicle (water) or estradiol (10nM) for 8 hours. As expected, FBXL16 overexpression significantly increased ERα-mediated transactivation upon E2 treatment (Figure 14).
Figure 13: FBXL16 regulates ERα expression in an E2-independent manner.

T47D cells were cultured in hormone-deprived media for 2 days. T47D cells were transfected with 50nM siControl or siFBXL16. 2 days after siRNA transfection, cells were treated with either vehicle or estradiol (10nM) for 24 hours. The expression levels of ERα, p-ERα, FBXL16, and GAPDH were detected by immunoblotting. Numbers below the blots indicate ERα and p-ERα protein level that were normalized to β-actin and were relative to siControl condition (arbitrarily set as 1).
Figure 14: FBXL16 upregulates ERE-luciferase activity mediated by ERα.

HeLa cells were co-transfected with a luciferase reporter plasmid (harboring estrogen response element), ERα, and a vector control or FBXL16 expression vector. After 24 hours of transfection, cells were treated with either vehicle (Veh) or estradiol (E2, 10nM) for 8 hours. Luciferase reporter assay was performed, and the relative luciferase activity (relative to cells transfected with vector control and treated with vehicle) was determined. Values in the bar graph represent the mean ± SE of three independent experiments. (One-way ANOVA test- **** p<0.0001; ** p<0.01). Expression of HA-tagged ERα and Flag-tagged FBXL16 were analyzed by western blotting using an HA antibody and an Flag antibody, respectively, as shown on the right.
FBXL16 knockdown downregulates mRNA levels of ERα target genes

To further validate the role of FBXL16 in ERα-mediated gene transcription, we silenced FBXL16 in T47D cells and measured the transcript levels of ERα target genes, including two classical ERα target genes pS2 (Shang et al., 2000) and c-myc (Cheng et al., 2006), and a noncanonical target gene cyclin D1 (Wong et al., 2001; Liu et al., 2002). Consistent with the effect of FBXL16 on ERE-driven luciferase activity, FBXL16 silencing significantly decreased mRNA levels of ERα target genes pS2, CCND1, and c-myc (Figure 15). The transcript level of ERα gene was also reduced upon FBXL16 knockdown, which is consistent with the autoregulation of ERα gene transcription by ERα protein (Barton and Shapiro, 1988; Castles et al., 1997).
Figure 15: Silencing of FBXL16 downregulates mRNA levels of ERα and its target genes.

T47D cells were transfected with 50 nM non-targeting siControl or siFBXL16. Two days post-transfection, RNA was extracted and the TaqMan-based RT-qPCR was performed to determine transcript levels of FBXL16, ERα, and ERα target genes (pS2, CCND1, and c-myc). The bar graph represents mean ± SE (Two-way ANOVA test- **** p<0.0001).
II. Regulation of ERα protein stability by FBXL16 and the underlying molecular mechanisms of this regulation

FBXL16 stabilizes ERα protein by preventing its proteasomal degradation

FBXL16 has been shown to regulate the expression of proteins by affecting their protein stability and ubiquitination. In a previous study from our lab, we discovered that FBXL16 upregulates c-myc and SRC-3 protein levels by decreasing their polyubiquitination and thus increasing protein stability (Morel et al., 2020b, p. 16). In contrast, a recent study reported that exogenously overexpressed FBXL16 binds to and initiates protein ubiquitination and degradation of HIF1α in triple-negative BCa (TNBC) cells, impeding HIF1α-mediated epithelial to mesenchymal transition (EMT) in TNBCs (Laws et al., 2020). Thus, we presumed that FBXL16 upregulates ERα protein stability, thereby increasing its protein level. To determine the effect of FBXL16 on ERα protein half-life (measurement of protein stability), we performed cycloheximide-based protein turnover assays. We silenced FBXL16 in T47D cells. 65 hours after siRNA transfection, we treated the cells with the ribosomal inhibitor cycloheximide (CHX) that stops de novo protein synthesis, and harvested cells 2-, 4-, 6-, and 8-hours post-treatment. Immunoblot analysis revealed that ERα protein was less stable with FBXL16 silencing (half-life: approximately 3 hours) than with control siRNA treatment (half-life: 6 hours) (Figure 16).

To confirm this observation, we further tested the effect of FBXL16 on ERα protein stability by transiently overexpressing eGFP (control) or FBXL16 using retrovirus transduction in T47D cells. The half-life of ERα was prolonged to 10 hours with FBXL16 overexpression versus 3.6 hours with control eGFP expression (Figure 17), suggesting FBXL16 overexpression drastically inhibited ERα protein degradation.
Figure 16: FBXL16 silencing promotes ERα degradation and decreases ERα protein half-life.

T47D cells were transfected with control siRNA (siControl) or siRNA against FBXL16 (siFBXL16). 65 hours post-transfection, cells were treated with 100μg/mL cycloheximide (CHX) for different times as indicated along with a non-treatment control (0-hour). The expression of ERα was analyzed by western blotting. ERα protein levels at each time point were normalized by the loading control (β-actin) and presented relative to non-treatment control (0-hour: arbitrarily set as 1). ERα half-life (t_{1/2}) was determined from the exponential curve equation calculated using a one-phase exponential decay model in GraphPad Prism 6 software. The graph (bottom panel) represents the mean ± SE of each time point from four independent experiments.
**Figure 17: FBXL16 overexpression stabilizes ERα protein.**

Control gene (eGFP) or FBXL16 was overexpressed in T47D cells using transiently retroviral transduction. After 48 hours of transduction, protein translation was inhibited by 100μg/mL cycloheximide (CHX) treatment for different times as indicated along with a non-treatment control (0-hour). ERα protein level at each time point was normalized to β-actin, and the normalized ERα protein is compared to 0-hour time (arbitrarily set as 1). ERα half-life ($t_{1/2}$) was determined from the exponential curve equation calculated using a one-phase exponential decay model in GraphPad Prism 6 software. The graph (bottom panel) represents the mean ± SE of each time point from three experiments.
Since ERα protein stability is well known to be regulated by the UPS, we also investigated if the effect of FBXL16 on ERα stability is through impeding its proteasomal degradation. For that, we silenced FBXL16 and treated cells with either vehicle (DMSO) or MG132, a proteasomal inhibitor. MG132 treatment increased ERα protein levels as expected. Importantly, FBXL16 silencing in MG132-treated cells did not result in a decrease in ERα level as in vehicle-treated cells, suggesting that FBXL16 affects proteasomal degradation of ERα (Figure 18).

FBXL16 interacts with ERα and inhibits ERα protein ubiquitination

We next investigated whether FBXL16 regulates ERα protein ubiquitination. First, we wanted to identify if ERα and FBXL16 interact. We co-overexpressed FBXL16 and ERα in 293T cells. We then pulled down overexpressed ERα using anti-Flag antibody-conjugated agarose beads. We saw that FBXL16 was co-immunoprecipitated with ERα. To confirm the interaction between ERα and FBXL16, we overexpressed HA-FBXL16 in MCF7 cells and immunoprecipitated FBXL16 using the anti-HA antibody-conjugated beads. Immunoblot analysis shows that endogenous ERα was co-immunoprecipitated with FBXL16, indicating the formation of a complex between the two proteins. Taken together, these results demonstrate that FBXL16 interacts with ERα (Figure 19).

Afterward, to test the effect of FBXL16 on ERα protein ubiquitination, Flag-ERα and HA-ubiquitin were transfected in HeLa cells together with either an empty vector or FBXL16. Ubiquitinated proteins were then immunoprecipitated using anti-HA antibody-conjugated beads and then immunoblotted with ERα antibody to detect ubiquitinated ERα. Indeed, FBXL16 overexpression decreased ERα polyubiquitination (Figure 20).
Figure 18: FBXL16 stabilizes ERα by preventing its proteasomal degradation.

FBXL16 was silenced by siRNA transfection in T47D cells. 3 days after transfection, cells were treated with vehicle or MG132 (10μM) for 6 hours. FBXL16 silencing and ERα protein changes were analyzed by western blotting. The quantification shows the mean ± SE of three individual experiments (One-way ANOVA test- ** p<0.01, * p<0.05, ns- not significant).
Figure 19: ERα interacts with FBXL16.

(A) Flag-tagged ERα and HA-tagged FBXL16 were co-overexpressed in 293T cells. ERα protein was immunoprecipitated using anti-flag antibody-conjugated beads, and FBXL16 and ERα were detected by immunoblotting. An immunoprecipitation using a mouse IgG was set as a control. (B) HA-tagged FBXL16 was overexpressed in MCF7 cells. After 48 hours of transfection, overexpressed FBXL16 was immunoprecipitated using anti-HA antibody-conjugated beads. Endogenous ERα was immunoblotted with an ERα specific antibody and FBXL16 was detected using an HA antibody.
Figure 20: FBXL16 decreases ERα ubiquitination.

HeLa cells were co-transfected with pMT-HA-ubiquitin and pCMV-FLAG-ERα plasmids together with either pSG5-empty vector or pSG5-FBXL16 expression vector. Cells were treated with a proteasome inhibitor (MG132) for 6 hours. HA-ubiquitin conjugates were immunoprecipitated using anti-HA affinity beads (HA-IP) and then immunoblotted with anti-ERα antibody to detect the ubiquitinated ERα protein. A control IP was performed with normal mouse IgG on protein A agarose beads using equal amounts of both cell lysates. The protein levels of FBXL16, ubiquitin, and ERα in the whole cell lysate were analyzed using anti-FBXL16, anti-HA, and anti-ERα antibodies, respectively.
FBXL16 decreases estradiol-induced ERα degradation by antagonizing the F-box protein FBXO45

We have identified that FBXL16 interacts with and stabilizes ERα protein and promotes its transcriptional activity. Next, we set out to investigate the mechanism of how FBXL16 decreases ERα ubiquitination and stabilizes it. Interestingly, estradiol not only activates ERα-mediated transactivation but induces its proteasomal degradation, two well-synchronized processes at transcription sites (Reid et al., 2003b). Several E3 ligases including SKP2, BRCA1, CHIP, and MDM2 are suggested to ubiquitinate and destabilize ERα (Saji et al., 2001b, p. 2; Fan, Park, and Nephew, 2005b; Calvo and Beato, 2011b; Zhou et al., 2014), whereas a few other ubiquitin ligases such as RNF31, TRIM56, SMURF1 have recently been shown to stabilize ERα protein and act as ERα coactivators (Zhu et al., 2014c, p. 31; Yang et al., 2018b; Xue et al., 2019b, p. 56). To date, the biochemical function of FBXL16 is largely unknown, and it is unclear if FBXL16 possesses SCF-E3 ligase activity. Given that FBXL16 was shown to stabilize c-myc by antagonizing FBW7 E3 ligase activity (Morel et al., 2020b, p. 16), we hypothesized that FBXL16 might interact with and antagonize another F-box E3-ligase specific to ERα, thereby promoting ERα stability. We focused on two F-box proteins, SKP2 (Bhatt et al., 2012b; Zhou et al., 2014, p. 2) and FBXO45 (Han et al., 2016a) which have been shown to degrade ERα.

First, we silenced SKP2 in MCF7 cells to verify its effect on ERα. However, SKP2 knockdown did not alter ERα protein levels although c-myc and p27 protein levels were increased upon SKP2 silencing as reported (von der Lehr et al., 2003; Nakayama et al., 2004, p. 2). Additionally, we silenced SKP2 and treated cells with cycloheximide to
identify its effect on ERα protein stability. Unexpectedly, we did not observe an increase in ERα protein stability upon SKP2 silencing (Figure 21).

We then set to test whether FBXL16 regulates ERα by affecting FBXO45, another F-Box protein shown to promote ERα degradation, particularly upon TSEC treatment (Han et al., 2016a). First, we aimed to confirm the effect of FBXO45 on ERα protein expression. We co-overexpressed ERα with increasing levels of FBXO45 in HeLa cells. As reported previously, the ERα protein expression level was downregulated gradually with increased FBXO45 expression (Figure 22).

We next examined the effect of FBXO45 on ERα protein stability in regular culture condition. We silenced FBXO45 in MCF7 cells and then treated cells with cycloheximide. The silencing of FBXO45 was confirmed by RT-qPCR due to the non-specificity of the FBXO45 antibody in western blotting. The effect of FBXO45 knockdown on ERα turnover was checked by measuring ERα protein expression over the next 24 hours after cycloheximide treatment. FBXO45 silencing in MCF7 did not show a clear effect on ERα protein level under regular culture condition (Figure 23).
Figure 21: SKP2 knockdown did not increase ERα protein expression level and stability.

(A) MCF7 cells were transfected with non-targeting control siRNA or siRNA against SKP2 (siSKP2). 3 days after the transfection, the protein levels of SKP2, ERα, c-myc, p27, and GAPDH were analyzed using immunoblotting. (B) SKP2 was silenced in MCF7 cells as shown in (A). 65 hours post-siRNA transfection, cells were treated with cycloheximide (100µg/ml) to inhibit protein synthesis. Samples were collected at different time points as indicated. ERα expression was analyzed using immunoblotting. The ERα protein level was normalized against the GAPDH level from the same sample. The ERα protein level at the 0-hour time point under each condition is arbitrarily set as 1. The relative ERα protein levels (indicated by numbers below the bands) at other time points were calculated by comparing to that of the 0-hour time point.
Figure 22: FBXO45 overexpression downregulates ERα protein expression.

HeLa cells were co-transfected with pcDNA-HA-ERα with an increasing amount of pCMV-flag-FBXO45. FBXO45 and ERα levels were checked by western blotting using anti-flag and anti-HA antibodies, respectively. The quantification shows the mean ± SE from three individual experiments (One-way ANOVA test- *** p<0.001, ** p<0.01, * p<0.05).
Figure 23: FBXO45 knockdown had little effect on ERα protein stability under regular culture condition.

MCF7 cells were transfected with non-targeting control siRNA or siRNA against FBXO45 under regular culture condition. Cells were treated with cycloheximide (100 µg/ml) for different times as indicated. ERα protein levels were analyzed using western blotting and the half-life ($t_{1/2}$) of ERα was determined using the one-phase decay model in GraphPad Prism 6 software. FBXO45 silencing was confirmed using RT-qPCR and FBXO45 gene expression levels were normalized to the housekeeping gene GAPDH.
FBXO45 was shown to be specifically involved in ligand-induced ERα degradation in both endometrial and BCa cells (Han et al., 2016a). To test if FBXL16 opposes this activity of FBXO45, we silenced FBXL16 or FBXO45 individually or doubly in T47D cells, followed by treatment with 10nM estradiol for indicated times. As expected, silencing of FBXL16 accelerated estradiol-mediated ERα degradation, whereas FBXO45 knockdown reduced estradiol-induced ERα degradation. However, the rate of E2-mediated ERα degradation under the condition of double knockdown of FBXL16 and FBXO45 was comparable to that of siControl, suggesting that FBXL16 decreases estradiol-induced ERα degradation by antagonizing FBXO45 (Figure 24).

We wondered whether there is interaction between FBXL16 and FBXO45 proteins. To test this, we overexpressed FBXO45 with either eGFP or FBXL16 in 293T cells. FBXL16 or eGFP (negative control) were then immunoprecipitated using anti-HA antibody-conjugated beads. We observed that FBXO45 was specifically pulled down with FBXL16 immunoprecipitation (Figure 25), indicating that FBXL16 interacts with FBXO45.
Figure 24: FBXL16 decreases estradiol-mediated ERα degradation by antagonizing FBXO45.

T47D cells were cultured in charcoal-stripped serum media for 2 days. Cells were transfected with either control siRNA (siControl), siFBXL16, or siFBXO45, or co-transfected with siFBXL16 and siFBXO45. After 70 hours of transfection, cells were treated with estradiol (10nM) for 2 hours to induce ERα degradation. Cell lysates were harvested at indicated time points and ERα, FBXL16, and GAPDH proteins were detected by western blotting. FBXO45 silencing was confirmed by RT-qPCR and the Ct values were normalized to the housekeeping gene GAPDH.
Figure 25: FBXO45 interacts with FBXL16.

293T cells were co-transfected with pCMV-flag-FBXO45 and either pSG5-HA-eGFP (control) or pSG5-HA-FBXL16. The overexpressed FBXL16 was immunoprecipitated using anti-HA affinity gel. A control IP was performed with normal mouse IgG on protein A agarose beads using equal amounts of both cell lysates. The protein levels of FBXL16 and FBXO45 were detected using anti-HA and anti-flag antibodies, respectively.
FBXL16 does not alter the interaction between FBXO45 and ERα

We then went on to investigate the detailed mechanism of how FBXL16 antagonizes FBXO45 to reduce E2-mediated ERα degradation. We postulate that FBXL16 may alter the interaction between FBXO45 (the E3 ligase) and ERα (the substrate). To investigate the role of FBXL16 in affecting the interaction between FBXO45 and ERα under estradiol stimulation condition, we cultured the cells in media containing charcoal-stripped serum. We then co-overexpressed pCMV-flag-FBXO45 with either pSG5-HA-EV (control) or pSG5-HA-FBXL16. The cells were stimulated with estradiol and overexpressed FBXO45 was pulled down using flag antibody-conjugated beads to check the interaction with endogenous ERα. FBXO45 and ERα are reported to interact with each other in both vehicle and estradiol-treated conditions (Han et al., 2016a). Consistently, we also observed the interaction of ERα with FBXO45 upon both vehicle and estradiol treatment. However, overexpression of FBXL16 did not cause any change in the amount of ERα immunoprecipitated with FBXO45. These results indicate that FBXL16 does not alter the interaction between FBXO45 and ERα (Figure 26).

FBXL16 does not alter the interaction between FBXO45 and its binding partner MYCBP2

FBXO45 is the only F-box protein that possesses a unique SPRY (SplA and ryanodine receptor) domain on the c-terminus. It is well established that FBXO45 forms an atypical ubiquitin E3 ligase by interacting with SKP1 and MYCBP2 (Myc binding protein 2, also known as Protein associated with Myc (PAM)). Several proteins like FBXW7, NMNAT2, N-cadherin, and Zeb2 are found to be ubiquitinated and degraded by the E3 ligase
**Figure 26: FBXL16 does not alter the interaction between FBXO45 and ERα.**

MCF7 cells were cultured in phenol-red free charcoal-stripped serum-containing media for 2 days. pCMV-flag-FBXO45 was co-overexpressed with either pSG5-HA-EV or pSG5-HA-FBXL16. After 48 hours of transfection, cells were treated with either vehicle or estradiol (10nM) for 1 hour. FBXO45 was immunoprecipitated by flag antibody-conjugated affinity gel beads. A control IP was performed with normal mouse IgG on protein A agarose beads using equal amounts of cell lysates. The specific pull-down of FBXL16 and ERα with FBXO45 IP was analyzed by immunoblotting. Numbers below the ERα blot indicate fold change in ERα levels relative to the control condition (EV- vehicle treated, arbitrarily set as 1).
FBXO45-MYCBP2-SKP1 complex (Zhu et al., 2014c; Desbois et al., 2018; Richter et al., 2020; Na et al., 2020). Thus, we investigated the possibility that FBXL16 hinders the interaction between MYCBP2 and FBXO45 and thereby impacts the functional E3 ligase complex formation, which may account for its antagonizing effect on FBXO45-mediated ERα degradation. We co-overexpressed FBXO45 with either control (eGFP) or two concentrations of FBXL16 in MCF7 cells. With flag antibody-conjugated beads, we immunoprecipitated the overexpressed FBXO45 in each condition and observed the endogenous MYCBP2 protein in the IP samples. We were able to confirm the interaction between FBXO45 and MYCBP2 in MCF7 cells. In addition, FBXO45 was found to interact with FBXL16 as expected. However, FBXL16 overexpression did not show a noticeable change in the interaction between FBXO45 and MYCBP2. Thus, we think that FBXL16 affect FBXO45 activity not by altering the interaction between FBXO45 and its binding partner MYCBP2 (Figure 27).
Figure 27: FBXL16 does not affect the interaction between FBXO45 and its binding partner, MYCBP2.

MCF7 cells were transfected with pCMV-flag-FBXO45 and pSG5-HA-eGFP or pSG5-HA-FBXL16 (4µg or 8µg). 2 days after the transfection, flag tagged FBXO45 was immunoprecipitated using flag antibody-conjugated affinity gel beads to check its interaction with MYCBP2 in the presence or absence of FBXL16. The protein levels of FBXO45, FBXL16, MYCBP2, and actin were detected by anti-flag, anti-HA, anti-MYCBP2, and anti-β-actin antibodies, respectively.
III. Role of FBXL16 in promoting BCa cell growth and altering therapeutic response to fulvestrant

FBXL16 knockdown suppresses ER⁺ BCa cell growth and decreases the expression of ERα-governed cell cycle regulators

ERα signaling is the central regulator of cell growth in ER⁺ cell lines. As FBXL16 regulates ERα protein stability and transcriptional activity, we wanted to determine the effect of FBXL16 on cell growth of ER⁺ cell lines. We silenced FBXL16 in two ER⁺ BCa cell lines, MCF7 and T47D, and measured cell growth over five days by DNA quantification. FBXL16 silencing significantly reduced cell growth in both MCF7 and T47D cells (Figure 28A, B). Additionally, we examined the effect of silencing FBXL16 on the expression of ERα target genes which are important cell cycle regulators in BCa cells, including cyclin D1, c-myc, and E2F1. Cyclin D1-CDK4 complex is shown to upregulate E2F transcription factors, which leads to transcription of cyclin E2 mRNA. Additionally, cyclin D1 sequesters p21^{Waf1/Cip1}/p27^{Kip1} (proteins that render cyclin E1-CDK2 in the inactive state), thereby activating the cyclin E1-CDK2 complex (Prall et al., 1997; Prall, Carroll, and Sutherland, 2001; Planas-Silva and Weinberg, 1997). c-myc negatively regulates p21^{Waf1/Cip1} transcription and increases Cdc25A expression which allows cyclin E-cdk2 activation and leads to a progression of the cell cycle from G1 to S phase (Prall et al., 1998; Mukherjee and Conrad, 2005). Overall, cyclin D1, c-myc, and E2F1 all contribute to the advancement of the cell cycle from G1 to S phase. The protein levels of cyclin D1, c-myc, and E2F1 were greatly reduced upon FBXL16 silencing (Figure 28C). Thus, we reason that FBXL16 promotes cell proliferation of ER⁺ BCa cells at least partly...
by upregulating the expression of these ERα target genes, including cyclin D1, c-myc, and E2F1 which are known cell cycle regulators.

**FBXL16 promotes BCa cell proliferation in a ligand-independent manner**

In addition to ligand (estradiol)-induced activation, ERα is regulated and can be even activated independent of hormone by signaling of protein kinases such as TKRs like epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), and insulin-like growth factor receptor (IGFR) (Kahlert et al., 2000; Britton et al., 2006; Arpino et al., 2008). Therefore, we wanted to determine the hormone dependence of FBXL16’s role in cell proliferation. FBXL16 was silenced by siRNA in MCF7 cells cultured in media with CSS. Cells were then treated with either vehicle or E2 (10nM) and cell growth was analyzed by measuring DNA content. FBXL16 silencing caused a significant reduction in cell growth in both vehicle and estradiol-treated cells, indicating that FBXL16 regulates cell growth in a ligand-independent manner most likely due to its direct effect on ERα protein expression level (Figure 29).
Figure 28: FBXL16 silencing reduces BCa cell growth and decreases the expression of ERα-governed cell cycle regulators.

(A) MCF7 and (B) T47D cells were transfected with negative control siRNA or siRNA targeting FBXL16. From one day after transfection, cell growth was measured daily for the next five days using the FluoReporter dsDNA quantification assay kit. The graphs represent the mean ± SE of three independent experiments (Two-way ANOVA test - *** p<0.001, ** p<0.01, * p<0.05); (C) Immunoblot analysis showing the protein expression of cyclin D1, E2F1, c-myc after FBXL16 silencing in MCF7 cells.
FBXL16 was silenced in MCF7 cells cultured in charcoal-stripped serum condition for 2 days. One day after the transfection, cells were treated with vehicle or E2 (10nM) every 24 hours. Cell growth was measured by fluorometric dsDNA quantification kit (Two-way ANOVA test: *** p<0.001).
FBXL16 promotes cell growth of ER⁺ BCa cells partially by upregulating ERα protein

Moreover, to ascertain that FBXL16 promotes cell growth of ER⁺ BCa cells by upregulating ERα protein, we determined if exogenous overexpression of ERα by viral transduction can rescue cell growth inhibition caused by FBXL16 knockdown. Indeed, we observed that restoration of ERα expression offset the decrease in cell growth caused by FBXL16 silencing (siFBXL16 + lenti-ERα versus siFBXL16 + lenti-ctrl, Figure 30). These results indicate that FBXL16 promotes cell growth through the upregulation of ERα protein.

FBXL16 positively regulates Y537S-ERα mutant protein expression and stability

Current challenges in treating ER⁺ BCa include the development of endocrine resistance. ERα is mutated in BCa, predominantly occurring post-endocrine therapy. Mutations in the ligand-binding domain of ERα protein not only promote BCa cell proliferation and metastasis but also make cancer cells irreversible to endocrine therapy (i.e., tamoxifen and fulvestrant) (Robinson et al., 2013a, p. 1; Toy et al., 2013a, p. 1; Jeselsohn et al., 2014). The most common clinically encountered mutations are Y537S and D538G within the ligand bind domain (LBD). They are gain-of-function mutations that induce constitutive activation of ERα which makes the growth of mutant cells ligand-independent. The LBD mutations also lead to a decrease in the binding of tamoxifen and fulvestrant to ERα conferring resistance to endocrine therapy (Li et al., 2013; Merenbakh-Lamin et al., 2013a). It would be therapeutically relevant if FBXL16 knockdown can considerably impact the proliferation of these mutant cell lines. To determine whether FBXL16 affects expression
Figure 30: FBXL16 promotes cell growth of ER+ BCa cells by upregulating ERα protein.

T47D cells were transfected with control siRNA (siControl) or siRNA against FBXL16 (siFBXL16) along with the lentiviral transduction of either pLJM1-EV or pLJM1-ERα. 24 hours post-transfection, cell growth was measured each day continuously for a total of 5 days using Fluoreporter dsDNA quantification kit (Two-way ANOVA test: ** p<0.01, **** p<0.0001). Silencing of FBXL16 and restoration of ERα were confirmed using western blotting.
levels of ERα LBD mutant protein, we silenced FBXL16 in MCF-7 cells harboring genome-edited ESR1 mutation Y537S and the targeted ESR1 wild type (TWT) cell clone. We found that similar to its effect on wild-type ERα, depletion of FBXL16 also led to a remarkable decrease in the protein level of ERαY537S (Figure 31).

FBXL16 increases the stability of wild-type ERα protein. Therefore, we then investigated the effect of FBXL16 on the stability of mutant ERα protein. FBXL16 was stably knocked down using the pSIH-shFBXL16 lentivirus transduction system in T47D cells expressing ERαY537S mutant. We subjected these cells to cycloheximide treatment for determining ERαY537S protein stability. We found that FBXL16 silencing greatly reduced the stability of ERαY537S mutant protein (Figure 32), suggesting that FBXL16 also promotes ERαY537S mutant protein stability and expression level.

FBXL16 knockdown decreases cell growth in both T47D/TWT-ERα and T47D/ERαY537S cell lines

Having known the role of FBXL16 in stabilizing and thus upregulating ERαY537S protein, we then examined the role of FBXL16 in cells expressing ERαY537S oncogenic protein. We transiently silenced FBXL16 in T47D/TWT-ERα and T47D/ERαY537S cells and measured cell growth by DNA content estimation. FBXL16 silencing caused significant growth reduction in both T47D/TWT-ERα and T47D/ERαY537S cells (Figure 33). To further confirm the importance of FBXL16 in the growth of cells expressing ERα (wild type or Y537S mutant), we cultured T47D/TWT-ERα or T47D/ERα-Y537S cells with stable knockdown of FBXL16 in a 3D soft agar environment. As reported previously (Harrod et al., 2017; Bahreini et al., 2017; Gates et al., 2018), T47D/ERα-Y537S cells
Figure 31: FBXL16 positively regulates expression levels of Y537S-ERα mutant protein.

FBXL16 was silenced using siRNA in MCF7/WT-ERα, MCF7/TWT-ERα, and MCF7/ERαY537S cells. Non-targeting siRNA served as a negative control. FBXL16, ERα, and SRC-3 expression were detected by western blotting.
Figure 32: FBXL16 knockdown decreases the stability of Y537S-ERα protein.

T47D cells harboring Y537S-ERα were used to generate stable cell lines expressing either shRNA against FBXL16 (shFBXL16) or shRNA against luciferase gene (shLuc). Cells with stable knockdown of FBXL16 (shFBXL16) and cells expressing shRNA against luciferase gene (shLuc) were subjected to cycloheximide treatment (100µg/ml) for indicated times. The expression of ERαY537S was analyzed using an ERα antibody by western blot. ERα protein level at each time point was normalized to the loading control (β-actin). The normalized ERα protein is compared to the ERα level at 0-hour time (arbitrarily set as 1). ERα half-life (t_{1/2}) was determined from the exponential curve equation calculated using a one-phase exponential decay model in GraphPad Prism 6 software. The graph (bottom panel) represents the mean ± SE for each time point from three independent experiments.
Figure 33: FBXL16 knockdown decreases growth of both T47D/TWT-ERα or T47D/ERα-Y537S cells.

T47D/TWT-ERα or T47D/ERα-Y537S cells were transiently transfected with siControl or siFBXL16. Five days after the transfection, cell growth was compared between T47D/TWT-ERα or T47D/ERα-Y537S cell lines using the dsDNA quantification assay kit. FBXL16 silencing and ERα protein levels were checked by western blotting. The graph represents the mean ± SE from four different experiments (n=16) (One-way ANOVA test: * p<0.05, ** p<0.01, **** p<0.0001).
showed significantly higher colony formation ability as compared to the T47D/TWT-ERα cells. Consistent with its role in cell growth under 2D culture condition, FBXL16 silencing significantly reduced colony formation in both T47D/TWT-ERα and T47D/ERα-Y537S cells (Figure 34).

**FBXL16 silencing accelerates fulvestrant mediated ERα degradation and increases fulvestrant responsiveness in T47D/TWT-ERα and T47D/ERα-Y537S cells**

Fulvestrant is the only FDA-approved SERD as a first-line endocrine agent for metastatic and locally advanced BCa and a second-line drug for advanced metastatic BCa that has progressed after initial tamoxifen or aromatase inhibitor treatment (Cardoso et al., 2019; Visvanathan et al., 2019). SERDs like fulvestrant are designed to degrade ERα (Wakeling, Dukes, and Bowler, 1991). As FBXL16 affects ERα stability and degradation, we expect that FBXL16 silencing would synergistically act with SERDs like fulvestrant in preventing BCa cell growth. To test if FBXL16 silencing affects fulvestrant-mediated ERα degradation, we silenced FBXL16 in T47D/TWT-ERα and T47D/ERα-Y537S cells and treated cells with fulvestrant (100nM) for 0, 30, 60, 90, and 120 minutes. As expected, fulvestrant induced ERα protein degradation in both WT ERα and ERαY537S expressing T47D cells. Moreover, ERαY537S was turned over slower than the WT ERα in response to fulvestrant treatment. Importantly, we observed a drastic increase in fulvestrant mediated ERα degradation upon FBXL16 silencing both in T47D/TWT-ERα and T47D/ERα-Y537S cells (Figure 35).
Figure 34: FBXL16 knockdown restricts anchorage-independent cell growth of T47D/TWT-ERα or T47D/ERα-Y537S cells.

T47D/TWT-ERα or T47D/ERα-Y537S cells with stable knockdown of FBXL16 were plated in 0.3% agarose in a 24-well plate at a seeding density of 3000 cells/well with a supporting bottom layer of 0.6% agarose. After three weeks of anchorage-independent growth, colonies were stained (images on the left and quantified using ImageJ software. The bar graph shows the mean ± SE from four independent experiments (n=12) (One-way ANOVA test: * p<0.05, ** p<0.01, *** p<0.001). Western blots confirm the stable knockdown of FBXL16 and reduction of ERα protein levels in cells plated for this experiment.
Cells with ERαY537S mutation show decreased response towards SERDs like fulvestrant, leading to reduced efficacy of fulvestrant in inhibiting BCa cell growth (Bahreini et al., 2017). We discovered that silencing of FBXL16 causes faster degradation of ERα upon fulvestrant treatment (Figure 35). This prompted us to test if targeting FBXL16 would increase the efficacy of fulvestrant in ER⁺ BCa cells with either WT ERα or ERαY537S. For that, we cultured T47D/TWT-ERα and T47D/ERα-Y537S cells with stable knockdown of FBXL16 in a 3D soft agar environment and treated these cells with increasing concentrations of fulvestrant. Consistent with the previous report, as compared to T47D/TWT-ERα, T47D/ERα-Y537S cells showed a significant decrease in responsiveness to fulvestrant (Figure 36). Importantly, the concentration of fulvestrant in inhibiting 50% of cell growth (IC50) in both TWT and Y537S cells was drastically lowered upon FBXL16 depletion (Figure 36). These results indicate that the silencing of FBXL16 sensitizes cells towards fulvestrant in restricting ER⁺ BCa cell growth.
Figure 35: FBXL16 silencing enhances fulvestrant mediated ERα degradation.

FBXL16 was silenced in T47D (A) TWT-ERα and (B) Y537S-ERα cells. 48 hours post-transfection, cells were treated with 100nM fulvestrant to induce ERα degradation and harvested at 0, 0.5, 1, 1.5, and 2 hours post-treatment. ERα protein levels at each time point were first normalized to β-actin loading control and then compared to that of 0 hr time point (arbitrarily set as 1) in each group (siControl or siFBXL16). Half-life was determined by comparing the relative amount of ERα over time after fulvestrant treatment using the
one-phase decay model. The graphs on the bottom panels show the mean ± SE from three independent experiments. Significant differences in the ERα protein levels at each time point were determined using a Two-way ANOVA test (* p<0.05, *** p<0.001).
Figure 36: Depletion of FBXL16 sensitizes ERα WT and ERα mutant T47D cells towards fulvestrant.

T47D TWT-ERα and Y537S-ERα cells with stable knockdown of FBXL16 were seeded at a density of 5000 cells/well in a 24-well plate with an upper growth layer of 0.3% agarose with complete culture media containing 5% charcoal-stripped serum. Cells were treated every 2 days with media containing 20 pM estradiol plus different concentrations of fulvestrant or vehicle (DMSO) as indicated. The colonies were allowed to grow for one month followed by staining and quantification (using ImageJ software). IC50 values for each condition were determined using a nonlinear regression model in GraphPad Prism 6. The graph represents the mean ± SE from two independent experiments (n=6). Significant differences in cell growth in each cell line upon FBXL16 knockdown was determined using the Two-way ANOVA test (* p<0.05, ** p<0.01). FBXL16 knockdown in cells was confirmed by immunoblotting.
DISCUSSION

I. Regulation of ERα protein expression and transcription activity by FBXL16

ERα belongs to the nuclear receptor transcription factor and is upregulated in 65-70% of total breast cancer cases (Nagaraj et al., 2012; Wang and Liu, 2015; Dai et al., 2015). ERα plays a critical developmental role in the brain and female reproductive system. It is predominantly expressed in the mammary gland, ovaries, uterus, and vagina in females and testes, seminal vesicles, and prostate in males (Bocchinfuso et al., 2000; Couse, Curtis Hewitt, and Korach, 2000; Walker and Korach, 2004). In addition to expression in sex organs, ERα mRNA is also expressed in smooth muscles, brain, heart, and liver. Early studies using the ERα knockout model showed infertility in female mice and decreased fertility in male mice (Walker and Korach, 2004). Insensitivity of the uterus to estrogen was at large the reason for infertility in the female ERα knockout mice (Lubahn et al., 1993). Even though ERα is dispensable for uterine development, it is essential for uterine maturity and function (Curtis Hewitt, Couse, and Korach, 2000). ERα knockout mice displayed immature follicular growth which resulted in a lack of ovulation (Couse and Korach, 1999; Couse et al., 2003). Of note, mammary gland growth in ERα knockout mice was the most impacted and glands couldn’t develop beyond the ductal rudiment stage (Bocchinfuso and Korach, 1997; Couse et al., 2003). On the contrary, a high level of ERα in breast epithelium poses an increased risk for breast cancer indicating the causative role of ERα in the initiation and progression of breast cancer (Ali and Coombes, 2000). The
hormone estradiol (E2) exerts its proliferative and mitogenic actions through binding and activating estrogen receptor alpha (ERα) (Yu et al., 2013; Leone, Busonero, and Acconcia, 2018; Sánchez et al., 2019). Hyperactivity of the E2-ERα axis is one of the prime causes of tumorigenesis and progression of ER+ breast cancer. Upregulation of ERα coactivators, mutations in ERα protein which increase its transcriptional activity, and post-translational modifications in ERα protein (phosphorylation, ubiquitination) contribute to an uncontrolled increase in ERα signaling (Lei et al., 2019). Inhibiting ERα signaling is the well-accepted treatment strategy for the treatment of ER+ breast cancer cases (Shao and Brown, 2004). Drugs designed to degrade ERα (selective estrogen receptor degraders-SERDs) like fulvestrant, AZD9496 show significant antitumor activity in both wild-type as well as constitutively active mutant ERα positive breast cancer (Yeh et al., 2013b; Savi et al., 2015b; Shomali et al., 2021). ERα ubiquitination is the major way of degrading ERα in breast cancer cells. Many E3 ligases, including some F-Box protein E3 ligases, act as coactivators of ERα which bind to ERα promoter, increase ERα transcription activity, and target ERα for degradation after the transcription cycle. In addition to E3 ligases which induce ERα turnover, some E3 ligase category proteins (RNF8, RNF31, SMURF1, TRIM56) stabilize ERα and prevent its degradation unfolding a new class of targets for the treatment of ER+ breast cancer (Zhu et al., 2014c, p. 31; Wang et al., 2017b, p. 8; Yang et al., 2018b, p. 1; Xue et al., 2019b, p. 56). However, few studies have reported E3 ligase(s) that can both increase ERα protein stability and expression level and act as ERα coactivators, thereby stimulating oncogenic ERα signaling for breast cancer progression. In the current study, we have identified a novel molecular regulation of ERα protein stability in ER+ breast cancer cells. We demonstrated that the F-box protein FBXL16
positively regulates ERα protein stability and expression level. Previous research has established the vital developmental role of FBXL16. FBXL16 depletion in mice leads to death right after birth (Dickinson et al., 2016b). In addition, FBXL16 might be involved in human cancers. FBXL16 is overexpressed in 14% of invasive breast carcinomas and 9% of lung adenocarcinoma patients. Previous reports showing FBXL16 as an E2F1 transcriptional target and a negative regulator of tumor suppressor gene, PP2A, hint toward the oncogenic role of FBXL16 in cancers (Sato et al., 2010b, p. 1; Honarpour et al., 2014b). FBXL16 has been demonstrated to promote the proliferation of lung cancer cells and breast cancer cells (Morel et al., 2020b, p. 16; Yang and Jing, 2021, p. 16). Moreover, the silencing of FBXL16 increased autophagy and cell apoptosis in breast cancer cells (Yang and Jing, 2021). Structurally, FBXL16 falls under the class of F-box proteins which are important substrate recognition components in the RING-type SCF E3 ligase complex. However, FBXL16 has not yet been shown biochemically to form a functional E3 ligase complex. FBXL16 possesses an F-box motif, which mediates the interaction with SKP1, and seven LRR domains, which are thought to aid in recruiting substrates (Morel et al., 2020b). However, FBXL16 doesn’t show a detectable interaction with CUL1 (Honarpour et al., 2014b; Liu et al., 2018b; Morel et al., 2020b), which implies that FBXL16 may have a different complex formation and function from other F-box E3 ligases like FBXW7 and FBXW1 (β-TRCP). From another study in our lab, we have shown that opposite to FBXW7 and β-TRCP, FBXL16 upregulates several oncoproteins targeted by SCF-E3 ligases, including c-myc, SRC-3, and β-catenin (Morel et al., 2020b). In this study, we observed that FBXL16 co-overexpressed with ERα in breast carcinoma patient samples as well as in breast cancer cell lines. While FBXL16 expression is not regulated by ERα, FBXL16
upregulates ERα protein expression levels by increasing its protein stability, revealing a new mechanism of ERα protein overexpression in breast cancer.

In addition to regulating ERα protein, we demonstrate that FBXL16 also upregulates the protein levels of SRC-3, a critical coactivator of ERα in breast cancer cells. These results are consistent with our findings of the upregulation of SRC-3 by FBX16 in lung cancer cell lines (Morel et al., 2020b). SRC-3 is critical for reproductive function including mammary gland development and is overexpressed in a large subset of breast, ovarian, and prostate cancer cases (Anzick et al., 1997, p. 1; Xu et al., 2000). Knockdown of SRC-3 resulted in a significant decrease in breast tumor formation and metastasis partially owing to downregulation in insulin-like growth factor-mediated activation of the PI3K- Akt pathway (Kuang et al., 2004; Torres-Arzayus et al., 2004). SRC-3 also exerts direct genomic action by binding to p300 and ERα to selectively activate ERα transcription activity at the promoter sites of ERα target genes (Zheng et al., 2005; Yi et al., 2015). In addition to recruitment of SRC-3, phosphorylation at the serine 118 site on ERα is also an indicator of ERα transcription activity (Chen et al., 2000, 2002). In our study, the positive regulation of ERα expression by FBXL16 also reflected in decreased phosphorylation at the S118 site in ERα and a significant reduction in ERα transcription activity measured by luciferase assay and gene expression of ERα target genes. The regulation of ERα expression and transcriptional activity by FBXL16 is ligand-independent. While it is inferred that the effect of FBXL16 on ERα transcription activity is mainly due to upregulation in ERα and SRC-3 protein levels, FBXL16 might be directly recruited to the ERα/coactivator complex on ERα target genes during transcription given that ERα-mediated gene transcription and ERα/coactivator complex turnover are coupled during the process. Further research needs
to be carried out to identify the detailed role of FBXL16 on ERα transcription activity and FBXL16 binding at ERα transcription sites.

Taken together, this study not only identifies FBXL16 as a positive regulator of ERα expression but also as a transcriptional coactivator of ERα. Additionally, the ligand-independent effect of FBXL16 on ERα substantiates it as a suitable target for the treatment of hormone-independent breast cancer cases which are irresponsive to endocrine therapy.

II. Regulation of ERα protein stability by FBXL16 and the underlying molecular mechanisms of this regulation

Nuclear receptors are involved in the transcription of key cell cycle regulators as well as genes involved in vital cellular processes, hence their expression is tightly controlled (Sever and Glass, 2013). One of the major pathways controlling ERα expression and turnover is the ubiquitin-proteasome system (UPS). ERα protein stability directly correlates with E2 genomic action and transcription activity of ERα, which is evidenced by an increase in expression of various E2-ERα-dependent genes upon stabilization of ERα (Powers et al., 2010). While many studies have identified different E3 ligases that ubiquitinate and stimulate ERα protein turnover, it is largely unknown how ERα and ERα LBD mutants are stabilized, leading to upregulation of ERα protein levels in breast cancer. Our study identified FBXL16 as a novel factor in stabilizing ERα and ERα LBD mutants in breast cancer cells. Mechanistically, we have found that FBXL16 increases the protein stability of ERα by decreasing its polyubiquitination and degradation. FBXL16 was first discovered as a transcriptional target of E2F1 (Sato et al., 2010b, p. 1). Regulation of substrate protein stability and ubiquitination by FBXL16 has been recently unraveled. Our
lab was the first to show that FBXL16 complexes with SKP1 and has the potential to regulate protein stability and ubiquitination via UPS (Morel et al., 2020b). We have demonstrated earlier that FBXL16 interacts with oncoproteins like c-myc, SRC-3, and β-catenin and inhibits their polyubiquitination by antagonizing their E3 ligases, FBXW7 (c-myc, SRC-3), and β-TrCP (β-catenin) (Morel et al., 2020b). However, another study revealed that FBXL16 overexpression induces protein ubiquitination and degradation of HIF1α independent of oxygen conditions (Kim et al., 2021, p. 16), but it is unclear which specific E3 ligase is involved. It remains to be investigated how FBXL16 exerts different effects on the protein stability of its targets. Investigation of the detailed mechanism of how FBXL16 stabilizes its targets may shed light on why FBXL16 has a differential effect on HIF1α ubiquitination. In the presence of the proteasome inhibitor MG132, ERα protein levels were not affected upon FBXL16 silencing, indicating that FBXL16 increases ERα protein stability by inhibiting its proteasome-dependent degradation.

To delineate the mechanism, we postulate that FBXL16 may antagonize an E3 ligase specific to ERα to decrease the ubiquitination of ERα. Our lab had previously shown that FBXL16 stabilizes c-myc protein by antagonizing an F-box E3 ligase, FBXW7. There was no competition between FBXL16 and FBXW7 for binding to the substrate, c-myc, nonetheless FBXL16 may directly impede the E3 ligase function/activity of FBXW7. Moreover, the deletion mutant FBXL16ΔFbox showed no interaction with SKP1 and FBXL16ΔLRR didn’t bind with c-myc. Furthermore, both these mutants lack the ability to stabilize c-myc n. These results suggest that both the F-box motif and LRR domain in FBXL16 are important for their role in stabilizing its targets (Morel et al., 2020b). To investigate whether FBXL16 stabilizes ERα by antagonizing the activity of another F-Box
protein E3 ligase, we looked into two F-box E3 ligases, FBXL1 (SKP2) and FBXO45 that were reported in the literature to induce ERα degradation. In the case of SKP2, contrary to the previous reports, we found that SKP2 knockdown using different siRNAs in different breast cell lines did not show a clear effect on ERα protein level. However, we uncovered a mechanism of FBXL16 in inhibiting estradiol-induced ERα degradation by antagonizing FBXO45. Upon binding to estradiol, ERα goes through a transcription cycle followed by ubiquitination and degradation of ERα (Nirmala and Thampan, 1995; Alarid, Bakopoulos, and Solodin, 1999). Different estrogen analogs, partial ERα agonists, or ERα antagonists modulate its ubiquitination and degradation differently (Wijayaratne and McDonnell, 2001). For instance, estradiol activates the protein kinase Src which phosphorylates ERα at Y537 residue leading to ERα’s binding to E6AP E3 ligase (Sun et al., 2012b). Our findings suggest that FBXO45 promotes estradiol-induced ERα degradation, consistent with the previous report (Han et al., 2016b). Importantly, FBXL16 drastically reduced FBXO45-mediated ERα degradation under E2 stimulation. Interestingly, FBXO45 did not affect ERα turnover in the absence of E2 stimulation, indicating that the effect of FBXO45 on ERα is ligand-specific. As reported, FBXO45 binds to ERα. We found that FBXL16 also interacts with FBXO45. However, FBXL16 does not interact with the FBXO45 binding partner, MYCBP2, which along with FBXO45 forms a functional E3 ligase complex. We did not observe any change in complex formation between FBXO45 and ERα by FBXL16 overexpression. Moreover, FBXL16 doesn’t seem to alter the interaction between FBXO45 and MYCBP2. These results suggest that FBXL16 does not affect the complex formation of FBXO45 with E3 ligase MYCBP2 and the substrate ERα. However, it is likely that FBXL16 might directly affect the ubiquitin conjugation activity of
FBXO45/MYCBP2 towards the substrate ERα. To identify that, we performed a ubiquitination assay with co-overexpression of control or FBXL16 vector and FBXO45. Either by immunoprecipitation of overexpressed ubiquitin or by pulldown of endogenous ERα, the polyubiquitination signal of ERα was not detectable. We reason that the E2-mediated ERα degradation may be a transient process or the polyubiquitination signal of ERα at the endogenous level of ERα is not enough to detect. In addition, we found that under co-overexpression condition, FBXL16 appears to decrease FBXO45 protein stability and expression level in HeLa cells (data not shown). However, the regulation of estradiol mediated ERα degradation needs to be investigated in ER+ BCa cell lines, as the complex formation between FBXL16 and FBXO45 to regulate ERα degradation may require specific co-factors of ERα signaling unique to BCa cell lines. Unfortunately, we were unable to confirm this under endogenous condition in that we could not find an FBXO45 antibody that is suitable for detecting endogenous FBXL45 by western blotting. It is noteworthy that FBXL16 stabilizes ERα protein under regular culture condition in media containing a minimum amount of steroid hormones. This suggests that FBXL16’s effect on ERα is not limited to the ligand stimulation conditions. The role of FBXL16 in antagonizing FBXO45 to diminish E2-induced ERα degradation is just part of the regulation of ERα protein by FBXL16. Further investigation of how FBXL16 stabilizes ERα under regular culture conditions without estradiol stimulation needs to be carried out with special attention to E3 ligases whose activity or expression gets inhibited by FBXL16. Although both E6AP and SKP2 were shown to promote ERα ubiquitination and proteasomal degradation, we found that FBXL16 doesn’t work via inhibiting E6AP or SKP2 to stabilize ERα protein. The E3 ligase(s) involved under this condition remains to
be identified. Lastly, the possibility that FBXL16 may impact ERα stability and ubiquitination via an indirect mechanism cannot be excluded.

In conclusion, our findings provide novel insights into the post-translational regulation of ERα expression and transcriptional activity. Specifically, our study identified FBXL16 as a new positive regulator of ERα by upregulating the stability and expression levels of both ERα and its coactivator SRC-3 in breast cancer cells.

III. Regulation of BCa cell growth and fulvestrant therapeutic response by FBXL16

ERα gene amplification and overexpression enhance ERα transcriptional activity, leading to increased expression of genes associated with cell cycle progression and proliferation (Fowler et al., 2004). Alterations in ERα signaling promotes BCa cells to grow under low concentrations of estradiol or even in a ligand-independent manner, leading to endocrine therapy resistance (Fowler et al., 2004, 2006). ERα target genes, such as c-myc, cyclin D1, E2F1, and cyclin E which play key roles in cell cycle progression and have been shown to impact the endocrine therapy responsiveness (Musgrove and Sutherland, 2009). For instance, overexpression of c-myc, cyclin D1, and/or cyclin E1 contributes to endocrine therapy resistance mainly via activation of cyclin-dependent kinases responsible for G1 phase progression or negative regulation of p21WAF1 and p27Kip1 (Butt et al., 2005). Downregulation of p21 and p27, which are CDK checkpoint inhibitors, is linked to tamoxifen irresponsiveness (Chu, Hengst, and Slingerland, 2008; Iida et al., 2019). An important study revealed that inhibiting CDK4/6 greatly reduced the growth of both ET-sensitive and ER-Resistant ER+ BCa cell lines. These findings led to the development of an important class of drugs, CDK4/6 inhibitors (Miller et al., 2011). Cyclin D1 activates
CDK4/6 to enable Rb-induced cell cycle progression through the G1 phase (Lukas, Bartkova, and Bartek, 1996). Of note, cyclin D1 amplification is detected in 58% of luminal B and 29% of luminal A breast cancer samples in the TCGA dataset (Cancer Genome Atlas Network, 2012). Hence, inhibition of cyclin D1 and/or CDK4/6 has been considered a therapeutic strategy for the treatment of ER+ breast cancer, in particular those with ET resistance. Currently, there are three FDA-approved CDK4/6 inhibitors, Palbociclib, ribociclib, and abemaciclib which are being used in ER+ breast cancer treatment (Hortobagyi et al., 2016; Finn et al., 2016; Goetz et al., 2017).

In another context, several mutations in ERα proteins have been identified in the metastatic cancer stage (Robinson et al., 2013b; Toy et al., 2013b; Merenbakh-Lamin et al., 2013b). It is assumed that these mutations are acquired due to selective pressure during the treatment of primary tumors with endocrine therapy (Ellis et al., 2012; Toy et al., 2013b, p. 1). Mutations in the ligand-binding domain such as D538G, Y537S, E380Q, Y537N, and Y537C are shown to make BCa cell growth hormone-independent and/or endocrine therapy-resistant leading to disease progression and metastasis (Johnston et al., 1995b; Gutierrez et al., 2005b; Toy et al., 2013b; Bahreini et al., 2017). Extensive clinical trial findings advocated that ERα mutations at sites Y537S, Y537N, D538G, and E380Q sites can serve as predictive markers for identifying endocrine therapy-resistant patients (Takeshita et al., 2016; Chandarlapaty et al., 2016). Selective estrogen receptor degraders like fulvestrant and AZD9496 are capable of inducing degradation of ERα mutants such as Y537S and D538G and may display an inhibitory effect on tumor growth in metastatic aggressive ER+ breast cancer patients (Weir et al., 2016; Toy et al., 2017b). It is believed that promoting ERα degradation by chemical compounds or inhibiting the coactivator or
upstream regulator which stabilizes ERα might be a successful approach in treating aggressive cases expressing mutant ERα proteins.

Even though fulvestrant is effective in the treatment of advanced ER+ breast cancer patients, many patients develop resistance to fulvestrant upon treatment. Many mechanisms and signaling pathways are thought to cause resistance to fulvestrant such as cyclin E2 amplification and cyclin E2/cdk2 hyperactivation (Kaminska et al., 2021), and upregulation of other mitogenic pathways (Hu et al., 2018; Ribas et al., 2018; Liu et al., 2019). In addition, a decrease in sensitivity of ERα protein (both wild type and LBD mutants) to SERDs in advanced breast cancers is also thought to be one of the mechanisms of SERD resistance. Therefore, it is important to identify regulators, such as FBXL16 revealed in our current study, that increase stability of WT and mutant ERα protein and decrease their sensitivity to SERDs such as fulvestrant. We can then design better therapeutics for treating advanced ET-resistant breast cancer.

ERα signaling is well documented to promote breast cancer development and progression. In this study, FBXL16 emerges as an important component of the ERα signaling for breast cancer cell growth. Previous studies have indicated FBXL16 as a potential oncogene since it was found to inhibit tumor suppressors like p14, p17, PP2A-B55, and FBXW7 (Sato et al., 2010b, p. 16; Honarpour et al., 2014b, p. 16; Morel et al., 2020b). Here, we identified an oncogenic role of FBXL16 in promoting breast cancer cell growth by upregulating ERα target genes which are important for cell cycle progression, such as cyclin D1, c-myc, E2F1. We demonstrated by a rescue experiment that this stimulatory role of FBXL16 on cell proliferation is partially by regulating ERα expression. In addition, we identified that FBXL16 also stabilized mutant ERα-Y537S protein, and knockdown of FBXL16 in cells
expressing ERα-Y537S protein led to a significant reduction in the 2D as well as anchorage-independent 3D cell growth. Moreover, ERα-Y537S breast cancer cells with FBXL16 knockdown displayed a significantly lower capacity for infiltrating in lungs as compared to the cells with control shRNA in mice. This discovers FBXL16 as a target for treating metastatic breast cancer cases with mutations in ERα protein. As FBXL16 and fulvestrant have opposite actions on ERα protein stability, we identified that silencing FBXL16 significantly increases the effectiveness of fulvestrant in inhibiting the growth of breast cancer cells expressing either wild-type ERα or mutant ERα-Y537S. Additional investigation is warranted to identify the detailed mechanism of how FBXL16 silencing promotes the efficacy of fulvestrant but these results are encouraging to predict that FBXL16 may promote fulvestrant resistance and targeting FBXL16 along with adjuvant treatment may benefit breast cancer cases displaying fulvestrant resistance.

IV. Conclusions
The data presented in this study revealed a novel regulatory mechanism governing the ERα protein stability and expression level in breast cancer. ERα is overexpressed in breast cancer and plays a critical role in promoting breast cancer growth and metastasis. Manipulating ERα protein stability and turnover is a highly sought-after approach for inhibiting ERα+ breast cancer growth. However, not much is known about the regulators of increasing ERα protein stability, leading to the upregulation of ERα protein levels in breast cancer. Our study identified the positive regulation of ERα protein stability by an F-box protein FBXL16. We have demonstrated that FBXL16 upregulates ERα protein stability and expression by reducing its protein ubiquitination and proteasomal
degradation. In the context of estradiol stimulation, FBXL16 antagonizes FBXO45-mediated ERα degradation induced by E2. Furthermore, we discovered that upregulation of ERα by FBXL16 increased the expression of ERα target genes and promoted breast cancer cell growth. Moreover, FBXL16 can also stabilize ERα-Y537S mutant protein, and silencing of FBXL16 impeded the growth and metastasis of BCa cells expressing ERα-Y537S. Furthermore, FBXL16 silencing greatly enhanced the effects of fulvestrant in not only stimulating the degradation of both WT-ERα and Y537S-ERα but also suppressing the growth of BCa cells expressing either wild type ERα or ERαY537S mutant. In conclusion, this study has identified FBXL16 as a novel positive regulator of ERα signaling in promoting tumor cell growth and fulvestrant resistance in ER+ breast cancer.
FUTURE PERSPECTIVE

This research study reveals a novel mechanism that regulates ERα protein stability via ubiquitination. The results here suggest that FBXL16 upregulates ERα to promote breast cancer cell growth. We discovered the regulation of ERα by FBXL16 at protein expression and stability, but we also observed a decrease in ERα transcript level with knockdown of FBXL16. However, ERα protein can regulate its own transcription (Castles et al., 1997). Further research is required to identify if FBXL16 directly regulates ERα transcript level or if it is an indirect outcome of FBXL16’s effect on ERα protein expression and stability. Performing a ChIP assay of FBXL16 binding on ERα gene or RNA stability assay by using actinomycin D for transcription inhibition would clarify whether the regulation of ERα transcript level by FBXL16 is direct or indirect.

Most importantly, it is remaining to be identified how FBXL16 regulates ERα protein ubiquitination and stability, particularly in the absence of estradiol stimulation. We postulated that FBXL16 may counteract the activity of another F-box protein which functions as an E3 ligase for ERα degradation. Thus far, our data shows that this is not through antagonizing the F-box protein SKP2, a known E3 ligase for ERα independent of E2 stimulation. Our study found that FBXL16 protects ERα against FBXO45-mediated degradation upon E2 stimulation. However, we still need to figure out the detailed mechanisms by which FBXL16 antagonizes the FBXO45-mediated E3 ligase activity.
towards ERα. FBXL16 might upregulate ERα protein stability by inhibiting the activity of another F-box protein or an E3 ligase of another category under regular culture conditions. In addition, the effect of FBXL16 on ERα stability may be indirect in the absence of estradiol stimulation. For example, FBXL16 may inhibit the kinase responsible for phosphorylating and signaling ERα for polyubiquitination given that ERα protein ubiquitination and stability are known to be regulated by a variety of kinases (Medunjanin et al., 2005; Grisouard et al., 2007; He et al., 2010; Medina and Wandosell, 2011; Giamas et al., 2011). All in all, identifying the detailed mechanism and complete regulatory axis between FBXL16 and ERα will help in designing a better targeting strategy for treating ER+ BCa, in particular those with co-overexpression of FBXL16 and ERα.

Additionally, this regulation of ERα needs to be investigated in vivo. Future studies in our lab aim to confirm the regulation of ERα by FBXL16 in breast tumor mouse models. For this purpose, we aim to utilize an MMTV-Cre mouse model for mammary gland-specific overexpression of FBXL16. This study intends to identify if FBXL16 overexpression specifically in the mammary gland upregulates ERα protein expression and stability, thereby promoting mammary gland tumor formation and progression. This model would also give us leverage to study the physiological role of FBXL16 in mammary gland development in mice.

Finally, additional investigation is needed to corroborate the role of FBXL16 in fulvestrant resistance. First, the interesting finding from the current study that silencing of FBXL16 increased the efficacy of fulvestrant in restricting cell growth in vitro needs to be confirmed in mouse models of ER+ breast cancer, including aforementioned genetically modified mouse models and xenograft tumor mouse models derived by breast cancer cells
expressing either wild type ERα or ERαLBD mutants. This effect should also be confirmed with other selective estrogen receptor degraders like AZD9496. Interestingly, our preliminary results identified that FBXL16 upregulates cyclin E2 protein expression and stability in breast cancer cells (data not shown). As cyclin E2 upregulation was shown to be associated with ET resistance in breast cancer, we hypothesize that upregulation of cyclin E2 protein by FBXL16 may be another mechanism by which FBXL16 promotes fulvestrant resistance in breast cancer cells. Taken together, our study poses FBXL16 as an oncogenic regulator of ERα signaling and a potentially important target for advanced ER+ breast tumors. The findings from this study and further investigation of the in vivo roles and the underlying molecular mechanism of FBXL16 in ER+ BCas will support the future development of pharmacological inhibitors (e.g., small molecule inhibitors) of FBXL16 for treating ER+ breast cancer, including those metastatic and/or ET-resistant cases.
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